The Generation of a Candidate Axial Precursor in Three Dimensional Aggregates of Mouse Embryonic Stem Cells

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This dissertation is submitted for the degree of

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To my parents & to Millie.
Acknowledgements

Looking back on how I reached this point, I would like to thank Steve Russell for first suggesting that I might have fun if I joined Alfonso’s lab for a short project in the summer of my second year as an undergraduate. Thank you, Alfonso, for taking me on and Penny, for persuading him to keep me! Little did I know then where this path would take me.

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Declaration of Collaborative Work

Some of the methods used and the results described in this thesis have been the outcome of collaborations with others. These contributions have been acknowledged with “Notes on Collaborative Work” or footnotes in the text and are listed below for clarity:

- Sections 2.5.1 & 2.5.2: Dr David Turner (Department of Genetics, University of Cambridge) assisted with the development of image analysis pipelines in Python.

- Section 3.2.1.3: Mehmet Girgin (École Polytechnique Fédérale de Lausanne, Switzerland) optimised the gastruloid protocol by moving the counting step to after the wash steps.

- Fig. 3.5: The gastruloids in this dataset were prepared and imaged jointly with Mehmet Girgin, but were analysed separately by the author.

- Fig. 4.6: The gastruloids in this dataset were prepared and imaged by Amy Horrell; images were processed and analysed by the author.

- Fig. 4.7: Dr Penny Hayward (Department of Genetics, University of Cambridge) assisted with the PCR and data analysis; Dr David Turner plotted the data.
• Section 4.2.4: Dr David Turner collected the datasets shown in Fig. 4.13 and marked the author’s ability to score images based on morphology (summarised in Table 4.2).

• Chapter 6: The approach and experimental regime were devised jointly between the author, Dr Ben Steventon (Department of Genetics, University of Cambridge) and Professor Alfonso Martinez Arias (Department of Genetics, University of Cambridge). Thanks are due to Dr Octavian Voiculescu (Department of Physiology, Anatomy & Neuroscience, University of Cambridge) for his tuition in the New Culture technique. Experimental samples were prepared for grafting by the author. The embryo culture and grafting steps of the xenotransplantations were performed jointly with Dr Ben Steventon. Image acquisition, quantification and analysis were performed by the author. Dr Ben Steventon also provided valuable input in the discussion and interpretation of the results.

• Section 7.2.3: Practical work and imaging was carried out equally with Mehmet Girgin; data analysis was performed by the author.

• Section 7.2.4: All practical work and imaging was undertaken by Mehmet Girgin; data analysis was performed by the author.
Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

It does not exceed the prescribed word limit for the Degree Committee of the School of the Biological Sciences.

Peter Baillie-Johnson
Cambridge, April 2017
Abstract

Textbook accounts of vertebrate embryonic development have been based largely upon experiments on amphibian embryos, which have shown that the tissues of the trunk and tail are organised from distinct precursors that existed during gastrulation. In the mouse and chick, however, retrospective clonal analyses and transplantation experiments have demonstrated that the amniote body instead arises progressively from a population of axial precursors that are common to both the neural and mesodermal tissues of the trunk and tail. For this reason, they are known as neuro-mesodermal progenitors (NMps). Detailed studies of NMps have been precluded by their lack of a unique gene expression profile and the technical difficulties associated with isolating them from the embryo. Mouse embryonic stem cells (ESCs) provide the possibility of instead deriving them in vitro. ESCs have been used to model developmental processes, partly through large cellular aggregates known as embryoid bodies. These structures do not, however, resemble the axial organisation of the embryo and they develop in a disordered manner. This thesis presents a novel culture system of small, three-dimensional aggregates of ESCs (gastruloids) that can recreate the events of early post-implantation development, including axial elongation. Gastruloids are the first ESC-based model for axial elongation morphogenesis; this body of work characterises their development and identifies a candidate population of NMps within their elongating tissues. Additionally, this work establishes a xenotransplantation assay for testing the functional properties of in vitro-derived NMp populations in the chicken embryo and applies it to NMps from
gastruloid cultures. The results of this assay show that gastruloids are a credible source of NMps \textit{in vitro} and therefore offer a new experimental means to interrogate their properties. The use of gastruloids to recreate embryonic development has implications for basic research as a synthetic system and for the therapeutic derivation of other embryonic progenitors through bioengineering.
Publications

The research presented in this thesis has contributed to the following publications, which can be found in the appendix (pp.287-347):

  - The images of elongating *Sox1::GFP*+ gastruloids in Chapter 3 Fig. 3.2B and Chapter 4 Fig. 4.15 are published as the middle panel of Fig. 7A in this paper.

  - The images of *Sox1::GFP*+ gastruloids cultured under different conditions in Chapter 3 Fig. 3.2 were published as Fig. 8A.
  - The bottom row of Chapter 4 Fig. 4.12 is shown as Fig. 8C in this paper.
  - The data shown in Chapter 4 Fig. 4.14 were published in an earlier form as Figs 9 & S7 in this paper.


  – Fig. 1.6 in Chapter 1 is reprinted directly from this paper, where it appears as Fig. 1.

  – The PCR data presented in Chapter 4 Fig. 4.7 have been replotted as a heatmap and appear as Fig. 4A in this pre-print manuscript.
  – The Gata6\textsuperscript{H2B\textit{Venus}} gastruloids shown in Chapter 4 Fig. 4.11A appear as Fig. 4C in this pre-print manuscript.
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<tr>
<td>ALK</td>
<td>Activin receptor-Like Kinase</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine Monophosphate-activated protein Kinase</td>
</tr>
<tr>
<td>AME</td>
<td>Axial Mesendoderm</td>
</tr>
<tr>
<td>AVE</td>
<td>Anterior Visceral Endoderm</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
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<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<td>Chi</td>
<td>CHIR99021 (Chiron)</td>
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<td>CLE</td>
<td>Caudal Lateral Epiblast</td>
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<td>CNH</td>
<td>Chordo-Neural Hinge</td>
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<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>DE</td>
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<td>1,1'-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate</td>
</tr>
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<td>Dorsomorphin H1</td>
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<tr>
<td>dpc</td>
<td>Days Post Coitum</td>
</tr>
<tr>
<td>DRC</td>
<td><em>mir-290-mCherry/mir-302-eGFP</em> (cell line)</td>
</tr>
<tr>
<td>DVE</td>
<td>Distal Visceral Endoderm</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EMT</td>
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<td>(mouse) Epiblast Stem Cell</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-Regulated Kinase</td>
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<tr>
<td>(m)ESC</td>
<td>(mouse) Embryonic Stem Cell</td>
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<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<td>Foetal Calf Serum/Foetal Bovine Serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<tr>
<td>(e)GFP</td>
<td>(enhanced) Green Fluorescent Protein</td>
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<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
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<td>GSK</td>
<td>Glycogen Synthase Kinase</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<td>Histone 2B</td>
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<tr>
<td>HH</td>
<td>Hamburger-Hamilton (stage)</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner Cell Mass</td>
</tr>
<tr>
<td>iPSC</td>
<td>induced Pluripotent Stem Cell</td>
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<td>Leukaemia Inhibitory Factor</td>
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<td>Lateral Plate Mesoderm</td>
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<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
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<td>NMp(s)</td>
<td>Neuro-Mesodermal Progenitor(s)</td>
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<td>Arginylglycylaspartic acid</td>
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<td>Tail Bud Mesoderm</td>
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<tr>
<td>TGF-(\beta)</td>
<td>Transforming Growth Factor Beta</td>
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<tr>
<td>SFEBq</td>
<td>Serum-Free Embryoid Body culture with Quick re-aggregation</td>
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<tr>
<td>(e)YFP</td>
<td>(enhanced) Yellow Fluorescent Protein</td>
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Chapter 1

Introduction

1.1 Principles of Development

The enigmatic question at the centre of developmental biology is how a recognisable individual can form from an apparently homogeneous, radially symmetrical zygote. The early development of every bilaterian embryo can be reduced to two fundamental processes: 1) the acquisition of the initial asymmetries that break the radial symmetry of the embryo and specify the body axes and 2) the co-ordination of cellular movements in relation to these axes that organises the future tissues of the embryo in space (i.e. gastrulation). The development of the Bilateria is far from uniform, however, since organisms from each of the major clades exploit a variety of mechanisms to achieve these two fundamental processes.

This chapter examines the variety of possible solutions to the problems of symmetry breaking, gastrulation and axial elongation and describes how these processes can be studied in vitro using cultures of stem cells and organoids. Uniting stem cell biology with embryology provides a valuable, deconstructed perspective on development that can ultimately be used to make new predictions about the processes that build an embryo.
1.1.1 Symmetry Breaking & Axis Specification

The first step in the development of the embryonic body plan is to break the initial symmetry of the oocyte and in doing so to specify the future body axes of the individual. It is helpful to consider the (now-dated) term of multiplicity (or complexity), which is initially an intrinsic, non-spatial feature of the oocyte that becomes an extensive spatial organisation of the early embryo, namely a recognisable body plan [128]. This subsection compares the transition from the zygote to a body plan across different organisms, specifically examining the origin and timing of the initial asymmetry. This ranges from maternally patterned oocytes in the case of some insects to interactions between the embryonic and extraembryonic tissues of mammalian embryos after implantation (see Figure 1.1).

Symmetry Breaking in Invertebrates

The *Drosophila* oocyte is a remarkable example since it is already endowed with the asymmetries that will define the body axes prior to fertilisation. Indeed, the oocytes of many insects have an overt geometric polarity due to an ellipsoid shape that will come to define the antero-posterior axis. In the case of *Drosophila*, maternally provided *bicoid* and *nanos* mRNAs prime the antero-posterior axis through their differential localisation to opposing poles of the oocyte [60]. On the activation of zygotic translation, the maternal mRNAs establish opposing protein gradients across the syncytium that inhibit the anterior expression of *caudal* and the posterior expression of *hunchback* mRNA respectively. In addition, the termini of the embryo are specified by the maternal localisation of Torso-like protein to the poles of the follicular epithelium. The dorso-ventral axis is established through the interaction between the forming oocyte and the follicular cells of the ovary following directional nuclear migration to the prospective dorsal side [60]. The oocyte is therefore primed with the prospective antero-posterior and dorso-ventral axes prior to fertilisation, entirely through maternally inherited transcripts and interaction with the maternal nurse cells. In this case, the information
Figure 1.1: The Timing of Symmetry Breaking Varies Across the Major Bilaterian Clades. Model organisms discussed in the text are shown on the right of the phylogeny, coloured according to when they break radial symmetry to establish their body axes. This can range from the pre-existing asymmetries in the oocyte (red, as in Drosophila) to organisms that use the point of fertilisation to break the symmetry of the zygote (orange, as in C.elegans). Many marine invertebrate embryos (e.g. Dentalium and Tubifex) establish their body axes during the early cleavage stages (green); this is also the case for some externally developing vertebrates (Xenopus and Zebrafish). Amniotes, (e.g. chicken and mouse) break symmetry after many rounds of cleavage (blue), shortly before gastrulation. Marine invertebrate development probably reflects the ancestral state of symmetry breaking through segregation of maternally inherited determinants, while insects and amniotes reflect adaptation to shorter and longer periods of development, respectively. Phylogeny reproduced from [107] with annotations added by the author.
that specifies the future body axes is already completely determined before fertilisation. The syncytial nature of the cleavage divisions in these species allows these early, pre-determined asymmetries to be translated into long-range polarity before cellularisation occurs.

The *C. elegans* oocyte shares a superficially similar geometric polarity to that of the *Drosophila* oocyte, but it is not molecularly pre-patterned to the same degree. The holoblastic pattern of cleavage in *C. elegans* precludes the formation of long-range protein gradients by partitioning the cytoplasm into separate cells. The oocyte is uniform along its length until the point of fertilisation, at which point the sperm pronucleus is transported to one end and so defines the prospective posterior [60]. The first cleavage divides the zygote eccentrically to produce a larger anterior cell and a smaller posterior one. The dorsoventral axis is specified at the second cleavage division, at which point one of the anterior daughter cells moves posteriorly to define the future dorsal side of the embryo [60]. *C. elegans* therefore derives its body axes from both extrinsic (the site of sperm entry) and intrinsic (the migration of the anterior daughter cell) asymmetry cues within the first two cleavage divisions. The development of asymmetry in *Drosophila* and *C. elegans* is entirely deterministic in these early stages.

Marine invertebrate embryos, the favourite subjects of early experimental embryologists, provide example systems in which the early specification of the body axes is at first regulative, but then becomes deterministic through the inheritance of cytoplasmic determinants [60]. Driesch demonstrated that sea urchin embryos at the 2-cell stage could give rise to identical, complete embryos on either the division of the blastomeres (1891) or through the fusion of two fertilised eggs (1900; also Bierens de Haan, 1913) [128]. These observations refuted the concept that the egg cytoplasm contained spatially localised determinants, since a whole embryo could be produced from a fraction of the cytoplasm (though Driesch himself did not interpret the results in this way).
1.1. PRINCIPLES OF DEVELOPMENT

Worm embryos such as *Tubifex* provide a similar example, since division of the egg along the animal-vegetal axis prior to the first cleavage generates a conjoined twinned embryo (reported by Penners, see [128]). This demonstrates that at early stages, each half of the egg is capable of producing a whole individual due to the determinants localised to each pole. Similarly, mollusc embryos such as *Dentalium* undergo a characteristic pattern of cleavage in which a polar lobe emerges from a vegetal cell on each division, passing cytoplasmic determinants into one daughter only. Manipulations of this pole plasm result in the loss of specific structures from the larva (reported by Wilson, see [128]).

In the case of ascidians, asymmetry is established very soon after fertilisation with the directional migration of the inner cytoplasm specifying the vegetal pole, followed by the appearance of a “yellow crescent” at the site of sperm entry which denotes the future ventral side. The first cleavage bisects this crescent to specify the left and the right sides of the embryo, with the antero-posterior and dorso-ventral planes defined by subsequent cleavages. The fate of each blastomere is determined by the complement of factors it inherits from the cytoplasm, which varies according to its position in the embryo [60]. The asymmetries underlying both the anteroposterior and dorsoventral axes are therefore established in the short period after fertilisation and prior to cleavage.

Collectively, the results from the marine invertebrates demonstrate that while the early embryo may be regulative, this property is lost during the cleavage stages, in which cytoplasmic determinants become important in specifying cell fates. The initial asymmetries required to form the body axes are likely to be maternally determined in the animal-vegetal direction, with dorso-ventral asymmetry defined by the asymmetric inheritance of polar cytoplasm through the early cleavage divisions. To return to the idea of developing complexity (or multiplicity), the invertebrate embryo can be regulative at spatially homogeneous stages, usually within the first few cleav-
ages, but its development becomes increasingly deterministic as the spatial complexity of the embryo increases.

The picture for the invertebrates discussed so far is one of early specification of the body axes, ranging from the oocyte to the early cleavage stages of the embryo, depending on when inherited determinants become localised. The underlying asymmetries are the result of intrinsic patterning as in *Drosophila* or from extrinsic cues such as the sperm entry point in nematodes and ascidians. Within the vertebrates, anamniotes such as zebrafish and *Xenopus* use similar systems to those already discussed, while amniotes such as the chicken and mouse differ substantially by their lack of inherited determinants. They therefore undergo many more rounds of cell division before the asymmetries become apparent.

**Symmetry Breaking in Vertebrates**

The development of asymmetry in the *Xenopus* embryo is similar to that of ascidians, since it takes its cues from the polarity of the oocyte and the sperm entry point. There is also a degree of congruence between the fate maps of these organisms [98]. The *Xenopus* zygote has an overt animal-vegetal polarity, which corresponds to the future dorsoventral axis in the pregastrula stage embryo (reviewed in [97]). After fertilisation, the cortical cytoplasm undergoes a $30^\circ$ rotation towards the point of sperm entry, revealing a “grey crescent” on the opposite side of the zygote that denotes the future site of cell invagination at gastrulation [60]. By the start of gastrulation, this position defines the future anteroposterior axis since the prime meridian that bisects Spemann’s organiser corresponds to the anterior midline of the embryo, which includes both dorsal and ventral derivatives [97]. The early development of the amphibian embryo is regulative, since blastomeres isolated at the two cell stage can give rise to complete individual embryos (Spemann 1903, see [128]) and newt embryos fused at the two-cell stage can produce chimaeric individuals (reported by Mangold & Seidel, 1927, see [128]).
The zebrafish oocyte also has an overt animal-vegetal polarity prior to fertilisation with a corresponding distribution of maternal factors. Unlike *Xenopus*, however, the second asymmetry cue that determines the site of gastrulation does not come from the site of sperm entry. An unknown cue causes the cortical microtubule-dependent movement of maternal dorsalising activity from the ventral region towards the animal pole [186]. The future dorsal side of the embryo is specified when these factors come to lie underneath one side of the cellular blastoderm. The initial asymmetry required for this process seems to rely on a transient array of parallel microtubules at the vegetal pole that forms within 20 minutes of fertilisation [87]. In this case, the asymmetry cue for the dorsoventral axis may be derived spontaneously from microtubule dynamics at the vegetal pole, while maternal factors are the effectors of this asymmetry in establishing the axis. In general terms, symmetry breaking in zebrafish is similar to in *Xenopus* since it relies on the directional transport of maternal factors following fertilisation.

Amniotes differ substantially from the examples discussed so far since the asymmetries that underpin the body axes arise well into the cleavage stages. Symmetry breaking in the early chicken embryo makes use of physical cues and maternal determinants to pattern an epiblast that comprises many thousands of cells [148]. By the time that the epiblast has formed, the two sides are distinguished by the direction of a potential difference across them, which results from the difference in pH between the basic albumen and the acidic subgerminal space. The experimental reversal of this potential difference can reverse the dorsoventral polarity of the epiblast (unpublished observations reviewed in [149]), suggesting that this is the asymmetry cue that specifies this axis. The fundamental asymmetry behind the anteroposterior axis is thought to derive from another physical cue: the combination of gravity and the rotation of the forming egg as it passes through the oviduct of the mother. This motion results in the asymmetric transport of yolk cytoplasmic factors to one end of the blastoderm, which is tilted in the direction of rotation. This
edge of the blastoderm becomes specified as the posterior marginal zone, the future site of primitive streak formation [148].

Mammalian embryos differ further still in acquiring asymmetries without the reliance on maternally inherited factors. This is due, in part, to adaptation to an internal mode of development as the embryos have no yolk and they must generate the tissues required to derive nutrition from the mother. Indeed, the polarity of the tissue produced by the first cleavage stages is used to specify the future embryonic and extraembryonic tissues. In the case of the mouse embryo, geometric asymmetry only becomes apparent at the blastocyst stage, where the formation of the blastocoel defines a rotationally symmetrical embryonic-abembryonic axis (illustrated in Chapter 4, Fig. 4.1). While this corresponds geometrically to the dorsoventral axis of the epiblast, the similarity does not extend to the fate of the cells. Anteroposterior asymmetry only becomes evident at the time of gastrulation, which arises from the combined interactions between the embryonic and extraembryonic tissues of the epiblast and visceral endoderm, respectively [186]. Molecular asymmetry first becomes evident in the extraembryonic tissues, which confer an anteroposterior identity on those of the epiblast through cell-cell interactions and the production of secreted factors. The polarity in the extraembryonic tissues arises from the directional proximal migration of the distal visceral endoderm and its neighbouring cells, but the underlying trigger for this asymmetry remains unknown. Like the chicken embryo, the mouse embryo is highly regulative and isolated individual blastomeres can form entire embryos up to the 8 cell stage, suggesting that maternal determinants do not play a role in assigning the body plan at these stages [186].

In summary, the asymmetries required to establish the fundamental body plan can arise from a range of intrinsic or extrinsic sources and they can be established at any point from pre-fertilisation to the onset of gastrulation. Many invertebrate embryos demonstrate that maternally inherited factors can dictate embryonic polarity at the oocyte stage and that these factors
1.1. PRINCIPLES OF DEVELOPMENT

can be inherited through the cleavage stages to specify different cell fates. In these cases, there is no reliance on extrinsic asymmetry cues and the axes are specified very early. *C. elegans*, ascidians and *Xenopus* demonstrate a reliance on sperm entry as an external asymmetry cue to complement the intrinsic asymmetry of their oocytes.

In those organisms which do not rely on maternally inherited factors, or are at a stage in which they have not yet been spatially localised, development is regulative and the embryo can tolerate experimental perturbations such as division or fusion. This form of experimental embryology has most frequently been used to describe cell fate specification, but it also raises the important point that the embryo has an intrinsic ability to pattern itself under quite different conditions to normal development. Given this degree of tolerance to perturbation, it is tempting to speculate that such “regulative embryos” could derive their asymmetry cues from a number of potential intrinsic or extrinsic sources. The mouse embryo provides an intriguing example, since polarity is first evident in the extraembryonic tissues and it is not yet fully understood how this originates. It is intriguing to consider that perhaps the mammalian epiblast in general shares this intrinsic ability to generate pattern. It should also be emphasised that regulation should be considered in extending deterministic models and observations from one model organism to another, which may in fact be less reliant on inherited determinants or instructive interactions (*e.g.* from the amphibian embryo to that of the mouse).

The development of highly regulative embryos such as the chicken, is exemplified by the formation of new individual embryonic axes if the chick blastoderm is divided into many parts [60][186]. This is substantially later than for the sea urchin or amphibian embryos, which do not form multiple individuals beyond the early cleavage stages. It is possible that this difference in the timing of individuation arises from the different life histories of these organisms. In the case of the externally developing invertebrate embryo, an
individual must form rapidly if it is to develop the capacity to feed and so derive nutrition for subsequent growth. In the case of the amniote embryo, the extra-embryonic tissues form early to secure an interaction with the maternal nutrient supply, whether that be through the yolk or the placenta. The formation of the individual therefore occurs at a later stage when the tissue architecture of the future nutrient supply is already established. Regarding the nature of the asymmetry cues in these different groups of organisms, it may be the case that highly regulative embryos make use of physical cues that are not required in rapidly developing organisms which instead rely on maternally inherited determinants. It is possible that the regulative nature of amniote development also carries an advantage in providing robustness to perturbations that may occur during the relatively long period of their development.

1.1.2 Gastrulation

With the exception of the more derived examples of *Drosophila* and *C. elegans*, many cleavage stage embryos are approximately radially symmetric about the animal-vegetal axis (Fig. 1.2). The next stage in the development of the bilaterian embryo is to elaborate this asymmetry into a body plan through the process of gastrulation, which positions the germ layers in relation to one another and generates the mesoderm. Arendt has given a detailed account of the evolution of gastrulation from an ancestral, sponge-like organism through to the wide range of bilaterally symmetric animals [2]. In comparing the development of these organisms, it becomes clear that the ancestral form of gastrulation is very widespread indeed and while organisms such as birds and mammals have some very derived phenomena, the underlying mechanisms are conserved.

Arendt cites Haeckel’s *gastrea* theory, which postulates that the ectoderm and mesoderm in all Metazoa are homologous due to their monophyletic origin from an ancestral gastrula-form organism [19]. Haeckel’s studies of
Figure 1.2: The Ancestral Bilaterian Blastula is Hollow and Radially Symmetric. The geometry of the blastula differs in size, geometry and the number of cells between different species, as indicated from the cartoons on the right (drawn from [2]). The presence of a hollow, spherical blastula in the three major bilaterian clades suggests that it is the ancestral morphology, with the *Drosophila* and zebrafish blastulae representing derived conditions that are adaptations to a store of yolk (through superficial and partial cleavage, respectively). Gastrulation must, therefore, proceed in different forms in different organisms, but the underlying movements of invagination are similar in all cases.
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sponges and medusae led him to conclude that all gastrulae arise from an
invagination of a group of cells in an initially spherical blastula to generate a
two-layered structure with an external opening at one end. Arendt demon-
strates that this is indeed the case for organisms across the major branches
of the Bilateria (polychaete worms, crustaceans and chordates, Fig. 1.2) and
presents the zebrafish and Drosophila embryos as more derived cases of adap-
tation to more yolk-rich embryos. Generally speaking, during the movements
of invagination the outer layer of ectoderm undergoes epiboly and spreads
from the animal pole as the inner layer of mesendoderm undergoes hypoboly
and spreads across the interior of the blastocoel. These movements are con-
served from sea urchins and basal chordates through to vertebrates, where in
Xenopus, hypoboly elongates the archenteron and in the chick, where a form
of epiboly is responsible for positioning the endoderm (hypoblast) underneath
the ectoderm (epiblast) [148]. There is some variation in Lophotrochozoan
embryos, which undergo a derived form of invagination where individual,
yolk-rich vegetal blastomeres enter the blastocoel and the overlying ectoderm
comes to overlie them via epiboly. Arendt suggests that this is likely to be
due to the pattern of spiral cleavage in these organisms which produces four
large vegetal blastomeres rather than a single-layered epithelium as in other
organisms [2]. In summary, the fundamental movements of gastrulation are
conserved from sponges through to the major branches of Bilaterians, though
the number of cells in the gastrula and the relative importance of hypoboly
and epiboly differs between organisms.

This level of conservation also extends to the molecular level through the
trio of Brachyury, the homeobox gene Caudal and a dependence on canonical
Wnt signalling during gastrulation. The expression of Brachyury and Caudal
(or their homologues) in the blastoporal region is strongly conserved from a
homologue in Cnidarian embryos through to polychaete worms and to acorn
worms, Amphioxus and vertebrates such as Xenopus [2]. The relationship
is more than simply correlative, since interfering with Brachyury function
impedes the cell movements of gastrulation in *Xenopus* [142], [134] and in the mouse [7]. Further upstream, canonical Wnt signalling plays a conserved role in activating *Brachyury* and *Caudal* expression at the blastopore from the Cnidaria (*Hydra, [77]*) to the Echinoderms ([78]) and to the vertebrates (*Xenopus*, [179]; mouse, [58]) (reviewed in [2]). In summary, the initiation of gastrulation occurs through a conserved mechanism of signalling and gene expression that is common to organisms which may otherwise differ in the exact nature of their gastrulation movements.

The formation of the mesoderm is another key component of the gastrulation process. In organisms such as *Amphioxus*, the mesoderm forms through enterocoely, a pouching-off of cells from the wall of the archenteron in a manner akin to the initiation of invagination at the blastopore. In vertebrates, the system is more derived, with the mesoderm emerging from the beginning of gastrulation as a mesenchyme, rather than an epithelium. These cells later undergo a secondary mesenchymal-to-epithelial transition to form the mesodermal compartments [2].

In summary, Arendt’s survey demonstrates that the movements and molecular mechanisms of gastrulation are fundamental to Metazoan development and that, at the gastrula stage, there are remarkable similarities even between distantly related organisms with very different adult body plans. Although there may be differences in the size and geometry of the gastrula between these organisms, the common mechanism is one of cell internalisation (whether as an epithelium or as a mesenchyme) using canonical Wnt signalling to induce changes in cell movement that are effected by *Brachyury* and *Caudal* expression.

There is also substantial variation in the morphology and geometry of gastrulae within the vertebrate clade. While the *Xenopus* embryo bears some resemblance to the ancestral *gastrea* form through its spherical morphology and animal-vegetal polarity, many avian and mammalian embryos are dis-
coid. Instead of forming a blastopore, through which the mesoderm and endoderm invaginates, these embryos form a primitive streak that extends centripetally (towards the centre of the disc). In the case of the chick, the disc-shaped epithelium of the epiblast becomes underlain with a layer of hypoblast prior to primitive streak formation. The primitive streak forms as the epiblast delaminates to form a layer of mesoderm between the epiblast and the hypoblast, extending centripetally (reviewed in [148]). This planar morphology of the gastrula is shared by most non-rodent and marsupial mammals, including higher primates such as the human [43]. In reviewing these organisms, Eakin and Behringer highlight the variation between the embryos of these species. In some cases such as the guinea pig, the embryonic component of the embryo becomes physically detached from the extraembryonic tissue. The amniotic cavity can also form through a process of cavitation, as in interstitially (deep) implanting organisms such as rodents or through folding, as is more common in superficially implanting organisms such as the hedgehog. Even the timing of gastrulation and somitogenesis varies considerably with respect to implantation; rodents implant prior to gastrulation while some hoofed mammals such as the horse form both pairs of limb buds prior to interaction with the maternal tissues (reviewed in [43]).

The rodent embryo\(^1\) forms an egg cylinder prior to gastrulation rather than a disc (illustrated in Chapter 4, Fig. 4.1), that is thought to be an adaptation to interstitial implantation and selection for smaller size [43]. In addition, the arrangement of germ layers in the gastrula is apparently “inverted,” with the endoderm forming on the external surface of the cup shaped epiblast, with the ectoderm at its centre. This arrangement can be compared to the structure of discoid embryos by conceptually flattening the cup-shaped epiblast into a disc [12]. This representation positions the epiblast above a layer of endoderm, with the mesoderm emerging effectively

\(^1\)Murine gastrulation is discussed in more detail in the introduction to Chapter 4 (Characterisation of Gastruloid Development).
from one edge of the disc, as in the chick. Comparing the embryos in this way highlights the similarity between the chick hypoblast and the mouse visceral endoderm in regulating the positioning of the primitive streak. Through single cell fate mapping of the prestreak and early streak epiblast, Lawson et al. note that the resulting fate map shares a great deal of similarity to those of the chick and urodele gastrulae when presented as a flattened projection [100]. Although the proportion of the epiblast that will form the different germ layers differs between these organisms, the overall topology between the tissues is the same, suggesting that topological relationships are conserved despite differences in size and geometry between the gastrulae.

From surveying gastrulation in this way, it can be thought of in general terms as a morphological solution to a geometric problem that results in the formation of the mesoderm and the positioning of the germ layers in relation to one another and to the body plan as a whole. This solution seems to be highly adaptable, with the mesoderm emerging as single cells or as groups, either before the formation of the definitive endoderm or coincident with it and across a variety of geometries (spherical, discoid or cup-shaped) and sizes of embryos (660 cells in the mouse, more than 20000 in the chick). In internally developing organisms, it can occur either before or after implantation. Despite these differences, the regulation of the process seems to be highly conserved, as does the eventual topology of the tissue types as is evident from fate mapping.

1.1.3 Axial Elongation & Anteroposterior Growth

Having positioned the germ layers in the correct relative positions with respect to the body plan, the embryo is completed by the differentiation of the germ layers into the tissues and the organs of the body\(^2\). With the exception of more derived cases (e.g. anurans and long germ band insects, see below), the precursors of the rostral tissues have formed by the end

\(^2\)The development of specific organs is not discussed in detail here.
of gastrulation but the more caudal tissues become allocated progressively through a process of posterior elongation [107]. The following discussion explores the division between the rostral and caudal tissues in more detail and considers the evidence for a conserved mode of posterior development.

**Different Modes of Vertebrate Head, Trunk and Tail Development**

The development of the vertebrate embryo can be divided into three phases according to the axial level of the forming tissues: cranial (or rostral), thoracic (primary development of a pre-anal trunk) and coccygeal (secondary development of a post-anal tail). The transition from thoracic to coccygeal development is evident at the histological level by the closure of the posterior neuropore and the termination of the gut at the anus. The transition from cranial to thoracic development is, however, less clear as the tissues are histologically continuous. Nevertheless, it is clear that the rostral and caudal tissues at this level have separate developmental origins that extend to the level of gene regulation (see Introduction to Chapter 5). In addition, comparisons between model organisms reveal that tissues at the same axial level can form through different modes of development.

Tissue re-arrangements play a central role in *Xenopus* development, both during gastrulation and immediately afterwards. In tracking the movement of groups of cells in gastrulating embryos, it is apparent that the tissues undergo a major extension through the directional convergence on the blastopore [89]. Furthermore, these tissue movements are autonomous as they continue when the tissues are cultured as sandwich explants [84] without a mechanical substrate [89]. These convergent extension movements correspond to the involution of the cells at gastrulation and the phase immediately afterwards in which the early neurula elongates along the antero-posterior axis. In preparing the explants, the earliest involuted prospective head mesoderm is removed; the elongating tissues that remain form the axial and paraxial mesoderm of the notochord and the somites as they extend [89]. DiI labelling
of the dorsal blastopore lip at mid- to late-gastrula stages marks the mesoderm of the trunk rather than that of the tail [63]. The cranial and trunk somites, therefore, arise in *Xenopus* as a result of the convergent extension movements during and after gastrulation from pre-existing tissue.

The tissues of the tail in *Xenopus* arise from the tailbud, which originates from the cells of the dorsal blastopore lip at the early neurula stage [63]. On labelling the dorsal blastopore lip and allowing the embryo to develop to the tadpole stage, labelled cells can be found throughout the notochord, interspersed with unlabelled cells, up to the tip of the tail. These results indicate that cell-rearrangements continue in this tissue up to the end of tail elongation, as a continuation of the mesodermal cell re-arrangements occurring at gastrulation [63]. Labelling small groups of cells in the tailbud has shown that their fates are highly mosaic, raising the possibility that some of the cells are multipotent [36]. In contrast to labelling the blastopore at the gastrula stages (which marks the tissues of the trunk), the development of the tailbud and its derivatives can be traced back to the neurula stage blastopore [63]. In summary, the *Xenopus* body is constructed through extensive cell re-arrangements: tissue from the gastrula forms the head, trunk and tail, while descendants of the early neurula contribute to the tail, with ongoing contribution from cells in the chordoneural hinge.

The head and trunk mesoderm form at different stages of gastrulation in the chicken embryo [148]. The former arises when the primitive streak is fully extended and the node forms, while the somitic mesoderm starts to form only once the node has started its apparent regression [148][15]. The start of node regression approximately correlates with the transition from cranial to trunk development since the first somite to form is at the level of the otic vesicle [75] (reviewed in [15]). The paraxial presomatic mesoderm of the trunk forms from the ingressions of epiblast cells neighbouring the anterior primitive streak during the regression of the node [127]. Unlike in *Xenopus* where the tailbud forms relatively quickly after gastrulation, the
tailbud of the chick embryo forms much later, after the node has completed its apparent regression along the streak. This point marks the transition from primary to secondary body development and the development of the tail. This transition is particularly evident in the neural tube, which forms through a process of folding at cranial and trunk levels and by cavitation in the tail\(^3\) [34]. Experiments in the chick determined that the transition occurs at the lumbosacral level and that secondary neural tube formation is independent of the more anterior tissue [34][33]. As is discussed in further detail below and in the introduction to Chapter 5 (Gastruloids as a Source of Axial Progenitors), the chick tail bud contains a population of self-renewing axial progenitors that contribute to both the paraxial mesoderm and the neural tube of the tail [111]. At the earlier stages of trunk development, an equivalent population is localised in the epiblast at the caudal border between the node and the primitive streak [26].

Axial development in the mouse embryo is qualitatively similar to that in the chick and is discussed in more detail at the level of the trunk and the tail in Chapter 5 (Gastruloids as a Source of Axial Progenitors). At the end of gastrulation there is already some degree of segregation in the mesoderm due to the cup-shaped geometry of the gastrula [148] (illustrated in Chapter 4, Fig. 4.2). At this stage, the streak is at its full extent and has formed the node distally; the first ingressed mesoderm of the heart and head has migrated across the lateral aspects of the cup and resides underneath the prospective anterior tissues [158]. The axial mesendoderm of the node extends anteriorly to underlie the forming tissues of the brain (reviewed in [3]). The mesodermal derivatives of the anterior half of the embryo therefore correspond approximately to those of the head. The later-ingressed mesoderm resides in the posterior half of the cup and will form axial and paraxial derivatives. The node begins its apparent regression through the elongation of the

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\(^3\)It is emphasised that secondary neurulation occurs in many vertebrate groups including in frogs and teleost fishes (reviewed in [71]).
notochord and convergence extension of the prospective paraxial mesoderm from the posterior. These movements account for the formation of the first six somites of the upper trunk. Once the node has started its apparent regression, the epiblast caudal and lateral to the node in the anterior primitive streak contributes to both the forming neural tube and paraxial mesoderm from a population of neuro-mesodermal progenitors (NMps), as in the chick [22]. The posterior neuropore closes once the node has reached the posterior limit of the streak and tail development proceeds from a population of NMps that are now localised in the chordoneural hinge (CNH) of the tailbud [21] (see Chapter 4, Fig. 4.4 and Chapter 5, Fig. 5.2).

To summarise, while the development of the Xenopus embryo occurs primarily through cell re-arrangements, amniote development relies instead on the proliferation of resident progenitor populations to form the trunk and tail (see Fig. 1.3). A common feature of all three systems is that the rostral mesoderm and neural tube arise from precursors that formed during gastrulation. In Xenopus, this process also accounts for the tissues of the trunk, through continued convergence extension movements. In the chicken and mouse embryos, the trunk tissues arise progressively as the node apparently regresses along the primitive streak. Convergence extension movements are important in elongating the primitive streak in the chick [148], but this corresponds to an effectively earlier stage of Xenopus development. Tail formation in all three species is similar at the morphological level as the progressive emergence of axial derivatives from the tail bud. While there is evidence for multipotent axial progenitors in the chordo-neural hinge of all three species, it is only in amniotes that they are thought to self-renew (see below). These differences are also reflected in the size of the newly formed somites, which become progressively smaller at more caudal levels in the Xenopus embryo but increase in size in the mouse and chicken [160].

At a conceptual level, it is important to consider the differences between the development of these apparently homologous structures in different model
Figure 1.3: Posterior Elongation is Predicted to Occur Primarily Through Cell Re-Arrangement in Externally Developing Vertebrates and Through the Proliferation of Neuro-Mesodermal Progenitors in Those That Develop Internally. Mechanisms of posterior growth are predicted from measurements of the length of the embryo and the posterior tissue volume [153]. The lamprey and zebrafish embryo increase in length with little change in posterior tissue volume, indicating that the elongation occurs primarily through re-arrangement of pre-existing tissue (black). The mouse and dogfish embryo increase in volume at the start of posterior elongation, suggesting a mechanism of cell proliferation from a self-renewing population of NMps. The development of the dogfish is effectively internal due to a rich yolk supply within a hard case. Externally developing organisms therefore rely primarily on cell re-arrangement during posterior growth while those that develop with a rich nutrient supply may rely on pools of NMps. The blue asterisk indicates an increase in posterior tissue volume in the zebrafish embryo at the final stages of tail development (Ben Steventon, personal communication). Phylogeny derived from [167], colours added by the author.
organisms since the results have implications for theories of germ-layer formation and the patterning of the nervous system. The presence of multipotent progenitors in the caudal lateral epiblast and the tailbud shows that some cells remain uncommitted to a particular germ layer after gastrulation until the end of tail elongation. The contribution of self-renewing, bipotent axial progenitors to extensive portions of the paraxial mesoderm and posterior neural tube in amniotes is not unlike that of the neural crest, which has been described as “the fourth germ layer” [69]. NMps therefore support calls to revise the concept of germ layers to correctly account for specific neural and mesodermal derivatives in the vertebrate embryo. While the development of the most caudal structures from the tailbud corresponds to Holmdahl’s description of secondary body development (reviewed in [71]), the vertebrate tailbud is not a homogenous blastema but in fact is highly structured at the histological and cellular level. This is illustrated by the chorodoneural hinge, a histological structure conserved from frogs to mice.\footnote{The CNH has been used to support the competing argument that the tailbud represents a continuation of the gastrulation process due to its descent from the dorsal blastopore lip [63] (reviewed in [71]).} The small scale photo-labelling used by Davis et al. demonstrates that this region is highly mosaic in \textit{Xenopus} since groups of fewer than 10 cells can contribute to all the developing axial tissues [36]. Finally, the progressive formation of the posterior nervous system in amniotes has implications for models of antero-posterior neural patterning. The activation-stabilisation-transformation model [150] (reviewed [55][151]) suggests that the spinal cord develops from the posteriorisation of the neural plate. While this may hold for the \textit{Xenopus} embryo, in which most of the tissue of the spinal cord is formed during gastrulation (and is therefore available for patterning), this cannot be extended to avian or mammalian embryos in which the tissue develops progressively. The presence of NMps in these organisms therefore calls for revisions to current models of neural patterning.
These differences in posterior growth may reflect different adaptations to the embryonic nutrient supply, as has been described for the process of symmetry breaking. In the case of *Xenopus*, cell re-arrangement may be a derived adaptation to the restricted energy supply of the yolk and the need to form a swimming, feeding tadpole just three days after fertilisation [63]. The following section reviews the evidence that the progressive posterior elongation of the amniote embryo may in fact resemble the ancestral state of axial growth.

Posterior Elongation is Conserved Across the Bilateria

In reviewing posterior elongation in the Bilateria, Martin and Kimelman describe how this pattern of growth is shared across the three major bilaterian clades (see Figure 1.4) and appears to be homologous, through the common regulation of *Caudal* expression by Wnt signalling [107]. They cite the examples of *Tribolium* (an insect) and the common house spider (a chelicerate) alongside the more familiar vertebrate examples of the zebrafish and the mouse (coloured green in Figure 1.4). Disrupting the function of either Wnt signalling or *Caudal* produces posterior truncation phenotypes in these organisms. Given this apparent homology, it appears that posterior elongation reflects an ancestral mode of body development. It is possible that long germ band insects such as *Drosophila* and amphibians such as *Xenopus* reflect more derived forms that have adapted to a rapid, external mode of development. In *Drosophila*, the body segments form simultaneously from an existing pool of ectoderm and mesoderm. *Xenopus* bears some similarity in the allocation of cells to the developing trunk and tail from precursors that were specified during gastrulation (reviewed in [71]). These organisms form attractive models for studying embryogenesis due to the fact that all axial levels are essentially accounted for by the end of gastrulation but they do not reflect the ancestral mode of development.

Martin and Kimelman identify an autoregulatory loop between Wnt signalling and *Brachyury* as a vertebrate-specific innovation that maintains the
Figure 1.4: Posterior Growth from a Wnt/Caudal-Expressing Growth Zone is Conserved Across the Major Bilaterian Clades. This phylogeny is reprinted from [107] and is coloured according to the presence (black, blue or green) or absence (red) of a posterior growth zone in developing bilaterian embryos. Clades that include one or more species with a posterior growth zone that expresses Wnt and Caudal are coloured blue; where there is functional data showing that these genes regulate posterior growth the clade is coloured green. The presence of a growth zone in the Lophotrochozoa, Ecdysozoa and Deuterostomia suggests that it is the ancestral mode of posterior development, with red clades representing examples of secondary loss of this mechanism.
identity of the posterior growth zone during elongation. In the *Xenopus* tail bud, a mosaic population of cells that were present during gastrulation become progressively recruited into the developing trunk and tail through a similar process of cell migration (reviewed in [71]). In this example, the tissues of the body essentially form from a pre-existing pool of tissue through extensive cell re-arrangement that elongates the body axis but does not increase the volume of the embryo through growth. The elongation of the zebrafish body is similar since it initially involves the recruitment and re-arrangement of lateral tissue, before an increase in the volume of the spinal cord and notochord during the final stages of the process [153].

The final stage of posterior elongation in the zebrafish appears to rely on a population of bipotent neuro-mesodermal progenitors that can contribute to both the paraxial mesoderm and spinal cord, as identified through single cell transplantations [108]. The capacity for these progenitors in the zebrafish to self-renew has not been experimentally determined, but given the late increase in the tissue volume of the tail, it would seem as if the proliferation of this population accounts for a relatively short extent of the body axis. The current model for the behaviour of this population is therefore one in which it proliferates and is expended during the final stages of tail development [153].

Unlike NMps in the zebrafish, clonal analyses in the mouse have not only demonstrated a common origin for the posterior neural tissues and paraxial mesoderm [175] but have also shown that the population contributes to these tissues at all post-occipital axial levels (see [110] for the spinal cord and [118] for the myotome). Transplantation experiments have shown that these cells reside in homologous regions of the mouse and chick embryo that can repeatedly colonise the axial tissues on serial transplantation (for the mouse, see [21]; for the chick, see [111]). It therefore seems as if the NMP population in amniotes is active at earlier stages of posterior elongation than in anamniotes; indeed the unsegmented region of the body undergoes an ini-
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The use of *in vitro* cultures of cells derived from the early embryo, namely embryonal carcinoma (EC) and embryonic stem (ES) cells, to study developmental processes. These cells can be grown as two-dimensional cultures or as three-dimensional aggregates and in both cases they can be directed towards specific cell fates through the exogenous modulation of cell signalling. In reviewing the use of cell cultures to mimic specific tissues and processes, it becomes clear that they provide an alternative perspective on the development of the embryo and are therefore useful models.
Figure 1.5: **Mouse Embryonic Stem Cell Systems Provide A Route to Studying Development *in vitro***. ESCs derived from the blastocyst can be used to produce 3D, *in vitro* cultures such as gastruloids, organoids and EBs. Grey dashed lines indicate attempts to “close the loop” from these cultures by using them to study development *in vitro*; gastruloids can recreate the events of early post-implantation development (cyan dashed arrow; see Chapter 4) while organoids represent short phases in the development of specific tissues (black dashed line). The introduction of a blastocyst, ESCs or EBs to an ectopic site can give rise to a teratoma, which is of historical importance in the derivation of ECCs and ESCs. This schematic was derived in part from one presented in [49]; see text for references to specific stages.
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1.2.1 Stem Cell Systems

Mouse ESCs are derived from the inner cell mass of the mouse blastocyst [49][109] and provide a useful experimental tool for studying mouse development in vitro (Fig. 1.5). They can be maintained indefinitely in culture and can contribute to all the tissues of the embryo and the extraembryonic tissues, as shown by the high level of chimaerism that results from their injection into host blastocysts [17] [9]. Their pluripotency is also apparent from their ability to form teratomas containing all three germ layers on injection into immunocompromised adult mice [109]. Consistent with these observations in vivo, ESCs can differentiate into derivatives of all three germ layers in vitro, including neurons, endoderm and cartilage [109] as well as the blood [181] and beating myocardial tissue [41]. In these cases, the ESCs were allowed to form aggregates of cells known as embryoid bodies in feeder-free or suspension cultures. Embryoid bodies have been a popular experimental tool for studying development thanks to the variety of differentiated cell types that can be derived in this way [39]. The differentiation of EBs is, however, a disordered process [135] that is not co-ordinated at an individual level; i.e. it is not organised according to a set of body axes. While one report describes the formation of a pole of Wnt signalling activity and Brachyury-GFP expression [163], it is not accompanied by an axial elongation of the kind that has previously been described for aggregates of EC cells [106].

ESC cultures also have been used in a directed manner to form specific tissue types through the addition of exogenous signalling factors or small molecule inhibitors. While some of these culture regimes have been developed empirically, others have taken their logic from the tissue interactions in the embryo itself as a form of “translational embryology” reviewed in [145] and [114]. Spence & Wells review the generation of pancreatic endocrine cells from ES cells, through a series of treatments that promote differentiation into

ECCs can be used to make chimaeras in the same way, but unlike ESCs they often give rise to tumours pre- or post-natally [17].
progressively more specialised endodermal derivatives. During the course of normal embryonic development, the pancreas emerges as an outpouching of the foregut in response to signals from the overlying mesoderm. The spatial component to these interactions is lost when deriving these tissues from ESCs in vitro, so the differentiation protocols instead rely on a temporal series of treatments. It is not as straightforward, however, as knowing which signals are present in the embryo and when, because cell fate decisions can be made in response to different doses of the same signal, for example. Spence and Wells highlight the case of Nodal signalling, which at high levels can promote differentiation into definitive endoderm but at low levels promotes mesodermal differentiation [145]. This problem is also identified by Murry & Keller, who in reviewing protocols for differentiating ES cells into cardiomyocytes in vitro highlight a biphasic response to Wnt/β-catenin signalling. Prior to primitive streak formation, Wnt signalling is procardiac but continued exposure to Wnt promotes differentiation into other mesodermal derivatives, indirectly inhibiting cardiac differentiation [114]. In addition, exogenous factors do not specifically suppress other cell types in the population so successive treatments are likely to be directing an ever-shrinking population towards the desired cell type. In the final stages of such a protocol, it is likely that cell-cell interactions become more important in developing the diversity of cell types that make up a functional tissue. At this point, the non-specific nature of exogenous treatments is likely to impede this diversification.

It is also worth noting that adherent ESC cultures can be used to model specific processes, such as the activation of Brachyury expression in the newly forming primitive streak. Turner et al. describe how exogenous stimulation of the Nodal/TGFβ and Wnt/β-catenin pathways triggers a pattern of cell behaviours that is consistent with accounts of the nascent mesendoderm in the primitive streak [173]. Cells stimulated in this way undergo an EMT-like event that is signified by Brachyury expression, loss of E-Cadherin from the membrane and an increase in cell motility that allows cells to move over a
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secreted layer of Fibronectin [173]. ESC cultures provide a degree of control over the exogenous signalling environment and are amenable to single cell and molecular analyses. By integrating different exogenous treatments with genetic changes to the ESCs, these authors could separate specific functions of Activin/Nodal and Wnt/β-catenin signals in regulating the initiation of the EMT and they describe how Brachyury seems to regulate the velocity of the migratory cells [173]. This study therefore exemplifies how the controlled environment of in vitro cell cultures can be used to provide new, precise insights on a specific developmental process which would otherwise be difficult to achieve by studying the embryo directly.

In summary, stem cell systems can be used to study specific tissues and developmental processes in vitro. The logic behind differentiation regimes is provided by a knowledge of the signalling interactions in development, but the complex relationship between signals, space and time makes directing differentiation a challenging test of our understanding. Nevertheless, in vitro differentiation allows the dynamics of signalling and tissue interactions to be observed in a controlled manner, which cannot be achieved in the embryo. The results of these observations and the empirical optimisation of differentiation protocols can be informative about developmental interactions that may previously have been overlooked and can be validated through re-examination of the embryo. This rationale has been extended by three dimensional cultures of stem cells known as organoids.

1.2.2 Organoid Cultures

Organoids are three dimensional cultures of stem cells that mimic the architecture and function of foetal or adult tissues in vitro\(^6\). A key feature of

\(^6\)The axially organised aggregates of ten Berge et al. [163], Marikawa et al. [106] and the gastruloid system presented in this thesis can be considered to be “embryonic organoids,” since they mimic the axial organisation of the embryo rather than a specific tissue or organ.
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organoid development is that they undergo three dimensional tissue morphogenesis, which cannot be recreated in two-dimensional cultures of adherent cells. In forming organoids from pluripotent stem cells, specific tissues can be produced by the same logic as used in directed differentiation approaches, namely exposing the cells to the same sequence of signals that would normally be present in the developing embryo. One example is the generation of organoids containing the sensory epithelium of the inner ear by directing embryonic stem cells through a succession of ectodermal lineages to form otic vesicle-like tissues [93]. Other examples of tissues generated from pluripotent stem cells include the stomach, intestine, liver, thyroid, lung, optic cup, cerebral cortex and pituitary gland (reviewed in [82] and [170]). Alternatively, organoid culture can be used to expand populations of adult stem cells, which can re-construct the stem cell niche and the architecture of the adult tissue when grown in this way. This approach has been used to generate in vitro cultures of mammary gland, liver, pancreas, intestine, tongue, lung, stomach and prostate gland tissue (reviewed in [82] and [170]).

A common feature of many organoid cultures is the use of Matrigel®, a mixture of extracellular matrix proteins secreted by the Engelbreth-Holm-Swarm sarcoma cell line. Encapsulating the cells within this matrix not only provides them with a three-dimensional support in which to grow but it may also substitute biochemically for the cell-cell contact with the mesenchyme that would normally neighbour the developing organ. It is apparent from the lists of tissues above that many organoids are endodermal or ectodermal tissues that are almost exclusively grown without mesodermal derivatives. The physical properties of the matrix may therefore be more important for the developing tissues than its biochemical activity. This form of minimal culture provides new insights on development by allowing the sufficient components of a given process to be identified. One example is in the formation of optic cup organoids, which undergo autonomous morphogenesis in the absence of the surrounding tissues that have been suggested to instruct this process in
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the embryo [46] (reviewed in [137] and below). This level of reductionism could never be achieved in the complex physical and biochemical microenvironment of the embryo. Huch and Koo caution, however, that reciprocal tissue interactions between the organ and the surrounding mesoderm cannot be studied in these systems and that this may limit their fidelity in recreating normal development. Liver organoids derived from adult stem cells, for example, only generate the endodermal derivatives and not the nearby mesoderm that is thought to be required in normal organogenesis [82].

Organoid culture is therefore a key experimental tool for studying the development and morphogenesis of these tissues in vitro, where the extracellular environment can be carefully controlled and genetic engineering can be used to manipulate cell-cell interactions within the tissues. This technique has also enabled the mechanisms of human organogenesis to be studied experimentally for the first time through the directed differentiation of human pluripotent stem cells (see editorial by Little [103]). Little also highlights the key challenge for organoid systems, namely whether they provide a sufficiently accurate model of normal development to provide new insights on general developmental principles [103]. The way to address this challenge will be to use organoids to generate testable predictions about development that can be explored in vivo. Negative results from these experiments still have value, however, in identifying components of the in vivo microenvironment that are necessary for a particular developmental process.

Collectively, two and three dimensional cultures of stem cells can provide new insights on the mechanisms that generate specific tissues and organs in the embryo. A fascinating aspect of organoid biology is their ability to generate tissue pattern and form spontaneously, outside of the context of the embryo. The following section describes how organoid development relates to our understanding of the mechanisms of tissue patterning and how processes that are closely associated in vivo can be uncoupled in vitro.
1.3 Mechanisms of Developmental Pattern- ing

In the period of development following gastrulation, the embryonic body plan is refined through the differentiation of the germ layers into specific tissues and organ primordia. Tissue pattern can be defined as the spatial component of this differentiation. This section reviews first the Wolpertian model of positional information as a means of achieving tissue patterning in relation to extrinsic cues and secondly self-organising systems such as reaction-diffusion models that can generate pattern intrinsically.

1.3.1 Morphogens and Positional Information

The most well-known conception of Wolpertian positional information is the French Flag Model, in which cells in a one dimensional field adopt three different fates in response to a gradient of an external signal across the field [185] (Fig. 1.6A). The cells respond to the signal in a concentration dependent manner: if the concentration “seen” by the cell is above a given threshold, it will adopt one fate; if it is below this threshold but above a second it will adopt the second fate and if it is lower than either threshold it will adopt the third fate. The signal, in a term coined by Alan Turing [168], is a morphogen as it elicits a concentration-dependent response and so provides the “positional information” required to pattern the field of cells.

1.3.2 Alternative Sources of Positional Information

It is clear, however, that developing systems do not rely exclusively on morphogens to generate spatial patterning, since cells can derive further information from a signal than simply its absolute concentration. One example is the patterning of the vertebrate neural tube, which is achieved through the integration of signals over time [95][136]. In these cases, information from external signals is decoded by a gene regulatory network that can measure
Figure 1.6: Tissue Patterning through Extrinsic and Intrinsic Sources of Positional Information. Cells within a field can derive positional information from an external source such as a morphogen (A), which triggers different cellular responses according to its concentration. Cells exposed to a concentration of morphogen above c1 adopt the green response, while those exposed to a concentration between c1 and c2 exhibit the red response. Cells that are exposed to a concentration that is beneath both thresholds show the blue response. Positional information can also be generated spontaneously through a reaction-diffusion system (B). Cells in the field secrete a slowly diffusing activatory molecule (green in B(c)) that can activate its own expression and a rapidly diffusing inhibitor (red in B(c)) that inhibits production of the activator. Random fluctuations in the expression of both molecules produces regions in which the local concentration of the activator exceeds that of the inhibitor, producing a local peak of expression (B(a)ii). This peak becomes self-reinforcing, since the inhibitor diffuses away from the peak more quickly than the activator. Additional peaks can form at a distance, where the levels of the inhibitor and activator are closer to the starting state (B(a)ii and (b)). The wavelength of the pattern is dependent on the boundaries of the tissue and the biochemical properties of the patterning molecules. This figure is reprinted directly from [170], Fig. 1.
features such as concentration (a classic French Flag Model) or exposure (in the vertebrate neural tube).

Spatial cues for tissue patterning do not necessarily have to come from an external source, however. Reaction-diffusion (R-D) systems such as those proposed by Turing [168] and Gierer and Meinhardt [59] are capable of producing emergent patterns through the spontaneous self-organisation of initially homogeneous parts (Fig. 1.6). In the case of a Turing system, these patterns arise through the interactions of a slowly-diffusing activatory molecule that can activate its own production (or reaction) and that of its partner, a rapidly-diffusing inhibitory molecule that inhibits this production (or reaction). To contextualise this within a biological system, both molecules can be considered to be diffusible extracellular factors produced by cells within a field. Random fluctuations in the production of both factors will result in regions in which the concentration of the activator exceeds that of the inhibitor. Here, the auto-activation of the activatory molecule will increase its local concentration, along with that of the inhibitor. This activation is not checked by the inhibitor, however, since it diffuses more quickly than the activator and so cannot accumulate. The result is a peak of expression in a group of cells, surrounded by groups in which the expression of the activator is repressed. Across the field as a whole, this produces a regular pattern from an initially homogeneous starting state, the exact nature of which is dictated by the size and shape of the tissue as well as the biochemical properties of the interacting molecules. This form of patterning is not necessarily incompatible with morphogen-mediated positional information, however. Green and Sharpe suggest three possible configurations of the two systems: R-D upstream of a morphogen gradient (i.e. only cells in the activated peak secrete a morphogen), R-D alongside a morphogen gradient, where the two systems pattern different features of the same field (such as the position and identity of the digits) and R-D downstream of a morphogen gradient, where the different regions of the “flag” differently tune the pattern produced by
1.3. MECHANISMS OF DEVELOPMENTAL PATTERNING

reaction-diffusion [66].

1.3.3 Genetically Encoded Self-Assembly

Organoids are challenging the established paradigm of Wolpertian positional information since they develop organised tissues in a spatially homogeneous external signalling environment. Organoid development is often referred to imprecisely as a “self-organising” process; this term, however, carries implications of self-organisation in the physical sense such as the reaction-diffusion system described above. In reviewing the organoid field, we made the distinction between self-organisation and self-assembly and contend that the majority of observable organoid development is in fact a process of self-assembly [170]. We define self-organisation as “the spontaneous emergence of order or asymmetry from an initially homogeneous starting population in an energy-dependent manner;” self-assembly is defined as “the formation of an ordered structure from non-equivalent parts as a system moves towards equilibrium [170].” To define these terms more generally in the context of construction (e.g. of a tissue), self-organisation requires a degree of upkeep to define its different parts, while self-assembly is the passive sorting of already distinctive parts. It is important to distinguish between the two since they reflect different processes in organoid development, namely the origin of a spatial pattern or the response to it.

We contend that many organoid systems undergo an initial phase of self-organisation that defines positions or regions within an initially homogeneous group of cells. This informational step is followed by changes in gene expression that make cells within the region different from each other and from the rest of the organoid. A phase of self-assembly now realises these differences morphologically in changing the shape of the tissue. In the case of the eye cup organoids [46], the self-organising stage demarcates the prospective primordium that will subsequently express Rx, a marker of the neural retina, at the optic vesicle stage. The tissue is regarded as self-assembling as by
the time the optic vesicle has formed, its components have already become non-equivalent. The process continues as the eye cup undergoes its characteristic morphogenesis. The formation of an intestinal organoid from a group of adult cells is by definition exclusively self-assembly, since the starting population of cells is not homogeneous. The association between unequal cells in the forming organoid provides the sufficient spatial cues for assembling the tissue into the familiar crypt structure. Self-organisation is uncommon in these cases where the starting population of cells is not homogeneous. It is worth noting that the dynamic heterogeneities in a population of ES cells may provide a substrate for developing pattern through self-organisation but they can be considered homogeneous in relation to their undifferentiated state at this stage. In summary, we propose that organoids develop through a process of genetically supervised self-assembly, where genetic programs encode the elements for emergent patterning through self-organisation before the subsequent self-assembly of the tissues [170].

1.3.4 The Self-Organisation of Morphogenetic Fields

The emergence of a tissue primordium within an initially uniform organoid is reminiscent of the precisely defined regions of the embryo that give rise to particular tissues, organs and appendages, known as “morphogenetic (or gradient) fields.” Field theory was developed in the early 20th Century by Gurwitsch, Spemann and Weiss, but it suffered a loss of popularity from the 1940s, possibly due to its abstract nature that was beyond the reach of experimental methods at the time (reviewed [37]). Gurwitsch’s conception of a field was more similar to the physical meaning, as an overall (often mechanical) feature of a tissue generated by its constituent cells (but not restricted to them) as the ordering principle behind morphogenesis (reviewed in [13]). By understanding the rules governing a particular field, Gurwitsch could predict the eventual form of the tissue. Belussov cautions, however, that while these predictions may have been formally correct, the underlying mechanisms are likely to be quite different (and possibly more simple) [13].
Spemann’s conception of the morphogenetic field originated from his work in defining the organiser as a “field of organisation” (reviewed in [13]). Paul Weiss formalised the concept of a field in the 1930s to include the following properties: 1) “When the mass of a field is reduced, the structure of the field is not affected”; 2) “the splitting of a field district leaves each half in possession of a complete proportionate field equivalent in structure to the original single field” and 3) “the fusion of two field districts can produce results based on the orientation of their axis” (reviewed in [13]). Weiss’ formalisation was no doubt informed by Harrison’s earlier work on the newt forelimb field, which showed that it could regulate to form an apparently normal forelimb after the removal of mesoderm from the centre of the field. Additionally, a divided forelimb field can give rise to two identical forelimbs on transplantation and two halved fields grafted together in the correct orientation give rise to an outwardly normal limb (reviewed [37]). Around the same time, Guenonot demonstrated that surgery of the newt branchial nerve could be used to induce the forelimb field or dorsal crest field (or correspondingly the sciatic nerve for the hindlimb or tail fields) into producing ectopic structures in the adult (reviewed [37]). Collectively, these historical findings define inducible regions of the embryo that can regulate for changes in their size or geometry and can undergo autonomous morphogenesis.

Evidence for the regulation of these fields suggests that cells within the field can in some way sense their position in the tissue as a whole and it is likely to be the same mechanism that restricts the size of these fields to their precise dimensions in the embryo. Given the ability of explanted or divided fields to develop in ectopic sites in the embryo, it is unlikely that this information is provided by an external signal; it is probably generated instead from within the field. This intrinsic regulation may include the establishment of morphogenetic signalling centres but it is likely that the underlying mechanism is in some way self-organising. There are clear parallels with the formation of organ primordia in organoids, which will now be discussed in
further detail from the perspective of field theory.

**Organoid Development is Locally Autonomous**

Sasai *et al.* review the autonomous development of eye cup, pituitary and intestinal organoids in the absence of spatially structured external signalling [137]. As mentioned above, the eye cup undergoes a characteristic progression of changes in morphology, starting with ectodermal evagination as a vesicle, followed by distal invagination to form a cup. The authors note how the size of the resulting cups appears to be species dependent, consistent with the respective sizes of the eye primordia in the mouse and human embryos [137], suggesting that the size of the structure as a whole is regulated. The morphogenesis of the optic cup field can be modelled using a set of simple rules (see below) in a manner not dissimilar to Gurwitsch’s theoretical models of shark brain morphogenesis (reviewed in [13]).

The example of pituitary organoids is more informative about field regulation. In the embryo, a single pouch forms through the interaction of the oral ectoderm and hypothalamic neuroectoderm. The number of placodes seems to be under tissue level regulation, since *Vax1* knockout mice develop a complete ectopic Rathke’s pouch (reviewed in [137]). Within the organoids, the pituitary placodes form randomly at points of contact between the ectoderm and the neurectoderm, but they appear to be regulated as they have a consistent size and shape. It is likely that the same mechanism that regulates the embryonic field (that is dysregulated in the *Vax1* knockout) also maintains the organised structure of these tissues in the organoids.

**Organoids Reflect Intrinsic Developmental Programs**

As mentioned above, the morphogenesis of eye cup organoids seems to obey a simple set of rules in the mouse, with an additional rule required for human cells ([46] [45]). The following rules can be considered to be steps in a simple morphogenetic program that sculpts the eye cup. The first step of
invagination is to increase the flexibility of the distal vesicular epithelium. Next, the cells in the hinge region undergo apical constriction, deflecting the distal tissue (prospective neural retina) into a concave epithelium. Thirdly, the neural retina expands tangentially within the overlying retinal pigmented epithelium, causing it to invaginate and so form the cup. Sasai et al. summarise this as a “simple internal program involving local mechanical changes [that is] sufficient to create characteristic epithelial deformation without external forces” [137]. This model is compelling in its simplicity since it does not invoke any external physical or biochemical factors and it clearly describes how a morphogenetic field can autonomously change shape according to internal rules.

Organoid Development is Regulative

The regulative nature of embryonic fields has not been explored in detail in organoid systems, but intestinal organoids provide an anecdotal example that is relevant to intestinal repair (reviewed in [82]). In organoids formed from primary tissue, it is routine to passage the culture every few days to maintain the organoids as small, non-necrotic tissues. In doing so, they are triturated into crypts and crypt fragments, which subsequently re-grow into complete tissues surrounding a central organoid lumen. This pattern of growth suggests that the disrupted crypts have the capacity to reform the missing portion of the organoids and may indicate an overall regulation of the tissue as a whole. This would be consistent with the behaviour of divided embryonic fields, albeit on a much smaller scale. In intestinal organoids derived from single cells, the progressive formation of morphologically consistent crypt structures suggests the presence of tissue level regulation from the earliest stages of growth.

In summary, the development of organoids and the regulation of their tissue architecture suggests that they form morphogenetic fields specific to their tissue architecture. Eiraku et al. have shown for the example of the eye cup
that the *in vitro* morphogenesis of this tissue bears very close similarity to the process *in vivo* and, importantly, that it can be explained by a simple set of internal rules. In considering the balance between self-organisation and self-assembly, the formation of the initial morphogenetic field may be a self-organising process in an initially equivalent group of cells, which is followed by their self-assembly to enact morphogenesis. Indeed, the plasticity of the self-organising state and its dependence on the size and shape of the tissue corresponds to the regulation of embryonic fields before apparent morphogenesis has begun.

### 1.3.5 Developmental Patterning through Permissive Self-Organisation

Sasai *et al.* describe how the eye cup organoids form from an intrinsic program that is independent of the surface ectoderm and periocular mesenchyme that were thought to regulate morphogenesis *in vivo*. They reconcile these findings with the lack of ectopic eye tissue in the embryo by suggesting a permissive model for optic cup formation. They suggest that the minimal physical and biochemical environment of organoid culture allows the intrinsic program of eye cup formation to run undisturbed, producing self-organised eye tissues without external instruction. They contend that the underlying network controlling this program could be sensitive to perturbation and it follows that the complex microenvironment of the embryo disrupts this network and so prevents the formation of ectopic eyes. Within the embryonic head, however, the co-ordinated development and interaction of the tissues produces an environment that is conducive to eye formation (reviewed in [137]). The *Drosophila* embryo provides two examples of permissive self-organisation in the formation of ectopic eyes [68] and wings [92] through the ectopic expression of *Eyeless/Pax6* and *vestigial* respectively in the imaginal discs. In both cases, the ectopic tissues form self-contained structures within a larger domain of expression, suggesting that they only emerge in regions where the combination of signals and mechanical inputs is conducive to their
To relate these observations to the summary of organoid patterning, perhaps it is the initial state of self-organisation that is sensitive to disruption, thereby preventing the formation of eye-fields in non-permissive environments. An appealing feature of this model is its apparent robustness, since it divides the developing embryo into smaller, autonomous interacting units that are responsible for their own development rather than being dependent on a set of extrinsic cues. In such a model, the embryo as a whole would be more resilient to injury or developmental defects than for a model in which cell fates are dependent on inductive interactions between tissues.

1.3.6 A Deconstructed View of Development

This chapter began by considering the range of different solutions that are employed by different organisms to achieve the processes of symmetry-breaking, gastrulation and axial elongation. Each process is likely to be adapted to the life history of the organism in question and collectively they show the diversity of mechanisms that can occur during development. In the case of the mammalian embryo, in vitro systems using cultures of stem cells have provided an alternative experimental route to uncovering how these mechanisms operate. Organoids in particular draw attention to the autonomy and regulation of embryonic patterning through their ability to recreate foetal or adult tissue architectures in vitro. These observations invite a deconstructed view of embryonic development when they are related back to the embryo, specifically one in which groups of tissues are separable parts that form according to internally encoded rules, in positions that are permissive to morphogenesis. To some extent, the mammalian embryo at organogenesis stages may develop from units that are akin to the imaginal discs of Drosophila, though they may not be physically discrete.
The question therefore arises as to whether the earlier processes of symmetry breaking and gastrulation can be recreated in vitro in an organoid system. Indeed, the morphogenesis of the eye cup has superficial similarity to the tissue movements of gastrulation in species with a blastopore. When the range of different mechanisms that are used by different organisms is considered alongside the regulative nature of the early amniote embryo, it is plausible that the regulatory programs controlling these processes may be accessible to in vitro experimentation. In the same way that organoid systems have prompted re-assessments of embryonic patterning, an “embryonic organoid” system would provide a new experimental platform from which to study these fundamental processes of embryonic development.

1.4 Thesis Overview

1.4.1 Hypothesis

ESCs have been used as an in vitro model system for development by studying their differentiation in monolayers or as three-dimensional embryoid bodies. Given the results of Marikawa et al. using aggregated ECCs to model axial elongation [106], we hypothesised that a similar level of morphogenesis could be achieved from cultures of ES cells. ten Berge et al. had reported polarised expression of Wnt signalling activity and Brachyury-GFP aggregates of ESCs [163]. The key distinction between the EC and ES cell aggregates was the starting number of cells, which was five times higher in the ten Berge et al. study. We therefore sought to derive smaller ES cell aggregates using the technique described by Marikawa et al. By doing so, we hoped to address the gap in the organoid field for an ES-cell derived model of individual embryonic development and axial elongation morphogenesis. Such a system could be used to study the properties of the murine neuro-mesodermal progenitor (NMp) population that is otherwise difficult to access in the embryo.
1.4.2 Aims & Scope of the Project

The aims of the project were as follows:

- To establish an *in vitro* model system of axial elongation morphogenesis from ES cells;
- To determine whether the tissues contain a candidate population of NMps;
- If present, to determine the properties of these NMps in comparison to those derived from 2D-cultures.

The scope of this project covers the capacity of mouse ES cells to recreate normal embryogenesis when cultured in three-dimensions, specifically focussing on the axial tissues of the trunk and tail. As is discussed in further detail in Chapter 4, the three-dimensional cultures do not form patterned tissues of the anterior nervous system, so the details of neural induction and patterning anterior to the spinal cord are beyond the scope of this work. The research presented in this thesis includes possible ways to extend the stem cell cultures through bioengineering but this is limited to three dimensional cultures. The details of more advanced patterning systems and their potential applications to two-dimensional cultures are beyond the scope of this work.

The expected outcome of this work was a robust and reproducible experimental model for axial elongation morphogenesis that could be used to study the regulation of the NMp population *in vitro*. In combining this model with bioengineering techniques, such a system could see application in the derivation of differentiated axial tissues such as skeletal muscle and spinal cord tissue that have thus far been difficult to access from 2D cultures.
1.4.3 Thesis Structure

The following chapter presents the technical details behind the ES cell-derived “gastruloid”7 system that we have developed and the tools that have been used to study them. Chapter 3 describes the development of the gastruloid system and the progress towards optimising its robustness and reproducibility. Chapter 4 is a detailed survey of the symmetry breaking, germ layer formation and axial elongation events that occur in the gastruloids and it discusses how the development of this in vitro system relates to that of the embryo.

In considering how gastruloids can be used to study axial elongation, Chapter 5 presents the evidence for an NMp-like population in the gastruloid tissues that emerges during the elongation phase of their growth. Chapter 6 describes the development and application of an assay for candidate populations of NMps, through transplantation to the chicken embryo. Candidate NMp populations that were generated by different methods were tested by xenotransplantation and were compared to pluripotent stem cell cultures.

Chapter 7 explores how bioengineering techniques could be used to extend the duration and tissue complexity of the gastruloid cultures, potentially as a means of propagating a population of NMps. Finally, Chapter 8 discusses the main findings from this research and contextualises them with current research into organoid culture and the in vitro-derivation of NMps. It goes on to explore how the development of the gastruloids relates to that of the embryo and their contribution to the emerging field of synthetic embryology.

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7Defined as a self-organising, three-dimensional aggregate of embryonic stem cells; see Chapter 3.
Chapter 2

Materials & Methods

The video protocol published in 2015 [5] contains details of ES cell culture, immunohistochemistry and microscopy that are paraphrased below. The gastroduoid culture protocol is as published, with the amendment of performing the cell counting step (2.4 in [5]) after the two PBS washes (2.5 and 2.6 in [5]).

2.1 Mouse Embryonic Stem Cell Culture

2.1.1 Media & Supplements

ES cells were routinely cultured on 0.1% gelatin (Sigma-Aldrich G1890-100G) precoated 25cm² tissue culture flasks (Grenier Bio One 690 175). Self-renewing cultures were maintained in ES+LIF medium, prepared from 500mL Glasgow’s Minimal Essential Medium (GMEM; Gibco 11710-035), 5mL sodium pyruvate (Invitrogen 11360-039), 5mL non-essential amino acids (Gibco/Invitrogen 11140-035), 5mL GlutaMAX (Gibco 35050-038), 1mL β-mercaptoethanol (Gibco/Invitrogen 31350-010), 50mL foetal bovine serum (Biosera FB-1090/500), 550µL Leukaemia Inhibitory Factor (Recombinant LIF, 1000 Units, produced in-house by the Wellcome Trust – Medical Research Council Cambridge Stem Cell Institute). Cell cultures were maintained in humidified incubators at 37°C with 5% CO₂.
Culture media used for differentiation or for gastruloid culture were prepared in a base medium of N2B27 (NDiff227®; StemCells, Inc. SCS-SF-NB-02) with additional factors as detailed in subsection 2.1.3. Stocks were defrosted at 37°C and were stored overnight at 4°C to allow any precipitated solutes to redissolve before use.

2.1.2 Routine Cell Culture Protocol

Growth media were exchanged daily, with the cells passaged to new flasks every second day. They were detached enzymatically by incubation with 0.05% Trypsin-EDTA (Gibco 25300-054) followed by trituration with a P1000 micropipette to produce a suspension of single cells. The Trypsin was neutralised with ES+LIF and the suspension was centrifuged at 1000RPM (∼170 x g) for five minutes. Following aspiration of the supernatant, the pellet was resuspended in 1mL ES+LIF. A fraction of the suspension was used to seed a new flask; typical passaging ratios were in the range of 1:4-1:10, depending on the confluency of the starting culture. In one experiment (see Chapter 3), Accutase® (Thermo Fisher Scientific A1110501) was used for detaching and dissociating the cells instead of Trypsin; this is indicated in the text.

Frozen stocks were prepared by expanding existing cultures to ∼70% confluence in 75cm² flasks (Grenier Bio-One 658 175). Expanded cultures were passaged into freezing medium (10% vol/vol DMSO (Sigma-Aldrich D2438) in ES+LIF) and were frozen as 1mL aliquots in cryotubes (Thermo-Fisher Scientific, 5012-0012) at -80°C overnight. Stocks were transferred to -150°C for long term storage. Newly defrosted cell lines were passaged twice before experimental use.

2.1.3 Cytokines, Growth Factors & Inhibitors

Commonly used ligands and small molecule modulators of cellular signalling pathways are described in Table 2.4, below which has been reproduced
2.1. **MESC CULTURE**

from [171].

Table 2.1: **Commonly Used Ligands and Small Molecule Inhibitors**
All supplied by Tocris Biosciences, except FGF2 (R&D Systems). See also Table 2.2.

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Stock Concentration</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHIR99021</td>
<td>Chi or Chiron</td>
<td>10mM</td>
<td>3µM</td>
</tr>
<tr>
<td>FGF2</td>
<td></td>
<td>100µg/mL</td>
<td>10ng/mL</td>
</tr>
<tr>
<td>Dorsomorphin H1</td>
<td>DMH1</td>
<td>5mM</td>
<td>0.5nM</td>
</tr>
<tr>
<td>IWP-2</td>
<td>PIN</td>
<td>5mM</td>
<td>1µM</td>
</tr>
<tr>
<td>SB431542</td>
<td>SB43</td>
<td>100mM</td>
<td>10µM</td>
</tr>
<tr>
<td>PD0325901</td>
<td>PD03</td>
<td>10mM</td>
<td>1µM</td>
</tr>
<tr>
<td>XAV939</td>
<td>TIN</td>
<td>10mM</td>
<td>1µM</td>
</tr>
</tbody>
</table>

2.1.4 **Cell Lines**

Frequently used cell lines are summarised in Table 2.3, below.

2.1.5 **Gastruloid Culture Protocol**

A detailed account of the gastruloid culture protocol including troubleshooting advice has been published online [5]. In brief, a single cell suspension was prepared from an adherent cell culture by incubation with 0.05% Trypsin-EDTA and trituration with a P1000 micropipette as in subsection 2.1.2. The suspension was neutralised with ES+LIF medium and centrifuged for 4 minutes at ∼170xg. The supernatant was aspirated and the pellet was washed by the addition of 10mL Phosphate Buffered Saline (PBS, with calcium and magnesium, Sigma-Aldrich D8662) and centrifugation as above. This step was repeated before the pellet was resuspended in N2B27. The density of the cell suspension was determined manually using an Improved Neubauer Haemocytometer (Hawksley AS1000) or automatically using a Moxi Z automated counter (Orflo Technologies, MXZ001). A subset of cells was resuspended in N2B27 at a density of 7.5-10 cells/µL to produce 300-400 cell
### Table 2.2: Functions of Commonly Used Small Molecule Inhibitors

<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHIR99021 (CT 99021)</td>
<td>Agonist of canonical Wnt signalling. Specific inhibitor of GSK3, thereby stabilising cytoplasmic β-catenin.</td>
<td>[6]</td>
</tr>
<tr>
<td>Dorsomorphin H1</td>
<td>Used as an inhibitor of BMP receptor type I signalling via ALK2/3/6 inhibition. Also a non-specific inhibitor of AMPK.</td>
<td>[64] [6] [193]</td>
</tr>
<tr>
<td>IWP-2</td>
<td>Blocks Wnt secretion by inhibiting Porcn, which adds an essential palmitoyl group to the Wnt molecule. No effect on Notch or Shh signalling, nor on protein secretion in general.</td>
<td>[28]</td>
</tr>
<tr>
<td>SB431542</td>
<td>Inhibitor of Activin and TGF-β signalling. Specifically inhibits ALK4/5/7; does not affect BMP signalling. Weakly inhibits the MAPK p38α at 10µM.</td>
<td>[86]</td>
</tr>
<tr>
<td>PD0325901</td>
<td>Inhibitor of Erk signalling, downstream of FGF stimulation. Specific, non-competitive inhibitor of MEK1 &amp; MEK2, thereby inhibiting activation of ERK1/2.</td>
<td>[6]</td>
</tr>
<tr>
<td>XAV939</td>
<td>Antagonist of canonical Wnt signalling. Specifically inhibits Tankyrase 1 and 2, thereby stabilising Axin and promoting the degradation of cytoplasmic β-catenin.</td>
<td>[81]</td>
</tr>
</tbody>
</table>
2.1. MESC CULTURE

Table 2.3: Cell Lines Used In This Study

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E14TG2A</td>
<td>E14</td>
<td>[79]</td>
</tr>
<tr>
<td>Gata6&lt;sup&gt;H2B-Venus&lt;/sup&gt;/+; ColA1&lt;sup&gt;TetO-Gata4-mCherry&lt;/sup&gt;/+; R26&lt;sup&gt;M2rtTA&lt;/sup&gt;/+</td>
<td>Gata6&lt;sup&gt;H2B-Venus&lt;/sup&gt;</td>
<td>[56]</td>
</tr>
<tr>
<td>CAG::GFP-GPI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mir-290-mCherry/mir-302-eGFP</td>
<td>DRC</td>
<td>[122]</td>
</tr>
<tr>
<td>Sox1&lt;sup&gt;GFP&lt;/sup&gt;/+</td>
<td>Sox1::GFP</td>
<td>[191]</td>
</tr>
<tr>
<td>Sox17::GFP; Ubiquitin::Tomato</td>
<td>Sox17::GFP</td>
<td>[117]</td>
</tr>
<tr>
<td>Brachyury&lt;sup&gt;GFP&lt;/sup&gt;/+ (“GFP-Bry”)</td>
<td>T/Bra::GFP</td>
<td>[51]</td>
</tr>
<tr>
<td>Tbx6&lt;sup&gt;tm2Pa&lt;/sup&gt;/+ (Knock-in of H2B-EYFP)</td>
<td>Tbx6::eYFP</td>
<td>[67]</td>
</tr>
<tr>
<td>TCF/Lef::mCherry</td>
<td>TLC2</td>
<td>[52],[50]</td>
</tr>
</tbody>
</table>

aggregates, respectively. The suspension was plated as 40µL droplets in U-bottomed sterile non-tissue culture treated 96-well plates (Grenier Bio-One 650185) and was left for 48 hours. At the 48 hour time point, 150µL of secondary medium was added to each well, which was exchanged for an equal volume of tertiary medium 24 hours later. The culture medium was exchanged again at the 96 hour time point. In each case, a dead volume of 40µL remained in each well to prevent aspiration of the aggregates. The 120 hour time point was typically used as an endpoint as the aggregates would begin to grow adherently on the plastic beyond this time.

2.1.6 Hydrogel Cultures

Matrigel® Cultures

Extended cultures were achieved by embedding the aggregates in droplets of ice-cold Matrigel® (Phenol Red-free, Growth Factor Reduced; Corning 356231). 10-50µL droplets were micropipetted onto the surface of a 6-well plate () on ice; aggregates were transferred into these droplets by micropipetting in a small volume of N2B27. The droplets were solidified by incubation at 37°C (with 5% CO₂) for 25 minutes, before 3mL of warm N2B27 was added.
to each well. The culture medium was exchanged daily.

“Chapter 7: Physical Inputs to Gastruloid Culture” describes the use of chemically defined microenvironments as an alternative to Matrigel®<sup>®</sup>, that were produced by polymerising the hydrogels within microfabricated PDMS culture wells. Each step in this process is summarised below.

**Preparation of PDMS Microwells**

PDMS was mixed with 10% curing agent and was degassed under vacuum as it set. Microwells were cut using a 4mm diameter biopsy punch before cleaning the PDMS blocks and bonding them to glass slides using oxygen plasma surface activation. Microwells were UV-sterilised before use.

**Preparation of PEG Hydrogels**

Hydrogels were prepared by mixing multiarm poly(ethylene glycol)–vinylsulphone (PEG-VS)–peptide conjugates with activated Factor XIII in a calcium containing buffer, as described previously [44]. The mechanical properties of the hydrogels were varied by changing the dilution of the PEG-VS macromers, expressed as a percentage volume/volume. Preliminary experiments showed that an appropriate range of dilutions is 0.7%-1.0%; stable hydrogels do not form below this range and they are too stiff to support morphogenesis above 1.0% PEG-VS (data not shown). Extracellular matrix proteins such as Laminin (100µg/mL) and the Fibronectin-derived short peptide, RGD (100µg/mL), can easily be incorporated before gelation. The addition of these components acts to dilute the gel further and can obstruct sites for cross-linking, with the result that these functionalised gels are less stiff than their minimal counterparts with the same percentage of PEG-VS.

Gastruloids were cultured within the hydrogels by adding them to the PEG monomer solution prior to polymerisation. The forming hydrogel was mixed thoroughly after the addition of Factor XIII and was transferred quickly into
the PDMS microwell before gelation was complete. The resulting hydrogel formed around the gastruloid tissues *in situ*.

### 2.2 Microscopy & Imaging

#### 2.2.1 Immunohistochemistry

The protocol for immunostaining the gastruloids was adapted from one originally provided by A.K. Hadjantonakis [131] and is summarised in the online protocol [5]. In brief, gastruloids were collected using a wide-bore P1000 micropipette and were washed twice in PBS. They were fixed by incubation in 4% paraformaldehyde (diluted in PBS; Sigma-Aldrich P6148, prepared as 12% stock in BES-Buffered Saline with calcium chloride) for one hour at room temperature or overnight at 4°C. The fixed tissues were washed three times for ten minutes in PBS at room temperature on a low-speed orbital shaker. Subsequent washes were in PBS supplemented with 10% Foetal Bovine Serum (FBS; Biosera FB-1090/500) and 0.2% Triton X-100 (Sigma-Aldrich X100) (PBSFT) for three ten-minute incubations as above. The aggregates were then left blocking in PBSFT at 4°C for at least one hour before an overnight incubation with the primary antibody mixture in PBSFT.

The aggregates were washed in 4°C PBSFT as follows: twice for 5 minutes, three times for 15 minutes and four to seven times for one hour. All washes were performed on a low-speed orbital shaker; the one hour incubations were maintained at 4°C. The aggregates were incubated overnight with the secondary antibody mixture in PBSFT at 4°C, protected from the light.

Following secondary antibody incubation, the aggregates were washed in 4°C PBS supplemented with 0.2% FBS (see above) and 0.2% Triton X-100 (see above) (PBT) twice for 5 minutes, then three times for 15 minutes, protected from the light. They were subsequently incubated for 30 minutes.
with a 1:1 solution of glycerol (Sigma-Aldrich G5516) to PBT, followed by 30 minutes in a 7:3 solution of glycerol:PBT at room temperature. The glycerol-PBT was replaced with mounting medium (formulation as described in [174]) and individual aggregates were pipetted in 17μL droplets onto a microscope slide (VWR Superfrost 631-0909). Spacers were made from four layers of double-sided tape and were placed at each end of the slide. A plastic coverslip was placed over the droplets and the slide was inverted for imaging.

Table 2.4: Table of Primary Antibodies used in Immunohistochemistry. All Alexa-conjugated secondary antibodies were supplied by Invitrogen and used at a dilution of 1:500.

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Raised In</th>
<th>Supplier</th>
<th>Cat. Number</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/Bra</td>
<td>Goat</td>
<td>Santa Cruz Biotech.</td>
<td>sc-17743</td>
<td>1:200</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td>Rat</td>
<td>Takara</td>
<td>M108</td>
<td>1:200</td>
</tr>
<tr>
<td>Sox17</td>
<td>Goat</td>
<td>R&amp;D Systems</td>
<td>AF1924</td>
<td>1:500</td>
</tr>
<tr>
<td>Sox2</td>
<td>Rabbit</td>
<td>Merck Millipore</td>
<td>AB5603</td>
<td>1:200</td>
</tr>
</tbody>
</table>

2.2.2 Microscopy

Widefield, single time points and time-lapse data were acquired at 5x magnification using a Zeiss AxioObserver.Z1 (Carl Zeiss, UK) in a humidified 37°C incubator, with the embryo cultures positioned on the lid of a six-well plate. An LED white-light illumination system (Laser2000, Kettering, UK) and a Filter Set 45 (Carl Zeiss Microscopy, Ltd., Cambridge, UK) filter cube were used to visualise the red fluorescence. Emitted light was recorded using a back-illuminated iXon888 Ultra EMCCD (Andor, UK) and the open-source Micro-Manager microscopy software (Vale Lab, UCSF, USA).

Confocal microscopy was performed as described in [174] on a Zeiss LSM700 laser scanning confocal microscope (Axiovert 200M body). Zen2010 (Zeiss) was used for data acquisition and the open-source FIJI ImageJ platform [139] was used for data analysis.
2.3 Chicken Embryo Culture

Fertilised chicken eggs were supplied to order by Winter Egg Farm, Royston, Hertfordshire and were stored in a 10°C humidified incubator for up to one week. The eggs were transferred to a humidified, rocking 37°C incubator for 24 hours prior to setting up embryo cultures. Embryos incubated in this way typically ranged from Hamburger and Hamilton Stage 4-9 ([70], 1951 reprinted 1993).

2.3.1 New Culture

Embryo cultures were prepared according to a modified version of New Culture [116] that uses glass rings with rectangular cross sections to support the explanted extraembryonic membranes within 35mm diameter bacterial Petri dishes (BD Falcon 351008) on a layer of thin albumen. Pannett-Compton saline was prepared to the formulation described in [178]. Embryo cultures were incubated in a humidified incubator at 37°C prior to grafting.

2.3.2 Fluorescent Labelling of Embryonic Tissues

Small regions of the chicken embryos were labelled for fate mapping experiments. In these cases, small amounts of CellTracker™ Red (C34552, Thermo Fisher Scientific, prepared in 20% sucrose (v/v)) were micropipetted onto the ectoderm around the node using mechanically drawn capillary needles and a mouth pipette (Sigma-Aldrich A5177). Tissues were labelled within a droplet of Panett-Compton saline that was aspirated after labelling to remove excess label. Labelled embryos were incubated, imaged and scored as for the grafted embryos as detailed below.

2.3.3 Fluorescent Labelling of Tissue Grafts

Labelled tissue grafts were prepared from both adherent cultures and gastruloid cultures. Adherent cells were detached mechanically using a cell
scraper in 2mL PBS (with calcium and magnesium) to lift intact colonies of cells that were transferred to an FBS-precoated 5mL FACS tube for centrifugation at $\sim 170\times g$ for five minutes. The supernatant was discarded and the pellet was washed by gentle resuspension in PBS and centrifugation as above. The pellet was gently resuspended in 500µL PBS without calcium and magnesium (Sigma-Aldrich D8537) and the colonies were labelled with DiI (Thermo-Fisher Scientific Vybrant® V22885, 1:100) for 25 minutes in the dark, on ice. The labelled cells were centrifuged at $\sim 170\times g$ for 5 minutes and the pellet was resuspended in 37°C PBS (with calcium and magnesium) for grafting as intact colonies of cells.

Gastruloid tissues were collected using a wide-bore P1000 micropipette and were dissected into small pieces using a hair loop tool and an eyebrow knife in warm N2B27. Dissected tissues were transferred to warm PBS in a 5mL FBS-precoated FACS tube and were washed in PBS and labelled as above. The labelled cells were grafted as intact clumps of tissue (without any form of dissociation).

Explants of chick node tissue were collected from embryo cultures (either mounted in a Petri dish or at the watch-glass stage) by dissecting a small square region around the node using an eyebrow knife or a tungsten needle. They were transferred to a 5mL FBS-precoated FACS tube and were labelled as above. The labelled cells were grafted as intact clumps of tissue (without any form of dissociation).

### 2.3.4 Xenotransplantation Protocol

The embryo cultures were screened prior to grafting and any that were developing abnormally or had flooded with albumen were discarded at this point. A few drops of Panett-Compton saline were pipetted onto the embryo and two pieces of labelled tissue were transferred into this droplet by mouth pipetting. An eyebrow knife tool or an electrolytically sharpened tungsten
2.4 MOLECULAR METHODS

2.4.1 qRT-PCR

qRT-PCRs were performed using the Quantifast SYBR Green PCR Kit (Qiagen 204054) [50]. The primers used are described in a table appended to this chapter. Populations of gastruloids were pelleted by sedimentation and RNA was isolated using the TRIzol® Reagent (Life Technologies 15596-018), following the manufacturer’s protocol. Gene expression data were normalised to the expression of the housekeeping gene PPIA.

2.5 Computational Methods

2.5.1 Quantification of Aggregate Fluorescence

Fluorescence traces were taken along the putative antero-posterior axis of the aggregates, or from the region of highest reporter expression to the region of lowest expression where polarity was not evident from morphology alone. Widefield images were quantified using the open-source FIJI ImageJ platform [139] by taking a line plot along this axis. Where necessary, a single kink was incorporated to accommodate the curvature of the tissue to give a spline. The line width was set to 10 pixels to smooth noise in the fluorescence measurements. The data were copied to Microsoft Excel, along
with the average background fluorescence value determined by the mean value of a line plot not passing through the aggregate. Collated data were plotted using a pipeline developed with Dr David Turner in the open source Project Jupyter iPython notebook and the Matplotlib library. Traces were represented as a normalised proportion of the length of each aggregate \( (x) \), with the intensity of the fluorescence normalised to the highest measurement in each dataset \( (y) \). The mean fluorescence trace was represented as a dark blue line, with the shaded blue region representing one standard deviation above and below the mean.

\[ \text{2.5.2 Quantification of Labelled Cells in the Chick} \]

Widefield images were analysed in the open-source FIJI ImageJ platform [139] and were stitched using a pairwise stitching plugin [126]. For most embryos, a single timepoint was taken at the start of the culture and a second approximately 18 hours later. In each experiment, a subset of six embryos was imaged every 20 minutes during this time.

Any embryos that were developing abnormally, or those where the grafted cells had been lost were excluded from further analysis. In brief, each set of images was qualitatively scored for the pattern of graft incorporation as described in Chapter 6 (A Xenotransplantation Assay for Candidate Axial Progenitors). Each graft was then scored at the endpoint by the tissues in which labelled cells could be identified and by the distance between the most rostral and most caudal labelled cell on each side of the midline. These measurements were recorded along with the initial size and absolute position of the graft (in relation to the medio-caudal edge of the node) immediately after grafting.

The quantified data were plotted using the open source Project Jupyter iPython notebook and the Matplotlib library. The position of each graft is presented as a set of co-ordinates in relation to the node at \((0,0)\), with
2.5. COMPUTATIONAL METHODS

the size of the point proportional to the size of the graft. The points are then coloured according either to the tissues in which labelled cells can be identified at the endpoint or the length of the axial contribution for each graft. Thanks are due to Dr David Turner for his assistance in developing the first incarnation of the image analysis pipeline.

Table 2.5: Primers used for qRT-PCR [171]

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axin2</td>
<td>CTAGACTACGGGCACTCAGGAA</td>
<td>GCTGGCAGACAGGACCATACA</td>
</tr>
<tr>
<td>BMP4</td>
<td>CTCAAGGGAGTGGAGATTGG</td>
<td>ATGCTTTGGGACTACGGTTTG</td>
</tr>
<tr>
<td>Cer1</td>
<td>GGAAACGCCATAAGTCTCCA</td>
<td>AGGGTCAGAATTTGCCATTTG</td>
</tr>
<tr>
<td>Chordin</td>
<td>GTGCCTCTCTGCTCTGTTCTTT</td>
<td>AGGAGTTGCATGGATATGG</td>
</tr>
<tr>
<td>Dkk1</td>
<td>CCATTCTGGCCAACTCTTTTC</td>
<td>CATTTCCCTCCCTTCCCAATAAC</td>
</tr>
<tr>
<td>FGF4</td>
<td>GGCCACTCAGAGAGATAGG</td>
<td>ACTTGGGCTCAAGCAGTAGG</td>
</tr>
<tr>
<td>FGF5</td>
<td>GCTCAATGATCAGAGAGGGA</td>
<td>TCAGCTGGTCTTGTAATGAG</td>
</tr>
<tr>
<td>FGF8</td>
<td>AGGACTGCTATTACAGAGAT</td>
<td>CATGTACCAGCCAACGTACT</td>
</tr>
<tr>
<td>Lefty1</td>
<td>AGGGTGCAAGACCTGAGCTG</td>
<td>GGAAGCAAGAGACACACAA</td>
</tr>
<tr>
<td>Nodal</td>
<td>AGGCACCTCCAGAAGAGGGA</td>
<td>GTGTCTGCCAAGCAGTACATCTC</td>
</tr>
<tr>
<td>Noggin</td>
<td>CCCATCATTTCCGAGTGAAG</td>
<td>CTCGCTAGAGGGTGTGGA</td>
</tr>
<tr>
<td>PPIA</td>
<td>TTACCCATCAAACCATTCTTCTTG</td>
<td>ACCCAAGAAGAGCAGTACGAGG</td>
</tr>
<tr>
<td>Spry4</td>
<td>ATGGTGAGATGTCGGATTG</td>
<td>GAGAGGGGAGCTACAGGAC</td>
</tr>
<tr>
<td>T/Bra</td>
<td>CTGGAGGACTTGCTTTTG</td>
<td>GTCCAGGGCTATGGAGG</td>
</tr>
<tr>
<td>Wnt3</td>
<td>CTAATGCTGGCCTGAGAGG</td>
<td>ACATGGTAGGAGAGTGCAGGC</td>
</tr>
<tr>
<td>Wnt3a</td>
<td>CATACAGGAGTGTCCTTGG</td>
<td>AATCCAGTGTTGGGTGGGM</td>
</tr>
</tbody>
</table>
CHAPTER 2. MATERIALS & METHODS
Chapter 3

Optimisation of Gastruloid Culture

3.1 Gastruloids as an Embryonic Stem Cell Model of Axial Elongation Morphogenesis

There is a clear demand for in vitro models of mammalian embryogenesis due to the ethical and technical challenges associated with the internal development of these organisms. While mESCs can produce all of the cell types of the embryo in vitro, they do not do so in a spatially organised manner when grown as two-dimensional cultures or as embryoid bodies [135][170]. The results of culturing embryonal carcinoma (EC) cells as small aggregates have demonstrated that axial elongation morphogenesis can be recreated in vitro [106], paving the way for the development of similar cultures of ES cells that can reveal their morphogenetic potential.

ES cells grown in suspension form embryoid bodies (EBs) that have been shown to contain derivatives of all three germ layers and can be directed to form differentiated tissues such as myocardium and the embryonic blood
This system has been used as a model for mouse embryogenesis and has particular strengths in isolating the precursor cells of different lineages and in assessing the impact of null mutations (reviewed in [39]). EBs do not, however, develop an overall organisation and instead they form differentiated tissues as locally ordered patches in an otherwise spheroid aggregate. They lack the axial organisation of the embryo that orientates each tissue in relation to the others, intrinsically limiting their use in studying the tissue-tissue interactions and morphogenesis of development.

This was demonstrated in a study by Sanjini et al., who followed the downregulation of Oct4 during EB differentiation and concluded that the pattern of differentiation is spatially random in EBs grown at a controlled size under standardised conditions. The main axial structures, indicated by expression of Brachyury and Foxa2 are often present as a single major concentration of cells but the tissue morphology is not predictable and these markers are also expressed by cells scattered throughout the aggregate. The authors conclude that overall the expression of specific markers follows a sequence and timing that corresponds to normal embryonic development but this relation does not extend to the overall morphology of the EBs [135].

Leahy et al. report a degree of polarised T/Brachyury expression in four day old EBs, as assessed by radioactive in situ hybridisation though the pattern and morphology is highly variable between aggregates [101]. The authors describe how the EBs undergo a progressive change in gene expression that corresponds to a maturation through the post-implantation, gastrulation and early organogenesis stages though the timing of these events does not correspond directly to embryonic development since it occurs at a slower rate [101]. Overall, the results of the in situ agree with the assessment of Sanjini et al. that the EBs are more like a mosaic of micro-regions than an axially organised structure with a basic body plan.
ten Berge et al. give a clear description of axial polarity in EBs, demonstrated through the polarised expression of Wnt signalling and Brachyury reporters [163]. They describe how BMP signals from the serum used in the aggregation of the cells activates endogenous Wnt and Nodal signalling in a localised region of the tissue, which becomes self-sustaining. Wnt signalling activity in this region is correlated with the upregulation of early mesendodermal markers at the expense of neur ectodermal genes. Cells in this region also express the genetic and cellular hallmarks of the epithelial-to-mesenchymal transition (EMT), demonstrating that the region of high Wnt signalling activity undergoes a local gastulation-like process. Experimental modulation of Wnt signalling with exogenous Wnt3a or Dkk1 shows that the initial polarisation is dependent on endogenous signalling and that this pathway modifies the balance between anterior neur ectodermal fates and posterior mesendodermal fates in EBs [163]. Despite the self-organising ability of the EBs to produce a region of co-localised Wnt signalling activity and Brachyury expression that undergoes a local EMT, they do not show any degree of axial elongation and remain as ovoid structures.

The next major advance in EB culture was achieved by Marikawa et al., who extended the findings discussed above by showing that aggregates of 200 P19 EC cells can undergo a consistent and synchronous series of changes in gene expression and morphology when cultured for six days under mesodermal differentiation conditions [106]. They report that the temporal order of gene expression corresponds to that of early post-implantation embryonic development and furthermore that gene expression is spatially organised along an antero-posterior axis in the aggregates. This expression pattern is coupled to a large-scale morphological change in the fifth and sixth days of the cultures that causes the aggregates to adopt an elongated morphology through convergent extension. Early mesodermal gene expression is shown to depend on Wnt3 and β-catenin, while the expression of more posterior markers and elongation morphogenesis are dependent on Wnt3a and Wnt5a. These find-
ings are consistent with the onset of expression of these genes in vivo and the phenotypes of their corresponding null mutations. The authors therefore present the P19 aggregate system as the first EC-based model for axial elongation morphogenesis and set a clear experimental target of applying the culture regime to embryonic or epiblast stem cells.

As described at the end of Chapter 1, the “gastruloid” system was developed as an ES cell model of axial morphogenesis by integrating the hanging drop approach used by Marikawa et al. with cultures of ESCs (instead of ECCs). The key distinction from the approach taken by ten Berge et al. was to lower the initial number of cells from 2000 cells to 400 cells per aggregate [163][106]. A gastruloid can therefore be defined as a self-organising, three-dimensional aggregate of embryonic stem cells, in this case from the mouse. To quote directly from our first publication on this system [176], we suggested the name “gastruloids” since they:

“...self-organise into polarised structures that exhibit collective behaviours reminiscent of those that cells exhibit in early mouse embryos, including symmetry breaking, axial organisation, germ-layer specification and cell behaviour, as well as axis elongation [176].”

These features of the gastruloid system are explored in more detail in Chapter 4; this chapter focusses instead on the development and optimisation of the system through the use of 96-well plate culture [47] and careful control over the initial size of the gastruloids.
3.2 Results

3.2.1 Refinements to the Protocol for Producing Gastruloids

A range of different cell lines was used to follow the development of antero-posterior polarity and the differentiation of specific germ layers in the gastruloids. The *Sox1::GFP* cell line is a reporter of neural differentiation, since *Sox1* is expressed specifically in the neur ectoderm until differentiation into neurons and glial cells [191][187]. The *Sox1::GFP* reporter was developed to follow the production of neur ectodermal progenitors in adherent cultures of ESCs [191]; it was therefore selected to follow neural differentiation in gastruloid cultures.

The *T/Bra::GFP* cell line was used to follow the acquisition of posterior identity in the gastruloids, as well as mesodermal differentiation. *T/Brachyury* is expressed in the epiblast adjacent to the primitive streak, in the cells of the streak itself and in the nascent mesoderm (although it is downregulated in more lateral mesoderm) [182][51]. It therefore consistently marks the posterior of the gastrulating embryo, as well as the newly formed mesoderm. At this point in development, there are no significant effects on pre-sacral mesoderm formation in *T/Brachyury* hemizygotes [74], such as cells of the *T/Bra::GFP* genotype.

Finally, the *Sox17::GFP* cell line was used to monitor endodermal differentiation in gastruloid cultures. *Sox17* is expressed in the primitive endoderm of the blastocyst [117] and becomes restricted to the extraembryonic visceral endoderm (VE) at pre-streak and early-streak stages [88]. During gastrulation, it continues to be expressed in the extraembryonic VE, as well as in the nascent endoderm subjacent to the anterior end of the primitive streak but it is notably not expressed in the embryonic VE [88]. After gastrulation, it becomes transiently expressed in the definitive endoderm of the prospective
foregut, before expression shifts to the mid- and hindgut endoderm [88]. By 9.0dpc, expression is absent from the posterior endoderm. *Sox17* is therefore an early and specific marker for the definitive endoderm at gastrulation stages [88].

### 3.2.1.1 SFEBq Culture Reliably Produces Self-Organising Aggregates

Initially, gastruloids were produced in hanging drop cultures, using 400 cells per 40µL drop of N2B27 (as in [106]). After the first 48 hours of aggregation, the droplets were washed into a common volume of secondary medium. Representative *Sox1::GFP* gastruloids produced using this method are shown in Figure 3.1, after 4 days’ culture in 3µM Chiron.

This technique had three major limitations: first, that aggregates would frequently contact one another and fuse in the common volume of secondary medium, as in Figure 3.1A’. Secondly, the resulting tissues showed considerable heterogeneity in size, partly due to the fusion events and possibly also due to the process of aggregation in the hanging drops, which may produce multiple small aggregates in some cases (see Fig. 3.1B). Thirdly, individual aggregates could not be followed over time as they were free to move within the culture medium, restricting the throughput of imaging techniques. The non-immobilised culture produced the additional problem that the culture medium could not be exchanged without losing a proportion of the aggregates in the transfer. All three problems were resolved by forming the gastruloids in U-bottomed 96-well plates from the same suspension of cells; a method known as Serum-Free Embryoid Body culture with Quick re-aggregation (SFEBq) [47] (see Fig. 3.1C). SFEBq culture allows individual aggregates to be cultured in isolation from one another, preventing any possible fusion and reducing the overall variability in size. The confines of each well make the plates tolerant to movement, allowing time-lapse microscopy to be performed across a large number of wells, thereby improving through-
Figure 3.1: **SFEBq Culture Reduces Variability in Gastruloid Size and Prevents Fusion.** Gastruloids were formed from the *Sox1::GFP* cell line using the hanging drop protocol (A,B) and SFEBq culture (C). (A) Representative aggregates after 4 days' exposure to 3µM Chiron in a common volume of medium. (A') Aggregates that have fused (lower two) and are contacting a third. (B) Aggregates after 4 days' culture in Activin and Chiron show considerable heterogeneity in size. (C) Aggregates treated as in (A) from separate wells of SFEBq culture cannot fuse and show greater consistency in size. Scale bars indicate 200µm throughout.
put and enabling quantitative analyses of large datasets. Additionally, the culture medium can be exchanged within each well, allowing more complex treatment regimes to be performed without losing the gastruloids.

3.2.1.2 A Second PBS Wash Improves Inter-Experimental Reproducibility

An anecdotal observation was made in April 2014 that inter-experimental variability was improved by the addition of a second PBS wash step. This effect may have been due to the removal of any residual serum carried over from the neutralisation of the Trypsin, which could be a source of BMP signalling during the first 48 hours of the culture.

3.2.1.3 Counting the Cell Suspension After the Washes Minimises Transfer Losses

A major refinement has been made to the published protocols by conducting the cell counting step after the two PBS washes instead of beforehand. This simple change removed a considerable source of inter-experimental variability, namely the transfer losses that occurred during the wash steps. The effect of this change is apparent when aggregates formed from the previously published method [5] are compared to those formed from 200 cells (see Table 3.1). The data for the T/Bra::GFP cell line were collated across a large number of experiments and were included as Supplementary Table 2 in a pre-printed manuscript [171]; those data for the revised method are taken from the aggregate size experiment in Figure 3.2. Data for the TCF/LEF:mCherry (TLC2) cell line are not shown.

Table 3.1 shows that the mean diameter of the aggregates produced from the published protocol is comparable to those produced from just 200 cells.

\[\text{Table 3.1} \]

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1The protocol incorporating the SFEBq culture method and the double wash with PBS was uploaded to the BioRxiv pre-print server in 2014 [4] and was later published in the Journal of Visualised Experiments as a more detailed video protocol [5].
3.2. RESULTS

Table 3.1: Comparison of Gastruloid Protocols Reveals Transfer Losses in Wash Steps. Error indicates ± one standard deviation for three replicate experiments in the published datasets and one replicate for the revised datasets.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Cell Line</th>
<th>Initial Number / cells</th>
<th>Diameter at 48 hours / µm</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Published</td>
<td>T/Bra::GFP</td>
<td>400</td>
<td>161.0 ±26.2</td>
<td>222</td>
</tr>
<tr>
<td>Revised</td>
<td>T/Bra::GFP</td>
<td>200</td>
<td>165.5 ±27.5</td>
<td>22</td>
</tr>
<tr>
<td>Published</td>
<td>TCF/LEF:mCherry</td>
<td>400</td>
<td>194.9 ±20.7</td>
<td>56</td>
</tr>
<tr>
<td>Revised</td>
<td>TCF/LEF:mCherry</td>
<td>200</td>
<td>193.1 ±29.3</td>
<td>24</td>
</tr>
</tbody>
</table>

when the counting step is moved to after the wash steps\(^2\). The correspondence between these data suggests that the actual number of cells contributing the aggregate from the published protocol is much lower than 400 cells and that up to 50\% of the pellet may in fact be lost in transfer.

3.2.2 Composition of the Culture Medium

Early experiments explored the effect of different signalling pathway manipulations on the eventual morphology of the aggregates. The results of these manipulations in Sox1::GFP aggregates are presented in Figure 3.2.

Maintaining the aggregates in N2B27 for five days produced a variety of morphologies that ranged from rounded, uniformly Sox1::GFP\(^+\) cases to others where the tissue had elongated and GFP expression was localised to specific regions of the gastruloid. Similar results were achieved through the inhibition of BMP signalling from 48-120 hours (DMH1 treatment), which produced a range of morphologies and strong activation of the reporter. Activation of canonical Wnt signalling through GSK3\(\beta\) inhibition (Chiron treat-

\(^2\)Collaborative Note: Thanks are due to Mehmet Ugur Girgin, who made this refinement during a collaborative research visit at the École Polytechnique Fédérale de Lausanne, Switzerland. This project was funded by a Development Travelling Fellowship through the Company of Biologists. All practical work and imaging was carried out equally with Mehmet, with data analysis performed separately by the author in Cambridge.
Figure 3.2: Manipulation of Signalling Pathways from 48 Hours Generates Aggregates with Different Morphologies. Gastruloids were produced from the Sox1::GFP cell line and were treated as indicated in (A). Upward arrows indicate activation of specific signalling pathways, while flat-ended horizontal arrows denote pathway inhibition (see Chapter 2, Table 2.2). (B) Representative morphologies at the 120 hour time point. Scale bars indicate 500µm throughout. Inhibition of MEK/ERK signalling by PD03 generates mostly spheroid, Sox1::GFP+ aggregates, while inhibition of Activin/Nodal signalling with SB43 produces complex, folded morphologies. Chiron treatment produces Sox1::GFP+ elongations, which are broader if treated from 48-120 hours. N2B27 or DMH1 treatment produces a range of morphologies and robust activation of the Sox1::GFP reporter. Images from this dataset have been published as Fig. 7A in [176] and Fig. 8A in [172].
3.2. RESULTS

ment) resulted in localised Sox1::GFP expression to an elongating region of the aggregate, which was broader following longer exposure to the inhibitor (compare “Chiron” to “Chi Pulse” in Fig. 3.2). The elongations were lost when the Chiron treatment was paired with inhibition of MEK signalling (PD03 treatment) or with inhibition of Activin/Nodal signalling (SB43 treatment). The former produced round, strongly Sox1::GFP+ aggregates while the latter often generated complex, folded structures. In all cases, there was greater morphological variation between the treatments than between individual aggregates from the same treatment. Exposure to Chiron from 48-72 hours after aggregation was chosen to investigate further as a model for axial morphogenesis since it frequently produced single elongations with a well-localised pattern of Sox1::GFP expression.

Having identified a pulse of Chiron from 48-72 hours as promoting the formation of single elongations, the effect of varying the dosage of Chiron at this time was explored. The results of different dosages are presented for the T/Bra::GFP cell line in Figure 3.3 and for the Sox17::GFP cell line in Figure 3.4.

The results of the qualitative morphological scorings (see Fig. 3.3A and Fig. 3.4) indicate that a 0.75µM dose of Chiron is sufficient to increase the frequency of single elongations from approximately 60% in untreated aggregates to 70%. Increasing the dose further produces an increased frequency of ovoid or spheroid aggregates at the 96 hour time point (for T/Bra::GFP and Sox17::GFP respectively). Quantifying the intensity and localisation of T/Bra::GFP fluorescence shows that the addition of 0.75µM Chiron is sufficient to increase the intensity of reporter activation in the elongating region of the aggregates (see Fig. 3.3B). Increasing the dosage further results in a broader distribution of T/Bra::GFP positive cells in the elongating tissue, shown as the peak of the curve shifting towards the right of the plots. Given these results, a 0.75µM pulse of Chiron treatment from 48-72 hours after aggregation may be an improved alternative to the 3µM treatment that has
Figure 3.3: 3µM Chiron Treatment from 48-72 Hours After Aggregation Promotes T/Bra::GFP Expression and Elongation. (A) Aggregates from the T/Bra::GFP cell line were treated as indicated from 48-72 hours and were qualitatively scored on their morphology at 96 hours. Low doses of Chiron (0.75-1.50µM) increase the fraction of singly elongated aggregates above the base frequency in N2B27. (B) Quantification of T/Bra::GFP fluorescence along the midline of the aggregates, from the region of highest signal to the region of the lowest. Chiron treatment increases the intensity of T/Bra::GFP expression and the proportion of the aggregate in which it is expressed. The filled blue region denotes one standard deviation either side of the mean fluorescence trace (blue line).
3.2. RESULTS

Figure 3.4: Low Doses of Chiron from 48-72 Hours Promote Elongation Morphogenesis in Sox17::GFP Aggregates. Gastruloids were treated as indicated and were qualitatively scored for their morphology at 96 hours. Treatment with 0.75µM Chiron increases the frequency of singly elongating aggregates, while higher doses appear to increase the proportion of spheroid morphologies.

become routine during the development of the gastruloid system and in the research presented in this thesis (discussed further in Section 3.3).

3.2.3 Gastruloids are Formed Optimally from 100-300 Cells

Aggregates were prepared from different numbers of T/Bra::GFP cells in order to quantify the effect of the starting number of cells on polarisation and elongation following a 3µM pulse of Chiron from 48-72 hours after plating. Images were collected every 24 hours for five days and the morphology of the aggregate in each image was scored according to the categories in Figure 3.5. Additionally, fluorescence measurements were taken along the midline of the aggregate, according to the method described in Chapter 2 (Materials & Methods). These measurements are summarised in Figure 3.6, where they have been normalised to the maximum fluorescence value and to the length of each aggregate (summarised in Table 3.2).
3.2.3.1 Larger Aggregates More Frequently Produce Double Elongations and Amorphous Structures

![Figure 3.5: The Frequency and Onset of Gastruloid Elongation Declines with Increasing Aggregate Size.](image)

Gastruloids were formed from different numbers of \( T/Brachyury::GFP \) cells and were treated with 3\( \mu \text{M} \) Chiron from 48-72 hours after plating. The gastruloids were qualitatively scored for their morphology every 24 hours over five days. Aggregates formed from 200 cells seem to pattern more quickly and produce a higher frequency of single elongations than those starting from 300 cells or more. Among aggregates formed from 300 to 500 cells, the frequency of double elongations increases with increasing size.

The majority of aggregates are spherical during the first 72 hours of development, regardless of their starting size (see Fig. 3.5). It is likely that the small fraction of ovoid aggregates in the first 48 hours of culture are the product of fusion events between the main aggregate and smaller satellites which have not fully fused with the tissue. An exception to this is the 200 cell condition which shows an increase in the proportion of ovoid aggregates by the 72 hour time point (top row in Fig. 3.5). This is likely to reflect...
the start of the elongation process, since satellite aggregates are much less common when starting from so few cells.

Following the 3μM pulse of Chiron from 48 to 72 hours, the aggregate morphologies start to deviate from simple spherical or ovoid structures, with a fraction of the aggregates in each case starting to develop single elongations. This fraction is very small, however, for the 500 cell condition, possibly reflecting a constraint on this morphogenetic process imposed by the large bulk of tissue. The frequency of single elongations at the 96 hour time point is highest in the 200 cell starting condition and appears to diminish with increasing numbers of cells with a corresponding increase in the number of spheroid or ovoid aggregates (fourth column in Fig. 3.5).

By the end point (120 hours), every aggregate has changed shape and it is clear that the 200 cell condition gives the highest proportion of aggregates with single elongations (fifth column in Fig. 3.5). The proportion of aggregates showing amorphous or doubly elongated morphologies increases in proportion to the initial cell number; a substantial proportion of the aggregates that were formed from 500 cells were amorphous or doubly elongated.

In summary, the qualitative morphological scoring demonstrates that smaller aggregates acquire an elongated morphology more quickly and more frequently than those formed from higher numbers of cells. Aggregates formed from more than 300 cells show an increasing frequency of double elongations or amorphous structures with increasing size. The effect of further reductions to the starting number of cells is presented in Figures 3.7, 3.8 and 3.9.

3.2.3.2 Larger Aggregates have Larger Brachyury-Expressing Domains but are Consequently Less Polarised

Aggregates formed from 200-500 cells show the same general trend in T/Bra::GFP reporter expression throughout the five days in culture (see
Figure 3.6: Aggregates Formed from Larger Numbers of T/Bra::GFP Cells Have Larger Brachyury-Expressing Domains and Show Secondary Poles of Expression. Gastruloids were formed from the T/Bra::GFP cell line and were treated with 3µM Chiron from 48-72 hours after plating. Fluorescence traces were measured and processed as detailed in Chapter 2 (Materials & Methods). The mean trace along the putative posterior-anterior axis is shown as a dark blue line, with one standard deviation above and below this line shown as the filled blue region. T/Bra::GFP expression is higher in larger aggregates at 72 hours and remains in a proportionally larger region of the aggregate at 96 hours. By 120 hours, the largest aggregates show infrequent secondary poles of expression. (n=20-24 for each condition in a single technical replicate; see Table 3.2).
3.2. RESULTS

Fig. 3.6). Expression of the reporter is substantially elevated during the 3µM Chiron treatment from 48-72 hours, before becoming polarised to the posterior (the left of each plot) over the remaining two days. The expression of the reporter, once polarised, reaches its highest level at the 96 hour time point and subsequently reduces in intensity. The difference between the starting numbers of cells are clear in the intensity of reporter activation at 72 hours, the size of the high Brachyury-expressing domain at 96 hours and finally the presence of secondary poles at 120 hours.

There seems to be a general trend towards higher levels of $T/Bra::GFP$ expression at the 72 hour time point with increasing starting numbers of cells after exposure to 3µM Chiron. A simple technical explanation is that the larger aggregates are likely to contain larger numbers of GFP-expressing cells, so they would be expected to show the highest levels of fluorescence due to the collection of light from multiple focal planes in the widefield imaging system. The larger standard deviation between the 200-cell aggregate samples suggests, however, that the response may be less robust in the smaller aggregates and may explain the lower mean fluorescence trace (compare top and bottom plots in the second column of Fig. 3.6).

Aggregates formed from 300 cells clearly show the resolution of the Brachyury expression into a single pole by the 96 hour time point (third column of Fig. 3.6). The relative size of this domain of high Brachyury expression seems to increase into greater proportions of the aggregates with increasing starting numbers of cells. It should be noted, however, that the fluorescence in some images at the 96 hour time point was saturating, so some caution is required in drawing conclusions about the size of the $T/Bra::GFP$-high pole. The opposite effect is also apparent for the 200-cell aggregates, which show a tight restriction of GFP signal to the posterior tissues with more variable levels of expression across the rest of the aggregate.
By the 120 hour time point, Brachyury expression has become restricted to the posterior-most quarter of the aggregate in all conditions. In keeping with the levels of expression at earlier points, the 200-cell aggregates retain the lowest level of Brachyury expression at this pole, while it is higher in larger aggregates. An important difference is clear in the anterior of the aggregates where those derived from 200 or 300 cells are Brachyury negative, while those derived from 500 cells infrequently show secondary peaks of expression. This reflects the occurrence of double elongations in the larger aggregates, which are largely absent from the 200 or 300 cell aggregates.

In summary, aggregates formed from 200 cells show lower levels of Brachyury expression throughout the culture but this fluorescence is restricted to a smaller proportion of each aggregate than those formed from 300-500 cells.

3.2.3.3 An Initial Size of 100 Cells is Required for Polarisation and Elongation

Aggregates were prepared from the T/Brachyury::GFP cell line using different starting numbers of cells per well (from 25-300 cells) and were treated with 3µM Chiron treatment from 48-72 hours after plating. The aggregates were imaged at the 96 and 116 hours after plating and were scored for both reporter expression (Fig. 3.7) and their morphology (Fig. 3.8). At these time points, aggregates formed from 200 cells or more were viable in all cases and a substantial fraction (>85%) showed clear T/Brachyury::GFP expression (see Fig.s 3.7 and 3.9). The viability of the aggregates declined with reducing initial numbers of cells, with 31% of the 50-cell aggregates scored as non-viable at the 116 hour time point due to the accumulation of large numbers of loosely-associated cells around a small central core (see Fig. 3.7). This was especially apparent for aggregates formed from 25 cells, in which many failed to aggregate (13/21 cases, data not shown), giving an overall viability of around 14% (data not shown). It is also clear that the fraction of viable aggregates which did not express the T/Brachyury::GFP reporter
Figure 3.7: *T/Brachyury::GFP* Aggregates Formed from Fewer than 100 Cells Show Reduced Viability and Reporter Expression. Imaged aggregates were scored according to their apparent viability and whether they expressed the *T/Bra::GFP* reporter at the 96- and 116-hour time points. Non-viable aggregates were indicated by large numbers of loose cells surrounding a central cluster of viable cells. The number of images scored in each case is indicated to the lower right of each chart. Viability and *T/Bra::GFP* expression is substantially lower in aggregates formed from fewer than 100 cells.
decreases with increasing initial size (light grey sectors in Fig. 3.7); this effect is quantified in more detail in Figure 3.9.

The results of qualitatively scoring the morphology of these aggregates are shown in Figure 3.8. Aggregates formed from 50 cells are frequently scored as

![Pie charts showing the morphology of aggregates formed from different initial numbers of T/Brachyury::GFP cells at 96 and 116 hours.](image)

Figure 3.8: Aggregates Formed from Fewer than 100 Cells do not Elongate. Aggregates formed from different initial numbers of T/Brachyury::GFP cells were scored for their morphology at the 96- and 116-hour time points according to the key indicated. The number of aggregates scored for each condition is shown to the lower right of each plot. Those formed from 50 cells retained a spheroid or ovoid morphology, while increasing the initial number of cells increased the frequency of single elongations at 116 hours.

spheroid or ovoid at these time points, indicating that they do not undergo the elongation morphogenesis that characterises those formed from larger numbers of cells. The high proportion of spheroid aggregates at the 116 hour time point is likely to be due to the poor viability of the aggregates, since many of those scored as “Non-Viable” (in Fig. 3.7) were scored as spheroids in this analysis (Fig. 3.8). It is clear, however, that the fraction of spheroid aggregates diminishes with increasing initial numbers of cells. As
the initial number of cells increases, it seems as if the elongation process becomes more co-ordinated. This is evident in the increasing proportion of ovoid aggregates (orange in Fig. 3.8) from each starting condition that become singly elongated (red in Fig. 3.8) between the two time points.

When the results from qualitatively scoring the aggregates for T/Brachyury expression and their morphology are taken together, it seems as if the 50-cell aggregates fall beneath a threshold for elongation morphogenesis since none adopts the elongated morphology at 116 hours. This threshold is likely to be between 50 and 100 cells, since a large proportion of the 100-cell aggregates express the reporter and go on to elongate. Increasing the initial number of cells further seems to improve viability and to increase the co-ordination of the elongation at the population level, suggesting that an initial number of around 200-300 cells may be optimal (see also Fig.s 3.5 and 3.6). In order to explore this polarisation effect further, the fluorescence signal of each aggregate was quantified using the method described in Chapter 2 (Materials & Methods); the results are presented in Figure 3.9.

Aggregates formed from 50 cells do not express T/Bra::GFP at these time points, with the exception of one case (upper grey line in the lowest left plot, Fig. 3.9; see also Fig. 3.7). Among aggregates formed from 100 cells, many show polarised expression of the reporter as indicated by the higher fluorescence on the left of the plots (Fig. 3.9). Some traces, however, remain at the baseline, suggesting that polarisation is less frequent in this case. This is consistent with the results of the morphological scoring, since a substantial fraction of the 100-cell aggregates go on to elongate but some remain spheroid (Fig. 3.8). When larger initial populations of cells are used, T/Brachyury::GFP is expressed at higher levels and as a greater proportion of the aggregate. Additionally, aggregates formed from 300 cells show some degree of polarisation at 96 hours in all cases (upper right plot, Fig. 3.9) as none is at the baseline on the left of the plot. Furthermore, the standard deviation of the fluorescence traces is smallest in this condition, suggesting
Figure 3.9: A Minimum Starting Number of 100 cells is Required for Robust Polarisation of T/Brachyury. Aggregates formed from different numbers of T/Bra::GFP cells were cultured with a Chiron pulse from 48-72 hours and were imaged at the 96 and 120 hour time points. The fluorescence signal for each aggregate was recorded along the midline from the region of highest to lowest signal; these traces are normalised to the highest fluorescence recorded and to the length of each trace. The mean trace is shown as a blue line; the standard deviation of the traces is indicated by the filled blue region. Aggregates formed from 50 cells are viable but do not show polarised expression of T/Brachyury. Polarised expression is evident among aggregates formed from 100 cells and it becomes more pronounced and robust with increasing initial size.
3.2. RESULTS

that it is most robust when this initial number of cells is used. It should be noted that the spike in expression in the 300 cell case at 116 hours is due to a “shared tail” type of double elongation, where the T/Bra expression falls at approximately 50% of the axial length when measured from one pole to another.

The lack of apparent polarisation in aggregates initially formed from 50 cells may, in part, be due to the viability effect described in Figure 3.7 but it also represents an inability of these aggregates to express the reporter and undergo elongation morphogenesis. The morphological scoring identified that aggregates formed from higher initial numbers of cells may polarise and elongate in a more coordinated manner (Fig. 3.8), which is supported by the lower standard deviation in the fluorescence traces of the larger aggregates (Fig. 3.9).

3.2.3.4 Towards an Optimal Size for Elongation Morphogenesis

Given the results of the qualitative and quantitative scoring described in the preceding sections, it seems appropriate to define the optimal culture as one that starts from 200-300 cells in the T/Brachyury::GFP cell line, since this produces the highest frequency of single elongations and a robust level of reporter activation. A summary of the length measurements used to produce the fluorescence traces in Figure 3.6 and Figure 3.9 (denoted by asterisks) is provided in Table 3.2, which can be used as a reference for future gastruloid cultures in this cell line. These data indicate that an optimal starting culture would have a mean diameter at the 48 hour time point of between 165\(\mu\)m and 215\(\mu\)m (bold in Table 3.2). Inter-experimental variability is likely to arise from differences in the viability or differentiation states of the starting cell cultures, which may vary according to the age of the culture (i.e. its number of passages), as well as from technical aspects of counting and handling the cells. This variability will give rise to differences in the starting size and state of the gastruloids, which have consequences for the processes of polarisation
CHAPTER 3. OPTIMISATION OF GASTRULOID CULTURE

and elongation. One way in which to check for these effects is to confirm that the size of the aggregates at the 48 hour time point falls within this optimal range.

3.2.4 Comparison of Dissociation Reagents

Producing gastruloids using this protocol depends on an accurate cell count and the quick re-aggregation of the cells, which Eiraku et al. identified as a potentially important feature of SFEBq culture due to the rapid restoration of cell-cell interactions [47]. An accurate count depends on the efficient dissociation of adherent cells into a single cell suspension but an effective dissociation reagent should not disrupt the cell surface proteins required to maintain intercellular adhesion if the cells are to re-aggregate quickly. The method used to dissociate the cells is therefore a target for optimisation. Routine ESC culture uses 0.05% Trypsin-EDTA, a mixture of porcine proteases and a chelator of divalent cations, to detach and dissociate adherent cells. The dissociation is arrested by the addition of FCS, which competes with the cell surface proteins for degradation. EDTA is included to sequester the calcium ions that are cofactors for cell adhesion proteins. As such, this treatment is considered to be harsh with respect to the integrity of cell surface proteins, but effective. Accutase® is a popular alternative for dissociating sensitive cell cultures since it uses a heat-sensitive mixture of proteolytic enzymes that is quickly inactivated at 37°C. It was therefore chosen as a possible alternative to Trypsin-EDTA, which could possibly promote more rapid cell re-aggregation by preserving the integrity of cell-cell adhesion proteins.

Aggregates of 300 cells were prepared from identical cultures of T/Bra::GFP cells which were dissociated either with 0.05% Trypsin-EDTA or with Accutase®. The aggregation phase of the gastruloid culture was recorded with time-lapse microscopy to investigate whether the dynamics of re-aggregation differed be-

\[\text{See https://www.thermofisher.com/order/catalog/product/25300054}\]

\[\text{See https://www.thermofisher.com/order/catalog/product/A1110501}\]
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Table 3.2: The Effect of Starting Cell Number on Aggregate Size. Errors denoted by \( \pm \) are one standard deviation above and below the mean. Asterisks indicate the data presented in Figures 3.6 and 3.9; bold type denotes measurements at the 48 hour timepoint.

<table>
<thead>
<tr>
<th>Starting Number</th>
<th>Time Point / hours</th>
<th>Mean Length / ( \mu \text{m} )</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 cells*</td>
<td>96</td>
<td>120.54 ±37.51</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>116</td>
<td>97.91 ±47.51</td>
<td>16</td>
</tr>
<tr>
<td>100 cells*</td>
<td>96</td>
<td>199.29 ±73.26</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>116</td>
<td>251.00 ±113.6</td>
<td>20</td>
</tr>
<tr>
<td>150 cells*</td>
<td>96</td>
<td>257.69 ±81.75</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>116</td>
<td>350.84 ±128.07</td>
<td>20</td>
</tr>
<tr>
<td>200 cells*</td>
<td>96</td>
<td>266.31 ±44.64</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>116</td>
<td>370.41 ±80.66</td>
<td>21</td>
</tr>
<tr>
<td>200 cells</td>
<td>24</td>
<td>133.50 ±10.13</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td><strong>165.64</strong> ±27.55</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>201.11 ±45.90</td>
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<td></td>
<td>96</td>
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<td>21</td>
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<tr>
<td></td>
<td>120</td>
<td>610.08 ±187.64</td>
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<td>300 cells*</td>
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<td>332.24 ±39.77</td>
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<td>300 cells</td>
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<td></td>
<td>96</td>
<td>382.46 ±25.65</td>
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<td></td>
<td>120</td>
<td>833.38 ±101.29</td>
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<td>500 cells</td>
<td>24</td>
<td>171.40 ±11.27</td>
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<td></td>
<td>48</td>
<td><strong>245.45</strong> ±20.19</td>
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<td>72</td>
<td>296.07 ±37.30</td>
<td>22</td>
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<td></td>
<td>96</td>
<td>384.78 ±31.91</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>862.40 ±152.45</td>
<td>22</td>
</tr>
</tbody>
</table>
between the two treatments (Table 3.3). The data were scored on the time of the last aggregation event, when the final satellite cluster of cells merges with the forming aggregate and the diameter of the gastruloid at 21.3 hours. The results of these measurements are summarised in Table 3.3. Neither the

Table 3.3: Cells Dissociated with Accutase® and Trypsin have Similar Re-Aggregation Dynamics.
Measurements taken from 8 time-lapse recordings in each case.

<table>
<thead>
<tr>
<th>Dissociation Agent</th>
<th>Mean Aggregation Time / hours ±1SD</th>
<th>Mean Diameter at 21.3 hours / µm ±1SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accutase®</td>
<td>15.46 ±4.61</td>
<td>117.68 ±6.46</td>
<td>8</td>
</tr>
<tr>
<td>Trypsin</td>
<td>18.00 ±2.31</td>
<td>122.41 ±8.60</td>
<td>8</td>
</tr>
</tbody>
</table>

rate at which the cells re-aggregate nor the diameter of the early gastruloids appears to differ between cell suspensions produced with Accutase® or with Trypsin; the data for each set of measurements almost overlaps within one standard deviation of the mean for each condition (see Table 3.3).

Although the initial stages of aggregation appeared to be unaffected by the choice of dissociation reagent, the morphology and polarisation of T/Bra::GFP expression were assessed at 117 hours after plating to check for a later effect on gastruloid development. The results of qualitative morphological scoring and quantification of the fluorescence traces along the posterior-anterior axis of the gastruloids are presented in Figure 3.10.

The range of morphologies represented in each condition was almost identical, demonstrating that the different dissociation reagents have no effect on the final morphology of the gastruloids (Fig. 3.10A). No double elongations were scored in either dataset. On quantifying the expression patterns of the T/Brachyury::GFP reporter along the midline of each aggregate, the polarisation and intensity of expression also appears identical between the conditions. Accutase and Trypsin are therefore interchangable in this protocol, where the incubation time with the dissociation reagent is short.
Figure 3.10: The Use of Accutase in Dissociation Does Not Affect the Frequency of Elongation nor the Degree of Polarisation at 117 Hours. (A) Morphological scoring of T/Bra::GFP gastruloids 117 hours after plating, prepared from identical cultures with either Trypsin (left) or Accutase (right) with a pulse of 3µM Chiron from 48-72 hours after aggregation. The distribution of morphologies is almost identical. (B) Quantification of T/Brachyury::GFP expression along the midline of the aggregate from the region of highest signal to the lowest. Each trace is normalised to the length of the aggregate and to the highest overall intensity recorded. The mean fluorescence trace is shown in blue, with one standard deviation either side of the mean in the filled blue region. The treatments produce the same degree of polarisation and expression of the reporter.
CHAPTER 3. OPTIMISATION OF GASTRULOID CULTURE

3.3 Discussion

The protocol used to produce the gastruloids [4] has undergone several refinements during the course of this research [5][169], bringing improvements in the reproducibility, throughput and control of the system. It is now known that early cultures were subject to transfer losses of up to 50%, which calls for a re-assessment of the initially reported observations on the effect of the initial number of cells in the aggregates. Figure 2 of the van den Brink et al. paper describes how aggregates formed from 200 cells do not elongate, while those formed from 800 cells can produce double elongations when grown in 3µM Chiron for 3 days. It now seems clear that the lower limit for forming aggregates that will elongate is closer to 50-100 cells, while double elongations can frequently occur in aggregates formed from 400 cells or more. Those aggregates produced from 200-300 cells seem optimal for studying axial elongation in this cell line and have a diameter at the 48 hour time point of 165-215µm. The results of these experiments suggest that there are clear thresholds to the patterning event, though they must be taken with the caveat that the lower limit to patterning may be imposed by the nature of the SFEBq method. The reduced viability in 25-50 cell aggregates may be due to the failure to form an aggregate in the relatively large volume of the culture well. The nature of the lower limit to patterning will become clearer through the use of more rapid aggregation in smaller culture volumes or within a three-dimensional substrate.

Nevertheless, the general observation still stands that the formation of axial pattern in aggregates of ES cells is size dependent, with larger aggregates becoming more disordered. Initially, this is apparent in the formation of multiple foci of Brachyury::GFP expression and subsequently, multiple elongations. This finding is in keeping with the observation of ten Berge et al. that larger EBs from the Axin2LacZ/+ cell line more frequently show multiple poles of Axin2 expression. The authors speculate that the EBs could be patterned by the progressive cell non-autonomous recruitment of cells to
3.3. DISCUSSION

a pole of signalling activity under the control of a longer range inhibitory signal that prevents the formation of multiple poles [163]. The observations from the gastruloids are consistent with the idea that there is an optimal length scale for the patterning process that may be defined by the action of a negative regulator of patterning, though the exact nature of this signal remains to be defined (see Chapter 5: Characterisation of Gastruloid Development). Nevertheless, this observation raises the possibility that gastruloid polarity might arise through self-organised patterning, such as the output of a Turing-like reaction-diffusion system [168] (reviewed in [170]). It is also interesting to note from Figure 3.9 that on average, the pole of \( T/Brax::GFP \) expression occupies a similar proportion of the aggregate (approximately 60% of its length), independent of the initial number of cells in the 100-300 cell range. This provides a potential indication that the process of polarisation is regulated with regard to the tissue as a whole, rather than locally.

It is also possible that the initial number of cells in the aggregate is in fact a proxy for another parameter which cannot be measured so easily, such as tissue mass or aggregate volume. It may be that the aggregates formed from 50 cells do not polarise as they have not yet crossed a threshold for the polarisation of \( T/Brachyury \) expression to occur. Above this point, the aggregates formed from 100-200 cells more frequently adopt an elongated morphology at the 96 hour time point than those formed from larger numbers of cells. This may be due to the simple fact that less cell movement or proliferation is required to change the shape of the whole tissue in a smaller aggregate. In larger aggregates, the excess of cells may constrain and so delay the extension of the tissue. It is possible that the smallest aggregates are able to undergo more rapid cell proliferation to reach a size that is conducive to patterning and elongation. This would be consistent with Snow and Tam’s observations of compensatory growth in mitomycin C-treated mouse embryos that can become overtly normal (though underweight) over 3-4 days, despite starting from around 10% of their normal size [144]. This response is described
as occurring from around E6.5-E10.5, during which the embryo is undergoing gastrulation and axial elongation, so it is not inconceivable that similar regulatory processes may be occurring in both systems.

It is surprising that the spontaneous elongation of small aggregates of mouse ES cells has not been reported previously, since it can occur without the addition of exogenous factors. Marikawa et al. suggest that the morphogenesis of P19 ECC aggregates may have previously been overlooked due to the small initial number of cells in each aggregate and tendency in the field at that time to culture the aggregates adherently after four days in culture. It is likely that the elongation morphogenesis observed in the gastruloids has been overlooked for the same reasons; EB cultures are often formed from many thousands of cells and can be grown adherently after aggregation. As the results of the initial cell number experiments have shown, tissues formed from more than 500 cells are likely to be disordered (Fig. 3.5), so only the smallest EBs may have shown this behaviour.

In refining the gastruloid system further, it is interesting to note that Marikawa et al. report that the P19 cell aggregates elongate at a frequency of 98.7%, while the gastruloids elongate in around 75% of cases. One parameter to improve is the dosage of Chiron, as the only exogenous factor used in the protocol. On reflection, a lower dosage of 0.75 µM may be appropriate for future studies, since it seems to produce the most frequent single elongations and robust polarisation of the T/Bra::GFP reporter. A 3 µM dose was used in the experiments throughout this thesis since it produced a strong T/Bra::GFP response across a wide region of the gastruloid (Fig. 3.3). At the time of the experiments, an excess dosage seemed appropriate as the consistency and reproducibility of the system were still being improved. Given the refinements to the protocol, there is now an argument for parsimony in using exogenous factors in order to allow the self-organising behaviour of the cells to unfold without external perturbation.
3.3. DISCUSSION

In conclusion, this chapter has described the development and optimisation of the gastruloid system as a robust and reproducible model for axial elongation morphogenesis, which has not previously been studied using ESCs. The gastruloid protocol was developed by integrating SFEBq culture [47] with the approach taken by Marikawa et al. [106] (Fig. 3.1) and was refined through collaboration with others (see Table 3.1). In its current form, the gastruloid system can be used for quantitative imaging experiments and it produces tissues with a reproducible organisation according to the three major body axes [169]. Explorations of the initial gastruloid size indicated that the optimal range for 96-well plate culture is between 100-300 cells per well. Below this threshold, viability appears to be poor (Fig. 3.7) while above it, the morphology of the gastruloids becomes disordered (Fig. 3.5). The following chapter considers the use of gastruloids in studying axial elongation morphogenesis in more detail by characterising their development, making relevant comparisons to that of the embryo at each stage.
Chapter 4

Characterisation of Gastruloid Development

4.1 Introduction

Chapter 1 provided a general overview of embryonic development in different model organisms but it did not describe that of the mouse in detail. This chapter describes the major events of early post-implantation mouse development which is an important reference for the events of gastruloid development described in Section 4.2. Early postimplantation development is illustrated in Figure 4.1, followed by gastrulation stages in Figure 4.2. Figure 4.3 illustrates the neural plate stages of embryogenesis, while Figure 4.4 summarises the axial elongation of the embryo during somitogenesis. The Discussion of this chapter identifies points of correspondence between gastruloids and the development of the embryo and highlights important differences between these systems.
CHAPTER 4. CHARACTERISATION OF GASTRULOID DEVELOPMENT

Gestational age (E/dpc)  
Theiler Stage (TS): Notes

4.5 (4.0-5.5)  
TS6: Attachment of blastocyst; primitive endoderm covers blastocoelic surface of the inner cell mass.

5.0 (4.5-6.0)  
TS7a: Implantation and egg cylinder formation; enlarged epiblast, primitive endoderm lines mural trophectoderm.

6.0 (5.5-6.5)  
TS8: Differentiation of the egg cylinder; Reichert’s membrane and proamniotic cavity form.

6.5 (6.25-6.75)  
TS9a: Pre-streak stage; embryonic anteroposterior axis becomes visible.

Figure 4.1: Attachment and Implantation Stages of Mouse Embryogenesis. Left, schematics of sagittal sections of the mouse embryo and extraembryonic tissues from the blastocyst stage (top) to the egg cylinder stage (bottom). Embryonic tissues are shown in pink; extraembryonic tissues in tan. Images adapted from EMAP Theiler Stage Definition under a Creative Commons Attribution Licence 3.0. Right, notes on staging from the eMouse Atlas Project [130], updated with reference to [99]. Bcl, Blastocoel; ExE, Extraembryonic Ectoderm; ICM, Inner Cell Mass; PrE, Primitive Endoderm; proAC, Proamniotic Cavity; RM, Reichert’s Membrane; TE, Trophectoderm; (A/D)VE, (Anterior/Distal)Visceral Endoderm.
4.1.1 Symmetry Breaking and Early Embryonic Patternning

Immediately after implantation, the mouse embryo is a radially symmetric egg cylinder about the proximo-distal axis, as defined by its attachment to the uterine wall at the proximal end of the cylinder. The inner cell mass (ICM) of the blastocyst cavitates to form an epithelium. The primitive endoderm, which once coated the blastocoelic surface of the ICM now proliferates to form a coating of visceral endoderm (VE) that surrounds the embryonic part of the egg cylinder (see Fig. 4.1). The radial symmetry of these tissues is broken by the specification of the antero-posterior axis, which thereby defines the body plan of the individual. This subsection describes how molecular asymmetries first emerge in the extraembryonic tissues before they become conferred upon the underlying epiblast, well in advance of the formation of the primitive streak which is the topic of the next subsection.

The first evidence of proximo-distal identity in the egg cylinder is the detection of \textit{Hex} transcripts in the visceral endoderm at the distal pole of the cylinder at E5.5 [165] (labelled DVE in Fig. 4.1). DiI labelling of these cells demonstrates that they migrate unilaterally to contribute to the \textit{Hex}-expressing anterior visceral endoderm (AVE) by E6.0, producing an overt molecular asymmetry in the extraembryonic tissues that precedes the formation of the primitive streak [165].

It is unclear what causes \textit{Hex} expression to become localised to the distal visceral endoderm (DVE), though Hiramatsu \textit{et al.} propose that the mechanical confinement of the peri-implantation embryo may be responsible. Using a PDMS microcavity culture system, the authors observe that transient lateral confinement induces a localised breach of the basement membrane at the distal pole of the embryo, with transmigration of epiblast cells into the \textit{Cer1}-expressing DVE [76]. A degree of mechanical confinement, comparable to that imposed by the uterine tissues, is essential for this process to occur.
It remains unclear, however, whether the specification of the DVE is wholly mediated by the stress distribution across the cup-shaped epithelium under confinement. An important caveat is that this mechanism is likely to be specific to the cup-shaped rodent embryo since hoofed mammals establish a complete body plan prior to implantation [43]. An alternative hypothesis is that the \textit{Lefty1}-expressing precursors of the DVE migrate away from the high proximal Wnt and Nodal activity to collect at the distal extremity of the egg cylinder (reviewed in [3]), though this behaviour has not been observed directly and would suggest that the cells are specified earlier than previously thought.

Once formed, the cells of the DVE begin to express negative regulators of Wnt, BMP and Nodal signalling such as Dkk, Cerl and Lefty1. The activity of these signalling pathways is highest proximally in the extraembryonic ectoderm and proximal VE, establishing a molecular proximo-distal polarity that may reflect graded signalling activity [3]. Following the proximal migration of the DVE, these cells and those of the surrounding VE continue to express these signalling antagonists, thereby demarcating a region of the epiblast for anterior neural development and restricting the propensity for primitive streak formation to the opposing proximal posterior side. The opposing configuration of the AVE and the future primitive streak is suggestive of opposing signalling centres that divide the developing embryo into anterior and posterior halves (see Fig. 4.2).

The involvement of the AVE in the development of the forebrain has been demonstrated by the removal of a small portion of the AVE during gastrulation, which selectively compromises forebrain development while leaving \textit{Stra7}/\textit{Gbx2} expression in the midbrain unaffected [164]. In addition, \textit{Hesx1} expression in the neurectoderm can be completely eliminated by the removal of the AVE during early or mid-gastrulation, but not during late gastrulation, indicating that the action of the AVE is coincident with the appearance of the primitive streak. The effect of AVE removal on forebrain development
could either be interpreted as the removal of a “forebrain inducer” or the conversion of forebrain tissue into another cell type by leaving the prospective forebrain exposed to other inductive signals.

The results of Thomas et al. [164][165] support the latter possibility: that the role of the AVE-derived signalling antagonists in the development of the rostral CNS may be instead to demarcate a region of the epiblast that is protected from the posteriorising signals at the streak. Evidence for a permissive, rather than instructive role is provided by transplantation and explant culture of the AVE: when the AVE or anterior epiblast is transplanted into the lateral portion of a late-streak stage embryo, they do not induce the ectopic expression of anterior neural markers [159]. Instead, Tam et al. describe how the anterior-most tissues of secondary body axes are only produced when a fragment of the posterior epiblast is grafted in addition to both of the anterior tissues [159]. The authors suggest that the AVE may act to prime a region of the anterior epiblast that is later induced by the streak-derived mesendoderm (provided by the posterior epiblast graft) to undergo forebrain morphogenesis. The action of both tissues, initially on opposing sides of the embryo, is required to correctly pattern the neuraxis. Consistent with this model, Kimura et al. demonstrate that explant cultures of embryonic ectoderm and AVE do not express anterior neural markers (such as Hesx1 and Six3) but that the AVE suppresses the expression of posterior genes (such as T and Cripto) [90].

The role of the AVE in regulating the primitive streak is evidenced by Cerl−/−; Lefty1−/− double mutant embryos (see Table 4.1), which have an enlarged anterior primitive streak and occasionally develop an ectopic primitive streak [124] (reviewed in [3]). The partial rescue of this phenotype in embryos that are also heterozygous for Nodal demonstrates that the AVE regulates the size and positioning of the primitive streak through the modulation of Nodal signalling [124]. The authors describe how the ectopic streak phenotype is not rescued in chimaeric embryos with compound mutant VE
and wild-type epiblast, demonstrating that *Cerl* and *Lefty1* are required in the extraembryonic tissue for the correct positioning of the streak.

An important feature of embryonic development is that anteroposterior polarity first becomes apparent in the extraembryonic tissues before it is conferred on the epiblast itself [8] [164]. This is exemplified by the expression of *Hesx1* in the AVE and subsequently in the underlying anterior neurectoderm and by Nodal posteriorly, which is first expressed in the proximal posterior VE, before it is expressed in the underlying epiblast. These patterns of expression are also suggestive of an ongoing role for the posterior visceral endoderm in maintaining the primitive streak once it has formed.

### 4.1.2 Gastrulation in the Mouse Embryo

Gastrulation in the mouse\(^1\) forms a layer of mesoderm between the apposing layers of ectoderm (epiblast) and endoderm (VE) through the localised EMT and migration of epiblast cells at the primitive streak (see Fig. 4.2). The primitive streak begins in a proximal posterior position at the junction of the extraembryonic and embryonic tissues and it spreads antero-distally towards the distal pole of the embryo. The appearance of the streak defines the midline of the embryo and dorso-ventral patterning of the mesoderm is achieved by the timing of cellular ingression through the streak [162].

Gastrulation converts the bilaminar cup of epiblast and visceral endoderm into a trilaminar structure through the emergence of the mesoderm between these two layers. The body plan of the embryo is co-ordinated through the primitive streak, which extends distally from the proximal posterior epiblast towards the prospective anterior. By the end of gastrulation, the non-ingressed epiblast that will form rostral nervous system and surface ectoderm is located on the anterior, internal side of the cup. Moving towards the ex-

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\(^{1}\) Tam and Gad provide a detailed description of this process [162], which deserves due credit as it has informed much of the following discussion.
4.1. INTRODUCTION

Table 4.1: Relevant Anterior-Posterior Patterning Mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{Lefty2}^{-/-}$</td>
<td>AVE still forms. From E7.5: Expanded primitive streak and excess mesoderm. Partially suppressed by Nodal$^{+//-}$.</td>
<td>[113]</td>
</tr>
<tr>
<td>$\text{Cerl}^{-/-}; \text{Lefty1}^{-/-}$</td>
<td>Expanded anterior primitive streak, lack of mid-streak fates, ectopic primitive streaks; partial rescue in Nodal$^{+//-}$ background.</td>
<td>[124]</td>
</tr>
<tr>
<td>$\text{Wnt3}^{-/-}$</td>
<td>Primitive streak does not form. AVE markers are expressed and correctly positioned.</td>
<td>[104]</td>
</tr>
<tr>
<td>$\text{Nodal}^{-/-}$</td>
<td>No AVE or primitive streak; gastrulation fails and mesoderm is largely absent. Extraembryonic and embryonic ectodermal hyperplasia.</td>
<td>[83] [194] [31] [32] [20]</td>
</tr>
<tr>
<td>$\text{Smad2}^{-/-}$</td>
<td>Deregulated expression of proximal posterior markers throughout the entire epiblast.</td>
<td>[20]</td>
</tr>
</tbody>
</table>
Gestational age (E/dpc)
Theiler Stage (TS): Notes

6.5 (6.25-7.0)
TS9b: Early streak; gastrulation starts, first evidence of mesoderm. Primitive streak at 50% extension to distal tip.

7.0 (6.5-7.25)
TS10a: Mid-streak; amniotic fold starts to form. Primitive streak at 75% extension to distal tip.

7.0 (6.75-7.75)
TS10b: Late streak, no (allantoic) bud. Anterior end of primitive streak at distal tip.

7.0 (6.75-7.75)
TS10c: Late streak, early bud; first evidence of the allantoic bud, node prominent but not exposed.

Figure 4.2: Gastrulation Stages of Mouse Embryogenesis. Left, schematics of sagittal sections of the mouse embryo and extraembryonic tissues from the early streak stage (top) to the late streak stage (bottom). The epiblast is shown in pink, the extraembryonic tissues in tan and the mesoderm in turquoise. The turquoise bar to the right of the embryo denotes the extent of primitive streak extension towards the distal tip. Images adapted from EMAP Theiler Stage Definition under a Creative Commons Attribution Licence 3.0. Right, notes on staging from the eMouse Atlas Project [130], updated with reference to [99].
ExE, Extraembryonic Ectoderm; No, Node; PS, Primitive Streak; (A/D)VE, (Anterior/Distal)Visceral Endoderm.
terior, the mesoderm surrounds the ectodermal tissue in the anterior and forms the bulk of the posterior tissue. On the outside of the cup, the visceral endoderm has mixed with and been displaced by the definitive endoderm that has emerged from the streak, that will go on to form the gut. The inverted configuration of these tissues is corrected by the morphogenesis of “turning” around a day later, which converts the tissues from a lordotic to a foetal arrangement (see Fig. 4.4). This subsection examines the mechanics of gastrulation at the primitive streak, before considering the extent to which cells fates are specified by this process.

Cells at the primitive streak locally degrade the basement membrane and undergo an EMT to migrate as a loose mesenchyme, forming a layer between the epiblast and the overlying endoderm. The epiblast anterior to the primitive streak proliferates rapidly, with a cell cycle time as short as three hours [143]. The mesoderm that forms first migrates proximally to contribute to the extraembryonic mesoderm and laterally, to form the cranial and cardiac mesoderm [158] (see Fig. 4.3). As more of the epiblast passes through the streak, the cardiac and cranial mesoderm migrates laterally and anteriorly, eventually adopting an anterior position. The mesoderm that emerges during this time forms progressively more posterior and more axial subtypes, producing the lateral plate mesoderm before the paraxial mesoderm at mid-to late-gastrulation, finally giving rise to the axial mesoderm of the notochord shortly before the node forms at the distal tip of the embryo. During the emergence of the mesoderm, the cells of the epiblast that will go on to form the surface ectoderm move posteriorly, towards the streak but do not pass through it. Coupled with the anteriorly directed extension of the streak, this gives an apparent anterior-ward “orthogonal rotation” to the embryonic tissues about the left-right axis. This tissue movement echoes the earlier changes in gene expression in the VE that convert the proximo-distal axis into the future antero-posterior axis.
The definitive endoderm (DE) is thought to form during gastrulation through the intercalation of epiblast cells into the VE layer at the mid-streak stage (reviewed [162]). The migration of these cells appears to be co-incident with that of the mesoderm. Consistent with the orthogonal rotation described earlier, the intercalation of the DE in the region immediately subjacent to the anterior primitive streak causes the VE to be displaced laterally and anteriorly (reviewed in [162]). In the same way that different mesodermal subtypes emerge at different stages of gastrulation, the first formed DE contributes to the foregut while cells that emerged later give rise to more posterior extents of the future gut.

The formation and extension of the primitive streak therefore positions distinct mesodermal and endodermal subtypes with respect to the ectoderm. The timing of ingression through the streak not only dictates this position in the antero-posterior direction but also to some extent in the medio-lateral and dorso-ventral directions. Lateral plate mesoderm emerges before the paraxial mesoderm and so occupies a position that is further from the midline and the future dorsal side of the embryo. The axial mesoderm, which emerges last, occupies the most medial, dorsal position after turning. Gastrulation is therefore responsible for both the correct positioning of the germ layers and finer regionalisation of prospective fates within these tissues.

It would therefore appear as if cells become specified through the ingression process and subsequent inductive interactions between neighbouring tissues, but fate mapping and transplantation experiments prove otherwise. In fate mapping the pre-streak and early-streak epiblast, Lawson et al. describe how the descendants of the labelled cells are not restricted to specific germ layers; the lineages are not, therefore, separated at the beginning of gastrulation [100]. As such, the fate maps produced for the pre-streak to early-streak epiblast do not have clearly defined boundaries, though the reproducible behaviour of specific regions of the epiblast indicates a degree of determinism at this stage of development. This is corroborated by the results of Tam et
al., who demonstrated that the anterior and posterior epiblast at 6.5dpc give similar contributions to the developing heart and cranial mesoderm on transplantation to the site of the heart precursors in the gastrula [158]. The potency of the epiblast is not, therefore, spatially regionalised in the way that fate maps would suggest, rather they indicate the relative probabilities of future tissue contributions from specific regions. Indeed, transplanting cells from the posterior epiblast to an anterior position causes these cells to contribute to the developing ectoderm [158].

The transplantation regime used by Tam et al. gives further insights into the role of gastrulation in influencing cell fate. On transplanting the posterior epiblast from a 6.5dpc donor to the equivalent position in a 7.5dpc host, the grafted cells no longer contribute to the extraembryonic mesoderm (as they do when transplanted orthotopically) and instead give rise exclusively to the embryonic component [158]. This suggests that cell fate is specified by the timing of ingression through the streak. Newly ingressed cells, however, retain a great deal of plasticity since returning nascent mesoderm at 7.5dpc to a younger posterior epiblast (6.5dpc) enables them to contribute to an expanded range of tissues. Specifically, the grafted cells additionally colonised the cranial mesoderm and somites, but notably not the lateral plate mesoderm [158]. These results suggest that gastrulation may restrict the potency of newly ingressed cells to some degree but it does not specify their fate, as they can be re-specified by a second ingression through the streak. Further details of cardiac fate specification are provided by transplantation directly into the heart field of the mesoderm. When posterior epiblast cells from an early streak stage gastrula are transplanted into the 7.5dpc prospective cardiac mesoderm, they participate in normal cardiac development. This result demonstrates that gastrulation can be bypassed for cardiac fate specification as the transplanted cells did not undergo ingression. Furthermore, moving nascent mesoderm from the distal region of an 7.0dpc embryo into the anterior region of a 7.5dpc embryo enhances its contribution to cardiac
tissue. This demonstrates that movement within the mesoderm itself is also dispensable for the specification of cardiac fate, which primarily occurs in the mesodermal heart field itself [158].

Fate maps of the mouse epiblast are invariably produced by correlating specific positions within the epiblast to their tissue contributions after gastrulation. Transplantation experiments and clonal analyses have shown, however, that the potency of the epiblast is not regionalised and instead it is the position of the cells that influences which tissues the cells will contribute to by dictating the order in which they ingress through the streak. The results of Tam et al. demonstrate that ingress through the streak does not result in a broad restriction of potency or specification of fate, rather these features of differentiation occur once the cells have adopted their final positions in the mesoderm at the end of gastrulation [158]. Gastrulation can, therefore, be considered to be a morphological process that positions the respective germ layers but one that does not determine cell fate (at least for the cardiac mesoderm discussed here). It effectively converts the position of cells within the radially symmetric epiblast to a set of interacting regions organised according to a bilateral body plan. The topological organisation of the different germ layers with respect to each other may be important for the correct regionalisation and patterning of cell fate through inductive tissue-tissue interactions that occur after gastrulation.

4.1.3 Development of the Rostral Nervous System

By the end of gastrulation, the migration of the mesoderm has positioned the different subtypes in relation to one another and the definitive endoderm has formed on the exterior of the embryo. The ectoderm that will go on to form the rostral nervous system (i.e. the tissues of the brain) remains almost in its original position in the distal anterior of the epiblast. As discussed in Subsection 4.1.1, the role of the AVE seems to be largely permissive in protecting a region of the epiblast from the signals of the forming primitive
4.1. INTRODUCTION

Gestational age (E/dpc)
Theiler Stage (TS): Notes

7.5 (7.0-8.0)
**TS11a**: Early pre-headfold; head process developing, amnion complete.

7.5 (7.25-8.0)
**TS11b**: Late pre-headfold; elongated allantoic bud.

7.5 (7.25-8.0)
**TS11c**: Early headfold. Neural groove along anterior midline. Node exposed as an indentation in posterior half of the A/P axis.

7.5 (7.25-8.25)
**TS11d**: Late headfold; foregut invaginates. Prominent headfolds and cardiogenic plate. 1st somite boundary forms.

Figure 4.3: Neural Plate Stages of Mouse Embryogenesis. Left, schematics of sagittal sections of the mouse embryo and extraembryonic tissues from the early pre-headfold stage (top) to the late headfold stage (bottom). The epiblast is shown in pink, the extraembryonic tissues in tan and the mesoderm in turquoise (including the extraembryonic mesoderm). Images adapted from EMAP Theiler Stage Definition under a Creative Commons Attribution Licence 3.0. Right, notes on staging from the eMouse Atlas Project [130], updated with reference to [99].

AB, Allantoic Bud; CP, Cardiogenic Plate; HF, Head-fold; No, Node.
streak, since it requires the derivatives of the primitive streak to induce anterior neural fates [159]. It therefore does not seem to have a direct inductive or ”organising” role in the same way that the early organiser of the amphibian embryo can induce the formation of secondary rostral tissues through inhibition of BMP signalling (reviewed in [190]). In dissecting the phenotype of Foxa\(^{-/}\) mutant embryos, Yang and Klingensmith describe separate roles for the AVE and the streak-derived axial mesendoderm (AME) in forebrain development in the mouse. The axial mesendoderm emerges from the anterior-most limit of the streak and extends anteriorly, coming to underlie the forming tissues of the rostral nervous system by the headfold stages [190] (see Fig. 4.3).

Foxa2 mutant embryos lack the tissues of the mid-gastrula organiser (the anterior tissues of the primitive-streak at mid-streak stages) and often show forebrain truncations. In these embryos, markers of the forebrain (Six3 and Hesx1) are only expressed weakly and in randomly positioned patches in approximately 27% of cases [190]. At pre-streak stages, these embryos do not seem to form the AVE correctly; instead markers of the AVE remain expressed at the distal tip of the embryo (19% of cases) or not at all (81% of cases) and BMP signalling is ectopically active throughout the embryo. Restoring gene function to the AVE only results in a partial rescue of the forebrain tissues, which form but are not maintained after neurulation. The maintenance of this tissue is achieved by the AME derived from the rostral node, which expresses some of the same genes as the AVE [165]. Experiments with explant cultures of neurectoderm demonstrated that exogenous BMP has an inhibitory effect on forebrain gene expression that can be blocked by co-culture with the AVE [190]. Yang and Klingensmith therefore suggest a model in which the AVE and mid-gastrula organiser promote forebrain development through BMP inhibition during the extension of the streak. Once the AME has formed, this maintains BMP inhibition in the overlying neurectoderm until at least E8.25 [190].
4.1.4 Formation of the Posterior Body

The development of the axial tissues in the mouse is discussed in more detail in the introduction to Chapter 5. At the completion of gastrulation, a relatively large proportion of the embryo will go on to form the rostral tissues of the brain, while a relatively small region will form the entire length of the spinal cord and its associated trunk mesoderm [151][152]. At this point, the notochord has started to form and is evident as the axial mesendoderm that underlies the forming neural plate. As the node apparently regresses, the notochord elongates through a combination of cell division, cell re-arrangement within the notochordal plate and cell accretion at the interface with the node [138]. This process is supplied by a resident population of cells in the antero-medial sector of the node (described in the chick in [140]). A similar progenitor population exists on the opposite side of the node at its border with the primitive streak (the node-streak border, NSB). Cells from this region and the neighbouring caudal lateral epiblast (CLE) contribute to both the forming neural tube and paraxial mesoderm of the forming body from a population of neuro-mesodermal progenitors (NMps) [22]. After the closure of the posterior neuropore, the NMp population continues to contribute to the axial tissues from the chordo-neural hinge (CNH) [21], progressively generating the tissues of the tail (see Fig. 4.1 and Chapter 5, Fig. 5.2). Posterior growth ceases by E13.5, by which point the embryo has a well formed set of limbs and a tail; the realisation of the body plan is complete.

In summary, the early post-implantation development of the mouse embryo is reliant on extensive interactions with the extraembryonic tissues in order to demarcate the tissues of the rostral nervous system and to position the primitive streak on the opposing, proximal posterior side of the embryo. Gastrulation positions different mesodermal subtypes in relation to the antero-posterior organisation of the embryo and the midline of the streak, with the timing of ingestion through the streak determining the dorso-ventral organisation of these tissues [161]. Ingression does not, however, determine
**Gestational age (E/dpc)**
**Theiler Stage (TS): Notes**

8.0 (7.75-8.5)
**TS12a:** 1-4 somites; allantois extends (not shown), 1st branchial arch forms, heart starts to form, foregut pocket visible.

8.0 (7.75-8.5)
**TS12b:** 5-7 somites; allantois contacts chorion (not shown). Linear heart tube forms, anterior neural folds elevate.

8.5 (8.0-9.25)
**TS13:** Turning; second branchial arch forms.

9.0 (8.5-9.75)
**TS14:** Formation & closure of anterior neuropore; 3rd branchial arch. Forelimb buds absent.

9.5 (9.0-10.5)
**TS15:** Formation of posterior neuropore; forelimb buds form, hindlimb buds absent.

10.0 (9.5-10.75)
**TS16:** Posterior neuropore closure; formation of hindlimb and tail buds.

Figure 4.4: **Somitogenesis Stages of Mouse Embryogenesis.** Left, schematics of sagittal sections of the mouse embryo and extraembryonic tissues from the 1-4 somite stage (top) to tailbud closure (bottom). Images adapted from EMAP Theiler Stage Definition under a Creative Commons Attribution Licence 3.0. Right, notes on staging from the eMouse Atlas Project [130], updated with reference to [99]. D, Dorsal; V, Ventral.
cell fates, which are acquired some time after cells have passed through the streak [158]. The rostral tissues of the embryo form from precursors that were present during gastrulation and through interactions with the axial mesendoderm, while the caudal tissues arise progressively from a population of axial progenitors [21] in the posterior growth zone.

With this description in mind, an organoid model of embryonic development would be expected to contain both embryonic and extra-embryonic tissues, to undergo an EMT-like process from a localised region and to elongate in the antero-posterior direction, first through re-arrangement of existing tissue and subsequently from a posterior growth zone. Section 4.2 describes how well the development of the gastruloids conforms to this description in recreating the events of early post-implantation development.

4.1.5 Following Development in vitro

This chapter characterises the development of the gastruloids over time by making use of fluorescent reporter cell lines to follow the activity of specific genes, pathways or states. One example to consider in more detail is the DRC cell line, developed by Parchem et al. [122], which allows the cellular transitions from pre-implantation to early gastrula stages to be followed in vitro.

The mir-290-mCherry/mir-302-eGFP line reports the expression of two micro-RNA clusters that are differently expressed by cells in different states of pluripotency (naïve/primed, respectively) and it has been used to monitor the re-acquisition of pluripotency during cellular reprogramming [122]. When assessed in vivo, the authors describe how all cells of the embryo expressed the reporters in a “sequential and stereotypical fashion.” ESCs derived from E3.5 ICM showed a heterogeneous mix of reporter states in serum + LIF culture, which subsequently progressed through the naïve (red), pre-primed (yellow) and primed (green) states as they differentiated. E5.5 epiblasts in culture showed the same progression from a pre-primed to primed state,
while EpiSCs derived from embryos expressed eGFP alone [122]. This system therefore provides a useful tool for instantaneously assessing the state of gastruloid progression through the culture protocol.

### 4.2 Results

#### 4.2.1 Gastruloids Can Spontaneously Break Symmetry and Polarise in the Absence of Extra-Embryonic Tissues (0-48 Hours)

Within the first 18 hours of gastruloid development, the single cell suspension coalesces in the bottom of each well to form a small aggregate of cells approximately 120 \( \mu \text{m} \) in diameter. Small satellite aggregates often join the main aggregate during this time. At this stage, it is composed of a heterogeneous mixture of cells, as evidenced by the presence of red, yellow and green cells from the \textit{mir-290-mCherry/mir-302-eGFP} (DRC) cell line (Fig. 4.5B and C). By the 24 hour time point, most of the cells in the main aggregate appear red (Fig. 4.24B), indicating that the cells of the aggregate remain in a naively pluripotent state at this early stage [122]. Over the subsequent 24 hours, the cells in the aggregate start to become pre-primed, as shown by the rising expression of the mir-302-eGFP reporter in this period (Fig. 4.24).

Aggregates at the 48 hour time point are morphologically spheroid but some show polarised gene expression. This is particularly clear for T/Brachyury, which is expressed in a uniform manner during the first 48 hours, but which often becomes localised to one side of the aggregate in this period, defining the future posterior tissue.

As described in Section 4.1.1, the posterior pole of the gastrula is associated with a region of elevated Wnt signalling activity. The \textit{TCF/Lef:mCherry} ES cell line uses six multimerised TCF/Lef binding sites upstream of a Histone
Figure 4.5: **Cellular Heterogeneity in Early Aggregates.** (A) Bright-field images demonstrating the progressive aggregation of a T/Bru::GFP single cell suspension within the first 24 hours of development. (B) Suspension of DRC cells 90 minutes into aggregation. Most cells are red (mir-290-mCherry⁺; mir-302-eGFP⁻), corresponding to naively pluripotent cells while others are yellow (mir-290-mCherry⁺; mir-302-eGFP⁺) to green (mir-290-mCherry⁻; mir-302-eGFP⁺), corresponding to a primed, epiblast-like state [122]. (C) Confocal section of a satellite aggregate 19 hours after plating, showing that these heterogeneities persist into the early aggregate. Scale bars correspond to 200µm in (A) and (B); 50µm in (C).
2B-mCherry fusion protein that is under the control of a minimal promoter to provide a live read-out of Wnt signalling activity [50]. In the embryo, the GFP variant of the reporter was detected first in the visceral endoderm layer of the pre-streak embryo, before becoming robustly expressed in the cells of the posterior epiblast, the primitive streak and the nascent mesoderm at gastrulation [52]. At the later 7-9 somite stage (8.5dpc), the reporter remained active in the presomitic mesoderm and the newly formed somites at the caudal end of the embryo, in addition to the posterior neural plate and some anterior neurectodermal derivatives [52]. The TCF/Lef:mCherry reporter therefore provides a read-out of posteriorly-localised Wnt signalling activity from the gastrula to early somitogenesis stages.

On forming gastruloids from the TCF/Lef:mCherry cell line, mCherry expression was found to follow the same pattern as that of T/Bra::GFP during the first 48 hours: it can often be found to be localised to one side of the aggregate (Fig. 4.6) while in others it is uniformly expressed\(^2\).

At the 48 hour time point, the T/Bra::GFP reporter was not detectable at levels above background in 4/11 cases. In one case, it was expressed uniformly throughout the aggregate and in six cases, it was clearly polarised to one side of the aggregate (the standard quantification method is described in Chapter 2: Materials & Methods). The mean fluorescence traces (blue in Fig. 4.6B and C) capture this pattern as the normalised fluorescence value is elevated above the background level in the first half of the aggregate (around 0-50\(\mu\)m in (B) since the mean diameter is 102\(\mu\)m; 0.0-0.5 in (C)).

This early polarity is also evident in the expression of the TCF/Lef:mCherry reporter line. At the 48 hour time point, it was expressed uniformly in 5/11 aggregates and in a polarised manner in 6/11 aggregates. As for the T/Bra::GFP reporter, quantifying the fluorescence across the aggregates

\(^2\)Thanks are due to Amy Horrell, who prepared and imaged these samples as part of her Master’s degree. Data processing and analysis were performed by the author.
Figure 4.6: Polarised Expression of T/Bra::GFP and TCF/LEF::mCherry at 48 Hours After Aggregation. Examples of polarised aggregates from the T/Bra::GFP and TCF/LEF::mCherry lines are presented in (A) and (D) respectively. (B) and (E) present the normalised fluorescence traces measured along a line bisecting the aggregate from putative posterior to anterior (yellow arrows in (A) and (D)) in two samples of 11 aggregates (see Chapter 2: Materials & Methods). The mean background-subtracted trace is shown in blue. The graphs in (C) and (F) show each trace normalised to both its length and to the highest recorded fluorescence value. The filled blue region represents one standard deviation above and below the mean fluorescence trace. The higher mean fluorescence on the left of each plot shows that the expression of these reporters is often polarised in the population at this time.
from the region of highest signal to the lowest (putative posterior to anterior), the mean fluorescence is higher in the first half of the aggregate, reflecting the tendency for the signal to be polarised at this time.

In order to test whether the polarisation during the first 48 hours is dependent on a visceral endoderm-like tissue, gastruloids were produced from a cell line that reports *Gata6* expression (*Gata6<sup>H2B-Venus</sup>). *Gata6* is the earliest known marker of the primitive endoderm lineage and its expression is maintained in the visceral endoderm up to gastrulation [56]. After gastrulation, *Gata6* expression is correlated with mesodermal and endodermal progenitors, notably those of the cardiac tissues and gut endoderm [56]. *In vivo*, the *Gata6<sup>H2B-Venus</sup>* reporter is strongly correlated with endogenous *Gata6* protein from preimplantation stages through to adult organs [56]. The reporter is not expressed in self-renewing cultures of ES cells but is activated upon endodermal differentiation *in vitro* [56]. On culturing the *Gata6<sup>H2B-Venus</sup>* ESCs as gastruloids, the reporter was not expressed during the period of polarisation from 0-48 hours after aggregation (Fig. 4.11; n=10 for the 24 and 48 hour time points). The lack of *Gata6<sup>H2B-Venus</sup>* reporter expression at this time indicates that the polarisation process occurs in the absence of a visceral endoderm-like tissue.

4.2.2 Exogenous Wnt Signalling Reinforces A Pre-Existing Pattern (48-72 Hours)

In order to further investigate whether any AVE-like tissue is present during this period of polarisation, aggregates were prepared from the *T/Bra::GFP* cell line for analysis by qRT-PCR at the 48 hour time point and after a further 24 hour treatment with either 3µM Chiron or an equivalent volume of DMSO (Fig. 4.7; 64 individual aggregates pooled per sample)<sup>3</sup>.

<sup>3</sup>Thanks are due to Dr Penny Hayward for her assistance in running the PCR and analysing the data and to Dr David Turner for producing the figure, which has previously been published in a pre-printed manuscript [171].
Figure 4.7: Gastruloid Gene Expression at 48 and 72 Hours Resembles that of the Embryo at the Onset of Gastrulation. 64 aggregates were pooled and lysed at the 48 hour time point and at the 72 hour time point, either with a 24 hour treatment with 3\(\mu\)M Chiron (C) or with an equivalent volume of DMSO (D). qRT-PCR expression data were normalised to the housekeeping gene *PPIA* and are representative of two replicate experiments. Error bars indicate the standard deviation of triplicate samples. This figure has previously featured in pre-print manuscripts in this form [171] and as a heatmap [169].
In keeping with the lack of Gata6 expression at the 48 hour time point, the PCR data indicate that the VE-produced signalling antagonists Dkk, Chordin and Noggin are not expressed at this time (not shown in Fig. 4.7) and that Cerberus is present at very low levels. The expression of FGF4, FGF5, Axin2, Wnt3, Nodal and Lefty1 at this time resembles the profile of gene expression in the E6.0 epiblast. This pattern becomes consolidated after 24 hours’ treatment with DMSO, with the opposing changes in expression of FGF4 and FGF5 reflecting a maturation of the epiblast.

The expression of targets of the Wnt signalling pathway increases following exposure to Chiron from 48 to 72 hours after aggregation. This is clear for Nodal, Lefty, Wnt3a, Dkk1, Axin2 and T/Brachyury (Fig. 4.7). The expression of Wnt3a and Dkk1 during this time are suggestive of the onset of gastrulation, suggesting that aggregates at the 72 hour time point might correspond approximately to the E7.0 embryo [171]. This is corroborated by the expression pattern of the DRC cell line (Fig. 4.24), which shows saturating levels of mir-302-eGFP expression and continued expression of the mir-290-mCherry reporter. mir-290 is no longer expressed in the epiblast from E7.5 in vivo [121], suggesting that the gastruloids at the 72 hour time point resemble the late-gastrulation stage epiblast.

Live imaging of the T/Brachyury::GFP reporter cell line shows that T/Brachyury becomes progressively activated across the aggregate. Expression spreads from the initial pole of expression at the 48 hour time point (approximately 20% of the aggregate’s length, see Fig. 4.8) to become expressed across the whole tissue 24 hours later. The Chiron treatment therefore builds upon the existing pattern in the aggregates at this time and by doing so, stabilises it.
Figure 4.8: 3µM Chiron Treatment Stabilises and Reinforces Pre-Existing T/Bra::GFP Expression from 48-72 Hours in Culture. (A) GFP signal from one T/Bra::GFP aggregate, shown every six hours from 48 to 72 hours in culture. The periphery of the aggregate is shown as a white dashed line, with a yellow line indicating the axis of polarity. (B) Corresponding measurements of T/Bra::GFP signal along the axis of polarity from highest to lowest signal. The mean background fluorescence value was subtracted from each trace and they have been normalised to the highest recorded value and to the length of the aggregate. T/Brachyury expression spreads and intensifies from initial expression in 20% of the aggregate at 48 hours to across its entire length by the 72 hour time point.
Figure 4.9: T/Bra::GFP Expression is Retained in the Posterior Elongating Region After Removal of Chiron. (A) The GFP signal from one T/Brachyury::GFP aggregate is shown at six hour intervals following the 24 hour pulse of 3µM Chiron treatment. The periphery of the aggregate is shown as a white dashed line and the axis of polarity, used to measure the fluorescence, is indicated in yellow. (B) Corresponding measurements of the T/Bra::GFP signal along this line. The mean background fluorescence has been subtracted from each value and the traces are normalised to both the maximum fluorescence recorded and the length of the aggregate. T/Brachyury expression is initially uniform but becomes progressively lost in the putative anterior region and retained in the tissue of the elongation.
Figure 4.10: **Gastruloids at the 72 hour Time Point Express Sox2, T/Brachyury and E-Cadherin.** Gastruloids were cultured with a pulse of 3µM Chiron from 48-72 hours and were fixed at the 72 hour time point. They were immunostained for T/Brachyury (green), E-Cadherin (white) and Sox2 (magenta). All three markers are expressed throughout the tissue, with some regionalisation into separate groups of cells. Scale bars represent 200µm throughout; expression levels are comparable to the 96 hour time point shown in Figure 4.18.
4.2.3 Antero-Posterior Polarity is Coupled to Elongation Morphogenesis (72-120 Hours)

The results of the qRT-PCR analysis (Fig. 4.7) and the development of the DRC gastruloids (4.24) suggested that the Chiron-treated gastruloids resembled the gastrulation stage epiblast at the 72 hour time point. In order to investigate whether this state is reflected in the regionalisation of the tissues, $T/Br::GFP$ gastruloids were exposed to 3µM Chiron from 48-72 hours and were fixed at the 72 hour time point. The gastruloids were stained for $T/Brachyury$ as an indicator of polarity, alongside E-Cadherin and Sox2, which are co-expressed in pluripotent cells prior to an EMT [173] (Fig. 4.10). At the 72 hour time point, $T/Brachyury$ expression is high and localised to one side of the aggregate and it can often be found in regions that co-express Sox2 and E-Cadherin. It therefore seems as if the gastruloids at this point resemble the epiblast at the point of cell ingression through the primitive streak; i.e. the $T/Brachyury^+$ cells may be starting to undergo an EMT.

After withdrawal of Chiron, the expression of $T/Bra::GFP$ remains high across the whole aggregate for approximately 12 hours, before it becomes polarised to one side by apparent loss from the future putative anterior region (Fig. 4.9). This pattern is shared by the Wnt signalling reporter (Fig.s 4.25A and 4.26A), as is expected with exogenous activation of this pathway. In both cases, polarised expression suggests that some cells of the aggregate are able to sustain high levels of Wnt signalling after Chiron withdrawal, indicating that asymmetries in cellular potential persist throughout the treatment. The retention of $T/Brachyury::GFP$ expression at a pole is coupled to a morphological change in the form of an elongation, which extends from this region (Fig. 4.9) and maintains expression of the reporter. This pattern is mirrored by the expression of the Gata6$^{H2B-Venus}$ reporter, which starts to be expressed at around 88 hours after aggregation (in 23/24 cases) and becomes localised to the cells of the putative anterior region (Fig. 4.11).
Figure 4.11: **Gata6\(^{H2B\text{Venus}}\) is Expressed After Aggregate Polarity has Been Established.** (A) Aggregates derived from the Gata6\(^{H2B\text{Venus}}\) cell line only begin to express the reporter after 48 hours in culture, by which point they have already established antero-posterior polarity. Subsequent time points are following a pulse of 3\(\mu\)M Chiron from 48-72 hours. Scale bars represent 200\(\mu\)m throughout. (B) Normalised fluorescence traces for the reporter along the posterior-anterior axis, where apparent. (C) Standard deviation about the mean for the traces in (B). \(n=10\) for the 24, 48, 96 and 115h time points; \(n=24\) for the 88h time point. The images in (A) have featured in a pre-print manuscript as Fig. 4C in [169].
During the elongation phase, aggregates formed from the DRC cell line show saturating levels of mir-302-eGFP expression (Fig. 4.24), suggesting that the tissues at this stage are younger than the E9.5 embryo, which loses expression of this micro-RNA cluster at this time [121]. In many cases, a small number of cells in the putative posterior show expression of the mir-290-mCherry reporter at this point. It has not been determined whether these cells retained expression of this cluster from 72 hours onwards, or whether they have lost and regained it.

Aggregates from the TCF/Lef:mCherry cell line were fixed and immunostained for T/Brachyury in order to correlate the expression patterns of these proteins. At the 120 hour time point, the domain of Wnt signalling activity is restricted to the elongating tissues, where it is expressed in a broad domain. T/Brachyury co-localises to the most posterior tissues of this region in a smaller domain at the tip of the elongation. The close correlation between the zone of Wnt signalling and that of T/Brachyury expression suggests that the elongating tissues correspond to the growing posterior end of the embryo (both a gastruloid and an embryo are shown in [176], Fig. 4).

4.2.4 Antero-Posterior Polarity Can Be Discerned by Inspection of Chiron-Treated Gastruloids at the 120-Hour Time Point

As described earlier, polarised T expression defines the putative posterior pole, as in the gastrulation stage embryo where it is expressed in the primitive streak [7]. During axial elongation, its expression is maintained in the notochord [182] and in the caudal tissues of the embryo (reviewed in [183]). This pattern is mirrored in the gastruloids, where at early stages it is expressed to one side of the spherical aggregates, while at later stages it becomes restricted to the end of the elongation. It is contended that during the elongation phase, the polarity of the gastruloids also becomes apparent
Figure 4.12: T/Brachyury Expression Co-Localises with Wnt Signalling Activity in the Elongating Region. TCF/Lef:mCherry gastruloids, cultured with 3µM Chiron treatment from 48-72 hours, were fixed at the 120 hour time point and were stained for Sox2 and T/Brachyury. Yellow arrows indicate the anterior-to-posterior extents of expression in each channel. Sox2 is expressed at very low levels in a broad domain across the elongation. TCF/Lef:mCherry expression localises to a smaller, more posterior, region while T/Brachyury is expressed highly in the most posterior tissues at the tip of the elongation. Images are average intensity projections of confocal z-stacks; scale bars represent 200µm throughout. The bottom row of this figure has been previously published as Fig. 8C in [172].
Figure 4.13: Poles of T/Bra::GFP Expression in Chiron-Treated Gastruloids Can Be Predicted from Phase Contrast Images Alone at the 120-Hour Time Point. Four sets of phase contrast images (A) were provided by Dr David Turner and were scored by the author for potential poles of T/Bra::GFP expression using optical and morphological cues alone. Some adjacent image pairs show overlapping images of the same gastruloid for stitching; in this case only one image was scored. The scores were marked by Dr Turner with reference to the corresponding GFP channel (B) independently of the author. The results of the scoring were categorised as True Positives, False Positives, True Negatives or False Negatives and are summarised in Table 4.2.
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Table 4.2: **Summary of Blind Scoring 120-Hour Chiron-Treated T/Bra::GFP Gastruloids on Phase Contrast Images Alone** Numbers beginning “04-” refer to specific datasets from Dr David Turner. The overall Precision and Recall metrics were calculated by pooling the scores from all four datasets. For examples of the scoring process, see Fig. 4.13.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>04-102</th>
<th>04-103</th>
<th>04-085</th>
<th>04-088</th>
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<td>12</td>
<td>12</td>
</tr>
<tr>
<td>True Negative</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>False Negative</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Overall Precision:</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.857</td>
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<tr>
<td><strong>Overall Recall:</strong></td>
<td></td>
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<td>0.938</td>
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</tbody>
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by phase contrast microscopy. Specifically, the surface appearance and optical brightness of the tissues changes: the putative anterior becomes opaque and is associated with a loose surface layer of cells, while the putative posterior is more translucent and appears smooth.

In order to test whether these surface properties are a reliable indicator of antero-posterior polarity (assessed by T/Bra expression), four small test datasets were scored blind by the author, who indicated the location of suspected regions of T/Bra::GFP expression on the phase contrast images alone (see red circles in Fig. 4.13A, or red crosses where no pole was suspected). These datasets were collected independently by Dr David Turner, who also marked whether the scoring was correct (blue ticks and red annotations in Fig. 4.13A). Independent data were selected for the test set to ensure that they were separate from the training set (*i.e.* any images previously collected by the author). The author did not see any images from the GFP channel until after all the data had been scored and marked (see Fig. 4.13B).

The results of scoring the test dataset are summarised in Table 4.2, with the Precision and Recall metrics calculated for the pooled results as “True
Positives ÷ All Positive Guesses” and “True Positives ÷ Total Poles Available to Score,” respectively. A perfectly precise method would have a Precision of 1.0 and would never call false positives (i.e. every guess would be correct). A perfect score for Recall would also be 1.0; in this case, the method identifies every pole in the dataset without calling any false negatives. The Precision of phase contrast classification was determined to be 0.857 while the Recall of the method was 0.938. A broad interpretation of these results is that the author can identify many potential poles in such a dataset, some of which are irrelevant to the true pattern of expression (i.e. are false positives), but with the result that a high proportion of the genuine poles are captured within all positive guesses.

4.2.5 MEK/ERK-Signalling is Required for the Establishment and Maintenance of the Elongations

*Sox1::GFP* aggregates were exposed to a variety of treatments in the 48-72 hour period and the 72-120 hour period to determine the signalling pathways involved in regulating the formation and extension of the elongations. The aggregates were imaged at the 120 hour time point and were scored qualitatively for the presence of any elongations (whether singly or as multiple elongations) following different treatments. The results of this scoring are presented in Figure 4.14.

Aggregates grown in the presence of 3µM Chiron in the 48-72 hour time period show a high frequency of elongations (C-N, 76.19%). The elongation of the embryo is dependent on continued FGF-signalling in the axial progenitor zone (reviewed in [183] and [73]) and FGFR1 mutants show caudal truncations, ectopic neural tubes and defects in paraxial mesoderm formation [183] (see Chapter 6). Exogenous FGF2 was therefore added to the cultures either following (C-F), co-incident with (CF-N, CF-F) or instead of the 48-72 hour Chiron treatment (F-F) to test whether it would promote the elongation process. In all cases, the frequency of elongations did not change
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Figure 4.14: MEK Signalling Inhibition From 48-72 Hours or 72-120 Hours Significantly Reduces the Frequency of Elongations. Aggregates from the Sox1::GFP cell line were treated as indicated in (A) and were qualitatively scored for their morphology at the 120 hour time point. (B) The percentage of elongating aggregates in each condition (whether as a single elongation or multiple ones). A statistical test of Binomial Proportions was used to compare pairs of treatments; n=21 per treatment. Aggregates exposed to the MEK inhibitor PD03 from 72 hours show a significantly reduced frequency of elongation (p=0.00068, ***), and similarly for exposure from 48-72 hours (p=0.00543, **), indicating a role for MEK1-ERK1/2-signalling in the establishment and maintenance of the elongations. The data presented in this figure have previously been published as Fig.s 9 and S7 in [172].
significantly when compared to the 48-72 hours Chiron condition (C-N). This indicates that the endogenous levels of FGF produced by the aggregates are sufficient to support the growth of the elongations, or that FGF-signalling is not required for this process.

In order to address the latter possibility, MEK/ERK signalling downstream of FGF receptor stimulation [120] was specifically inhibited using PD03; either following the Chiron treatment (C-P, Fig. 4.14) or co-incident with it (CP-N, Fig. 4.14). At a 1µM dosage, PD03 specifically inhibits MEK1 [6] and so disrupts the transcriptional response to FGF stimulation [120]. It does not, however, inhibit other pathways downstream of the activated receptor such as the pro-survival effects of activated PI3K/AKT pathway, which stimulates cell growth and proliferation, nor the increase in intracellular calcium levels triggered by PLCγ signalling [120]. Furthermore, the inhibition of MEK/ERK signalling with PD03 also affects the activity of other growth factor receptor tyrosine kinase signalling pathways that converge on the MAPK cascade, such as PDGF signalling.

On treating the gastruloids with PD03, the frequency of elongations was significantly reduced. This effect is clear whether the inhibition is restricted to the 48-72 hour period (C-N vs. CP-N) or whether it is continuous from 72 hours (C-N vs. C-P). This suggests that MEK1-ERK1/2 signalling is essential in establishing the elongations in the early phase of the culture, before the aggregates have started to change shape and in maintaining the elongation once morphogenesis has begun.

Collectively, the independence from exogenous FGF stimulation and the loss of elongations following PD03 treatment supports the possibility that endogenous FGF signalling is sufficient to support the development of the gastruloids. These results do not, however, rule out the possible action of other growth factor-receptor tyrosine kinase pathways that converge on MEK1-ERK1/2 signalling in regulating the elongation process. Further work using
specific, receptor-level inhibition of FGF signalling will be required to determine conclusively whether the elongations are regulated by this signalling pathway.

Canonical Wnt signalling was also selected as a candidate regulator of axial extension in the gastruloids since it remains active in the caudal end of the elongating gastruloids (see Figure 4.25), as it does in the embryo [154]. Furthermore, genetic experiments have demonstrated its fundamental roles in embryonic axial extension and segmentation. Wnt3a−/− mutants, for example, show an axial truncation phenotype caudal to the forelimb bud at E9.5 [154]. A more severe axial truncation phenotype is caused by the T/Bra-driven conditional inactivation of β-catenin, which also prevents the segmentation of the paraxial mesoderm into somites [42].

The small molecule inhibitor XAV939 was used to specifically inhibit Tankyrase 1 & 2 and so reduce the cytoplasmic concentrations of β-catenin through stabilisation of Axin [81]. Exposing the gastruloids to XAV939 from 48-72 hours (FX-N) or from 72-120 hours (C-X) did not produce a statistically significant change in the elongation frequency in comparison to untreated gastruloids (C-N). Further work will be required to determine whether manipulating canonical Wnt signalling at different stages of gastruloid development has any effect on their axial extension; in the 48-72 hour and 72-120 hour periods, this does not appear to be the case.

4.2.6 All Three Germ Layers are Represented in the Elongating Tissues

The tissue composition of the elongating region was determined by comparing the expression patterns of fluorescent reporter cell lines for the different germ layers. Gastruloids were formed from the Sox1::GFP [191], Tbx6::eYFP [67] and the Sox17::GFP [117] cell lines to report the neural, mesodermal and endodermal tissues respectively.
The \textit{Tbx6::eYFP} reporter cell line \[67\] was selected to follow mesodermal differentiation since \textit{Tbx6} is expressed in an overlapping but nonidentical domain with \textit{T/Brachyury} during gastrulation, where it marks the streak and the newly formed paraxial mesoderm of the future trunk \[24\]. After gastrulation, \textit{Tbx6} expression becomes highly restricted to the presomitic mesoderm of the caudal region of the embryo and is lost as this tissue segments into somites \[24\]. \textit{Tbx6} continues to be expressed in the unsegmented region of the tailbud until 12.5dpc \[24\]. It is therefore suitable as a specific marker of the paraxial mesoderm from gastrulation until the final day of tail development (from 7.0-12.5dpc). Gastruloids from each cell line were cultured with a pulse of 3\,$\mu$M Chiron from 48-72 hours and were imaged at the 120 hour time point. The results are presented in Figures 4.15, 4.16 and 4.17.

From the examples in Figure 4.15, Sox1::GFP appears to be expressed in the elongating region of the gastruloids, with small, discrete groups of cells in the putative anterior tissues. The expression of the reporter does not seem to be uniform throughout this tissue, however, since in some cases it appears to be lower in the tip of the elongation (bottom left in Fig. 4.15) or along the middle of the elongating tissue (top left in Fig. 4.15). This reporter suggests that a substantial part of the elongation has started to differentiate as neural tissue by this point.

The \textit{Tbx6::eYFP} reporter appears to be expressed throughout the aggregate, with low levels of expression in the elongating region and high expression in the putative anterior tissues (Fig. 4.16). In some cases, the anterior expression is localised to the edges of the gastruloid and to the loosely associated cells that adhere to its surface, suggesting that the reporter is expressed in cells on the exterior of the tissue. This reporter suggests that the gastruloids are forming tissue with a paraxial mesodermal identity and it is possible that this contributes to the external layer of cells that is loosely associated with the putative anterior.
Figure 4.15: The Neural Marker Sox1::GFP is Expressed in the Elongation and in Small Regions of the Putative Anterior. Sox1::GFP gastruloids were cultured with a pulse of Chiron from 48-72 hours and were imaged at the 120 hour time point. Sox1::GFP expression is localised to the elongating region and with reduced expression at the tip of the elongation in some cases. Scale bars represent 500 µm throughout. Images from this dataset have been published as Fig. 7A in [176].
Figure 4.16: The Mesodermal Marker $\text{Tbx6}^{\text{H2B-eYFP}}$ is Expressed at Low Levels in the Elongation and at High Levels in Cells Surrounding the Putative Anterior. $\text{Tbx6}^{\text{H2B-eYFP}}$ gastruloids were treated with a pulse of Chiron from 48-72 hours and were imaged at the 122 hour time point. Expression of the reporter is low in the elongating region (right of each image) and is high in the cells surrounding the putative anterior of the gastruloid (left of each image). Scale bars represent 200$\mu$m throughout.
Figure 4.17: The Endodermal Marker Sox17::GFP is Expressed in the Elongating Region and in the Putative Anterior. Sox17::GFP gastruloids were cultured with either a pulse of Chiron from 48-72 hours (A) or in N2B27 continuously (B) and were imaged at the 120 hour time point. In Chiron-treated gastruloids, expression of the reporter is confined to the more anterior part of the elongation, with small groups of positive cells in the putative anterior. In the N2B27-treated gastruloids, the reporter is still expressed in the elongations (where present) and expression is higher in the anterior tissues. Scale bars represent 200μm throughout.
In contrast, the *Sox17::GFP* reporter appears to be expressed internally in the more anterior part of the elongation and into the anterior tissues (Fig. 4.17). In gastruloids treated with Chiron, the reporter is expressed in small, discrete groups of cells in the middle part of the elongation at a distance from the tip. In gastruloids cultured continuously in N2B27, the reporter appears to be more highly expressed in the anterior tissues but this expression still extends into the elongation, where it is present. These results suggest an endodermal component to the elongating tissue and taken with the results from the other reporters demonstrates that the elongations contain derivatives of all three germ layers.

In order to look more closely at the tissue organisation of the elongating region, gastruloids grown with a pulse of Chiron from 48–72 hours were fixed at the 96 and 120 hour time points for immunostaining. E-Cadherin was chosen as a particular protein of interest, since it is expressed early in the epiblast and later continues to be expressed in all proliferating epithelial tissues derived from the ectoderm and the endoderm [155]. Neural and mesodermal tissues, generally speaking, do not express E-Cadherin [155]. By examining E-Cadherin expression alongside Sox2, residual epiblast-like (or ectodermal) cells can be identified. Similarly, co-expression of Sox17 with E-Cadherin indicates likely definitive endodermal cells. In addition, its localisation to the cell membrane allows continuous epithelial tissues to be visualised; providing an indication of the overall organisation of the tissue.

Figure 4.18 shows the expression pattern of Sox2, T/Brachyury and E-Cadherin in *T/Bra::GFP* gastruloids at the 96 hour time point. The aggregates are orientated according to their polarity, with the highest T/Brachyury expression on the right of each image (defining the putative posterior). In each case, this region is associated with low Sox2 expression (magenta) in cells displaced a short distance from the tip of the elongation. T/Brachyury expression (green) is low and confined to the tip region. In many cases, the region of Sox2 and T/Brachyury co-localisation towards the tip of the
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Elongation also expressed E-cadherin (white).

The majority of the aggregate does not seem to express E-Cadherin, although in some cases it is highly expressed in coherent parts of the putative anterior tissues (see top right image in Fig. 4.18). In other cases, it is confined to small groups of cells in the anterior (bottom right, Fig. 4.18). In these examples, Sox2 is also highly expressed in a subset of the E-Cadherin+ cells.

A similar distribution of E-Cadherin expression is seen at the 120 hour time point, which is shown along with staining for Fibronectin in Figure 4.19. Imaging near the surface of the gastruloid shows widespread E-Cadherin expression (red) in the elongating tissue (as assessed morphologically, see Fig. 4.19(A’) and (B’)), but the level of expression is lower at the tip (D). Integrating this observation with the expression of the $T/Brachyury::GFP$ reporter (Fig. 4.26) suggests that this E-Cadherin-low region is correlated with the T/Brachyury-expressing tissue.

Confocal sections at a deeper level in the tissue show small groups of more anterior cells that express E-cadherin (red, Fig. 4.19(A) and (B)) and are encapsulated by Fibronectin (white, Fig. 4.19(C)). Aggregates stained at the 96 hour time point suggest that these groups of cells could also be expressing Sox2, though it is possible that expression has been downregulated 24 hours later.

In order to test whether the E-Cadherin+ population had a definitive endodermal identity, gastruloids grown with or without Chiron treatment were fixed at the 120 hour time point and were stained for E-Cadherin and Sox17 proteins (Fig. 4.20). Among Chiron-treated aggregates, E-Cadherin expression (red) follows the pattern described previously: it is expressed in the elongating tissue but at a low level (if at all) in the tip of the elongation,
Figure 4.18: **Sox2**$^+$ Cells are Associated with E-Cadherin Expression at the 96 hour Time Point. Gastruloids were cultured with a pulse of 3µM Chiron from 48-72 hours and were fixed at the 120 hour time point. They were immunostained for T/Brachyury (green), E-Cadherin (white) and Sox2 (magenta). Sox2 expression is correlated with the E-Cadherin$^+$ regions, including groups of cells in the prospective anterior (left of each image). Scale bars represent 200µm throughout; expression levels are comparable to Figure 4.10.
Figure 4.19: **E-Cadherin is Expressed in the Elongation and in Small Groups of Cells at the 120 hour Time Point.** Gastruloids cultured with a 3µM Chiron pulse from 48-72 hours were fixed at the 120 hour time point and were immunostained for E-Cadherin (red) and Fibronectin (white). The gastruloids in (A) and (B) show a similar pattern of expression, with a large region of E-Cadherin$^+$ cells in the main part of the elongation ((A’) and (B’)) and smaller, isolated groups of cells present towards the prospective anterior ((A) and (B)). Closer examination shows that these groups of cells are surrounded by Fibronectin (C) and that E-Cadherin is expressed at lower levels in the tip of the elongation (D). Scale bars represent 200µm throughout.
with occasional groups of cells in the putative anterior. Sox17 (white nuclei) is expressed in small numbers of cells at the surface of the aggregate in the elongating region and in larger numbers throughout the E-Cadherin$^+$ domain. The bottom right image in Figure 4.20A shows that some of the small groups of E-Cadherin$^+$ cells in the putative anterior tissue also express Sox17. These results suggest that a large proportion of the E-Cadherin$^+$ tissue is differentiating as endoderm in the elongating region and as small groups of endodermal cells in the anterior domain. They are therefore in agreement with the widefield images of the *Sox17::GFP* reporter cell line shown in Figure 4.17; additionally both experiments indicate that Sox17 is not expressed in the tip of the elongation. Gastruloids cultured continuously in N2B27 (Fig. 4.20(B)) share the correlated expression of E-Cadherin and Sox17 in more coherent regions of tissue than in gastruloids treated with Chiron. This is consistent with the widefield observations of the *Sox17::GFP* reporter line which showed higher anterior expression in the N2B27-treated gastruloids.

Given the persistence of Sox2$^+$, E-Cadherin$^+$ tissue in the gastruloids at the 96 and 120 hour time points, it was not unreasonable to suspect that some of these cells might correspond to residual pluripotent tissue. Chiron-treated gastruloids were therefore fixed at the 120 hour time point and were stained for two other markers of pluripotency, Nanog and Oct4 (Fig. 4.21). Oct4 was undetectable in all cases (Fig. 4.22) but the possibility that it is expressed cannot be excluded without a positive control. Nanog expression (white) was evident in varying proportions of the gastruloid, ranging from a subset of cells in the elongating tissue with a second group more anteriorly (left in Fig. 4.21A) to being expressed at a very low level in fewer than 10 cells in the elongation (right in Fig. 4.21A). Among aggregates grown continuously in N2B27, the pattern of Nanog expression was variable (Fig. 4.21B); where the gastruloids elongated, Nanog$^+$ cells were present in both the putative anterior and posterior of the aggregate (Fig. s 4.21).
Figure 4.20: Sox17\(^+\) Cells are Associated with the E-Cadherin\(^+\) Regions but are Absent at the Tip of the Elongation at the 120 hour Time Point. Gastruloids were cultured either with a 3\(\mu\)M pulse of Chiron from 48-72 hours (A) or continuously in N2B27 (B) and were fixed at the 120 hour time point. They were immunostained for E-Cadherin (red) and Sox17 (white); secondary-only controls are shown in (C). Sox17\(^+\) cells are always found in or alongside the E-Cadherin\(^+\) region but not within the tip of the elongation. Gastruloids cultured in N2B27 show larger and more coherent regions of Sox17\(^+\) tissue. Scale bars represent 200\(\mu\)m throughout.
Figure 4.21: Gastruloids Retain Expression of Nanog at the 120 hour Time Point
Gastruloids were cultured either with 3µM pulse of Chiron from 48-72 hours (A) or continuously in N2B27 (B) and were fixed at the 120 hour time point. Fixed aggregates were stained with the nuclear marker Hoechst (blue) and with an antibody against Nanog (white); a secondary only control is shown in (C). Confocal stacks are represented here as mean average intensity z-projections. Gastruloids grown under either condition retain cells that express Nanog; when grown with a Chiron pulse, they can be found in the elongating region of the tissue. Scale bars represent 200µm throughout.
4.2. RESULTS

Figure 4.22: Nanog$^+$ Cells in 120 hour Gastruloids do not Co-Express Oct4. Gastruloids were cultured with a 3µM pulse of Chiron from 48-72 hours (A) or continuously in N2B27 (B) and were fixed at the 120 hour time point. They were immunostained for Nanog and Oct4 and with the nuclear stain Hoechst. Confocal z-stacks are presented as mean average intensity projections for each channel. The small groups of Nanog$^+$ cells in the gastruloids do not co-express Oct4 in either condition.
To summarise, the tissue composition of the gastruloids at the 120 hour time point is a complex mix of all three germ layers, which overlap substantially in the elongating region. More anteriorly, discrete groups of cells have identities that can more easily be ascribed to particular germ layers. An attempt to summarise these results as a schematic is shown in Figure 4.23 below.

4.3 Discussion

The results presented in this chapter form an account of gastruloid development that has similarities to that of the embryo but the tissues also show a number of important differences. This section reviews these points of agreement and disagreement in the context of the in vitro systems that informed the gastruloid approach (Chapter 3: Optimisation of Gastruloid Culture) and in regards to current theories of embryonic patterning.

During the first 48 hours of gastruloid development, the tissues are composed of a heterogenous mix of cells corresponding to different states of pluripotency. The heterogeneity in mir-290-mCherry/mir-302-eGFP expression (Fig. 4.5B and C) is consistent with the account of Parchem et al., who describe these cells in serum + LIF culture as “...express[ing] high levels of mir-290-mCherry” and with “…a small fraction of cells [that] expressed both reporters or eGFP alone, suggestive of low levels of differentiation and consistent with heterogeneity in LIF-only conditions” [122]. By the 48 hour time point, the gastruloids have spontaneously developed polarised expression of T/Bra::GFP and TCF/Lef:mCherry (Fig. 4.6); it is possible that the heterogeneities in differentiation during the first 48 hours of development are important in establishing this polarity.

\*The widefield nature of the imaging data lacks any information about tissue organisation in the Z-direction. Additionally, the expression patterns of tissue markers cannot be orientated in relation to one another without clear dorso-ventral landmarks. One solution is to examine multiple markers simultaneously by immunohistochemistry (see [169]).
4.3. DISCUSSION

E-Cadherin  
Nanog  
Sox1 (neural)  
Sox17 (endoderm)  
Tbx6 (mesoderm)  
Brachyury (mesoderm)  
Sox2 (neural)  
Gata6 (mesoderm)

Tip region expresses 
T/Bra::GFP and includes 
candidate NMp population.

Figure 4.23: Elongating Gastruloids at 120 hours Contain All Three Germ Layers. Elongating gastruloids have a morphological polarity with a rounded, putative anterior domain and an elongated putative posterior domain. The tip of the elongation expresses T/Brachyury (green), which diminishes anteriorly. Sox2 (pink) appears to be expressed in a band slightly anterior to the tip of the elongation, overlapping with the T/Brachyury-expressing region. It is also expressed in small groups of cells in the E-Cadherin+ (grey) tissue in the anterior. Small groups of Nanog+ cells can be found near the tip of the elongation. Sox1 (blue) is expressed broadly across the elongating region and as discrete groups of cells in the anterior. Sox17 (purple) is expressed in association with the E-Cadherin+ tissue in a region that extends from the anterior into the elongation. Tbx6 (yellow) is expressed at low levels near the tip of the elongation and at higher levels in the surface cells that are loosely associated with the anterior tissues. Gata6 expression (orange) also follows this pattern but with Gata6H2B-Venus + cells within the anterior tissue.
Figure 4.24: Summary of DRC Gastruloid Development with 3µM Chiron Treatment from 48-72 Hours After Aggregation. (A) Representative aggregates for each time point. Despite the saturated green channel at the 72h and 102h time points, it is clear that the aggregates progress from a mostly red, naïve state through a yellow stage to a green, primed state from 24-72 hours of development. This progression is quantified in (B) as fluorescence traces in either the green channel (top row) or the red channel (bottom row), normalised to the highest overall fluorescence value for each and to the length of each aggregate. The mean trace is shown as a green or red line in each case, with one standard deviation either side of the mean shown in the filled blue region. n=6 for the 24 & 48 hour time points; n=8 for the 72 & 102 hour time points.
Figure 4.25: Summary of TCF/Lef:mCherry Gastruloid Development with 3µM Chiron Treatment from 48-72 Hours After Aggregation. (A) Representative aggregates for each time point indicated. Wnt signalling activity is initially uniform, then becomes localised to the putative posterior region which elongates from around 96 hours. (B) Fluorescence traces for aggregates from each time point, measured along the midline of the aggregate from the region of highest signal to the region of the lowest. The average background value has been subtracted from each trace and it has been normalised to the highest value of the dataset. The mean fluorescence trace is shown in blue. (C) The normalised fluorescence traces, each normalised to the length of the aggregate. The mean fluorescence trace is indicated as a blue line, with one standard deviation either side of the mean shown as the filled blue region. n=11 for the 48 hour time point; n=8 otherwise. Scale bars in (A) correspond to 200µm.
Figure 4.26: Summary of T/Bra::GFP Gastruloid Development with 3\(\mu\)M Chiron treatment from 48-72 Hours After Aggregation. (A) Representative aggregates for each time point indicated. (B) Fluorescence traces for aggregates from each time point, measured along the midline of the aggregate from the region of highest signal to the region of the lowest. The average background value has been subtracted from each trace and it has been normalised to the highest value of the dataset. The mean fluorescence trace is shown in blue. (C) The normalised fluorescence traces, each normalised to the length of the aggregate. The mean fluorescence trace is indicated as a blue line, with one standard deviation either side of the mean shown as the filled blue region. Scale bars in (A) correspond to 200\(\mu\)m.
4.3. DISCUSSION

qRT-PCR analysis of gastruloids at the 48 and 72 hour time points (Fig. 4.7) showed that they resemble the pre-streak epiblast at 48 hours, consistent with the expression of both *mir-290-mCherry* and *mir-302-eGFP* in the DRC cell line (Fig. 4.24). The spontaneous polarisation event seems to take place in the absence of any AVE-like tissue as indicated by the lack of expression of *Chordin, Noggin* and *Dkk1* as assayed by qRT-PCR (not shown in Fig. 4.7; *Cerberus* is expressed at low levels). This is corroborated by the lack of *Gata6^{H2B-Venus}* expression at this time, as assayed with the reporter cell line (Fig. 4.11). This result stands in opposition to the well-established hypothesis that the AVE in the mouse is responsible for breaking the radial symmetry of the epiblast prior to primitive streak formation, in an evolutionarily conserved patterning event. It is possible to reconcile these results through a model of the embryo where the AVE acts to reproducibly bias what is otherwise a spontaneous process of polarisation. In the gastruloid cultures which apparently lack an AVE-like tissue, the process can unfold in an unconstrained fashion. The observation that this polarisation event is apparently dependent on the starting size of the aggregates (Chapter 3: Optimisation of Gastruloid Culture) fits with a model of self-organised patterning, such as that which could arise from a simple reaction-diffusion system (see [170]). Such a system could be able to spontaneously generate pattern, as is the case with the formation of the putative antero-posterior axis in the gastruloids and would have a defined length-scale that determines the size of the tissue that can be patterned. The results of the qRT-PCR analysis at the 72 hour time point following 24 hours’ treatment with 3µM Chiron suggests Nodal and Lefty as attractive candidates for such a system, since they are a diffusible activator-inhibitor pair\(^5\).

\(^{5}\)Unpublished experiments from David Turner suggest that this may not be the case since Nodal\(^{-/-}\) gastruloids can still polarise when presented with a spatially homogeneous exogenous source of Nodal. There may be a further layer of interactions that determines a competence to respond to Nodal signalling that is not dependent on Nodal itself.
Following the activation of the T/Bra::GFP reporter during the Chiron treatment shows that it is progressively activated throughout the gastruloid (Fig. 4.8), extending across the tissue from the pole that pre-existed at the 48 hour time point. By 72 hours, expression has become uniform and on withdrawal of Chiron it becomes confined to the elongating portion of the gastruloid that retains elevated Wnt signalling activity (Fig.s 4.9 and 4.12). Immunostaining at this point suggests that the majority of the tissue is epiblast like, expressing Sox2 and E-Cadherin, with T/Brachyury detected in localised regions. There may therefore be some perdurance of the GFP when T/Brachyury expression is in fact more localised. The co-localisation of T/Brachyury with elevated Wnt signalling is reminiscent of the primitive streak of the embryo and later its elongating caudal domain.

During the elongation phase of gastruloid development, T/Brachyury expression and Wnt signalling activity become localised to the putative posterior end (Fig.s 4.26 and 4.25) of the elongating tissue. There are clear similarities between the gene expression patterns observed in the gastruloids and those reported by Marikawa et al. for small aggregates of P19 embryonal carcinoma cells which recreate axial elongation morphogenesis [106]. Whole mount in situ hybridisations for T/Brachyury and members of the Wnt family show that they are localised to the tip of the elongating tissue.

At the 96 hour time point, the putative posterior pole contains an overlapping region of Sox2 and T/Brachyury expression that is a candidate source of neuro-mesodermal progenitors that seems to persist to the 120 hour time point (Chapter 5: Gastruloids as a Source of Axial Progenitors). The Sox1::GFP and Tbx6::eYFP reporter lines demonstrate that the gastruloids develop both neural and paraxial mesodermal tissue in this region (Fig.s 4.15 and 4.16). The elongations also appear to contain a source of endoderm, as assessed by co-expression of E-Cadherin and Sox17 at the 120 hour time point (Fig. 4.20), which extends more anteriorly. It is interesting to note that the arrangement of the germ layers appears to be inverted with
4.3. DISCUSSION

respect to the gastrula-stage mouse embryo, since the endoderm is present on the inside of the tissue and the paraxial mesoderm resides outermost. A possible explanation for this is that the prospective posterior tissues resemble a later stage in development during the formation of the trunk, which would predict the endodermal tissue in this region to resemble the midgut to hindgut. This has not yet been experimentally verified. Intriguingly, the persistence of Nanog-expressing cells in the putative posterior of the gastruloids (see Fig. 4.21) is suggestive of possible primordial germ cell identity, though this will have to be confirmed by examining specific molecular markers such as Stella [133]. A striking difference to the tissue organisation of the embryo is that the gastruloids lack a notochord, as evidenced by the absence of an antero-posterior stripe of T/Brachyury, suggesting that the tissues lack an axial mesodermal component.

As for the identity of the anterior tissues, small groups of cells retain expression of Sox2 and E-Cadherin at the 96 hour time point (see Fig. 4.18), which could have a residual epiblast or ectodermal identity. At the 120 hour time point, some of these small groups express Sox17 and are demarcated from their surrounding tissues with a layer of Fibronectin (see Fig. 4.20 and 4.19), suggesting that they are groups of endodermal cells. These data are apparently corroborated by the Sox17::GFP fluorescent reporter (see Fig. 4.17). It is interesting to note that the putative anterior region does not widely express Sox1::GFP (see Fig. 4.15), as would be expected in the embryo. A possible reason for this is the apparent lack of an AVE-like tissue, which in the embryo would demarcate a region of the epiblast as ectoderm by inhibiting the posteriorising signals from the streak. In the absence of these signals from the AVE and through the exogenous activation of canonical Wnt signalling with Chiron, the gastruloid tissues may become posteriorised. This notion is supported by the results of ten Berge et al. who describe how

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6Recent unpublished work suggests that there is a degree of dorso-ventral organisation to the three germ layers in the elongating tissue in cultures that have been extended to the 168 hour time point [169].
exogenous Wnt3a treatment of axially polarised EBs promotes the expression of early mesendodermal markers at the expense of neuroectodermal markers, as assessed by RT-PCR and the loss of prospective anterior Otx2 expression in immunostained samples. A source of ectoderm for anterior neural tissues may therefore be lost. In addition, the absence of a notochord-like stripe of T/Brachyury::GFP expression suggests that the gastruloid tissues lack the axial mesoderm that is required to embellish the anterior ectoderm with the pattern of the rostral nervous system (reviewed in [3]). A posterior identity for the Sox1::GFP+ tissues in the elongation remains to be experimentally determined.

Details of the antero-posterior identity of the gastruloid tissues could be obtained by further use of the DRC reporter cell line. The mir-302-eGFP reporter remains expressed through gastrulation until around E9.5 [122], when it becomes restricted to the anterior nervous system [121]. It is likely that the downregulation of the mir-302-eGFP reporter will only become apparent after the 120 hour time point, since this corresponds to five days of differentiation from a heterogeneous, but approximately naïve starting state. Following the development of longer term gastruloid cultures, this cell line could offer an appropriate system in which to assess the extent of anterior neural tissue in the aggregates. Furthermore, the authors describe how the expression of mir-302-eGFP is largely absent by E10.5 and undetectable at the mRNA level by E11.5, offering further points of possible correspondence between the development of gastruloids and the embryo. It may even offer an indication of germ cell identity, since mir-290 is expressed in the gonads of both sexes at E12.5, when it is lost in females but persists in males to E14.5 [112]. This reporter line may therefore be a useful tool if integrated with longer-term approaches to gastruloid culture (Chapter 7: Physical Inputs to Gastruloid Culture).
4.3. DISCUSSION

4.3.1 Interactions Between Wnt and Nodal Drive Spontaneous Symmetry Breaking

It is notable that the gastruloids can self-organise a pole of Wnt signalling and T/Brachyury expression in the first 48 hours of development in the absence of the exogenous signalling factors that would be expected to instruct this process. ten Berge et al. have previously described a similar pattern of axial self-organisation in EBs derived from 2000 ESCs and reflect that this pattern is likely to arise stochastically before being amplified and stabilised to form a pole of signalling activity [163]. They further speculate that Wnt and Nodal signals are likely to be responsible for the progressive recruitment of cells to this pole of activity, which spreads throughout the EB over the first five days of development, possibly under the control of a longer range inhibitory signal [163]. While it is likely that the underlying patterning mechanism is the same in both systems, it is particularly interesting to note that the process in the EBs requires exogenous BMP, Wnt or Nodal signalling to initiate polarisation, while the gastruloid cultures do not require exogenous treatments. The signal for the EB cultures is normally provided by BMPs present in the serum in which they are aggregated, as shown by the absence of patterning in the presence of Noggin or in serum and growth factor-free conditions [163]. The gastruloids, on the other hand, are routinely formed in serum-free culture conditions and the results of the PCR analysis show that they do not express BMP4 at the 48 or 72 hour time points (Fig. 4.7). It is possible that the process of dissociating the cells activates Wnt signalling at a low level due to the release of previously sequestered β-catenin from adherens junctions and further that the small number of cells in each gastruloid allows an initially stochastic pattern to be consolidated into a self-sustaining pole of signalling activity.
4.3.2 Gastruloid Development Corresponds to Embryonic Development

Gastruloids recreate a number of general features of embryonic development, namely symmetry breaking, germ layer organisation and axial elongation. These results occur in the correct order and result in a structure with a defined single antero-posterior axis that undergoes a consistent and reproducible pattern of morphogenesis. A direct correspondence can be drawn between the timing of these events in each system, which is supported by the results from this chapter. This alignment is represented schematically in Figure 4.27.

Taking the results from this chapter together, the gastruloids at the 48 hour time point appear to have an early epiblast-like identity, which matures to a late-epiblast/early gastrula stage by 72 hours. The correspondence with the embryo would suggest that the subsequent elongation of the gastruloids can be divided into two phases: the first as a short phase of convergence extension that corresponds to the formation of the first six somites and the second as a longer phase of re-arrangement and growth from a population of neuro-mesodermal progenitors retained in the caudal end of the tissue. This alignment predicts that extending the gastruloid cultures to later time points could not only enable axial tissue such as the spinal cord to be derived in vitro but also potentially to access the products of early organogenesis.

This alignment does, however, make a number of assumptions about gastruloid development. First, it is assumed to progress at a constant rate, which may not be the case. As has been shown in Chapter 3 (Optimisation of Gastruloid Culture), the formation of the elongations is dependent on the number of cells in the aggregate. It may be the case that gastruloids formed from fewer cells than is optimal undergo a form of compensatory growth prior to polarisation and may delay the polarisation process, as occurs in the embryo [125]. Anectodal observations so far indicate that this is not
4.3. DISCUSSION

Figure 4.27: Gastruloid Development Directly Corresponds to Early Post-Implantation Embryonic Development. The main events of early post-implantation development, namely symmetry-breaking, gastrulation and posterior elongation occur with the same timing in vitro as they do in vivo. The gastruloids break symmetry at a stage when they apparently have an early epiblast identity, as assayed by the results from the DRC reporter line. From 48-72 hours, they correspond to the late epiblast/early gastrula, as shown by the downregulation of FGF4 and upregulation of FGF5, Wnt3 and Nodal indicated by qRT-PCR. From around 80 hours, the gastruloids deform and elongate, retaining T/Bra::GFP and TCF/Lef:mCherry at the putative posterior pole. The first phase of this process is likely to correspond to the convergence extension of the embryo in forming the first six somites; beyond the 110 hour period it is likely to proceed from a candidate population of neuro-mesodermal progenitors that are the subject of Chapter 5 (Gastruloids as a Source of Axial Progenitors).
the case, but it reflects a degree of plasticity in the developmental timeline for the embryo. The second assumption (which is related to the first) is that gastruloid development is directly proportional to embryonic development. It is perhaps more surprising that the timing of events is apparently the same between the very different physical and biochemical environments \textit{in vivo} and \textit{in vitro}. It could be argued that the unconstrained \textit{in vitro} environment could allow the developmental process to unfold more rapidly, though the converse may also be true that the process may be delayed by the absence of instructive interactions between embryonic or extraembryonic tissues. Thirdly, it is assumed that the expression of the reporter constructs used for the gastruloid cultures obeys the same pattern of expression as it would do in the embryo. It is possible that \textit{in vitro} systems are missing a level of embryonic regulation, but this is difficult to test experimentally.

Nevertheless, the resemblance between gastruloid development and embryonic development allows them to be used as a new experimental system for studying developmental mechanisms. Importantly, the reproducibility and throughput of this system enables experimental approaches that could never be used for embryos, such as large scale screening for particular phenotypes or effects. The points at which the gastruloids differ from the embryo highlight features of the \textit{in vivo} system that are important for development and those that can be isolated. This deconstructed approach provides new insights on development that could never be derived from studying the embryo alone. Examples include the spontaneous polarisation of the epiblast-like gastruloids in the absence of the extraembryonic tissues and the uncoupling of antero-posterior axis specification from anterior neural development [176].

In conclusion, the chapter shows that gastruloids can spontaneously break radial symmetry to form a putative antero-posterior axis, which undergoes a reproducible axial elongation over the following two days of development. All three germ layers are represented in the gastruloids, suggesting that this process corresponds to the axial elongation of the embryo. The first aim stated
in Chapter 1, to establish an ESC model of axial elongation morphogenesis, has therefore been satisfied.
CHAPTER 4. CHARACTERISATION OF GASTRULOID DEVELOPMENT
Chapter 5

Gastruloids as a Source of Axial Progenitors

5.1 Introduction

As discussed in Chapter 1, the head and the body of the vertebrate embryo have separate developmental origins. One example is found in the rostro-caudal organisation of the vertebrate central nervous system, where the specialised tissues of the brain and spinal cord form physically continuous but functionally distinct structures. While most of the tissues of the head are formed during gastrulation, the following period of progressive posterior elongation accounts for the post-occipital axial levels. This chapter begins by examining the evidence for a population of axial stem cells, or neuro-mesodermal progenitors (NMps), in the Amniote embryo that contributes progressively to the axial tissues of the trunk and tail. The separation between cranial and post-occipital development in the mouse is discussed first, before the properties of their respective NMp populations are considered in more detail. This chapter goes on to review efforts to generate NMp-like cells \textit{in vitro} and presents experimental evidence for a candidate population of NMp-like cells in the putative posterior end of elongating Chiron-treated gastruloids.
5.1.1 Cranial and Post-Occipital Development in the Mouse

Fate maps of the late gastrula show that a relatively large proportion of the ectoderm is fated to become the tissues of the brain, while a much smaller territory contains the progenitors of the spinal cord [148]. Underlying these tissues, a substantial fraction of the mesoderm will contribute to the cranial mesenchyme [160], along with the axial mesoderm that extends anteriorly [148]. A relatively small proportion of the posterior half of the embryo is fated to form the paraxial mesoderm of the trunk [160]. With respect to the final proportions of the body, the gastrula therefore represents primarily the developing rostral tissues, while the majority of the trunk and the entirety of the tail form progressively following gastrulation (in a similar manner to the chick, discussed in Chapter 1).

The cranial paraxial mesoderm is specified from mesoderm that ingressed during gastrulation but more caudally, this tissue forms primarily from invagination of cells from the epiblast alongside the primitive streak [160]. This process of contribution from the caudal lateral epiblast (CLE) accounts for the formation of the somites of the trunk, with non-ingressing cells integrating into the posterior neural tube. The tailbud forms after closure of the posterior neuropore at 9.6dpc, once the node has reached the caudal limit of the primitive streak. The segmentation of the presomitic mesoderm continues to form the somites of the tail, which are derived from continued proliferation in the tailbud. In summary, the neural tube and paraxial mesoderm of the head are derived primarily from precursors present at gastrulation stages of development, while the tissues of the trunk and tail emerge progressively from the caudal lateral epiblast and tail bud through ongoing invagination and proliferation. Table 5.1 describes posterior truncation phenotypes in which the tissues of the head and anterior trunk form approximately normally but where subsequent development is disrupted.
The differences between rostral and caudal development extend to the regulatory level, which in the nervous system are exemplified by the N2 and N1 enhancers of Sox2. These regions regulate Sox2 expression in the developing anterior and posterior neural plate respectively in both the chicken and the mouse [156][157]. Though they are histologically continuous, these regions of the nervous system have different modes of growth, which is uniform in the anterior neural plate but a directional elongation in the forming spinal cord. This elongation requires a different response to signalling that is reflected in the properties of the enhancer [156], since the growing posterior neural plate depends on Wnt and FGF signalling from its posterior end (reviewed in [94]). The role of the Sox2-N1 enhancer in regulating the neural versus mesodermal fate decision in NMps is discussed in more detail in Subsection 5.1.4.

5.1.2 Amniote Axial Development Proceeds via a Bipotent, Self-Renewing Neuro-Mesodermal Progenitor

Two lines of investigation have suggested the existence of a bipotent neuro-mesodermal progenitor in amniotes: retrospective clonal analyses have shown a shared, post-gastrulation origin for the paraxial mesoderm and posterior neural tube and transplantation experiments have identified apparently self-renewing regions in the posterior of the developing embryo.

5.1.2.1 Evidence from Retrospective Clonal Analyses

As described in Section 5.1.1, a relatively small territory of the post-gastrulation Amniote embryo is fated to produce the axial tissues such as the spinal cord [151]. Given the slow and progressive development of the trunk and tail tissues in these species, several possible models have been proposed for the origin of these tissues (discussed in [118] and [110]). One possibility is a simple re-arrangement of existing tissues by convergence ex-
### Table 5.1: Posterior Developmental Phenotypes in the Mouse.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Reference(s)</th>
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<tbody>
<tr>
<td>$Cdx2^{-/-}$</td>
<td>Reduced PSM, abnormal somites (5+, &lt;17 form). Incomplete rescue with exogenous FGF8a.</td>
<td>[27] [177]</td>
</tr>
<tr>
<td>$Cdx2^{+/+}; Cdx4^{-/-}$</td>
<td>Reduced PSM; truncation posterior to the hindlimb buds. (Non-cell autonomous defect).</td>
<td>[192][16]</td>
</tr>
<tr>
<td>$Cdx1^{-/-}; Cdx2^{-/-}; Cdx4^{-/-}$</td>
<td>Posterior truncation anterior to the forelimb buds (&lt;5 somites). Lethal prior to E10.5.</td>
<td>[177]</td>
</tr>
<tr>
<td>$T/Brachyury^{+/+}$</td>
<td>Truncated tail, abnormal posterior vertebrae.</td>
<td>[40][62][30]</td>
</tr>
<tr>
<td>$T/Brachyury^{-/-}$</td>
<td>Truncated (~6th somite), lethal.</td>
<td>[30] [189] (rev. [94][15])</td>
</tr>
<tr>
<td>$Tbx6^{-/-}$</td>
<td>Ectopic neural tubes (posterior to 6th somite).</td>
<td>[25] (rev. [94])</td>
</tr>
<tr>
<td>$Wnt3a^{-/-}$</td>
<td>Truncated (~7th-9th somite).</td>
<td>[154] (rev. [94][15])</td>
</tr>
</tbody>
</table>
tension and cell intercalation during gastrulation. Another possibility is that a pool of precursor cells is regionalised before the formation of the axis and that this early pattern subsequently expands to form the final arrangement of tissues. A further possibility is a common pool of progenitors for the whole axis, which may either change over time to be specific to particular regions of the axis, or which may be a permanent pool of axial stem cells. Retrospective clonal analyses have provided a means to distinguish between these different models through the use of \( \text{lacZ} \) constructs, which infrequently generate \( \text{lacZ} \) labelled clones by intragenic homologous recombination. The results of labelling the axial tissues of the spinal cord [110] and the myotome [118] are discussed separately below.

The myotome includes the segmented paraxial mesoderm that will go on to form the musculature of the trunk and tail. Among embryos collected at E11.5, the \( \text{lacZ} \) labelled clones can be divided into separate classes of long clones and short clones, which are collectively continuous across the 42 body segments analysed (see Fig. 5.1A). This indicates that a single labelled cell can generate progeny throughout the axis and so excludes a model of regionalised progenitors that are restricted to form particular segments. Importantly, the clonal complexity of each segment, i.e., the number of times it is labelled by clones, increases in a rostro-caudal gradient. This refutes a model of simple tissue re-arrangement, since this would require a biased distribution of cells in the progenitor pool towards posterior-levels. Furthermore, the frequency of the long clones is uniform in relation to their axial position, suggesting that the precursor pool is not structured. The authors conclude that the pattern of clonal labelling is consistent with a permanent pool of asymmetrically dividing progenitors for the myotome that may be localised near the anterior primitive streak [118].

Similar results were obtained for clonal labelling in the tissues of the central nervous system at E12.5. In the spinal cord, the frequency of clonal labels with exponentially increasing length was found to increase, in contrast
Figure 5.1: The Pattern of Clonal Labelling in the Axial Tissues is Consistent with a Population of Self-Renewing Progenitors. Published data from clonal labelling experiments, ordered by the most anterior segment labelled in each clone and presented with the antero-posterior axis on the y-axis. (A) Results from [118]; (B) Results from [110], spinal cord clones are in the cyan region; (C) Results from [175] and (D) Schematic of generic labelling pattern. In all cases, the clones are clonally continuous along the antero-posterior axis, they show posterior polarity (ie. have a variable rostral limit) and increasing clonal complexity from anterior to posterior.
to the results in the brain. In addition, the pattern of spinal cord clones was polarised, with an increasing clonal complexity (see above) towards the caudal end of the embryo (see Fig. 5.1B). Many of these clones labelled the most caudal segments and instead had a variable anterior limit. These observations are similar to those for the myotome and they strongly support the possibility of a self-renewing population of stem cells for the spinal cord that would produce the observed clonal distribution [110].

These studies raised the question of whether the hypothesised progenitor pool may be common to both tissues. Tzouanacou et al. present an undirected approach using a similar laacZ system inserted into the ubiquitously expressed Rosa26 locus instead of using a tissue specific promoter [175]. In this study, the authors found that a large proportion of neurectodermal clones also had labelled derivatives in the mesoderm and that some clones were restricted solely to these lineages (hereafter N-M clones). When taken with the finding that the smallest such clone comprises only 24 cells, it is likely that these tissues share a common progenitor that persists at least until late gastrulation. Furthermore, comparison between E8.5 and E10.5 embryos indicates that the progenitor is continually present in this period since the frequency of N-M clones increases where the frequency of those restricted to single lineages remains approximately constant. In line with the findings of Mathis and Nicolas ([118], [110]), the N-M clones are clonally continuous, they show posterior polarity (ie. they frequently label the most caudal extent, with a variable rostral limit) and demonstrate increasing clonal complexity from anterior to posterior (see Fig. 5.1C). The authors conclude that the observed pattern of clonal labelling can best be explained by a resident population of self-renewing neuro-mesodermal stem cells that undergoes a degree of expansion and re-arrangement during trunk development [175]. These findings are corroborated by transplantation experiments that are discussed below.
5.1.2.2 Evidence from Labelling and Transplantation Experiments

Figure 5.2 shows the key embryonic locations that are tested in the following transplantation experiments. At E8.5, the region of interest is around the node, namely the rostral node, the node-streak border (NSB), the anterior primitive streak and the epiblast that is lateral to the streak (caudal lateral epiblast, CLE). An equivalent region, Zone C, has been described in the chick embryo [26]. At E10.5, the region of interest is just caudal to the end of the notochord and ventral to the most caudal extent of the neural tube, known as the chordoneural hinge (CNH; also in the chick, see [111]). Immediately caudal to this is the mesenchyme of the tail bud mesoderm (TBM). The transcription factors Sox2 and Brachyury are co-expressed in these regions during axial elongation and their co-expression is undetectable once it is complete, so these genes are frequently used to describe populations of cells that will harbour the NMps [188].

Sox2/Brachyury double positive cells have first been described in the ectoderm lateral to the primitive streak at E7.5 [188]. Functional evidence for a resident population of axial progenitors has been described from E8.5 through DiI labelling of the primitive streak [184] and grafting of transgenically labelled cells [22]. Embryos labelled in the streak and node often retained labelled cells into the tail bud 48h later, raising the possibility of a resident population of cells that contributes to large extents of the axis [184]. Homotopic transplantation of the node-streak border and the caudal lateral epiblast showed that these regions contribute to the chordoneural hinge (CNH) region of the tail bud. Furthermore, heterotopic grafting of node or streak explants to the node-streak border frequently resulted in contribution to the CNH, suggesting that the signalling environment of this region may act as a niche for axial progenitors. Finally, there is topological continuity of gene expression domains from node-streak border to the CNH [22]. These findings are consistent with the authors’ previous analysis that the cells immediately abutting the node demonstrate stem-cell like properties on grafting.
5.1. INTRODUCTION

Figure 5.2: Axial Progenitor Regions in the Mouse Embryo at E8.5 and E10.5. (A) At E8.5, axial progenitors are located in the Node-Streak Border (NSB, red) and the Caudal Lateral Epiblast (CLE, blue). (B) At E10.5, axial progenitors are located exclusively in the Chordoneural Hinge (CNH, red), between the tissues of the notochord and neural tube. Figure adapted from [188].

[21]. Heterotopic grafting of the cells of the rostral part of the caudal lateral epiblast to the node-streak border has shown that they colonise the CNH as frequently as the cells of the border, indicating they contain an equivalent population of adaptable NMps rather than being functionally distinct [188].

During the formation of the trunk from E8.5-E9.5, the population of Sox2 and Brachyury double-positive cells undergoes a Wnt/β-catenin-dependent expansion by approximately 2.8 fold, before starting a progressive four day decline [188]. Following the closure of the posterior neuropore at around
E10, the NMp population is localised in the chordoneural hinge (CNH) of the tailbud. The most compelling evidence for a stem cell population in this region is that it can be serially transplanted through the node-streak border of younger unlabelled embryos for up to three generations. Not only do these cells contribute to a considerable extent of the axial tissue but they also re-colonise the CNH in each case [21]. This suggests that the cells of the CNH can form more rostral axial tissues as well as self-renewing. Control grafts of the tail bud mesoderm, located just caudally to the CNH, did not show this behaviour.

Over the final three days of tail development (E10.5-E13.5), expression of Wnt3a, Cdx2, Fgf8 and T/Brachyury declines in the caudal tip of the tail [22]. The NMps retain the potential to form axial tissues in heterochronic grafts, but the changing pattern of gene expression could reflect a maturation of the progenitor zone that will eventually terminate axial elongation [22]. The lifespan of the embryonic NMp population is therefore only around five to six days, though the results of the serial transplantation demonstrate that they retain their potential up to this point. In addition, the behaviour of grafted NMps depends on their position in the node-streak border [188], suggesting that they can be directed to form neural or mesodermal tissues by signals in their environment.

In summary, the combined approaches of retrospective clonal analysis, tissue labelling and transplantation suggest that a population of progenitors resides in the rostral node and anterior primitive streak position early in posterior elongation that can later be found in the chordoneural hinge of the tailbud. The progenitors are bipotent and contribute to both the neural tube and paraxial mesoderm of the developing trunk and tail. In addition, serial transplantation experiments have shown that the progenitors can apparently self-renew in a manner that is dependent upon their environment, or niche. The self-renewing properties of the progenitors has raised the possibility of their propagation in vitro, which has attracted considerable attention due to
their contribution to all post-occipital levels of the embryonic body.

5.1.3 *In vitro* Derivation of NMp-like Cells

The NMp populations *in vivo* are correlated with regions of *Sox2* and *T/Brachyury* co-expression in the mouse [188] (see also Fig. 3 in [73]) and chicken embryo [119]. This expression profile has therefore been used to assess the efficacy of culture regimes designed to produce NMps *in vitro*. A key caveat is that this gene expression profile alone is not sufficient to define cells as NMps; indeed around 14% of the self-renewing mEpiSCs co-express *Sox2* and *Brachyury* at a given time [166]. It is therefore important to assess candidate NMp populations on their expression of other genes and ultimately on their function to behave as axial progenitor cells *in vivo*.

A common feature of the NMp differentiation protocols is a pulse of Wnt signalling through an exogenous Wnt agonist such as Chiron [65][172][166]. We have previously shown that a pulse of Chiron, acting in concert with endogenous FGF signalling, can direct adherent cultures of mouse embryonic stem cells towards a neuro-mesodermal progenitor-like state [172]. This culture regime produces a morphological change in the form of an elongation when it is used in gastruloid culture (see Chapters 4 and 6). Tsakiridis *et al.* describe how, in cultures of mouse epiblast stem cells, the pulse of Wnt signalling acts to generate a mixture of committed mesendodermal and neuro-mesodermal progenitors from an initially primitive-streak biased sub-population of the original culture [166]. The pulse of Chiron upregulates expression of *Brachyury*, as well as Hox genes from paralogous groups 5-9, which are active late in gastrulation. On grafting the Chiron treated cells to the node-streak border of the mouse embryo, they can incorporate efficiently into the paraxial mesoderm and in a small number of cases, also to the neural tube [166].
Similar results are obtained by Gouti et al., who describe the formation of Brachyury and Sox2 co-expressing cells in cultures of mouse embryonic stem cells that have been grown in FGF2 and treated with a pulse of Wnt signalling [65]. The resulting cell cultures can be steered towards a paraxial mesoderm identity with continued exposure to Chiron, as assessed by upregulation of Tbx6, or to a posterior neural identity with retinoic acid and an agonist of sonic hedgehog signalling, as assessed by expression of Sox1 and Sox2. They confirm that these cells can differentiate appropriately into the chick neural tube and somitic tissue in vivo. The culture protocol is extended to cultures of mouse epiblast stem cells and human embryonic stem cells by extending the length of exposure to FGF2 and Chiron [65].

In each case, the identity of the candidate progenitors is judged by profiles of gene expression at the level of populations and by grafting groups of cells to recipient embryos. Single cell analysis and demonstration of serial transplantation will be required to show bipotency and self-renewal in these populations.

5.1.4 Signals Regulating Fate Decisions in NMps & The Progenitor Niche

The key point of regulation in the neural versus mesodermal fate decision of NMps is the N1 enhancer of the Sox2 gene [157]. The N1 enhancer is active throughout the CLE and in response to the synergistic activity of Wnt and FGF signalling and it becomes precociously active in response to BMP inhibition [157]. Takemoto et al. revisit the phenotype of Tbx6−/− mutant embryos, which have ectopic neural tubes in the paraxial mesodermal compartment and demonstrate that they arise from the ectopic activation of Sox2. This phenotype is rescued by the specific deletion of the N1 enhancer but the lack of mesodermal or endodermal marker gene expression in the paraxial tissues of Tbx6−/−; Sox2ΔN1/ΔN1 embryos shows that Tbx6 is required for correct mesodermal development. The ectopic neural development in
Tbx6−/− embryos shows that Tbx6 has a role in repressing Sox2 activation, though this is shown to be indirect through repression of Wnt3a [157].

Collectively, these results suggest a model in which the N1 enhancer is active in the CLE, but where cells are prevented from activating Sox2 by BMP signalling. As the regressing node approaches these cells, BMP signalling is alleviated by inhibitors secreted from the node, thereby allowing cells in the epiblast to activate Sox2 and to contribute to the caudal neural plate. Cells from the CLE that ingress through the streak instead move out of the region of high Wnt signalling and so deactivate the N1 enhancer. Commitment to a mesodermal fate is reinforced through inhibition of Wnt3a by Tbx6 and its role in promoting mesodermal differentiation [157] (reviewed in [94]).

A further level of complexity is added by the regulation of the NMp niche in the posterior growth zone. Cambray and Wilson describe how the gene expression profile of the mouse NMp population changes over time [22], yet older cells can still contribute to more anterior axial levels upon heterochronic transplantation into younger embryos [21]. These experiments suggest that the NMp niche “ages” reversibly during axial elongation and that the properties of the NMps are determined by features of the niche rather than the absolute age of the cells (reviewed in [115]). Transplantation experiments have shown that the mouse Cdx genes play a crucial role in maintaining a signalling-dependent niche for axial progenitors during posterior elongation (reviewed in [177]). On grafting Cdx2+/−; Cdx4−/− (hereafter Cdx2/4) axial progenitors into a wild-type node-streak border, their posterior truncation phenotype was fully rescued, indicating that the defect in the mutant cells is non-cell autonomous and that they are not intrinsically impaired in their ability to produce the axial tissues [16]. Bialecka et al. show that the grafted mutant cells show nuclear activated β-catenin, indicating that they are responsive to canonical Wnt signalling in the wild-type niche. They take this observation in hand with previous genetic experiments in which the truncation phenotype of Cdx2/4 mutants was partially rescued in a Lef1 gain-of-
Figure 5.3: **Summary of Regulation of State and Fate of NMps.** (A) Levels of Wnt, FGF and BMP signalling increase in the anterior-posterior direction, while Retinoic Acid (RA) signalling is highest in the somites and absent in the posterior growth zone. (B) BMP signalling in the Caudal Lateral Epiblast (CLE) prevents the N1 enhancer from activating Sox2. More anteriorly, BMP levels are lower due to the influence of inhibitors secreted by the tissues of the node. Cells remaining in the CLE can activate Sox2 expression and differentiate as neural tissue. Cells that have ingressed through the streak into the presomitic mesoderm are exposed to lower levels of Wnt signalling and begin to express Tbx6, which indirectly inhibits neural differentiation by further inhibiting Wnt signalling. (C) The interactions regulating the posterior growth zone change over time. During trunk development, a positive feedback interaction between Cdx2, mid-cluster Hox genes and Wnt signalling maintains the signalling environment of the posterior growth zone. After the trunk/tail transition, the expression of the caudal-most Hox genes inhibits the positive feedback loop to bring about the termination of axial extension. In parallel, levels of RA in the posterior growth zone rise, in part due to the downregulation of Cyp26a1. Figure compiled from information in [16], [94], [156], [157], [177] and [192].
function background [192] and deduce that the mutant cells are “defective in the production of, but not the response to Wnt signals” [16]. Comparing the partial rescue in the Lef1 gain-of-function background with the complete rescue on transplantation to a wild-type host suggests that canonical Wnt signalling may not be the only feature of the axial progenitor niche that is disrupted in Cdx2/4 mutants.

Cdx1-/;Cdx2-/;Cdx4-/ mutant embryos show a complete posterior truncation caudal to the fifth somite and a disrupted signalling environment in the posterior growth zone [177]. FGF signalling is lost in this region and the source-sink gradient of retinoic acid signalling between the forming somites and the posterior growth zone is never established. T/Brachyury expression is indistinguishable from the control embryos at the end of gastrulation (E7.5), but no new mesoderm emerges from the primitive streak at E8.5 in the triple mutant embryos [177], demonstrating that loss of function of these genes specifically affects post-occipital development. The phenotypic similarity between the triple Cdx mutant and that of the Fgfr1-/ mutant led van Rooijen et al. to test whether exogenous FGF8a can rescue the phenotype of Cdx2-/ mutants, which was partially successful. Taking these observations together, the authors suggest that the phenotype of the Cdx triple mutants may be attributed in part to their loss of FGF signalling from the posterior growth zone [177]. The Cdx genes therefore play a crucial role in the maintenance of the signalling environment of the posterior growth zone during post-occipital development.

In addition to the activity of the Cdx genes in maintaining the axial progenitor niche, the Hox genes add spatial and temporal regulation to posterior elongation as “mediators or collaborators of Cdx” gene function [192]. Strikingly, the posterior truncation phenotype of Cdx2/4 mutants can be partially rescued through the transgenic expression of trunk-level Hox genes, which now form a short tail [192]. Young et al. emphasise that the Hox genes expressed in this case do not act through the remaining active allele or
Cdx2, nor through activating expression its downstream targets, indicating that trunk Hox genes and Cdx2 regulate the posterior growth zone separately. This does not extend to Hox genes from paralog group 13, which instead produce axial truncation phenotypes that resemble Cdx2/4 mutants on precocious expression in the trunk [192]. Transgenic expression of Cdx genes cannot rescue the truncation phenotype, further suggesting a degree of separation between Hox and Cdx regulation of the posterior growth zone. Given these observations, Young et al. suggest a model in which the trunk Hox genes act co-operatively with the Cdx genes to maintain the posterior growth zone through a positive feedback loop with Wnt3a. Following the trunk/tail transition, they suggest that the expression of paralog group 13 Hox genes terminates axial elongation by reducing the level of Wnt signalling in this zone. There are clear parallels between the regulation of the growth zone by Hox, Cdx and Wnt and the dynamics of retinoic acid signalling; Cdx2 and Cyp26a1 expression are correlated but a causal link has not yet been established between the two.

In summary, the axial progenitor population is regulated at the level of the neural versus mesodermal fate decision through the Sox2 N1 enhancer and the Wnt and BMP signalling pathways in the caudal lateral epiblast and the primitive streak. The posterior growth zone is maintained during trunk formation through the action of the Cdx and trunk Hox genes through positive feedback on Wnt signalling. After the trunk/tail transition, axial elongation is terminated by the changes in the signalling environment in the posterior growth zone. Retinoic acid signalling increases in this region due to shortening of the PSM (moving the growth zone relatively closer the source of RA in the somites) and a reduction in the expression of Cyp26a1 in the growth zone. In parallel, the expression of the paralog group 13 Hox genes inhibits the positive feedback loop with Wnt signalling in this region, causing its levels to decline. The Hox genes therefore co-ordinate the production of neural and mesodermal tissues from the posterior growth zone with their
axial level through differential regulation of Wnt signalling.

5.1.5 Defining Characteristics of NMps

Although NMps lack a uniquely expressed marker, it is possible to establish a set of necessary criteria that a candidate population of progenitors is expected to achieve. These cells would co-express low levels of Sox2 and Brachyury and would exist in a region (or environment) with high Wnt and FGF and low RA signalling. Functional testing must demonstrate behaviour that is consistent with bipotency and self-renewal. Bipotency would be indicated by contributions to both the paraxial mesoderm and neural tube, while self-renewal would be suggested by long axial contributions from the posterior growth zone and serial transplantation. Ideally, these functional tests would be extended to the single cell level to unequivocally resolve the properties of the cells.

The preceding chapter described how gastruloids treated with a Chiron pulse, which produces a candidate population of NMps in adherent cultures [172], reproducibly elongate along a putative antero-posterior axis. Through alignment of gene expression in gastruloid development, it appears that their elongation corresponds to the initial extension of the embryo at the transition to post-occipital development. These observations therefore suggested that the gastruloids at the 120 hour time point may harbour a population of NMps in their elongating posterior tissue and that they may, therefore, represent the only existing source of NMps in three-dimensional culture. The following sections of this chapter present the case for a candidate population of NMps in the gastruloid tissues, which is functionally tested by transplantation into the chicken embryo in Chapter 6 (A Xenotransplantation Assay for Candidate Axial Progenitors).
5.2 Results

5.2.1 Sox2 and Brachyury are Co-Expressed in the Elongating Region of the Gastruloids

As described above, a key feature of in vitro-derived axial progenitors is their co-expression of Sox2 and Brachyury. The localisation of these proteins in the gastruloids was determined in fixed samples by immunohistochemistry (Fig. 5.4; see also Chapter 4, Fig. 4.13 for co-expression at the 96 hour time point). On staining gastruloids at the full extent of their elongations (around 120 hours after aggregation), Brachyury expression can be seen increasing towards the tip of the elongation. This is supported by results from the T/Bra::GFP reporter cell line, which shows Brachyury expression localised to the elongating region (see Chapter 3: Optimisation of Gastruloid Culture and Chapter 4: Characterisation of Gastruloid Development). Sox2 expression, on the other hand, is highest in a part of the elongation that is slightly offset from the tip in the most developed examples. The region of overlapping expression is around 50µm from the tip and has low expression of Sox2 and Brachyury (Fig. 5.4); reminiscent of the arrangement in the embryo at the node-streak border and later at the chordoneural hinge.

5.2.2 Increased Cell Proliferation in the Putative Posterior Tissue

As described in Section 5.1.1, the cranial tissues are primarily derived through convergent extension of progenitors that were present during gastrulation, while the post-occipital tissues form progressively through proliferation from a posterior growth zone. It is possible that the elongation of the gastruloid tissues arises through convergence and extension, cell proliferation or both processes. In order to determine whether the Sox2\(^+\), Brachyury\(^+\) region was a site of heightened cell proliferation, gastruloids grown with a 3µM Chiron pulse were incubated with the thymidine analog BrdU and were
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Figure 5.4: **Sox2 and Brachyury are Co-Expressed at the Tip of the Elongation.** Sox1-GFP Gastruloids were fixed and stained 120 hours after aggregation; the images presented here are average intensity projections of 75µm deep confocal stacks. Brachyury expression increases moving towards the tip of the elongation, while Sox2 expression is highest slightly more anteriorly.
Figure 5.5: DNA Synthesis is Heightened in the Putative Posterior Region. Gastruloids were grown with 3μM Chiron treatment from 48-72 hours and were incubated with BrdU for 4.5 hours prior to fixation at the 120 hour time point. Nuclear BrdU signal is detectable in scattered cells throughout the Sox2\(^+\) region which co-localises with Brachyury in the putative posterior during elongation (see Fig. 5.4). The non-uniform spatial distribution of BrdU\(^+\) nuclei suggests that DNA synthesis occurs more rapidly in the putative posterior, which would be supportive of heightened cell proliferation in this region. See also Fig.s 5.6 and 5.7.
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Figure 5.6: 1-Hour Incubations with BrdU Do Not Produce A Detectable Signal in Gastruloids at the 120-Hour Time Point. Gastruloids were grown with a pulse of 3µM Chiron from 48-72 hours and were incubated with BrdU for 1 hour prior to fixation at 120 hours. No signal is detected above background levels for the anti-BrdU antibody after DNA denaturation and co-staining for Sox2, indicating that such a short incubation does not allow sufficient BrdU to be incorporated to give a detectable signal. See also Fig.s 5.5 and 5.7.
Figure 5.7: 20-Hour BrdU Incubations Result in Widespread Cell Labelling in Gastruloids at the 120-Hour Time Point. Gastruloids were grown with a pulse of 3µM Chiron from 48-72 hours and were incubated for 20 hours with BrdU before fixation at the 120-hour time point. After DNA denaturation and staining for BrdU and Sox2, the signal from the anti-BrdU antibody was bright and widespread throughout the tissue. This indicates that nearly every cell had synthesised new DNA during the 20 hour exposure to BrdU. See also Fig.s 5.5 and 5.6.
fixed at the 120-hour time point (Fig.s 5.5, 5.6 and 5.7). Proliferating cells incorporate BrdU into newly synthesised DNA, which can be detected immunologically. On incubating the gastruloids with BrdU from 119-120 hours, no BrdU$^+$ nuclei could be detected (Fig. 5.6). This indicates either that cell proliferation is synchronous and that it occurs less than once per hour, or more likely that this short incubation does not allow sufficient BrdU to be incorporated to give a detectable signal with immunohistochemistry. An alternative approach would be to use a marker of cell proliferation such as Ki67, which does not rely on a live-cell incubation step to mark proliferating cells. In contrast to the lack of labelling with a 1-hour BrdU incubation, an incubation from 100-120 hours marked almost every nucleus (Fig. 5.7); indicating that nearly all of the gastruloid tissues have synthesised new DNA in this time.

On incubating the gastruloids with BrdU from 115.5-120 hours, scattered BrdU$^+$ nuclei were present in a region correlated with Sox2$^+$ cells (magenta in Fig. 5.5). Given the correlation between Sox2 and Brachyury at this stage (see Figure 5.4), the region of heightened DNA synthesis is correlated by extension to the putative posterior region. Cell proliferation in elongating gastruloids may be similarly non-uniform along the antero-posterior axis. The differences in DNA synthesis suggest that the elongation may arise at least in part from cell proliferation in the posterior region. If the gastruloid tissues correspond to the early trunk of the embryo, they may therefore contain a resident population of axial progenitors.

5.2.3 Morphogenesis of the Posterior Tissue

The immunostaining data presented in Figure 5.4 suggested that the candidate progenitor population was localised within the tip of the elongation, raising the question of whether the elongation was formed from this region alone or from re-arrangement of the gastruloid as a whole. This was tested by cutting the gastruloid in half at the base of the elongation using an eye-
Figure 5.8: Explanted Posterior Tip Tissue Infrequently Continues to Elongate in Suspension. Sox1::GFP gastruloids were cut in the elongating region to give posterior tip explants that were subsequently cultured in suspension in N2B27. Five days after cutting (A), one of the explants had elongated substantially while the other explants had changed little in size or shape. Widefield microscopy six days after cutting (B) showed that much of the explanted tissues expressed Sox1::GFP. In the single elongating case, closer inspection (C) showed that most of the tissue expressed Sox1::GFP and that it had an apparent lumen, reminiscent of the neural tube.
brow knife and a hair-loop tool. Initially, explanted tissues were cultured in suspension in N2B27 (see Fig. 5.8) which demonstrated that the putative posterior tissues can continue to elongate in culture, albeit at a very low (<1%) frequency\(^1\). It is difficult, however, to assess the increase in length without images of the gastruloids prior to resection and without images of the corresponding putative anterior fragments.

On embedding the posterior tissues in Matrigel®, the frequency of continued elongation was much higher (7/9 cases, see Fig. 5.9) and occurred within 48 hours of resection. Brachyury expression in the explants was maintained up to around 120 hours and was subsequently lost. In the cases analysed above, the time that this downregulation occurred varied from 117 hours up to the end of imaging at 167 hours, when the growth of the tissue had become disordered due to cell migration into the Matrigel® (see Chapter 7: Physical Inputs to Gastruloid Culture). In all cases, the expression of Brachyury was localised to a region corresponding to the original posterior tip and to small groups of cells that migrated into the gel (Fig. 5.9A).

These results raised the question of whether this elongating behaviour is restricted to the posterior explants, or whether the anterior portions would also elongate, perhaps in response to the cutting procedure. To examine this possibility, corresponding anterior and posterior tissues were embedded in neighbouring droplets of Matrigel® and were imaged by time-lapse microscopy (Figure 5.9B). The anterior explants begin to elongate but cannot maintain their structure and produce round masses of cells by 114 hours after aggregation (in 8/10 cases, 2/10 retain an elongation (Fig. 5.10B)). All of the posterior tip explants (10/10) continued to elongate and retained their structure by the 114 hour endpoint (Fig. 5.10C). Beyond this time, the explants did not increase further in length and the cells started to migrate into the gel (as in top right panel of 5.9A).

\(^{1}\)Attempts to reproduce this observation in suspension cultures failed to reproduce the full extent of the elongation after resection of ~100 gastruloid tips.
Figure 5.9: Explanted Posterior Tissues Continue to Elongate in Matrigel®. (A) Posterior tip explants from the T/Bra::GFP cell line were embedded in Matrigel® at 96 hours after aggregation and were measured by time lapse microscopy. Brachyury continues to be expressed as the tip elongates but growth becomes disordered by 144 hours. Dashed lines indicate the gastruloid boundary. (B) Corresponding anterior and posterior explants from the same gastruloid were embedded in neighbouring droplets of Matrigel®. Directional elongation is restricted to the posterior explants.
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Quantitative measurement of explant morphogenesis shows the growth dynamics more clearly (see Fig. 5.10). In many cases, the anterior explants initially elongate but do not sustain the increase in length; they either stop elongating or start to shrink after around 13 hours in the matrix. The posterior explants, on the other hand, maintain a constant rate of elongation that can be fitted with a linear trendline, corresponding to an increase of around 6µm/hour throughout this 18 hour window. When whole gastruloids are embedded and measured, the average profile can be fitted with a polynomial trendline. This indicates that although the initial elongation may be rapid and comparable to the explanted posterior tissue, elongation eventually becomes constrained and the rate decreases. The morphogenesis of whole gastruloids in Matrigel® can therefore be attributed to the posterior tissue region, with an initial contribution from the anterior tissue that may later become restrictive. It therefore seems as if the posterior region, which contains the domain of Sox2 and Brachyury co-expression, is responsible for the elongated tissues of the explants and whole gastruloids when they are embedded in Matrigel®.

In summary, the results presented in this chapter show that the putative posterior end of Chiron-treated gastruloids at 120 hours expresses both T/Brachyury and Sox2 (Fig. 5.4). The regions in which these proteins can be detected appears to overlap in a region set slightly anteriorly to the posterior end of the elongation, which can be considered as the site of the candidate NMp population. Broadly speaking, DNA synthesis is higher in the elongating tissue but the BrdU incorporation assay did not detect a specific proliferative group of cells (Fig. 5.5). The posterior tissue has qualitatively different properties to the anterior tissue when cultured as explants in Matrigel®, since it can continue to elongate where the anterior tissue cannot (Fig. 5.9). On quantifying the elongation of the explants, it appears that the continued elongation of whole gastruloids can be attributed to that of the posterior tissue alone (Fig. 5.10). Collectively, these results suggest
Figure 5.10: **Morphogenesis in Matrigel® can be Attributed to the Posterior Tissue.** (A) Explants and whole gastruloids embedded in Matrigel® were measured over 18 hours by time lapse microscopy. Their change in length, \( \Delta L \), was measured as a straight line or two straight line segments along the longest axis. Dotted lines in (B,C,D) indicate the average profiles. (B) Anterior explants initially increase in length but do not continue to elongate. (C) Posterior explants elongate at a constant rate that can be fitted with a linear trendline. (D) Whole gastruloids show an initially constant rate of elongation that subsequently slows.
that the gastruloids contain a population of NMps that can contribute to the elongation of the tissue through proliferation and that they may be responsible for the different properties of the anterior and posterior tissues in explant cultures. The properties of the posterior region were tested further in Chapter 6 by transplantation to the caudal lateral epiblast of the chicken embryo.

5.3 Discussion

The results presented in this chapter demonstrate that the anterior and posterior gastruloid tissues differ qualitatively in terms of gene expression, proliferation and morphogenesis. The posterior of the gastruloids is a region of Sox2 and T/Brachyury co-expression (see Fig. 5.4), which has been previously used to signify candidate axial progenitor populations in adherent cultures due to its correlation with the NMp population in the embryo. The posterior tissue therefore fulfills this necessary, but not sufficient, condition for a candidate axial precursor population. This region may also be supportive of heightened cell proliferation compared to the anterior, since 4.5-hour incubations with BrdU showed that DNA synthesis is non-uniform in the gastruloids at this time-scale (Fig. 5.5). The pattern of BrdU incorporation does not, however, indicate a clear regionalisation of potential progenitor cells; longer Matrigel®-embedded cultures may be required to identify a specific region of the tissue that maintains elevated DNA synthesis during the elongation. Alternatively, morphogenesis at this time may primarily arise from convergence and extension, though single cell-resolution tracking and the use of specific markers of cell proliferation (such as Ki67) will be required to demonstrate this conclusively.

On embedding corresponding anterior and posterior tissues in Matrigel®, it is clear that the posterior tip is functionally distinct from the corresponding anterior region in its ability to continue elongation morphogenesis (Fig. s
It is interesting to note that the elongation of the explants is dependent on their physico-chemical environment, as Matrigel® embedded cultures elongate much more frequently than those in suspension. Measurement of the change in axial length of these tissues with time indicates that the elongation of intact gastruloids in Matrigel® can be attributed to the elongation of the posterior tissues, with a possibly restrictive influence of the anterior tissue (see Fig. 5.10). This could possibly be due to the distribution of tension across the tissue, which may restrict the extension of an elongation that is continuous with a large anterior mass of cells. Alternatively, the high concentration of Matrigel® may impose an elastic resistance to the extension of the tissue that is greater around the larger intact gastruloids than it is around a smaller tissue explant. In either case, the posterior tissues retain T/Brachyury::GFP signal in a small population of cells during the elongation in Matrigel® and the loss of its expression is co-incident with the cessation of extension.

These observations suggest that the initial elongation of the gastruloids is likely to correspond to the post-gastrulation convergence extension movements in the embryo that establish the occipital tissues. This is consistent with the alignment between gastruloid development and that of the embryo presented in Chapter 5, which would suggest that the 96 hour time point corresponds approximately to the end of gastrulation at E7.5. The transition to trunk and tail development (from around E8.5) would occur around the 120 hour time point when the cultures are embedded in Matrigel®; subsequent elongation is likely to correspond to the progressive posterior elongation that has been described in the embryo. This is consistent with the observation of a Sox2+ T/Brachyury+ region in the gastruloids at this time, that would correspond to the caudal lateral epiblast in the embryo. The Sox2+ T/Brachyury+ population in the embryo has been shown to expand

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2The effect of embedding elongating gastruloids in different concentrations of Matrigel® is explored in Chapter 7: Physical Inputs to Gastruloid Culture.
through the first stages of posterior elongation (E8.5-E10.5) before diminish-
ing to extinction (at E13.5) [188]. The elongation of the gastruloids therefore appears to correspond to the first stages of trunk development. They are probably limited from elongating further by technical features of Matrigel® culture, which may be inappropriately matched mechanically to the elonga-
tion of the tissues. The question therefore arises as to how long the process of elongation can be maintained in physically embedded cultures and whether synthetic environments provide an inert alternative to the use of Matrigel® (see Chapter 7). Biological limitations to continued elongation may include the lack of a notochord, as a source of mechanical support to the developing tissue and the lack of an enclosing layer of ectoderm that could prevent the potentially disruptive invasion of cells into the matrix. These missing fea-
tures may also explain why the tissues do not seem to produce somites, since the paraxial presomitic mesoderm cannot be held in close association to the axial tissues that may help to organise it into a coherent layer.

In summary, the elongating region of the gastruloids contains a candidate population of NMps but further work is required to more precisely describe the balance between cell proliferation and re-arrangement in axial elonga-
tion morphogenesis in this system. The continued elongation of the poste-
rior tissues seems to be dependent on the physico-chemical environment of Matrigel®, though its biochemical and mechanical properties may not be optimally matched to the developing tissues. While the candidate NMP pop-
ulation satisfies the necessary condition of Sox2 and Brachyury co-expression, functional testing of bipotency and self-renewal is required to conclusively de-
fine them as NMps. The following chapter begins to address these features of the population by transplanting them into the CLE of the developing chicken embryo.
Chapter 6

A Xenotransplantation Assay for Candidate Axial Progenitors

6.1 Introduction

Approaches to deriving NMps \textit{in vitro} have been deemed successful if they generate cells that co-express \textit{Sox2} and \textit{T/Brachyury} [65][166][172]. While this profile correlates with the NMp-containing regions of the embryo [188][119], co-expression of these genes is a necessary but not sufficient condition for defining a population of NMps (see Chapter 5). The properties of the embryonic population have been determined through retrospective clonal analysis and transplantation experiments, which have demonstrated that they are bipotent and are capable of self-renewal (respectively). Although some \textit{in vitro} approaches have been validated by transplanting candidate NMps into the mouse [166] and chicken [65] embryos, these studies have been limited to analyses of their tissue contributions at the endpoint. These studies lack information about the way in which the cells are incorporated into the developing axial tissues, which can be obtained by time-lapse microscopy. This approach specifically addresses the question of whether the
cells reside in the axial progenitor niche during posterior elongation which has not been answered by previous studies.

Transplantation of mESC or mEpiSC-derived candidate NMps to the mouse embryo is the most stringent assay of their properties. Immediately after gastrulation, however, the mouse embryo is small and cup-shaped, making transplantation to the node-streak border or caudal lateral epiblast highly technically demanding. In addition, the need to culture the embryos in suspension precludes the use of a live imaging approach. The chicken embryo was therefore chosen as a suitable alternative since its planar structure and attachment to the vitelline membrane make it amenable to both transplantation and time-lapse imaging. This chapter presents the development of a xenotransplantation assay for assessing the properties of candidate murine NMp populations in the developing chicken embryo through live imaging and endpoint-analysis.

6.1.1 Rationale

The aim of this assay was to test the bipotency of a population of candidate NMps, assessed by their contribution to the developing neural and mesodermal tissues and to make inferences about their capacity for self-renewal from the length of the tissue contributions. In addition, time-lapse microscopy provided an indication of the cells’ ability to occupy the endogenous axial progenitor niche by assessing their pattern of integration during posterior elongation.

There are experimental precedents for producing mouse-chick chimaeras through microsurgery, notably in determining the processes regulating the development of the somites and neural tube. Fontaine-Perus et al. have shown that groups of somites from an 8-9dpc mouse donor can differentiate appropriately and migrate into the limb bud when grafted into 2 day old chick embryos [54]. They have also shown that neural tube explants from
8-9dpc mouse donors can undergo neurogenesis and can prompt myogenesis in the surrounding chick tissues when grafted into 1.5-2 day old chick embryos. These authors describe the chick embryo as “...a privileged environment facilitating access to the developmental potentials of normal or defective mammalian cells” [53], making it an appropriate choice of host for this assay. Chicken embryos have previously been used to test the capacity of in vitro-derived NMps to contribute to neural and mesodermal tissues by injection to a site lateral to the node at HH Stage 9 [65]. Similar results have been obtained by grafting in vitro-derived NMps into the anterior and mid-streak regions of late streak stage mouse embryos [166].

Three criteria for defining a population of NMps can be defined from their expected behaviour in the xenotransplantation assay. First, they would be expected to contribute labelled cells to both the neural and mesodermal axial tissues from a single graft. Secondly, these contributions would be spread across a long extent of the body axis [21][22]. Thirdly, labelled cells would be expected to associate with the node throughout the elongation of the embryo since Cambray and Wilson describe how “...prospective fate mapping studies consistently point to the node region ... as a site for resident cells throughout axis elongation” [22]. The results of transplanting in vitro-derived NMps to mouse and chicken embryos have thus far only shown the incorporation of small groups of cells at the end of the incubation. By using live imaging in this assay, it is possible to determine how these grafted cells incorporate into the developing embryonic tissues during posterior elongation and therefore the extent to which a population satisfies these criteria.

6.2 Note on Collaborative Work

The results presented in this chapter are the product of an ongoing collaboration with Dr Ben Steventon (Department of Genetics, University of Cambridge, UK). The approach and experimental regime were devised jointly
between the author, Dr Steventon and Professor Martinez Arias. Thanks are
due to Dr Octavian Voiculescu (Department of Physiology, Development &
Neuroscience, University of Cambridge, UK) for his tuition in the New Cul-
ture technique and for his assistance with a set of preliminary experiments
that are not described in this chapter.

Experimental samples were prepared by the author for grafting as de-
scribed in the following section. The embryo culture and grafting steps were
performed jointly with Dr Steventon. Image acquisition, quantification and
analysis were performed by the author. Thanks are due to Dr David Turner
for his assistance in presenting the data and to Dr Steventon for his input in
the discussion and interpretation of the results.

6.3 Materials & Methods

Chapter 2 (Materials and Methods) contains a complete account of the ex-
perimental procedures used in this chapter to prepare the host embryos and
to label the grafted tissues. In brief, fertilised chicken eggs were incubated
at 37°C for 24 hours before the embryos were collected and maintained in
modified New Cultures [116]. Experimental tissues were collected as intact
colonies (from adherent cultures of cells) or small fragments (of dissected
gastruloids or chicken node explants) and were labelled with DiI

1Preliminary experiments showed that enzymatic dissociation of the tissues prior to
grafting confounds their ability to reside in the region associated with the node after
transplantation (data not shown). All of the experimental tissues presented in this chapter
were detached or dissected using solely mechanical means.

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were detached or dissected using solely mechanical means.
6.4. RESULTS

displacements from the caudal boundary of the node at the midline. The
grafts were also scored qualitatively for their pattern of incorporation, using
the endpoint images in conjunction with the time-lapse data (see Table 6.1).
Figure 6.1 summarises how these measurements are made and presented as
points on scatter plots. This technique was used for both the fate mapping
experiment that labelled groups of cells in the intact embryo and in the
analysis of grafting experiments.

The development of the host embryos was scored according to their Ham-
burger & Hamilton (HH) stage [70]. Datasets for each experimental condition
are presented in each case across all host stages and separated by stage.

<table>
<thead>
<tr>
<th>Score</th>
<th>Graft Behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Node with Spreading”</td>
<td>The grafted cells spread from their original site and are found in association with the node at the endpoint.</td>
</tr>
<tr>
<td>“Spreading”</td>
<td>The grafted cells spread from their original site within the axial tissues.</td>
</tr>
<tr>
<td>“Lateral Contribution”</td>
<td>The grafted cells are found exclusively in the lateral tissues of the embryo at the endpoint.</td>
</tr>
<tr>
<td>“Graft Remains as Blobs”</td>
<td>The grafted cells do not spread from their original position and remain as a discrete group.</td>
</tr>
</tbody>
</table>

6.4 Results

6.4.1 Fate Mapping the Axial Progenitor Zone

A first step in the development of the xenotransplantation assay was to
establish a fate map of the caudal lateral epiblast in the chicken embryo.
This fate map could then be used to define an appropriate region of the
Figure 6.1: Measurement Scheme for Scoring Label/Graft Position and Contribution. (A) The position of the labelled cells was measured as an axial displacement from the caudal boundary of the node ("a") and as a medio-lateral displacement from this point ("L"). The diameter of the group of labelled cells was also recorded ("d"). After 15-18 hours of incubation, the distance between the most rostral and most caudal labelled cells was measured as the axial length of the contribution. The yellow dashed lines in (B) show how these measurements relate to imaging data. (C) The starting position of each graft was plotted on Cartesian co-ordinates, with the node at the origin and the axial ("a") and lateral ("L") displacements as the x and y axes, respectively. The size of each point was proportional to the starting size of the graft ("d"). Identical plots were coloured according to either the length of the contribution (left panel) or the contributions of the labelled cells to the different tissue compartments (right panel).
chicken embryo in which to graft the candidate NMps. Small regions of the CLE were labelled with a lipophilic fluorescent dye and the position of the labelled cells was scored after 15 hours. The region of interest was determined by the positions that contributed labelled cells to both neural and mesodermal tissues across long extents of the rostro-caudal axis. The results of this tissue labelling experiment are presented in Figure 6.2, with the target region bounded by a dashed line.

When scored for the fate of the labelled cells, it is clear that cells immediately lateral to the node or a short distance rostral or caudal to it can give rise to labelled cells in both the somitic mesoderm and neural tube (orange points) and, additionally, the node (red points). These contributions are used to define a bounded region, marked by the dashed lines, that can be used to test the ability of grafted cells to produce both of these tissues. Labels placed caudal or lateral to this region are more likely to mark the lateral plate mesoderm (LPM, blue). Conversely, labelled cells rostro-medial to this region are more likely to contribute to the neural tube (maroon). Labelled cells throughout this region can contribute to the somitic mesoderm (green), reflecting their position lateral to the midline. The pattern of more lateral mesodermal fates at more caudal levels of the streak has been widely described, including with single cell labelling approaches [140]. Overall, these results are consistent with the fate map produced by Psychoyos & Stern [127] using a similar method of cell labelling that identified the anterior quarter of the primitive streak as a region that contributes to the medial somitic mesoderm and to neural tissue.

Having scored each graft by its tissue contributions, the points were recoloured according to the length of each contribution. A lower threshold of 500µm was chosen to represent short contributions of less than 3 somites in length. In order to determine an appropriate threshold for defining “long” contributions to the axial tissues, the data presented in Figure 6.2B were recoloured according to different upper thresholds (Fig. 6.3). When a threshold
Figure 6.2: Fate Mapping Defines the Axial Progenitor Region Lat-
eral to the Node. (A) Grafts scored by their contributions to the indicated tissues ((P)SM, (pre)somitic mesoderm; LPM, lateral plate mesoderm; NT, neural tube; N, node). (B) Grafts coloured by the length of their axial contributions. In both cases, the orange vertical line denotes the level of the node. A rectangular region bounded by the dashed box was defined by those grafts that contribute to both neural and mesodermal derivatives across a long antero-posterior extent.
Figure 6.3: **Defining a Threshold for Classifying “Long” Axial Contributions.** The data from the fluorescent cell labelling experiment in Figure 6.2 have been recoloured according to different upper thresholds for defining “Long” labels: $1500\mu m$ in (C) and $2000\mu m$ in (D). The threshold in (C) captures all 24 of the labels scored as “Node with Spreading,” while that in (D) excludes 10 of these labels. A threshold of $1750\mu m$ was used for the analysis of the grafted tissues.
of 1500µm was used (Fig. 6.3C), all of the labels that contributed cells to the region around the node were included within the bounded region, while a more stringent threshold of 2000µm (Fig. 6.3D) excludes 10 of these labels. The threshold of 1750µm used in Figure 6.2B was therefore chosen as an appropriate definition for “long” contributions.

When coloured according to these thresholds, it is clear that labelled cells in the bounded region frequently contribute to long stretches of the axis. It is notable that some of the labelled cells in this region only spread over short extents of the axis but this demonstrates that only a subset of cells in this region will interact with the axial progenitor niche. An interesting feature of the data is that the long axial contributions only arise from HH Stage 6 onwards. While additional grafts are required to confirm this effect, this is consistent with the fact that many of tissues labelled in this region at earlier stages are fated to contribute to the anterior neural and mesodermal tissues over limited extents of the axis; the first somites begin to form between HH Stages 6 and 7. A further possibility is that the axial progenitor niche only arises at these later stages and so labelled cells in this region could produce long axial contributions if they mark the progenitors.

These results are corroborated by heterotopic transplantation of the chick node and its neighbouring cells (the endogenous NMp population) to this region (see Fig. 6.4). The grafted cells spread posteriorly as the node regresses, with a small group of cells apparently leaving the site of the graft to reside at the posterior growth zone during this process.

To aid the visual interpretation of the experimental results, it is possible to make a number of predictions about how hypothetical cell populations would behave in this assay. A summary of these predictions is presented in Figure 6.5, coloured according to the scheme that is used consistently throughout. The plots in the left hand column of Figure 6.5 are coloured according to the predicted tissue contributions of the grafts: shades of green for mesoder-
A Stage 8- embryo was grafted with explanted node tissue from a donor embryo of similar age at positions caudal and lateral to the node. (A) The location of the grafts immediately after grafting. (A’) The embryo after 15 hours’ incubation. Labelled cells are present on both sides of the somitic mesoderm, with possible neural tube contribution from the lower graft. A large clump of grafted cells remains at the level of the node. (B) Time course of grafted cell integration. White arrows in the right panel indicate the position of the caudal-most labelled cells. A small group of cells appears to leave the caudal limit of the graft as the node passes, which remains resident in the posterior growth zone. Dashed yellow lines indicate the approximate boundary of the unsegmented posterior region; scale bars represent 500 µm throughout.
mal derivatives and maroon for neural tissues. Mixed contributions, where both tissue types are labelled, are shown in yellow and shades of orange. Grafts that are committed to either mesodermal or neural lineages would produce only green or maroon plots, respectively. A population of NMps, however, would contain examples of both tissue types as well as some mixed contributions (yellow and orange).

The plots on the right of Figure 6.5 are coloured according to the length of each tissue contribution: blue for short (<500µm), orange for medium (500-1500µm) and red for long (>1500µm) axial extents. Populations of cells that are committed to either lineage will only produce short to medium (orange to blue) contributions, while those that behave as progenitors can give medium to long (red) contributions. A population of NMps would, therefore, give medium to long (orange to red) tissue contributions.

Given this scoring system, a population of mesodermal progenitors (Fig. 6.5A) would be represented by mostly mesodermal (green) tissue contributions and medium to long (orange to red) axial lengths. A population of cells committed to mesodermal lineages (Fig. 6.5B) would give the same pattern of tissue contributions but would be restricted to short to medium (blue to orange) axial lengths. Similarly, a population of neural progenitors (Fig. 6.5C) would produce mostly neural (maroon) tissue contributions across medium to long (orange to red) axial lengths. Cells committed to neural fates (Fig. 6.5D) would show the same pattern of tissue contributions but would be limited to short to medium (blue to orange) axial extents. Finally, a population of NMps (Fig. 6.5E) would contribute to both neural and mesodermal tissues, either specifically to each tissue type (green or maroon) or to both from a single graft (yellow and orange). These contributions would be spread over medium to long (orange to red) axial lengths. Although this picture is an exaggerated one, it helps to classify the behaviour of the experimental grafts into clear categories that can be used to make predictions about the proportions of NMp-like cells in each case.
6.4. RESULTS

It is worth noting from the results of the heterotopic node transplants that these long contributions would arise through interaction with the regressing node (see Fig. 6.4). A population of grafted NMps would also be expected to contribute to the presomitic mesoderm in the unsegmented portion of the axis.

6.4.2 Testing Populations of Candidate NMps

The grafts that were tested with this assay can be divided into two groups: those from pluripotent cell cultures that have not been directed towards a particular lineage (and so are a form of negative control) and candidate NMps from adherent and gastruloid cultures (see Table 6.2). The cultures of pluripotent cells were ESCs in serum+LIF conditions (“ES”) and early or late\(^2\) EpiSCs (in FGF+Activin+XAV939, “EPI+XAV” or FGF+Activin, “EPI”). As mentioned above, the pluripotent cell cultures were included as a form of negative control since they have not been differentiated towards an axial progenitor state and they can contribute separately to both lineages since they are pluripotent.

<table>
<thead>
<tr>
<th>Pluripotent Cells</th>
<th>2D Candidate NMps</th>
<th>Gastruloid Candidate NMps</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES</td>
<td>ES NMp G</td>
<td>G N2B27</td>
</tr>
<tr>
<td>EPI</td>
<td>ES NMp T</td>
<td>G Ant</td>
</tr>
<tr>
<td>EPI + XAV</td>
<td>EPI F CF</td>
<td>G Post</td>
</tr>
<tr>
<td></td>
<td>EPI CF CF</td>
<td></td>
</tr>
</tbody>
</table>

The candidate NMps derived in 2D culture include those formed from ESCs using either the protocol published by Gouti et al. (“ES NMp G”) [65]

\(^2\)Early EpiSCs are grown with an inhibitor of canonical Wnt signalling and so reflect the pre-gastrulation epiblast, while those cultured without Wnt inhibition correspond to a more advanced developmental state.
Figure 6.5: Mock Data Illustrate the Predicted Behaviour of Different Classes of Cells in this Assay. (A)-(E) represent different hypothetical cell populations that could be grafted in this assay. Mock data for each population are shown coloured for their tissue contributions (left column) or the length of each contribution (right column). Grafts composed primarily of progenitors for either the mesodermal (A) or neural (C) tissues would be expected to make medium to long contributions, restricted to each tissue type. Grafts comprising committed cells for either mesodermal (B) or neural (D) tissues would be expected to contribute to only short to medium extents of each tissue. A candidate NMp population (E) would be predicted to contribute to both neural and mesodermal tissues either singly or in combination across medium-long extents of the axis.
or by Turner et al. (“ES NMp T”) [172]. Also included are those formed from EpiSCs using either the protocol of Tsakiridis et al. (“EPI F CF”) [166] or Lippmann et al. (“EPI CF CF”) [102].

Finally, candidate NMp populations derived from gastruloid culture were tested with this assay, including those that had been grown continuously in N2B27 (“G N2B27”) or as separate anterior or posterior fragments of gastruloids grown with a pulse of 3 μM Chiron from 48-72 hours (“G Ant” and “G Post”) [172].

Having grafted populations of cells from each of these conditions into recipient chicken embryos, the patterns of incorporation were qualitatively scored and were quantified. The results of qualitatively scoring the grafts are presented in Table 6.3. For ease of interpretation, the quantifications of the grafts are presented consistently between conditions, as described previously for the fate mapping experiment (Fig. 6.2). The following subsections consider the results of grafting populations of cells from each type of culture in turn (pluripotent cells, 6.4.2.1; 2D NMps 6.4.2.2, 6.4.2.3; gastruloid NMps 6.4.2.4).

6.4.2.1 Pluripotent Stem Cell Cultures

The results of grafting self-renewing cultures of ESCs and EpiSCs are presented in Figures 6.6, 6.7 and 6.8. It is apparent from Table 6.3 that the ESC grafts integrate efficiently, with every graft spreading in some way. This is contrasted by the EpiSC grafts, which do not integrate efficiently and instead frequently remain as blobs of labelled cells.

The grafted ESCs produce mostly mesodermal contributions (green and blue in Fig. 6.6), with one graft producing a long axial contribution that includes labelled cells in the neural tube, the somitic mesoderm and the region

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3 All gastruloid samples were collected for grafting at the 120 hour time point.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Node with Spreading /%</th>
<th>Spreading /%</th>
<th>Lateral Contribution /%</th>
<th>Remains as Blobs /%</th>
<th>n</th>
<th>Contribution to PSM?</th>
<th>Cells at the Node?</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES</td>
<td>27.3</td>
<td>72.7</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>EPI</td>
<td>0</td>
<td>28.6</td>
<td>0</td>
<td>71.4</td>
<td>14</td>
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<td>No</td>
</tr>
<tr>
<td>EPI+XAV</td>
<td>0</td>
<td>44.4</td>
<td>0</td>
<td>55.6</td>
<td>9</td>
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<td>No</td>
</tr>
<tr>
<td>ES NMp G</td>
<td>36.4</td>
<td>63.6</td>
<td>0</td>
<td>0</td>
<td>11</td>
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<td>Yes</td>
</tr>
<tr>
<td>ES NMp T</td>
<td>66.7</td>
<td>33.3</td>
<td>0</td>
<td>0</td>
<td>12</td>
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<tr>
<td>EPI F CF</td>
<td>21.7</td>
<td>69.6</td>
<td>0</td>
<td>8.7</td>
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<tr>
<td>EPI CF CF</td>
<td>0</td>
<td>76.2</td>
<td>0</td>
<td>23.8</td>
<td>21</td>
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<td>No</td>
</tr>
<tr>
<td>G N2B27</td>
<td>52.0</td>
<td>32.0</td>
<td>0</td>
<td>16.0</td>
<td>25</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>G Ant</td>
<td>12.5</td>
<td>56.3</td>
<td>25.0</td>
<td>6.3</td>
<td>16</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>G Post</td>
<td>40.0</td>
<td>55.0</td>
<td>0</td>
<td>5.0</td>
<td>20</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
around the node. For the two grafts that produce mid-length contributions in the somites and region around the node, it is noted that the number of cells at the node is very small (data not shown). Using the predictions made in Figure 6.5, the behaviour of the ESC grafts appears consistent with a population of putative mesodermal progenitors. A more likely interpretation, however, is that the pluripotent cells have a tendency to differentiate to mesoderm in response to the high Wnt and FGF signals of the CLE in the embryo. The pluripotent nature of the cells is also reflected in the small proportion of cells that are additionally competent to contribute to neural tissue.

The late EpiSCs (-XAV) behave in a similar way to the ESCs when grafted to the CLE: they contribute mostly to the mesodermal compartments with an infrequent mixed neural and somitic mesodermal contribution (see Fig. 6.7). It is clear, however, that the majority of EpiSC grafts produce short contributions, as reflected in the high proportion of grafts that remain as blobs for these conditions (71.4%, see Table 6.3). It should be emphasised that the EpiSC grafts do not contribute to the PSM or the region around the node; the only caudal spreading observed was after the node had passed (data not shown). It is also notable that the graft with a mixed contribution remained as a group of cells near the midline, rather than providing a neural contribution to an axial level other than that of the graft. In relation to the predicted behaviour illustrated in Figure 6.5, these cells resemble a mesodermal committed population, though as for the ESC grafts this probably reflects a differentiation response to the signalling environment of the CLE.

In the case of the early EpiSCs (+XAV) in Figure 6.8, it is clear that many of the grafts seem to give mixed neuro-mesodermal contributions. It is likely, however, that the three most rostral grafts are cases where the cells remain as a short, discrete group (55.6% of cases for EPI+XAV, see Table 6.3). Looking specifically at the PSM contribution, this graft remains as a 62\(\mu\)m group, so it is unlikely that the grafted cells are integrating efficiently into this tissue (data not shown). It is notable, however, that the most lateral
graft that contributed to both the somitic mesoderm and the neural tube demonstrated a transient engagement with the node as it regressed, pulling the cells into the neural tube (data not shown). These results predict, using the classes described in Figure 6.5 that early EpiSCs could be a population of mixed neural committed and mesodermal committed cells.

The results of this assay suggest that the pluripotent stem cells become progressively poorer at colonising both the neural and mesodermal tissue compartments with more advanced developmental stage. The ESC grafts could contribute to the PSM and showed engagement with the node, where neither of the EpiSC grafts did so (given the caveat for the small Epi+XAV graft described above). The behaviour of all three datasets resembles that predicted for populations of mesodermal progenitors, with the EpiSC-derived grafts seeming more committed than those derived from ESCs.

6.4.2.2 Adherent NMp Cultures (ESCs)

The results of grafting two-dimensional cultures of candidate NMps are presented in Figures 6.9, 6.10, 6.11 and 6.12. At a first glance, candidate NMps produced according to the protocol published by Gouti et al. seem to behave in a similar manner to cultures of pluripotent ES cells as they integrate well and they can contribute to the PSM and the node region (see Table 6.3). They are frequently found in the mesodermal tissues, with two instances of mixed neural and mesodermal contributions (one mid-length and one long). On closer inspection, it is clear that the candidate NMp grafts more frequently interact with the node than cultures of ES cells, albeit transiently in some cases (5/11 grafts versus 3/11 grafts). Given the frequency of medium-length contributions, the 2D ESC-derived NMps produced using the Gouti et al. protocol behave in a manner consistent with a population of mostly mesodermal progenitors with a small proportion that can also contribute to the neural tube (see Fig. 6.5).
Figure 6.6: Xenotransplanted Embryonic Stem Cells Can Infrequently Produce Long Neuro-Mesodermal Contributions. (A) Grafts scored by their contributions to the indicated tissues and (B) the length of their axial contributions (abbreviations and classification as in Figure 6.2). These grafted ESCs produced one long mixed contribution and many mid-length mesodermal contributions.
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Figure 6.7: Xenotransplanted Epiblast Stem Cells Produce Short, Mesodermal Contributions. (A) Grafts scored by their contributions to the indicated tissues and (B) the length of their axial contributions (abbreviations and classification as in Figure 6.2). Grafted EpiSCs do not contribute to the PSM or the node; instead, they are mostly found in the SM and LPM.
Figure 6.8: Xenotransplanted Early Epiblast Stem Cells Can Produce Short, Mixed Neuro-Mesodermal Contributions. (A) Grafts scored by their contributions to the indicated tissues and (B) the length of their axial contributions (abbreviations and classification as in Fig. 6.2). Many of the grafts produce short stretches of mixed neural and mesodermal tissue, often at the same axial level (data not shown).
Candidate NMps generated using the method published by Turner et al. (ES NMp T) contribute to a similar set of tissues to those derived using the Gouti et al. protocol (ES NMp G) and can frequently be found in the node region and the PSM (see Table 6.3). It is clear, however, that the proportion of mixed neuro-mesodermal contributions is higher than for the ES NMp G condition (6/12 grafts versus 2/11 grafts) and that three of the grafts spread across a long portion of the axis (red in Fig. 6.10B). It is noted that labelled cells from some of the grafts clearly associated with the regressing node, though this interaction was transient in some cases. Overall, these data bear a closer resemblance to the early epiblast stem cells (see Fig. 6.8) than to the starting ES cell cultures. According to the predictions made in Figure 6.5, the behaviour of 2D NMps derived using the Turner et al. protocol is consistent with that of a population of neuro-mesodermal progenitors.

### 6.4.2.3 Adherent NMp Cultures (EpiSCs)

Candidate NMp populations were also derived from two-dimensional cultures of epiblast stem cells, with or without Chiron treatment on the second day of differentiation (“Epi F CF” and “Epi CF CF”, respectively). Although grafts from both treatments often contributed labelled cells to the PSM, only the “Epi F CF” contributed to the node region (3/23 grafts, see Table 6.3). Two grafts from this condition showed transient associations with the node during its regression, leaving labelled cells in the somitic mesoderm and presomitic mesoderm (data not shown). Despite frequent mixed neural and mesodermal contributions for this case, they are all short to mid-length and a small number of grafts do not spread from their starting position (8.7%, see Table 6.3). The behaviour of 2D EpiSC-derived NMps produced using the Tsakiridis et al. protocol is therefore consistent with that predicted for a mixed population of committed neural and committed mesodermal cells.

The inclusion of Chiron on the second day of differentiation (“Epi CF CF”) seems to produce cells that less frequently contribute to both neural
and mesodermal tissues and are not found in the node region. These cells can still be found in the PSM, however (see Table 6.3). There appears to be a higher number of neural contributions for this condition but this is could to be due to higher sampling of the rostro-medial part of the bounded region than for the “Epi F CF” condition. Additionally, it is noted that some of these grafts merged at the midline to give apparent neural contributions, with some including the tissues of the head (data not shown). It is also clear from Figure 6.12B that all of the grafts produce short to mid-length contributions, some of which may be the result of the grafts remaining intact at the graft site (23.8% of cases for this condition, see Table 6.3). The behaviour of 2D EpiSC-derived NMps produced using the Lippman et al. protocol is also consistent with a mixed population of neural-committed and mesodermal-committed cells.

In summary, the protocols for generating NMp-like cells from ES cells can produce populations that interact with the node as it regresses and that give long, mixed neural and mesodermal contributions. This is not the case for NMps that are derived in 2D culture from EpiSCs, which start from a state of pluripotency that corresponds to the post-implantation epiblast rather than the blastocyst. The “Epi F CF” protocol can also produce cells that give mixed neural and mesodermal contributions that include the node region, but none of these contributions is long. Cells from the “Epi CF CF” protocol do not seem to interact with the node and only infrequently contribute to both neural and mesodermal tissues. It therefore seems as if the epiblast derived candidate NMPs are more committed progenitors for each tissue than those derived from ES cells, which contribute to longer portions of the axis, though this may be confounded by differences in integration between the two cell types.
Figure 6.9: Xenotransplanted ES-Derived NMps Frequently Contribute to the Node Region. ES cells were treated according to the protocol published by Gouti et al. [65]. (A) Grafts scored by their contributions to the indicated tissues and (B) the length of their axial contributions (abbreviations and classification as in Figure 6.2). Labelled cells are frequently found in the mesodermal compartments and often additionally in the node region. Contributions to both neural and mesodermal compartments are infrequent but can be long.
Figure 6.10: Xenotransplanted ES-Derived NMps Can Produce Long Neuro-Mesodermal Contributions. ES cells were treated according to the protocol published by Turner et al. [172]. (A) Grafts scored by their contributions to the indicated tissues and (B) the length of their axial contributions (abbreviations and classification as in Figure 6.2). These grafts frequently contribute to both neural and mesodermal compartments across long regions of the axis.
Figure 6.11: Xenotransplanted EpiSC-derived NMps Can Produce Mid-Length Neuro-Mesodermal Contributions. EpiSCs were grown without Chiron treatment on the second day of differentiation. (A) Grafts scored by their contributions to the indicated tissues and (B) the length of their axial contributions (abbreviations and classification as in Figure 6.2). These cells often contribute to both neural and mesodermal tissues but only across mid-length extents of the axis. Labelled cells can also be found in the PSM and in the region around the node.
Figure 6.12: Chiron-Treated EpiSC NMps Infrequently Produce Neuro-Mesodermal Contributions and Do Not Contribute to the Node Region. EpiSCs were grown as in Figure 6.11, but with Chiron treatment on the second day of differentiation. (A) Grafts scored by their contributions to the indicated tissues and (B) the length of their axial contributions (abbreviations and classification as in Figure 6.2). Under these conditions, the cells do not contribute to the region around the node and infrequently contribute to both neural and mesodermal tissues.
6.4.2.4 Grafts Derived from Gastruloids

Gastruloids were prepared as described by Turner et al. [172] with or without Chiron treatment from 48-72 hours of the culture and were grafted as dissected tissue fragments at the 120 hour time point. Gastruloids grown in N2B27 were dissected into small pieces, while those that had been treated with Chiron were separated into putative anterior and posterior halves before they were further divided for grafting. The results of these grafts are presented in Figures 6.13, 6.14 and 6.15.

Strikingly, grafts from gastruloids grown continuously in N2B27 frequently produce long neuro-mesodermal contributions to the chick axis that include the region around the node (see Fig. 6.13). Labelled cells were frequently observed to leave the main part of the graft to reside in the region of the node as it regressed (data not shown, but represented in Table 6.3). The result of this interaction was often to draw labelled cells into the neural tube, which may explain why none of the grafts contributed to the PSM. While a high proportion of the grafts interacted with the node (52%, see Table 6.3), 16% remained intact at the graft site, which may account for some of the short, mixed neural and mesodermal contributions. Collectively, this pattern of incorporations is consistent with a population of neuro-mesodermal progenitors (see Fig. 6.5) and the high frequency of long integrations suggests that NMp-like cells comprise a greater proportion of the grafted population than for adherent NMp cultures.

The Chiron-treated gastruloids, which have been identified as a possible source of NMps in vitro [172] (and see Chapter 5: Gastruloids as a Source of Axial Progenitors), were divided into putative anterior and posterior halves according to their morphology. On grafting the anterior portions, labelled cells were infrequently found to contribute to both neural and mesodermal tissues. Two of these contributions were short (<500 µm) and the remaining one was classed as medium-length (516.39 µm). All other contributions were
mesodermal (including the PSM) and additionally with labelled cells in the region around the node in two cases. None of grafts produced a long axial contribution. The behaviour of grafts from this tissue is therefore consistent with a population of primarily mesodermal committed cells, with a small proportion of neural committed cells (see Fig. 6.5).

On grafting the posterior portions, labelled cells were observed to interact with the node during its regression to give long and medium length contributions that included the region around the node (6/20 grafts) and labelled cells could be found in the PSM (see Table 6.3). In contrast to the anterior explants, the mixed neural and mesodermal contributions included medium to long extents of the axis. Given that all three length classes were represented in this data, the behaviour of these grafts is consistent with the predicted behaviour of a tissue comprising a small population of neuro-mesodermal progenitors and a larger proportion of committed mesodermal cells.

In summary, explants from gastruloids grown continuously in N2B27 showed the most frequent long neuro-mesodermal contributions but they did not contribute labelled cells to the PSM. The posterior explants showed a lesser degree of interaction with the node (see Table 6.3) and less frequent neuro-mesodermal contributions but they did contribute labelled cells to the PSM. Both the N2B27-treated and the posterior Chiron-treated explants are therefore candidates for populations of neuro-mesodermal progenitors in vitro.

6.4.2.5 Concluding Remarks

The results of the fate mapping experiment (Fig. 6.2) defined a small region lateral to the node in which labelled groups of cells contributed to the somitic mesoderm and neural tube across long extents of the axis (>1750µm). This region does not give rise exclusively to long neuro-mesodermal contributions and it in fact includes short contributions that are restricted to specific tissues. The seemingly mosaic nature of this region could arise from the
Figure 6.13: Xenotransplanted N2B27-Treated Gastruloid Explants Frequently Produce Long Neuro-Mesodermal Contributions. (A) Grafts scored by their contributions to the indicated tissues and (B) the length of their axial contributions (abbreviations and classification as in Figure 6.2). Labelled cells are frequently found in both neural and mesodermal tissue compartments and additionally in the node region. Contributions to the PSM are notably absent. Many of the tissue contributions are long.
Figure 6.14: Xenotransplanted Anterior Explants from 3 μM Chiron-Treated Gastruloids Infrequently Produce Medium-Length Neuro-Mesodermal Contributions. (A) Grafts scored by their contributions to the indicated tissues and (B) the length of their axial contributions (abbreviations and classification as in Figure 6.2). Labelled cells are frequently found in the mesodermal tissues and infrequently also in the node region. They are infrequently found in short-medium stretches of both neural and mesodermal tissue compartments.
Figure 6.15: Xenotransplanted Posterior Explants from 3µM Chiron-Treated Gastruloids Infrequently Produce Long Neuro-Mesodermal Contributions. (A) Grafts scored by their contributions to the indicated tissues and (B) the length of their axial contributions (abbreviations and classification as in Figure 6.2). Labelled cells are often found in the node region and can contribute to long stretches of both neural and mesodermal tissues.
cells within it having a particular probability of contributing to the posterior growth zone. Two identically placed labels may therefore produce contributions of different lengths. An alternative explanation is that the point size in these representations is slightly smaller than the actual size of the labels, which have a mean diameter of around 120µm. The coverage of the bounded region and the overlap between similarly placed labels is therefore under-represented in these figures.

When cultures of pluripotent stem cells were grafted to the bounded region (Fig.s 6.6, 6.8 and 6.7), they often contributed to medium-length stretches of the mesodermal tissues and infrequently gave rise to mixed neuro-mesodermal contributions. For the ESC grafts, this mixed contribution was long. ESCs have therefore been ascribed to the “Mesodermal Progenitor” class in Figure 6.16. Labelled ESCs could be found in the PSM and in the region associated with the node, while labelled EpiSCs could not. This may be due to the high proportion of the EpiSC grafts that remained as intact groups near the original graft site (more than 50% in each case, see Table 6.3), which is a relevant confounding factor in assessing their potential to integrate into the developing axial tissues. For this reason, EpiSCs have been assigned to the “Mesodermal Committed” class in Figure 6.16.

On grafting candidate NMp populations generated from 2D cultures, those produced from ESCs could frequently interact with the node to produce long neuro-mesodermal tissue contributions. Comparing the results of the different protocols suggests that those derived using the protocol published by Gouti et al. have a more mesodermal identity than those from the Turner et al. protocol. The former have therefore been attributed to the “Mesodermal Progenitor” class in Figure 6.16 while the latter are in the “NMp” class (Fig. 6.16). For NMps derived from epiblast stem cells, only the “Epi F CF” condition was able to contribute labelled cells to the node region and neither condition produced any long axial contributions, though this could be due to poorer integration into the epiblast from these tissues. Due to the
short axial contributions, the EpiSC-derived NMps are placed between the “Neural Committed” and “Mesodermal Committed” classes in Figure 6.16. All of the candidate NMp populations contributed cells to the PSM.

On grafting explants of gastruloid tissues, those from continuous cultures in N2B27 produced the most frequent long contributions to both neural and mesodermal tissues, with labelled cells frequently being retained in the region of the node as it regressed. These explants did not contribute labelled cells to the PSM. Given the long, primarily neural contributions of N2B27-treated gastruloids, they have been placed on the neural side of the “NMp” class in Figure 6.16. Anterior tissues from Chiron-treated gastruloids infrequently contributed cells to both neural and mesodermal tissues but only across short axial lengths (around 500\(\mu m\)). All other grafts produced short to medium-length mesodermal contributions, sometimes including the node region. They have therefore been grouped with the EpiSCs in the “Mesodermal Committed” class in Figure 6.16. The explanted posterior tissues infrequently contributed cells to long stretches of the neural and mesodermal tissues and to the region around the node. For this reason, they are included with the N2B27-treated gastruloids and the ES-derived NMps (Turner et al. protocol) in the “NMp” class in Figure 6.16. Both the anterior and posterior gastruloid explants contributed cells to the PSM.

Taken together, these observations would suggest that ES cells treated according to the protocol published by Turner et al. [172] are a convincing candidate for an in vitro population of NMps since they can produce long neural and mesodermal contributions, they engage with the node as it regresses and they can contribute to the PSM when grafted to this region of the embryo. The behaviour of the N2B27-treated gastruloid tissues is striking, but without continued contribution to the somitic mesoderm via the PSM, it is possible that these grafts produce an ongoing contribution to the neural tissue with limited mesodermal contributions at more rostral axial levels. Alternatively, it is known that these gastruloids still generate
Figure 6.16: **Summary of Experimental Grafts.** The experimental grafts can be separated into broad classes, according to the predictions made in Figure 6.5. ESCs behave like mesodermal progenitors, while EpiSCs show shorter integrations and are assumed to be more committed. Candidate NMps derived in 2D culture from ES cells using the Turner et al. protocol behave as NMps, while those generated using the Gouti et al. protocol behave in a manner consistent with that predicted for mesodermal progenitors in this assay. Candidate NMps generated from EpiSCs behaved as predicted for mixed populations of neural and mesodermal committed cells. Tissues from gastruloids grown in N2B27 behaved as predicted for NMps, with a possible bias towards neural tissue in the caudal region. Anterior tissues from Chiron-treated gastruloids behaved as predicted for a mesodermal committed population, while those from the posterior end behaved as predicted for NMps.
morphologically identical elongations to the Chiron-treated gastruloids, albeit at a lower frequency, so there is also the possibility that some of the N2B27 tissue explants included Sox2 and Brachyury co-expressing regions. Notwithstanding this caveat, the treatment regime and the frequency of neural contributions is suggestive of a capacity of these explants to produce neural tissue. The posterior part of the chiron-treated gastruloids seems to produce longer neuro-mesodermal contributions than the anterior explants, but the frequency of these contributions is low. The posterior explants nevertheless also fulfill the additional criteria of interacting with the node as it regresses and contributing to the PSM.

6.5 Discussion

The results of this assay have demonstrated that different populations of cells grafted into this region of the chicken embryo will behave differently in terms of the tissues that they contribute to, the length of these contributions and their behaviour in relation to the node as it regresses. The populations of pluripotent cells (ESCs and EpiSCs) produced patterns of graft incorporation that are largely consistent with those predicted for mesodermal progenitors and mesodermal committed cells. This could be attributed to the cells’ response to an environment that is rich in Wnt and FGF signals, though this will need to be confirmed by the observed expression of specific markers of mesodermal differentiation. Candidate NMps derived from ES cells using the Turner protocol [172] behave in a manner that is consistent with that predicted for a population of NMps. Those derived from ESCs using the Gouti et al. protocol bear a closer resemblance to mesodermal progenitors in this assay. Candidate NMps from cultures of EpiSCs seemed committed to each lineage and did not produce the long grafts that would be predicted for progenitor cells. Grafts from the anterior Chiron-treated gastruloid tissues resemble a population of mostly mesoderm committed cells. Grafts from either the corresponding posterior regions or from gastruloids grown contin-
 massively in N2B27 showed behaviour consistent with a population of NMps, albeit with frequent contributions to the mesoderm from the Chiron-treated tissues. This could be the result of the Chiron treatment, which could increase the proportion of mesodermal tissue that would also be included in the graft, while the N2B27-cultured tissues may have a larger neural component. These conclusions are, however, limited by a number of confounding factors which are not related to the intrinsic potential of the cells and could affect their ability to incorporate into the embryo.

6.5.1 Integrity of the Grafted Tissues

It is clear from Table 6.3 and Figures 6.6, 6.8 and 6.7 that the pluripotent cell populations differ greatly in their ability to integrate into the embryo and contribute to neural and mesodermal tissues. Each population can produce these derivatives in culture and it is therefore unsurprising that the ESCs can infrequently contribute to long stretches of both neural and mesodermal tissues from a population of pluripotent cells. It is possible that the epithelial nature of the epiblast stem cell grafts reduced their ability to integrate efficiently into the tissues of the chick, while the more mesenchymal ES cell grafts spread efficiently within the tissues. This is an important caveat to the statement that the more advanced pluripotent cells become progressively poorer at contributing to the neural and mesodermal tissues of the embryo. Poor incorporation may explain the high proportion of the epiblast grafts that remain as discrete groups and only contribute to very short stretches of the axis. This may also be the case for the candidate NMp populations that were derived from cultures of epiblast stem cells that showed a similar pattern of incorporation and short to medium-length contributions.

6.5.2 Limitations of the Assay

As alluded to above, a major limitation of this assay is that it does not currently allow the behaviour of these cells to be tested at the resolution of
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single cells. For the purposes of the scoring, and as illustrated by ESC grafts, this assay cannot resolve a long neuro-mesodermal contribution produced by a mixed population of separate neural and mesodermal progenitors from a contribution produced by a population of bipotent axial progenitors. The additional criteria of retention in the region around the node and of contribution to the PSM offer further indications of possible NMp properties but this assay cannot absolutely define them. Further work will be required to follow the incorporation of these cells at the single cell level using high resolution microscopy and clonal labelling approaches. Only then can the question of bipotency and self-renewal in these grafts be definitively resolved. Nevertheless, it is clear that this assay can be used to make predictions on the proportions of neuro-mesodermal progenitors in candidate populations of cells.

As a first pass approach, this study simply scored tissue contributions by the localisation of the fluorescent signal of the labelled cells using wide-field microscopy. This has the effect of over-estimating the number of short mixed neural and mesodermal contributions in the cases where the grafted cells have converged from each side to the midline, giving the appearance of contribution to both tissues. Detailed histology will be required to determine precisely in which tissues the labelled cells can be found and to determine how efficiently the grafts integrate into the host tissues. It will be particularly important to confirm that the grafted cells are being introduced correctly into the epiblast layer and that they are not simply spreading within the mesodermal compartment, since this would preclude their ability to generate any neural tissues. An additional layer of detail is required to determine what proportion of the grafted tissue is viable after culture in the embryo and whether it differentiates appropriately for each tissue compartment; an immunofluorescence approach for fixed tissues would be appropriate.

The rectangular region of the embryo that was targeted in this study was identified from the collated results of the fate mapping experiment in Figure
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6.2. The boundaries of this domain were set to enclose the largest region in which grafted cells would produce long contributions to both neural and mesodermal tissues, using data from all stages tested. It is worth noting that the axial progenitor domain is unlikely to be rectangular and it is likely to change in both position and shape at different stages of axial development. It is also possible that the sampling of this region may have affected the interpretation of the results in each case. The gastruloid grafts are an example that warrants revisiting, since the grafts from cultures grown with N2B27 continuously are targeted entirely within the bounded region, while both sets of explants from the Chiron-treated tissues are distributed over the caudal limit of the region. A more complete sampling of the bounded region may resolve the differences between these tissues more clearly.

A lateral position was chosen in preference to a medial one as a means of increasing the throughput of the assay but a regime of medial grafts is a clear target for further study that would provide a clearer indication of axial progenitor behaviour. In reviewing the results of tissue transplantations in the mouse embryo, Cambray and Wilson describe how neural and mesodermal progenitors overlap extensively in the ectoderm layer lateral to the node and the rostral primitive streak [22] and that this region makes a minor contribution to the chordoneural hinge in addition to the cells of the node-streak border. Grafts targeted to this region may, therefore be more likely to contribute to both the neural tube and paraxial mesoderm than from a lateral position. In reality, it is likely that those cells that interact with the region around the node are drawn into such a position as it regresses. Cambray and Wilson also raise the important point that the fate map of the chicken axial progenitor zone has the same rostro-caudal arrangement as in the mouse, but that a much smaller proportion of the chick streak produces somites (only the most rostral 20%) and that the region from 50-80% of its length is destined to produce lateral mesoderm. The fate map of the chick primitive streak at the 7 somite stage, they summarise, corresponds to a much earlier stage in
the mouse embryo, closer to the late streak stage at E7.5 [22]. The results of the fate mapping in Figure 6.2 seem to capture the diversity of somitic and lateral fates across this region but a relevant consideration is that the grafting regime may also include heterochronic effects between the grafts and their hosts.

In previously published fate maps of the chick node, it is noted that the rostral part of the node and the anterior portion of the primitive streak contribute to the medial part of the somites, with the lateral parts being derived from a rostral portion of the primitive streak [140] [127]. A candidate NMp population would be expected to produce the medial paraxial mesoderm and it might therefore be profitable to score the grafts that contribute to the somitic mesoderm based on their medio-lateral location. This analysis will, however, depend on accurate histological analysis of the tissues.

It is worth restating that every type of graft tested in this assay gave at least one mixed contribution to both the neural and mesodermal compartments of the embryo. In the same way that a population of NMps cannot be defined by co-expression of Sox2 and Brachyury alone, the contribution of a grafted population to both the neural and mesodermal tissues of an embryo does not demonstrate bipotency. The bar for such validation experiments needs to be set higher using functional criteria such as retention in the axial progenitor niche around the node and in contribution to the undifferentiated regions of the PSM and the caudal-most neural tube; this assay presents a means of testing for these properties. The results presented in this study begin to capture these additional criteria through qualitative scoring but closer examination of the axial levels of each contribution is required to resolve these more subtle differences.

In conclusion, the results of this assay show that the putative posterior end of the Chiron-treated gastruloids behave in a manner that is consistent with the endogenous population of NMps in the chick and with previously
published populations of *in vitro*-derived NMps [172][65]. This region has qualitatively different properties to the corresponding anterior tissue, which behaves more like a mesodermally committed population. The observation that gastruloids cultured continuously in N2B27 can frequently give NMp-like behaviour in this assay is consistent with the observation that the gastruloids can elongate in culture and it invites closer investigation of their tissue composition at this stage. It remains to be seen whether the three dimensional context of the gastruloid progenitors affects their properties, but it is possible that the tissues of the aggregate provide them with a niche that is otherwise absent from adherent cultures.
Chapter 7

Physical Inputs to Gastruloid Culture

7.1 Introduction

The results presented in Chapter 5 describe a candidate population of neuro-mesodermal progenitor (NMP) cells in the elongating region of the gastruloids. A major limitation to the propagation of the NMP population is the adhesion of the growing gastruloid tissues to the plastic culture plate around 120 hours after aggregation. A possible solution is to embed the gastruloids within a synthetic matrix that provides mechanical support to the growing tissues but does not promote adherent tissue growth. This chapter will first explore the mechanical environment of the post-gastrulation mouse embryo, before considering possible approaches to tissue embedding from the embryoid body (EB) and organoid fields. The results presented in this chapter demonstrate that Matrigel® provides a means of extending the culture of the elongations and that spontaneously hydrolysing poly(ethylene glycol)-acrylate hydrogels are a chemically defined alternative to this synthetic extracellular matrix.
7.1.1 Physical Inputs to Embryonic Morphogenesis

Many investigations into the embryonic mechanical microenvironment have naturally focussed around implantation. Less is known, however, about local microenvironments in the post-gastrulation mouse embryo, possibly due to the added difficulty of obtaining embryos at this stage and the technical demands of making mechanical measurements at the tissue level instead of at the whole-embryo scale. From the perspective of the candidate NMP population, the key questions are: 1) to what extent can axial cells sense their mechanical environment? and 2) is there a physical component to axial elongation of the embryo? Each will be considered in turn before a discussion of how a synthetic system could be engineered to culture and propagate these tissues.

The extracellular matrix (ECM) is the most immediate mechanical environment for the cells in a tissue and it has its own physico-chemical properties that can change over time. Cells can sense the stiffness of the ECM and in the case of human mesenchymal stem cells, this can strongly influence their cell fate specification in synergy with the action of soluble factors (Engler et al. [48]). Loganathan et al. hypothesise that “time-dependent material property change is an emergent morphogenetic mechanism” [105]. When coupled with the observations of Engler et al., this could provide a physical basis for generating tissue pattern over time as different groups of cells respond to a changing, shared environment.

Cells are also sensitive to tension that may be transmitted through the ECM [105]. The notochord is an axial tissue derived from the node that extends by cell proliferation, accretion and re-arrangement as the body axis elongates [138]. Imuta et al. contend that the correct re-arrangement of cells in the notochord is dependent on tension across the embryo. This is generated by the isotropic expansion of the amniotic cavity, which separates the embryo from the extra-embryonic tissues [85]. They tested this hypothesis by piercing
the amniotic cavity with a microcapillary needle, releasing the tension across the embryo. The resulting embryos developed a shortened body axis with mis-orientation of cell division in the notochord, although the final somite number was normal [85]. These results suggest that the elongation of this axial tissue relies on a degree of antero-posterior tension to correctly orientate cellular re-arrangements.

The notochord is not the main driving force underlying axial elongation in the chick, however. Bénazéraf et al. demonstrated through laser ablation that bilateral deletion of the posterior part of the presomitic mesoderm (PSM) slows the rate of axial elongation to a greater extent than the loss of any of the axial structures or anterior PSM [14]. By tracking the movement of individual cells and the ECM, the authors found that motility was highest in this region and decreased more anteriorly, but was non-directional. They used genetic and pharmacological manipulations to demonstrate a causal link between FGF signalling and cell motility in this region. The posterior tissues produce a gradient of FGF8 that, through modulation of cell motility in a graded fashion, can establish the observed pattern of increasing cell density moving towards the anterior. They speculate that the lateral constraints imposed by the more anterior PSM bias tissue expansion to the posterior end of the embryo, thereby promoting axial elongation [14]. These results can be coupled to the findings of Girós et al. that the formation and sensing of a functional Fibronectin ECM is needed for correct PSM cell migration and axial elongation to occur ([61], reviewed in [105]). Collectively, these results suggest that the cells in the growing posterior end of the embryo are receptive to the extracellular signalling and mechanical environments and that the interaction between the two may generate a posteriorly directed tissue expansion that drives axial elongation. Bénazéraf and Pourquié concede that direct mechanical measurement of the axial tissues will be required to reinforce this link between tissue mechanics and the dynamics of elongation [15]. From the perspective of propagating an elongating axial tissue in vitro,
it is clear that it will require a carefully designed microenvironment to do so.

### 7.1.2 Engineering Synthetic Microenvironments

Artificial extracellular matrices form an integral part of many 3D culture systems, not only as a scaffold in which to culture cells but also as a source of biochemical signals. One of the most popular is Corning® Matrigel®, a complex mix of extracellular matrix proteins derived from the Engelbreth-Holm Swarm mouse sarcoma line. It is well-suited to embedding live cells since it forms a soft gel at temperatures above 10°C. It is in routine use across the organoids field and sees application in cerebral, optic cup, intestinal, stomach, colonic, lung and liver organoid cultures (reviewed in [170]). A major limitation, however, is that Matrigel® is not completely defined and it shows considerable batch-to-batch variability. The incomplete definition of Matrigel® and its tumour origin currently precludes its use in clinical applications.

It is therefore desirable to identify chemically defined alternatives to Matrigel® in order to move the field towards therapeutic applications. Simple examples include methylcellulose, used to promote the formation of haematopoietic lineages in embryoid body (EB) cultures ([181], reviewed in [39]) and agarose, which has been shown to improve the efficiency of human EB formation, growth and differentiation in comparison to suspension cultures [147]. These cases indicate that it is not necessarily a protein component of Matrigel® that makes it such a successful platform for 3D cell culture, but that there is also the simple physical component of the mechanical properties of the matrix. It is also possible that semi-solid media may promote the local enrichment of factors secreted by the tissue that promote differentiation.

A further refinement in engineering a synthetic tissue microenvironment is to build upon the simplicity of these defined matrices by including additional components in a controlled manner. This enables extracellular matrix
components to be tested combinatorially to build up a fully defined, optimal culture environment. Ehrbar et al. present a particularly flexible approach that uses Factor XIII, a transglutaminase enzyme from the blood clotting cascade, to cross-link peptide-conjugated macromers of poly(ethylene glycol) [44]. This system also undergoes gelation under physiological conditions and can be readily customised to include a variety of peptide linkers with different biochemical activities. A range of gels can therefore be engineered that have the same physical characteristics but can range from being completely biochemically inert to sharing many of the properties of a tissue in vivo.

A final layer of complexity in engineering a synthetic microenvironment is the global culture environment outside the embedded tissue. This can range from a static arrangement of tissues such as the air-liquid interface culture used to generate kidney organoids (reviewed in [170]) to dynamic bioreactor cultures such as those used to generate cerebral organoids ([96], reviewed in [170]). Dynamic culture environments can affect the growth and fate decisions of cultured cells through hydrodynamic forces and increased nutrient and gas exchange. Carpenedo et al. describe how a rotary culture environment “formed EBs more efficiently, demonstrated higher cell and EB yield, and exhibited improved homogeneity of size and shape...” [23]. Rotary EB cultures have also been shown to express higher levels of ECM proteins and proteases, suggesting that they undergo more active remodelling than their counterparts in static culture [57]. Furthermore, rotary cultures show less necrosis (possibly due to their smaller and more uniform size) and expression of a wider range of growth factors, suggesting that their eventual fate might be affected by their global culture environment [57], [23].

The key feature of these environments is that they increase the rate of nutrient and gas exchange through mass transport of the culture medium. This allows tissues to be cultured beyond the limit of purely diffusive transport, which is estimated to be around 100-150µm (reviewed in [91]) and which likely explains the necrotic core of large EBs in static cultures. It is worth
noting that the latest stages of mouse embryonic development can only be reached \textit{in vitro} in a shaken culture environment [29]. The \textit{in vitro}-cultured mouse embryos described in this paper are a striking example of the extent of development that could be reached in a synthetic system, given appropriate culture conditions. These small-scale conditions impose a hydrodynamic shear stress on the tissues that is comparable to the environment within a stirred bioreactor culture (reviewed in [91]), which would provide the means to efficiently increase the scale of the cultures to an industrial level. In doing so, it will be important to strike a balance between the effect of hydrodynamic shear forces required to mix the medium and the risk of aggregate agglomeration to ensure that the culture remains relatively homogeneous (reviewed in [91]).

It is clear that developing tissues in the embryo are sensitive to their mechanical microenvironment and that artificial extracellular matrices provide the means to probe these interactions. The organoid field has demonstrated through its use of Matrigel\textsuperscript{®} how critical these interactions are in patterning tissues cultured \textit{in vitro}. The goal of this chapter, therefore, is to integrate Matrigel\textsuperscript{®} into the gastruloid culture in order to provide a degree of mechanical support to the developing elongations. A further aim is to test chemically defined PEG hydrogel matrices to identify the key biomechanical properties of the ECM that affect the elongation of the gastruloids such that their growth can be maintained.

\section{7.2 Results}

\subsection{7.2.1 Sensitivity to the Time of Embedding in Matrigel\textsuperscript{®}}

Suspension culture of the gastruloids is limited to around 120 hours after aggregation, at which point they begin to form an adhesion to the plastic of the culture well. The main aim of integrating Matrigel\textsuperscript{®} into the gastruloid protocol was to provide a means of extending the culture beyond this point.
From the perspective of the gastruloids as a source of NMps, extending the culture could allow the population to be propagated if the structure of the gastruloids can be maintained in the long term.

Regarding the experimental approach, gastruloids were prepared from the \textit{T/Bra::GFP} and the \textit{GPI-GFP} cell lines and were embedded in droplets of neat Matrigel\textsuperscript{®} at different times in the culture. The \textit{T/Bra::GFP} cell line was chosen as a means of monitoring posterior identity during the embedded cultures, as indicated by continued local expression of the reporter. Cells from the \textit{GPI-GFP} line have GFP anchored to the outer leaflet of the plasma membrane by an attached lipid modification \cite{129}, allowing the structure of the tissue to be assessed by live imaging\textsuperscript{1}. Initial experiments adapted the embedding protocol described in \cite{96} to prepare the aggregates within floating droplets. Subsequent experiments used an immobilised culture where the droplets were cast on the surface of a 6-well plate for ease of imaging. The results of embedding aggregates in neat Matrigel\textsuperscript{®} at the 72 hour, 96 hour and 120 hour time points are represented in Figure 7.1, below.

When aggregates are embedded before the phase of elongation, ie. at the 72-hour time point, they form many outgrowths into the matrix (see panel B). Similarly, embedding during the early stages of the elongation at the 96-hour time point results in widespread cell migration into the gel before the elongation can reach its full extent. Only by embedding the aggregates at the 120-hour time point, by which stage they have reached the limit of their growth in suspension culture, can they go on to elongate further in the gel. \textit{Brachyury} expression is maintained during the course of this elongation but is later lost at the 168-hour time point. This result mimics the loss of

\textsuperscript{1}The GFP channel is not shown in Figures 7.3, 7.4, 7.5, 7.6 and 7.7 since the cell membranes are poorly resolved by low magnification widefield microscopy.
Figure 7.1: **Embedding Gastruloids Prior to 120 Hours Results in Disordered Growth.** Gastruloids were embedded in neat Matrigel® at the points indicated in (A). The results of embedding at the 72 hour time point are shown in panel (B) and those from 96 hours are shown in panel (C). In each case, the aggregates grow into the gel and lose their elongating structure. When embedded at 120 hours (Panel (D)), the aggregates continue their directional elongation before starting to grow into the gel at around 150 hours.
**7.2. RESULTS**

*Brachyury* expression that occurs when the aggregates grown in suspension begin to grow adherently on the surface of the culture well.

The images presented in Panel D of Figure 7.1 are shown with the corresponding *Brachyury*::GFP signal in Panel B of Figure 7.2. Panel C shows equivalent gastruloids from the *Tbx6*::eYFP cell line embedded under the same conditions, suggesting that this pattern of continued elongation may be a general phenomenon.

![Figure 7.2](image)

**Figure 7.2: Embedding Gastruloids in Matrigel® at 120 Hours Extends the Elongation Phase to 150 Hours.** (A) The treatment regime and the point of embedding at 120 hours. (B) A *T/Bra::GFP* aggregate embedded in a droplet of neat Matrigel® elongates for a further 30 hours, before the cells of the tissue start to invade the matrix. (C) A *Tbx6::eYFP* aggregate embedded under the same conditions also shows continued, directional growth. Cell migration into the gel begins at the anterior (*Brachyury*-negative) pole of the aggregate.
7.2.2 Cell Migration is Dependent on the Concentration of Matrigel®

The aim of this experiment was to test whether the continued elongation and the migration of cells from the putative anterior pole is sensitive to different concentrations of Matrigel®. This is an important target for optimisation, since the invasion of cells into the gel can disrupt the structure of the gastruloid tissues and so limit the duration of embedded cultures. Aliquots of Matrigel® were diluted in PBS to different final concentrations before aggregates were embedded in 20µL droplets for imaging. Figures 7.3-7.7 show the patterns of growth in concentrations of Matrigel® ranging from 20%-100%.

Figure 7.3: Gastruloids Embedded in 20% Matrigel® Undergo Rapid Cell Migration. GPI-GFP gastruloids were embedded in 20% Matrigel® at the 120 hour time point and were imaged over 50 hours. Cells started to migrate through the gel within 3 hours of embedding and continued to migrate, eventually disrupting the structure of the gastruloids.
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Figure 7.4: Gastruloids Embedded in 40% Matrigel® Continue to Elongate for up to 20 Hours. GPI-GFP gastruloids were embedded in 40% Matrigel® at the 120 hour time point and were imaged over 50 hours. Tissue elongation continued for around 20 hours, after which point cells started to migrate from the putative anterior and the elongating tissue retracted.

The results presented above suggest that the optimal concentration of Matrigel® for extending the gastruloid cultures is approximately 60%. At lower concentrations, cells throughout the gastruloid start to invade the gel (see 20%, Fig. 7.3) within the first 20 hours of embedding the tissues. The migration can be as early as within 3 hours for gastruloids cultured in 20% Matrigel®. A concentration of 40% seems to be conductive to the elongation of the tissue, which reaches its maximum length by 143 hours before it starts to retract. Gastruloids embedded in 60% Matrigel® show continued elongations that are maintained for up to 30 hours. Cell migration from the putative anterior starts 10-20 hours after embedding in this condition. At higher concentrations of Matrigel®, the elongations can be maintained for a similar length of time but the morphology of the elongating tissue is broader and shorter, suggesting that the gel might become restrictive at this concentration. Embedding the aggregates in 100% Matrigel® delays cell migration from the putative anterior to after approximately 20 hours of embedding.
Gastruloids Embedded in 60% Matrigel® Continue to Elongate for up to 40 Hours. GPI-GFP gastruloids were embedded in 60% Matrigel® at the 120 hour time point and were imaged over 50 hours. Tissue elongation continued for up to 40 hours before the elongation started to retract. Cell migration from the putative anterior started within 10-20 hours of embedding and in one example was not accompanied by elongation.
Figure 7.6: Gastruloids Embedded in 80% Matrigel® Continue to Elongate for up to 30 Hours. GPI-GFP gastruloids were embedded in 80% Matrigel® at the 120 hour time point and were imaged over 50 hours. Tissue elongation continued for up to 30 hours before it started the tissue started to retract. In some cases, the elongations were broader and shorter than for lower concentrations of Matrigel®. Cell migration from the putative anterior started within 10-20 hours of embedding.
GPI-GFP gastruloids were embedded in Matrigel® at the 120 hour time point and were imaged over 50 hours. The tissue continued to elongate for up to 20 hours before it started to retract. Cell migration from the putative anterior started approximately 20 hours after embedding.
The elongating tissues, however, reach their maximum length at this early stage before they start to retract, again suggesting that stiffer matrices might become restrictive. In summary, the 60% Matrigel® condition provides the longest period of continued elongation, though stiffer gels delay the migration of the cells until approximately 20 hours after embedding.

### 7.2.3 Gastruloids do not Elongate in PEG-VS Hydrogels

#### 7.2.3.1 Note on Collaborative Work

The data presented in this section and in Section 7.2.4 were collected in collaboration with Mehmet Ugur Girgin, in the laboratory of Professor Matthias Lutolf at the École Polytechnique Fédérale de Lausanne, Switzerland. This project was funded by a Development Travelling Fellowship through the Company of Biologists. All practical work and imaging in this section was carried out equally with Mehmet, with data analysis performed separately in Cambridge. The experiment presented in Section 7.2.4 was devised together but Mehmet conducted all the practical work. It is included in this chapter for completeness as a conclusion to our collaborative project.

#### 7.2.3.2 Aims & Experimental Approach

A major aim of the research visit was to test chemically defined alternatives to Matrigel® for extending aggregate culture beyond 120-hours, in the manner that has been described earlier in this chapter. A key aim was to identify a specific, minimal set of culture conditions that would permit the continued elongation by progressively increasing the complexity of the microenvironment.

The basic experimental approach was to embed gastruloids within defined poly(ethylene glycol) (PEG) matrices inside small poly(dimethyl siloxane) (PDMS) microwells (see Chapter 2: Materials & Methods). This arrangement allowed very soft matrices to be tested without disrupting them during
medium changes, as would be the case with an exposed droplet on a surface.

Aggregates of \textit{T/Bra::GFP} cells were embedded 110 hours after aggregation in either 0.8% or 1.0% PEG-VS hydrogels with or without additional Laminin. They were imaged after embedding, then every 24 hours for two days. The results are presented in Figures 7.8, 7.9, 7.10, and 7.11.

\textbf{Figure 7.8: 0.8% PEG-VS Hydrogels do not Support Continued Elongation.} 110-hour aggregates were embedded in a 0.8% PEG-VS hydrogel and were imaged over 48 hours. Instead of continuing to elongate as in Matrigel\textsuperscript{®}, the tissues instead form broad structures and lose Brachyury expression by the 134 hour time point (data not shown).
Figure 7.9: **110-Hour Aggregates can Develop Complex Epithelia in 0.8% PEG-VS Hydrogels with Laminin.** The majority of aggregates do not continue to elongate in the manner that has been described for Matrigel® when they are embedded in 0.8% PEG-VS with Laminin at 110 hours. A notable exception is shown in the top row, where a complex series of epithelia appears to develop and rotate with time. As for the condition without Laminin, Brachyury expression has been lost by the 134-hour timepoint and is not regained (data not shown).
Figure 7.10: 1.0% PEG-VS Hydrogels do not Support Continued Elongation. 110-hour aggregates were embedded in 1.0% PEG-VS and were imaged over 48 hours. Instead of continuing to elongate as has been described for Matrigel®-embedded cultures, the tissues instead form broad epithelia and lose expression of Brachyury by 134 hours (data not shown).
7.2. RESULTS

In all cases, it is clear that the aggregates lose expression of Brachyury and do not continue to elongate; instead, they form epithelial tissues. These observations indicate that these matrices are soft enough to permit the expansion of the growing tissue, but that they may be too stiff to allow the continued growth of the elongation as seen in Matrigel®. It is unclear whether the inclusion of Laminin in the matrices affects the development of tissue polarity due to the small sample size and the difficulty in maintaining aggregate viability during the embedding process. Figure 7.9 would suggest that Laminin does promote the formation of epithelial tissues, while Figure 7.10 suggests that they can form in a stiff environment in the absence of the protein. Further experiments will be required to determine how the mechanical microenvironment determines the structure of the forming tissues in this region.

Figure 7.11: **110-Hour Aggregates Embedded in 1.0% PEG-VS can Develop Epithelia but do not Continue to Elongate.** When embedded at this time, the elongations do not continue to grow as has been described in Matrigel®, but instead form broad epithelial tissues. As for the other matrices tested in this experiment, Brachyury expression is lost by 134 hours (data not shown).
7.2.4 PEG-Acrylate Hydrogels as an Alternative to Matrigel®

Following the end of the research visit, Mehmet has successfully cultured elongating aggregates in dynamically softening PEG-Acrylate hydrogels. The cross-linked polymer network undergoes spontaneous hydrolysis over time, resulting in a progressive degradation of the gel. By mixing PEG-Acrylate and PEG-VS macromers in different proportions, the properties of the gels can be tuned to leave a range of soft, stable gels after the hydrolysis has occurred. Aggregates of Brachyury::GFP cells were embedded 100 hours after aggregation in a range of matrices and were imaged over the following 63 hours. The results of these experiments are summarised in Table 7.2.4.

<table>
<thead>
<tr>
<th>Matrix Composition</th>
<th>T/Bra::GFP Exp. at 143h (+/-)</th>
<th>Elongated at 143h (+/-)</th>
<th>T/Bra::GFP Exp. at 163h (+/-)</th>
<th>Elongated at 163h (+/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Matrigel®</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1.5% PEG (100% -Ac)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.0% PEG (100% -Ac)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.5% PEG (100% -Ac)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1.5% PEG (75% -Ac, 25% -VS)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.0% PEG (75% -Ac, 25% -VS)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 7.1: Summary of Culturing 100-Hour T/Bra::GFP Aggregates in PEG-Acrylate Hydrogels. 2.5% PEG-Acrylate Hydrogels maintain both Brachyury expression and morphogenesis until 163 hours and are therefore a suitable alternative to Matrigel®.
These results suggest that the softening dynamics of a 2.5% PEG-Acrylate hydrogel are suitable for maintaining Brachyury expression and the growth of the elongations. It seems as if hydrogels with lower concentrations of PEG-Acrylate soften too rapidly to support the maintenance of the elongation, while those containing a small amount of PEG-VS may remain too stiff. It is clear from the images in Figure 7.12 that the 2.5% PEG-Acrylate matrix also enables cell migration from the anterior of the aggregate, demonstrating that this cell behaviour is dependent on the properties of the mechanical microenvironment rather than on interactions with the extracellular matrix proteins of Matrigel®. It will be important to reproduce these results but at this stage they are a promising indication that the PEG-Acrylate system is a suitable alternative to Matrigel®.

Figure 7.12: 2.5% PEG-Acrylate Hydrogels Extend Gastruloid Culture. (A) Microfabricated chips in small Petri dishes with gastruloids cultured in PEG hydrogels (pink droplets). (B) A 120 hour gastruloid embedded 2.5% PEG-Acrylate continues to grow and maintains Brachyury expression (green). (C) For comparison, a 120 hour gastruloid embedded in the biologically-derived matrix, Matrigel®.

7.3 Discussion

The introduction of this chapter set out two key questions to be addressed by published data, namely whether the cells of the axial tissues can sense their mechanical environments and whether there is consequently a physical
component to the process of axial elongation. The results presented here demonstrate that the gastruloid tissues are highly sensitive to the time at which they are embedded in Matrigel® and will only continue to elongate directionally when embedded around the 120-hour time point and not sooner. In addition, this behaviour is dependent on the concentration of Matrigel® as the gastruloids undergo non-directional cell migration in low concentrations (20%, Fig. 7.3) but will continue to elongate at higher concentrations (Fig.s 7.4, 7.5, 7.6 and 7.7). This is corroborated by results from the PEG-hydrogel-embedded cultures that suggest that the stiffness and degradability of the matrix are important physical characteristics for the continued growth of the tissues. By extension, the key feature of Matrigel® in this system may be a physical characteristic rather than a biochemical one, as equivalent elongations can be achieved in 2.5% PEG-Acrylate hydrogels (Fig. 7.12).

Matrigel®-embedded cultures from the 120-hour time point are shown to maintain Brachyury expression until the 168-hour time point and elongate substantially over this period. The PEG-VS hydrogels did not provide a suitable alternative since Brachyury expression was quickly lost and the elongations instead formed broad epithelia. It seems as if the gel environment is soft enough to permit initial tissue expansion, but that it is too stiff to support the continued elongation that characterises the Matrigel® cultures. In accordance with this, the dynamically softening PEG-Acrylate gels provided a suitable environment with the maintenance of the elongation. In this case, Brachyury expression was maintained at least as long as in the Matrigel®-embedded cultures. It seems as if the expression of Brachyury in this region is affected by the surrounding mechanical environment and that the dynamics of the gel softening process are matched to the growth of the elongation. The exact correspondence between these processes remains to be described and is a good target for optimisation. This could be achieved through micromechanical measurements of the growing tissue and the degradation of the matrix, as well as empirical approaches that test a range of
PEG-VS/PEG-Ac mixtures that will soften to different degrees. The results of this optimisation may shed light on the mechanical features that regulate the posterior growth zone that are very difficult to measure in the embryo. In summary, the stiffness of the matrix is a key biomechanical property that affects the elongation of the gastruloids in PEG-hydrogel cultures.

From a practical perspective, both Matrigel® and the PEG-Acrylate hydrogels provide a means of extending the culture beyond its limits in suspension to generate larger elongations and more mature tissues. The results presented in Figure 7.5 indicate that a lower concentration of 60% Matrigel® may be an appropriate refinement to embedded cultures, reducing the consumption of this reagent. The main advantage offered by the PEG-Acrylate system is that it is chemically defined and not derived from a transformed cell line and is, in principle, amenable to clinical applications. If aggregate embedding becomes an important stage in the derivation of particular cell types in the future, this may become a particularly important finding for the translation of this experimental approach. It is particularly interesting to note, however, that the extensive cell migration from the anterior is a limiting factor for both synthetic extracellular matrices. It is likely that the migratory behaviour of these cells is an inescapable response to their mechanical environment, rather than being biochemically induced by the ECM proteins in Matrigel®, since it is observed in soft, inert hydrogels as well as biochemically active ones. Further experiments are certainly required to characterise these cells and to identify how they respond to their environment, with a view to controlling their growth in the future.

In terms of taking these bioengineering approaches forward, it will be important to first refine the handling of the aggregate tissues during the preparation of the hydrogels to balance sample throughput with aggregate viability. As it stands, the role of Laminin in the PEG-VS hydrogels is unclear due to the small number of viable aggregates in this study, making this an appropriate question to revisit. A further improvement will be the use of
second-generation PEG macromers that are the subject of current research in Professor Lutolf’s laboratory. These macromers are engineered to carry additional side arms, which provides additional sides for functionalization with ECM peptides or for cross-linking. The result will be a system of gels that will be capable of gelation at lower percentages of PEG (below the current lower limit of around 0.7%) and that will have quite different physical properties. With these advances in hand, it will be relatively straightforward to explore the effects of additional ECM components on the continued growth that has now been established in the PEG-Acrylate system.

A final point to consider and one that is emphasised by Loganathan et al., is that the ECM is not simply a passive scaffold for cell migration but a material that changes its physical characteristics over time and undergoes its own flows during morphogenesis [105]. Novel bioengineering methods can address the first feature but the large scale flows remain difficult to engineer. A possible solution may involve the use of complex microenvironments that are built up from differently composed matrices and that integrate directional physical forces such as hydrodynamic flows into the culture. The process of devising such an environment and the resulting effects on tissue morphogenesis will help describe the properties of the ECM in vivo and will provide predictions as to which mechanical features are important during development.
Chapter 8

Discussion

The aims of this research were summarised in Chapter 1 as:

- To establish an *in vitro* model system of axial elongation morphogenesis from ES cells;
- To determine whether the tissues contain a candidate population of NMps;
- If present, to determine the properties of these NMps in comparison to those derived from 2D-cultures.

The results presented in the preceding chapters show that all three aims have been met. Chapters 3 and 4 demonstrated that aggregates of mouse embryonic stem cells can autonomously undergo axial elongation morphogenesis when formed from approximately 100-400 cells. Strikingly, early gastruloids acquire putative antero-posterior polarity in the absence of the extraembryonic tissues that are thought to instruct this process *in vivo*. A direct correspondence can be drawn between the development of the gastruloids and that of the early post-implantation embryo, suggesting that the gastruloids are faithfully recreating the course of normal development. Taking this in hand with the apparent reproducibility of the elongations, the gastruloids offer a new model system for studying axial elongation morphogenesis *in vitro.*
In relation to the second aim, Chapter 5 showed that the putative posterior elongations of the gastruloids contain a region of Sox2 and Brachyury co-expression that resembles the posterior growth zone of the embryo that harbours the NMp population. Explant cultures showed that this region was capable of autonomous elongation when cultured in Matrigel®. Chapter 6 described the development of an assay that used the chicken embryo to functionally test the behaviour of populations of candidate NMps. This assay showed that candidate NMp populations derived from ES cells could contribute cells to both the neural tube and paraxial mesoderm across substantial extents of the chick axis. While this was also possible from self-renewing cultures of ES cells, the frequency of long contributions was higher in cells differentiated as NMps and they often included groups of cells that remained resident at the node. Posterior explants from Chiron-treated gastruloids were found to behave in a manner consistent with the published in vitro-derived NMps, indicating that the elongating tissue does indeed harbour an axial progenitor population. Gastruloids grown continuously in N2B27 frequently gave long contributions to the axial neural and paraxial mesodermal tissues, often including cells resident in the region around the node. While this suggests that they may be competent to form NMps, N2B27-cultured gastruloids do not contribute to the PSM so their potential may be more limited. The properties of gastruloid-derived NMps are quantitatively comparable to NMps derived from 2D cultures (i.e. they give similar length neural and mesodermal contributions at a similar frequency) but they qualitatively differ on the extent of their interaction with the region around the node, with the gastruloid tissues occupying this region for longer extents of the posterior elongation process. To address the third aim, NMps derived from gastruloids therefore appear to more frequently occupy the axial progenitor niche than cells derived from comparable 2D cultures.

Having satisfied the aims specified in the introduction, the gastruloid system was extended in Chapter 7 by integrating it with bioengineered hy-
drogels to support elongation morphogenesis. In keeping with the alignment with embryonic development presented in Chapter 4, the gastruloids continue to elongate when cultured either in Matrigel® or PEG-Acrylate hydrogels, demonstrating that mechanical support is a key input in maintaining tissue elongation in vitro. These results are important preliminary findings for advancing this experimental system in the future (discussed in more detail below).

8.0.1 Contributions

The gastruloid system is the first three-dimensional mESC-based model of axial elongation morphogenesis. It is directly comparable to the results of Marikawa et al., who have used aggregates of embryonal carcinoma (EC) cells for the same purpose [106] but it represents a considerable advance on existing accounts of axial patterning in embryoid bodies (see ten Berge et al., [163]) which did not undergo elongation.

In relating the findings from the gastruloids to the development of the embryo, new insights have been gained on the separability of developmental processes that could not have been gained from in vivo studies. Firstly, their spontaneous polarisation in the absence of extraembryonic tissues has suggests that the role of the AVE in vivo may in fact be to bias the orientation of an otherwise spontaneous process to position the primitive streak in the correct relative position to the tissue demarcated to become the brain. Second, the lack of a notochord in the elongating gastruloid tissues suggests that the process of posterior elongation can be separated from the development of the axial mesoderm. Third, it follows that without the AVE or axial mesoderm, the gastruloid tissues represent only the post-occipital tissue, demonstrating that rostral and caudal development can be separated in vitro. These observations demonstrate the value of this in vitro system in generating new, testable predictions that can be validated through careful experiments in the embryo.
CHAPTER 8. DISCUSSION

The generation of a candidate NMp population in three-dimensional culture provides new opportunities to study the tissue interactions regulating the fate choices made by these cells in self-renewing or forming either neural or mesodermal derivatives. The gastruloid system provides a minimal environment in which to explore these interactions which are otherwise complicated by the physical and biochemical complexity of the embryonic tissues. The three-dimensional nature of gastruloid culture allows the relationship between the NMp population and the process of posterior growth to be explored in a way that cannot be accessed from two-dimensional in vitro systems. Furthermore, it is possible that the NMp population in the gastruloids occupies a niche with similar architecture to that found in the embryo. Further histological analysis will be required to determine if this is the case, but if so, it could allow the signals regulating the NMp state to be studied.

Finally, integrating hydrogels into the gastruloid cultures provides the field with a route to deriving tissues that resemble the mouse embryo at around E9.5, when the primordia of the major organs start to form. Bioengineered gastruloid cultures may therefore offer the means to derive progenitors for specific tissues or organs in a three-dimensional context. The identification of PEG-Acrylate hydrogels as an alternative to Matrigel® is an important finding since their physical and biochemical properties can be controlled in spatially or temporally defined manner. By combining the gastruloid tissues with a bioengineered culture environment at an advanced stage, it may be possible to produce spatially patterned axial tissues in vitro. Unlike Matrigel®, synthetic PEG hydrogels are chemically defined and are therefore suitable for clinical applications, which may become an important consideration in the future.

8.0.1.1 Contribution to the Field

The research presented in this thesis provides a new experimental model for axial elongation morphogenesis and an alternative route to deriving neuro-
mesodermal progenitors in three dimensional culture.

8.0.2 Implications - First Steps Towards Synthetic Embryology

On considering the gastruloids in the context of the organoids field as a whole, it is apparent that tissues and organs can be engineered \textit{in vitro} to mimic embryonic development. Examples for the mouse include structures resembling blastocysts ("blastoids," [132]), peri-implantation and post-implantation embryos [176] [72]. Human ES cells have also been grown in micropatterned cultures that self-organise into separate germ-layers but with a radial, rather than bilateral, symmetry [180]. To borrow a concept from the field of synthetic biology, this research forms a type of synthetic embryology, in which an embryonic system is built from the bottom-up using standardised or well-defined parts. Davies has reviewed the use of synthetic techniques in developmental biology and identified two advantages to this approach; it provides the means to verify developmental principles and reveals new perspectives on specific processes [35]. Implicit in Davies’ discussion is the fact that minimally constructed systems demonstrate the sufficient components of a process that are difficult to identify by genetic means alone.

\textit{In vitro} cultures of mouse and human embryos provide important precedents for this emerging field since they demonstrate the possible extent to which an engineered system could develop. Mouse blastocysts can be grown on a plastic surface through early somitogenesis [80] to the limb bud stage [29], when the primordia of the major organs are apparent. When provided with the correct culture media, heightened oxygenation and physical agitation, the mouse embryo can therefore complete the first half of its gestation in the absence of the maternal tissues. More recent studies have adapted this technique to integrate it with live imaging, which has revealed previously undiscovered mechanisms responsible for the formation of the amniotic cavity [10][11]. This approach has also been used to examine the peri-implantation
development of human embryos [38][141] which develop structures akin to the amniotic and yolk sac cavities when grown in vitro, though these studies have been limited to pre-gastrulation stages by law.

Efforts to recreate embryogenesis from cultures of pluripotent stem cells can be divided into assembled (or constructed) and self-assembled (deconstructed) approaches. On assembling mouse embryonic stem cells into an aggregate in Matrigel® resembling the peri-implantation epiblast, the tissue undergoes a similar cavitation process to that described in the embryo [11]. This also occurs in combinations of trophoblast stem cell and embryonic stem cell aggregates in Matrigel® [72]. The important feature of these assembled tissues is that they are built to the specifications of the embryo, with respect to the relative number and position of the components. Gastruloids and micropatterned cultures fall into the self-assembled, or deconstructed, category since they are produced in a relatively unguided, abstract manner that does not necessarily resemble the tissue organisation of the embryo. Furthermore, the rationale behind these systems is to start from a defined, minimal set of components under controlled starting conditions rather than seeking to recreate the complex uterine environment.

A synthetic embryo generated through either approach would not only represent a notable scientific achievement but would also see application in basic research by allowing higher throughput experiments to be designed that would otherwise be impossible (e.g. genetic or pharmacological screens). The degree to which such a result will be informative on specific developmental mechanisms will, however, depend on the approach that is taken. An assembled (or constructed) approach would be informed by our current understanding of development (e.g. the morphology and cell signalling environment of the embryo) and while it might highlight areas in which our understanding is lacking, it may not offer many novel mechanistic insights as the approach is somewhat circular. A self-assembled (deconstructed) approach, while more abstract in its form, may instead hold value in the identi-
fication of the minimal set of components for a specific process. While genetic approaches have been a powerful way to identify the necessary components in development, they do not so easily identify those that are sufficient for it to occur. Additionally, it is possible that self-assembled approaches can uncover developmental potential that is not realised (or perhaps is not apparent) in vivo\(^1\). It remains to be seen whether a structure representing a complete set of embryonic tissues can be formed entirely through controlled self-assembly under minimal conditions; it will likely be the case that either a degree of construction will be required or some external instruction from a bioengineered culture environment.

The broader questions of how an embryo should be defined and protected under the law are beyond the scope of this discussion, but recent experiments with in vitro cultured human embryos have raised issues that are pertinent to the translation of the gastruloid system to cultures of human cells. The 2008 amendment to the Human Fertilisation and Embryology Authority Act\(^2\) extended its definition of an embryo to include those derived in vitro by any means. The central question for synthetic approaches to embryology, therefore, regards this definition: at what point does an assembly of tissues become an embryo? While the 14-day limit would apply to such a structure, Aach et al. raise the important point that synthetic approaches may be able to bypass this stage in a shorter space of time [1]. Instead, the authors propose that future regulations are based on the development of “morally significant structures” that may arise from the use of specific techniques. Establishing which structures are morally significant requires a detailed understanding of the underlying biology, which, unfortunately, we do not yet have. The argument therefore becomes circular, since improving our understanding of the

\(^1\) One example is antero-posterior symmetry breaking in gastruloids lacking the AVE, another is the germ-layer organisation of micropatterned human pluripotent cell cultures in the absence of a primitive streak [180]

development of these tissues in the embryo will require exactly the sort of experimentation that the legislation is being designed to regulate, either \textit{in vivo} or in a synthetic system. This is especially true for regulations based on the developmental potential of an \textit{in vitro} system, which can only be determined through its realisation.

While systems assembled in the same configuration as the early embryo may fall under this regulation, the gastruloid system may offer an alternative route that is regulated instead as a culture of stem cells. The mouse gastruloids currently resemble the early post-implantation development of the embryo, but notably without the tissues of the brain or those required to interact with the mother. The sensory capacity of such a structure is therefore intrinsically limited, as is its ability to develop \textit{in vitro} (or even if introduced into the uterus of a surrogate female). It will remain to be seen whether gastruloids from human cells have a similarly limited complement of tissues, but if so, they might present an ethically acceptable route to studying early post-occipital human development using cultures of stem cells. It is worth noting that integrating such a system with reprogramming technology could offer a particularly interesting realisation of re-acquired developmental potential. It is cautioned, however, that generating the full set of embryonic tissues in this way would effectively be a form of cloning.

As Daniel St Johnston recently commented in “A Renaissance in Developmental Biology” [146], the value of organoid systems in uncovering developmental mechanisms will become realised by integrating them with iPS cell lines and CRISPR/Cas9 genome editing. It is hoped that this will be no different for mouse and human gastruloids and that they might provide new, comparative insights on the regulation of axial elongation in these species. To quote Martin Pera \textit{et al.}, “the extent to which these structures ... actually resemble the early post-implantation embryo will determine not only their usefulness as models for mammalian development, but also the level of public scrutiny that research on them will attract” [123].


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Appended Publications
Symmetry breaking, germ layer specification and axial organisation in aggregates of mouse embryonic stem cells

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ABSTRACT

Mouse embryonic stem cells (mESCs) are clonal populations derived from preimplantation mouse embryos that can be propagated in vitro and, when placed into blastocysts, contribute to all tissues of the embryo and integrate into the normal morphogenetic processes, i.e. they are pluripotent. However, although they can be steered to differentiate in vitro into all cell types of the organism, they cannot organise themselves into structures that resemble embryos. When aggregated into embryoid bodies they develop disorganised masses of different cell types with little spatial coherence. An exception to this rule is the emergence of retinas and anterior cortex-like structures under minimal culture conditions. These structures emerge from the cultures without any axial organisation. Here, we report that small aggregates of mESCs, of about 300 cells, self-organise into polarised structures that exhibit collective behaviours reminiscent of those that cells exhibit in early mouse embryos, including symmetry breaking, axial organisation, germ layer specification and cell behaviour, as well as axis elongation. The responses are signal specific and uncouple processes that in the embryo are tightly associated, such as specification of the anteroposterior axis and anterior neural development, or endoderm specification and axial elongation. We discuss the meaning and implications of these observations and the potential uses of these structures which, because of their behaviour, we suggest to call 'gastruloids'.

KEY WORDS: Mouse, Gastrulation, Self-organisation, Symmetry breaking, Polarisation, Axial elongation, Endoderm, Mesoderm, Neural ectoderm, Pattern formation, Live cell imaging

INTRODUCTION

The emergence of asymmetries within a mass of otherwise equivalent cells is the starting event in the development and patterning of all embryos, and results in the establishment of a coordinate system that cells use as a reference to generate the main axes of an organism. In animal embryos the axial organisation acts as a reference for the process of gastrulation, a choreographed feat as these structures emerge without a recognisable reference coordinate system. An explanation for this observation might lie in the intrinsic tendency of mouse ESCs (mESCs) to develop anterior neural fates (Tropépe et al., 2001; Turner et al., 2014c; Watanabe et al., 2005; Wataya et al., 2008). In contrast to these observations, there are no reports of the emergence of axial structures in EBs, even though in culture it is possible to obtain progenitor cells for mesodermal and endodermal structures (Gradue et al., 2006; Kouskoff et al., 2005; Kubo et al., 2004) that exhibit some of the morphogenetic properties of the embryo (Turner et al., 2014b), and signalling can elicit a degree of polarised gene expression in EBs (ten Berge et al., 2008). One exception was reported in a study of P19 embryo carcinoma (EC) cells. Under differentiation conditions, EBs made from these cells can organise themselves into polarised and extending structures resembling gastrulating embryos (Marikawa et al., 2009). Such large-scale organisation has not been described in ESCs.

Here we show that small aggregates of mESCs undergo a symmetry-breaking event in culture and that, under conditions that
promote the formation of mesendoderm in embryos, they exhibit polarised expression of the endoderm marker Sox17 (Kanai-Azuma et al., 2002) and FoxA2 (Monaghan et al., 1993; Sasaki and Hogan, 1993) and of the PS and early mesoderm marker brachyury (Bra, or T) (Herrmann, 1991). Over time, Bra expression becomes restricted to a small population of cells at a tip of the aggregate, which acts as a source of cells that express Tbx6, a mesoderm gene (Chapman et al., 1996), and these cells are extruded from the main body of the aggregate in a process that is reminiscent of some of the movements of gastrulation. For this reason, we call these aggregates ‘gastruloids’ and show that, although for the most part they are autonomous in their development, the culture conditions influence the cell types that develop within them. We compare the behaviour of these aggregates with that of embryos and discuss their potential as a new experimental system with which to study mechanisms of early mammalian development.

RESULTS
Symmetry breaking in differentiating EBs
The observation that P19 EC cells are able to form polarised, elongated structures during differentiation (Marikawa et al., 2009) prompted us to seek culture conditions in which EBs derived from mESCs would develop similar structures. When cells were placed in a serum and LIF hanging drop culture, cells formed aggregates and, after removal of LIF, a small proportion changed their morphology to an ovoid appearance, although any further suggestion of elongation was never apparent (data not shown). In order to stimulate the emergence of PS features, we used culture conditions that steer the cells towards this fate in adherent culture (Gadue et al., 2006; Turner et al., 2014b,c) and exposed EBs of different sizes to N2B27 for 2 days followed by continuous treatment with both activin A (Act) and CHIR99021 (Chi), a Wnt/β-catenin signalling agonist (Act/Chi conditions) (supplementary material Fig. S1A; see Materials and Methods for details). The initial EBs contained ~800-1000 cells and during the first phase of aggregate formation in N2B27 we noticed that, in contrast to cells hanging in serum and LIF where only one aggregate was formed per drop (supplementary material Fig. S1D), cells in N2B27 formed multiple aggregates of variable sizes per drop (supplementary material Fig. S1C). Following the change of medium into Act/Chi conditions, the aggregates dispersed and over time we observed an increasing number of aggregates adopting a shape that differed from their original spherical appearance (supplementary material Fig. S1E-F); some displayed an ovoid shape (supplementary material Fig. S1E).
resembling what has been described previously for EBs when β-catenin is activated (see Figure 4B in ten Berge et al., 2008). However, we also observed clear elongation in a few aggregates (supplementary material Fig. S1E) and, by the fourth day in Act/Chi, a median of 30% of aggregates exhibited a polarised, elongated morphology. The reduction in the proportion of cells displaying elongated aggregates at later time points reflected an increase in cells displaying a differentiated phenotype in addition to an increase in an apoptotic appearance (data not shown). The aggregates needed to be in suspension for shape changes to occur (Baillie-Johnson et al., 2014).

These results indicate that it is possible to elicit symmetry breaking and polarisation in aggregates of ESCs.

**The effect of signals and aggregate size on polarisation**

The heterogeneous response of the EBs to our experimental treatment could be due to several factors and we decided to focus on three that we deemed to be most influential in the outcome of the experiment: (1) the composition of the culture medium; (2) the timing of exposure; and (3) the initial size of the aggregates. In these experiments we moved from culturing the aggregates in hanging drop to 96-well plates, as this allowed us to arrange for one, and only one, aggregate to develop in each well, minimising the possibility of fusions (for details see Baillie-Johnson et al., 2014). The aggregates were first placed in N2B27 for 2 days and assayed on the fifth day of culture.

If, after the initial 2 days in N2B27, the aggregates are left in this medium then we observe a range of morphologies, with 20-30% exhibiting some polarisation. When signals (Act, Chi and BMP) are applied continuously from the third day of differentiation, the response is signal specific: in Act/Chi many of the aggregates exhibit a weak elongation, whereas continuous exposure to Act alone elicits a variable number of short protrusions or invaginations per aggregate; on its own Chi triggers a smaller number of longer and broader protrusions (Fig. 1A,B, protocol P2). In all cases there is a variability in the response of the aggregate to a particular culture condition that changes with the cell line, although the structure that emerges is specific and recognisable for each of the signals (Fig. 1B).

Using Act and Chi in combination (Act/Chi) or individually, we next tested the effect of changing the timing of exposure to signals (Fig. 1A,B, protocols P1-3). We began by restricting the exposure of these signals from differentiation days 2-5 (Fig. 1A, protocol P2) to days 2-3, 3-4 and 4-5 (Fig. 1A, protocols P3, P4 and P5, respectively), and returning the aggregates to N2B27 for the duration of the timecourse. Limiting the exposure to the third day (48-72 h) triggered the most reproducible response, with greater than 70% of the aggregates undergoing similar morphological changes (Fig. 1C,C'). Under these conditions, Act alone produces a number of small, broad protrusions and invaginations from a large oval. Addition of Chi to the Act reduces the number of invaginations and, in many instances, elicits a single elongation of ∼40-60 µm that is attached to a broad mesenchymal-like structure at the distal end of the aggregate (Fig. 1C,C'). On its own a short exposure to Chi consistently elicits a single elongation without clear protrusions or invaginations. Exposure to the different signals limited to either the second or the fourth day of culture produced more variable responses, and many aggregates that did not respond (Fig. 1). These results suggest that, during the third day of differentiation, ESCs are in a competent state to efficiently interpret signals in the medium.

As exposure to Chi from the third day elicited a simple and consistent response in the form of an elongation, we used this experimental condition as the basis to analyse the effect of the initial size of the aggregates on their polarisation (Fig. 2; supplementary material Fig. S2A-D). Starting with different numbers of cells revealed that only small aggregates, of ∼300-100 cells, exhibit the elongation effectively. Smaller aggregates (∼200 cells) either grow or remain small and exhibit slow growth; when they grow, they tend to produce the elongation. Larger aggregates (∼600 cells) grow in the disorganised and symmetrical manner that is regularly reported for EBs.

These results indicate that EBs configured from mESCs are capable of elongation in the manner that has been described for P19 EC cells (Marikawa et al., 2009) and that this behaviour is associated with specific culture conditions.

**Spatial and temporal patterns of gene expression in polarising EBs**

The elongated aggregates resemble structures that have been described in sea urchin and amphibian embryos (Holtfreter, 1933; Horstadius, 1939; Ishihara et al., 1982; Keller and Danilchik, 1988) or when animal caps from Xenopus embryos are exposed to Activin.
These comparisons suggested to us that the elongated bodies might be recapitulating some of the early events associated with gastrulation. If this were the case, the cells involved in generating the protrusions might represent mesendodermal tissue. To address this and exclude the possibility that the protrusion is simply a mechanical response to the size and shape of the aggregates without a specific fate (i.e. that there is no correspondence between structure and fate), we analysed the expression of genes associated with early differentiation in culture and in embryos (Fig. 3). To begin with we analysed the expression of Sox17 (Figs 3 and 4), a marker of primitive and definitive endoderm (Kanai-Azuma et al., 2002), and of Bra (Fig. 4), a gene associated with the specification of endoderm and mesoderm in the PS (Herrmann, 1991), using fluorescent reporter ES cell lines for both genes (Fehling et al., 2003; Niakan et al., 2010). Aggregate formation and staining with Sox17 and Bra antibodies confirmed that both lines are faithful reporters of the expression of the genes (supplementary material Fig. S3) (Turner et al., 2014b).

Following a transient exposure during day 3 to either Act or Act/Chi we observe expression of Sox17::GFP mostly in clusters of cells which, in the presence of Act/Chi, tend to lie within the elongating region (Fig. 3A). Confocal optical sections through the aggregates revealed them to be multi-layered structures (Fig. 3A, A′) with a number of aggregates displaying internal cavities and localised indentations or pits on their surface (Fig. 3B, B′; supplementary material Movie 1). Sox17 expression is, for the most part, restricted to the external cells and is associated with E-cadherin (Fig. 3A), as it is in the embryo. The amount of Sox17 expression increases with the time of exposure and requires Act, as exposure to Chi alone reduces the levels of expression and the number of expressing cells (Fig. 3A). In all cases, Sox17-expressing cells tend to invaginate, retain E-cadherin expression and form vesicles near the surface of the aggregate (Fig. 3B; see also Fig. 7F).

Further analysis of aggregates exposed to Act and, particularly, to Act/Chi revealed expression of Bra (Fig. 4A,B), Sox17 (Fig. 3 and Fig. 4B) and FoxA2 (Fig. 4B), which are all associated with the PS, localised towards the extending tip of the aggregate; Bra and Sox17 are expressed in a mutually exclusive pattern (Fig. 4B). Whereas the expression of Sox17 coincides with that of FoxA2 (Fig. 4B), that of Bra correlates with a high level of β-catenin transcriptional activity as demonstrated in a TCF/LEF::GFP (TGL) reporter cell line (Fig. 4C). These patterns of expression are reminiscent of those in gastrulating embryos, in which Bra and Wnt/β-catenin signalling can be observed in the PS (Fig. 4D) (Ferrer-Vaquer et al., 2010). Taken together, these observations suggest that the aggregates formed from mESCs undergo morphogenetic movements that resemble the early stages of gastrulation.

**Imaging symmetry-breaking events in real time in the aggregates**

In order to monitor the emergence of the polarised expression of Sox17 and Bra, we performed live cell microscopy on E14-Tg2A, Sox17::GFP and Bra::GFP transcriptional reporter cell lines. First, aggregates exposed to N2B27 for 5 days with a 24 h pulse of either Act (n = 10), Chi (n = 5), or Act/Chi (n = 14) between 48 and 72 h and imaged by confocal microscopy (GPI-GFP channel not shown). The expression of the indicated markers on the surface of the aggregates is shown in A′, with the corresponding orthogonal view through the aggregate in A′. The arrows in A′ indicate the e-section shown in A and A′. Note how the expression of Sox17 is localised to the surface of the aggregate. (B,B′) A representative aggregate from GPI-GFP mESCs exposed to N2B27 was imaged at the end of the treatment after being fixed and stained for E-cadherin and Sox17 (B); the boxed region is enlarged to show E-cadherin (B′). The depressions that are associated with Sox17 expression and high levels of E-cadherin. (C) Section through an E7.5 embryo stained for Sox17 and with DAPI.
we imaged the formation of the aggregates from a cell suspension of 
mESCs in N2B27 for 48 h (Fig. 5A; supplementary material 
Movie 2). Individual cells or clusters containing small numbers of 
cells were found to coalesce into larger aggregates due to a 
combination of the spatial constraints of the round-bottomed culture 
wells and active cell movement towards the aggregate (Fig. 5A; 
supplementary material Movie 2). During this time we do not 
observe expression of visceral endoderm (VE) markers, such as 
Gata6 (not shown) or Sox17, suggesting that the aggregates are 
composed exclusively of embryonic tissues. We then focused on the 
Act/Chi conditions, recording the emergence of the fluorescence 
from the time of transfer from the N2B27 medium into Act/Chi 
(Fig. 5B,D) and then at later stages when the aggregates were more 
advanced (Fig. 5C,E; supplementary material Movies 2-4). In both 
cases, we observe the development of polarised gene expression 
over time, but the patterns are different for each of the genes. 

In the case of Sox17 (Fig. 5B,C), after ∼32 h in Act/Chi we observe 
an initial pattern of scattered cells expressing the reporter intermingled 
with Bra-expressing cells (Fig. 5B,D; supplementary material 
Movies 3 and 4). The videos suggest that the definitive, polarised 
expression pattern is established from the aggregation of Sox17-
expressing cells on one side of the aggregate, which then proliferate 
and at 96 h can be seen to be associated with the elongation. Clusters 
of Sox17-expressing cells can be seen to move inside the aggregate, 
which would be consistent with the invaginations described above 
(Fig. 4B,C). In the case of Bra (Fig. 5D,E; supplementary material 
Movies 5 and 7), Bra::GFP was initially expressed transiently across 
the whole aggregate (79 h in secondary medium; Fig. 5E) before 
becoming restricted to a small region (Fig. 4E, Fig. 5D,E). Downregulation 
of the reporter in other regions of the aggregate appeared to be undertaken by individual cells not within the region of 
high expression. As time progressed, the aggregate increased in size 
and maintained the expression of Bra::GFP within one region 
(Fig. 5E; supplementary material Movie 5). These results suggest that 
symmetry breaking and polarisation of gene expression are a feature of 
these aggregates elicited by different signals.

**Signalling and pattern formation during aggregate 
differentiation**

In the early postimplantation epiblast, cell fate assignments are 
triggered by interactions between BMP, Nodal, Wnt signalling and 
their antagonists, and lead to the partitioning of the embryo into 
anteior neuroectodermal and posterior mesendodermal populations 
(Arnold and Robertson, 2009; Pfister et al., 2007; Tam and Loebel, 
2007). To expand our studies beyond mesendoderm, we used a Sox1::
GFP reporter ES cell line to monitor neural development (Ying et al., 
2003), and a TBX6::EYFP line to follow the emergence of mesoderm 
(see Materials and Methods). We also included BMP (see also 
Fig. 1C) in the repertoire of signals, as it plays a role in the early stages 
of embryonic patterning (Arnold and Robertson, 2009; Tam and 
Loebel, 2007). In these experiments, aggregates were exposed to the 
signals either during the third day of differentiation and then returned 
to N2B27 for a further 2 days, or for the last 3 days of the experiment 
(as summarised in Fig. 6). When cells are left in N2B27, for the most part they do not undergo 
any specific morphogenetic process and express Sox1::GFP 
throughout the aggregate (Fig. 6 and Fig. 7A), although in ∼10% of 
cases we observe some polarised Bra expression (not shown). The pattern of Sox1::GFP expression does not change when inhibitors of 
Nodal/Activin (SB43) or MEK (PD03) are added to the medium from 
day 3 (not shown) and is consistent with the observation that mESCs 
placed in N2B27 will develop, mainly, as neural precursors (Ying et al., 
2003). Also consistent with known inputs of signalling on neural 
development (Andoniadou and Martinez-Barbera, 2013; Turner et al., 
2014c), Sox1::GFP expression was suppressed by exposure to Act, 
although a few foci of expression remained in some aggregates (Fig. 6).
The response of genes associated with endoderm (Sox17) and mesoderm (Tbx6) to the different signals is summarised in Fig. 6 (examples of expression patterns are shown in Fig. 7). The pattern of responses mirrors that of embryos to the same signals (Figs 6 and 7). For example, Act suppresses mesoderm and promotes endoderm, whereas BMP promotes mostly mesoderm and Chi is able to elicit all germ layers (Fig. 6). In all cases the different cell types emerge as continuous and polarised groups of expressing cells: a pulse of Chi leads to an increase in TBX6::EYFP expression (Fig. 7B) and polarised β-catenin transcriptional activity (Fig. 7C) and Bra::GFP expression (Fig. 7D). We also observe interactions between the different signals; thus, BMP appears to quench the effects of Chi on Sox1 expression, and Act suppresses the effects of BMP on Tbx6 expression (not shown). Prolonged exposure to a signal or signal combination tends to increase the response in terms of expression but has a negative effect on polarisation of the expression (Fig. 6 and data not shown).

The exposure to a pulse of Chi led to an elongation that, surprisingly, exhibits Sox1::GFP expression in the elongating cells (Fig. 7A), with Bra expression restricted to the tip of the elongate in a small region that does not express Sox1::GFP (Fig. 7A, Chi pulse insets; Fig. 7D). The elongated region exhibits a complex structure, with most of the cells expressing Sox2 and, often, Sox17 in vesicles that form near the surface and have lower levels of Sox2 (Fig. 7F); Sox1 expression is non-overlapping with that of Sox17. In the non-elongated region we observe low levels of Sox2 expression. This arrangement is reminiscent of the situation in the embryo, where the endoderm, which expresses Sox17 and Sox2 (Wood and Episkopou, 1999), lies underneath the developing nervous system (Fig. 7G). In addition, the aggregates express TBX6, which is usually associated with mesoderm formation (Chapman et al., 1996), and appear to recapitulate events associated with axial extension (see Turner et al., 2014a). The aggregates lack a notochord, which, in the embryo, lies between the nervous system and the gut. An important feature of the development of these aggregates is the timing of the events, which is reliable and reproducible from experiment to experiment: the Sox17 expression precedes and initially overlaps with Bra expression and the extrusion of cells, and Tbx6 expression follows a few hours later (see Fig. 8) (Turner et al., 2014a).

These results complement the morphological changes described above and are consistent with what is known about the early events in the embryo, namely the existence of a pre-proneural basal state in the epiblast with the mesendoderm being specified by BMP, Nodal/Act and Wnt signalling (Turner et al., 2014c). N2B27 appears to be a transitional medium in which cells can adopt a primary neural fate (Turner et al., 2014c). Furthermore, in the aggregates, as in the embryo, Act initiates endoderm development (Sox17) and BMP initiates mesoderm (Tbx6) development.

Cell movement in polarised aggregates

When Sox17::GFP aggregates were cultured in Act/Chi for 120 h, we observed cells that were being extruded from a region adjacent to the primary focal point of reporter expression (Fig. 8A; supplementary material Movie 6). As time progressed, the frequency of this event...
increased and many more cells were seen to emerge from the same point. Close observation revealed two types of movement: Sox17-expressing cells appeared to move inside the aggregate, close to its wall, whereas others, not expressing Sox17, moved towards the outside (supplementary material Movie 6). Cell movements can also be observed after exposure to Chi and in other cell lines such as Bra::GFP (Fig. 7D and Fig. 8B; supplementary material Movie 7) and TBX6::EYFP (Fig. 7B and Fig. 8C; supplementary material Movies 8 and 9). Movies show that the extruded cells stem from the region of Bra::GFP expression (Fig. 7B) and that they express Tbx6 (Fig. 8C,C′; supplementary material Movies 8 and 9), suggesting that they are mesodermal. The extruded cells produce floating trails in the medium or attach to the main body of the aggregate (Fig. 7B and Fig. 8C′) and, when they attach, maintain expression of Tbx6, suggesting that continuing Tbx6 expression requires some substrate that can only be provided by other cells. It appears as if the cells prefer to attach to the ‘anterior’ section of the aggregate, suggesting that there are differences between the two regions.

We do not observe filopodia or lamellipodia in the cells leaving the aggregate but observe the emergence of blebs (Fig. 8D,D′; supplementary material Movie 10), which have been associated with cell movements during gastrulation in zebrafish (Paluch and Raz, 2013).

DISCUSSION

We have shown that under defined culture conditions aggregates of mESCs undergo processes that resemble the collective behaviours of cells in early mouse embryos: symmetry breaking, axial organisation, germ layer specification, gastrulation and axis elongation. This is surprising in light of the fact that EBs are commonly used in differentiation experiments and yet, with two exceptions, there have been no reports of similar behaviours. One of the exceptions is an account of elongation and polarised gene expression in aggregates of P19 EC cells exposed to serum (Marikawa et al., 2009). The second is a report of weak polarisation of Bra expression in EBs of mESCs exposed to agonists of Wnt signalling (ten Berge et al., 2008), which resembles the early stages of what we report here. It is possible that similar partial polarisation events occur at low frequency in EBs but are generally overlooked. We believe that the consistency and magnitude of the behaviour that we observe in our aggregates are founded, principally, in two aspects of our experimental protocol: the sequence of culture conditions that we use and the initial number of cells in the aggregate.

In our adherent cultures we had noticed that exposure of differentiating ESCs to N2B27 for 2 days results in a homogeneous response to external signals (Turner et al., 2014c). We reasoned that this treatment allows all cells in the culture to enter a state resembling the postimplantation epiblast, where they become competent to respond to signals (Sterneckert et al., 2010; Turner et al., 2014c), and it is for this reason that we used this protocol as the basis for our experiments. The second element in our protocol that differs from standard procedures concerns the number of cells in the initial aggregate, which appears to be a critical variable in the experiments. Aggregates above or below 300±100 cells (average aggregate diameter of 100 μm) will either not develop or do so into amorphous masses of cells, characteristic of the EB protocols in current use. This size of 300±100 cells is reminiscent of that of early postimplantation embryos and perhaps defines an optimal length scale for a unique outcome of the biochemical reactions that mediate symmetry breaking and polarisation. Experiments searching for conditions that mimic the emergence of the postimplantation epiblast from mESCs also find that the number of starting cells is a critical parameter of the process (Bedzhow and Zernicka-Goetz, 2014). A surprising conclusion from these observations that will need to be pursued is that early patterning events do not scale easily and this is in agreement with recent observations on the emergence of germ layers in micropatterns of human ESCs (Warmflash et al., 2014).

Our observations raise many questions about symmetry breaking in early embryos, the ability of cell ensembles to respond to signals and the different behaviour of mESCs in adherent and three-dimensional cultures. For reasons of space, here we shall focus on two specific issues concerning how the system that we have
Symmetry breaking and axis specification

Our results reveal that, under appropriate culture conditions, aggregates of mESCs have an intrinsic ability for symmetry breaking and stable polarisation of gene expression. This pattern resembles events in the embryo at \( \sim E6.0 \) with some, perhaps informative, differences.

In the embryo, the initial localisation of the PS can be identified as a focus of Bra expression in the proximal posterior region of the embryo (Wilkinson et al., 1990) and its specification follows a sequence of events associated with the localisation of ligands for BMP, Nodal and Wnt signalling to the same region (reviewed by Arnold and Robertson, 2009; Pfister et al., 2007; Rossant and Tam, 2009; Tam and Loebel, 2007). This process requires first the specification and localisation of the anterior visceral endoderm (AVE) to the prospective anterior region of the conceptus, where it acts as a source of antagonists of Wnt, BMP and Nodal signalling (Arkell and Tam, 2012). It is thought that the action of the AVE positions or restricts the PS to the opposite end of the epiblast (Perea-Gomez et al., 2004; Perea-Gómez, 2014; Rivera-Pérez and Magnuson, 2005). Our results raise questions about the actual role of the AVE, since they show that a stable axis, as reflected by localised expression of Bra, Sox17 and FoxA2, can be initiated without external influences. In our experiments the signals are ubiquitous and so the symmetry-breaking event must be intrinsic to the aggregates, raising the possibility that a similar spontaneous event takes place in the embryo. This conclusion is at odds with the large body of experimental evidence suggesting that the anteroposterior axis requires a sequence of interactions between extraembryonic and embryonic tissues (Rossant and Tam, 2009).

One way to reconcile our observations with those of the genetic analysis of early development would be to entertain the possibility that the function of the AVE is not to break the symmetry of the embryo but rather to ensure that an event that can happen spontaneously has a reproducible outcome, i.e. the AVE ensures the maintenance of a region primed for anterior neural development at the opposite pole to that of the PS and, more importantly, endows this region with an anterior neural fate potential (Albazerchi and Stern, 2012).
This suggests that it is possible to uncouple symmetry breaking and anterior neural specification. The latter requires suppression of Nodal, BMP and Wnt signalling (Andoniadou and Martinez-Barbera, 2013; Stern, 2005) and this, in terms of patterning anterior and posterior domains, can only be achieved by a localised source which, in the embryo, is provided by the AVE. Consistent with this, aggregates maintained in N2B27, or in N2B27 in the presence of BMP inhibitors, for the most part remain symmetrical and express a neural fate, probably mimicking the specification of anterior neural fate under these conditions (Eiraku et al., 2011). In the future it will be interesting to provide localised inhibition of BMP and Nodal in aggregates exposed to Act, BMP and Chi to try to obtain both anterior neural and mesendodermal fates in the same group of cells. However, this only provides a partial explanation, as embryos lacking a VE develop symmetrically (Perea-Gómez et al., 1999; Waldrip et al., 1998), suggesting that our experimental conditions might be generating a situation that does not occur in the embryo. One explanation is that, in addition to maintaining a proneuroectodermal region, a key function of the AVE is to bias a spontaneous symmetry-breaking event that is intrinsic to the epiblast. Our experiments might be creating these imbalances by an excess of specific signals in the medium, a hypothesis that will be of interest to test in further experiments.

The events that lead to symmetry breaking remain out of the scope of this work; however, our observations provide some hints as to their constraints. There is clearly a defined length scale to the process, as only aggregates of a certain size undergo the unique event. The symmetry-breaking event must contain an activating and an inhibitory component that are linked, i.e. once the process has started it can inhibit itself within a certain length scale to make the process unique (Meinhardt, 2012). In support of this suggestion, there is evidence for the potential to generate multiple PSs or axes from a single embryo in mouse (Merrill et al., 2004; Perea-Gomez et al., 2002) and chicken (Bertocchini and Stern, 2002; Bertocchini et al., 2004) but only one emerges in the embryo. The possibility that limitation of signalling range plays a role can be gauged in our experiments, which show that persistent signalling can give rise to multiple patterning foci.

Finally, and in the context of symmetry breaking, our experimental system underpins a well-known connection between Wnt signalling and axial elongation (Martin and Kimelman, 2009; Petersen and Reddien, 2009) and provides an opportunity to probe into its mechanism.

**Gastrulation in culture?**

In the mouse embryo, one of the consequences of the formation of an anteroposterior axis is the localisation of the start of gastrulation to the posterior proximal region, a process that will generate the primordia for the endoderm and the mesoderm as well as reveal the axial organisation of the embryo (Novotschin and Hadjantonakis, 2010; Ramkumar and Anderson, 2011; Tam and Gad, 2004). The start of this process is manifest in the localisation of the expression of BMP, Nodal and Wnt3 to this region and, more significantly, of Bra to the emergent PS (Herrmann, 1991; Pfister et al., 2007). A central feature of this structure is an epithelial-to-mesenchymal transition (EMT), which, under the control of specific signals, leads to a germ layer-specific behaviour: in the endoderm, cells re-epithelialise (Burtischer and Lickert, 2009; Kwon et al., 2008; Lewis and Tam, 2006), whereas in the mesoderm they become highly mesenchymal (Nakaya and Sheng, 2008). As a result of these movements the three germ layers are distributed relative to each other. We observe related behaviours when our aggregates are exposed to Act, BMP and, in particular, Wnt/β-catenin. On its own, Act treatment elicits the expression of the endodermal marker Sox17.
in a group of cells that express E-cadherin and form coherent epithelial groups on the outer edges of the aggregate, as they do in the embryo. By contrast, BMP and especially Wnt/β-catenin favour the extrusion of cells from a domain that expresses Bra and TBX6, a gene associated with paraxial mesoderm (Chapman et al., 1996). These observations suggest that our culture system recapitulates some of the features of gastrulation, even though the behaviour of the mesodermal-like cells is the reverse of that in the embryo, where cells move inward rather than outward. It is likely that this topological switch reflects the architecture of the aggregates.

At the end of gastrulation, amniote embryos undergo a process of axial extension that generates the spinal cord and the paraxial mesoderm from a population of stem-like cells located in the distal end of the embryo (Kondoh and Takemoto, 2012; Wilson et al., 2009). This process relies on Wnt signalling and a localised source of Bra expression at the tip of the extension (reviewed by Wilson et al., 2009). We observe that transient exposure of the aggregates to Chi is able to elicit this structure (see also Turner et al., 2014a).

Furthermore, in some of these aggregates we observe endoderm elements. Although these do not speciate into endodermal cells in the aggregates, they do form epithelial groups and intrude, with the elongation starting sometime during day 4, as proposed in an earlier study (Turner et al., 2014a,b,c). Primary antibodies used for immunofluorescence were: goat anti-Bra (Santa Cruz Biotechnology, sc-17743; 1:200), goat anti-β-catenin transcriptional reporter TCF/LEF::mCherry (Faunes et al., 2013; Ferrer-Vaquer et al., 2010), TBX6::EYFP (this is a knock-in into the Thbx6 locus; A.-K.H. and S.N.) and CAG::GPI-GFP (referred to hereafter as GPI-GFP) (Rhee et al., 2006).

Aggregate culture and imaging

A detailed protocol for the growth of the aggregates, with trouble-shooting, is provided elsewhere (Baillie-Johnson et al., 2014). Images in Fig. 1 were generated by manipulating the brightness and contrast of pictures of the aggregates in addition to edge detection; the outlines were enhanced manually through tracing. The original unprocessed images of the aggregates are provided in supplementary material Fig. S1I,H, N2B27 (NDiff) was sourced from StemCells (USA) and tissue culture slides for monolayer imaging were obtained from Bids (Germany). All experimental conditions were repeated at least twice.

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Competing interests

The authors declare no competing financial interests.

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References


Wnt/β-catenin and FGF signalling direct the specification and maintenance of a neuromesodermal axial progenitor in ensembles of mouse embryonic stem cells

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ABSTRACT
The development of the central nervous system is known to result from two sequential events. First, an inductive event of the mesoderm on the overlying ectoderm that generates a neural plate that, after rolling into a neural tube, acts as the main source of neural progenitors. Second, the axial regionalization of the neural plate that will result in the specification of neurons with different anteroposterior identities. Although this description of the process applies with ease to amphibians and fish, it is more difficult to confirm in amniote embryos. Here, a specialized population of cells emerges at the end of gastrulation that, under the influence of Wnt and FGF signalling, expands and generates the spinal cord and the paraxial mesoderm. This population is known as the long-term neuromesodermal precursor (NMP). Here, we show that controlled increases of Wnt/β-catenin and FGF signalling during adherent culture differentiation of mouse embryonic stem cells (mESCs) generates a population with many of the properties of the NMP. A single-cell analysis of gene expression within this population reveals signatures that are characteristic of stem cell populations. Furthermore, when this activation is triggered in three-dimensional aggregates of mESCs, the population self-organizes macroscopically and undergoes growth and axial elongation that mimics some of the features of the embryonic spinal cord and paraxial mesoderm. We use both adherent and three-dimensional cultures of mESCs to probe the establishment and maintenance of NMPs and their differentiation.

KEY WORDS: Wnt signalling, Mesodermal, Morphogenesis, Neural, Stem cells

INTRODUCTION
The nervous system comprises a cellular network that processes sensory and motor information to generate patterns of activity and behaviour in the organism. At the centre of this network there is a large number of interconnected neurons that shape behaviours. In mammals, the basic framework of the network is engineered during development from a sheet of neuroepithelial progenitors that arise in an anteroposterior sequence as the body plan unfolds. This process can be followed through the expression of members of the Sox2b family of transcription factors (Kamachi and Kondoh, 2013): Sox1, Sox2 and Sox3 (Pevny et al., 1998; Graham et al., 2003).

An important feature of the nervous system is its specialization along the anteroposterior axis, which is most obvious in the structural and functional differences between the fore-, mid- and hindbrain, and the spinal cord. The emergence of these differences is thought to occur in two steps. According to the ‘activation/transformation’ hypothesis (Nieuwkoop et al., 1952; Nieuwkoop and Nigtevecht, 1954) the neural plate is first specified, presumably by BMP antagonism, with anterior characteristics; subsequently, posterior fates, including those in the spiral cord, emerge by the action of a gradient of one or more signalling molecules (Mangold, 1933; Nieuwkoop et al., 1952; Saxén and Toivonen, 1961; reviewed by Stem et al., 2006). Experiments in Xenopus have led to the suggestion that the transformative influence is provided by a gradient of Wnt/β-catenin signalling (Kiecker and Niehrs, 2001). In contrast to the situation in frogs, where fates are assigned on an existing neural plate, there is evidence that in amniotes the development of the cranial and hindbrain regions, and of the spinal cord, are temporally and spatially separate (reviewed by Wilson et al., 2009; Kondoh and Takemoto, 2012). While the anterior central nervous system emerges from a neuroectodermal progenitor population following neural induction (reviewed by Andoniadou and Martinez-Barbera, 2013), work in chickens and mice has shown that the spinal cord is derived from a specialized self-renewing precursor population located within the growing caudal end of the embryo (Brown and Storey, 2000; Mathis and Nicolas, 2000; Mathis et al., 2001; Cambray and Wilson, 2002, 2007; Delfino-Machín et al., 2005; Tsouanacou et al., 2009; Nowotschin et al., 2012). Cells within this population exhibit some features of stem/progenitor cells (Mathis...
and Nicolas, 2000; Roszko et al., 2007; Tzouanacou et al., 2009) and can give rise to mesodermal and neural progenitors of different posterior axial levels (Brown and Storey, 2000; Cambrey and Wilson, 2002; Tzouanacou et al., 2009; Tsakiridis et al., 2014). These cells, named long-term axial neuromesodermal precursors (NMPs), can be identified because they co-express markers of the primitive streak (Br) and neuroectoderm (Sox2), as well as the homeobox gene Ssx1/ 

**RESULTS**

Wnt/β-catenin signalling promotes the emergence of posterior axial fates in adherent mESC differentiation

In the postimplantation epiblast of the mouse embryo, cells become allocated to one of two prospective fates: anterior neuroectoderm (aNESC) that will give rise to the anterior nervous system; or, posteriorly, to a rapidly expanding population that gives rise to the mesoderm and the endoderm through the primitive streak (Arnold and Robertson, 2009; Rossant and Tam, 2009). Aspects of this decision-making event can be modelled in ESC cultures where fates are controlled by extracellular signals. Thus, differentiation in the presence of nodal, activin, BMP and Wnt/β-catenin signalling promotes the emergence of a primitive streak-like population (Gadue et al., 2005), whereas culture in N2B27 or serum and retinoic acid (RA) generates neural precursors that can be monitored in the expression of Sox2 and a Sox1-GFP reporter (Ying et al., 2003; Abranches et al., 2009). After 2 days of differentiation in N2B27, mESCs commit either to a neural or to a mesendodermal fate in a signal-dependent manner (Thomson et al., 2011; Turner et al., 2014b). This commitment is associated with a transient rise in Wnt/β-catenin signalling (Faunes et al., 2013) (supplementary material Fig. S1) that can be shown to promote both fates, depending on the levels of nodal and activin: together with high levels it promotes mesendodermal fates; in the context of low levels, it is required for neural differentiation (Turner et al., 2014b), as shown by the effects of inhibition of Wnt signalling (Fig. 1B). This suggests that the activation of Wnt signalling observed during the second day is part of a general priming of the differentiated state and raises the question about its role in the development of the nervous system.
Wnt/β-catenin signalling has been shown to promote the maintenance of neural progenitors and their maturation into neurons (Zeclner et al., 2003; Otero et al., 2004; Kim et al., 2009; Slawny and O Shea, 2011); this could account for the effects of Wnt/β-catenin signalling during neural differentiation. However, Wnt/β-catenin signalling also plays a role in the specification of axial identities in developing embryos (Kiecker and Niehrs, 2001; Nordström et al., 2002, 2006; Mazzoni et al., 2013) and we have tested whether this is also the case during mESC differentiation.

mESCs differentiating in N2B27 adopt a neural fate and, after 5 days, express high levels of genes that are characteristic of mid/hindbrain (En1, Otx2), but lack expression of spinal cord identifiers (Hoxc5, Hoxc6, Hoxc9) (Fig. 1C). However, exposure to Chiron after 2 days in N2B27 elicits the expression of thoracic Hox genes in the absence of anterior ones (Fig. 1C), indicating that β-catenin promotes the development of posterior fates in mESCs. To test whether this effect required persistent signalling, cells were exposed to Chiron for shorter periods of time. Exposure of mESCs to Chiron between 48 and 72 h of differentiation elicits the emergence of thoracic spinal cord identities at the expense of anterior fates (Fig. 1C). A difference between the continuous and pulsed exposure to Chiron can be observed in the cell types that emerge from this population: in both cases, Chiron suppresses the differentiation of anterior identities but, in the case of continuous exposure, in addition to neural development Chiron promotes mesoderm differentiation, as shown by the expression of Tbx6 (Fig. 1C). These results are in agreement with the notion that high levels of Wnt signalling lead to the emergence of a cell population with posterior identities in the epiblast (Mazzoni et al., 2013; Tsakiridis et al., 2014) and raises the issue of the mechanism through which this is achieved.

Exposure of mESCs to Chiron between 48 and 72 h elicits the appearance of clusters of cells that co-express Sox2 and Bra (Fig. 2A,B), a signature that is characteristic of a population of cells that have been suggested to act as a precursor of the spinal cord and the paraxial mesoderm in the embryo, the neuromesodermal axial precursor (NMP) (reviewed by Wilson et al., 2009; Kondoh and Takemoto, 2012). Addition of SB43, a TGFβi antagonist, simultaneously with Chiron, abolishes the expression of Bra, and thus of the Sox2+ Bra− subpopulation. A similar effect is observed if FGF signalling is inhibited by exposure to PD03, an inhibitor of MEK. This suggests that the population is associated with the specification of mesendodermal fates that have been shown to be the source of this population.

Analysis of gene expression in cells 72 h after exposure to Chiron between 48 and 72 h shows that they express Sox1 and Sox2, as well as markers associated with the NMP: Bra, Nkx1.2 and Hoxb8 (Delfino-Machin et al., 2005) (Fig. 2C). These cells do not express markers of anterior neural fate, e.g. En1 and Otx2, but exhibit low levels of posterior ones, e.g. Hoxb1, Hoxc5, Hoxc6 and Hox9 (Fig. 2C). This signature requires ERK and nodal/activin signalling (Fig. 2), and is dependent on Chiron, as cells differentiating in N2B27 for the same period of time do not express Bra or posterior markers, but do express anterior fates.

To study the emergence of the Sox2+Bra−Nkx1.2+ population in the context of neural differentiation, we analysed gene expression in subpopulations of cells expressing Sox1::GFP, a marker of mature neural progenitors. After 3 days differentiating in N2B27, Sox1::GFP-expressing cells exhibit a complex expression profile with a prominent negative population (Fig. 3A). Addition of Chiron during the third day
reduces the Sox1::GFP high population in favour of the negative one (Fig. 3A). To search for the NMp gene expression signature within this profile, we used FACs to sort cells with no, low or high levels of Sox1::GFP, and analysed them for the presence of the gene expression signature of the NMp. The three subpopulations expressed Sox2, with lowest levels in the negative population, but could be distinguished by the levels of expression of Bra and Nkx1.2, which were highest in the negative population, noticeable in the low and absent in the high (Fig. 3B). Immunocytochemical analysis of the three populations reveals differences in the proportion of Sox2+ Bra+ cells, with the Sox1::GFP negative population having the highest, with all Bra+ cells expressing Sox2 (Fig. 3C). These results indicate that exposure of mESCs to Chiron during day 3 of differentiation induces the appearance of a population that has many of the hallmarks of the NMp that has been postulated to emerge during the late stages of gastrulation.

A window of competence for the emergence of the axial progenitor

To determine whether there is a period of competence for the induction of the Sox2+ Bra+ population, we altered the timing of the
exposure to Chiron (Fig. 4A). Treatment of the differentiating cell population between 72 and 96 h reduces the number of Sox2⁺ Bra⁺ cells and this number is further reduced if the exposure occurs between 96 and 120 h (Fig. 4). When mESCs that had been exposed to Chiron (Fig. 4B) between 48 and 72 h of differentiation are returned to N2B27, the Sox2⁺ Bra⁺ population decays rapidly over the next 2 days, and cannot be found by day 5 (Fig. 4). In both cases, the cells maintain Sox2 expression. These results suggest that there is a sensitive period on the third day of differentiation in which this population can be induced and that, in the absence of signalling, the population is not maintained and adopts a neural fate.

**Single-cell analysis of ESC-derived NMps**

To gather further information about the presence of NMp-like cells in the negative and low Sox1::GFP populations of mESCs that had been exposed to Chiron, we sorted 192 single cells from both subpopulations (96 negative and 96 low). After a strict quality control, we selected 89 cells (53⁺, see supplementary material Fig. S4 for details) and analysed them for expression of 85 genes, including pluripotency markers, lineage markers, axial identifiers and reporters of signalling pathway activity (see Materials and Methods, Fig. 5 and supplementary material Fig. S4). We first tried to order the cells using the conventional NMp signature: T/Bra, Sox2, Nkx1.2 in a correlation analysis (Fig. 5, see also supplementary material Figs S4 and S5). Most of the cells analysed have detectable levels of Nkx1.2, with the expression of Bra being heterogeneous and slightly correlated with the levels of Nkx1.2 (correlation coefficient of ∼0.3, P < 0.001). Surprisingly, the expression of Sox2 mRNA is low in all cells except for a small group and exhibits no significant correlation with the expression of Nkx1.2; this is not a failure to amplify the mRNA as the pluripotent population that can be observed within the Sox1::GFP-negative population exhibits high levels of Sox2 expression (Fig. 5). A search for genes whose expression is correlated with Nkx1.2 and Bra identified Wnt3a, Fgf8, Foxb1, Lin28 and Mbd3 (Fig. 5 and supplementary material Figs S4 and S5). In the embryo, Fgf8 and Wnt3a are expressed in the area in which NMps reside (Delfino-Machín et al., 2005) and thus validate our analysis. Foxb1 is a member of the Fkh family of transcription factors that has been reported to be expressed in the NMp region during elongation (Ang et al., 1993; Zhao et al., 2007), and thus represents a novel marker for the NMp. However, Lin28, a microRNA-binding protein (Viswanathan and Daley, 2010; Shyh-Chang and Daley, 2013), and Mbd3, a core member of the NurD chromatin remodelling complex (Kaji et al., 2006; Hu and Wade, 2012), have been associated with pluripotency and reprogramming, and might represent a signature of ‘stemness’. Next, we performed different dimensionality reduction analyses of the data, including principle component analysis (PCA) and linear discriminant analysis (LDA), but these methods were unable to capture structural properties of the cell population, most probably due to the heterogeneous nature of the data. The method that performed best was multidimensional scaling (Fig. 6), which was capable of separating the Sox1::GFP-negative and -low populations. From this analysis we can conclude that the two populations are very similar but that the negative cells contain a subpopulation that has not yet completed a transition from the pluripotent epiblast, as evidenced by a prominent group of cells with high levels of all pluripotency markers, including Sox2. The analysis also allows visualization of differences in gene expression between the cells from the two populations (Fig. 6).

The NMp population does not exhibit widespread expression of lineage markers and lacks clear expression of axial markers, except for Hoxb1, which is active in the NMp region in the embryo (van de Ven et al., 2011). Interestingly, amidst the analysed population we
observed five cells that express Tbx6, Pdgfra and Dll1 and are likely to be mesodermal, and 40 cells that express Sox1, Sox2 and Tbx6 expression. Only the Sox1::GFP originally negative population gave rise to Tbx6-expressing cells and the number of Tbx6-positive cells was enhanced in the presence of FGF (Fig. 7E). This analysis suggests that, in culture, cells within the NMp-like population are in a state that reflects the situation in the embryo in terms of signalling requirements, particularly FGF and Wnt, and can differentiate into both neural and paraxial mesodermal precursors in a signal-dependent manner.

**FGF and Wnt signalling are active in NMps**

The single-cell analysis reveals expression of targets of FGF (Spry2, Spry4) and Wnt (e.g. Left1, Dkk and Axin2) signalling in cells that express Nkx1.2, an indication of FGF/ERK and Wnt/β-catenin signalling in this population. This is consistent with results from embryos (Storey et al., 1998; Mathis et al., 2001) and with the requirements for these signalling pathways in the specification of the NMp population (Fig. 2A,C). By contrast, there is no clear indication of BMP or nodal signalling (supplementary material Fig. S4). These observations led us to test the roles of FGF in the specification and differentiation of the NMp population.

Exposure to both Chiron and FGF between 48 and 72 h leads to a strong suppression of Sox1, a decrease in Sox2 levels and an increase in Bra expression relative to Chiron alone (Fig. 7B,C). This results in an increased number of NMps after exposure to these signals and suggests that, in combination with Wnt/β-catenin signalling, FGF promotes early progenitors at the expense of differentiating cells that are likely to become mesoderm. During differentiation, continuous exposure to both FGF and Chiron between 48 and 120 h amplifies the emergence of Wnt/β-catenin-dependent mesodermal derivatives, as reflected in Tbx6 expression (Fig. 7). To determine whether this is due to an effect on the NMps, Sox1::GFP-negative and -low cells were sorted from pulsed exposure to either Chiron or Chiron and FGF, and each fraction was further cultured in either Chiron or Chiron and FGF. After one and two passages, cells were tested for Sox1, Sox2 and Tbx6 expression. The latter result is in agreement with previous observations that differentiation of embryoid bodies in the absence of external signals leads to the development of neural tissue (Watanabe et al., 2005; Wataya et al., 2008).

When the aggregates are cultured in conditions that elicit the NMp-like population in adherent culture, they exhibit a polarized expression of Bra and undergo a polarized elongation that, after 2 further days in N2B27, has become 200-300 μm long and 20-40 μm wide (Figs 8 and 9). The elongated region expresses Sox1::GFP and Sox2, while the tip exhibits high levels of Bra and of Wnt signalling (Fig. 8). Expression of Bra is confined to the population at the tip, indicating that the elongation lacks a notochord (Fig. 8C). This configuration is very reminiscent of the situation in the caudal lateral epithelial of the developing embryo, where the NMp population is thought to reside between stages 7.5 and 11.0 (Wilson et al., 2009).

To test for the presence of paraxial mesoderm in these aggregates, we used mESC carrying a YFP reporter for Tbx6 (van den Brink et al., 2014). As the aggregate begins to elongate, after it has expressed Bra and Sox1 we observe mesenchymal-like cells that express Tbx6::EYFP in the elongating region (Fig. 9A). Most of these cells leave the aggregate and either float in the medium or attach, preferentially, to the opposite end to the elongating tissue; only cells that attach to the aggregate maintain expression of the reporter (Fig. 9). These results suggest that, when elicited in three dimensions, the NMps can organize themselves into structures that resemble the initial steps of spinal cord development and can be maintained autonomously for a number of days.

**FGF regulates the extension of the aggregates**

We also tested the role of FGF in the organization and development of the elongates (Fig. 9). Suppression of MEK signalling from 72 to 120 h prevented the growth of the elongations (Fig. 9B′), indicating that it is necessary to support the growth once it has started. Addition of FGF during this period did not have a significant effect, indicating that the endogenous levels produced by the aggregates are sufficient.
This effect was also seen for Wnt/FGF inhibition during the 48–72 h period (Fig. 9D), consistent with our observations from adherent culture that FGF signalling is necessary for the establishment of the elongation. Blocking Wnt/β-catenin signalling also affects the elongation, consistent with the effect of mutants in Wnt signalling on axial development in the embryo (Aulehla et al., 2008; Dunty et al., 2008; Aulehla and Pourquie, 2010).

**DISCUSSION**

We have shown that mESCs differentiated in adherent culture under the influence of Wnt/β-catenin signalling give rise to cells with the signature and potential of the axial NMPs that have been described in the embryo as giving rise to both paraxial mesoderm and neural progenitors. The impact of Wnt/β-catenin signalling in the emergence of this population is most effective on day 3 of differentiation, when mESCs have been shown to transit through a stage that is similar to that of the post-implantation epiblast (Sterneckert et al., 2010; Turner et al., 2014b). This adds support to a recent report that EpiSCs exposed to Wnt signalling develop primitive streak fates, as well as a small subpopulation with the characteristics of the NMP (Tsakiridis et al., 2014).

The NMP-like population that emerges in adherent culture cannot be expanded efficiently under our culture conditions and tends to differentiate into neural precursors and paraxial mesoderm. Applying the same conditions to a 3D system of mESC aggregates that we have developed to study the early stages of embryogenesis, we observed the emergence of a phenotypically similar population that now is maintained for at least 3 days, and drives the polarized elongation of a structure containing, principally, neural progenitors; the maintenance and elongation of this population is driven by FGF signalling. These observations support the suggestion that the spinal cord is derived from an axial progenitor population and provide an experimental system, the aggregates of mESCs, to identify and dissect the networks that establish, maintain and differentiate this population (see also the accompanying paper in this issue: van den Brink et al., 2014). This system is complementary to those that already exist that can generate forebrain and retina (Eiraku et al., 2008, 2011; Sasai et al., 2012; Lancaster et al., 2013; Sasai, 2013), and it should allow the study of the mechanisms that pattern axial derivatives, in particular the nervous system (Sasai et al., 2012).

**Origin of the NMP population**

An important unresolved issue in developmental biology relates to the embryological origin of the spinal cord in amniotes, whether its progenitor population emerges from a posteriorization of an anteriorly fated neural plate or as a separate population, induced de novo in the primitive streak, which does not go through an anterior fate (Stern et al., 2006; Wills et al., 2010). The first notion is derived from classical experiments in frogs (Nieuwkoop et al.,...
1952) and finds support from modern work with *Xenopus* and zebrafish. However, in amniotes, the origin of the spinal cord anlage and the MEK inhibitor PD03, or 3 days with the BMP inhibitor DMH1. Aggregates were imaged on day 5 by wide-field epifluorescence microscopy. The phase-contrast and fluorescence images shown are representative examples. Aggregates exposed to a 24 h pulse of Chiron are able to show a single large extension containing Sox1::GFP-expressing cells with a region at the tip that is negative for the fluorescence reporter. (B) Wnt reporter TLC2 mESC aggregates differentiated in N2B27 with a 24 h pulse of Chiron on day 3 and imaged on day 5. (C) Aggregate as in B fixed and immunostained for brachyury (white) and Sox2 (green). The fluorescent reporter is expressed predominately in the aggregate extension corresponding to a Sox2+ Bra+ region. The white arrowhead indicates the region of highest Sox2 expression. Hoechst was used to label the nuclei. Scale bars: 500 µm in A; 200 µm in B, C.

The NMP population emerges within the mouse node-streak border (NSB) at the end of gastrulation and careful lineage-tracing experiments have identified a subpopulation in the regressing node that acts as a source of progenitors, at least for the ventral nervous system (Cambray and Wilson, 2002, 2007). However, removal of the node in chicken and mouse embryos does not affect axial elongation. Genetic or mechanical ablation of the node in mouse embryos before it regresses results in the loss of the notochord and the floor plate, but it does not impair the emergence of a neural tube with spinal cord characteristics, though it is smaller and lacks motor neurons; the paraxial mesoderm is also affected in these embryos (Ang and Rossant, 1994; Weinstein et al., 1994; Davidson et al., 1999; Klingensmith et al., 1999). This observation suggests the existence of alternative sources of progenitors for the spinal cord that are located laterally to the NSB. The elongations that we observe in our aggregates support this possibility as they lack a notochord.

In our experiments, we have observed that signalling on the third day of differentiation is crucial for the commitment of cells to particular lineages both in adherent and 3D culture. At this time, differentiating mESCs transit through an epiblast-like state and therefore our results parallel recent ones showing that transient activation of Wnt signalling in EpiSCs can generate various primitive streak-related populations (Tsakiridis et al., 2014). Altogether, these observations favour the possibility that, at least in culture, an NMP-like population can be generated from a primitive streak-primed epiblast-like population without the need for an anteriorly fated neural template. This is supported by the observation that differentiation of mESCs continuously exposed to...
activin and Chiron, which suppresses the neural fate, results in the appearance of some Sox2+ Brα+ cells (Fig. 1A).

Signalling and the specification and differentiation of the NMp population
In both adherent cultures and aggregates, the appearance of the Sox2+ Brα+ population is associated with Wnt/b-catenin, as well as with FGF/ERK signalling. Once the population has been established, it appears to use cell-autonomous gene regulatory networks fuelled, as in the embryo, by FGF/ERK signalling for its maintenance and differentiation. Consistent with this, blockage of FGF/ERK signalling after the establishment of the primordium, inhibits its elongation, although it does not affect neural cells generated prior to the inhibition. The details of the structure of this population remain to be elucidated. Retrospective clonal analysis in embryos has suggested the presence of a small, stem cell-like population, with dual potential (Tzouanacou et al., 2009), but this remains to be confirmed. Preliminary results of clonal analysis of the NMp-like population generated in adherent culture have been obtained (P.C.H. and D.A.T., unpublished).

Single-cell analysis of a population enriched for NMps reveals a number of features that will require further examination. Most strikingly, we observe a variation in the levels of Brα mRNA expression in the population and a generally low level of Sox2 expression, which FGF makes very low. These heterogeneities contrast with the more homogeneous and correlated expression of the corresponding proteins. The discrepancy raises the possibility of dynamic transcription time averaging at the level of the population in some of the identifiers of the NMp. Such a situation has been described before for some elements of the pluripotency network (Munoz Descalzo et al., 2013) and might represent a general feature of stem/progenitor cell populations (Martinez Arias and Brickman, 2011). The single-cell analysis also reveals the expression of one new gene in the NMp: FoxB1. This gene had been associated with the development of the diencephalon but lineage tracing and gene expression place it within the NMp population (Zhao et al., 2007). Finally, this analysis confirms that the population exhibits high FGF and Wnt signalling, and identifies two genes associated with pluripotency, Lin28 and Mbd3, expressed in these cells, suggesting that they might share some features with mESCs.

In the future, it will be important to explore the properties and long-term renewal and differentiation potential of the NMp-like cells that we have generated in culture and in the 3D aggregates, and compare them with those that have been recently obtained from EpiSCs (Tsakiridis et al., 2014). In this regard, we notice that, under the same conditions, the NMps in the aggregates are maintained over a few days in a manner that they are not in adherent culture. This is likely to be due to the ability of the cells in the aggregate to generate their own niche. It will be interesting to investigate the differences between the two systems to identify the factors that create these differences.

Note added in proof
While this paper was being reviewed Gouti et al. (2014) have reported a population of cells similar to the one described here during the differentiation of both mouse and human ES cells.

MATERIALS AND METHODS
Routine monolayer cell culture, differentiation and aggregate formation
E14-Tg2A, TCF/LEF::mCherry (TLC2), Sox1::GFP, TBX6::EYFP and Brα::GFP mESCs were grown on tissue-culture plastic dishes as described previously (Faunes et al., 2013; Turner et al., 2014b). For differentiation experiments, cells were plated at a density of 4×104 cells/cm2 in a base medium of N2B27 (ND10 227, StemCells) supplemented with combinations of activin A (100 ng/ml), CHIR99021 (Chiron; 3 μM), XAV939 (1 μM), SB431542 (SB43; 10 μM), BMP4 (1 ng/ml), FGF2 (2.5 ng/ml), PD0325901 (PD03; 1 μM) and dorsomorphin-H1 (DM; 0.5 μM). Differentiation medium was replaced daily to reduce the influence of increased concentrations of secreted factors.

To generate aggregates, 40 μl droplets of a 1×106 cells/ml solution (in N2B27) were pipetted into each well of a sterile, non-tissue-culture treated, 96-well plate with a multichannel pipette. After 48 h, 150 μl of differentiation medium was added directly to the cells and 150 μl was replaced daily. It was essential that during medium replacements, cells were agitated by the forceful ejection of medium to prevent their adhering to the plate surface; medium was not changed during the initial 48 h of aggregate formation. For a full protocol and troubleshooting, see Basille-Johnson et al. (2014).

Flow cytometry and microscopy
Cells were analysed for GFP fluorescence using an LSR Fortessa (BD Biosciences) with a 488 nm laser and emission was measured using a 530/30 filter. Forward- and side-scatter properties alongside DAPI exclusion (405 nm laser and emission at 450/50) were used to select live, single cells for analysis in FlowJo (TreeStar). Data were analysed using FlowJo software. Sox1::GFP mESCs were sorted according to their GFP fluorescence in a MoFlo sorter (Beckman Coulter) using the same laser and filter sets described above. Immunofluorescence was performed as described previously (Turner et al., 2014a); a list of primary antibodies and staining conditions, as well as details of data capture are also fully described previously (Turner et al., 2014a).

Epifluorescence, time-lapse imaging of adherent cells was performed with an AxioObserver inverted microscope (Carl Zeiss) in a humidified CO2 incubator (37°C, 5% CO2). Images were captured every 10 min for the required duration using a 20× LD Plan-Neofluar 0.4 NA Ph2 objective with correction collar adjusted for imaging through plastic. All media were changed daily. An LED white-light system (Laser2000, Kettering, UK) provided illumination. The filter cubes GFP-182A-ZHE (Semrock) and Filter Set 45 (Carl Zeiss) were used for GFP and RFP, respectively. Emitted light was recorded using an AxioCam MRm and recorded with Axiovision release 4.8.2. For live imaging of cell aggregates, aggregates within a 35 mm non-tissue-culture grade bacterial dish were imaged every 10 min within a humidified (37°C, 5% CO2) Biotostation IM (Nikon). Analysis performed using Fiji (Schindelin et al., 2012).

Quantitative RT-PCR
Protocols used are as described previously (Faunes et al., 2013) (see supplementary material Figs S2 and S3). The primers used are available in supplementary material Table S2. The population qRT-PCR experiments are from data from one experiment (each sample analysed in triplicate) and are reflective of two independent experiments, whereas, due to constraints of sample availability, the sorted subpopulation qRT-PCR data are from one experiment with each sample analysed in duplicate or triplicate where possible. The Sox1+GFP population profiles gained in each condition have been analysed on multiple occasions and are both reproducible and stable.

Fluidigm
A list of primers and details of the protocols followed for the Fluidigm analysis have been described previously (Turner et al., 2014b) and are available in supplementary material Table S3 and supplementary materials and methods.

Acknowledgements
We thank James Briscoe and Val Wilson for sharing unpublished information and, together with Ben Steventon and Kate Storey, for constructive and helpful discussions throughout this work; Berthe Gottgens and Christina Pina for help with the Fluidigm single-cell analysis; Nigel Miller for help with the flow cytometry; Sofia S. Nowotschin and Anna Katerina Hadjiantoniakas for the Tbx6 reporter; Christopher Budjan for help with the immunostaining; and Tina Balayo for assistance with the tissue culture of the aggregates.
Competing interests
The authors declare no competing financial interests.

Author contributions
A.M.A., D.A.T. and P.C.H. conceived the project; D.A.T., P.H., P.B.-J., R.B. and F.F. performed the experiments; P.R. and P.C.H. analysed the data; A.M.A. and D.A.T. wrote the manuscript.

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References


Organoids and the genetically encoded self-assembly of embryonic stem cells

David A. Turner*, Peter Baillie-Johnson and Alfonso Martinez Arias*

Understanding the mechanisms of early embryonic patterning and the timely allocation of specific cells to embryonic regions and fates as well as their development into tissues and organs, is a fundamental problem in Developmental Biology. The classical explanation for this process had been built around the notion of positional information. Accordingly the programmed appearance of sources of Morphogens at localized positions within a field of cells directs their differentiation. Recently, the development of organs and tissues from unpatterned and initially identical stem cells (adult and embryonic) has challenged the need for positional information and even the integrity of the embryo, for pattern formation. Here we review the emerging area of organoid biology from the perspective of Developmental Biology. We argue that the events underlying the development of these systems are not purely linked to self-organization, as often suggested, but rather to a process of genetically encoded self-assembly where genetic programs encode and control the emergence of biological structures.

Keywords: development; organoids; positional information; reaction-diffusion; self-assembly; self-organization

Introduction

Embryonic development transforms a single celled zygote into a collection of multicellular tissues and organs arranged into structures we call organisms. A key element in this transformation is the ordered generation of cellular diversity which depends on the progressive allocation of cells to specific fates and their self-assembly into three dimensional structures according to emergent rules encoded in those fates. This process depends on programs encoded in, and decoded by, signaling and transcriptional networks. For the last 50 years our understanding of how these molecular devices organize cells in space and time has been dominated by the notion of Positional Information (Fig. 1A). Introduced by Lewis Wolpert in 1969 [1], Positional Information states that in a developing organism cells acquire fates by “reading and interpreting” molecular instructions encoded in diffusible substances which, following a terminology introduced by Alan Turing, are known as Morphogens [2]. A most important element of Positional Information is the notion that Morphogens diffuse from a fixed source across a cellular field thus creating a concentration gradient with different concentrations evoking different responses in the underlying cells i.e. the position of a cell relative to the source of the Morphogen is transformed into a fate. Genetic analysis of pattern formation in Drosophila identified genes whose products could be cajoled into mediating Positional Information through a classic Wolpertian mechanism [3], a notion that was later extended to other organisms. However, the observation that Wingless, a leading member of the Wnt gene family and an influential Morphogen, does not work at a distance in Drosophila [4, 5] and that time of exposure to, and concentration of, a Morphogen are interchangeable variables for patterning fields of cells [6], has invited a reflection on the role of gradients in pattern formation. An alternative to Positional Information preceded Wolpert’s ideas and was put forward by Alan Turing. In his seminal paper of 1952 he showed how, under certain conditions, random heterogeneities in chemically interacting diffusible substances could generate patterns without a pre-existing organisation (Fig. 1B) i.e. they could act as agents of self-organization [2]. A few years later, in an independent study, Gierer and Meinhardt proposed a formally equivalent solution to the problem of
spatial patterning in biological systems \[7\]. Turing’s ideas, a theoretical proof of principle, were difficult for biologists. This together with the geometric and intuitive design of *Drosophila* development as well as the appeal of the Wolpertian Morphogen metaphor to explain the patterning of the vertebrate limb \[8, 9\], led developmental biologists to embrace *Positional Information* rather than Turing-based mechanisms as a basis for the patterning of cells during developments.

The last few years have produced a large number of observations that cells can organise themselves into recognisable patterns without a fixed reference. These observations are difficult to relate to the classical views of pattern formation and suggest that, perhaps, the classical notion of *Positional Information* is in need of a revision. The ability of cell ensembles to organize themselves into patterns resembling those that arise in embryos finds a surprising extreme in the experimental ability to coax stem cells into building different structures, from an eye cup \[10\] to an intestine \[11\]. In particular, embryonic stem cells (ESCs) can be steered into specific tissues and organs with surprising ease. This ability has been referred to as self-organization and, by implication, evokes notions of Turing-like mechanisms (Box 1). Whereas these observations have been hastily discussed in the context of regenerative medicine, it may be the case that they are telling us more about both Development itself and how we shall be able to use this information practically. In this essay, we shall discuss these novel observations from the perspective of Developmental Biology. We shall question the notion that organoids exclusively represent examples of self-organization and suggest that they reveal interactions between cells and underlying genetic programs that encode emergent properties of developmental systems.

**Anterior neural as a primary fate in stem cells and embryoid bodies**

Stem cells (SCs) have the defining characteristics of self-renewal and the ability to differentiate into specialized cell types. Generally there are two classes of SCs, embryonic (ESC) and somatic (adult), the former being derived from the inner cell mass (ICM) of the pre-implantation embryo \[12, 13\] are able to generate all the tissues of the embryo proper, whilst the latter sustain the homeostasis and fuel repair mechanisms of differentiated tissues and organs \[14\]. The ability of SCs to be maintained in culture and their propensity to differentiate into the different cell types of the developing organism has resulted in their use as a model system for investigating biological processes such as early developmental events, self-organization, tissue homeostasis, and repair.

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**Figure 1.** Patterning tissues through *Positional Information* or a Reaction-Diffusion System. \(A\): Patterning through *Positional Information*. Secretion of a Morphogen from a fixed source results in a graded distribution of the signal through the tissue. This signal is interpreted by cells where their fate depends on defined concentrations of the Morphogen \((c_1, c_2)\). \(B\): Patterning through Reaction-diffusion (R-D) systems (Turing). In Turing’s R-D model, two genes interact where one activates itself (green) as well as its inhibitor (red; \(c\)). Critically, since the activator diffuses slowly with respect to the inhibitor, the inhibitor is unable to provide enough negative feedback to counter the autoinduction of the activator at the point of initiation. This results in sharp peaks centred around regions of inhibition \((a-i, b-i)\). As the levels of inhibitor decrease around these local maxima, other peaks can form until the expression of these genes dynamically alters to produce a regular oscillatory pattern \((a-iii, b-iii)\). The wavelength of these oscillations depends on the size and shape of the tissue being patterned, where the concentration of specific substances produced by these oscillations may determine the specific fates a tissue will adopt. Figure part adapted from \[91\] and \[97\].
Classically, experiments using SCs have relied on two-dimensional culture techniques, where cells are grown on plastic dishes as a monolayer (Fig. 2A). ESCs for example, are traditionally cultured on either a bed of feeder cells (which provide a number of factors that maintain their pluripotency e.g. LIF in the case of ESCs) or adherent substrates (such as fibronectin or gelatin) (Fig. 2A). Although this two-dimensional culture method has been exceptionally useful as a foundation for understanding many cellular processes, it cannot recapitulate the three-dimensional environment cells are exposed to in vivo [15]. Allowing cells to grow in three dimensions reveals a potential for them to assemble spatially organized patterns (Fig. 2B–D). Early pioneering studies from the laboratory of Howard Green and colleagues showed how cultured primary human skin cells on a bed of irradiated 3T3 cells could form a stratified squamous epithelium [16], presumably derived and maintained by the SCs present within the primary tissue [17]. Furthermore, mechanically supported cultures of primary keratinocytes from the skin or oesophagus (Fig. 2D) can generate fully stratified, organized epithelia upon their making contact with an air-liquid interface [15, 18, 19] (Table 1). The mechanical support provided by artificial matrices and scaffolds also allows primary endothelial cells to generate blood vessels with tissue architecture not dissimilar from their in vitro situation [20].

Culturing ESCs in high density, non-adherent, suspension culture, gives rise to aggregates that form three-dimensional structures termed Embryoid Bodies (EBs; Fig. 2C) [21]. ESCs differentiated in this manner, typically requiring many thousands of ESCs which grow in a largely disordered manner, are able to progress towards further stages of early embryo development [21]. This therefore provides an attractive system for deriving a number of embryological cell types, some of which are not easy to obtain in adherent culture e.g. blood [22] and cardiac [23]. Sometimes, sorting of cell types with different characteristics can be observed within a single EB as in the case of endoderm [24] (Fig. 2B). Whereas the emerging organoid field also relies on three-dimensional suspension culture, these organoids are typically studied as

**Box 1**

**Definition of Terms**

**Genetic Program:** In Developmental Biology, a genetic program is a temporal sequence of changes of state of a cell or cell population, brought about by the decoding of a temporal order of gene expression scripted in the genome.

**Self-Assembly:** The formation of an ordered structure from non-equivalent parts as a system moves towards equilibrium.

**Self-Organization:** The spontaneous emergence of order or asymmetry from an initially homogeneous starting population that occurs in an energy-dependent manner.

**Genetically-Encoded Self-Assembly:** A genetic program that contains cell autonomous instructions as well as signalling events which can induce emergent properties.

**Figure 2.** Comparison of culture methods. Schematics of the typical culture methods utilised for the differentiation of SCs. **A:** Cells grown as a monolayer on a bed of feeders or surfaces coated with substances such as gelatin or fibronectin. In the case of ESCs, specific culture conditions can direct their differentiation towards anterior neural (i), a primitive streak (PS) population (e.g. T/Bra-expressing cells [66]); ii, derivatives of the germ layers (iii) and a neuromesodermal progenitor (NMP) population for axial tissues such as the spinal cord and paraxial mesoderm [74, 98]. **B:** Mechanically supported culture allows the further differentiation of primary tissues such as human keratinocytes. Upon contact with an air-liquid interface and over a period of weeks, cells differentiate and self-assemble to form a fully stratified tissue (adapted from [15]). **C:** Embryoid bodies (EBs) can either be generated on low-adherence tissue-culture plastic or through hanging drop culture (pictured). In the latter case, droplets of ESCs are suspended above PBS or water and cultured for a number of days. Haematopoietic progenitors (i) [23] and cardiomyocytes (ii) [22] have been produced through EB culture. EBs typically show organised gene expression (i), however polarized, elongated structures have been formed by this method using low numbers of EC cells [75]. **D:** More modern techniques producing “Gastruloids” (i) and the serum-free floating culture of embryoid-body-like aggregates with quick reaggregation (SFEbBs) [27] (ii) have been successful in generation of structures that mimic a number of early developmental processes (axial elongation, polarisation; i) as well as the generation of self-assembling and patterned organoids such as the optic cup (iii). In the case of the latter organoids, cells are usually embedded in Matrigel and occasionally transferred to bacterial dishes once aggregation has occurred. See Table 1 for details on the culture methods and time for organoid formation.
intact structures throughout their development rather than continued culture in two dimensions following initial EB formation.

However, though sometimes pockets of spatial organization can be found in EBs [25], these are not structured in the manner of the organs in embryos. Building on this observation and making use of fundamental principles of developmental biology, Sasai and his team were able to generate optic cups from ESC aggregates in the absence of external mechanical inputs in around nine days [26].

### Table 1. Comparison of the culture techniques, generation time, and plating densities of organoids

<table>
<thead>
<tr>
<th>Organoid Origin</th>
<th>No. Plated cells/tissues</th>
<th>Method/Comments</th>
<th>Time to generation (days)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortical Neurones</td>
<td>hESCs</td>
<td>3 × 10⁶ cells</td>
<td>SFEBq; low-adhesion U-bottomed plates</td>
<td>10–20</td>
</tr>
<tr>
<td>Optic cup</td>
<td>mESCs</td>
<td>3 × 10⁴ cells</td>
<td>SFEBq, Matrigel embedding; low-adhesion U-bottomed plates</td>
<td>∼9</td>
</tr>
<tr>
<td>hESCs</td>
<td>9 × 10⁶ cells</td>
<td>SFEBq; low-adhesion V-bottomed plates; Matrigel embedding day 2; transfer to petridish day 12; SFEBq; low-adhesion plates; Matrigel embedding</td>
<td>∼24</td>
<td>[10]</td>
</tr>
<tr>
<td>Inner Ear</td>
<td>mESCs</td>
<td>3 × 10⁶ cells</td>
<td>E Bs generated in low-adhesion U-bottomed plates; embedded in Matrigel and cultured in spinning bioreactor</td>
<td>14–24</td>
</tr>
<tr>
<td>Cerebral</td>
<td>mESCs</td>
<td>2 × 10⁶ cells</td>
<td>E Bs generated in low-adhesion U-bottomed plates; embedded in Matrigel and cultured in spinning bioreactor</td>
<td>18–24</td>
</tr>
<tr>
<td>Neural Tube</td>
<td>mESCs</td>
<td>1 cell</td>
<td>Cells (5 × 10⁴ cells) in N2B27 embedded in Matrigel, spread evenly over glass-bottomed MatTek dishes; organoids form from single cells</td>
<td>&lt;10</td>
</tr>
<tr>
<td>The viscera</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestine</td>
<td>Crypts (m)</td>
<td>500 Crypts</td>
<td>Matrigel embedding; Single LGR5⁺ forms organoids; enhanced with Paneth cell co-culture</td>
<td>8–14</td>
</tr>
<tr>
<td>LGR5⁺ SC (m)</td>
<td>1 cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LGR5⁺ SC; Paneth (m)</td>
<td>500 cells each</td>
<td>Monolayer differentiation towards hindgut; formed spheroids embedded in Matrigel</td>
<td>14–28</td>
<td>[51, 52]</td>
</tr>
<tr>
<td>Colon</td>
<td>Crypts (m, h)</td>
<td>500 Crypts</td>
<td>Matrigel Embedding; single LGR5⁺ SCs can form organoids if anoikis is inhibited in first 2 days</td>
<td>7–10</td>
</tr>
<tr>
<td>LGR5⁺ SC (m)</td>
<td>1 × 10⁶ cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>Gastric glands (m)</td>
<td>100 glands</td>
<td>Matrigel embedding</td>
<td>7–10</td>
</tr>
<tr>
<td>LGR5⁺ SC (m)</td>
<td>50 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>hESCs/IPSCs</td>
<td>50 spheroids</td>
<td>Monolayer differentiation towards posterior foregut; spheroids embedded in Matrigel</td>
<td>28</td>
</tr>
<tr>
<td>Kidney</td>
<td>hESCs</td>
<td>50 spheroids</td>
<td>Monolayer differentiation towards anterior foregut; spheroids embedded in Matrigel</td>
<td>65–110</td>
</tr>
<tr>
<td>Liver</td>
<td>hPSCs</td>
<td>1 × 10⁶ cells</td>
<td>Monolayer differentiation towards intermediate mesoderm, dissociation and culture in Air-Liquid interface after 18 days in culture</td>
<td>4</td>
</tr>
<tr>
<td>Biliary Ducts (m)</td>
<td>100 glands</td>
<td>Matrigel Embedding</td>
<td>7</td>
<td>[95]</td>
</tr>
<tr>
<td>LGR5⁺ SCs (m)</td>
<td>1 cell</td>
<td>Matrigel Embedding</td>
<td>19</td>
<td>[95]</td>
</tr>
<tr>
<td>Embryo</td>
<td>'Gastruloids'</td>
<td>mESCs</td>
<td>Cells plated in low-adhesion, U-bottomed plates</td>
<td>4–5</td>
</tr>
<tr>
<td>Other</td>
<td>Primary keratinocytes (h)</td>
<td>3 × 10⁶ cells</td>
<td>Air-liquid interface culture</td>
<td>∼21</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>Oesophageal fibroblasts (m/h)</td>
<td>2.5 × 10⁶ cells</td>
<td>Fibroblasts embedded in collagen/matrigel; Oesophageal keratinocytes (4 × 10⁶) added after seven days; Air-liquid interface culture</td>
<td>11–13</td>
</tr>
<tr>
<td>Blood vessels</td>
<td>HUVECs</td>
<td>4.5 × 10⁴ cells</td>
<td>Cells seeded into collagen microvessels (mechanical support)</td>
<td>7–14</td>
</tr>
</tbody>
</table>

Information on selected organoids from a number of tissues, broadly grouped into three categories, is given for the tissue origin (e.g. ESC, adult SC, tissue fragments etc.), number of cells or individual cellular units (crypts or cell spheroids) used to generate the organoid and the time taken to form the organoid structure. Time to formation is taken as the amount of time forming organoid structure, not the total time in culture. This is particularly important in the case of the visceral organoids from SCs, where cells are first directed to specific lineages before Matrigel embedding and organoid formation. ESCs: embryonic stem cells; SCs: stem cells; (h): human; (m): mouse; SFEBq: serum-free floating culture of embryoid-body-like aggregates with quick reaggregation; HUVECs: Human Umbilical Ven Endothelial Cells; hMSCs: human Mesenchymal Stem Cells.
culture, approximately 80% of the aggregates form a retina anlage at a specific position with an almost perfect organization. In doing so, it is conceivable that the ESCs generate an underlying pattern through a self-organizing process. However, Sasai and colleagues stressed that the contribution of self-organization to this structure is the information required for self-assembly – as soon as the cells start to express Rx (a marker of retina tissue), they begin to assemble themselves, changing their properties in a genetically predictable manner. Over the following 4 days, this emerging tissue arranges itself into a well-formed optic cup through mechanically and biochemically imposed changes to the tissue. These observations allowed the authors to identify self-organization as a means to form highly ordered structures from an unpatterned cellular ensemble, neatly describing the origin of the anlage [26]. In the same cultures it is often possible to observe the emergence of anterior brain structures: through tweaking of the culture conditions, diverse structures of the forebrain, which are the source of the optic primordia, are generated efficiently, although not predictably [26]. Interestingly, the essence of these protocols tends to be the suppression of most external signals [27] and reflect a (developmental) tendency of the ESCs to develop these structures [28–31]. These observations are presaged in classical experiments with Xenopus embryos in which animal caps, if left in simple medium, differentiate into forebrain with the occasional emergence of eye tissues [32–35], suggesting that the anterior neural fate might be a universal primary fate in development.

The emergence of a complex structure such as an eye cup from a collection of cells without an external reference is, at first sight very surprising. One possible explanation for this observation is that within the large numbers of cells undergoing anterior neural development in the culture, one or a few of them might, just by chance, activate the eye cup genetic program in an environment which amplifies it and takes it to term. This situation would be reminiscent of the emergence of compound eyes in Drosophila upon ectopic activation of the Eyeless/Pax6 transcription factor [36]. In these experiments, the result of this misexpression is the production of well-formed and histologically complete compound eye structures. However while the ectopic expression occurs in large spatial domains across whole imaginal discs, the ectopic eye tissue occupies a smaller domain, generally in the same place within each disc, and tends to form contained structures. This observation, which has also been made for the emergence of ectopic wings [37], indicates that the development of specific structures relies not only on the activation of a specific cell-autonomous genetic program, but also on the convergence of specific signals and mechanical inputs that restrict the potential to develop the genetic program. The ectopic transcription factor can only act where there is a constellation of signals which will drive its activity [37, 38]. It is possible that the same happens with the mouse ESC-derived eye cups, though in this case it is a stochastic event within a very large cell population in culture and highlights the relationship between environment, transcription and, probably mechanics.

The emergence of eye cups from ESCs contrasts with the events associated with the in vitro derivation of regionalized cerebral cortex in organoid culture [39]. In this case, the whole structure develops into a mixture of locally organized tissues that are proposed to interact with one another, but there is no global organization as in the previous examples. Where the results of the neural cyst cultures showed that the organoids are capable of generating a rudimentary axis, there is no overall co-ordination in the growth of these cortical tissues, perhaps indicating that local tissue interactions may play a greater role in patterning in this case. It is worth noting that the period of culture is much longer in these aggregates than the organoids described so far (Table 1); this may be partially a property of the human stem cell system but it could also reflect a much longer period of self-assembly, following local tissue self-organization.

Mesendodermal organoids can emerge from adult and embryonic stem cells

A number of in vitro models have been established which generate organoids of visceral tissues either directly from pluripotent SCs (ESC and iPSC) or from adult SCs, primary cells, dissociated tissues or organ slices [15] (Table 1). To date, the pancreas [40], kidney [41–44], thyroid [45], liver [46], various regions of the gastrointestinal (GI) tract [11, 47–52] and respiratory system [53, 54] have been described. Similar to the examples for anterior structures (above), the time taken to generate these structures can vary following the initial plating event. Once more the structures are said to develop primarily through self-assembly but the self-assembly of the tissues is subtly different compared with anterior, neural organoids (above) as well as between visceral organoids formed from either embryonic or adult stem cells.

Visceral organoids from adult tissue

During the generation of GI-tract organoids, the initial approaches relied on isolating crypts from either the intestine [11], colon [48], or stomach [49] and embedding them in matrigel (Table 1). As these crypts contain LGR5-expressing crypt base columnar (CBC) cells, the adult SC population, which are able to re-generate all epithelial lineages of its respective tissue [55], it is therefore no surprise that over time, the crypts containing these cells are key to the regeneration of the tissue structure in vitro. Indeed, as the three dimensional culture takes shape, further crypt domains are generated within the matrigel culture, forming an organoid with multiple crypts interspaced by a villus-like epithelium that surrounds a central lumen by approximately fourteen days. Generally, the initial culture period of these organoids from the isolation of the adult SC population to the initiation of organoid development takes approximately eight days in culture, much more rapid than those observed with most anterior structures (Table 1). This rapid development may be attributed to the initial patterning and positional information that is inherent in the isolated crypts, where the signalling environment is permissive for their self-assembly. In support of this, whereas single LGR5⁺ cells can only
generate organoids approximately 6% of the time [11], co-culture of LGR5⁺ cells with Paneth cells (500 cells each) is much more efficient [47], suggesting that the heterogeneities created through the co-culture of these cells may facilitate the initial self-organizing pattern formation: e.g. Turing-like mechanisms (Fig. IB) may allow these self-assembling processes to progress more efficiently as the initial asymmetry is already established.

**Visceral organoids from ESCs**

The generation of organoids for visceral tissues directly from embryonic or induced pluripotent SCs requires a different approach. In these conditions, pluripotent SCs are guided sequentially towards lineages that are the primordia for their tissues by supplying in vitro the chemical signals that would be received during development (i.e. directed towards the definitive endoderm for tissues derived from the primitive gut tube [56] or towards the primitive streak/mesoderm for derivatives of the kidney [42, 43]). For example, differentiation of ESCs towards the definitive endodermal lineage with a 3 day treatment of high dose Activin, followed by a further 4 days of specific differentiation signals can guide them towards posterior foregut (stomach [57], liver [46]), the hindgut (intestine [51, 52]) or the anterior foregut (lung [53]) lineages. Following this initial treatment, spheroids spontaneously form and upon their transfer to matrigel and organoid culture conditions, are able to form their respective organoids on a time scale similar to the time taken in the formation of anterior structures (See above and Table 1).

The period of time required to generate the correct information for self-assembly of these organoids may only take place once a suitable signalling niche has been generated by directed differentiation. For example, during the formation of liver organoids from human iPSCs [46], after their differentiation towards definitive endoderm, the cells require co-culture with Human Umbilical Vein Endothelial Cells (HUVECs) and human mesenchymal stem cells (MSCs) for cell coalescence and liver organoid formation [46]; this has marked similarities with the co-culture of Paneth cells with LGR5⁺ SCs described above [47]. In general, multiple rounds of signalling factors may generate the cellular heterogeneities required for symmetry-breaking events to occur and, as subsequent cell fates are acquired, different genetic programs may be activated over time that facilitate the organization and patterning of the tissues prior to the self-assembling events.

In summary, a common feature in the examples of anterior and visceral organoids is the generation of complex structures through an initial phase of generation and organization of different cell types which can vary depending on the tissue lineage and whether single SCs are used or isolated sub-structures of tissues (e.g. intestinal crypts). This initial phase specifies a particular pattern or region before a longer phase of self-assembly builds upon this pattern. In terms of single cells, they are capable of generating positional information de novo in order to achieve this construction. However, they are not dependent on an external source of a Morphogen (evidenced by the fact that these cultures take place in a signalling environment that is assumed to be uniform) but may be able to generate subsequent lineages that generate and secrete their own patterning factors.

**Gastrulation as a result of symmetry breaking within an ensemble of embryonic stem cells**

The process of gastrulation represents a crucial event in animal development as it transforms a mass of similar cells into the physical outline of an organism with recognisable body axes and germ layers (the seeds for the different tissues and organs) [58]. This process is common to all animal embryos and is driven by a conserved set of molecular interactions which lay down a transcriptional map upon an otherwise phenotypically similar group of cells [58, 59]. This map acts as a cue for a complex choreography in which groups of cells move from the outside to the inside of the embryo to give rise to the mesoderm and the endoderm. The physical implementation of this process depends on the geometry of the embryo. For example, in amphibia it involves the ordered invagination of cell populations through an orifice in a ball of cells, the blastopore [60], while in birds and mammals, gastrulation creates a dynamic longitudinal furrow within an epithelial disc (chicken, rabbit and human) [61–63] or cylinder (rodents) [64], known as the Primitive Streak. In all cases, cells undergoing gastrulation express the T-box transcription factor T/Brachyury (T/Bra) [65] and follow an orientation with respect to some global axial system that has been laid down in the embryo. T/Bra integrates spatial and temporal signals at the level of individual cells [66], promotes their movement [66–68] and, together with those signals, implements specific fates. The outcome of gastrulation is the assignment of different cells to the anteroposterior and dorsoventral axes of the organism and the localization of the endoderm and mesoderm to the inside and the ectoderm to the outside of the embryo, respectively. The detailed spatiotemporal correlation of these processes and their relationship to gene expression highlight how remarkable it is that eye cups [10, 26], intestines [51, 52] and pancreas [40] can emerge from SCs without a coordinate system.

Cells in adherent culture can be made to recapitulate some features associated with gastrulation [66, 69]. For example, one of the hallmarks of the process, rapid cell movement associated with transient T/Bra gene expression, can be observed in cultures of ESCs differentiating in the presence of Activin/Nodal and Wnt signalling [66]. The timing and sequence of these events is very similar to those in the embryo [64, 70]. Furthermore, human ESCs arranged on micropatterned discs and induced to differentiate by BMP4 give rise to a radially symmetric pattern of gene expression with an arrangement that attempts to mirror the topological organisation in the embryo [69]; from the outside to the inside: extraembryonic (Cdx2), Endoderm (Sox17), mesoderm (T/Bra) and neural (Sox2). This organization would be a flattened projection of the mouse cylinder and also of the arrangement in a human embryo. In this arrangement, the adhesion to the substrate prevents morphogenesis suggesting that it is possible to separate the morphogenetic movements from...
the fate, a conclusion that had been obtained from experiments in chicken embryos suggesting that the morphogenetic movements are there to make an embryo. Two intriguing observations of the micropatterned hESCs are that the process does not scale and that the pattern appears to use the boundaries as a reference [69].

The radial symmetry of the micropatterned hESCs contrasts with the anisotropic and polarised patterns of activity of cells in early embryos and begs the question of the origin of these asymmetries. One possibility is that these asymmetries require a three dimensional organisation but it may be that they reflect a combination of dimensionality, movement and localised cues. Indeed, in embryos, cells become endowed with such asymmetries very early in development and might be registered in manners that allow them to move (or they are endowed with the ability to move). For example, when explants are made from the region around the organiser of Xenopus embryos, the cells undergo polarised convergence extension [71], supporting the notion of an intrinsic “navigation system.”

As in the case of the retina from ESCs, not only the fates, but also the mechanical properties of the system are autonomously encoded. This view is clearly demonstrated in experiments in which Xenopus animal caps treated with Activin, which if undisturbed would develop into neuroectoderm, will not only turn into mesoderm but will organize themselves into polar structures that resemble the exogastrulae characteristic of Keller sandwiches [71]. Furthermore, if one takes these structures that have been exposed to Activin, disaggregates them and lets the cells aggregate, they will form structures that resemble the original ones [72] i.e. the system can reassemble itself. For these reasons it is perhaps not surprising that ESCs can organize themselves into similar structures.

Experiments with three dimensional aggregates of mouse ESCs reveal an intrinsic tendency to break symmetry [25, 73, 74]. For example, when large EBs are exposed to Wnt signalling, occasionally they exhibit axial organization in the form of an asymmetric activation of Wnt signalling and expression of T/Bra [25]; although the causes for this are not known. However, a first glimpse of consistent axial organization of ESCs was reported in P19 Embryo Carcinoma cells [75]. When EBs of P19 cells are placed in serum, they organize into structures that very much resemble exogastrulae with a polarised extension that expresses T/Bra in a Wnt-dependent manner [75]. Furthermore, gene expression analysis of these aggregates indicates that they differentiate further and express mesodermal genes much as they would do in the embryo [75]. Recently, mouse ESCs have been shown to be able to undergo reproducibly similar patterning events [73, 74] (Fig. 3). Analysis of the cause underlying this behaviour revealed a need for an initial critical cell number and the requirement for Wnt signalling for the elongation [73]. In addition, these events are associated with a process that resembles gastrulation, whereby cells move from a defined position and with a direction [73]. Furthermore, filming of the emergence of these structures over time shows how the symmetry is broken and how they evolve [73]. The development of these structures, called Gastruloids (Fig. 3), leads to axial extensions in a manner that parallel similar events in embryos.

In contrast with the adult organoids, Gastruloids can be followed over time with good temporal resolution [73]. The picture that emerges from these observations is one of cell autonomous molecular processes that unfold over time to produce asymmetry and spatially ordered structures.

### Genetically encoded self-assembly

There is a widespread view that the ability of stem cells to generate tissues and organs is an example of self-organization. We believe that such statements confuse self-organization with self-assembly (Box 1). For example, in some instances it has been suggested that a criterion for self-organization is the ability of a system to put itself together after its structure has been disrupted [76]. As in these instances there might be a “memory” of the original arrangement in the elements that are produced by the destruction of the original order, this might be better described as an example of self-assembly. Such processes have been observed in many instances (e.g. vertebrate limbs and insect imaginal discs) and are used as a basis for the assembly of some organs from stem cells in the form of natural scaffolds around which different parts arrange themselves [43]. In contrast with these processes, a canonical self-organizing system achieves dissipative, nonequilibrium order at the global
level through local interactions within a collection of its components [77]. This can be induced by internal and external factors, but critically order is lost upon removal of the source of energy [77] (see also [27] in the context of biological systems). Classical examples of self-organizing systems are spontaneous magnetisation [78], lasers [79], the Belousov-Zhabotinski reaction (classic Turing patterns) [80, 81] and in biology, bird flocking [82]. The emergence of organoids does not belong to either of these classes as in the initial phases of the process it is not clear that all the components of the system (i.e. the cells), are equivalent-populations of SCs, particularly ESCs which are dynamically heterogeneous. While these heterogeneities can be the source of signals that promote patterning in a Turing like mechanism, the ingredients for the final structure are not present in the starting population and emerge in an ordered manner over time. Furthermore, removal of the trigger does not result in the decay of the structure.

The heterogeneities inherent to the organoid systems and the genetic encoding of the process associated with their evolution in culture, make the point that these are not self-organizing systems in the sense of classical physical systems. In all cases, the biological systems evolve through local interactions that are encoded in their components and unfold over time (see above). The intrinsically encoded governance of the process is an essential element of these systems and leads to what James Briscoe has called “supervised self-organization” (personal communication). This highlights that while there is an underlying element of progressive self-organization in the process, this stems from the unfolding of a genetic program within ill-understood physically constrained conditions (Fig. 4). A related thought has been emphasised by Sasai and colleagues e.g. the curvature of the optic cup and the forces that lead to its symmetry breaking in the emerging structure are autonomous to the structure [26]; this is important to understand. It is clear that the genetic programs encode elements that can mediate emergent properties by interacting with the environment, other cells or the mechanics that results from their packing and this encoding and its feedback on the cell autonomous events is, probably, the key to the process. The divergence of programs leads to the generation of interacting sets of cells but the gene expression programs are autonomous. Thus it is possible to observe these programs in the differentiation of ESCs and less clearly in the intestinal organoids. We would suggest that we should talk about genetically supervised self-organization or, perhaps more properly, genetically encoded self-assembly (Fig. 4B2) (Box 1).

**Programs of gene activity lead to the autonomous emergence of tissues and organs**

An important question from these studies is not only whether the resulting structures are the same as those that emerge in embryos but whether the process that leads to them is the same as that followed by cells in embryos. The emergence of a floor plate (FP) in differentiating neural cysts highlights many of the elements of this discussion [83].

In the embryo, the generation of motoneurones depends on the activity of a source of Shh that is located on the ventral side of the neural tube [84]. This pattern is induced early in development by the underlying notochord and strategically positions the neural progenitors next to the somitic mesoderm progenitors of the muscles that they will innervate. Under mechanically and chemically controlled conditions ESCs can be coax to differentiate into neuroepithelial structures with a lumen that resembles a neural tube [83]. Surprisingly, when treated with Retinoic Acid, a patch of Sonic Hedgehog (Shh)-producing floor-plate emerges in around 45% of the cysts. The FP begins to form around three days, after which it matures (as indicated by the expression of ARX at day 7) and patterns the rest of the neural tissue, forming ten distinct layers by day nine. There is no notochord in the culture suggesting that a floor plate is an integral part of the “neural tube genetic program” and that when activated, within the right length scale, cell interactions mediated by signalling molecules, lead to the emergence of a single structure [83].

How then is it possible to have such a precise structure autonomously? It may be that in the experimental culture conditions, the threshold for the intrinsic patterning process is very low which allows it to occur with ease (Fig. 5A1); in the fact that 4/207 cysts presented with two FPs demonstrates that there is the potential to specify more than one site within a cyst [83]. If one could provide a fixed axial reference to these cysts, it is likely that the FP would appear in a different position in different cysts (Fig. 5Bii) which contrasts with the situation in the embryo where the FP is always precisely positioned with regard to the somites and, importantly, the notochord (Fig. 5Bii). We surmise that the difference between the embryo and the culture lies in the threshold needed to
trigger the emergence of the FP. The culture reveals an intrinsic ability of the system, genetically encoded, to generate a floor plate in vitro in the absence of a notochord (which acts as the source for Shh-mediated ventral-dorsal patterning) may be due to the differences in a threshold level (RT\(_{(i)}\), RT\(_{(ii)}\)) that integrate them at a higher level. Organoids might represent a way to focus on such systems and their mechanisms: the ones that act autonomously to generate particular organs and systems, and those that integrate them at a higher level.

Conclusions

There might be surprises ahead as the molecular mechanisms underlying the generation of organoids might be different from those mediating the corresponding organs in vivo. The organoids in their own way represent a new challenge to the molecular systems that underlie pattern formation and we might find that although the final structures are very similar to those produced in embryos, their paths are different. In probing this, we shall learn how to harness the molecular systems to “replicate” tissues and organs in vitro. At this, the interface between genetically encoded self-assembled organoids with designer bioengineering [90] promises much, but the harvesting of this interaction will bring about an interesting reassessment of developmental biology, more focused on molecular mechanisms than on patterns. One of the lessons already learned from these studies is a reconciliation of Turing-driven mechanisms and Wolpertian positional information [91] as it is clear that the former drives the emergence of localised signalling sources that, when stabilised, act as references for patterning: positional information is a result of genetically encoded self-assembly. There are more to come.

This research invites a consideration of the moral and ethical issues associated with these structures, in particular the embryonic ones [92], but is imperative that this goes hand in hand with the development of robust and reproducible experimental systems in which we understand the cellular and molecular events that fuel these structures.

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References


Gastruloids develop the three body axes in the absence of extraembryonic tissues and spatially localised signalling

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Abstract

Establishment of the three body axes is a critical step during animal development. In mammals, genetic studies have shown that a combination of precisely deployed signals from extraembryonic tissues position the anteroposterior axis (AP) within the embryo and lead to the emergence of the dorsoventral (DV) and left-right (LR) axes. We have used Gastruloids, embryonic organoids, as a model system to understand this process and find that they are able to develop AP, DV and LR axes as well as to undergo axial elongation in a manner that mirrors embryos. The Gastruloids can be grown for 160 hours and form derivatives from ectoderm, mesoderm and endoderm. We focus on the AP axis and show that in the Gastruloids this axis is registered in the expression of T/Bra at one pole that corresponds to the tip of the elongation. We find that localisation of T/Bra expression depends on the combined activities of Wnt/β-Catenin and Nodal/Smad2,3 signalling, and that BMP signalling is dispensable for this process. Furthermore, AP axis specification occurs in the absence of both extraembryonic tissues and of localised sources of signalling. Our experiments show that Nodal, together with Wnt/β-Catenin signalling, is essential for the expression of T/Bra but that Wnt signalling has a separable activity in the elongation of the axis. The results lead us to suggest that, in the embryo, the role of the extraembryonic tissues might not be to induce the axes but to bias an intrinsic ability of the embryo to break its initial symmetry and organise its axes.

One sentence summary

Culture of aggregates of defined number of Embryonic Stem cells leads to self-organised embryo-like structures which, in the absence of localised signalling from extra embryonic tissues and under the autonomous influence of Wnt and Nodal signalling, develop the three main axes of the body.

Key Words Gastruloids, anteroposterior, dorsoventral, left-right, embryonic axes, organoids.

1 Introduction

The establishment of the anteroposterior (AP) and dorsoventral (DV) axes during the early stages of animal development is a fundamental patterning event that guides the spatial organisation of tissues and organs. Although this process differs from one organism to another, in all cases it involves a break in an initial molecular or cellular symmetry resulting in the precise positioning of signalling centres that will drive subsequent patterning events. Dipteran and avian embryos provide extreme examples of the strategies associated with these processes. For example, in Drosophila, the symmetry is broken before fertilisation within a single cell, the oocyte, which acquires information for both the AP and DV axes. This occurs through interactions with surrounding support cells that control processes of RNA and protein localisation which then serve as references for the rapid patterning of the embryo as the zygote turns into a multicellular system (2, 3). On the other hand, in chickens the processes take place within the developing embryo, within a homogeneous multicellular system that lacks external references (4, 5). In mammalian embryos, the axes are also established within a homogeneous cellular system, the epiblast, but in this case they are under the influence of an initial symmetry breaking event that takes place within the

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Figure 1: The anteroposterior, dorsoventral and left-right axes are clearly defined in Gastruloids and develop a Node-like region. Sox1::GFP (A) and Nodal::YFP reporter (B) Gastruloids pulsed with Chi (48-72hAA) and stained with Hoechst and anti-GFP with either (A) T/Bra (red) and Sox2 (Blue) or (B) Sox2 (red) at 120h AA. Hoechst is not shown in (A). Nodal is clearly expressed opposite an extending plate of Sox2 expression and T/Bra is observed at the most posterior region of the Gastruloid. In both examples, 3D projections are displayed. Taken in combination with supplemental figure S1, the expression of different genes and reporters can be used to map axes of the embryo onto the Gastruloid. (C) Gastruloids formed from Wnt/β-Catenin (TLC2), Smad2/3 and Nodal::YFP reporters lines following a 48-72h Chi pulse are shown. Insert shows the quantification of reporter expression for the TLC2 (red) and Bra::GFP (green) Gastruloids in a posterior to anterior direction. Stimulation results in activation of the TLC2 reporter with highest expression at the posterior pole. Interestingly, lateral expression of the Smad2/3 reporter is observed in a large proportion of Gastruloids, indicating the initiation of a LR axis. Nodal expression by 120h is confined to the posterior region indicating a prospective Node-like region. (D) A small number of Smad2/3 reporter Gastruloids at 120h show expression in two regions at the elongated tip at a low frequency. Over time one side is down-regulated similar to the expression of Nodal in the perinodal crown cells of the embryo (see Fig. 1C&F in ref (1)).
extraembryonic tissues which is then transferred to the developing embryo (5–8).

Efforts to understand the molecular mechanisms that pattern early embryos have relied on genetic approaches such as perturbation of the process by genetic mutations and a correlation between specific processes and molecular events as highlighted by the genes (9, 10). Although successful, these approaches have limitations as they often conflate correlation and causation, and importantly cannot probe the role of mechanical forces that have been shown to play a role in the early events (11, 12). This suggests a need for a complementary experimental system in which, for example, rather than removing components we attempt to build tissues and organs from cells and learn what the minimal conditions are that allow this (13). We have recently established a non-adherent culture system for mouse Embryonic Stem Cells (ESCs) in which small aggregates of defined numbers of cells undergo symmetry breaking, polarisation of gene expression and axial development in a reproducible manner that mirrors events in embryos (14–16). We call these polarised aggregates Gastruloids and have suggested that they provide a versatile and useful system to analyse the mechanisms that mediate cell fate assignments and pattern formation in mammalian embryos (16, 17).

In this study we show that Gastruloids develop the three main axes of a mouse embryo (AP, DV and left-right (LR)) in the absence of extraembryonic tissues and investigate the underlying molecular causes for this event. Unlike the embryo, this process does not require BMP signalling but relies on interactions between Nodal and Wnt signalling that are recorded in the expression of the transcription factor T/Brachyury (T/Bra). Furthermore we show that localisation of Nodal, which is widely held as essential for the establishment of the AP axis, is not required for the polarisation of T/Bra expression. Our results provide novel insights into the patterning of the embryo and suggest that a similar spontaneous symmetry breaking event may occur in the embryo where biases from the extraembryonic tissues, ensure its reproducible location at the site of gastrulation.

2 Results

2.1 Axial organisation in Gastruloids

Our previous studies using Gastruloids revealed them to have an AP axis with the expression of T/Bra located towards one end that will lead an elongation process (14, 16, 18). To follow on from these observations and to determine whether other markers of the embryonic axis are present, we cultured Gastruloids for 120h, probed for a range of axis-specific markers by immunofluorescence, and mapped the expression domain of reporters for the two major signalling pathways involved in axial organisation in the embryo: Wnt/β-catenin (Tcf/LEF) and Nodal (Smad2,3) signalling (Fig. 1 and fig. S1 and Materials and Methods). At 120h After Aggregation (AA) Gastruloids, which have been exposed to the Wnt signalling agonist CHI99201 (Chi) (19) between 48 and 72h AA, are clearly polarised, with localised expression of T/Bra (Fig. 1A) and Cdx2 (fig. S1A) at one end of the protruding tip. They also exhibit a shallow gradient of Wnt signalling away from the T/Bra expressing region (Fig. 1C top and insert; fig. S1C, top). The positioning of T/Bra expression suggests that this region is similar to the tail bud of an embryo supporting our previous observations that Gastruloids have AP axial organisation (20–22) where T/Bra and Cdx2 define the posterior domain. Correlating the expression of Sox2, a Sox1::GFP reporter (23) and a Nodal::YFP transcriptional reporter (24) (Fig. 1A,B, fig. S1A), we observe an additional, perpendicular axis where high levels of expression of the neural development markers Sox1 and Sox2 extend away from the T/Bra-expressing tip on one side of the Gastruloid and a tight clustering of Nodal::YFP expression directly opposite (Fig. 1A,B; fig. S1A). This second, orthogonal axis has a strong similarity with the DV axis of the early embryo, and suggests that Gastruloids are capable of generating a Node-like structure (Fig. 1C (bottom); fig. S1B,C), a conjecture supported by two observations. Firstly, the ventral expression of Nodal::YFP exhibits a weak but noticeable asymmetric expression similar to that of the Smad2,3 reporter (Fig. 1C, middle and lower; Fig. 1D, top). Secondly, the Smad2,3 activity around the putative node-like region evolves from a symmetric to a bilaterally asymmetry emerging over time (Fig. 1C, top, middle and insert and Fig. 1D). Shaking of the Gastruloids from 120h AA allows the cultures to proceed until 160h AA and reveal a more complex organisation in coherent structures over 1mm in length (Fig. 2). The AP and DV organisations are maintained and the Sox1 expressing tissue exhibits a bent morphology reminiscent of the organisation of the developing spinal cord (Fig. 2B). Inside, we observe a long tubular epithelium (Fig. 2B,C); these cells express low levels of Sox2 and high levels of Sox17 and therefore are likely to correspond to endodermal derivatives.

Taken together, these results suggest that by 120h AA, Chiron treated Gastruloids have an organisation that resembles the posterior region of the embryo and exhibit its three primary axes. The lack of Sox1 expression at the most anterior region suggests that Gastruloids lack brain or head structures and, in this sense, they are very similar to gain-of-function β-Catenin mutants (25–27), consistent with their having been exposed to high levels of Wnt signalling in the early stages of development.

2.2 Wnt/β-catenin signalling provides robustness to the polarisation of T/Bra expression

To understand the emergence of Gastruloid axial organisation, we focused on the AP axis and monitored the expression of T/Bra over time from the moment
Figure 2: Gastruloids in shaking culture develop complex interior structures reminiscent of the primitive gut. (A) Gastruloids are maintained in normal suspension culture from 0-120h with a pulse of Chi between 48 and 72h. Gastruloids are then shaken in the incubator from 120h-168h (see materials and methods). (B,C) Bright-field and immunofluorescence images of Gastruloids fixed at 144h (Bi, C) and 168h (Bii), and stained with DAPI (Blue) and Sox2 (magenta), co-expressing Sox1::GFP (Green) and T/Bra::mCherry (red). 3D reconstruction of the 168h Gastruloid reveals the spatial arrangement of Sox1, Sox2 and T/Bra as well as complex internal structures which expressing markers suggestive of a primitive gut tube.

To garner an understanding of the heterogeneities in the levels of T/Bra::GFP expression over time, we quantified the fluorescence levels of the reporter in a posterior to anterior direction along the spine of the Gastruloids (Fig. 3A; fig. S2A,B). We notice that the changes in shape and patterns of gene expression are highly reproducible and this allows us to extract quantitative information about expression and morphogenesis at single-time-points or at regular intervals over time. Exposure of the Gastruloids to Chi during 48 and 72h AA results in a tighter distribution of all the measured variables and a higher level of sustained fluorescence than when they are exposed to DMSO (the standard deviation is indicated by the light blue shading about the mean of fluorescence; Fig. 3B,C; fig. S2C). Stimulation with recombinant Wnt3a is able to substitute for Chi and results in less variability in the fluorescence signal and a more rapid acquisition of an elongated morphology (Fig. 3B,C; fig. S2D).

The quantitative analysis allowed us to classify the morphology of the Gastruloids in an objective manner through the use of two-dimensional moment invariants, Hu Moments (29) in place of the previously acquired shape descriptors. This technique provides the assessment and recognition of two-dimensional geometrical patterns that are independent to the orientation, rotation, size and position (29).
Figure 3: Wnt/β-Catenin signalling stabilises and enhances spontaneous symmetry-breaking and polarisation events in Gastruloids. (A) Expression of T/Bra::GFP in Gastruloids at 24 and 48h prior to the Chi pulse (left), and examples (i-iv) of Gastruloids following a DMSO or Chi pulse. Chi stimulation increases the robustness of the response and reproducibility of the phenotype. Experimental design and the AP orientation of the Gastruloids indicated (top right). (B) Quantification of T/Bra::GFP reporter expression in individual Gastruloids over time from one replicate experiment following DMSO, Chi or Wnt3A treatment as indicated. The maximum length of each Gastruloid is rescaled to 1 unit and the fluorescence is normalised to the maximum fluorescence from the Chi condition. Wnt3A condition taken from a different experiment (indicated by hashed horizontal line). Vertical line in each plot marks the peak max and the corresponding coordinates demote the position of this value. (C) Heat maps indicating the average fluorescence (fluorescence norm.), the average area taken up by the standard deviation (StDev Area), average length and the roundness (an indication of Gastruloid deformation from spherical) of the Gastruloids in the indicated conditions and time-points from the traces in Fig. 3B (Refer to fig. S2 and Materials and Methods for further details). (D) PCA of the Hu Moments of the Gastruloids in B and C. (E) Live imaging of one representative Gastruloid subjected to a pulse of DMSO (top) or Chi (bottom) between 48 and 72h AA. The length of the Gastruloid is indicated by the ordinate (posterior = 0µm), time on the abscissa and the fluorescence intensity of the reporter in colour. Early time-points (24-72h AA) imaged with a higher power objective. (F) PCA of the Hu Moments of a single Gastruloid in DMSO or Chi with examples of the shapes at the indicated time-points. Time is indicated by the heat-map.
Gastruloids do not express genes associated with extraembryonic tissues and progressively activate posterior markers. (A) Quantitative RT-PCR (qPCR) analysis of Gastruloids at 24, 48 and 72h AA for genes associated with the Epiblast, Extraembryonic tissues or those expressed in both tissues. (B) Examples of Gastruloids expressing reporter genes for the Wnt/β-Catenin (TLC2), Nodal (Smad2,3) and BMP (Smad1,5,7) pathways treated as indicated. No BMP signalling is observed under normal conditions (48-72h Chi), but the reporter is responsive to exogenously applied BMP. (C) Gastruloids up-regulate the expression of the GATA6::H2B-Venus reporter at ∼88h and maintain the expression in the non-elongating (anterior) region over time.

Nodal::YFP Gastruloids treated with Chi, Wnt3a or Nodal (with their corresponding vehicle controls) were determined following binary image processing of their bright-field channel and analysed by principal component analysis (PCA; Fig. 3D). Spherical Gastruloids from all conditions were found to accumulate tightly, without much variation, and almost all control and Nodal treated Gastruloids were found in this region (Fig. 3D). Upon treatment with Chi or Wnt3a, Gastruloids developed a degree of elongation which could be well separated with the first principal component (PC1). The second principal component was able to distinguish between Chi and Wnt-treated Gastruloids. We also noticed a correlation between PC3 and the elongation potential (Fig. 3D). Using a Sox17::GFP line (30) which reveals endodermal progenitors, we observe that at 120h, Sox17 expressing cells localise anterior to the T/Bra expression domain following the Chi pulse (fig. S3) in the elongating Gastruloid, probably creating the progenitors of the tube that can be observed at 160h AA (Fig. 2C).

Live imaging of the T/Bra::GFP reporter throughout the process confirms that Chi enhances an intrinsically polarised expression but also reveals a global transient response to the Chi pulse throughout the Gastruloid which relaxes to the original position after the pulse (Fig. 3E; supplemental movie M1,2). The gradual change in morphology of a single Gastruloid in either DMSO or Chi conditions can be captured by PCA of the Hu Moments (Fig. 3F). The two conditions can be clearly distinguished: DMSO treatment clusters all time-points to one region, whereas over time, Chi treated Gastruloids have a well defined trajectory through out the plot (Fig. 3F).

Taken together, these results suggest that Gastruloids have an intrinsic symmetry breaking ability that is reflected in the expression of T/Bra and made robust and stable by Wnt/β-catenin signalling.

2.3 Extraembryonic tissues are not required for Axial organisation in Gastruloids

In the embryo, the spatial restriction of T/Bra expression is concomitant with the establishment of the AP axis and the onset of gastrulation at the posterior end of the embryo (31). Genetic analysis has shown that this pattern arises from interactions between signalling systems symmetrically deployed in the extraembryonic tissues: while the embryo expresses Nodal and, as gastrulation begins, Wnt3 and later Wnt3a, the extraembryonic tissues express and secrete BMP (trophoectoderm) and antagonists for Nodal, Wnt and BMP signalling (visceral endoderm) (7). Interactions between these proteins within the epiblast result in the localisation of T/Bra expression to the
Figure 5: Nodal signalling absolutely required for T/Bra induction and correct patterning. (A) Gastruloids stimulated with Chi, SB43, Chi + SB43 or Nodal alone between 48 and 72h AA, or subjected to either vehicle or SB43 pre-treatment (24-48h AA) prior to a Chi, Nodal or Chi+Nodal pulse (48-72h AA). Normalised fluorescence traces shown per condition with the average (blue line) and standard deviation (light blue shading) of Gastruloid fluorescence shown following the indicated treatment regimes. (B) The average fluorescence, area of Standard deviation, length and roundness are represented as heat maps (right side). SB43 treatment blocks the expression of Bra::GFP and cannot be rescued by Chi co-stimulation. Inhibition of Nodal signalling has a positive influence on axial length and elongation morphology suggesting that Nodal modulates axial extension (refer to fig. S4 and S5 for further details).
proximal-posterior region of the embryo.

To determine the mechanism whereby Gastruloids are patterned along the AP axis and compare the process with that taking place in embryos, we first analyzed the expression of several genes involved in the AP patterning at 48h AA, when we first observe signs of polarisation in gene expression (Fig. 4A). At this stage, Gastruloids expressed Fgf4, Fgf5, Axin2, Wnt3, Nodal, and Cripto, all of which are expressed in the epiblast in the embryo (Fig. 4A). We also detect low levels of Lefty1 (Fig. 4A), which in the embryo is expressed mainly in the extraembryonic tissues but also in the epiblast as gastrulation begins. On the other hand, we do not detect expression of genes associated with extraembryonic tissues e.g. BMP4, Dkk, Furin, Lrp5 and Dab, with very low levels of Cerberus (Fig. 4A).

By 72h AA in N2B27 we observed increases in expression of Nodal, Lefty1 and Fgf5, decreases in Fgf4 and the emergence, at low levels, of Wnt3a (Fig. 4A). Some of these patterns are Wnt/β-Catenin signalling dependent as exposure to Chi during 48 to 72h AA leads to a clear increase in Nodal, Lefty1 and Wnt3a as well as of the Wnt/β-catenin targets Axin2, Dkk and Cripto.

These observations support the original contention that Gastruloids are made up exclusively of embryonic cells. This conclusion is reinforced by the absence of BMP expression or signalling (Fig. 4B, right), and also by the lack of GATA6 expression during the first 72h of culture (Fig. 4C), which in the early embryo is associated with the Visceral Endoderm.

The patterns of gene expression at different times AA, together with the timing of the cell behaviours associated with gastrulation that we have described before (14–16, 18), provide landmarks to correlate the development of the Gastruloids with that of embryos. They suggest that 48h AA corresponds to the onset of gastrulation in the E6.0 embryo and 72h AA is an approximation to the E7.0.

2.4 Nodal signalling promotes T/Bra expression

The expression of signalling reporters suggests that by 48h AA, Gastruloids are being patterned through an intrinsic mechanism which relies on Nodal and Wnt signalling (Fig. 3, 4B). To gain insights into this requirement and probe the relationship between the two pathways, we exposed Gastruloids to agonists and antagonists of both signalling pathways before or at the time of exposure to Chi. Treatment with the Nodal ALK4 receptor inhibitor SB431542 (SB43) (32) between 48-72h AA in the absence of Chi abolished both the expression of T/Bra::GFP and the elongation, with Gastruloids remaining essentially spherical (Fig. 5, fig. S4). Co-treatment with Chi and SB43 (48-72h) severely reduces the levels of fluorescence and greatly impacts the ability of the Gastruloids to elongate in a typical manner, with a large degree of variation between experimental replicates (Fig. 5, fig. S4). These results suggest an absolute requirement for Nodal signalling in the expression of T/Bra. To identify a temporal element to this requirement, we pre-treated Gastruloids with SB43 between 24 and 48h before pulsing them with Chi (48-72h). These Gastruloids are delayed in expressing T/Bra::GFP and the levels show higher degree of variation in terms of the location and expression of T/Bra within individuals; however their ability to elongate is not affected and occasionally enhanced relative to the Chi control (Fig. 5, fig. S4). These results confirm a requirement for Nodal in the expression of T/Bra and suggest that it is possible to separate the axial elongation from T/Bra expression.

Addition of Nodal alone or with Chiron during 48 and 72h AA results in an increase in T/Bra expression, however the elongation is severely reduced with respect to Chi alone, with Gastruloids tending to remain spheroid or ovoid (Fig. 5, fig. S4). This suggests a synergy between the two signalling events. To test this further we tried to rescue the effects of Nodal inhibition on T/Bra expression during 24 and 48hrs. Gastruloids treated with SB43 during 24-48hAA followed by Chi and Nodal co-stimulation between 48 and 72h AA, show enhanced levels of fluorescence compared to Chi and Nodal co-stimulation alone (Fig. 5, fig. S5). Additionally, the increased elongation that was observed with SB43 (24-48h) to Chi (48-72h) treatment is suppressed in this condition, indicating that increased Nodal signalling at this period negatively impacts the elongation, similar to single Nodal stimulation (48-72h; Fig. 5, fig. S5). These results demonstrate an absolute requirement for Nodal signalling in the expression of T/Bra and its requirement for precise modulation in its levels at specific phases for the elongation. Furthermore, they suggest a negative impact of Nodal signalling on axial elongation.

2.5 Wnt signalling promotes T/Bra expression and axial elongation in Gastruloids

To test the role of Wnt signalling in the patterning process, Gastruloids were treated in different regimes with either recombinant Wnt3a or its antagonist Dkk1, as well as with small molecule inhibitors of Wnt signalling (IWP3 that affects secretion of all Wnt proteins (33) and XAV939 that increases β-Catenin degradation through Tankyrase inhibition (34); Fig. 6). As demonstrated above, Wnt3a is able to substitute more than adequately for Chi during the 48-72h AA period and reduces the fluorescence heterogeneity between individual Gastruloids (Figs. 3B,C, 6A,B). Pre-treatment with Wnt3a prior to a pulse of Chi enhanced the expression of T/Bra::GFP, reduced expression heterogeneity and generated an elongated phenotype more rapidly than controls (Fig. 6). By contrast, pre-treatment with Dkk1, XAV939 or IWP3 before Chi exposure results in a delayed and variable expression of T/Bra (Fig. 6 and fig. S6, S7); however we observe differences in the response to Dkk1 and IWP3, which target Wnt expression and receptor binding, compared to
Figure 6: Wnt/β-Catenin inhibition delays but does not inhibit Bra::GFP expression. Bra::GRP Gastruloids stimulated with a pulse of Chi (48-72h AA) following pre-treatment with vehicle IWP3, XAV939, DKK or Wnt3a. Fluorescence traces (A) and heatmaps of the data (B) are shown. Blocking secretion of Wnt proteins with IWP3 effectively abolishes Bra::GFP expression until 96h AA, whereby highly heterogeneous expression is observed. Interestingly, the pulse of Chi can partially rescue Bra::GFP expression at 72h following XAV939 pre-treatment, indicating the requirement for wnt protein secretion in maintenance of expression. Wnt3a pre-treatment reduces the heterogeneity of the response, better defines the pole of expression and maintains high Bra expression for longer than controls (refer to fig. S6 and S7 for further details).

XAV939, that targets active β-catenin (Fig. 6, and fig. S6, S7). This suggests a requirement for non-canonical Wnt signalling in T/Bra::GFP maintenance as reductions in Wnt expression (IWP3) or receptor interaction (Dkk1) have a more dramatic effect than reductions in β-Catenin activity (XAV939) (Fig. 6). These results reveal that Wnt signalling is essential and the primary signal required for the elongation of Gastruloids but that it cooperates with Nodal in the control of T/Bra expression and polarisation.

A synergy between Nodal and Wnt signalling during axial organisation has been reported in other organisms (35–37) and is supported by our results which, in addition, suggest different roles for each signalling system. Nodal is essential for the onset of T/Bra expression and Wnt/β-catenin signalling does provide amplification and robustness to the response and also promotes Nodal expression in a positive feedback for the process. Our results also demonstrate a requirement for tight temporal regulation of Nodal signalling to allow full elongations to occur.

2.6 Wnt/β-catenin can generate multiple axes in a Nodal dependent manner

To further delimit the requirements for Wnt/β-Catenin signalling, we exposed aggregates for 24h at different periods from 24, 42, 48, 52 and 72 h AA and analysed elongation and T/Bra expression (Fig. 7; DAT and AMA: manuscript in preparation). The experiments reveal that the 48-72h period is critical for both the elongation and correct patterning of the Gastruloids. While in all cases there is localised T/Bra::GFP expression and tissue elongation, exposure during the 48-72h period is the most effective (Fig. 7A,B). In the course of these experiments we observed that exposures to Wnt signalling between 24-72 h AA, led to
Figure 7: Wnt/β-Catenin signalling between 48-72h AA is essential for the correct position and expression of T/Bra. (A) Examples of the morphology and expression of T/Bra::GFP Gastruloids stimulated with Chi between 24-72h (top), 48-72h (middle) and 48-96h (bottom) AA and (B) the corresponding fluorescence and shape-descriptor quantification. (C) PCA of the Hu Moments of the Gastruloids. Multiple poles of expression and stunted elongations are observed when Chi is applied between 24-72h AA whereas longer, later stimulation (48-96h) results wider Gastruloids and less well defined T/Bra::GFP expression, compared with the 48-72h control.

Together these results reveal two overlapping events centred around the 48h AA time which we have mapped to approximately E6.0 in the embryo. In the 24-48h period there is autonomous axial organisation from within the Gastruloid which is stabilised through Wnt/β-catenin signalling but critically dependent on Nodal signalling. Following this period (after 48h), it is essential that Nodal signalling is tightly regulated as it negatively impacts the elongation potential of the Gastruloid and long exposures abolish elongation without altering the expression localisation of T/Bra::GFP. This highlights the period between 24 and 48h as critical for axial establishment, which is then consolidated in the period after 48h AA.

2.7 A polarised source of Nodal signalling is not required for Gastruloid patterning

Exposure of Gastruloids to Nodal during 48-72h AA does not lead to overall expression of T/Bra suggesting that, like Wnt signalling, a localised source of Nodal may not be required for its effect. We tested this hypothesis using Nodal mutant mESCs (1) (Fig. 8).

When aggregated in standard conditions and grown in N2B27 supplemented with the appropriate vehicle controls, Nodal mutant Gastruloids remain spherical or ovoid, exhibit a number of protrusions and by 120h AA a large proportion (~90%) have developed small bulbous structures at varying locations (Fig. 8A,B). These Gastruloids resemble those treated with SB43 and confirm the absolute requirement for Nodal in symmetry breaking. We then attempted to rescue this Gastruloids with various signalling regimes. Addition of Nodal (24-48h AA) reduces the frequency of protrusions but the number is not significantly different from the control (Fig. 8B). Treatment with Chi (48-72h) leads to an increase in the proportion of elongated Gastruloids (~50%) supporting a role for Wnt signalling in elongation (Fig. 8A). However the average number of protrusions was similar to controls, with some showing four or more protrusions; the size of the protrusions was also increased relative to the control, but not statistically significant (Fig. 8B). Application of Nodal (24-48h) followed by Chi (48-72h) drastically increased the proportion of Gastruloids displaying an elongated, non-protrusion phenotype (0 to 50%) and the number of protrusions was greatly reduced, but not eliminated, compared with the Vehicle to Chi and Vehicle to DMSO controls. Immunofluorescence revealed that Nodal mutant Gastruloids treated with Chi were unable to up-regulate the posterior markers T/Bra and Cdx2 compared with previous observations (Fig. 1). However, addition of Nodal prior to the Chi pulse rescued the patterning and location of the reporters (Fig. 8C).

To assess whether the timing and duration of Nodal addition are important for the rescue of the Nodal mutant phenotype, Nodal was applied between 48-72,
Figure 8: Tight temporal regulation of Nodal signalling is required for axial elongation and proper Axial patterning. (A) Examples of Nodal-/- Gastruloids pulsed with either DMSO or Chi (48-72h AA) following a pulse of the vehicle or 100ng/ml recombinant Nodal (24-48h) and (B) the quantification of morphology (pie charts, right). In the absence of Nodal signalling, a number of protrusions are evident which are suppressed by Nodal pre-stimulation, and Chi stimulation enhances an elongated phenotype but does not suppress protrusions. The wild-type phenotype can be rescued if Chi treated Gastruloids have been previously exposed to Nodal. Addition of Nodal at different time-points is not able to rescue the elongations (left and fig. S8). (C) Quantification of the number of protrusions observed in each experimental condition. Significance determined following Mann-Whitney U test followed by Bonferroni adjustment, comparing selected columns. (D) Immunofluorescence of Nodal-/- Gastruloids pulsed with Chi (48-72h) following pre-treatment with vehicle or 100 ng/ml Nodal (24-48h) and stained at 120h with Hoechst (blue), Brachyury (Green) and CDX2 (Red). Nodal addition rescues axial patterning. Later addition of Nodal has less of an effect on the patterning (see fig. S8).

In summary, a localised region of Nodal expression is not required for Gastruloid patterning. However, a tight control of Nodal signalling prior to the Chi pulse is necessary to rescue the Nodal mutants in terms of morphology and AP axis patterning.

3 Discussion

We find that Gastruloids, mouse embryonic organoids, develop an embryo-like axial organisation in the absence of external patterned influences over a period of five days in culture. Significantly they organise themselves in the absence of extraembryonic tissues, which have been shown to drive axial organisation during embryogenesis. This observation leads us to suggest that in vivo, the role of the extraembryonic tissues might not be to induce axial organisation but rather to bias an intrinsically driven symmetry breaking event similar to the one we report here. The deployment of signalling centres around the embryo thus provides a robust source of spatial information that positions the onset of gastrulation in a defined and reproducible location. This ensures that the first cells to exit the primitive streak can easily access the extraembryonic ectoderm, which lies adjacent to them, to form the allantois and primitive blood. If the symmetry breaking was stochastic it would be difficult to link gastrulation to the interactions with extraembryonic tissues. Our suggestion is supported by the observation that in the absence of extraembryonic signals, the embryo still exhibits a degree of patterning and axial organisation, though somewhat variable.

A most important consequence of the symmetry breaking event in the embryo is the polarised onset of T/Bra expression that will define the initiation of gastrulation and, later, lead to axial elongation. In Gastruloids this is recapitulated through interactions between Nodal and Wnt signalling that promote the expression and localisation of T/Bra expression between 24 and 48h AA. The activity of signalling reporters in neutral culture medium suggests that ligands for these signalling pathways are expressed in the differentiating cells, something confirmed by RNA expression. Thus the early patterning of the ESC aggre-
gate is driven by intrinsic Wnt and Nodal signalling but its stabilisation requires high levels of Wnt signalling between 48 and 72h AA that boost the expression of Nodal and T/Bra, thus providing a positive feedback for the process. An interpretation of our results is that Nodal provides the initial input on the expression of T/Bra and the organisation of an AP axis but that these effects are enhanced and consolidated by Wnt/β-catenin signalling. This possibility is supported by the observation that in the embryo T/Bra expression is initiated and localised in the absence of Wnt signalling, though this pattern is not robust (44). Similar interactions between Nodal and Wnt/β-catenin signalling have been described in chick and frog embryos (35–37) and we have also shown them to occur in an adherent culture system of Primitive Streak formation (45). At the molecular level this synergy is supported by reports of molecular interactions between Smad2,3 and β-catenin in the regulatory regions of genes expressed in the Primitive Streak and specifically of Nodal and T/Bra (46–48).

Mechanisms to explain how Nodal leads to symmetry breaking during AP axis formation often invoke Reaction-Diffusion mechanisms (49–51). Accordingly, interactions between Nodal and its inhibitor and downstream target Lefty, lead to the asymmetric localisation of both and to the asymmetric expression of target genes e.g. T/Bra. Surprisingly we observe that ubiquitous exposure to Nodal leads to polarisation of T/Bra expression in the aggregate and, moreover, that this will occur when high ubiquitous concentrations of Nodal are provided to a Nodal mutant Gastruloid (45).

This observation challenges many of our current notions about patterning driven by Nodal and demonstrates that Nodal needs not be localised to generate an axis. One possible explanation that is consistent with our results is that Nodal signalling initiates the expression of T/Bra but that it is not involved in its refinement and maintenance which depends on a positive feedback between Wnt/β-catenin signalling and T/Bra (45). Indeed, several Wnt genes are known to be downstream targets of T/Bra (52) which, in turn, is a target of Wnt/β-catenin signalling which thus provides the elements of a positive feedback loop that could be involved in the patterning and localisation of T/Bra and its downstream targets. The absence of a requirement for a localised Nodal signal is supported by the lack of a localisation of Nodal signalling and expression in the Gastruloids and also in the embryo, where at the time of gastrulation Nodal is expressed in most of the circumference on the embryo (53).

Our results also highlight that, in addition to, and independently of its role in T/Bra expression and of its interactions with Nodal/Smad2/3 signalling, Wnt/β-catenin signalling is central to axial elongation. This provides an independent proof of this well established phylogenetic relationship (54). Timing of exposure suggests two different phases to this involvement. Long exposures to Wnt signalling early (24-72h AA; E5.0-E7.0 in the embryo) can lead to multiple axes, only some of which express T/Bra; this mirrors situations with gain of function of Wnt signalling (55, 56). Increased activity later on (48-96h AA; E6.0-E8.0) results in abolition of the polarity and ubiquitous expression of T/Bra. These observations highlight two temporally separate activities of Wnt. A first one in the establishment and enhancement of the AP axis, probably together with precisely controlled Nodal signalling, followed by a second phase of stabilisation of the pattern and the elongation of the axis. As in the case of Nodal but in a more manifest manner, the observation that a localised source of Wnt/β-catenin activity is not necessary for the polarisation of T/Bra expression and the elongation of the Gastruloid, questions the widespread notion for a role of Wnt signalling gradients in pattern formation and supports views in which the function of Wnt signalling is to control the signal to noise ratio of events induced by other means (57, 58).

An important feature of the Gastruloids is the absence of anterior structures. In this regard they resemble Smad2,3 (59) or Dkk (27) mutants and show that it is possible to orientate an axis without an identifiable head or brain. A likely cause of this deficiency is a combination of the exposure to high levels of Wnt signalling which will suppress anterior development (56, 60) and the lack of a prechordal plate which is essential for neural induction (61). This observation would suggest that while signalling from the extraembryonic tissues might not be strictly necessary for the establishment of an AP axis, it might be essential not only for the reliable positioning of the initiation of gastrulation but also for the location of the brain at the opposite pole.

Over the last few years a number of experimental systems have emerged in which ESCs are spatially patterned and each of them can make a contribution to our understanding of the connection between cell fate assignments and the polarisation of the embryo (62–64). The system that we have developed has some advantages in particular, its reproducibility and robustness allow it to be used in long term studies and screens. Furthermore, the observations that we report here, particularly their ability to be cultured long term and the observation that in addition to the AP and DV axes they can develop an LR asymmetry and generate a node-like structure, suggest that they are a useful model system beyond the early stages of development.

References


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5 Materials and Methods

5.1 Cell lines and routine cell culture

AR8::mCherry (Smad2,3 reporter) (65), T/Bra::GFP (28), GATA6::H2B-Venus (66), IBRE4-TA-Cerulean (Smad1,5,7) (65), Nodal浓合体YFP (Nodal::YFP reporter) (24), Nodal−/− (67), Sox1::GFP (23), Sox1/eGFP Bra/mCherry double reporter (SBR) (68), Sox17::GFP (30) and TCF/LEF::mCherry (TLC2) (69, 70) were cultured in GMEM supplemented with LIF, foetal bovine serum, Non-Essential Amino Acids, Glutamax, Sodium Pyruvate and β-mercaptoethanol (ESL medium) on gelatinised tissue-culture flasks and passed every second day as previously described (ESL medium) on gelatinised tissue-culture flasks and passed every second day as previously described (18) and when required, images were stitched using the Pairwise Stitching plugin in FIJI (73). Widefield, single-time point images of Gastruloids were acquired using a Zeiss AxioObserver.Z1 (Carl Zeiss, UK) and data visualisation/analysis performed using Velocity. The z-stacks were acquired of at least 4 Gastruloids per condition with a z-interval of 0.5µm for a maximum of 42.2µm. Images were analysed using the ImageJ image processing package FIJI (73).

Widesfield, single-time point images of Gastruloids were acquired using a Zeiss AxioObserver.Z1 (Carl Zeiss, UK) and data visualisation/analysis performed using Velocity. The z-stacks were acquired of at least 4 Gastruloids per condition with a z-interval of 0.5µm for a maximum of 42.2µm. Images were analysed using the ImageJ image processing package FIJI (73).

5.2 Immunofluorescence, Microscopy and data analysis

Gastruloids were fixed and stained for as required according to the protocol previously described (18). Hoechst3342 was used to mark the nuclei (see table S2 for the antibodies used and their dilutions) except for the images in Fig. 2 where DAPI was used. Confocal z-stacks of Gastruloids were generated using an LSM700 (Zeiss) on a Zeiss Axiovert 200 M using a 40 EC Plan-NeoFluar 1.3 NA DIC oil-immersion objective. Hoechst3342, Alexa-488, -568 and -633 were sequentially excited with 405, 488, 555 and 639 nm diode lasers respectively as previously described (45). Data capture was carried out using Zen2010 v6 (Carl Zeiss Microscopy Ltd, Cambridge UK) and data visualisation/analysis performed using Velocity. The z-stacks were acquired of at least 4 Gastruloids per condition with a z-interval of 0.5µm for a maximum of 42.2µm. Images were analysed using the ImageJ image processing package FIJI (73).

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Fluorescence levels were normalised to the maxi-
um obtained in following Chi stimulation, and the maximum length of each Gastruloid was rescaled 1 unit. Average fluorescence traces of Gastruloids S.D. are shown in the main figures, and the raw data and individual traces in the supplemental data. For live imaging experiments, each well of a 96-well plate containing individual Gastruloids were imaged as described above using both the 20x (24-72h) and the 10x (72-96h) objectives, and images captured every 30 min for a maximum of 96h (120h AA). All images were analysed in FIJI (73) using the LOI interpolator (75) with the LOI set as described above.

Data processing, graph plotting, statistical analysis and Hu Moments analysis was performed in the Jupyter IPython notebook environment (76, 77) using the following principle modules: Matplotlib (78, 79), NumPy & SciPy (80–82), tifffile (83), Statsmodels (84) and Pandas (85). All code is freely available upon request.

5.3 Gastruloid culture and application of specific signals

Aggregates of mouse ESCs were generated using an optimised version of the previously described protocol (14, 18). Mouse ESCs harvested from tissue-culture flasks were centrifuged and washed twice in warm PBS. After the final wash, the pellet was resuspended in 3ml warm N2B27 and cell concentration determined using a Moxi™Z automated cell counter with curve-fitting (Orflo Technologies). The number of cells required to generate Gastruloids of ~150µm in diameter by 48h (optimised for each cell line, ~300 cells; table S3) was then plated in 40µl droplets of N2B27 in round-bottomed 96-well plates. Counting cells after washing in PBS in this way instead of prior to the washes (as described previously (14, 18)) results in the number of cells required for Gastruloid formation being ~100 fewer than previously described as fewer are lost during washing. See table S3 for the number of cells required for each cell line.

In experiments which required the addition of specific factors to Gastruloids on the second day of aggregation (24-48h), 20µl medium was carefully removed with a multichannel pipette, and 20µl of N2B27 containing twice the concentration of the required factors was added. This method was preferable to the addition of smaller volumes containing higher concentrations of agonist/antagonists, as the data from these experiments showed more variation between Gastruloids (DAT, PB-J, AMA unpublished). Control experiments showed that replacement of half the medium at this stage did not significantly alter the ability of Gastruloids to respond to signals on the third day (DAT, PB-J, AMA unpublished). The next day, 150µl fresh N2B27 was added to each of the wells with a multichannel pipette and left for no more than 30 min to wash the Gastruloids; a time delay ensured that sample loss was prevented. Following washing, 150µl N2B27 containing the required factors was then applied. The small molecules used in this study and their concentrations are described in table S4.

To prolong the culture period, individual Gastruloids were transferred to low-attachment 24 well plates in 700µl of fresh N2B27 at 120h and cultured on an incubator-compatible shaker for 48h at 50 rpm. 400µl of medium was replenished at 144h and Gastruloids were fixed at 160h.

5.4 Quantitative RT-PCR

Gastruloids (n = ~64 per time-point) from T/Bra::GFP mouse ESCs, subjected to a Chi or DMSO pulse (between 48 and 72h AA), harvested at 48 or 72h AA, trypsinised, pelleted and RNA extracted using the RNeasy Mini kit (Qiagen, 74104) according to the manufacturers instruction as previously described (72). Samples were normalised to the housekeeping gene PPIA. The sequences for the primers are described in table S5.

5.5 Orientation of Gastruloids

To define the AP orientation of Gastruloids, we have assigned the point of T/Bra::GFP expression as the ‘Posterior’, as the primitive streak, which forms in the posterior of embryo, is the site of T/Bra expression in the embryo (20–22).

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Supplementary Figure 1: Expression of axial markers in Gastruloids. Gastruloids from Nodal::YFP (A) and Sox1::GFP (B) mESCs stained for anti YFP (green) and either CDX2 (A) or T/Bra (B) (red) at 120h AA. 3D projection shown above each image. The TLC2 (top), Smad2,3 (middle) and Nodal::YFP (bottom) reporter at 120h (C) are shown as 120h AA. Magnified region for the Nodal reporter indicates a node-like region. (D) Stereo images of the Gastruloids from Fig.1A and B.
Supplementary Figure 2: Quantification of Gastruloid Fluorescence. Expression of the TLC2 reporter (A) and the Nodal::YFP reporter (B) after 48h in culture. (C,D) Expression of the T/Bra::GFP reporter at the indicated time-points (DMSO or Chi (C) and Chi or Wnt3a (D) stimulation) prior to length normalisation (top) and following normalisation of the length to from 0 to 1 (middle). The bottom panel shows the length and roundness of the Gastruloids in the indicated conditions.
Supplementary Figure 3: Sox17::GFP is expressed anterior to the elongating region of the Gastruloids at 120h AA. Gastruloids made from Sox17::GFP mESCs were grown in standard conditions, pulsed with Chi between 48 and 72h AA and imaged by widefield microscopy. Two examples from two replicate experiments are shown, indicating an expression domain more anterior to the elongating posterior region. The posterior of the Gastruloid is orientated towards the base of the figure. Scale bar indicates 100µm. Fluorescence levels are not directly quantitatively comparable between experimental replicates due to differences in exposure times and objectives used.
Supplementary Figure 4: Quantifying the Effect of modulating Nodal signalling in Gastruloids (#1). (A) examples of T/Bra::GFP reporter expression in Gastruloids treated as indicated. (B, C, D) Quantification of the reporter expression at the indicated time-points prior to length normalisation (B) and following normalisation of the length from 0 and 1 (C). The length and roundness of the Gastruloids in the indicated conditions (D). Vertical line and coordinates in C correspond to the location and position of the peak maximum. Scale bar indicates 100 µm.
Supplementary Figure 5: Quantifying the Effect of modulating Nodal signalling in Gastruloids (#2). (A) examples of T/Bra::GFP reporter expression in Gastruloids treated as indicated. (B, C, D) Quantification of the reporter expression prior to length normalisation (B) and following normalisation of the length from 0 and 1 (C). The length and roundness of the Gastruloids in the indicated conditions (D). Vertical line and coordinates in C correspond to the location and position of the peak maximum. Scale bar indicates 100 µm.
Supplementary Figure 6: Quantifying the Effect of modulating Wnt/β-Catenin signalling in Gastruloids (#1).
(A) examples of T/Bra::GFP reporter expression in Gastruloids treated as indicated. (B, C, D) Quantification of the reporter expression prior to length normalisation (B) and following normalisation of the length from 0 and 1 (C). The length and roundness of the Gastruloids in the indicated conditions (D). Vertical line and coordinates in C correspond to the location and position of the peak maximum. Scale bar indicates 100 µm.
Supplementary Figure 7: Quantifying the Effect of modulating Wnt/β-Catenin signalling in Gastruloids (#2). (A) examples of T/Bra::GFP reporter expression in Gastruloids treated as indicated. (B, C, D) Quantification of the reporter expression prior to length normalisation (B) and following normalisation of the length from 0 and 1 (C). The length and roundness of the Gastruloids in the indicated conditions (D). Vertical line and coordinates in C correspond to the location and position of the peak maximum. Scale bar indicates 100µm.
Supplementary Figure 8: Modulation of Nodal signalling in Nodal mutants. (A) Examples of Gastruloids treated with Chi between 48 and 72h with a 24h pulse of either vehicle or Nodal at the indicated time-points (24-48h, 48-72h, 72-96h and 24-72h AA). Pie charts indicated the proportion which do not show protrusions (no), show protrusions (yes), show protrusions with a defined AP axis (yes+APaxis) or dont show protrusions but still have a defined AP axis (no+APaxis). The schematic for the time-course is indicated on the right of the panel. (B) Quantification of the area of the protrusions in the indicated experimental conditions. Significance determined following Mann-Whitney U test followed by Bonferroni adjustment, comparing selected columns.
5.7 Supplemental Movies

**M1** T/Bra::GFP expression in *Gastruloids* following DMSO treatment (48-72h AA). *Gastruloids* made from T/Bra::GFP mESCs stimulated with a mock pulse of DMSO and imaged by wide-field microscopy from 24h to 120h AA every 20 min. The 20x objective was used between 24 and 72h, followed by the 10x objective from 72h to the end of the experiment. Quantification of both the length and fluorescence as a function of time can be seen in Fig. 3E (top).

**M2** T/Bra::GFP expression in *Gastruloids* following Chi treatment (48-72h AA). *Gastruloids* made from T/Bra::GFP mESCs stimulated with a pulse of Chi and imaged by wide-field microscopy from 24h to 120h AA every 20 min. The 20x objective was used between 24 and 72h, followed by the 10x objective from 72h to the end of the experiment. Quantification of both the length and fluorescence as a function of time can be seen in Fig. 3E (bottom).
5.8 Supplemental Tables

<table>
<thead>
<tr>
<th>Condition</th>
<th>No Expression</th>
<th>Polarised</th>
<th>Ubiquitous</th>
<th>Spherical</th>
<th>Ovoid</th>
<th>Elongated</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>24h N2B27</td>
<td>26.8 (21.5)</td>
<td>62.5 (16.1)</td>
<td>10.7 (15.2)</td>
<td>100.0 (0.0)</td>
<td>0.0 (0.0)</td>
<td>0.0 (0.0)</td>
<td>112</td>
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<tr>
<td>48h</td>
<td>23.7 (13.2)</td>
<td>74.1 (11.8)</td>
<td>2.2 (3.4)</td>
<td>67.0 (9.4)</td>
<td>33.0 (9.4)</td>
<td>0.0 (0.0)</td>
<td>140</td>
</tr>
<tr>
<td>DMSO</td>
<td>3.6 (-)</td>
<td>89.3 (-)</td>
<td>7.1 (-)</td>
<td>10.7 (-)</td>
<td>85.7 (-)</td>
<td>3.6 (-)</td>
<td>28</td>
</tr>
<tr>
<td>72h Chi</td>
<td>0 (-)</td>
<td>91.2 (11.7)</td>
<td>8.8 (11.7)</td>
<td>23.3 (18.2)</td>
<td>52.9 (18.1)</td>
<td>23.8 (26.3)</td>
<td>82</td>
</tr>
</tbody>
</table>

Table 1: Expression phenotype of T/Bra::GFP mESCs. The proportion of T/Bra::GFP Gastruloids not expressing the reporter (No Expression) or displaying either Polarised or Ubiquitous expression at 24, 48 and 72h AA followed by a pulse of DMSO or Chi (72h). The standard deviation is shown in brackets and the number of Gastruloids analysed (n) are shown.

<table>
<thead>
<tr>
<th>Species</th>
<th>Dilution</th>
<th>Cat. Number</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>Primary</td>
<td>Brachury</td>
<td>Goat 1:200 sc-17743</td>
<td>Santa Cruz Biotechnologies</td>
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<tr>
<td>Nanog</td>
<td>Mouse 1:300 14-5761-80</td>
<td>e-Biosciences</td>
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<tr>
<td>GFP</td>
<td>Rabbit 1:500 A11122</td>
<td>Molecular Probes</td>
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<tr>
<td>Secondary</td>
<td>Goat-A633 Donkey 1:500 A21082</td>
<td>Molecular Probes</td>
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<td>Mouse-A568 Donkey 1:500 A10037</td>
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<td></td>
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<tr>
<td>Rabbit-A488 Donkey 1:500 A21206</td>
<td>Molecular Probes</td>
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<td></td>
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<tr>
<td>Hoechst3342 n/a 1:1000 H3570</td>
<td>Invitrogen (ThermoFisher)</td>
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Table 2: Antibodies and their concentrations used for Gastruloid immunofluorescence with the associated supplier details.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Reference</th>
<th>Cells/40µl</th>
<th>48h Diameter (µm)</th>
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<tbody>
<tr>
<td>AR8::mCherry</td>
<td>(65)</td>
<td>450</td>
<td>182.7 ±17.3 (n = 83)</td>
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<tr>
<td>T/Bra::GFP</td>
<td>(28)</td>
<td>300</td>
<td>161.0 ±26.2 (n = 222)</td>
</tr>
<tr>
<td>GATA6::H2B-Venus</td>
<td>(66)</td>
<td>300</td>
<td>300 ± (n = 10)</td>
</tr>
<tr>
<td>IBRE4::mCerulean</td>
<td>(65)</td>
<td>400</td>
<td>152.6 ±12.2 (n = 39)</td>
</tr>
<tr>
<td>NodalcondHBE::YFP</td>
<td>(24)</td>
<td>400</td>
<td>138.7 ±16.1 (n = 124)</td>
</tr>
<tr>
<td>Nodal-/-</td>
<td>(67)</td>
<td>300</td>
<td>181.6 ±23.7 (n = 251)</td>
</tr>
<tr>
<td>Sox1::GFP</td>
<td>(23)</td>
<td>400</td>
<td>112.8 ±18.4 (n = 42)</td>
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<tr>
<td>Sox1::GFP/Bra::mCherry</td>
<td>(68)</td>
<td>300</td>
<td>N.D.</td>
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<tr>
<td>sox17::GFP</td>
<td>(30)</td>
<td>400</td>
<td>N.D.</td>
</tr>
<tr>
<td>TLC2</td>
<td>(69, 70)</td>
<td>200-300</td>
<td>194.9 ±20.7 (n = 56)</td>
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Table 3: Cell lines used and numbers of cells required for Gastruloid culture. The average diameter of the Gastruloids at 48h AA is indicated with the standard deviation and the number of Gastruloids measured. N.D.: not done
<table>
<thead>
<tr>
<th>Small Molecule/Recombinant protein</th>
<th>Ref.</th>
<th>Working Conc.</th>
<th>Stock Conc.</th>
<th>Cat. Number</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>CHI99201</td>
<td>(19)</td>
<td>3µM</td>
<td>10mM</td>
<td>4423</td>
<td>Tocris Biosciences</td>
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<tr>
<td>IWP3</td>
<td>(33)</td>
<td>1µM</td>
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<td>04-0034</td>
<td>Stemgent</td>
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<tr>
<td>SB431542</td>
<td>(32)</td>
<td>10µM</td>
<td>100mM</td>
<td>1614</td>
<td>Tocris Biosciences</td>
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<tr>
<td>XAV939</td>
<td>(34)</td>
<td>1µM</td>
<td>10mM</td>
<td>HY-15147</td>
<td>MedChem Express</td>
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<td>BMP4</td>
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<td>1ng/ml</td>
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<td>DKK</td>
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<td>200ng/ml</td>
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<td>Nodal</td>
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<td>1µg/ml</td>
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<tr>
<td>Wnt3a</td>
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<td>100ng/ml</td>
<td>40µg/ml</td>
<td>1324-WN-002</td>
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Table 4: Concentrations of Small molecules and recombinant proteins used in this study. Conc: Concentration

<table>
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<tr>
<th>Gene</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
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<tr>
<td>Axin2</td>
<td>CTAGACTACGGCCATCAGGAA</td>
<td>GCTGGCAGACAGGACATACA</td>
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<tr>
<td>Bmp4</td>
<td>CTCAGGGGATGCAGGAGTGG</td>
<td>ATGCTGGCGACTACGTTTGG</td>
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<tr>
<td>Cer1</td>
<td>GCCAACGCCATAGTCTCCA</td>
<td>AGGGCTGAAATTGCCCATTG</td>
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<td>Chordin</td>
<td>GTGCCCTGCTGGCTGGCTT</td>
<td>AGGAGTTCGCGATGATATGG</td>
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<td>Dkk1</td>
<td>CCATTTGCCGCCAACCTTCTTC</td>
<td>CATTCCCCTCCCTCCAATAAC</td>
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<td>Fgf4</td>
<td>GGGCACCTCAGAGATAGAG</td>
<td>ACTTGCGCTCAAGCAGTAGG</td>
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<td>Fgf5</td>
<td>GCTCAATGATCAGAAGAGAGA</td>
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<td>Fgf8</td>
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<td>Lefty1</td>
<td>AGGGTGACAGATGACTTG</td>
<td>GGAAGCAAGAGCAGACACACA</td>
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<td>Nodal</td>
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<td>GTGCTGGCAAAGCATACATTC</td>
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<tr>
<td>Noggin</td>
<td>CCCATCATTTGCGACTGTAAG</td>
<td>CTCGCTAGAACTGTCACCTGAA</td>
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<tr>
<td>ppiA</td>
<td>TTACCATCAAACCATTTCTTG</td>
<td>AACCCAAAAGAATTCAGTAGACG</td>
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<tr>
<td>SPRY4</td>
<td>ATGGGTGGAGATTCAGG</td>
<td>GGAGGGGGAGCTACAGAGAC</td>
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<td>T/Bra</td>
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<td>Wnt3</td>
<td>CTAATGCTGGCTTGCAGAG</td>
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<tr>
<td>Wnt3a</td>
<td>CATAAGCAGTGTGCTGCTGGA</td>
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Table 5: Primer Sequences used for qRT-PCR.