Endodermal Patterning in *Xenopus laevis*

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Preface

This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others except as specified in the text and acknowledgements.

The present dissertation is not substantially the same as any other submitted for qualification at any other University.

This thesis is divided into 5 chapters, contains 18 figures and 6 tables. It also complies with the Biology Degree Committee specifications.

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First of all, to the ones who know me better! Obrigado, a meu pai e minha mãe. My heart is with you in all moments. They understand me better than anyone, and have always supported me.

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Thank you, for helping me with comments and moments.

Here, I am left with two pages and not even half-done! Friend, thank you…

Yours,

Those who fail to plan
Plan to fail…

George Hewell

"Caminhante não há caminho, o caminho faz-se ao andar"

Antonio Machado

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List of Abbreviations

MBT – Mid Blastula Transition

cDNA – Complementary DNA (Nucleic Acid)

EST – Expressed sequence tag

FGF – Fibroblast Growth Factor

WMISH – Whole mount *in situ* hybridisation

ISH - *in situ* hybridisation

HMG – High Mobility Group (Transcription factor domain)

Dpf – Days post fertilization

DLP – Dorsal Lateral Plate

VBI – Ventral Blood Island

P-Sp – para-aortic splanchnopleura

AGM – aorta-gonad-mesonephros

Shh – Sonic Hedgehog

PCR – Polymerase Chain Reaction

Please note:

st = Stage

Nieuwkoop and Faber staging of *Xenopus laevis* is used throughout the text

Hlxbox8 = *Pdx1*

Abstract

The endoderm is the inner germ layer of the vertebrate embryo from which the respiratory and digestive systems are derived. These include organs such as the liver, pancreas, stomach, lungs and intestine. Recent research has helped our understanding of early vertebrate endoderm specification and terminal differentiation of specific endodermal lineages. However, very little is known about the molecular mechanisms that control endoderm patterning and morphogenesis during vertebrate development.

As a way to identify genes involved in these elusive steps of development I performed a differential hybridisation screen on a macroarray tailbud ventral midgut cDNA library coupled with *in situ* hybridisation analysis. My aim was to identify and characterise new regionally expressed endodermal genes in *Xenopus laevis*, a classic embryological model organism.

Here, I report the identification and characterisation of a dozen novel regionally expressed endoderm genes. At tailbud stages their expression patterns fall into three re-occurring domains, anterior ventral midgut endoderm, posterior endoderm and dorsal endoderm. In addition, regional expression of some of these genes is observable at gastrula stages, during endoderm specification. These are the first early stable endodermal markers for different regions of the gastrula endoderm. This suggests that the earliest steps in endoderm patterning are concurrent with endoderm specification.

Furthermore, I describe the identification of a mesodermal transcription factor, which appears to be expressed in early embryonic macrophages – a poorly characterised embryonic cell population.

I present an overview of endoderm development together with the results from my screen. Overall, these results reveal an unexpected degree of early endodermal patterning and assist our understanding of the link between early and late events of vertebrate endoderm development. In addition, this work provides us with new and very useful markers for the study of endodermal patterning, but also perhaps some key developmental regulators of endodermal formation.

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CHAPTER ONE – ENDODERM DEVELOPMENT

0.Outline

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0. Outline

When an embryo gastrulates, three germ layers are established, ectoderm, mesoderm and endoderm. The ectoderm will give rise to the nervous system and the epidermis. The mesoderm will become muscle, skeleton, kidney and blood. The endoderm will give rise to the epithelial lining of the gut and contribute to internal organs associated with the digestive tract and the respiratory system. In this chapter an overview of endoderm development is presented, focusing on specification, patterning and organogenesis.

Many molecules known to take part in endoderm development have conserved roles across phyla and orthologous genes are required for similar steps of development. The early steps of endoderm development are well known in *Xenopus*; however, the following developmental steps are not so well characterized. Using *Xenopus*, I hope to contribute to the understanding of subsequent steps of vertebrate endodermal patterning. The work presented here sheds light on new molecules and their possible functional relevance in endoderm patterning.

Xenopus is a classical embryological model organism used to study vertebrate development. It combines external embryonic development and well-documented embryology with many molecular biology tools that have made possible many breakthrough discoveries in the mechanisms of embryonic development.

To find out more about how the endoderm is patterned, I started a screen for genes that are differentially expressed within the endoderm. The goal was to identify novel endodermal patterning markers, their characterisation and possibly the identification of novel endoderm development regulators. The criterion used to look for such genes was to identify differentially expressed genes in the endoderm of a tailbud *Xenopus* embryo when patterning is believed to occur. Differential screens are also discussed in this introductory chapter.

1.Overview of Endoderm Development

Endoderm development is less understood than the development of other germ layers. The endoderm is defined as the innermost metazoan germ layer giving rise to the gut and associated organs. The respiratory and digestive systems derive from the endoderm and are fundamental for organism survival. Non-vertebrate endoderm development has been reviewed elsewhere (Stainier, 2002). Vertebrate endoderm formation has been studied mainly in mouse (*Mus musculus*), zebrafish (*Danio rerio*), chick (*Gallus gallus*) and frog (*Xenopus laevis*) (Dale, 1999; Grapin-Botton and Melton, 2000; Shivdasani, 2002; Stainier, 2002; Wells and Melton, 1999). Although the anatomy, morphology and morphogenesis in early steps of endoderm development varies among different organisms, the molecular players that control endoderm development share homologies and seem to be regulated in similar ways (Grapin-Botton and Melton, 2000) (Figure 1.1). Thus, a cross species comparison of endoderm development remains useful and necessary, in order to assist our knowledge on endoderm development.

Vertebrate endoderm development can be described in five continuous and overlapping stages: 1) specification or formation of the endoderm; 2) patterning of the pluripotent endoderm; 3) the induction and specification of organ specific lineages; 4) the commitment of endodermal cells to a specific fate; 5) and finally, the terminal cellular differentiation coupled with organ morphogenesis. The main aim of this thesis is to bridge the current gap between the understanding of endoderm specification and terminal differentiation. For that purpose, I have searched for differentially expressed genes within the endoderm during the period endodermal patterning must occur.

Endoderm Fate Maps

Fate maps are necessary tools to understand cells developmental history. Fate maps describe what cells will eventually differentiate into during normal development, and they present a topographic projection of later developmental fate in earlier embryonic stages. Due to their large embryo size and external development, *Xenopus* have well characterized embryonic fate maps (Dale and Slack, 1987; Moody, 1987a; Moody, 1987b). Unlike *C. elegans, Xenopus* shares with other animals a non-deterministic fate map. That is, we cannot predict exactly what a cell will become, but we can ascribe one or several prospective fates to the progeny of that cell, and this is possible due to a reproducible projection of fate from very early embryonic stages.

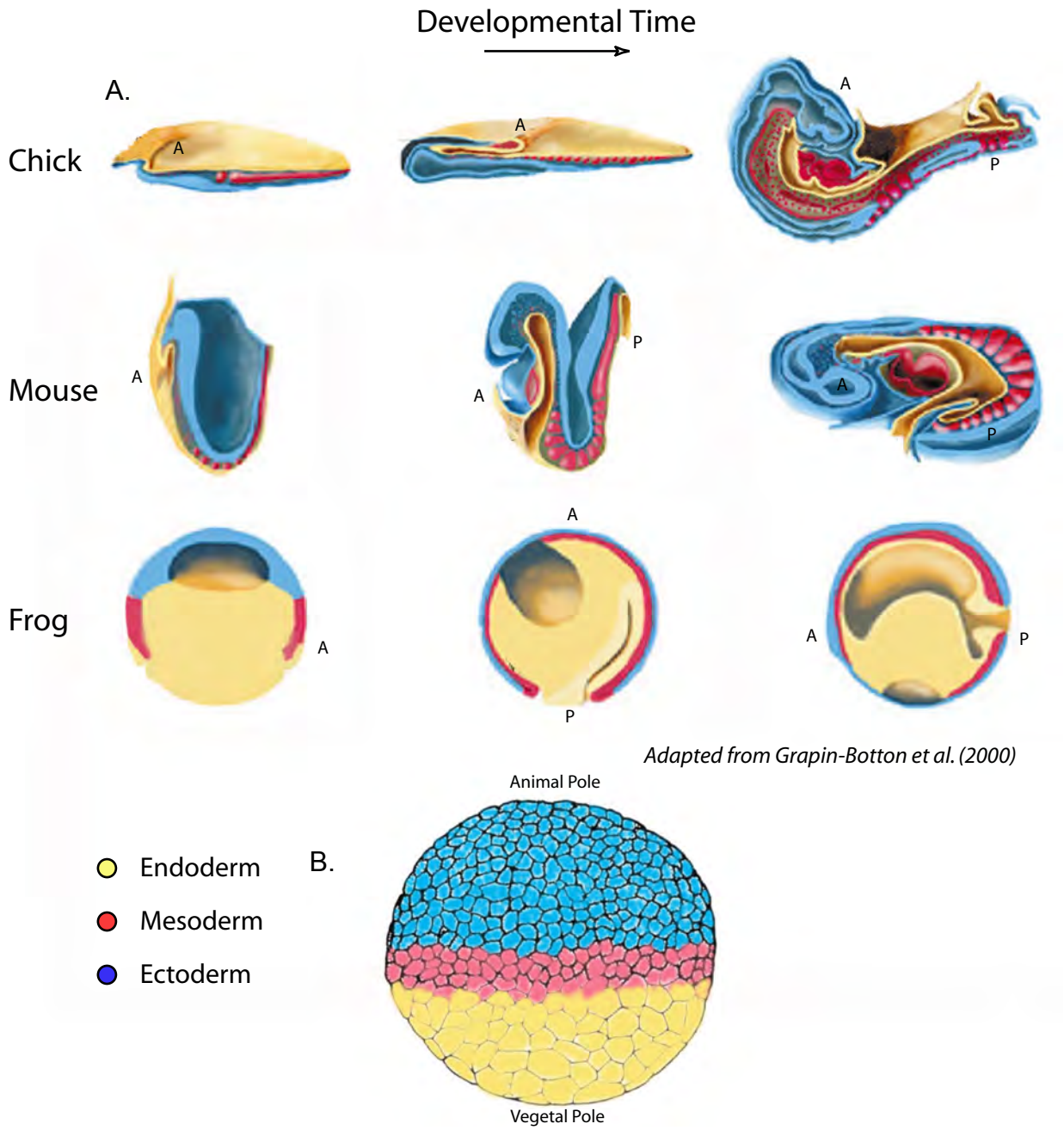


Figure 1.1 - Vertebrate Endoderm Development. **A.**) Early developmental stages of chick, mouse and frog. The different germ layers are color coded. Although gastrulation movements vary, the result is very similar, with several tissues organized in the anterior-posterior, dorso-ventral, and right-left axes. In *Xenopus*, a blastula, gastrula and neurula stage embryo are shown. **B.**) Prospective ectodermal, mesodermal and endodermal areas of the *Xenopus* blastula. A=Anterior, P=Posterior.

60% of the volume of a 32-cell *Xenopus laevis* embryo will become endoderm (Dale and Slack, 1987). However, the fate of endodermal cells is difficult to follow since, after gastrulation, the endoderm is internalized in a non-transparent embryo. Fate maps show that all blastomeres at 32-cell stage can contribute to all germ layers, but there is topographic projection of the animal-vegetal axis onto future germ layers. The vegetal most blastomeres are fated to become endoderm, the animal ones will become ectoderm, and mesoderm will form in between (Figure 1.1). Moreover, dorsal 32-cell blastomeres will form more anterior tailbud tissues and ventral more posterior tissues (Dale and Slack, 1987; Moody, 1987b). Epiboly and gastrulation morphogenetic movements account for the differential distribution of 32-cell blastomere progeny along the tailbud anterior-posterior axis (Bauer *et al.*, 1994; Keller, 1991; Keller *et al.*, 2003; Keller, 1975; Keller, 1976; Keller *et al.*, 1985; Winklbauer and Schurfeld, 1999). Using these fate maps, one can follow the fate of bottle cells, or see how dorsal gastrula blastomeres give rise to the anterior endoderm. Keller fate maps, in particular the one of the endoderm deep layer, are invaluable to understand the formation of the archenteron, the archenteron roof originating from dorsal cells, and the archenteron floor originating from ventral endoderm cells (Keller, 1976) (Figure 1.2).

Recently, fate maps that project early neurula endoderm and mesoderm into pre-feeding late tadpole were constructed (Chalmers, 1999; Chalmers and Slack, 2000). Such fate maps reveal which parts of the neurula endoderm, and the associated mesoderm, gives rise to the feeding tadpole internal organs. *Xenopus* endoderm was fluorescently labelled, explanted into same stage host embryos, and fate mapped to gut coiling stages (Figure 1.3). Roughly, the neurula endoderm can be divided into three parts in the anterior to posterior direction. The most anterior third is fated to become pharynx, brancheal derivatives, trachea, lungs, liver, gall bladder, pancreas and bile duct. Indeed, a great diversity of organs originates from a small cell population. The middle third will give rise to the pancreatic buds, stomach and the proximal (most anterior) small intestine, together with the external coil of the small intestine. The posterior third of the neurula endoderm is fated to become the large intestine. It is interesting to notice that neurula dorsal endoderm will contribute to more anterior organs of the gut than neurula ventral endoderm, in agreement with earlier fate maps.

Development of the gut endoderm is closely associated with the development of lateral plate mesoderm. Is important to know when the two tissues come into alignment. At neurula, the endoderm fate map does not coincide with the lateral plate mesoderm fate map (stage 14) (Chalmers and Slack, 2000). However, they later come into alignment, the mesodermal and endodermal contributions to gut tube coincide by stage 23 (Muller *et al.*, 2003). No endoderm

patterning molecular markers existed to analyse the interactions between the mesoderm and endoderm at these stages (stage 14 to 23).

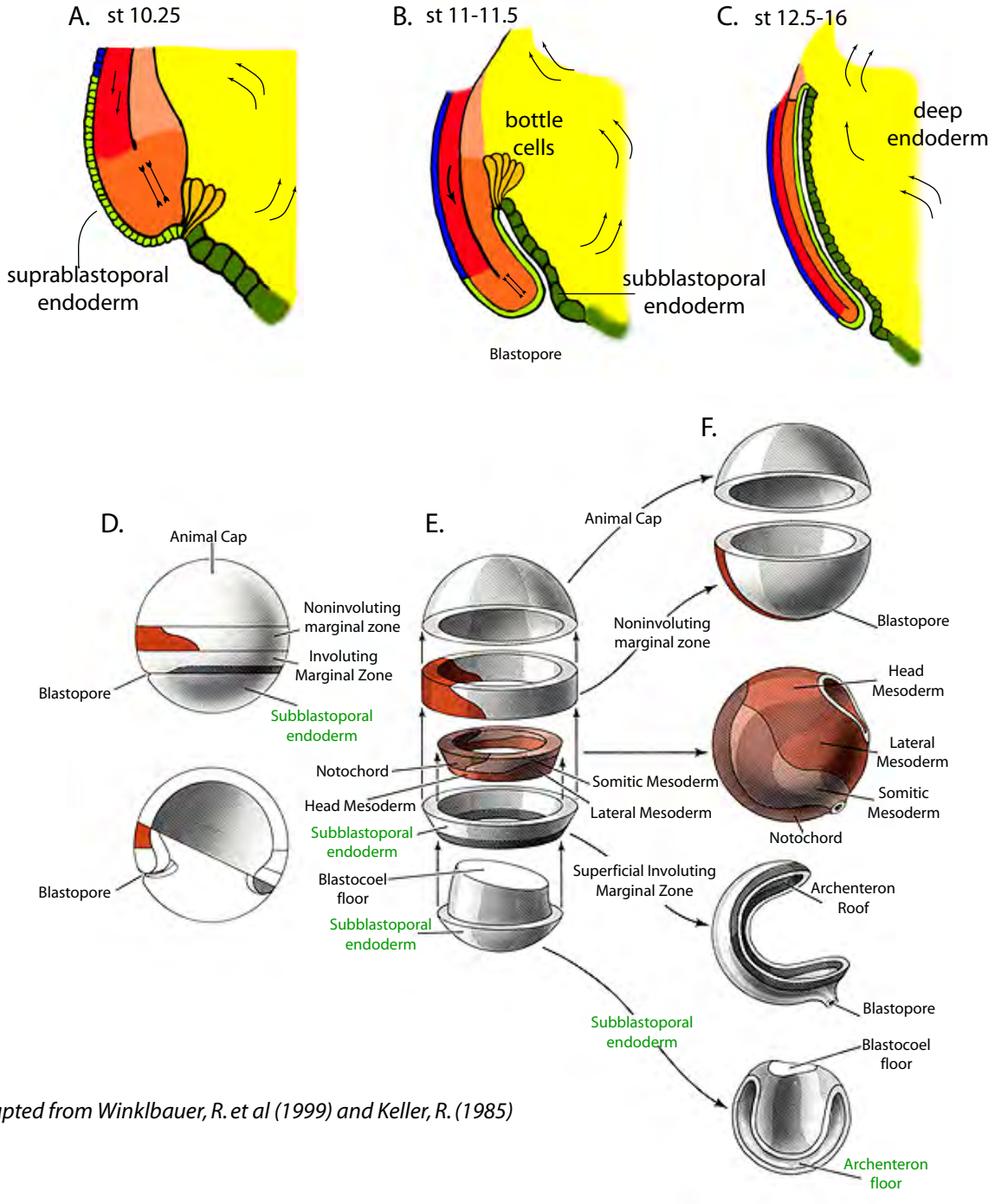
Fate maps are invaluable to our understanding of vertebrate development. *Xenopus,* more than any other vertebrate, has thorough fate maps that project the fate of cells of the early blastula into gut coiling stages, contributing to our knowledge of endoderm cell developmental history, from very early blastula up to a functional gut tube.

Endodermal Cell Types and Behaviours

The gastrula endoderm already contains four distinguishable types of endodermal cells. These include the bottle cells, the large yolky cells, the suprablastoporal endoderm cells and the subblastoporal endoderm cells. The two latter ones are part of an epithelium. Bottle cells are the first differentiated cell type visible in the endoderm, and are specialized motile cells. Bottle cells form around the ring of the blastopore lip and migrate in front of the archenteron. They appear at the onset of blastopore formation and can be identified by the pigment contraction on their apical surface (Figure 1.2). The suprablastoporal endoderm possesses organizer properties, and it is able to induce cell behaviours necessary for gastrulation (Shih and Keller, 1992). The suprablastoporal endoderm will give rise to the roof of the archenteron and the subblastoporal endoderm will give rise to the archenteron floor (Keller, 1975) (Figure 1.2).

The large yolky endodermal cells constitute the remaining endodermal tissue. Amongst them, the cells in the dorsal-anterior endoderm present distinct cellular behaviour and express *Hex* and *Cerberus* (Zorn *et al.*, 1999). The dorsal most endoderm is the first tissue to show the vegetal rotation movements characteristic of gastrulation, during which these cells are pushed upwards forming the involuting leading edge (Winklbauer and Schurfeld, 1999). This tissue will migrate along the dorsal midline to become the most anterior endoderm that gives rise to the foregut. Because of the internal nature of the endoderm, very little is known, of the endoderm morphogenetic movements or the cell differentiation that occurs past these stages (Keller *et al.*, 2003).

Already within the gastrula endoderm one can find different cell types and specific behaviours, and it is not a naïve tissue. Which on itself is indicative of some degree of patterning. Its development past these early gastrulation stages is largely uncharacterized.



Adapted from Winklbauer, R. et al (1999) and Keller, R. (1985)

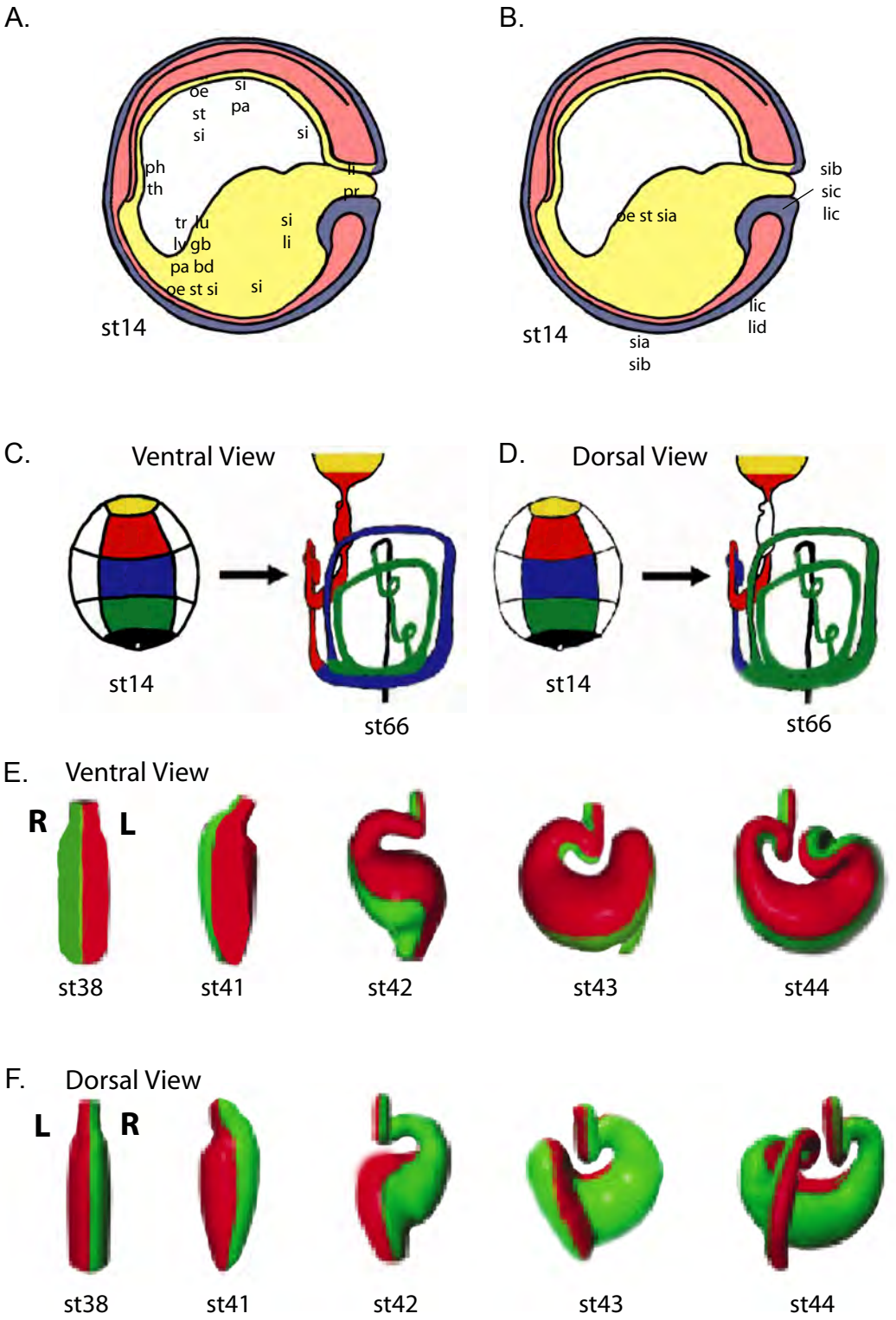
Figure 1.2 - *Xenopus* gastrulation, endodermal cell types and behaviors. **A to C**) Endodermal cell movements in the gastrula. Mid-sagital section of gastrulation embryos in close up of the blastopore region. Ectoderm in blue, mesoderm in red and orange and endoderm in yellow or green. Bottle cells in pale orange. Bulk cell movements are illustrated by arrows, convergent extension is illustrated by inverted end-arrows. The stages shown are Nieuwkoop and Faber. **D to F**) Illustration of the gastrulation movements. **D**) *Xenopus* blastula fate maps, **E**) "Exploded" blastula and, **F**) Movements of the germ layers. One can follow through these maps the development of every tissue in the embryo, at this stage *Xenopus* has very good fate maps when compared with other vertebrates.

Endoderm Morphogenesis

During *Xenopus* gastrulation and neurulation, the mesoderm and ectoderm exhibit distinguishable morphology and cellular behaviours (Keller *et al.*, 2003). In contrast, endoderm morphogenetic movements are much less understood, but they must occur. At this stage, we only know that the endoderm is responsible for vegetal rotation movements necessary for proper gastrulation, and that the endoderm is likely to account for the elongation of the embryo at tailbud stages (Gerhart and Keller, 1986; Keller, 1991; Keller, 1975; Keller, 1976; Keller *et al.*, 1985; Winklbauer and Schurfeld, 1999).

In amniotes, the gut tube is formed by the migration of both the anterior and posterior intestine portal, and organs generally develop by budding from the main tube (Bellairs, 1998; Grapin-Botton and Melton, 2000; Kaufman, 1999; Wells and Melton, 1999). In *Xenopus*, a gut tube has to be formed from a flat layer of endodermal cells (the archenteron roof) and a bulky mass of endodermal cells with several cell diameters (from the archenteron floor to the ventral side). A model was proposed for the morphogenesis of the central part of the gut tube. The large yolky cells are incorporated into the future gut epithelium by radial intercalation, while the archenteron narrows. However, in the middle part of the embryo, the archenteron reopens forming the gut lumen (Chalmers and Slack, 2000). A recent study in zebrafish, reveals that the anterior endodermal organs are assembled from individual anlagen (Wallace and Pack, 2003; Warga and Nusslein-Volhard, 1999). However, bud formation is always the first morphological manifestation of a patterned endoderm, which is only visible in late tadpoles (*Xenopus*), long after patterning and organ induction has occurred.

After gut tube formation, the next developmental step is the coiling of the gut. At this stage, the mesenchyme associates closely with the epithelia of the forming gut tube, and the nodal pathway is involved in asymmetric organ development (Branford *et al.*, 2000; Dagle *et al.*, 2003), but it is not known how these affect the coiling of the gut. The *Xenopus* gut coiling movements from 3 to 7 day tadpoles have been described in detail and a system has been devised to classify abnormalities (Branford *et al.*, 2000; Chalmers and Slack, 1998) (Figure 1.3). Left-right asymmetric gut coiling and organ formation has been followed from stage 23 to 45 and it was found that both right and left sides of the embryo contribute equally to organ formation (Muller *et al.*, 2003). In zebrafish gut coiling is directed by lateral plate mesoderm, but it is only a matter of speculation if *Xenopus* uses similar processes (Horne-Badovinac *et al.*, 2003).



Adapted from Chalmers et al (2000) and Muller et al (2003)

Figure 1.3 - Endoderm Fate Maps and the Coiling of the Gut. **A**) Neurula stage 14 endoderm fate map and, **B**) Neurula stage 14 mesoderm fate map (mid-sagital sections). **C**) Ventral and, **D**) Dorsal projections of stage 14 endodermal regions of the tadpole gut. Regions are color coded. **E**) Ventral and, **F**) Dorsal view of the gut coiling movements from stage 38 onwards . On a ventral view, rotation in the coiling of the gut is always in an anti-clockwise direction. ph, pharynx; tn, tongue; tr, trachea; lu, lungs; lv, liver; gb, gall bladder; pa, pancreas; bd, bile duct; oe, oesophagus; st, stomach; si, small intestine; li, large intestine; (mesoderm) sia, proximal small intestine; sib, external coil of small intestine; sic, internal coil of small intestine; lic, internal coil of the large intestine; lid, distal large intestine; pr, proctodeum. Anterior to the top in C to F, anterior to the left in A and B. R is right, L is left.

2. Endoderm Specification

During development cells restrict their developmental potential. It is known that vegetal pole cells can contribute to all germ layers before mid-blastula. However, a stage 10 single labelled vegetal pole blastomere transplanted into the blastocoel roof of another embryo is already committed to become endoderm. Blastomeres isolated before stage 10, demonstrate greater potency than their normal fate. That means prospective endodermal cells are not committed to become endoderm before the beginning of gastrulation (stage 10). The process of commitment to form endodermal tissues is progressive; prospective endodermal cells lose first the capacity to become ectoderm, and then the capacity to form mesoderm (Wylie *et al.*, 1987). Classical experiments also tell us that explant cultures of vegetal pole cells will give rise to poorly differentiated endoderm-like tissues, while at the same stage of development, prospective mesoderm and ectoderm will not differentiate (Nieuwkoop, 1997). This data reveals a degree of determination in the prospective endodermal cells. In addition, in the absence of an organizer – as in UV treated embryos where cortical rotation is inhibited – the resulting embryos develop as "belly" pieces, while there is no mesodermal or ectodermal differentiation.

In summary, endoderm is specified at the beginning of gastrulation, and it has been recognized that urodelean and anuran blastulas and early gastrulas endoderm have higher differentiation potential than same stage prospective mesoderm and ectoderm (Nieuwkoop, 1969). However, we do not know if cells in the gastrula endoderm have some degree of positional information, if it is stable or not, and at what point does the endoderm obtain that pattern and by what means.

A multi-step model of *Xenopus* endoderm specification is currently accepted. The first step is dependent on the maternal determinant, *VegT*. The second step, activated by *VegT*, is dependent on a TGF-β type signal, and is believed to be nodal (Agius *et al.*, 2000; Yasuo and Lemaire, 1999). Nodal signalling is central to the pathway of endoderm specification in all vertebrates examined so far (Schier, 2003; Schier and Shen, 2000). Intriguingly, this pathway does not appear to be important in the formation of invertebrate endoderm. Although, it is likely that other signalling pathways are also involved in vertebrate endoderm specification, there is very little data support this view (Stainier, 2002). The nodal signal is responsible for the maintenance of the expression and action of many other factors necessary for endoderm development, as for example, *Sox17* (Clements and Woodland, 2003) (Figure 1.4)

In *Xenopus*, *VegT* initiates endoderm specification. *VegT* initiates the expression of several *Xnr's*. (Xanthos *et al.*, 2002; Xanthos *et al.*, 2001). No functional homologs of *VegT*

are described for other model organisms. However, in the mouse nodal induction in the epiblast is dependent on another T-box gene, *eomesodermin,* which is expressed in the extra embryonic ectoderm (Brennan *et al.*, 2001; Russ *et al.*, 2000; Stainier, 2002). In zebrafish, the onset of the nodal signalling cascade is believed to originate from the maternal deposited nodal, *squint* (Feldman *et al.*, 1998), since the known homolog of *VegT*, *spadetail,* does not seem to regulate nodal or *Sox17* expression (Griffin *et al.*, 1998). Therefore, the initial activation of endoderm specification pathway upstream of nodal signalling does not seem to be conserved among vertebrates. So far, one *nodal* gene has been identified in the mouse (Conlon *et al.*, 1994), two in Zebrafish (*squint* and *cyclops*) (Feldman *et al.*, 1998) and five mesendodermal inducing nodals have been identified in *Xenopus* (*Xnr1, Xnr2, Xnr4, Xnr5* and *Xnr6*) (Jones *et al.*, 1995; Onuma *et al.*, 2002; Takahashi *et al.*, 2000), all of which have been implicated in endoderm specification.

Nodal signalling affects directly or indirectly four families of transcription factors known to be essential for endoderm development (Shivdasani, 2002; Stainier, 2002). These are: 1) the paired-class homeobox Mix type factors; 2) the high mobility group proteins (HMG), *Sox17* and *Casanova*; 3) the zinc finger transcription factors, *Gata4/5/6* and, 4) the forkhead transcription factors, of the FoxA family (*foxA1, foxA2*, and *foxA3*). The epistatic relations between them have not been worked out in detail for all vertebrates studied (Figure 1.4). Endoderm specification is associated with a choice between becoming endoderm or mesoderm (Kimelman and Griffin, 2000; Warga and Nusslein-Volhard, 1999; Wells and Melton, 1999). Nodal is both required for the formation of endoderm and mesoderm. Currently, it is believed that the choice to follow an endodermal fate is assured by high levels of nodal signalling, and the action of several transcription factors downstream of nodal pathway; such as *Mixer* (Henry and Melton, 1998), *Sox17*, or *Gata5* (Alexander and Stainier, 1999; Aoki *et al.*, 2002; Reiter *et al.*, 1999; Reiter *et al.*, 2001; Shivdasani, 2002; Stainier, 2002; Tam *et al.*, 2003; Weber *et al.*, 2000; Xanthos *et al.*, 2001) (Figure 1.4).

VegT – Activation of the Endoderm Specification Pathway

VegT was identified by several independent groups attempting to find genes involved in mesodermal and endodermal formation (Horb and Thomsen, 1997; Lustig *et al.*, 1996; Stennard *et al.*, 1996; Zhang and King, 1996). VegT is a T-box transcription factor. Two isoforms of VegT exist, a maternal and a zygotic (Stennard *et al.*, 1999). Maternal *VegT* is deposited in vegetal pole of oocytes at a cortical location (Horb and Thomsen, 1997; Zhang *et*

al., 1998). Zygotic transcription of *VegT* can be induced by TGF-β signals all around the marginal zone in the future mesoderm.

The maternal *VegT* isoform is necessary for endoderm specification (Xanthos *et al.*, 2001; Zhang and King, 1996). Depletion of *VegT* function has been achieved using antisense oligos and by a dominant negative approach. In *VegT* depleted oocytes, endoderm does not form, and the mesoderm now forms at the vegetal pole (Xanthos *et al.*, 2001). *VegT* is at the hierarchical top of the endoderm specification pathway. *VegT* is required for the expression of *Xnr's* and several other endoderm specific genes necessary for the acquisition of the endodermal fate; these include *Bix1, Bix2, Bix4, Milk, Mixer, Mix.1, Mix.2, Gata4, Gata5, Gata6, Endodermin, Xlim-1, Hex, Cerberus* and *Sox17* (Xanthos *et al.*, 2001). The VegT-Engrailed (VegT:En) dominant negative form of VegT causes a less severe phenotype. Ventral vegetal injections of VegT:En impair the development of anterior mesodermal structures. Dorsal vegetal injections inhibit the development of posterior mesodermal derivatives (Horb and Thomsen, 1997), revealing a role of VegT, as a likely secondary effect, in controlling mesodermal patterning.

In summary, in amphibians, *VegT* initiates both a cell autonomous and a cell non-autonomous pathway of endodermal specification. *VegT* initiates the expression of many transcription factors required for endoderm formation. At the same time, *VegT* induces the expression of *Xnr's*, which is the signalling component necessary for the maintenance of the endodermal fate. *VegT* mRNA injections rescue *VegT* depleted embryos as do nodal related genes. Dominant negative form of *Xnr2* blocks the ability of *VegT* mRNA to rescue *VegT* depleted embryos, demonstrating the necessity of intercellular signalling for endoderm specification. The pathway that starts with *VegT* rapidly becomes extremely complex due to the number endodermal transcription factors induced by *VegT* and the interactions among them.

TGF-β Signalling in Endoderm Development

Nodals are a TGF-β type ligand (Transforming Growth Factor). The TGF-β signalling pathway is used during embryonic development and adult tissue homeostasis (Massague and Chen, 2000; Shi and Massague, 2003). Upon extra cellular ligand binding to the type II receptor, the type I receptor is recruited. The type II membrane receptor is then able to phosphorylate the type I receptor rendering it active. The active complex phosphorylates intracellular Smads, the effectors of the TGF-β pathway. Upon activation, Smads bind co-

Smad proteins and other cofactors, and specifically activate their transcriptional targets. The family of TGF-β signalling molecules includes activins, antivins, TGF-β's, BMP's and Nodals. Activin was the first TGF-β ligand shown to be able to induce endoderm and mesoderm (Smith *et al.*, 1990), and several other TGF-β signalling members can act as mesendoderm inducers, including *activinB, Xnr1, Xnr2, Xnr4, Xnr5, Xnr6, derrière* and *bVg1* (Harland and Gerhart, 1997). Loss of a functional TGF-β pathway can be achieved using of a dominant negative receptor (Hemmati-Brivanlou and Melton, 1992), in which embryos do not develop endoderm or mesoderm. These results implicate TGF-β signalling in the establishment of the mesendodermal fate (Hemmati-Brivanlou and Melton, 1992). In summary, endoderm only forms if a TGF-β signalling pathway is functional.

Nodal requirement for endoderm formation was discovered in mouse *nodal* null embryos, which do not form definitive endoderm, primitive streak and have very few mesodermal cells (Conlon *et al.*, 1994). *Nodal* signalling has many other functions in the embryo such as: correct positioning of the anterior-posterior axis; midline patterning; mesendodermal induction; left-right asymmetric development and; development of the vasculature, lungs and stomach (Brennan *et al.*, 2001; Lowe *et al.*, 2001). Nodal signalling is central for endoderm development of the vertebrate embryo, as shown in mouse, fish and frogs (Schier, 2003; Schier and Shen, 2000; Whitman, 2001). *Smad2* is believed to be the intracellular transducer of *nodal* signalling and *Smad2* null embryos do not form definitive endoderm (Waldrip *et al.*, 1998). Moreover, graded *nodal* signalling governs cell fate decisions within the mouse organizer (Varlet *et al.*, 1997; Vincent *et al.*, 2003). This is achieved through genes like Mixer/Bon and FoxH1/Sur, which regulate the outcome of *nodal* signalling (Kunwar *et al.*, 2003). Nodals are the TGF-β ligands necessary to endoderm specification, and their levels influence the fate choice between endoderm and mesoderm.

In the frog, the nodal related genes *Xnr1, Xnr2, Xnr4, Xnr5 and Xnr6* are the functional homologs of *nodal*. *Xnr's* role in mesendodermal induction and patterning was first established with the use of cleavage mutants of *Xnr2,* which inhibit the formation of mesendodermal derivatives (Osada and Wright, 1999). More recently, *Xnr5* and *Xnr6* were established as having the inductive properties of the Nieuwkoop centre (Takahashi *et al.*, 2000), and it has been shown that the several *Xnr's* have overlapping roles in the regulation of mesendodermal formation (Onuma *et al.*, 2002). *Xnr's* functions can be inhibited either with nodal cleavage mutants or with inhibitors such as *Cerberus.* In brief, *Xnr's* are believed to be responsible for the induction and maintenance of the endoderm by inducing the expression of

genes necessary for endoderm formation (Agius *et al.*, 2000; Clements *et al.*, 1999; Jones *et al.*, 1995; Yasuo and Lemaire, 1999).

Endodermal Transcription Factors

Endoderm specification and maintenance requires the function of several genes. Some are exclusively expressed in the endoderm, but others are also expressed in other tissues. In *Xenopus,* the following endodermal regulators are known, *Sox17α/β* (Hudson *et al.*, 1997), *Mixer* (Henry and Melton, 1998), *Mix.1* (Rosa, 1989), *Mix.2* (Vize, 1996), *Milk* (*Bix.2*) (Ecochard *et al.*, 1998), *Gata4/5/6* (Patient and McGhee, 2002), and *Bix1/3/4* (Casey *et al.*, 1999; Saka *et al.*, 2000; Tada *et al.*, 1998). All are downstream of *VegT* and expressed in the prospective endodermal tissue (Xanthos *et al.*, 2001), both in *Xenopus laevis* and in *Xenopus tropicalis* (D'Souza *et al.*, 2003).

Mix-like Homeobox Genes

Mixer contains a homeobox motif and it is required for endoderm specification (Henry and Melton, 1998). *Mixer* induces endodermal fates at the expense of mesoderm. Over expression of *Mixer* induces the endodermal markers in a concentration dependent manner, while injection of a dominant negative fusion protein Mixer:Engrailed blocks endoderm development. *Mixer* is transiently expressed at the beginning of gastrulation, when vegetal pole cells become committed to the endodermal fate, and is speculated to be involved in the establishment of the boundary between mesoderm and the endoderm. *Sox17β* is inducible by *Mixer* but the reverse is not true (Henry and Melton, 1998). In zebrafish, the *Mixer* related homeobox gene *mezzo* and *bonnie and clyde* are also involved in endoderm specification (Kikuchi *et al.*, 2000; Poulain and Lepage, 2002).

The Mix genes, *Mix.1* and *Mix.2*, are related to *Mixer* and also contain a homeobox domain. Their expression becomes restricted to the endoderm (Rosa, 1989; Vize, 1996). The VegT cascade regulates the expression of both genes. However, *Mix.1* is not able to induce endoderm. Instead, over expression of *Mix.1* suppresses mesoderm formation, while inhibition of *Mix.1* results in the ectopic expression of *Xbra,* a mesodermal marker. It seems that the Mix genes are involved in the suppression of mesodermal fates. *Mix.2* is activated by P-Smad2&4, effectors of the TGF-β pathway, when associated with the forkhead gene FoxH1 (Carlsson and Mahlapuu, 2002).

The Bix gene family, *Bix1*, *Bix2* (*Milk*), *Bix3* and *Bix4*, was identified in a screen for targets of T-box genes (Tada *et al.*, 1998). The Bix gene family, like *Mixer*, induce endoderm

specific genes. All the Bix genes, except *Bix3*, are direct transcriptional targets of *VegT* and act cell autonomously in the establishment of endoderm (Casey *et al.*, 1999; Ecochard *et al.*, 1998; Tada *et al.*, 1998). Furthermore, nodal signalling regulates all Bix genes.

Sox17 (HMG Proteins)

Sox17 and Casanova are two HMG proteins necessary for vertebrate endoderm formation. *Sox17α/β* was initially identified in *Xenopus*. So far, *Casanova* has only been identified in zebrafish. Zebrafish *Casanova* is directly upstream of *Sox17,* being both necessary and sufficient for *Sox17* regulation (Kikuchi *et al.*, 2001). *Sox17* is expressed exclusively in the gastrula prospective endoderm, and regulates the expression of several endoderm genes. In *Xenopus*, *VegT* and nodals affect *Sox17* expression and its activity. Over expression of *Sox17* diverts cells towards an endodermal fate, and loss-of-function studies reveal that *Sox17* is required for endoderm formation (Clements *et al.*, 2003; Clements and Woodland, 2000; Engleka *et al.*, 2001; Hudson *et al.*, 1997). $Sox17^{-/-}$ (null) mice form very little or no definitive endoderm, with its developmental replacement by extra embryonic tissues (Kanai-Azuma *et al.*, 2002; Tam *et al.*, 2003). Recently, *Sox17* was found to interact with β-catenin to regulate the transcription of many genes necessary to the specification of the endoderm (Sinner *et al.*, 2004).

Gata Transcription Factors

Unlike nodals, which seem to be only present in vertebrates, zinc finger transcription factors, *Gata4/5/6* seem to be widely conserved throughout evolution in the specification of the endomesodermal fate. The sea urchin *Gata-E* is responsible for the activation of a complex hierarchy of genes involved in endoderm formation in sea urchin (Davidson *et al.*, 2002). In higher organisms, GATA factors are also involved in other developmental processes such as blood and heart development (Patient and McGhee, 2002). Genetic evidence of the involvement of GATA factors in the pathway of endoderm formation came from the analysis of the *Gata5/faust* zebrafish mutant. This mutant presents abnormalities in the morphogenesis of the heart, gut and associated gut organs (Patient and McGhee, 2002; Reiter *et al.*, 1999; Reiter *et al.*, 2001; Stainier, 2002; Weber *et al.*, 2000). *Gata5/Faust* is also upstream of *Casanova* in the endoderm specification pathway (Figure 1.4). In *Xenopus, Gata4/5/6* are expressed in the endoderm, and *Gata4/5* have been shown to induce the expression of genes necessary for endoderm formation and under the influence of a TGF-β signal (Shoichet *et al.*, 2000; Weber *et al.*, 2000).

Forkhead Genes

The *foxA* family members, *foxA1/A2/A3,* are also involved in endoderm formation, apart from their other functions (Kaestner *et al.*, 2000). GATA and Forkhead transcription factors have been shown to cooperate in the regulation of the transcription of *albumin in vivo*, a liver specific gene (Bossard and Zaret, 1998; Gualdi *et al.*, 1996). *HNF3β/axial/foxA2* is a forkhead gene essential for endoderm and primitive streak formation in the mouse (Ang and Rossant, 1994; Ang *et al.*, 1993; Dufort *et al.*, 1998; Levinson-Dushnik and Benvenisty, 1997; Wu *et al.*, 1997). All mice FoxA null have an endodermal phenotype (Carlsson and Mahlapuu, 2002). In *Xenopus,* the forkhead genes, *Foxa1* and *Foxa2,* are direct transcriptional targets of *Sox17* and β-catenin and necessary to endoderm formation (Sinner *et al.*, 2004).

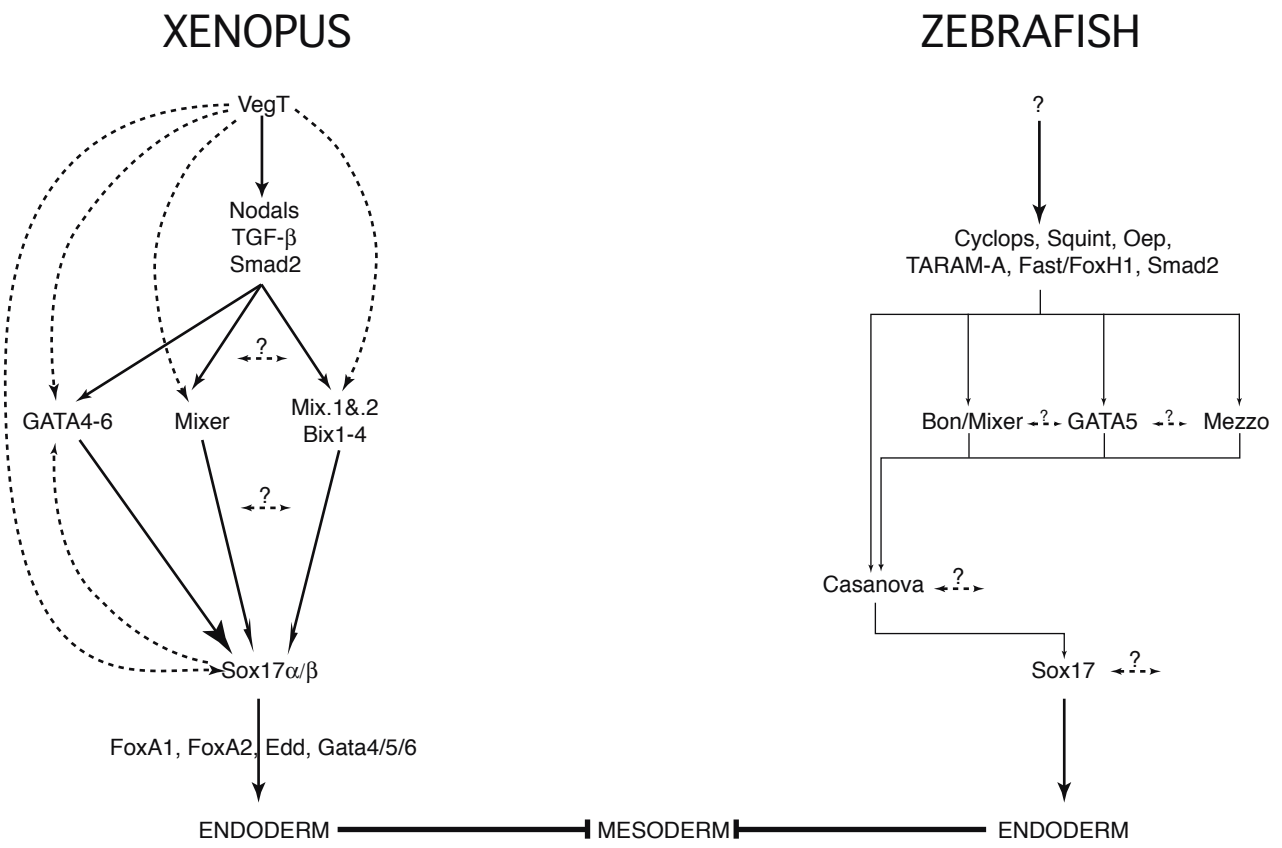
Other Signalling Pathways

Very little data is published on how signalling pathways, such as the Wnt's, Notch-Delta, FGF's, BMP's or Retinoic Acid, affect endoderm development. The organizer is known to control several of these pathways (De Robertis *et al.*, 2000; Harland and Gerhart, 1997), and appears to have a poorly characterized endodermal inducing activity (Sasai *et al.*, 1996). In addition, BMP's, Wnt's and FGF's are active in the endoderm at early gastrula stages, since their activities can be detected by *P-Smad1*, *β-catenin* and phosphorylated *MAPK* (Schohl and Fagotto, 2002).

Extra cellular BMP antagonists, *chordin* and *noggin,* are known to drive dorsalization of the mesoderm and neuralization of the ectoderm. Interestingly, *Xenopus* animal caps can follow endodermal differentiation, tested by the expression of *endodermin*, if co-injected in BMP antagonists and FGF dominant negative receptor (Sasai *et al.*, 1996). Recently, BMP signalling has been implicated in the patterning of the Zebrafish endoderm, and the control of the expression of *her5*. *Her5* on the other hand controls the number forerunner cells, the most anterior mesendodermal cells (Tiso *et al.*, 2002).

In frogs, it has been known that FGF inhibition down regulates the expression of *Pdx1* a pancreatic marker (Gamer and Wright, 1995; Henry *et al.*, 1996). FGF signalling also affects the expression of gut specific epitope marker 4G6 (Jones, 1993). Furthermore, FGF factors have been shown to induce posterior endodermal differentiation in 7.5dpc mice (Wells and Melton, 1999; Wells and Melton, 2000).

However, these reports are only scarce references to the influence of several signalling pathways in endoderm development. Wnt, BMP, and FGF signalling is very likely to influence the specification and patterning of endodermal cells. However, up to now, the paucity of early regional endodermal markers delayed the study of endodermal specification and patterning.



Adapted from Xanthos et al., (2001); Shivdasani, R. (2002) and Poulain et al., (2002)

Figure 1.4 - Models of Endoderm Specification Pathway in *Xenopus* and zebrafish. Question marks indicate the likelihood of unknown players. These models are very likely incomplete. In zebrafish, some epistatic relations are established, however, those are not resolved in *Xenopus.* In *Xenopus,* the endoderm specification pathway starts with *VegT*, which directly or indirectly afects the expression of many the downstream players. Orthologous genes are shown at the same level in the pathways.

3. Endoderm Patterning

In order to attain fully functional digestive and respiratory systems, the next developmental step is the patterning of the pluripotent endoderm. The future gut tube, made of the epithelium derived from the endoderm and the mesenchyme (splanchnic mesoderm), has to be patterned to allow the differentiation of the many different cell types along the tube, as well as the organogenesis of the auxiliary organs. Vertebrate endodermal regionalization is sometimes defined as a reversible commitment step, of a sub-region to a specific fate (Horb and Slack, 2001). I will discuss patterning or regionalization in a broader sense, i.e., as the acquisition of different properties in a sub region of an otherwise homogeneous tissue. Such patterning can be observed by the existence of compartments and boundaries, by the appearance of different morphologies, by the existence of domains of signalling activity, by domains of gene expression or by differences in the potency, competence*, or commitment of a specific group of cells or tissue.

Compartments and boundaries are well characterized in *Drosophila* development (Dahmann and Basler, 1999; Irvine and Rauskolb, 2001). Morphological boundaries have been described in the gut of the feeding *Xenopus* tadpole, and are characterized in chick (Roberts *et al.*, 1998; Roberts, 2000; Roberts *et al.*, 1995). However, sharp differences between different organ epithelia appear at late stages of development. The existent 32-cell fate map also reveals one endodermal boundary, which is not frequently mentioned in the literature. This boundary lies, in the words of the authors, in the cleavage lines between the progeny of the most vegetal tier (Dale and Slack, 1987). But at present, boundaries or compartments have not been well characterized in vertebrate endoderm development

An obvious suspect responsible for endoderm patterning is the organizer tissue. Transplantation of organizer tissue induces a complete secondary axis containing differentiated endoderm, mesoderm and ectoderm. (Gimlich, 1985; Gimlich and Gerhart, 1984; Gimlich and Gerhart, 1986; Nieuwkoop, 1969; Nieuwkoop, 1973; Nieuwkoop, 1977; Nieuwkoop, 1997). The amphibian organizer is itself divided in head and trunk organizer (Bouwmeester and Leyns, 1997), and is a source of many signalling molecules and developmental regulators (De Robertis *et al.*, 2000; Harland and Gerhart, 1997), and therefore likely to be involved in endodermal patterning. However, it is not known how the organizer influences the patterning of the endoderm.

* Ability of the cell or tissue to follow a differentiation pathway, and not others.

Early Endodermal Patterning

In the *Xenopus* gastrula several genes are regionally expressed in the anterior endoderm. These are; *Hex, Cerberus, Dickkof (dkk1),* and *Frzb* (Bouwmeester *et al.*, 1996; Kazanskaya *et al.*, 2000; Leyns *et al.*, 1997; Newman *et al.*, 1997; Sasai *et al.*, 1994; Wang *et al.*, 1997b). This dorso-anterior endoderm tissue will form the leading edge of the migrating endoderm, and will become the foregut. Many of these genes have identified signalling roles, and their localized expression is indicative of an endodermal pattern. However, they are not good patterning markers because their expression is transient.

Although *VegT* is expressed equally throughout the vegetal mass, several of *VegT* downstream targets such as *Hex, Cerberus* or *Xlim1* show asymmetric expression in the anterior endodermal region (Taira *et al.*, 1992; Xanthos *et al.*, 2001). Although, this suggests that *VegT* does not regulate their asymmetric expression, the differential gene expression indicates some degree of endodermal patterning when specification is still occurring.

At the end of zebrafish gastrulation, comparison between the expression of *axial* and *Sox17*, show differential gene expression among the anterior and the posterior endodermal cells. *Axial* is only expressed in the anterior endodermal two-thirds, while *Sox17* is expressed throughout. Furthermore, the expression of *her5* at 30% epiboly is restricted to the endmost mesendoderm, which shows that some degree of patterning is already molecularly visible while endoderm specification is still occurring (Alexander and Stainier, 1999; Bally-Cuif *et al.*, 2000). Hence, it seems that the patterning of the endoderm is concomitant with the maintenance of the endodermal fate.

Furthermore, the *Hex* and *Cerberus* expressing anterior mesendoderm is patterned at the same time and by the same signals that specify the organizer, TGF-β and β-catenin (Xanthos *et al.*, 2002; Zorn *et al.*, 1999).

Another form of generating a pattern is the active displacement of a maternal component. *XBic-C* is deposited as a maternal animal to vegetal mRNA gradient that is displaced towards the dorsal side of the embryo after fertilization. *XBic-C* is capable of inducing endoderm without inducing mesoderm. To date, the role of *XBic-C* in endodermal patterning is uncharacterised, and we do not know how *XBic-C* gradient is displaced. *XBic-C* is the frog homologue of *Drosophila Bicaudal,* a RNA binding protein involved in the anterior-posterior patterning of the *Drosophila* oocyte (Saffman *et al.*, 1998; Wessely and De Robertis, 2000; Wessely *et al.*, 2001).

During gastrulation, both *Xenopus* and zebrafish gene patterns of expression are indicative of the existence of a molecular pattern along the prospective anterior-posterior axis, which must be the result of a patterning mechanism. Overall, this suggests that the process of endodermal patterning occurs during gastrulation and is concomitant with the process of endodermal specification and endodermal fate maintenance.

Endodermal patterning is far less understood than that of mesoderm or ectoderm. However, all the signalling pathways, TGF-β (*Smad2*), FGF (*MAPK*), BMP (*Smad1*) and *β-catenin* are active in different endodermal domains in *Xenopus* gastrulas and neurulas (Schohl and Fagotto, 2002). In particular, the TGF-β signalling pathway has been shown to be active in a graded form, in the gastrula endoderm. *P-Smad2* activity, the nuclear effector of TGF-β signalling, was shown to move in a wave from dorsal to ventral (Lee *et al.*, 2001; Schohl and Fagotto, 2002). Therefore, establishing different domains of signalling intensity in the gastrula endoderm.

In addition, *β-catenin* and TGF-β's are responsible for the induction and establishment of the anterior endomesoderm (Hashimoto-Partyka *et al.*, 2003; Schohl and Fagotto, 2002), and cooperate to establish *Cerberus* and *Hex* expression in dorsal-anterior endodermal progenitors (Bouwmeester *et al.*, 1996; Newman *et al.*, 1997). The anterior endoderm is specified to express *Cerberus* and *Hex* by stage 8 (Zorn *et al.*, 1999). However, the expression of *Cerberus* and *Hex* in early blastula is dependent on cell-cell signalling that occurs after stage 8. *β-catenin* and *TGF-β* signalling are necessary for the establishment of the anterior endomesoderm, and during gastrulation BMP antagonists are required for its maintenance (Zorn *et al.*, 1999). These signalling events use in fact the same signalling pathways known to be active in mesodermal patterning, and the establishment of the Spemann organizer. This data shows the involvement of signalling pathways in the formation of gene expression domains, is evidence for a patterned endoderm, and that signalling is used for its establishment.

Cerberus expressing cells are part of the organizer, and *Cerberus* inhibits Wnt, Nodal and BMP signalling (Bouwmeester *et al.*, 1996; Piccolo *et al.*, 1999). *Cerberus* over expression induces extra liver and heart tissue on rare occasions, an unusual phenotype in other duplicating axis molecules. *Hex* is expressed in the deep endoderm and in the suprablastoporal involuting endoderm in an overlapping pattern with *Cerberus* (Newman *et al.*, 1997). *Hex*'s function is necessary in both of these tissues for the development of axial structures and correct neural patterning (Jones *et al.*, 1999; Smithers and Jones, 2002). The frog deep anterior endoderm and the suprablastoporal endoderm are thought to have similar

properties to the mouse anterior visceral endoderm and anterior definitive endoderm, respectively. $Hex^{-/-}$ mice have defects in liver and thyroid development and deficient patterning of anterior neural structures, which is patterned by the anterior visceral and definitive endoderm (Bogue *et al.*, 2000; Brickman *et al.*, 2000; Martinez Barbera *et al.*, 2000; Thomas *et al.*, 1998). In summary, the dorsal-anterior endoderm has several signalling properties, which other endodermal regions do not present, and is necessary for the patterning of the embryo.

Analyses of hypomorphic nodal mutants support the view that TGF-β signalling establishes an anterior-posterior pattern in the mouse endoderm (Vincent *et al.*, 2003). In mouse, only one nodal gene is present, and nodal null embryos arrest development at early stages (Conlon *et al.*, 1994). But high levels of nodal signalling seem to be required for the establishment of anterior endodermal fates, and lower nodal signalling levels are required for posterior endodermal fates (Vincent *et al.*, 2003). In *Xenopus, Derrière,* a TGF-β member is another example of a signalling molecule believed to be involved in the specification of posterior fates, and which is also able to induce endoderm (Sun *et al.*, 1999).

Mice posterior endoderm patterning is also known to be under the influence of FGF signalling. Embryonic day 7.5 mouse endoderm is patterned by soluble factors from adjacent germ layers. At this early stage endoderm will only express regional markers of differentiation if in contact with adjacent tissue. However, differentiation is not dependent on contact but on FGF diffusible growth factors. Amongst all the growth factors tested, FGF4 mimics the induced regional markers of endodermal differentiation in a concentration dependent manner (Wells and Melton, 2000). FGF signalling is a posteriorizing signal expressed in the mouse primitive streak.

In zebrafish, *her5*, a *hairy/enhancer of split*-related gene is expressed in a sub-population of dorsal endodermal cells, roughly equivalent to the dorsal endomesoderm of *Xenopus* and also fated to become pharynx and anterior tissues. This family of transcription factors is known to control cell fate decisions in other organisms. In zebrafish, *Her5* controls the number and fate of the anterior most mesendodermal cell progenitors (Bally-Cuif *et al.*, 2000). In addition, expression of *her5* is under the regulation of BMP signalling. Interference with the levels of BMP signalling, using *chordino* and *swirl* mutants, defective respectively in Chordin and BMP2b activities, alters the expression of *her5*. Excess BMP signalling reduces the number of *her5* expressing cells, while removal of BMP signalling enlarges the *her5* cell population (Tiso *et al.*, 2002). Therefore, it is reasonable to assume this example as another signalling mechanism (BMP) used to pattern the anterior endoderm.

In summary, the endoderm acquires a distinct molecular anterior-posterior pattern during gastrulation. Currently, it is unknown how stable this regionalization is, its implications, or how exactly it is achieved. Subsequent endoderm development will rely on interactions with adjacent tissues. Overall, patterning of the endoderm depends on early gastrula signalling mechanisms and the stabilization of an initial pattern by interactions with the associated developing mesoderm. Both the differential activity of signalling pathways, and specific gene expression domains during gastrulation evoke the existence of a pattern in the gastrula endoderm of all vertebrates examined.

Mesoderm Patterning the Endoderm

In *Xenopus*, the literature has many examples of how the mesoderm can influence the differentiation of the endoderm, but no molecules have been implied in the process (Okada, 1953; Okada, 1955a; Okada, 1955b; Okada, 1955c; Okada, 1955d; Okada, 1960; Takada, 1960a; Takada, 1960b; Yasugi, 1993). In chick and mice endodermal differentiation is also controlled by interactions with the mesenchyme (Haffen *et al.*, 1987; Kedinger *et al.*, 1998; Kedinger *et al.*, 1986; Ratineau *et al.*, 2003), but again research has not been able to explain the molecular mechanisms responsible for this.

Initial studies on *Xenopus* implicated TGF-β and FGF signalling (Gamer and Wright, 1995; Henry *et al.*, 1996) in the establishment of the expression of *Pdx1* (Wright *et al.*, 1988) and *IFABP* (Shi and Hayes, 1994). These endodermal patterning markers define the developing pancreas and the posterior intestine of tadpoles. The use of inhibitory constructs of both TGF-β and FGF pathways blocks the correct specification of pancreas. The expression of *Pdx1* and *IFABP* was independent of mesoderm and only dependent on cortical rotation (Henry *et al.*, 1996). The interpretation of these results supported an early autonomous specification and pattern of the endoderm.

Regional specification was re-investigated using stage 15 and 25 *Xenopus* endodermal explants, and the same molecular markers, *Pdx1* and *IFABP*. Such explants, assayed at stage 42, only express the endodermal markers *Pdx1, IFABP, XCad2* if cultured in the presence of adjacent mesoderm. The absence of regionally expressed markers in mesoderm free endodermal explants was indicative of the necessity of mesoderm for endoderm regionalization (Horb and Slack, 2001). Therefore, the authors claimed that endoderm regionalization is a non-autonomous process, dependent on the action of the mesoderm. However, endoderm explants free from mesoderm did express anterior-posterior character, in

spite of the lack of expression of patterning markers, since posterior endoderm explants elongated much more than did anterior endoderm explants (Horb, 2000; Horb and Slack, 2001).

The status of endoderm regionalization was also studied through explant recombination and histological observations made at stage 40 and stage 46 (Zeynali *et al.*, 2000). After stage 22, if pieces of anterior endodermal tissue and associated mesoderm are transplanted to a new location in the embryo, they will develop according with their initial fate. However, if the endodermal core is transplanted before stage 28 to a more posterior location, it will acquire posterior characteristics. This shows that the fate of the endodermal core is labile until stage 28 and that the associated mesoderm retains some information necessary to re-specify the endoderm (Zeynali *et al.*, 2000). Thus, the initial pattern only becomes stable enough to allow autonomous differentiation by stage 28.

Overall, these results show that the initial pattern of the endoderm is not stable and requires the contact of the mesoderm for its stabilization, and that mesoderm-free endoderm explants express their anterior-posterior character in culture, in spite of not expressing patterning markers. Lastly, endoderm can be re-specified when in contact with ectopic mesoderm until relatively late tailbud stages.

The limitation of recombination and explant studies is that the analysis relies on the appearance of morphological characteristics or the use of molecular markers expressed much later than the interaction between tissues. This has been particularly true for studies in *Xenopus* where the organ specific markers are not expressed until day 3-4 of development, but patterning events are likely to have occurred at least one day earlier. In fact, for *Xenopus,* the endodermal markers previously available, like *IFABP* or *Pdx1,* are only expressed at tadpole stages (Figure 3.1A) (Horb, 2000; Zorn and Mason, 2001).

The first step to uncover the importance of the mesoderm, in the determination of a stable endodermal fate, is to know when both tissues become in contact. As we have seen, the fate map of both tissues is not in alignment at neurula stages (stage 14) (Chalmers and Slack, 2000). From here onwards, the lateral plate mesoderm will continue its overall ventral migration and the endoderm will extend along the anterior-posterior axis. At stage 23-24 the endoderm is already in alignment with its associated mesoderm (Muller *et al.*, 2003). However, the two germ layers have been in contact, where interactions are likely to have occurred.

The above-mentioned results are in agreement with experiments in axolotls where endoderm differentiation was found to be labile until neurula stages (Okada, 1960). Similarly,

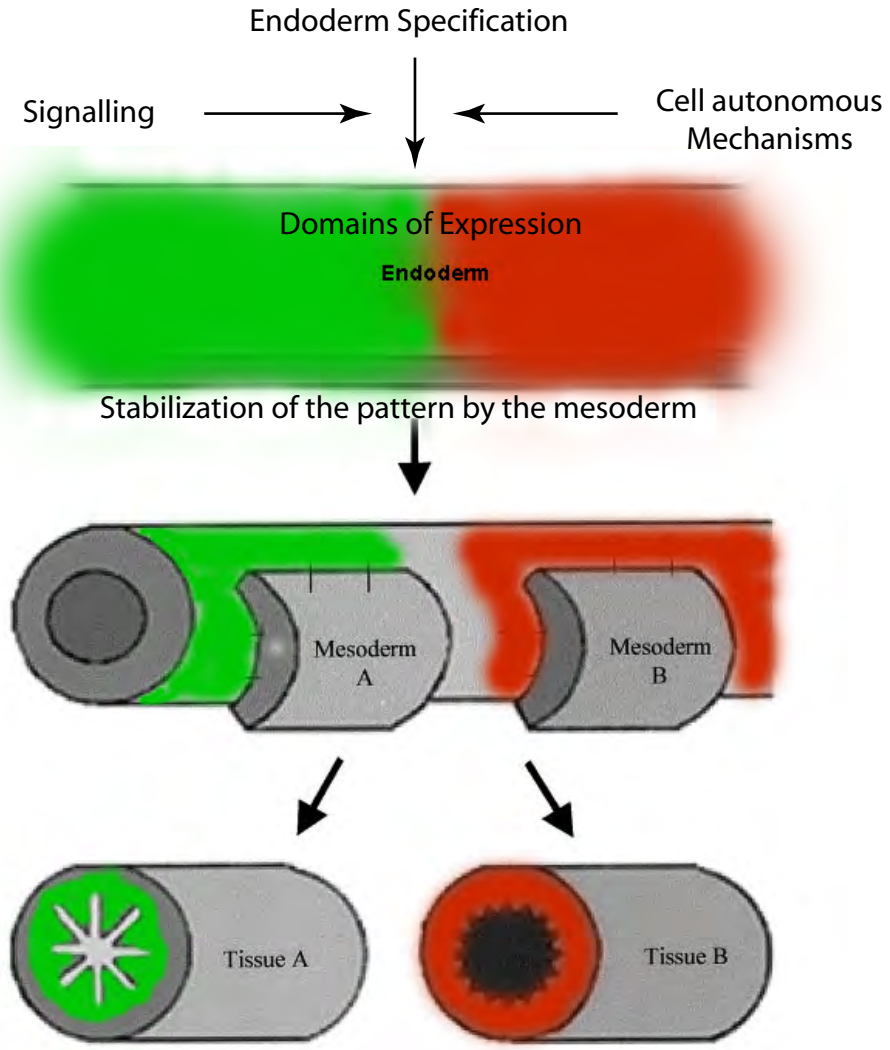
explanted chick gut endoderm differentiates autonomously to its proper region-specific morphology between embryonic days 3.5 to 6. However, the endodermal tissue can be influenced by heterotypic mesoderm and alters its differentiation up to embryonic day 9 (Roberts *et al.*, 1998). Most of the data available on the interaction between the endoderm epithelium and the mesenchyme derive from studies in chick. Full differentiation along the gut tube depends on both mesoderm and endoderm (Rawdon, 2001). Overall, considerable evidence suggests that the endodermal layer of the gut tube is regionally patterned from an early stage, but require the contact with mesoderm to differentiate into specialized structures and specific cell types, demonstrating the importance of continuous development between both the endoderm and mesoderm germ layers (Rawdon, 2001; Roberts *et al.*, 1998; Roberts, 2000; Roberts *et al.*, 1995).

The influence of the mesoderm in regionalization of the vertebrate endoderm is revealed by recombination and extirpation experiments with notochord (Cleaver and Krieg, 2001). The notochord defines the chordate phylum, and it is rod-like structure derived from axial mesoderm spatially located between the neural tube and dorsal endoderm in the axial midline. Notochord tissue develops in close association with dorsal endoderm and both tissues are in contact for most of early development. The role of notochord in the patterning of the neural tube and mesoderm derivatives such as the somites has been established, and therefore it is also likely to pattern the endoderm (Cleaver and Krieg, 2001). In *Xenopus,* the notochord induces the hypochord from dorsal endodermal cells. The hypochord is a transient structure under laying the notochord believed to be necessary for the induction of dorsal aorta. The hypochord is only present in frogs and fish, being a transient structure that disappears by stage 41. The hypochord is derived from dorsal endoderm cells. The nature of the notochord molecular signal inducing the hypochord is not known. Removal of the notochord at stage 13 blocks the development of the hypochord. Still, if the removal of the notochord is done at stage 18 the hypochord develops normally in the absence of the notochord. In addition, transplantations of an additional notochord induce more hypochord tissue. Thus, the notochord specifies hypochord from dorsal endoderm at early neurula stages. However, not all endoderm is able to respond to the contact of the notochord. The endoderm competent to respond to the notochord inductive signal is restricted to the most dorsal endoderm. Transplantation of notochord tissue onto lateral endoderm does not result in induction of hypochord tissue. Therefore, demonstrating differences in the potential and competence from dorsal to ventro-lateral endoderm. That is, a pattern is obvious in the most dorsal endodermal cells, as they are the only ones able to respond to the notochord-inducing signal (Cleaver *et al.*, 2000).

In chick, notochord transplantation experiments reinforce the idea that not all endoderm is able to respond to same notochord signals. The posterior endoderm does not respond in the same way to the environment provided by the notochord. Also demonstrating an anterior-posterior pattern in the chick endoderm. In summary, studies on the notochord patterning effects on the endoderm reveal that not all endoderm is able to respond to the same signals, demonstrating different potentials and competence along the dorso-ventral and anterior-posterior axis of the endoderm. In addition, it has been shown that further development of the endoderm is dependent on *Shh* signalling, between the notochord and endoderm (Cleaver and Krieg, 2001; Ramalho-Santos *et al.*, 2000).

In conclusion, several lines of evidence point to the establishment of a pattern during vertebrate endoderm gastrulation. Although with rare exceptions this patterning is poorly characterized. At least, three signalling pathways cooperate in the establishment of anterior endodermal fates, VegT, TGF-β and β-*catenin*. FGF signalling and lower levels of TGF-β signalling appear to be responsible for posterior endodermal fates. However, we do not know how stable this pattern is, or how one can follow its development. Many inductive events must occur in the neurula endoderm, and my screen is aimed at providing markers for these stages of endoderm development.

We have known for long that mesoderm is necessary for the development of the endoderm, and that heterotypic mesodermal transplantation can re-specify endodermal fates. We also know that in *Xenopus,* the endodermal pattern analysed by the expression of *Pdx1, IFABP* and *Xcad2,* is only stable at stage 28. We do not know what are the mechanisms that re-enforce or modify this initial pattern. My screen targeted a time window that correlates with the events that are important to the modification or stabilization of the initial endodermal pattern.



Adapted from Horb et al. (2001)

Figure 1.5 - Model for the patterning of the endoderm during vertebrate development. Concurrent with endoderm specification, the endoderm is subdivided into domains of gene expression by largely unknown mechanisms. Later those domains of expression are later stabilized by interactions with adjacent tissues.

4. Endodermal Organogenesis

The endoderm germ layer gives rise to the epithelium lining of the digestive tract, respiratory system and associated organs. These associated organs are from the anterior to the posterior, the thyroid, parathyroid, thymus, lungs, liver, gallbladder, pancreas and caecum. To those we add the oesophagus, stomach, small and large intestine as being part of the gut tube itself. Two concentric layers of tissue form the gut tube, the inner is endodermally derived and the outer mesodermally derived.

The development of each of these organs is dependent on signalling and interactions between neighbouring tissues that ultimately influence cell specific differentiation, proliferation, morphogenesis and function (Grapin-Botton and Melton, 2000). Although certain genes can affect the development of almost every endodermal organ known, the development of an organ relies on signalling cascades and the coordinated action of many genes (Grapin-Botton and Melton, 2000; Wells and Melton, 1999). Briefly, I will mention the examples of hepatic, pancreatic and gut organogenesis.

Gut Organogenesis

The way the gut tube forms varies across phyla. Mouse and chick form a gut tube by invagination and migration of the intestinal portals (Grapin-Botton and Melton, 2000). In *Xenopus* the gut tube forms by radial intercalation, which re-opens the archenteron (Chalmers, 1999). In zebrafish, the gut tube is assembled from individual organ anlagen and rearrangement of newly polarized cells (Wallace and Pack, 2003). During development the mesenchyme associates closely with the gut tube endoderm, and is responsible for the gut coiling movements, as been recently studied in *PKCγ* zebrafish mutants, in which the coiling of the gut is random (Horne-Badovinac *et al.*, 2001; Horne-Badovinac *et al.*, 2003).

The epithelium of the gut tube can acquire new characteristics if associated with different mesenchymes (Rawdon, 2001; Roberts, 2000). In chick, *Sonic hedgehog*, *Bmp4* and members of the *Hox* gene family have a role in establishing region-specific endoderm differentiation (Roberts *et al.*, 1998).

Hepatic Organogenesis

The liver is a major endodermally derived organ consisting only of four major cell types, which are hepatocytes, Kupfer cells, stellate cells and endothelial cells. However, liver organogenesis is complex and still poorly understood (Arias, 2001). The liver of birds and

mammals arises from a proliferating bud on the ventral endoderm, which invades the septum transversum mesenchyme (Le Douarin, 1975). In mice, hepatic development occurs closely associated with neighbouring mesodermal tissues, the heart and the septum transversum under the influence of FGF and BMP signalling. Cardiac mesoderm initiates the liver gene program through FGF signalling that is maintained by BMP signalling from the septum transversum the tissue that is invaded by the hepatic cords (Cascio and Zaret, 1991; Cirillo *et al.*, 2002; Gualdi *et al.*, 1996; Jung *et al.*, 1999; Rossi *et al.*, 2001; Zaret, 1998; Zaret, 1999; Zaret, 2000; Zaret, 2001; Zaret, 2002).

Organs develop from presumptive overlapping territories in the early embryo, like for instance the liver and the ventral pancreas (Grapin-Botton and Melton, 2000). Initially, a domain of competence must be established within the endoderm. In the case of the liver, several *Fox* and *GATA* transcription factors are expressed earlier in the endoderm, and are likely to contribute to the establishment of competence for the expression of liver specific genes such as *albumin*. Later other events, as for instance, FGF's emanating from the cardiac tissue induce a hepatic fate from bipotential precursors (Deutsch *et al.*, 2001; Jung *et al.*, 1999). To further illustrate the complexity of organogenesis, many of these inductive events are bidirectional, as the heart itself is induced by presumptive liver tissue (Nascone and Mercola, 1995), and liver and pancreas develop in coordination. In addition, after hepatic induction endothelial cells contact the developing liver and drive its growth, morphogenesis and differentiation (Lammert *et al.*, 2003; Matsumoto *et al.*, 2001). In summary, organogenesis is the result of many temporally distinct interactions between cells and tissues and the combination of many signalling events, that only now we begin to understand (Duncan, 2003; Ober *et al.*, 2003).

Pancreatic Organogenesis

In contrast to the liver, the pancreas contains many cell types necessary for the pancreatic endocrine and exocrine functions, such as β-, α-cells, and PP-cells. However, the pancreas and the liver share a common bipotential precursor. FGF signalling from the heart, diverts prospective liver cells to express *Shh,* which in turn inhibits the pancreatic fate (Deutsch *et al.*, 2001). Under experimental conditions the liver can be converted in pancreas and vice-versa (Grompe, 2003; Horb *et al.*, 2003; Shen *et al.*, 2003). The pancreas arises also through budding of the gut tube, but unlike the liver, from two or three buds. One bud originates dorsally and one or two other buds ventrally, depending on the organism studied. Many transcription factors are known to be pancreas lineage specific (*Pdx1, Isl1, Pax6, Pax4*), and necessary for pancreatic development (Edlund, 1998).

The notochord is involved in the determination of pancreatic tissue in chick (Kim *et al.*, 1997; Slack, 1995). Analysis of pancreatic development demonstrates that notochord signalling is necessary for the expression of pancreatic markers such as, *HNF3β*, *Pax6*, *Islet-1*, *Glucagon*, *Pdx1*, *Insulin* and *Carboxypeptidase A*. Pancreatic development is also dependent on the interactions with other tissues besides the notochord (Apelqvist *et al.*, 1997). The notochord downregulates the expression of *sonic hedgehog* (*SHH*), that induces the expression of activin Bβ and FGF, which are necessary for pancreatic differentiation (Hebrok *et al.*, 1998). Again, a fully functional organ is obtained when the endoderm and the adjacent mesodermal tissues interact. Signalling events have a major role in defining the poorly characterized cellular responses of proliferation, differentiation and morphogenesis.

The inductions that occur before organogenesis are poorly characterized in vertebrate endoderm development. The existence of patterning markers at the time when organ induction occurs will assist our knowledge and our future studies of endoderm development.

5. Differential Screens

Many regulators of development have been isolated by the use of molecular cloning techniques. For more than 20 years we have been cloning differentially expressed genes in amphibians (Sargent and Dawid, 1983). Subtractive cloning has been a successful cloning strategy, providing regulators of development such as *chordin*, *Sox17* or *Cerberus* (Bouwmeester *et al.*, 1996; Hudson *et al.*, 1997; Sasai *et al.*, 1994).

I have decided to find new endodermal patterning markers using a differential screening strategy, because looking for differential expressed genes is the best way to find tissue specific genes. Here, I present some theoretical background of differential screening and on macroarrays, they will be important to understand my screening criteria.

Our concept of cellular gene expression had its foundations laid in the 60's when messenger mRNA was described, the genetic code deciphered and the mechanisms of protein synthesis unravelled (Ermolaeva *et al.*, 1998; Jacob and Monod, 1961; Nirenberg and Matthaei, 1961). From these, concepts such as, mRNA abundance, mRNA functional class, "house-keeping" ubiquitous mRNAs, and transcript cell specificity, were a small step away. To find differentially expressed genes, one has first to know how many genes do cells express, which could be differentially expressed, and how can we select them.

In mammalian cell lines reassociation kinetics experiments indicate the expression of 10,000 to 30,000 genes (Sambrook and Russell, 2001). Brain cells express as many as 100,000 genes (Bantle and Hahn, 1976). Probably these are overestimates since genome sequencing reveals only 30,000 genes in humans, 14,000 in the fly and 20,000 in the worm (Levine and Tjian, 2003). Currently, it is believed that 20% of the cell mRNA population is composed of abundant transcripts (12,000 to 1,000 copies per cell), 25% of medium abundance mRNA (1,000 to 100 copies per cell) and the remaining 50% consists of low abundance transcripts (Sambrook and Russell, 2001). But, how many of those are specific? This will depend on how related are the tissues or cell types. Reassociation kinetics measurements between different mRNA populations yield values as low as 2% of specific transcripts for closely related B and T lymphocytes or 17% for comparison between adult liver and oviduct mRNA populations (Axel *et al.*, 1976; Galau *et al.*, 1974; Sagerstrom *et al.*, 1997). Generally, different mammalian cell lines reach values of specificity around 20% and these can be as high as 56% for different stages of sea urchin development (Galau *et al.*, 1974). Differences are both quantitative and qualitative. That is, cells may express or not a certain gene or that gene can be up or down-regulated. Lastly, differentially expressed genes reside in any abundance class, illustrating the difficulty of finding differentially expressed genes.

Macroarrays are nylon membranes with 'printed' cDNA colonies at high-density in a regular array. Macroarray major uses include gene discovery and expression profiling (Jordan, 2002; Lennon and Lehrach, 1991). Macroarrays have been surpassed in expression profiling by microarrays, a newer and more effective technology (Lockhart and Winzeler, 2000). Although based in the same principles, macro- and microarrays should not be confused. They are simply different technologies. At the time I started this work microarrays where not yet available. As microarrays become more available and reliable it is likely that embryonic gene expression profiling will become common practice (Livesey, 2002). cDNA macroarray have been used successfully in gene discovery, particularly in 'non-genetic' model organisms, like sea urchin (Ransick *et al.*, 2002; Rast *et al.*, 2000; Rast *et al.*, 2002). However, macroarrays can serve at most as semi-quantitative tools (Dickmeis *et al.*, 2001). Reasons for the non-quantitative abilities of the macroarrays include among others; irregular clone size, clone unknown identity, unknown size of target sequences, uneven printing, radioactive probe construction, no control for label incorporation, and the use of large volumes of hybridisation volume in non-flat surfaces.

At the time I started my project, macroarrays were the best available technology to do the profiling of many complex probes, and therefore the technology of choice to identify putatively differentially expressed genes in the endoderm of *Xenopus* embryos. Differential screens, searching for genes expressed in probes enriched with endodermal transcripts and absent on probes with a high mesodermal content, are likely to provide novel endodermal markers. As we have seen the lack of markers of endodermal patterning, at neurula and tailbud stages, have impaired our knowledge of endoderm development.

CHAPTER TWO – MATERIAL AND METHODS

1. Material and Reagents

2. Screening protocols
Construction of the Libraries
Construction of the Complex Probes
Improvements to Probe Construction
Removal of Ubiquitous Sequences
Hybridisation Procedures
Acquisition of Hybridisation Data
3. Pattern of Expression Characterization
Whole Mount "In Situ" Hybridisation
Photography
Sectioning
4. Identification and Sequence Characterization
Cluster Searches

1.Material and Reagents

Suppliers of reagents were Roche-Boehringer Mannheim, Ambion, Quiagen, Merck, Sigma, NEN Life Sciences, Bio-Rad, BDH, ICN Biomedicals, Life Technologies-Gibco BRL, Invitrogen, among others. The Wellcome CR UK Gurdon Institute provided all the reagents and materials. The source of the reagent is referred in detail if, in my opinion, that source is crucial for the results in which the method was applied. Embryos were obtained with standard procedures (Sive HL, 2000), and according to the Home Office Regulations. Care of adult animals was performed in the Wellcome CR UK Gurdon Institute. *Xenopus laevis* female ovulation was hormonally induced and embryos were fertilized "in vitro", as current laboratorial practice (Sive HL, 2000). Embryos were reared in 0.1x MBS or 0.1x NMR, staged according to Nieuwkoop and Faber, which is used throughout this thesis, and allowed to develop at a preferred temperature of 18º C (Nieuwkoop, 1967). All oligonucleotides were obtained from Sigma-Genosys and the sequencing reactions were performed by the Department of Biochemistry, Cambridge University. Table 2.1 lists the primers used.

2. Screening protocols

The protocols used are standard molecular biology protocols, commonly used and performed according to Sambrook and Russell (2001). The methods described below were necessary for the execution of a differential hybridisation screen on macroarray ventral midgut cDNA library filters. The screen strategy is described in chapter 3 (page 46-48). Briefly, the differential screen consisted of two steps; 1) complex probe profiling and selection of differentially expressed candidate clones, 2) verification of the pattern of expression of candidate clones by whole mount *in situ* hybridisation (Figure 3.1). A complex probe profile is obtained when a complex probe is hybridised to the macroarray filters. Complex probes represent all the RNA expressed in the tissue. I have named them complex probes, in contrast to probes made from a finite and known number of nucleotide sequences. Several different complex probes were used throughout the screen, but the complex probes used in the final selection criteria are shown in table 3.2.

Construction of the Libraries

With the purpose of screening for genes specifically expressed in the midgut region of the early tadpole, the lab produced a ventral midgut arrayed cDNA library. Presumptive midgut regions were micro-dissected from 2000 stage 22-24 *Xenopus laevis* embryos (Figure 3.1B). The tissue used for the library contained a high proportion of endoderm but ectoderm and mesoderm were also present. Total RNA was extracted using a proteinase K digestion method. PolyA$^+$ was isolated on oligo-dT columns. cDNA was oligo-dT primed and directionally cloned with EcoR1 at 5'end and Not1 at the 3'end into pCS107 (Grammer *et al.*, 2000). The resulting cDNA library contained $3.5x10^6$ recombinants with an average insert size of 1.4 Kb. In all of my handling of the library, which at present consisted in the picking of more than 1500 clones, I have detected very few clones with no insert. I am therefore confident to say that less than 1% of clones in the library have no cDNA insert. During the screening procedures I have also made use of other libraries existent in the lab, all of which had the mRNA extracted by a proteinase K digestion method, size selected, oligo-dT primed and directionally cloned into the respective vectors (Nigel Garrett or Dr. Aaron Zorn).

The resulting presumptive liver library was macroarrayed onto nylon filters, using a Qbot kindly provided by Doug Melton (Harvard & http://www.genetix.co.uk/). 55,296 bacterial colonies were printed in 3 filters of 22 x 22cm, each containing 6 fields of 24 x 16 squares. Each square contains 8 clones printed in a duplicate 4 x 4 array. The orientation of the duplicates and their position corresponds to a specific well of the cDNA library plates. The

arrayed library was kept at –70^{o} C in 384 (24 x16) well plates, each of the three resulting filters contained 18,432 colonies spotted in duplicate in a four by four array. I call a spot to each printed cDNA clone in the macroarray.

Construction of the Complex Probes

Complex probes represent the mRNA population expressed in a tissue. Hybridisation of radio labelled complex probes onto macroarrays provides a graphic representation of the mRNA population (transcriptome). Primarily, radio labelled cDNA complex probes were prepared from adult tissue RNA. Total RNA was obtained from adult organs extracts using 3M LiCl, 6M Urea, 10mM sodium acetate, pH 7.5, 0.1% SDS and 0.5% β-mercaptoethanol, overnight at 4^{0}C. PolyA^{+} mRNA was isolated using NucleoTrap kit (Clontech). ^{33}P labelled cDNA was produced with SuperScript™ II (GibcoBRL–Life Technologies) using between 5 and 10 μg of polyA^{+} mRNA in each reaction, a high concentration of polyA^{+}. The first strand synthesis step was oligo-dT primed, and random hexamers were also used to reduce 3' bias, achieve better yield and longer products. The resulting cDNA was purified by alkaline hydrolysis at 68^{0}C for 20min, buffered with TRIS pH 8.0 and purified on Bio-Rad Micro Bio-Spin® 6 columns. An aliquot of the complex probe was size gel verified, and radiolabel incorporation was calculated. Typically, 6 to 9 complex radio labelled cDNA probes from such reactions were used in a single hybridisation experiment in a volume up to 25 ml (15-20 ml, preferred).

Improvements to Probe Construction

High amounts of polyA^{+} are difficult to obtain from embryonic material. A second method of complex probe synthesis was used for embryonic tissues. Synthetic mRNA obtained by *in vitro* transcription of entire embryonic cDNA libraries. This synthetic mRNA was used to produce a ^{33}P labelled cDNA as described above. cDNA libraries were grown for less than five hours and DNA plasmid purified. The DNA was divided and linearized with two different restriction enzymes to avoid digestion of transcripts containing one of the restriction sites. Linearized templates were pooled together and transcribed (Ambion MEGAscript) producing more than 50 μg of synthetic mRNA. RNA polymerase (Ambion MEGAscript) offers a linear method of amplification maintaining the RNA initial complexity, illustrating a major concern of maintaining the initial complexity and representativity of the mRNA sample.

Removal of Ubiquitous Sequences

During the initial screening steps, the retrieval of ubiquitously expressed cDNAs was a major problem. Therefore, I tried to increase the efficiency by eliminating such ubiquitous cDNAs. Initially, I tried to reduce the percentage of ubiquitous sequences in the initial mRNA population. Others achieved good results using subtracted probes (Sagerstrom *et al.*, 1997). However, in my hands the low yield (μg) obtained from of the resulting subtracted mRNA population, and the following first strand cDNA synthesis was inadequate for hybridisation onto macroarray filters. The resulting probe was not sufficient to drive a complete hybridisation, biased in size and in relative abundances.

By selecting 126 pre-tested ubiquitous clones from the initial rounds of screening, and hybridising them to the filters, I was able to eliminate approximately 20% of the macroarray ubiquitous sequences from future selections (4,000 in 18,000). A probe containing 126 ubiquitous individual cDNAs was used. cDNA inserts from these 126 clones were ^{32}P radio labelled during PCR amplification with T7 and SP6 primers. PCR products were pooled, digested with EcoRI and NotI to remove vector end sequence, purified on Bio-Rad Micro Bio-Spin ® 6 columns and denatured prior to hybridisation as described below.

Hybridisation Procedures

Macroarray filter hybridisations were performed overnight (>14 hours) at 42^0C in 15 to 25 mls of either formamide hybridisation buffer (50% formamide, 5x SSC, 2x Denhart's, 0.1% SDS and 100μg/ml denatured Salmon Sperm DNA), or commercial available buffers (Ambion ULTRAHyb) according to manufacturer instructions. Macroarray filters were washed three times 45 minutes in 0.1x SSC, 1% SDS at 65ºC. Filters were always pre-hybridised (>6 hours). The use of mesh between filters improves results but increases final volume of hybridisation solution necessary. Macroarray filters were wrapped in saran wrap on a flat surface and exposed to the phosphoimager screens attempting saturation of stronger hybridisation signals. Phosphoimages were analysed by Storm840 (Molecular Dynamics) at 50 μm resolution (maximum) and exported in digital format as .tiff files.

Acquisition of Hybridisation Data

Imported phosphoimager files (.tiff) of the hybridisation profile are accessible by the software. Complex probe hybridisation profile data and clone hybridisation signal intensity was acquired using VisualGrid® software (GPC Biotech-Germany). Pixel intensities are

measured in a circle over the expected area of the printed clone, added up, and standardized in a scale of 0 to 255. The grid defines the expected area over the printed clone. The grid for macroarray analysis was superimposed on the hybridisation profile, until correlation between signal intensities of spot duplicates was higher than 97%. The value for signal intensity for each clone was defined as the average intensity of the duplicate spots. Background levels were found on the brightest area of the array and used to define empirical thresholds for the presence and absence of signal. To verify the empirical thresholds, at least 50 random spots in the array were visually examined. Signal intensity thresholds defined absence or presence of signal in a hybridisation profile, and their definition is described in chapter 3. Signal intensity of every spot on the macroarray was exported to an Excel spreadsheet as a relative value in a scale of 0 to 255. Excel spreadsheet from each experiment was compiled in a master file used to the selection procedures, were several complex probes can be compared simultaneously (Figure 3.2).

TABLE 2.1- Primers used

Primer Name	Sequence
Vito_Az107	5'-GCCGGTGTTCTACAGACTAGG
Vito_Az108	5'-ATGCTTCTCCCACCCATCAGG
Vito_Az109	5'-GGTGTAGACAACAGATACAATTC
Vito_Az110	5'-GCTGCTGACTTGTGTTTCAGGA
Vito_Az111	5'-CGTGCCTTCCAGTTCAACACTTC
Vito_Az112	5'-ACCGCTTTGGAGCCATGTTCATC
Vito_Az113	5'-CAAACCTGCCCGACCAGCGGGT
Vito_Az115	5'-GTGGTTCAGGGTGGCATTGGT
Vito_Az116	5'-GCTTACAGTGGGACATTAAGT
257_RC120	5'-GATCTGATGGACTAGAGAAACTTGTG
712_RC117	5'- GCTGTCCTTGTTGTATTCTGC
712_RC118	5'- CACACCCACAATAACAGAGAC
712_RC119	5'- TGATGATGATGATGACGACC
1012_RC121	5'- CCTCCACCCTTCTTTCATAC
1012_RC122	5'- TGAGTATGAAAGAAGGGTGG
846_RC123	5'- GCCAATCAGTAGCAACTTACAAGC
846_RC124	5'- TGTCTGTATGGGAACGGCTG
847_RC125	5'- ACTTCACCCACACCCCATAG
847_RC126	5'- TGCCTGTCTCTGCTCTAAAC
846_RC127	5'- GGTAAGGATGTAGGCACCTG
846_RC128	5'- CCGCAATACAAGTCTATGGC
846_RC129	5'- AAATGGTTGGGTCCTCTG
846_RC130	5'- TCATTTCCCACCTCTTCTC
847_rc131	5'- CAGAACTTCCTTACGCTTCG
847_rc132	5'- GACTGAATCTGAGAAGCAACAG
141_rc133	5'-AAAGGGCAAGACAGATGC
141_rc134	5'- CATGAGTCAAATTATTCTCTCA
176_rc135	5'- GCATTGGTTATGACCCAGG
176_rc136	5'- CCTAACAACAGAACAATGGG
141_az554	5'- AAGCCCCAGTAGGTAAGAGG
tFet_az555	5'- GAGATGGTATCACCAGTT
tFet_az556	5'- GGACAGAACAGTTGGTTT
SpiB_rc140	5'- TCACTCTGACCAAATCGG
SpiB_rc141	5'- TTCCAGGAGCCTTTACGAG
spiB_rc142	5'- ACCCGAAAGATTTGCGTG
spiB_rc143	5'- GGTCATTTTCTTGCGGTTG
SpiBa_rc144	5'- TATTAGCGAGAGGACACCG
SpiBa_rc145	5'- TAAGCACTGTCTTCTCGCC
XtSpiB_rc146	5'-TCAGGTGTCCCCCTGCCCAC
XtSpiB_rc147	5'-CGGAGAGAAGTTCTCATCTGA

3. Pattern of Expression Characterisation

Putatively differentially expressed candidates were selected from the library plates in the –70º C freezer. Differentially expressed candidates were defined based on the criteria of selection defined in chapter 3, and the analysis of the complex probe hybridisation profiles and signal intensities recorded on the master file. Digoxygenin labelled antisense mRNA probes were prepared and tested for each individual clone. 1 μl of bacterial culture was used to PCR amplify the clone template using T7/SP6 primers, PCR products were gel analysed and used to produce digoxygenin labelled antisense *in situ* probes using T7 polymerase. Whole mount *in situ* hybridisations (WMISH) were performed in baskets on embryos from stage 18 to 28 (Sive HL, 2000). After WMISH, the restricted patterns of expression found were noted, and their position on the macroarray was used to improve selection criteria from the master file (see results Chapter 3).

Whole Mount "in situ" Hybridisation

Clones in the library, which had a restricted or specific pattern of expression with my PCR digoxygenin probes, were re-selected from the frozen library, and new digoxygenin antisense probes prepared. Whole mount *in situ* hybridisations were performed in vials, specially when using sectioned embryos or dissected guts (Sive HL, 2000). Sectioning after whole mount *in situ* hybridisation procedures was preferred to *in situ* hybridisation in sections. When necessary, pre-bisected embryos were subjected to *in situ* procedures (Faure *et al.*, 2000). *In situ* hybridisations on dissected guts were used to characterize gene expression at later stages (Chalmers *et al.*, 2000).

Photography

Embryos were re-fixed in MEMFA for 1 to 2 hours, and bleached (0.5x SSC, 5% Formaldehyde; 2 to 10% H_2O_2) under strong light with agitation (Sive HL, 2000). After completed bleaching, embryos were placed in 1x PBS and photographed with a digital camera attached to a Leica stereoscope (Leica MZ APO). Cleared embryos were obtained by dehydration in a methanol series and placed on a 2:1 solution of Benzyl Alcohol:Benzyl Benzoate (Murray's).

Sectioning

Whole mount *in situ* embryos were sectioned using a vibratome (40 μm), but imbedded in gel albumen prior to sectioning (4.4g/L gelatin, 270g/L albumen, 180g/L sucrose) and cross-linked with 25% gluteraldehyde (Sive HL, 2000).

4. Identification and Sequence Characterisation

When a clone with a specific pattern of expression was found, its sequence was blasted against the non-redundant and EST databases (Altschul *et al.*, 1997). If both 5' and 3' sequences did not overlap, primers were designed at regular intervals and the insert sequenced. From the blast database searches two outcomes resulted, either the sequenced clone was already known or it was an uncharacterised EST. On the first case, it was noted if the clone contained the full length coding sequence and its identity. On the second case, where the clone sequence was unknown the EST clusters for both 5' and 3' were assembled whenever possible. In the case of clones of the same gene, the longest clone was chosen for sequencing, based on the clustering of the EST data and homology searches. Table 2.1 lists the primers used.

CHAPTER THREE - SCREENING FOR ENDODERM SPECIFIC GENES

1. Introduction

2.Overall Strategy

3. Pre-screening Procedures
The Midgut Library
Preliminary Screen
Representativity of the Array
4. Results

1. Introduction

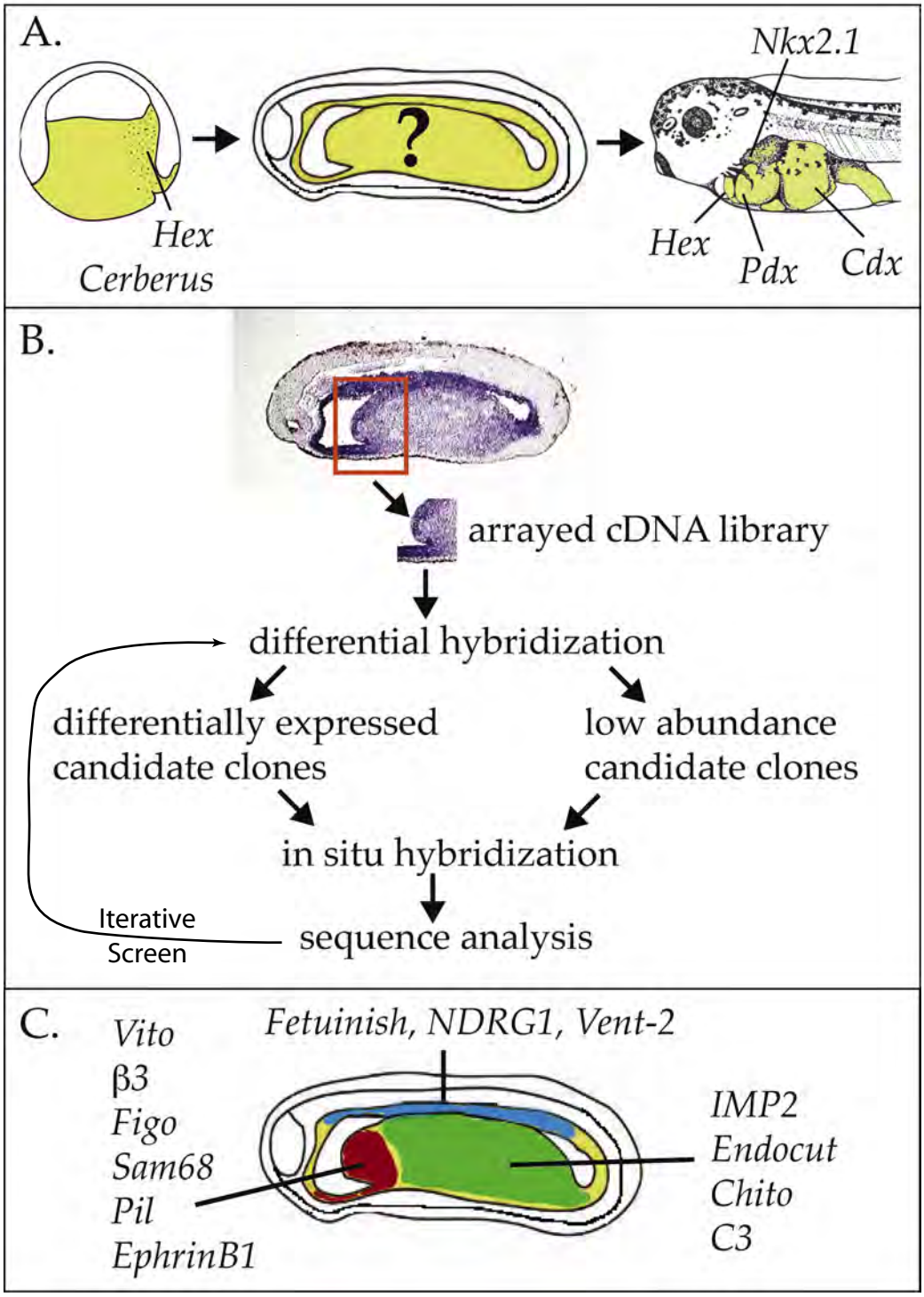
Very little is known about the signals that pattern the endoderm, because few specific markers are available from the end of gastrulation up to tailbud stages. Cellular differentiation along the gut tube is likely to be a consequence of differential gene expression. Obtaining information about the overall process of endoderm development will rely, at first, on specific molecular markers (Figure 3.1). Here, I describe a differential hybridisation screen designed to find novel genes involved in endoderm development. My screen proved to be an efficient gene-discovery tool and provided new markers for the study of endoderm patterning, and perhaps novel endodermal regulators.

I made use of an arrayed ventral midgut macroarray cDNA library. This tissue is fated to give rise to the liver, pancreas, stomach and lungs, for example. Indeed, it represents a small cell population from where many organs arise. Several genes responsible for the development of these organs must be expressed in this tissue.

The screen strategy included hybridisation of complex probes onto a ventral midgut macroarray library to identify differentially expressed candidate genes. Hybridisation profiles of complex probes derived from embryonic and adult tissues were compared to allow selection of differentially expressed candidate genes. Radio labelled probes constructed from mRNA of different tissues, both ‘positive’ and ‘negative’, were hybridised to the macroarray (Table 3.2). Positive probes are enriched in endodermal transcripts and negative probes are low in endoderm specific transcripts. The resulting hybridisation profiles and individual clone hybridisation signal intensities were systematically analysed. Such a systematic analysis provided lists of candidates clones likely to be differentially expressed in the endoderm.

A secondary *in situ* hybridisation screen followed selection of differentially expressed candidates. After several iterative rounds of selection a final hit rate* of ~5% was obtained in the final *in situ* hybridisations. This represented a four or five fold higher success rate when compared with similar screens that did not use a differential hybridisation pre-screen (Figure 3.1). More than 30 restricted patterns of expression were found in all 3 germ layers. Amongst these, I found 11 specific endodermal patterns of expression, and their characterisation is dealt with in chapter 4, as well as a discussion of the screen results.

*Number of specific patterns of expression found per clones tested



Adapted from Costa et al., 2003

Figure 3.1 - Schematic view of screening approach and results. My strategy is illustrated in B, and the results in C. **A)** Endoderm patterning at tailbud stages is not well understood, because of the previous lack of markers for the neurula and tailbud endoderm. **B)** My screening strategy. See text for details on methodology. The screen proceeded by many rounds of improvements, that I call iterative. **C)** The results obtained, the re-occurring domains of expression, address the lack of endodermal markers at a stage when patterning must occur. Yellow=endoderm, Green=posterior, Blue=dorsal and Red=ventral midgut expression domains in the tadpole endoderm.

2. Overall Screening Strategy

My screening strategy consisted of three basic steps. The first step included the profiling of several complex probes in order to select differential expressed clones from our midgut macroarrayed cDNA library. The secondary screening step consisted in the verification of the pattern of expression of my candidates by whole mount *in situ* hybridisation. The last step was iterative, and consisted in the use of the hybridisation profile data, from candidates in which specific expression was verified, to improve my next selections of candidate clones (Figure 3.1B).

The overall screening goal was to identify genes expressed in different regions of the endoderm that would be tools to study endodermal patterning. The use of high-density cDNA macroarrays permitted the comparison of several hybridisation profiles, and the record of individual clone signal intensities on all complex probes, which permitted their simultaneous comparison (Figure 3.2). Analysis of the macroarray hybridisation profiles permitted the selection of candidates, which were detected in the positive probes and absent in the negative probes. Hybridisation signal intensity of every individual clone was recorded for all probes on a master file (Figure 3.2). Signal intensity data was acquired through specialized software, VisualGrid™ (GPC, Germany), and exported to Excell (Microsoft™) datasheets where it was compared.

These procedures allowed me to be systematic throughout, and record all clone signal intensities, allowing an iterative selection process for differentially expressed candidates (Figure 3.2). An advantage of my strategy consisted in the ability of selecting candidate clones not only based on the criteria of presence or absence on a single probe but many probes. In addition, my criteria of selection could now be based on relative levels of expression, clone signal intensity, within the same probe, which albeit not quantitative permitted a more sensible approach to the selection of candidate clones, when compared with traditional colony lift differential screens.

The secondary screening step consisted in the construction of antisense digoxygenin labelled RNA probes for each individual candidate. Whole mount *in situ* hybridisations were performed to verify expression. Specific patterns of expression found, and the recorded data about the candidate clones confirmed to be differentially expressed was used to improve the selection of the next round of candidate clones to be tested. Several candidate clones (~20) were not only subject to *in situ* hybridisation procedures but to sequencing as well. Sequence information, hybridisation profile, clone signal intensity, and pattern of expression were used together to guide and improve my selection criteria. The selection criteria used at the end of the screen are described in the results of this chapter (page 53-55).

I have made use of complex probes constructed from different tissues. These tissues include; the midgut tissue itself, fertilized egg mRNA, mRNA extracted from the rest of the embryos where the midgut was extracted, and mRNA extracted from adult organs, liver, stomach, intestine, and blood. I made use of positive probes, such as adult liver probe, that is enriched in endodermal transcripts, since 80% of the adult liver cells are endodermally derived hepatocytes. And compared them with negative probes, i.e., probes made from organs with a high mesodermal component, such as the heart or blood.

I have also used a probe of 126 pre-tested ubiquitous clones containing among others Ef-1α, rRNAs and Cold-Inducible Protein, genes known to be ubiquitous and expressed at high levels.

To determine if a gene was expressed or not, I defined cut-off values or signal intensity thresholds for presence and absence of a signal in the hybridisation profile of a complex probe. Signal intensity thresholds were determined empirically as described below. The value of signal intensity for each individual clone is measure in a scale of 0 to 255. Background signal was evaluated in the brightest area of the macroarray. Signal intensity values lower than background were rendered as undetected clones, establishing the threshold for absence.

For the establishment of the presence threshold, approximately 50 clones were visually examined in the hybridisation profile. Clones which intensity values were 2 fold or higher above background were selected, and examined visually. Visual examination determined if this clones could be confidently assigned as positive in the context of local background. Whenever necessary values were increased until clones could be assigned as positive, establishing the threshold for presence.

At the end of every round of *in situ* hybridisation, many candidates were ubiquitously expressed. A radio labelled probe was made from 126 of these ubiquitous clones and hybridised to the macroarray. In this way, we eliminated from my future selections more than 20% of the clones printed in the macroarray. A major improvement in the overall hit rate was achieved by the elimination of the ubiquitous clones.

The macroarray information about the hybridisation behaviour of the newly found differentially expressed clones was used to improve following rounds of selection, by attempting to select candidate clones with similar hybridisation behaviour across all of the probe hybridisation profiles recorded. Overall, I refer to this as the iterative screening procedure which accounts for the improved hit rate on differentially expressed clones. By the end of the screen, we were retrieving specific patterns of expression in one out of 10 to 20 candidate clones tested by in situ hybridisation. We can compare this with the estimated rate of one specific pattern of expression retrieved per 100 to 200 candidates tested in the

preliminary screen (see below – pre-screening procedures). More than 20 clones presented a eye or cement gland specific expression pattern, because this tissue is already quite differentiated and represented in the library, but no further work on them is presented in this thesis. For an illustration of the differential expression clones retrieved during the screen see Table 3.1 and Figure 3.3.

3. Pre-screening Procedures

The Midgut Library

The library was made from ~2000 ventral midgut explants. It contains presumptive liver, heart, lung and stomach. In fact, this region of the embryo is fated to give rise to several organs (Figure 3.1 B). It is mostly made from endodermal tissue, but it also contains mesoderm and ectoderm. ~55000 individual bacterial colonies were printed in three filters (18,432 colonies each filter). It is estimated that those library clones represent approximately 80% of the complexity of the genes found in those tissues (Galau *et al.*, 1974). During the construction of the library no amplification steps were made, maintaining the relative abundance of all transcripts. The only steps of the library construction procedure affecting the relative abundance of transcripts, and consequently the representativity of the library, are related to the size selection, and with the less efficient synthesis of long transcripts. However, transcripts over 4 Kb are present in the array and only first strand synthesis smaller than 500 bp were discarded.

Preliminary Screen

A preliminary screen using the un-arrayed midgut library had been previously performed in the lab, with the same goal, to identify endodermal specific genes. However, a classical approach was taken, and screening of the library was done through colony lifts. As a negative selection probe, mRNA from the rest of the embryo (minus midgut) was used to construct a complex probe. 820 individual cDNA clones without signal in the negative probe were used to perform whole mount *in situ* hybridisations. From 820 clones used, present in the midgut library and absent from the rest of the embryo, 66 (8%) showed restricted patterns of expression, but only 7 were endoderm specific* (0.8%). Of these 7 only *EphrinB1* and *Chito* were not found in the macroarray screen (see chapter 4).

This preliminary screen revealed few endodermal specific genes, ~0.8%, in spite of the endodermal bias of the library. In addition, after sequencing, all differentially expressed clones belonged to the class of highly abundant transcripts, and were pulled-out more than once. Although interesting genes could be found, I needed a more systematic way of screening the midgut library.

* Restricted expression – meaning not ubiquitous, for instance mainly anterior and ventral.
Specific expression – expression on a specific tissue. (i.e., liver, heart, eye, etc…)

Representativity of the Array

To verify the relative abundance and position of specific transcripts in the macroarray library, I made use of radio labelled probes for *Hex*, *Ef1α*, *HNF3β/FoxA2* and *Vito* (also found in our preliminary screen).

Hex is a divergent homeobox gene expressed in the tailbud ventral foregut endoderm (Newman *et al.*, 1997). Earlier, *Hex* is transiently expressed in the involuting mesendoderm. As development proceeds *Hex* expression is restricted to developing liver bud and the presumptive gall bladder (Zorn and Mason, 2001). *Hex* was originally isolated from a whole embryo cDNA library at low abundance (1 in 10^6 clones). I have found 7 *Hex* clones in the macroarray presumptive midgut library, all of which were confirmed by sequencing (1 in 10^4 clones). Hence, the midgut library is enriched in genes expressed in the ventral tailbud midgut, like the *Hex* transcription factor, by about 50 to 250 fold.

Ef-1α is ubiquitously expressed in all cells (Krieg *et al.*, 1989). *Ef-1α* is an elongation factor, part of the protein synthesis machinery. *Ef-1α* radio labelled probe hybridises with variable degrees of signal intensity to approximately 2% of the arrayed clones printed in the macroarray. In comparison, of all *Xenopus tropicalis* cDNA libraries sequenced so far, (>100,000 EST's) approximately 1% of all EST's cluster as *Ef-1α* sequences (Mike Gilkrist, personal communication), a number not very different 2%. I must conclude then, that ubiquitous sequences are present at high level in the library, and one gene, when present in multiple copies, can be detected at different signal intensities within a simple probe.

FoxA2/HNF3β is a forkhead family transcription factor expressed in the anterior most part of all three germ layers during gastrulation and neurulation (Ruiz i Altaba and Jessell, 1992). It is also expressed in the endodermal tissues giving rise to the pharynx, thyroid and liver, and it is expressed in the tissues that gave rise to the macroarray. A *FoxA2/HNF3β* radio labelled probe detected 21 library clones at low stringency. Sequencing of 16 of these clones revealed that only 2 were indeed *FoxA2/HNF3β*, while the others were forkhead related sequences. This confirms that regulatory molecules are present in the library and can also be detected at low stringency.

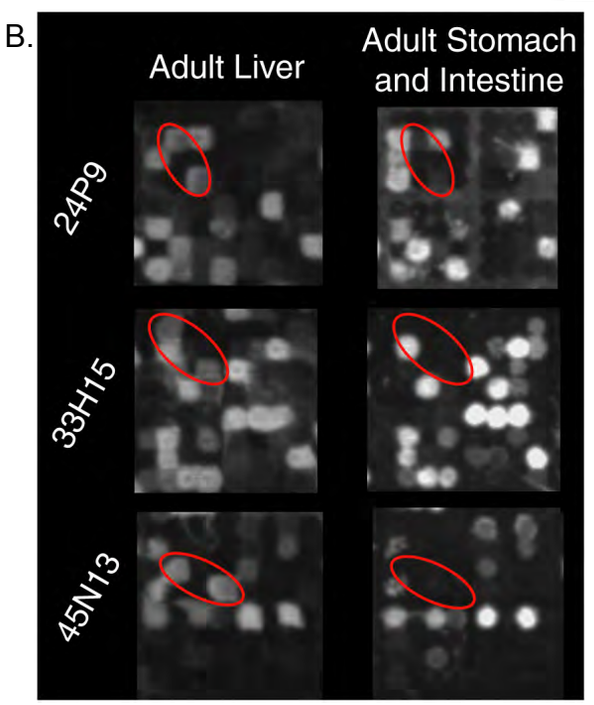
Vito is expressed at high levels in the presumptive midgut tissues during gastrulation through tailbud stages (Chapter 4). *Vito* was at the time uncharacterised, and has no ascribed function. Its relative abundance in the library is of 1 per 10^3 clones of the library. The *Na/K*

ATPase β_3 (Costa, 2003) subunit is another gene expressed in the tadpole midgut. The *Na/K ATPase* β_3 subunit is 10 fold less abundant than the *Vito* transcripts (1 per 10^4). The relative abundance and the position of these clones in the library help me estimating the sensitivity of hybridisation profiles. That is, if *Vito* clones and not β_3 would be detected in a hybridisation profile derived from midgut tissue, I would be detecting transcripts of an order of 10^{-4} (i.e., 1 in 10,000).

With this data in hand and the correlations that can be drawn between them I can estimate the sensitivity of my hybridisation profiles and design selection criteria that avoid testing ubiquitous clones, and increase the likelihood of finding endoderm specific transcripts, like *Hex*.

A.

Clone		Screen ID	Midgut	Rest of Embryo	Midgut	Tail	Adult Liver	Adult Stomach and Intestine
"041 P 10"	7	391	35				89	111
"035 P 1"	6		35	51			130.5	170
"024 P 9"	4		174	178.5			97.5	51
"028 L 5"	5		174	145.5			50.5	55
"033 A 3"	6		156	176			152	91.5
"020 G 11"	4		138	107			92.5	132
"008 O 7"	2		121	48.5			39	49
"033 H 15"	6	477	174	230			92.5	31.5
"010 P 16"	2		121	33.5			19.5	30
"020 B 9"	4		86.5	94.5			74.5	92.5
"022 M 17"	4		86.5	38.5			57.5	56.5
"017 O 20"	3	257/Endocut	35	43.5			116.5	49.5
"016 I 23"	3		86.5	58.5			74	22.5
"027 E 4"	5		86.5	119.5			52.5	70
"036 O 13"	6		86.5	114.5			41	56.5
"004 N 24"	1		86.5	33.5			28	30.5
"045 N 13"	8	506	104	163			129.5	30
"031 E 12"	6		35	51			39	56.5
"027 D 14"	5		35	53.5			139.5	185.5
"025 D 19"	5		35	31			138	111
"041 O 7"	7		35	38.5			136.5	105
"022 C 4"	4		35	41			37.5	45
"014 G 9"	3		35	31			36.5	40.5
"006 G 7"	1	153/Vito	35	36	173.5	52.5	53	53.5



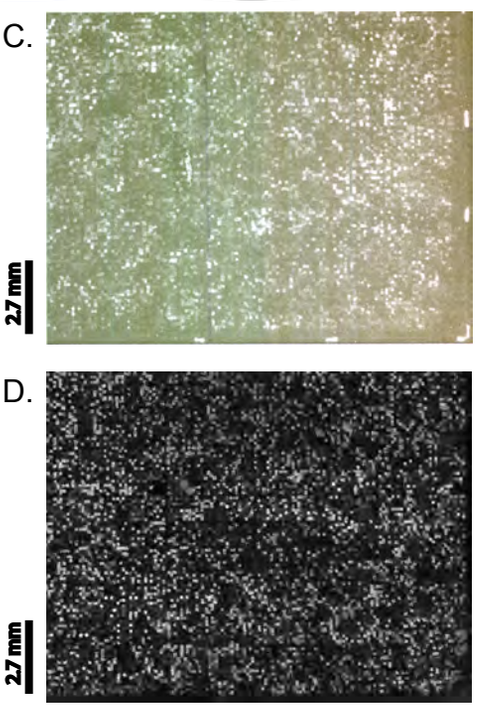


Figure 3.2 - Screening data and hybridisation profiles. **A)** A sample of the master file. The master file is a database containing all clone signal intensity values recorded from all the complex probes used. Rows are from left to right, library clone identification, orientation in the macroarray, my screen identification number, and data from the probes used in the final selection criteria. Probes enriched in endodermal transcripts (positive) were always compared with probes low in endoderm specific transcripts (negative). Purple arrowheads show complex probes derived from embryonic tissues, and yellow arrowheads show complex probes derived from adult tissues. The data is also color coded to assist the identification of differentially expressed clones. On positive probes, clone signal intensity values above the presence threshold were colored green. On negative probes, clone signal intensity values above presence threshold were colored red. The data circled in red is shown in B. Data circled in blue illustrates clones detected as differentially expressed, that is, detected in positive probe and absent in negative probe. Data circled in black shows genes detected at low level in my profiling experiments and can indeed represent differentially expressed genes, such as *Vito*. **B)** Example of how the data circled in red in the master file sample looks like in the 'real' hybridisation profile. **C and D)** Same area of the macroarray hybridised with two different complex probes from the same tissue illustrate the quality difference in the beginning and the end of my screening.

4. Results

I present the criteria used for selection of candidate clones based on my hybridisation profiling data. These criteria of selection resulted from several rounds of improvements done during the screen, and account only for the final part of the screen. I refer to final selection criteria as the way I have selected clones, I consider good candidates to be differentially expressed in the tailbud endoderm. The probes used for the final selection criteria are shown in Table 3.2.

I designated two of the cDNA probes as "positive", since they contained a high proportion of endoderm-enriched sequences. These were the presumptive midgut and adult liver. The three "negative" cDNA probes were made from the tail of stage 28 embryos, a mixture of adult organs such as heart, blood, and stomach, which are made up mainly of mesodermal tissue, and a set of 126 individual cDNAs that I determined as ubiquitous by *in situ* hybridisation. After comparing the hybridisation patterns from all five probes on one filter (18,432 clones) I was able to classify clones into several categories (Table 3.3). On one hand, I eliminated 10755 clones that were present in at least one or more of the negative probes. Out of the 6051 clones that were clearly expressed in the positive probes, 5717 were common to the "negative" pool. Therefore 334 clones, approximately 2% of the array, were judged as being differentially expressed and detected only with the positive probes (Table 3.3).

In addition to these clones, a second pool of 912 clones (5% of the array) was detected consistently below background levels in all five probes. Therefore, the majority of this class represented rare mRNAs expressed in the presumptive midgut, since they were present in the library. Finally, I was unable to assign ~35% of the array as present or absent in the "positive" or "negative" probes because their signal intensity fluctuated between thresholds of presence and absence. In summary, I was able to eliminate ~60% (10755) of the clones as ubiquitous or not enriched in the endoderm, while I identified 2 and 5% of the array, depending on the selection criteria, as good candidates for being endoderm specific or enriched.

I selected the two pools of clones for the secondary whole mount *in situ* hybridisation part of the screen. The pool of the 2% (334) candidate clones was seen as differentially expressed in the tailbud midgut and adult liver based on my hybridisation profiling data. Of the 334 candidate clones, I performed 126 *in situ* hybridisations. From these, I found 26 clones (20%) with spatially restricted expression patterns. The remainder of the clones were either ubiquitously expressed or undetected (Table 3.3). The spatially restricted expression patterns included clones whose expression was enriched in neural tissue, mesoderm, cement gland,

eye, blood islands, and 14 clones with endoderm specific or enriched expression. Subsequent sequence analysis indicated that some clones represented the same gene. I selected five novel uncharacterized endodermal genes; *Complement C3*; *β_3 ATPase subunit*; *IMP2*; *Vito* and *Endocut,* which are characterized in the following chapter.

The 5% pool, 912 candidate clones, were seen as low abundance transcripts likely to be specific to the library and, therefore, the tissue where it originated from, the tailbud midgut. These candidate clones were consistently below the level of detection in all hybridisation profiles. The rational for the selection of these clones was based on the notion that these may represent rare transcripts, as we might expect for regulatory genes, and that we know are expressed in the midgut library. *Hex* fell into this category of clones. I was never able to detect *Hex* clones on the filters with a midgut probe population, even though I knew that *Hex* sequences were present in the probe at a low level. Therefore, similar rare regulatory genes, which are below the level of sensitivity in my differential screen, are likely to be present in this pool of 912 clones.

From this pool of 912 low abundant clones, 432 were assayed by whole mount *in situ* hybridisations (Table 3.3). Of these, 119 clones (27.5%) had spatially restricted expression patterns, only ten of them being endoderm specific. After sequence analysis, I selected six different genes whose endodermal expression had not been previously described, *NDRG1*, *Figo*, *Sam68*, *Pil*, *Vent-2* and *Fetuinish,* and describe them in the following chapter. The remaining genes identified in this pool, which I did not characterize further include, GATA-4 (Jiang and Evans, 1996), BMP7 (Wang *et al.*, 1997a), and the α1 ATPase subunit (Davies *et al.*, 1996).

The cDNAs I found in the pool of low abundance clones, 5% pool, appeared to encode regulatory proteins, such as transcription factors, postulated secreted proteins, and possible inter-cellular signalling proteins. In contrast, the abundant differentially expressed clones, 2% pool, many of which were isolated more than once, encoded metabolic enzymes, proteases, and membrane proteins.

Table 3.1 - Interesting Patterns of Expression Retrieved from the Screen

Screen ID	Array pos.	Initial description of the Pattern of Expression	Sequence
72	"120 K 6"	Presumptive Liver Region	Vito
93	"087 E 11"	Ectoderm	
109	"104P22"	Cement Gland	
138	"001 C 7"	Neural crest and Endoderm	Complement C3
141	"001 D 20"	Neural Crest and Liver Region	
176	"001 J 3"	Blood Islands	SpiB
186	"001 K 8"	Similar to foxA2/HNF3b	
210	"001 P 5"	Eye and Neural Tube	
247	"017 F 11"	Scattered Surface Cells - Cilliated Cells?	
257	"017 O 20"	Posterior Endoderm	Endocut / Darmin
477	"033 H 15"	Similar to foxA2/HNF3b	
486	"047 O 23"	Neural Plate and Eye	
489	"047 M 23"	Middle of the eye	
491	"047 K 22"	Anterior mesendoderm	
546	"040 E 22"	Border of Neural folds	
676	"020 P 7"	Neural Tube, eye, Kidney and somites	
677	"020 P 8"	Neural Crest and Scattered Cells	
701	"044 O 7"	Liver Diverticulum, Kidney	
712	"002 C 4"	Dorsal Endoderm, Cilliated Cells	Fetuinish
714	"002 D 14"	Small patch in front of Head	
718	"002 E 1"	Notochord and Border Endoderm	
719	"002 E 4"	Hatching Gland	
725	"002 G 2"	Somites	
727	"002 G 4"	Endoderm	
764	"002 N 19"	Hatching Gland and Endoderm	
846	"008 I 18"		NDRG1
847	"008 I 19"	Presumptive Liver Region	Figo
897	"008 P 7"	Notochord with two dots in front of Head	
904	"009 O 5"	Somites	
934	"012 M 12"	Presumptive Liver Region	BMP7
945	"014 B 6"	Neural	
1006	"014 P 14"	Heart or Liver	Sam68
1012	"015 B 1"	Presumptive Liver Region and Somites	Pil
1057		Cement Gland, Tip tail, Dorsal Endoderm	XVent-2
1090	"020 L 14"	Two dots in front of head	
1093	"020 L 7"	Mesoderm, presumptive nephric region	CGRP-RCP

Eye or Cement Gland Specific Patterns: 31D24; 29N24; 29G22; 10P16; 20N16; 2B1; 2B2; 2K8; 2L4; 2M16; 2M2; 2O21; 2P23; 4P11;4P23; 4P4; 8A1; 8C2; 8C4; 8J8; 14D2
EphrinB1, IMP2, DG42, Gata4, α1 ATPase, β3 ATPase and Chito are not listed here.

Figure 3.3

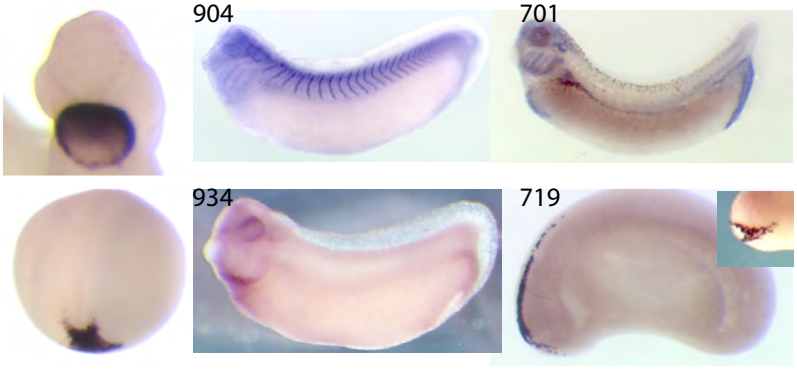


Figure 3.3 - Different expression patterns obtained in the course of the screen. These were not characterised because of their non-endodermal expression. Numbers indicate the screen identification number in Table 3.1. Or in the case of the left most panels they represent one example of one cement gland specific pattern of expression.

Table 3.2 - Complex Probes Used for the Final Selection Criteria

Positive Probes	Negative Probes
Midgut library	Tail library
Adult Liver	Adult Stomach, Intestine, Heart
	126 pre-tested Ubiquitous Clones

Table 3.3 Results from Final Selection Criteria and respective WMISH

cDNA clones	number	% of array	
Total in the array	18,432	100%	
Hybridized with "negative " probes:			
(stage 28 tail / adult stomach + heart + intestine /126 ubiquitous cDNAs)	10,755	58%	
Hybridized with "positive" probes: (stage 22-24 midgut / adult liver)	6,051	33%	
Hybridized to both "negative" and "positive" probes	5,717	31%	
Ambiguous clones with uncertian hybridization signals	~6,500	35%	
Differentially expressed in the "positive" probes	334	2%	% of in situs
Differentially expressed clones selected for in situ hybridization	126		100%
Spatially restricted expression patterns	26 of 126		20%
Restricted expression in the endoderm	14 of 126		11%
Low abundance clones - never detected with any probe	912	5%	% of in situs
Low abundance clones selected for in situ hybridization	432		100%
Spatially restricted expression patterns	119 of 432		28%
Restricted expression in the endoderm	10 of 432		2%

CHAPTER FOUR – ENDODERMAL DOMAINS OF EXPRESSION

0.Abstract

1.Introduction

2. Results

2.1.Ventral Midgut Expression Domain
Vito
β3-subunit Na^+/K^+ ATPase
Figo
Sam68
Pil
EphrinB1
2.2.Posterior Endoderm Expression Domain
Endocut
Complement C3
IMP2 & Chito
2.3.Dorsal Endoderm Expression Domain
Fetuinish
NDRG1
Vent-2

2.4.Early Expression of Endodermal Markers
2.5.Expression in Organ Buds

3.Preliminary Functional Studies

4.Discussion
Screening Strategy and Efficiency
Differential Screening Sensitivity
Reproducibility of Hybridisation Profiles
Improving the Screen
Expression of Endodermal Genes
Domains of Expression – Endodermal Patterning
Future Directions – Endodermal Patterning
Future Directions – Functional Analysis

0.Abstract

My screening identified novel endodermal genes expressed in the *Xenopus laevis* endoderm. I present the molecular nature of those genes and their pattern of expression. Interestingly, these genes are expressed in three reoccurring sub-domains of the endoderm, which had not been previously reported. The existence of such early endodermal expression domains indicates some degree of patterning in the gastrula, neurula and tailbud endoderm. Some of these genes might be involved in the control of endoderm development.

1.Introduction

My secondary *in situ* hybridisation screen identified a list of clones in which a specific or restricted pattern of expression was observed (Table 3.1). This list contains clones that were detected in all germ layers, and not only in the endoderm. The screen provided more than 20 clones expressed in tissues other than the endoderm, such as eye, neural tube or cement gland. The reasons for finding clones expressed in tissues other than the endoderm include; 1) the tissue used to construct the library, and the hybridisation probes used for profiling, were not purely endodermal. 2) It is possible that the endoderm is a less differentiated tissue, that is, it contains less differentiated cells when compared with the eye or cement gland, and therefore may contain less endodermal specific transcripts. Besides the mesodermal expression of the transcription factor *SpiB* (see chapter 5), and the endodermally expressed genes (Table 4.1), I do not describe here any of these other genes. My goal was to perform an analysis of endoderm development. Hence, I have focused on the characterization of the endodermally expressed clones (Costa *et al.*, 2003).

Table 4.1 Characterized cDNAs with Endodermal Expression

Name	Accession number of full length clone	Unigene Cluster	pFAM number	Domains / Homology	Number of isolates	Category of Selection
Vito	AY260728-30	Xl.6392	00474	Sodium solute transporter	3	Differential
ß3 ATPase	M37788	Xl.6045	00287	Na/K ATPase ß3 subunit	2	Differential
EphrinB1	U31427	Xl.302	00812	Ephrin B1 or Xlerk	1	Differential[1]
IMP2	BC042315	Xl.2579	00478	Inosine-5'-monophosphate Dehydrogenase	2	Differential
Chito	AY260731	Xl.6837	00704	Glycosyl Hydrolases / di-N-acetylchitobiase	1	Differential[1]
Endocut	BC045077	Xl.6024	01546	Glutamate Carboxypeptidase M20	3	Differential
C3	U19253	Xl.2209	00207	Complement C3	2	Differential
DG42*	M22249	n.d.	n.d.	hyaluronan synthase 1	2	Differential
Gata4*	U45453	n.d.	n.d.	Zinc finger transcription factor	1	Undetected
Figo	AY277253	none	none	unknown	1	Undetected
Sam68	AY260734	Xl.5538	00013	RNA-binding KH Domain / Sam68	1	Undetected
Pil	AY260733	Xl.8891	12370	Protein Phosphatase Inhibitor / IPP-1	2	Undetected
Fetuinish	AY260732	Xl.5948	00031	cystatin protease inhibitor / Fetuin-ß	1	Undetected
NDRG1	BC046693	Xl.18152	03096	NDRG /N-myc downstream regulated gene 1	1	Undetected
Vent-2	X98849	Xl.699	00046	Homeodomain transcription factor	1	Undetected
BMP7*	U40034	n.d.	n.d.	Bone morphogenetic protein	1	Undetected
α1ATPase*	U49238	n.d.	n.d.	Na/K ATPase α1 subunit	1	Undetected

[1]. Clones isolated in the preliminary differential screen; * Clones previously whose expression was known; n.d. = not determined.

Table 4.2 - Endoderm specificity and non-endodermal expression of the genes characterized

	onset	end	Endoderm marker for	Other Tissues Tailbud 22-26	Endoderm marker for	Other Tissues Early tadpole 28-33	Endoderm marker for	Other Tissues Tadpole 35-39
Vito	11	35	*Ventral Midgut*	Not detected	*Pres. Liver, gb*	Tail tip and Pronephros	-	Not detected
ß3	11	40	*Ventral Midgut*	Neural Tube, Somites, Proctodeum	*Pres. Liver, gb*	Neural Tube, Somites, Proctodeum	-	Neural Tube
Figo	>20	40	*Ventral Midgut*	Not detected	*Endoderm*	Pronephros	*Liver*	Pronephros
Sam68	>20	42*	*Ventral Midgut*	Not detected	*Gall bladder*	Head, Neural Tube	-	Head, Neural
Pil	22	40	*Ventral Midgut*	Somites	*Pres. Liver*	Somites	-	Not detected
EphrinB1	8a	n.d.	*Ubiquitous*	(see previous publications)	*Liver diverticulum*	Head, Brancheal Arches	*Liver*	Head, Foregut, Presumptive Liver
Endocut	11	42*	*Post. Middgut*	Not detected	*Post. Midgut*	Not detected	*Pres. Intestine*	Not detected
C3	16	42*	*Post. Midgut*	Neural Crest	*Post. Midgut*	Neural Crest	*Pres. Intestine*	Not detected
IMP2	>20	42*	*Post. Middgut*	Not detected	*Post. Midgut*	Not detected	*Pres. Intestine*	Not detected
Chito	>20	42*	*Post. Middgut*	Not detected	*Post. Midgut*	Not detected	*Pres. Intestine*	Not detected
Fetuinish	16	42*	*Foregut*	Epidermal Cells	*Pres. Liver*	Not detected	*Pres. Liver*	Not detected
NDRG1	>20	42*	*Dorsal End.*	Mesoderm and Pronephros	-	Brancheal arches, mesoderm, pronephros, eye	*Liver diverticulum*	Pronephric duct, dorsal eye, brain boundary
Vent-2	<11	42*	*Dorsal End.*	(see text)	*Dorsal End.*	(see text)	-	(see text)

Table 4.2 - Pattern of expression of the genes characterised. Genes are color coded as in figure 3.1C. Red for ventral midgut, green for the posterior endodermal domain and blue for the dorsal endoderm expression domain. Expression analysis was done until stage 42 (42*). The pattern of expression of *Chito* and *IMP2* has not been reproduced.

2.Results

The clones characterized here are novel endodermal markers. The expression of these 13 markers was observed in re-occurring endodermal territories or domains*. Table 4.1 describes the accession numbers for all the cDNAs with endodermal expression found, as well as their Unigene cluster number, pFAM number (gene family), the identified protein structural motif and the most similar protein present in the database. Full-length coding sequences were isolated for *Vito*, and two of its splice variants, for *Endocut,* and for *Fetuinish*. All other sequence information was retrieved from the sequence of partial clones and the identification of the Unigene cluster. Some of the isolated clones represented the same gene; this is more common in the 'differential' category of selection, 2% pool (see chapter 3), where the selection criterion yields transcripts of higher abundance (Table 4.1).

The patterns of expression of a transcript tend to be highly dynamic both spatially and temporally (Figures 4.1 to 4.5). Although mostly endodermal, some of the genes are expressed in other tissues. Table 4.2 describes the details of when and where the gene expression was detected. I have only characterized the expression until stage 42, which is the initial gut coiling stage.

While characterizing the expression of these clones, I realized that they were expressed in re-occurring patterns at tailbud stage, which I classified as domains of expression. Such domains of expression were unknown and unexpected, since the specification of different fates in the endoderm was thought to occur much later, around stage 28 (Horb and Slack, 2001; Zeynali *et al.*, 2000). The presence of these domains implies a degree of positional information not previously recognized in the early tailbud endoderm. Figure 3.1C illustrates the localization of such domains and their markers. Such findings clearly illustrate the usefulness of our screening strategy, as it unravelled new patterning markers for the early endoderm. The domains found are; the ventral midgut, posterior endoderm, and dorsal endoderm expression domains, and their respective markers are described below.

2.1.Ventral Midgut Expression Domain

I found 6 genes (*Vito, β_3-ATPase subunit, Figo, Sam68, Pil*, and *EphrinB1*) whose common characteristic is the expression in the midgut endoderm at tailbud and early tadpole stages (Figure 4.1). This region is fated to give rise to liver, lung, pancreas, gallbladder and stomach (Chalmers and Slack, 2000). The genes *Fetuinish* and *NDRG1* could be included in this domain of expression because they also exhibit a later ventral midgut expression, but I

* Domains of expression: area of the embryo to which the expression of 2 or more genes is restricted.

have included them in a third domain of expression due to their earlier strong dorsal endodermal expression.

Vito

The full-length *Vito* mRNA is predicted to encode a novel seven pass transmembrane, Na^+/solute symporter protein. Using Na^+ chemiosmotic energy this class of proteins is thought to be involved in the cellular import of small molecules such as vitamins, ions or amino acids (University of California San Diego Transport Protein Database entry: TC 2.A.21 - http://tcdb.ucsd.edu/tcdb/background.php). All the *Vito* clones we initially identified were partial cDNAs, and subsequently we have isolated six full-length cDNAs approximately 3.1 Kb in length. These represented two different mRNA splice variants, encoding 623 and 403 amino acid proteins respectively, with alternative carboxyl ends (Acession no., AY260728, AY260729, AY260730). The protein most similar to *Vito* in the database was an uncharacterised human apical iodine transporter (Accession no., NP_666018), 67% identical to *Vito* at the amino acid level. The function of *Vito,* or related solute transporters, during embryonic development is unknown. At tailbud stages, *Vito* transcripts are abundant in the ventral midgut region, which makes it an excellent marker of the ventral midgut tissue. As development proceeds, *Vito* transcripts become restricted to the liver diverticulum and ultimately to the presumptive gallbladder by stage 35 (Figure 4.1). Other sites of expression include the pronepheros and the tip of the tail.

β_3-subunit of the Sodium/Potassium ATPase

Expression of the *β_3-subunit* of the Sodium/Potassium ATPase has previously been described in the brain and neural tube (Good *et al.*, 1990; Messenger and Warner, 2000). The *β_3-ATPase subunit* is described here because is also strongly expressed in the tailbud ventral midgut endoderm, which had not been reported before. Like *Vito*, the endodermal expression of β_3 *ATPase subunit* is progressively restricted to the presumptive gallbladder by stage 37 (Figure 4.1).

Figo

The clone that we call *Figo*, appears to represent a partial cDNA with no obvious structural motifs, and only one other EST in the database. *Figo* is expressed in the ventral midgut endoderm at tailbud stages. *Figo* transcripts are then detected more broadly

throughout the endoderm at stages 28-33. By stage 35, *Figo* transcripts are once again restricted to the hepatic primordium and strong expression is also observed in the pronepheros (Figure 4.1).

Sam68

We have isolated a partial clone that appears to encode *Xenopus Sam68* (Accession no. AY260734). Mammalian *Sam68* is a RNA-binding KH domain protein postulated to play a role in growth control. *Sam68* was originally identified as a target of Src tyrosine kinase in mitotic cells (Fumagalli *et al.*, 1994; Taylor and Shalloway, 1994). *Xenopus Sam68* has not previously been reported, but EST database searches and analysis of the Unigene cluster (Xl.5538) suggests a putative protein 77% identical at the amino-acid level to rat *Sam68*. *Xenopus Sam68* expression is first strongly detected in the anterior ventral endoderm at tailbud stages, which becomes resolved to the presumptive gallbladder by stage 32 (Figure 4.1 arrow). *Sam68* transcripts are also strongly expressed in the developing head tissue and neural tube.

Pil

The *Xenopus Pil* (Phosphatase Inhibitor Like) cDNA appears to be full length and encode a novel 185 amino acid protein, which is 50% identical at the amino acid level to human protein phosphatase inhibitor 1 (PPI-1). *PPI-1* is known to inhibit the activity of protein phosphatase 1 (PP1) in response to cAMP in many biological contexts (Oliver and Shenolikar, 1998). *Pil* transcripts are first detected at late neurula where they are restricted to the ventral midgut endoderm and somites throughout development, and then down regulated by late tadpole stages (Figure 4.1).

EphrinB1

EphrinB1, also known as *Xlerk-2*, a ligand for the Eph family of tyrosine kinase receptors is already known in *Xenopus laevis* (Helbling *et al.*, 1999; Jones *et al.*, 1997). I include *EphrinB1* here, because of its presumptive hepatic expression which has not previously been described (Figure 4.1). Ephrins and their receptors counterparts have been implicated in a variety of biological functions such as vascular development, tissue-border formation, cell migration, tissue patterning, axon guidance, and regulation of cell adhesion (Kullander and Klein, 2002).

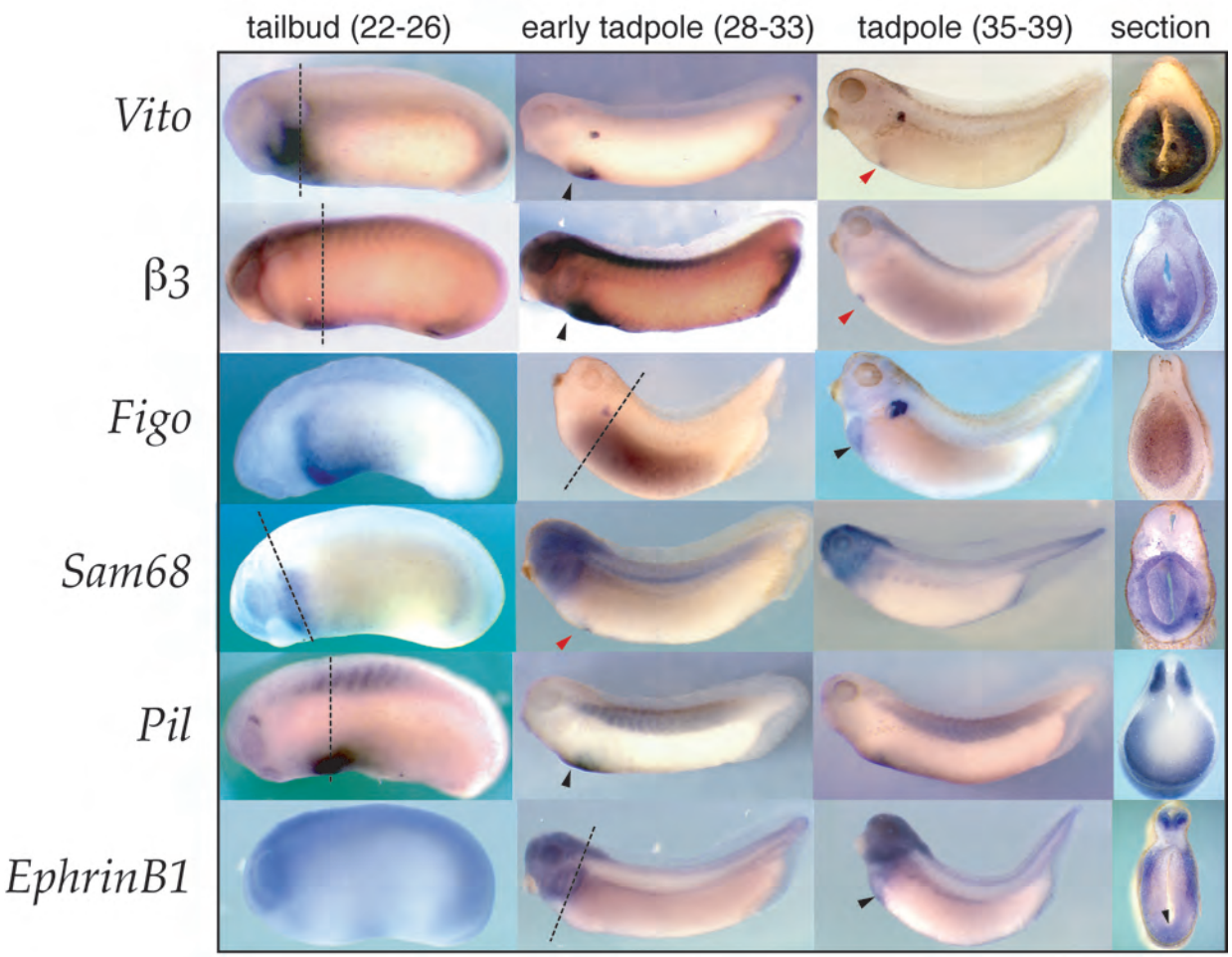


Figure 4.1- Ventral midgut expression domain. *Vito,* β3, *Figo, Sam68, Pil* and *EphrinB1 in situ* hybridisation from stages 22 to 39. Black arrowheads indicate expression in the liver diverticulum, and red arrowhead show expression in the prospective gall-bladder. The thin black line indicates the position along the anterior-posterior where cross sections, shown in the right, were taken. For the whole embryos, dorsal to the top, anterior to the right. In the sections, dorsal is to the top.

In summary, the 6 genes described above all share a restricted endodermal expression in the midgut, fated to give rise to liver, stomach, pancreas and lungs (Chalmers and Slack, 2000). Another general feature of these genes is that the ventral midgut expression at early tailbud is restricted as development proceeds, in several cases just to the gall bladder precursors, indicating that additional patterning is likely to refine cell fates between stages 22 to 35. This is consistent with the idea that patterning is a continuous process, happening as morphogenesis occurs.

2.2 Posterior Endoderm Expression Domain

Four of the genes we identified had almost identical posterior endoderm restricted expression patterns (Figure 4.2). Three of them are excluded from the anterior endoderm to the presumptive midgut. Based on the fate map, this region of the embryo is predicted to give rise to the intestine immediately posterior to the stomach (Chalmers and Slack, 2000). Blast homology searches, analysis of protein motif databases and EST Unigene clusters revealed these three posterior genes encoded; *IMP2* (inosine-5'-monophosphate dehydrogenase 2), *Chito* a novel di-N-acetylchitobiase, and *Endocut* a glutamate carboxypeptidase, also characterised by others (Chen *et al.*, 2003; Pera *et al.*, 2003). I also describe the *Xenopus* homologue of *complement C3* (Lambris *et al.*, 1995) in this section due to its similar strong posterior endodermal expression, although it is also expressed in the ventral midgut earlier (Figure 4.2).

Endocut

Full-length *Endocut* encodes a glutamate carboxypeptidase (Accession no. AY66869/ BC045077). The predicted *Endocut* protein contains a M20 domain common in metalloproteases (Rawlings and Barrett, 1995). Our expression analysis revealed that *Endocut* transcripts are robustly expressed in the posterior endoderm throughout tailbud and tadpole stages, but they are excluded from the midgut and anterior endoderm (Figure 4.2). This expression is virtually indistinguishable from *IMP2* and *Chito*. Proteases have been shown to be involved in development by cleavage of pro-active signalling factors rendering them active. My initial functional experiments by over expression have shown no obvious phenotype, and the same as been reported by others (Pera *et al.*, 2003). The importance of *Endocut* in posterior endoderm development is unclear at the moment.

Complement C3

Complement component C3 is a serum protein produced by the liver, and it is well known as a pivotal protein in the immune system. Its functions include defence against infection, immune responses, and the disposal of cellular waste (Halkier, 1991; Walport, 2001a; Walport, 2001b). The embryonic expression of *Xenopus C3* has not been reported (Lambris *et al.*, 1995). *C3* expression is first observed in the neural crest at stage 16 (Figure 4.4). By stage 24, expression is seen in the neural crest along the entire anterior posterior axis (Figure 4.2 arrow). By stage 28, two domains of endodermal expression are observed in a pattern that is reminiscent of other adult liver enzymes, such as *fibrinogen* and *AMBP* (Zorn and Mason, 2001). The first domain is restricted to the liver diverticulum. Immediately posterior to the liver diverticulum there is a band of endoderm, which gives rise to the stomach and pancreas, that has low or no *C3* expression. The second domain of expression is posterior to the presumptive stomach, and indistinguishable from *Endocut.*

IMP2 & Chito

Sequence analysis revealed that I had isolated a 566 bp clone corresponding to the 3'untranslated region of Xenopus *Inosine-5'-monophosphate dehydrogenase 2* (IMP2, Accession no. BC042315), which was deposited in the database by the *Xenopus* full-length cDNA sequencing project (Table 4.1). In mammals, two IMP isoenzymes exist, and this metabolic enzyme is required for the synthesis of guanine nucleotides (enzyme classification number: EC.1.1.1.205). The deletion of mouse *IMP2* by homologous recombination is early embryonic lethal, but the reason for this is unclear since deletion of IMP1 is not lethal (Gu *et al.,* 2000). *Xenopus* IMP2 was previously uncharacterised and we found that it was exclusively expressed in the endoderm posterior to the presumptive midgut (Figure 4.2).

The *Chito* cDNA appears to be full length (~1.2 Kb) and encodes an uncharacterized di-N-acetylchitobiase (Accession no. BC041322) most similar at the amino acid level (62%) to rat lysosomal glycosidase di-N-acetylchitobiase (Accession no. AAA40924, enzyme classification number EC.3.2.1.-). Di-N-acetylchitobiases are known to be involved in processing of glycoproteins (Liu *et al.*, 1999). The importance of finding such a specific endodermal expression for an enzyme thought to be required in all cells is unknown. *Chito* is first expressed at the tailbud stage in the posterior endoderm in a pattern similar to *IMP2* and *Endocut* (Figure 4.2). However, I have not been able to reproduce the pattern of expression obtained for these two cDNA's.

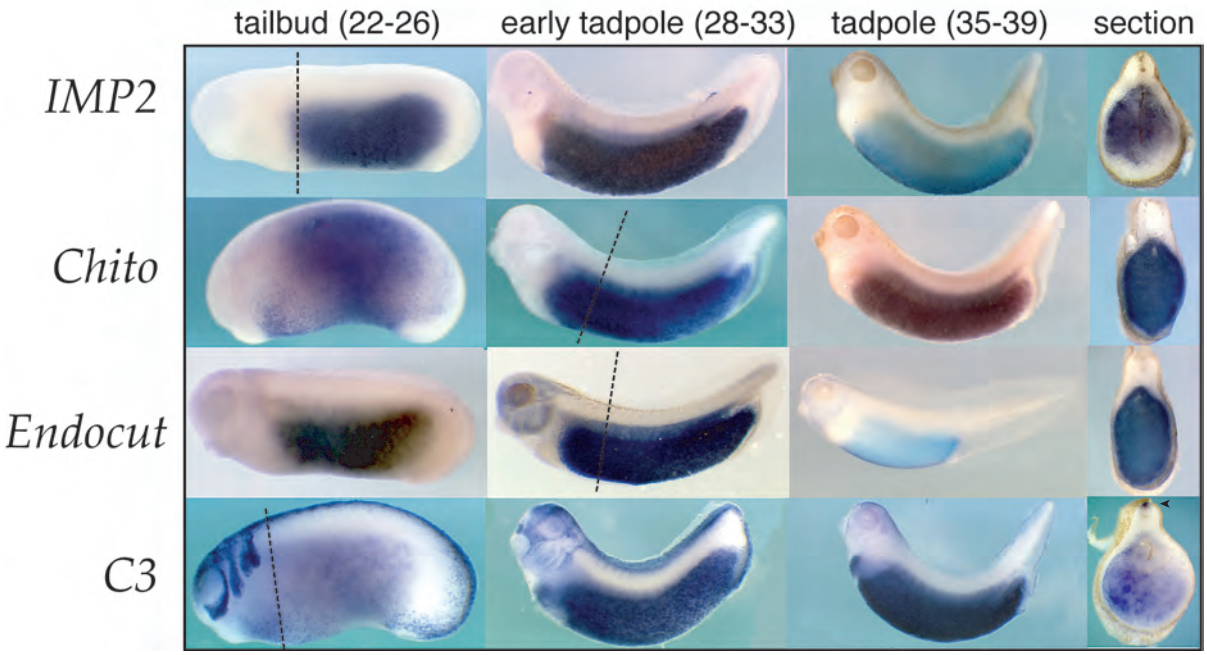


Figure 4.2 - Posterior endoderm expression domain. Whole mount *in situ* hybridisation with antisense *Chito, IMP2, Endocut,* and *Complement C3,* from stage 22 to 39. Fine black line shows the plane of the section, presented on the right. For whole embryos left is anterior, top is dorsal. Black arrowhead in the C3 section points to the staining in the neural crest. I have not been able to reproduce the *Chito* and *IMP2* pattern of expression.

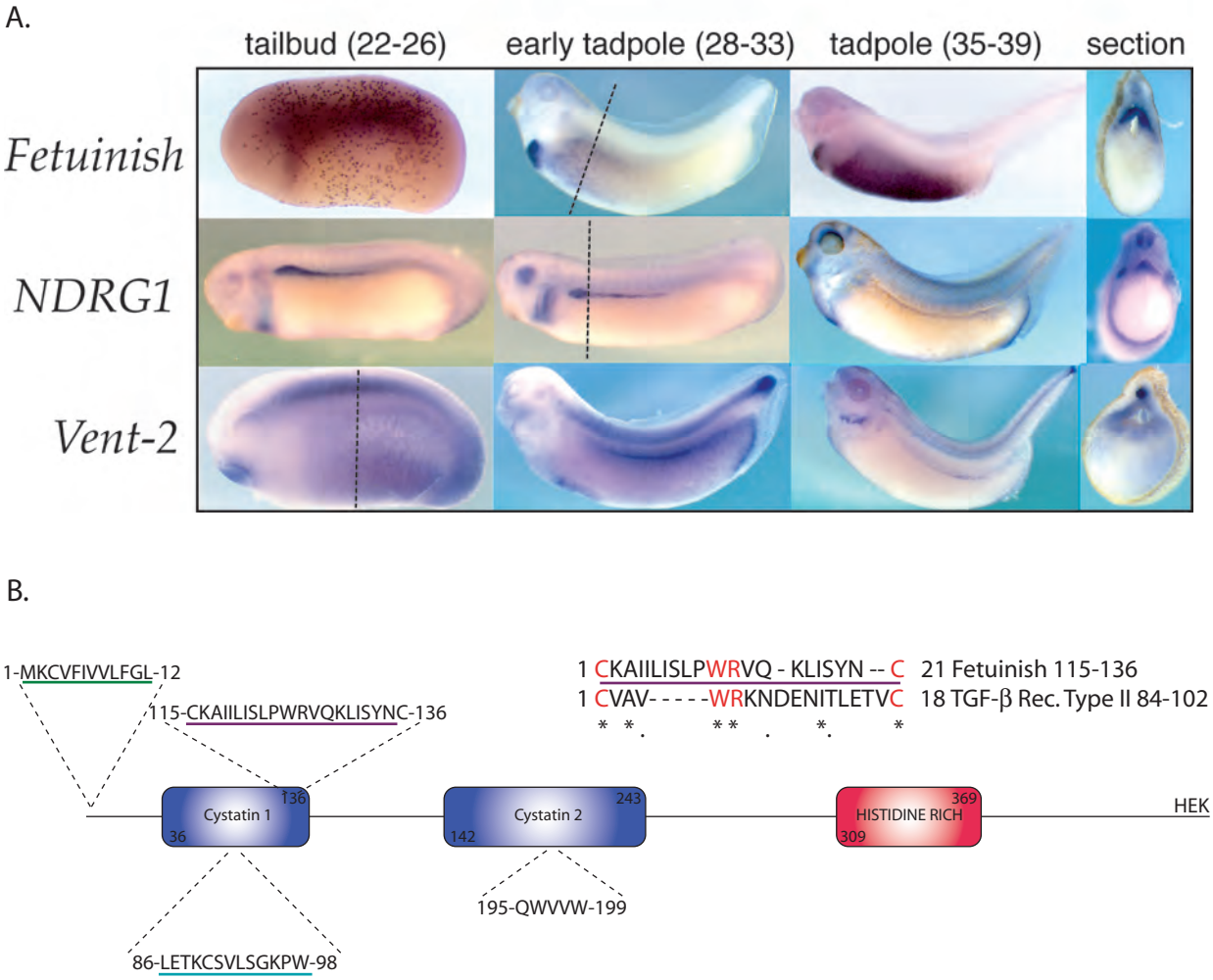


Figure 4.3 - Dorsal endoderm domain of expression. **A)** Genes expressed in the dorsal endoderm at tailbud and tadpole stages (22 to 39), *Fetuinish*, *NDRG1*, and *Vent-2*. Fine black line shows the plane of section taken and presented on the right. Scattered stain in the tailbud *Fetuinish* embryo are epithelial cells in an albino embryo. All other embryos were bleached. Dorsal endoderm staining is very difficult to observe in whole mount procedures, the likely reason for which the endodermal expression of *Vent-2* had not been reported. **B)** Molecular domains of *Xenopus laevis Fetuinish*, peculiar motifs are highlited , in green the secretion motif, in light blue the Fetuin family signature, in light purple the low conserved stretch with homology to the human type II TGF-β receptor. Homology is shown with red letters meaning functionally conserved aminoacids. Fetuinish has many peculiar motifs that contrast with its unknown funtion, its one of the few molecules with two cystatin domains, and contains a histidine rich region with 50 histidines in a stretch of 60 aminoacids. For whole embryos, anterior is to the left, dorsal to the top.

2.3 Dorsal Endodermal Expression Domain

Three of the genes we isolated (*Fetuinish, NDRG1,* and *Vent-2*) shared a dorsal to ventral gradient of expression at tailbud stages, highest in the dorsal endoderm underlying the notochord (Figure 4.3). Based on the available endoderm fate map, the dorsal endoderm is fated to be, in an anterior-posterior direction, or according to the time of involution through blastopore lip, posterior pharynx, oesophagus, stomach, dorsal pancreas and small intestine. The available fate map shows that these cells are not fated to become liver (Chalmers and Slack, 2000; Keller, 1975; Keller, 1976).

Fetuinish

Our full-length *Fetuinish* cDNA (~1.5 Kb) is similar to sequences submitted by the *Xenopus* full-length cDNA sequencing project and the Pieler group (Chen *et al.*, 2003). Overall 6 point substitutions exist in all of the available DNA sequences, 3 of which are silent (Accession no. BC043891), encoding a secreted protein with two cystatin domains and a histidine rich domain (Figure 4.3B). Cystatin domains are present in proteins that inhibit cysteine protease activity and present an array of biological functions. The closest homologues to *Fetuinish* are the cystatin family members, fetuins. These molecules are involved in processes as diverse as inhibition of the insulin receptor tyrosine kinase, osteogenesis, and TGF-β signalling inhibition (Brown *et al.*, 1992; Demetriou *et al.*, 1996; Olivier *et al.*, 2000; Szweras *et al.*, 2002). The predicted *Fetuinish* protein is 33% identical at the amino acid level to human *fetuinβ* and 38% identical at the amino acid level to HRG (Histidine Rich Glycoprotein), both of which are proteins of yet unknown function. At tailbud stage, *Fetuinish* transcripts are detected in the dorsal endoderm beneath the notochord, except at the level of the midgut/liver diverticulum, where expression extends ventrally (Figure 4.3). At this stage in development, *Fetuinish* is also expressed in ciliated epithelial cells. By stage 28, its expression pattern changes to resemble that of *complement C3*, with transcripts abundant in the liver diverticulum, and at a lower level, in the posterior endoderm.

NDRG1

Human *NDGR1* (N-myc down regulated gene 1) encodes a protein implicated in cell differentiation, and was first discovered in neoplastic cells because it was down regulated by the proto-oncogenes c-myc and n-myc (Kokame *et al.*, 1996; Strieder and Lutz, 2002; Zhou *et al.*, 2001). The full length *Xenopus NDGR1* was recently reported by the *Xenopus* full length

cDNA sequencing project (Accession no. BC0436693), but its embryonic expression has not been documented. *NDGR1* transcripts were localised to the dorsal endoderm beneath the notochord, the neural tube, and the nephric region, and weakly in the brachial arches. In tadpole embryos, *NDGR1* mRNA was also detected in the dorsal eye, brain, liver diverticulum and pancreatic midgut endoderm (similar to the pancreatic marker *Pdx1*), and proctodeum precursors (Figure 4.3).

Vent-2

The homeodomain transcription factor *Vent-2* (*Xom, Xbr-1, Vox*) is well known for its role in early mesoderm formation (Ladher *et al.*, 1996; Onichtchouk *et al.*, 1996; Papalopulu and Kintner, 1996; Schmidt *et al.*, 1996). While *Vent-2* has been studied in the ventral mesoderm, notochord, brachial arches, dorsal eye and the tail tip, its expression in the dorsal endoderm has not previously been reported. We detect *Vent-2* mRNA in a dorsal-ventral gradient in the tailbud endoderm (Figure 4.3). *Vent-2* is also expressed in the liver diverticulum, and in isolated guts at low level in the ventral pancreatic bud.

2.4.Early Expression of Endodermal Markers

Since three distinct domains of gene expression exist in the tailbud embryo, I next asked how early in development these markers might be used to visualize endodermal patterning. I examined the expression of all 13 markers in gastrula and neurula stage bisected embryos by *in situ* hybridisation to reveal the deep endoderm tissue. Four of the genes, *Vito*, *β3-ATPase subunit*, *Endocut* and *Fetuinish* are expressed at gastrula or neurula stages (Figure 4.4).

Vito and *β3-ATPase subunit* are expressed in the gastrula anterior endoderm, similar to *Hex* (Zorn *et al.*, 1999), and in the entire circumference of the blastopore lip superficial endoderm (Figure 4.4). In addition, *β3-ATPase subunit* transcripts are detected in the deep endoderm at the mesoderm/endoderm boundary, and in the notochord and neural tube of the stage 20 embryos. In contrast to *Vito* and *β3-ATPase subunit*, *Endocut* is expressed in the deep yolky endoderm cells of the gastrula, but not in the superficial layer of the blastopore lip. Others have found the same pattern of expression, supporting my findings (Chen *et al.*, 2003; Pera *et al.*, 2003). This expression pattern is reminiscent of Gata4/5/6, which is also expressed in the deep, but not the superficial endoderm (D'Souza *et al.*, 2003; Weber *et al.*, 2000). By neurula stage, *Endocut* is already excluded from the anterior endoderm and localised to the middle of the deep endoderm, like the late *Sox17α* expression. Finally, *Fetuinish* is first detected in the dorsal endoderm as early as stage 15 (Figure 4.4). Thus, as

early as mid neurulation, stage 15-18, the ventral midgut, posterior and dorsal endoderm expression domains appear mutually exclusive. Furthermore, these gene expression domains appear to mark regions of the embryo with different developmental potential, as predicted by the endoderm fate maps (Chalmers and Slack, 2000; Keller, 1975; Keller, 1976). For example, based on the fate maps, the superficial, *Vito*-expressing gastrulating endoderm gives rise to the *Vito*-expressing anterior ventral tissue, which in turn gives rise to the liver, pancreas and lung precursors; The *Endocut*-expressing deep endoderm, on the other hand, gives rise to the future intestine. Thus, the endoderm appears to have a significant degree of positional information long before regional specification is thought to occur.

2.5.Expression in Organ Buds

I was also interested in finding out if the tailbud gene expression domains predicted later organs of the gut tube. Lineage labelling is the definitive test of this; nonetheless, the examination of gene expression with the knowledge of the fate map can also be informative. We found that *NDGR1*, *Sam68*, *Fetuinish*, *Vent*-2, *Endocut* and *C3* were all expressed in the stage 42-gut tube (Figure 4.5). *Sam68* was expressed in the ventral midgut at tailbud stages, whereas *NDRG1* and *Vent-2* were in the dorsal domain. Yet, all three were later expressed in the pancreatic buds and the stomach in a pattern resembling *Pdx1* (Zorn and Mason, 2001). In contrast to *NDGR1* and *Vent-2*, the two other dorsal expressed genes, *Fetuinish* is expressed in the liver bud and weakly in the intestine, but not in the stomach and pancreas. *NDRG1* and *Sam68* were also expressed in the proctodeum. Thus, for these genes, there was not a strict correlation between where they were expressed at tailbud and the organ buds they were expressed in later. However, a strong predictive correlation was observed for the posteriorly expressed genes. Of these, *Endocut* and *C3* were strongly expressed in the developing intestine posterior to the stomach and pancreas, consistent with the idea that at tailbud stage they already mark the presumptive intestine (Figure 4.5).

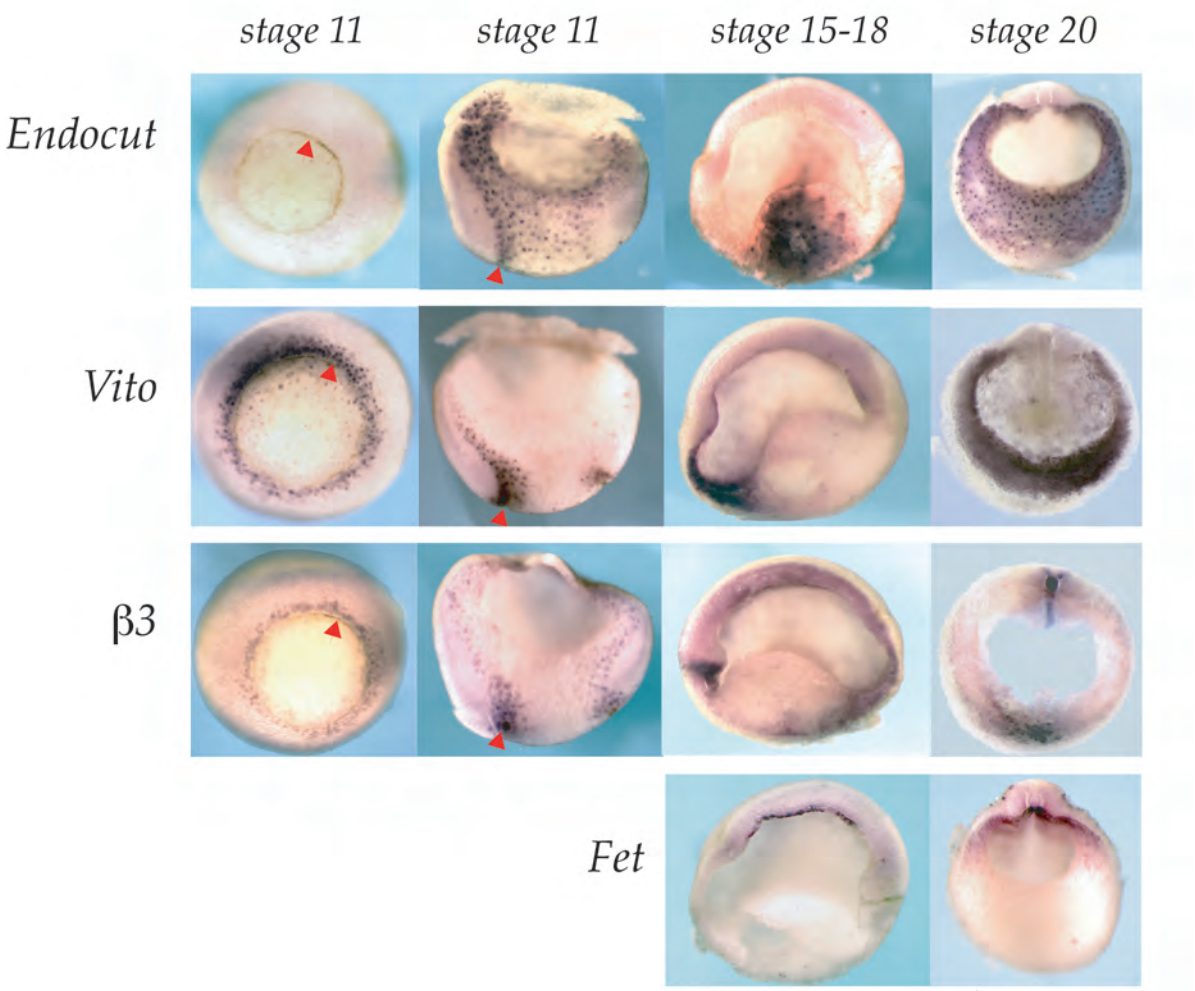


Figure 4.4 - Patterning in the gastrula endoderm. Expression of endodermal patterning markers during gastrulation and neurulation. Differential and stable gene expression is seen in the suprablastoporal and subblastoporal endoderm. *In situ* hybridization in bissected embryos with antisense *Endocut*, β3, *Fetuinish* and *Vito*. Blastopore is shown with red arrowhead. From left to right, ventral views of stage 11 embryos, mid-sagital sections of stage 11 (dorsal to the left), mid-sagital sections of neurula stages (anterior to the left), and cross-section of stage 20 embryos (dorsal is to the top).

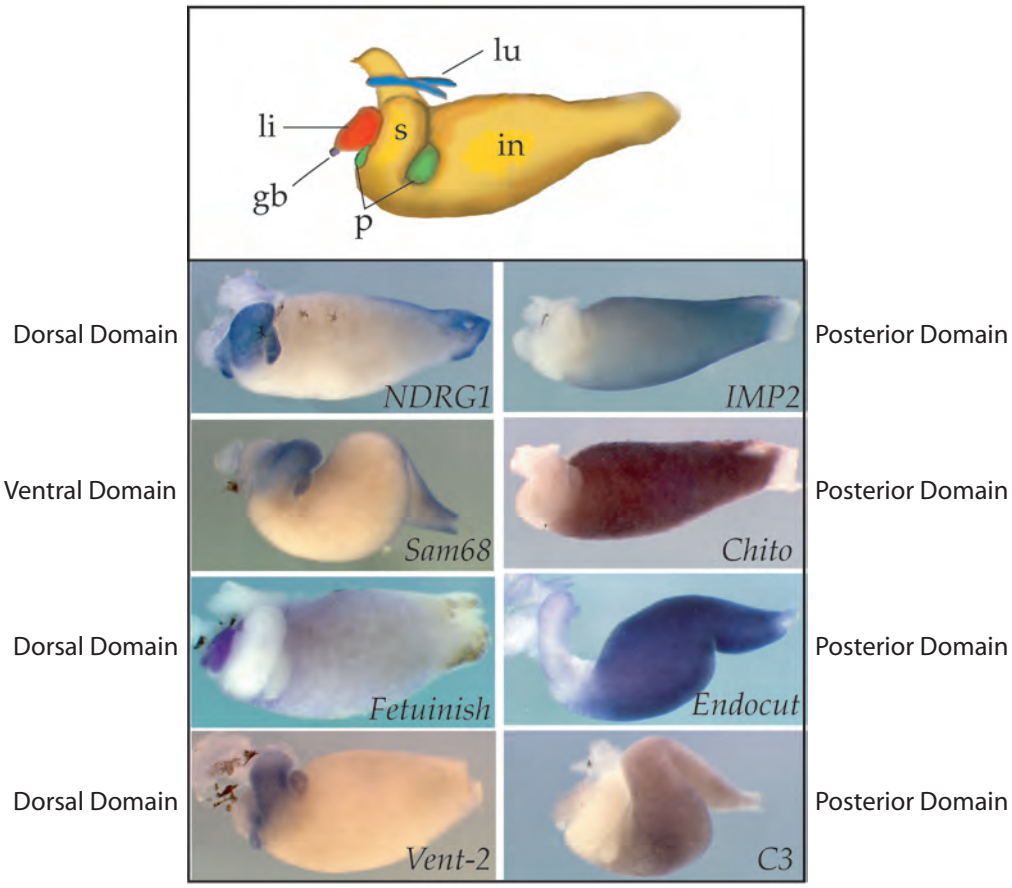


Figure 4.5 - Expression of endodermal genes at organogenesis. Budding stages in the tadpole dissected gut, stage 41/2. Gene names in the insets. li=liver bud, p=pancreatic buds, lu=lung buds, s=stomach, gb=gallbladder, in=intestine.

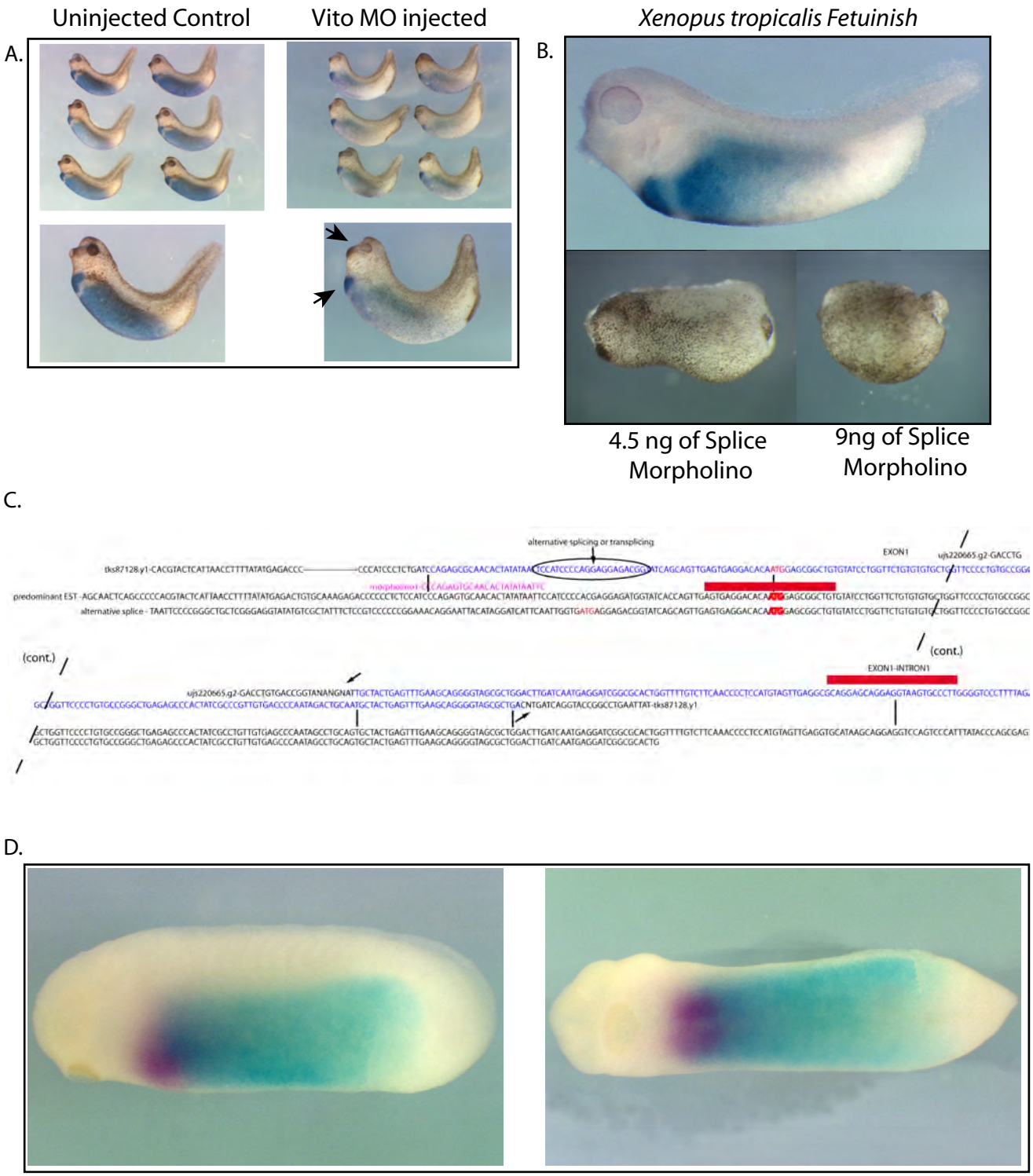


Figure 4.6 - Preliminary functional experiments. **A**) Pilot depletion of *Vito* function, 21ng of *Vito* morpholino were injected at 1 cell stage, and assayed for *Fetuinish* expression at stage 37/8. Arrows point to anterior detects. **B**) Pattern of expression of *Xenopus tropicalis Fetuinish* is very similar to the *Xenopus laevis* (top panel compare with A). Pilot depletion of *tFet (X. tropicalis Fetuinish)* function, shows a severe phenotype, from anterior defects to failure in the closure of the blastopore (bottom panels). Embryos do not survive past stage 24/6, soon after the expression of *tFet* is first detected, with a exploding head phenotype. **C**) Morpholino design based on partial genomic sequence and EST data. Arrows point to low quality sequence, red bars are morpholinos that show phenotype when injected, pink morpholino is a control morpholino. **D**) Endodermal Boundaries. Double whole mount *in situ* hybridisation with the *Vito* and *Endocut* antisense probes. Lateral view on the left and ventral view on the right panel. The domains of *Vito* and *Endocut* do not overlap at any stage of development analysed.

3. Preliminary Functional Studies

Although incomplete, here I summarise the preliminary functional analysis on three genes, *Vito, Fetuinish,* and *Endocut,* representative of the three domains of endodermal expression presented above.

Vito is a strong marker of the presumptive hepatic tissue. SSF transporters, the family of proteins that *Vito* is part of, do not have characterised functions in embryonic development. *Vito* loss-of-function reveals a phenotype with anterior defects, which is in agreement with its pattern of expression (Figure 4.6). However, I still need to verify the robustness and specificity of the phenotype. I could achieve this with the design of novel morpholinos, together with dose response and rescue experiments. I would also like to test if there is interference between the *Vito* loss-of-function and levels of RA signalling.

In order to simplify my loss-of-function approach, I decided to perform *Fetuinish* functional studies in *Xenopus tropicalis* (*tFet)*. I have characterised *tFet* sequence and pattern of expression, which shares many features with the *X. laevis* homolog. With the available genomic sequence and EST data, I was able to design morpholino oligos targeted at the alternative start translation sites and splice junctions (Figure 4.6C). The phenotype obtained with the splice junction morpholino (Figure 4.6B and C) shows loose adherent cells on the dorsal midline, failure to close the blastopore, shorten axis, and at higher doses a surprising 'exploding head phenotype', obtained when expression of *tFet* is detected on dorsal endodermal cells. Much is left undone, the robustness and specificity of the phenotype remains to be evaluated. Moreover, to establish what exact morphologic structures are affected in the morphants would require histological analysis.

Simultaneously, two other independent groups have identified *Endocut* (*Darmin)* (Chen *et al.*, 2003; Pera *et al.*, 2003). Like other over expression studies, of *Fetuinish* and *Vito*, gain-of-function by microinjection of synthetic mRNA does not show gross morphological changes in embryo development (Pera *et al.*, 2003). However, characterization of extra cellular proteases in development is very likely to reveal interesting features of endoderm development, since its known that *Endodermin*, is also an uncharacterised protease, exclusively expressed in the endoderm and *Xolloid,* yet another protease, is known to have a signalling role (Dale, 2000).

4. Discussion

My differential screen on a ventral midgut cDNA library was aimed at finding genes specifically expressed within the endoderm. The study of endoderm development has lagged behind the study of other germ layers, particularly because of the paucity of regionally expressed molecular markers. The screen allowed the characterization of at least 11* new molecules that are regionally expressed in endodermal tissues. Endodermally expressed clones were selected from ~18,000 macroarrayed clones in two steps. At the end of the whole mount *in situ* hybridization analysis step, I achieved a success rate of 4.3%. That is, I found that approximately 1 in every 20 clones tested showed restricted endodermal expression. The analysis of the expression of all these molecules reveals three previously unrecognized endodermal expression domains. The onset of expression for some of these molecules, already at gastrula stages, indicates the existence of early endodermal patterning. In addition, to this already fruitful approach we might have identified regulatory molecules involved in endoderm development, like *Fetuinish*. Hence, the screen contributed in several ways to the extension of our knowledge on how the endoderm is patterned and develops after the initial stages of endoderm specification.

Screening Strategy and Efficiency

The screening strategy is a fundamental key for success (Figure 3.1 B). The results presented here are due to several rounds of improvements. The iterative nature of our procedures was probably the factor that most contributed to achieve a great number of endodermally expressed clones. – At first sight, endoderm regionally expressed genes should be no more difficult to find than regionally expressed markers in other tissues. However, in a large scale *in situ* hybridisation screen of a whole embryo neurula cDNA library, from where clones were chosen randomly, only four cDNAs out of 1765 *in situs* (0.2%) exhibited endodermal restricted expression (Gawantka *et al.*, 1998), such number is low when compared with other germ layers, or neural tissue (>5%), demonstrating the difficulties of finding regionally expressed endodermal clones. – The iterative screening procedure was only possible after the establishment of a systematic recording of the macroarray hybridisation profiles in database files (VisualGrid™ & Excell™). The measure of the hybridisation signal intensities for each clone and its informatic record permitted the comparison of all different probes at the same time. The systematic way in which I was able to compare several complex

* 13 if we consider *IMP2* and *Chito*. At the moment, I cannot reproduce their pattern of expression.

probes permitted to overcome the initial difficulties involved in finding clones with restricted endodermal expression.

At the end of each whole mount *in situ* hybridisation secondary step, I made an evaluation of the number and qualitative nature (i.e., sequencing) of the patterns of expression found. After tracing back these clones to the original hybridisation profiles, this permitted the modification of either the complex probes used, or the criteria for selection of candidate clones (i.e., primary screening step). Overall, ~2,000 individual cDNA's were tested. The iterative procedures permitted an increase of more than five fold in efficiency. That is, regionally expressed clones were found 5 times more frequently at the end of the screen than when I started.

Moreover, the final selection criteria were based on hybridisation profiles of better quality (Figure 3.2C). The quality of the complex probe hybridisation profile is dependent on many variables among which I include; experience, perhaps the most critical, labelling efficiency, level of background signal, initial amount of mRNA sample used, similarity between the complex probe and the arrayed library, etc… The quality of the hybridisation profile can be evaluated indirectly, by the percentage of clones in the macroarray with signal intensity above the presence threshold intensity value. Such value started as 12% in initial experiments, and was close to 50% in the probes used for final selection criteria (Table 3.2).

More biological reasons account for a successful screen. The tissue chosen to construct the library, an endodermally enriched tissue, the ventral midgut tissue of stage 22-24 *Xenopus laevis* embryos, at a stage when patterning should occur (Nieuwkoop, 1967), accounted for an enrichment of 50 fold in endoderm specific transcripts, as measured by *Hex* clone abundance in the library.

My profiling experiments were also successful because I have compared significantly different tissues, representing endodermally enriched versus tissues low in endodermally specific transcripts, therefore increasing the possibility of finding differences among them. In addition, complex probe construction did not affect representativity of the initial tissue. No PCR amplification or subtraction methods were used, which are known to change the representativity of the initial tissue. Moreover, the removal of ubiquitous genes resulted in a more effective selection of candidate clones. A useful solution was the elimination of ubiquitous genes individually, by knowing the position previously tested ubiquitous cDNAs occupy in the array, which was only feasible using macroarrays. Ubiquitously expressed genes are a major problem for gene-discovery, with their position in the macroarray known, I was able to eliminate more than 20% of the clones printed in the macroarray from future selections.

Together all the above-mentioned factors added to the criteria of selection used in the choice of candidate clones resulted in an efficient screen.

Different results are expected for the two criterions that I have used for selection of candidate clones. The first criterion relied on the selection of clones detected in positive but absent in negative probes (2% pool). The second criterion relied on the selection of clones specific to the library but undetected in all profiling experiments (5% pool).

The first criteria should yield more abundant clones, as they were detected in our profiles (see discussion on sensitivity), but a higher percentage of endodermally expressed clones within the restricted patterns of expression found. Indeed, this was the case. cDNAs detected in the differential category were normally isolated more than once (Table 4.1), and 11% of the restricted patterns of expression found were endodermal (Table 3.3).

The strategy of selecting low abundance transcripts from a specific tissue library had been successfully used in the mouse (Neidhardt *et al.*, 2000). The second criterion, based on the selection of low abundance, but library specific transcripts, is likely to reveal regulatory molecules, such as *Hex*. As I effectively, did not perform a differential screen using this criterion one expects to find less endodermal transcripts within the restricted patterns of expression found. Again, this was the case. With this criterion of selection I found, transcription factors, RNA binding proteins, signalling factors and phosphatases (Table 4.1). It was also true, that only 2% of the restricted patterns of expression found with this criterion were endodermal and that most cDNAs were isolated once (Table 3.3 and Table 4.1).

It is clear that the combination of the two criteria gave us better possibilities of success.

A direct comparison to other screens aimed at finding endodermally specific genes is not possible, as no similar efforts have been reported. Thus, the overall efficiency of the screen can only be compared with an initial hit rate of 1 in 100 clones tested in our preliminary screen, to 1 in 20 clones tested in the final stages of the screen. This is particularly satisfactory, when unbiased screens have a hit rate of 0.2% for endoderm-restricted patterns of expression (Gawantka *et al.*, 1998).

Differential Screening Sensitivity

When compared with microarrays or other techniques of transcriptome profiling, macroarrays do have inherent problems of sensitivity. They are due, to the high volumes of hybridisation necessary, the large amounts of mRNA sample necessary, uneven printed array clone size and level of probe radiolabel incorporation. Quantitative answers can be provided with statistical measurements and consistent internal controls which are both outside of the scope of the gene-discovery work and were not included when the printing of the filters.

There are a few but solid examples on how semi-quantitative use of macroarrays can be achieved (Dickmeis *et al.*, 2001; Ransick *et al.*, 2002; Rast *et al.*, 2000; Rast *et al.*, 2002). However, the level of sensitivity of my profiling experiments can be estimated indirectly by the position of known clones. The two following examples will clarify what I mean.

Hex is expressed at low abundance in the ventral midgut endoderm of the *Xenopus* embryo. For comparison purposes, I was trying to find genes like *Hex*. Of all 7 *Hex* clones in the arrayed library, I could not confidently detect any in all probe hybridisation profiles. I therefore have to conclude that the sensitivity achieved was inadequate for the detection of low abundance genes. If one considers *Vito,* a more abundant gene present at the frequency of one copy per 10^3 clones in the library, the signal intensity values of the 55 *Vito* clones fluctuated around background values and the presence threshold in the positive complex probes (Figure 3.2). However, on occasions, the value for signal intensity for *Vito* clones was above the presence threshold. This means, that I was able to detect transcripts of average abundance, like *Vito,* at the limit of sensitivity, and conclude that it was possible to retrieve differentially expressed genes from the procedures outlined above.

Reproducibility of Hybridisation Profiles

In order to select candidate clones based on complex probe hybridisation profiles, one must assure their reproducibility. Due to the iterative nature of the screen no two identical profiling experiments were made. Therefore, reproducibility has to be tested indirectly, by comparing hybridisation profiles of similar probes or by analysis of the level of detection of known genes in several hybridisation profiles.

The large majority (>95%) of the rRNA, *Ef-1*α and other ubiquitous genes, known to be abundant, were found in the detectable fraction of clones in all probes hybridisation profiles analysed, that is with signal intensities above the presence threshold. In addition, when comparing two probes made from the same midgut library tissue, where probe 1 had ~2,000 clones confidently above the presence threshold, and probe 2 had ~4,000 clones above presence threshold, I could verify that 99% of the clones detected in probe 1 were also detected in probe 2. Moreover, when I verified all the *Ef-1*α clones in the array (~2%), I could detect at least 95% of them in all of the hybridisation profiles used for the final selection criteria (Table 3.2).

Based on these three examples, I estimate a reproducibility of approximately 95%, for the signal intensities detected above the presence threshold. Clearly, signals of lower intensity are expected to be less reproducible, but my differential screening criteria dealt only with high intensity signals and therefore highly reproducible.

Improving the Screen

At the time I started this project no microarray slides were available for *Xenopus*. At present, and in spite the incomplete coverage of the genome, it would be more sensible to use this technology to search for novel endodermally expressed genes because of its increased sensitivity.

However, the present screen could be improved in several ways. Surely, the limiting step was the number of *in situ* hybridisations necessary to perform; my priority would be the automation of the secondary step. Then, I would try to reduce the 35% of the cDNAs on the macroarray that had signal intensities that cannot be confidently defined as positive or negative, i.e., between background and the presence threshold. This probably would be time consuming, as it could involve statistic analysis, construction of new probes, perhaps subtracted, and the re-array of the library without ubiquitous clones.

Endodermal Domains of Expression

The genes described on this chapter are all endodermal markers (Figures 4.1 to 4.3). They are either novel or their endodermal pattern of expression had not been characterized. Half of these genes have no ascribed biological functions (*Vito, Figo, Pil, Endocut, Fetuinish and NDRG1*). The remaining genes were described but have not been linked with endodermal development (e.g., *Vent-2* in mesodermal patterning and *EphrinB1* in cell-cell recognition and signalling) (Kullander and Klein, 2002; Onichtchouk *et al.*, 1996; Onichtchouk *et al.*, 1998). I have identified at least 11 genes that are useful markers of endoderm patterning in the *Xenopus* tailbud embryo. Analysis of their patterns of expression reveals that as early as the neurula stage, the endoderm is already subdivided into three distinct gene expression domains. These domains are the ventral midgut, the posterior endoderm, and the dorsal endoderm. These expression domains appear to mark tissue territories previously predicted by the neurula endoderm fate map, long before the expression of the commonly used endodermal patterning markers such as *Pdx1*, *Cdx2*, *Nkx2.5*, *IFABP* and *albumin*. The existence of these three domains reflects a molecularly sub-divided endoderm at neurula stages, and a previously unknown degree of endodermal patterning.

The expression of genes in the gastrula and neurula endoderm is of particular interest to study early patterning events (Figure 4.4). The stable expression of these markers at early stages of endoderm development renders them as evidence for a molecularly patterned endoderm at gastrula. Moreover, now it will be possible to examine the molecular

mechanisms responsible for these patterning events. Although domains of expression were known within the gastrula endoderm, either by the transient expression of *Hex* and *Cerberus* in the anterior endoderm or the transient expression of GATA factors in the deep endoderm of the gastrula, *Vito* and *Endocut* are particularly useful because their stable expression throughout development (Costa *et al.*, 2003) (Figure 4.4)

Genes such as *Hex, Cerberus, Vito, Endocut, Gata5 or Gata6, Goosecoid* and *Chordin* are expressed in the gastrula endoderm in overlapping but with somehow distinct patterns of expression (Zorn *et al.*, 1999, and this work). Overall, all of these point to a great diversity of gene expression of the gastrula endoderm, which resolve into stable domains of expression in the tailbud endoderm. These results demonstrate that positional information in the gastrula endoderm translates into domains of expression well before stable regional specification is thought to occur under the influence of the mesoderm (Horb and Slack, 2001).

The expression of most genes described is not strictly exclusive of endodermal tissues (Table 4.2). However, *Endocut, Vito* and *Fetuinish* are specific markers and expressed in the posterior, ventral midgut and dorsal endodermal domains. At all stages analysed they are not expressed in any other tissues, exceptions being *Fetuinish* is expressed on ciliated cells of albino embryos, and *Vito* in the nephric region. The expression of these three markers in complementary parts of the tailbud embryo makes of them useful tools to examine patterning during tailbud stages in *Xenopus,* and reveals specific markers for these sub-endodermal domains; at a time where no other markers are available (Figure 3.1C).

Expression analysis of these genes reveals very dynamic patterns of expression. *Vito, Endocut* and *β3* are expressed in the gastrula endoderm. All of the remaining genes are expressed in the tailbud endoderm. Within the anterior ventral midgut domain, a progressive localization of transcripts is seen as development proceeds. Generally, this is expected as presumptive organs have overlapping territories, which become restricted as development continues (Grapin-Botton and Melton, 2000), and many different organs are believe to originate from this embryonic area (Chalmers and Slack, 2000). In particular for the presumptive liver markers, *Vito* and *β3*, as development continues to tadpole stages the expression of these genes gets restricted to the gallbladder (Figure 4.1 arrows). The posterior endodermal domain expression shows a good predictive correlation with the genes that later are expressed in the presumptive intestine (Figure 4.5). Within the dorsal domain, expression patterns are again more dynamic and the same predictive correlation is not found, but expression of all these genes (*Fetuinish, NDRG1, Vent-2)* is anterior to the stomach in the coiled gut (Figure 4.5). Hence, I also found molecular markers capable of following the development of several organ primordia within the endodermal domains.

Endodermal Patterning

The domains of expression are distributed among the anterior-posterior and dorsal-ventral axis. The anterior-posterior domain is first seen during gastrulation, stage 11, in the form of expression either in the subblastoporal endoderm or in the suprablatoporal endoderm. *Endocut* is expressed in the subblastoporal endoderm. *Vito* and the *β_3-ATPase subunit* are expressed suprablastoporal endoderm (Figure 4.4). Since this is a stage when endoderm specification is believed to occur, a molecularly patterned endoderm is seen already during endoderm specification.

The distribution in complementary and almost exclusive domains of gene expression in the gastrula is maintained at tailbud stages in an anterior-posterior direction, ventral midgut to posterior tailbud endoderm (Figure 3.1C). These expression domains do not seem to overlap, but no clear boundary is visible between them. At present we do not know, what are the mechanisms responsible for the establishment of these domains of expression, or if a boundary exists between them (Figure 4.6D).

My results can be seen in the light of two groups of authors. The first group, initiated by Holtfreter, proposes an amphibian gastrula endoderm already with some regional character (Holtfreter, 1938a; Holtfreter, 1938b). Holtfreter explanted endoderm from both anuran and urodelean species, and observed that it was capable of differentiation according with its prospective fate in the absence of other tissues. The second group of authors, initiated by Okada, reinforces the idea of the inductive character of the mesoderm necessary for endoderm differentiation, and that isolated endoderm is not capable of differentiation without the associated mesoderm (Okada, 1953; Okada, 1955a; Okada, 1955b; Okada, 1955c; Okada, 1955d; Okada, 1957; Okada, 1960). However, all of the authors refer to potency differences in sub-regions of the endoderm, either under the influence of the mesenchyme or under simple explant conditions.

In summary, I found evidence for a molecularly patterned endoderm well before stable regional specification occurs by the action of the mesoderm. We know that the endodermal pattern only becomes stable by the action of the mesoderm (Horb and Slack, 2001). We also know that association with heterologous mesoderm can modify the fate of an endodermal region. This suggests that the early pattern described here, has to be stabilized by some yet unknown mechanism, and that these early domains of expression possibly account for the different potencies and prospective fates of different domains of the endoderm. However, the question of how stable is this early pattern still remains, and if it is a consequence of the action of the organizer.

Furthermore, the idea of a patterned endoderm during gastrulation is not new (Clements *et al.*, 2001). It has been put forward as an interpretation of the differential expression of *Pdx1* and *IFABP* in endoderm gastrula explants (Gamer and Wright, 1995; Henry *et al.*, 1996). *Hex, Cerberus* or *Gata5/6* are expressed in the anterior endoderm well before stage 11, the time at which I detect *Vito* and *Endocut*. In addition, the literature refers the endoderm as the germ layer with the highest differential potential in explant culture (Nieuwkoop, 1997). It would also be unlikely that the endodermal cells, that surround the organizer would not be influenced by the many patterning molecules expressed there. Moreover, the organizer possesses not only a neural inducing activity and mesoderm dorsalizing activity but a gut inducing one as well (Sasai *et al.*, 1996). Together, these indicate the likelihood of the action of patterning mechanisms on early gastrula and neurula endoderm, in particular derived from the organizer, which action is poorly characterized in the endoderm.

The dorsal to ventral pattern is observed at later stages when compared with the anterior-posterior pattern. Through the expression of *Fetuinish*, in the dorsal most endodermal cells, first observed by stage 16, one can predict the existence of a complementary ventral expression domain. *Fetuinish* transcripts are detected strongly in the epithelial layer of the archenteron roof (Figure 4.4). Although no gene expression was detected in the archenteron floor, it is likely that *Fetuinish* pattern of expression demonstrates the molecular differences existing between the two tissues. At present we do not know what establishes this dorsal domain of expression. However, at neurula stages, the archenteron roof is in close contact with the notochord, which is an established signalling centre. Then, it is possible to hypothesise, that the dorsal endoderm expression domain could be established under the influence of the notochord (Cleaver and Krieg, 2001).

Future Directions – Endodermal Patterning

I have identified two major lines in which endodermal patterning could be further studied. At present, there is no indication of what mechanism may establish the endodermal pattern observed with the molecular markers presented here. The organizer, and its control over signalling in the embryo, is a likely candidate as the source of anterior-posterior endodermal patterning mechanisms. It would be interesting to manipulate the different signalling pathways active in the gastrula, and investigate how do they affect the early endodermal pattern we observe.

The dorsal domain that we see later, that I hypothesise would be under the control of the notochord, could be tested by extirpation and recombination and by manipulation of *Shh*

signalling cascade, that we know influences endoderm development in other model organisms. With these markers in hand we will be able to study of the labile nature of endoderm determination.

The sharp delimitation, complementary domains of expression, and almost mutually exclusive expression of these markers throughout endoderm development, raises the question of an endodermal boundary. Sharp delimitations are also found between the mesoderm and endoderm at these stages (e.g., *Xbra, Mixer* and *Sox17*). It is clear that at gastrulation, and then neurulation, the endoderm acquires specific and diverse patterns of gene expression, and these are maintained until organogenesis stages. It would be interesting to lineage label cells in these domains and follow their development, as a way to follow the determination of their developmental fate and when its developmental fate is established.

Ongoing Functional Analysis

The traditional approach to the study of gene function in amphibian development is based in microinjection over expression studies. Here, functional interactions are likely to show phenotypes. For later stages of development the traditional approach is less likely to show specific phenotypes, due to the increasing complexity of tissues and targeting difficulties. Accordingly, my pilot over expression experiments on three candidates of choice – *Vito*, *Fetuinish* and *Endocut* – show no obvious phenotypes. Others support my findings in the *Endocut* over expression experiments (*Darmin*) (Pera *et al.*, 2003) (Figure 4.6).

However, other approaches are available to advance our knowledge in the function of these three genes, *Vito, Endocut* and *Fetuinish*. I present my working hypotheses. *Vito* is a membrane protein and possibly an exchange pump as it contains SSF domain. Members of this family exchange Na^+ ions for vitamins. Retinoid acid (RA) has long been know as capable of acting as morphogen, and capable of inducing anterior fates. *Vito* is strongly expressed in anterior tissues. Interestingly, RA is a vitamin A derivative, molecularly very similar to the solutes transported by SSF domain proteins. A working hypothesis towards *Vito* functional studies would be to test its function as a retinoid transporter. With six full-length sequences available, morpholino's were designed towards the putative translation start Metionine. The morphants present anterior defects and perhaps alteration of liver bud size (Figure 4.6). Since *Vito* is strongly expressed in these anterior tissues, such phenotype certainly needs a much more careful analysis.

Endocut is a secreted protease (also called *Darmin*). Other proteases, like *Xolloid*, are involved in signalling and the patterning of the mesoderm and ectoderm (Dale, 2000). *Endodermin* is another example of a protease of unknown function (Sasai *et al.*, 1996). Many times extra cellular signalling is dependent on ligand activation through proteolytic cleavage. It would be interesting to investigate if *Endocut* has similar proteolytic activating properties and with which specificity. Moreover, mouse FGF signalling is known to be necessary for the specification of posterior endodermal fates (Wells and Melton, 2000). Then, the hypothesis to test would be; does FGF signalling establishes the posterior endoderm expression domain?

Fetuinish is another secreted protein and a candidate for a regulator of liver development. Its pattern of expression is strongly associated with presumptive hepatic tissue (Figure 4.3 and 4.5), but information on *Fetuinish* or similar molecules is scarce. However, the existent information on its sequence is sufficient to formulate two initial hypotheses. *Fetuinish* might be involved in embryonic developmental signalling in two ways (Figure 4.3B). Activity of *Fetuin,* a molecule similar to *Fetuinish,* in adult tissues reflects BMP signalling inhibition, which probably arises from the cystatin inhibitor domains (Brown *et al.*, 1992; Demetriou *et al.*, 1996; Heiss *et al.*, 2003; Szweras *et al.*, 2002). Cystatins were initially characterized has protease inhibitors. In addition, it is worth mentioning a poorly conserved stretch (Figure 4.3 – purple line), close to the conserved region of the fetuin signature, which possesses a 'hidden' TGF-β like binding domain, with the active aminoacids used in the human TGF-β type II receptor being conserved (Figure 4.3B). The hypothesis to test is then, is *Fetuinish* regulating TGF-β signalling extra cellularly? If so, which are the protein domains involved in the regulation, the cystatin domains or the TGF-β 'hidden' motif, or both?

The preliminary results obtained with depletion of *Xenopus tropicalis Fetuinish (tFet)* function, show an unexpected phenotype, which is in agreement with a postulated function as a protease inhibitor. Morphants die soon after the time when expression of *tFet* is detected in the dorsal endoderm domain with an exploding head phenotype.

CHAPTER FIVE - SPIB

0.Abstract

1.Introduction

ETS Transcription Factors
SpiB Homologs and their Functions
Haematopoietic Development
Xenopus Haematopoiesis
Early Embryonic Macrophages

2.Results
2.1. Cloning of SpiB
2.2. SpiB Pattern of Expression

3.Discussion

Xenopus SpiB is the Homolog of Mice SpiB
SpiB is expressed in Embryonic Macrophages
SpiB and the Haemangioblast
SpiB and Endothelial cells
SpiB and Blood
SpiB and the Evolution of the Adaptative Immune System
Future work

0.Abstract

SpiB is a member of the ETS family of transcription factors. *SpiB* had already been cloned in *Xenopus* but its embryonic expression was undocumented. I isolated it in my screen for genes expressed in the ventral midgut region. Here, I describe the embryonic expression pattern of *SpiB* and the two paralogs in *Xenopus laevis, SpiBa* and *SpiBb.* The transcription factor *SpiB* is expressed in a salt and pepper pattern in the Ventral Blood Islands (VBI) of the *Xenopus* tailbud and early tadpole embryo. Mammalian *SpiB* is exclusively expressed in lymphoid cells, the effectors of adaptative immunity, and has a function in B cell differentiation, cells that are specialized in antibody production. B cells or lymphoid precursors do not originate from the mammalian yolk sac or the amphibian equivalent, the VBI. The apparent inconsistency between the characterised function and expression of mouse *SpiB*, and the expression of *Xenopus SpiB,* when mouse and *Xenopus* share many common features of blood development, was a reason to further investigate the expression of *SpiB*.

For much of early development, *SpiB* is expressed in a similar pattern to *XPOX2*, a recently identified marker for embryonic macrophages in *Xenopus laevis.* Embryonic macrophages are a poorly characterized cell type. Embryonic macrophages are migratory cells that differentiate very early in development, and for which analogous cell types exist across phyla. I have identified *SpiB* as a novel marker for embryonic macrophages. I also

discuss the link between *SpiB* expressing cells, the haemangioblast, the progenitor of haematopoietic cells, and the angioblast, the progenitor of endothelial cells.

I decided to study SpiB expression, also because SpiB is a transcription factor of a family known to be involved in cellular fate decisions in the haematopoietic system. Its expression at early stages of development reveals an embryonic phase of haematopoietic development, and in particular, of an uncharacterized population of embryonic macrophages, likely to have several developmental functions in the early embryo.

In summary, *SpiB* function in *Xenopus* is unknown and might be different from its mouse homolog. From my analysis we now know that amphibian *SpiB* is a marker for early embryonic macrophages, and not a B cell marker. Embryonic macrophages are primitive myeloid cells. The primitive wave of myelopoiesis is not well defined in the vertebrate embryo, and the study of *SpiB* in amphibians might help our understanding of how the haematopoietic system evolved, and of the function of the primitive wave of myelopoiesis, and its functions in the embryo.

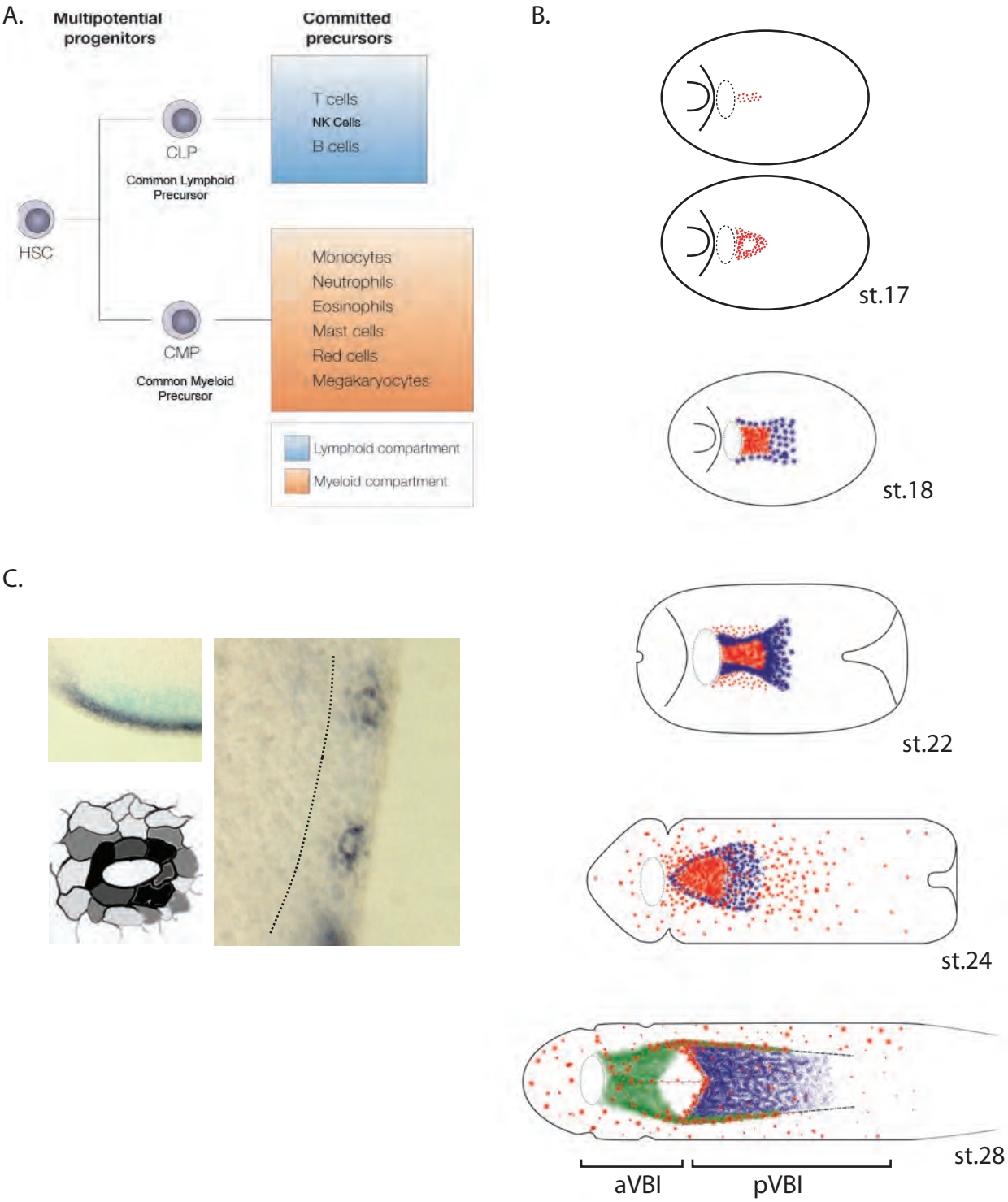


Figure 5.1 - Adult haematopoiesis and relations between *SpiB* and haematopoiesis. **A**) Adult haematopoiesis. The HSC (Haematopoietic Stem Cell) gives rise to the lymphoid and myeloid lineages, blue and orange, respectively. Monocytes are the progenitors of adult macrophages. B cells are derived from the lymphoid compartment and are first observed in the developing liver (*Xenopus*), the major site of embryonic haematopoiesis in anurans. Two sites, the VBI (Ventral Blood Island) and DLP (Dorsal Lateral Plate), have been described as harboring HSCs (Zon, L. 2001). **B**) *Xenopus laevis* ventral views with drawings of the domains of expression of SCL (blood - blue), Xmsr (endothelial - green) and SpiB (red). There is also an anterior VBI (aVBI) and a posterior VBI (pVBI), originating from the dorsal and ventral gastrula mesoderm (Walmsey *et al.*, 2001). The posterior VBI is characterized by a "V-shape" (blue) defined by the expression of SCL and αT-globin. **C)** In a clockwise direction, SpiB continuous expression in the ventral region of a stage 20 embryo. Hollow SpiB cell clusters in the ventro-lateral mesodermal region of a stage 30 embryo, and one illustration of what I understand by hollow clusters. The dashed line indicates the mesoderm-endoderm boundary.

1.Introduction

Mammalian *SpiB* is expressed only in lymphocytes, and is required for B cell differentiation, which are lymphocytes specialized in antibody production (Su *et al.*, 1996). Concomitant with differentiation away from the lymphoid progenitor, *SpiB* is down regulated in T cells, but up regulated in B cells. *SpiB* null mice have immune deficiencies derived from abnormal B cell development and maintenance of its germinal centres (Su *et al.*, 1997; Su *et al.*, 1996). Since *SpiB* is one of few lymphocyte specific genes, homologs have been cloned in several model organisms in order to study the evolution of the adaptative immune system, which is only executed by lymphocytes. I isolated *SpiB* in my screen for genes enriched in the ventral foregut. *Xenopus SpiB* is known but its pattern of expression has been undocumented (Shintani *et al.*, 2000).

ETS Transcription Factors

SpiB is a member of the ETS family of transcription factors. ETS transcription factors were initially discovered in birds (Ets-1, is the founder member). ETS transcription factors are unique to metazoans and currently there are more than 30 members, which can be further classified into their subfamilies (PEA3, SPI, TCF, ERF, ELG, YAN, ETS, ELF, ERG, TEL) (Sharrocks, 2001; Sharrocks *et al.*, 1997). The SPI subfamily comprises four transcription factors, *PU.1/Spi1*, *SpiB*, *SpiC* and *SpiD*. Very little is known about *SpiC* and *SpiD*. The ETS domain, characteristic of the ETS transcription factors, is a variant to the WINGED HELIX-TURN-HELIX domain. ETS proteins can contain other domains such as PEST, Pointed, D-domains, B-boxes and conserved phosphorylation sites. The activity and specific biological function of ETS transcription factors depends critically on their regulatory partners (i.e., *AML-1, Pax5, MafB, GATA1*) and cellular context, providing specific biological activities (Sharrocks, 2001). ETS transcription factors can act as transcriptional activators or repressors (Sharrocks, 2001; Theoleyre *et al.*, 2004). Nothing is known about *SpiB* partners or its function in *Xenopus* (Figure 5.3G).

ETS transcription factors are regulated by phosphorylation and are nuclear effectors downstream of tyrosine kinase receptor signalling. Phosphorylation of ETS transcription factors has been observed by Mitogen Activated Protein Kinase (MAPK), Casein Kinase II (CK II) and calcium dependent kinases such as, calmodulin-dependent kinase II (CaMK II) (Cowley and Graves, 2000; Li *et al.*, 2000; Oikawa and Yamada, 2003; Sharrocks, 2001; Wasylyk *et al.*, 1998). Two other ETS transcription factors, ER81 and PEA3, have been

described downstream of FGF signalling during embryonic development (Munchberg and Steinbeisser, 1999; Roehl and Nusslein-Volhard, 2001). Although phosphorylation of *Xenopus SpiB* has not been studied, amphibian *SpiB* contains several putative phosphorylation sites detected by homology with known used phosphorylation sites in mouse *SpiB,* and that regulate *SpiB* protein stability (Ray-Gallet and Moreau-Gachelin, 1999) (Figure 5.3).

SpiB Homologs and their Functions

ETS transcription factors have been linked with many biological processes including haematopoiesis, vasculogenesis, angiogenesis, and the specification of neuronal cells. ETS proteins have a role regulating cellular activities such as proliferation, senescence, apoptosis and differentiation (Sharrocks, 2001).

Mouse *Spi1/PU.1* (Klemsz *et al.*, 1990) is related to mouse *SpiB* (75% over the ETS domain) and shares several *in vitro* biochemical properties (Ray *et al.*, 1992; Shintani *et al.*, 2000; Su *et al.*, 1996). The SPI sub-family of ETS proteins, which is found only in vertebrates, contains very divergent ETS transcription factors (Chen *et al.*, 1998; Dahl *et al.*, 2002). *Spi1/PU.1* is expressed in several lineages during embryonic and adult haematopoiesis (Anderson *et al.*, 1999; Schebesta *et al.*, 2002). *Spi1/PU.1* null mice die after birth due to severe septicaemia, a consequence of the lack of mature macrophages, B and T cells, demonstrating that *Spi1/PU.1* is essential for the formation of both myeloid and lymphoid lineages (McKercher *et al.*, 1996; Scott *et al.*, 1994). The $SpiB^{-/-}$ phenotype is milder and just reveals deficiencies in the maturation of B cells. *SpiB* and *Spi1/PU.1* exhibit partial redundancy depending on their cellular context. *SpiB* can functionally replace *Spi1/PU.1* in myeloid development but not in lymphoid development and both transcription factors act synergistically to regulate B cell survival (Dahl *et al.*, 2002; Garrett-Sinha *et al.*, 2001; Garrett-Sinha *et al.*, 1999; Hu *et al.*, 2001). The mouse *Spi1/PU.1* transcription factor has an established role in adult myelopoiesis, and the differentiation of the mononuclear phagocyte system (Figure 5.1A). However, yolk sac derived macrophages (also denominated primitive or embryonic) are not affected in *Spi1/PU.1* null mice (Lichanska *et al.*, 1999). The functions of *Spi1/PU.1* and *SpiB* have not been addressed in *Xenopus.*

Some ETS transcription factors regulate cellular motility in the context of angiogenesis and vasculogenesis. In cell culture, the over expression of the ETS domain from *PU.1, Ets1* or *Ets2* increases adhesion and impairs migration and proliferation of endothelial cells. *In vivo* studies using antisense oligos against *Ets1* result in a dose-dependent inhibition of

angiogenesis (Lelievre *et al.*, 2001; Sharrocks, 2001). Over expression of *XlErg* and *Xl-fli,* two other ETS transcription factors, results in ectopic endothelial differentiation (Baltzinger *et al.*, 1999). In summary, ETS transcription factors have been implicated in the control of cellular migration by modifying cellular adhesive properties, and endothelial development and it is not known if *SpiB* participates in similar processes, as we will see embryonic macrophages are also migrating cells.

Haematopoietic Development

Blood is a mesodermal derivative. Haematopoiesis (blood formation) is reasonably well understood in mammals. Haematopoietic Stem Cells (HSC) give rise to all adult blood cell types (Figure 5.1A). All lymphoid and myeloid precursors originate from the HSC. The lymphoid compartment consists of B, T and NK (Natural Killer) cells. The myeloid precursor gives rise to monocytes, neutrophils, eosinophils, mast cells, erythrocytes (red blood cell) and megakaryocytes. Monocytes give rise to adult mature macrophages. During mammalian adult life the HSCs reside in the bone marrow and differentiation of specific lineages occurs in other haematopoietic organs (spleen or thymus). These pathways of differentiation are generally named definitive haematopoiesis (Orkin, 2000).

However, embryonic haematopoiesis is very different from adult haematopoiesis. During embryonic development two waves of haematopoiesis, a primitive and a definitive one, give rise to different cell types. This is most obvious in erythropoiesis (red blood cells formation), with primitive and definitive erythrocytes expressing different globins (Godin and Cumano, 2002). Embryonic haematopoiesis occurs through waves of migration and colonization of several haematopoietic organs in the embryo. Mouse blood formation is first detected in the yolk sac at 7.5dpc, in the blood islands. Blood islands are clusters of erythrocytes, endothelial cells and macrophage-like cells (Cuadros *et al.*, 1992; Gilbert, 2000; Godin and Cumano, 2002; Lichanska *et al.*, 1999). Because blood development is always found closely associated with endothelial cells the existence of a precursor of both lineages, the haemangioblast, has been postulated *in vivo*, and is characterized *in vitro*. The haemangioblast is defined as a mesoderm cell that gives rise to the HSC and endothelial progenitors (Ciau-Uitz *et al.*, 2000; Robb and Elefanty, 1998; Walmsley *et al.*, 2002).

In mouse, colonization of haematopoietic organs by HSCs happens around the onset of circulation. Before the onset of blood circulation two locations of HSC development exist in mouse, the yolk sac (YS) and the Aorta Gonad Mesonephros (AGM). Although, HSC derived

from these two sites have the same *in vitro* potential, that is, immune Long Term Reconstitution activity (LTR), *in vivo* the yolk sac derived HSC do not give rise to the definitive lineage precursors, perhaps due to their HSC specific micro-environment (Godin and Cumano, 2002). After the onset of blood circulation both HSC from the AGM and YS colonize the liver, a major haematopoietic organ during fetal life. Cell type specific precursors then migrate to other haematopoietic organs (Figure 5.2A).

Mouse *SpiB* is believed to be B cell specific, and the B cell precursors first detected in the fetal liver are derived from the AGM region. No B cell precursors have been detected in the yolk sac (de Andres *et al.*, 2002). In fact, mammalian lymphopoiesis originates only from the HSCs of the AGM (Cumano *et al.*, 1996; Cumano *et al.*, 2001; Godin and Cumano, 2002; Tavian *et al.*, 2001). In summary, mammalian definitive B cells derive from HSC in the p-Sp*/AGM region, colonize the liver during fetal life and during adulthood reside in the bone marrow and mature in the spleen. No primitive B cells are known to exist, or no B cell precursors differentiate from the yolk sac.

Interestingly, lymphocytes (B and T cells) are the only effectors of the adaptative immune system, which is present only in jawed vertebrates (Matsunaga and Rahman, 1998). To date, SPI genes were only found in vertebrates and are good candidates to study the evolution of the immune system (Figure 5.2). On the contrary, the innate immune system develops in all animals, even in the ones without a proper circulatory system, as the fly. The innate immune system function is executed by the myeloid lineage. Although not yet firmly established, myelopoiesis also appears to occur in two waves, a primitive and a definitive one (Lichanska *et al.*, 1999; Lichanska and Hume, 2000). The primitive wave of myelopoiesis gives rise to the embryonic macrophages (Shepard and Zon, 2000) (Figure 5.2).

Xenopus Haematopoiesis

Xenopus, like all vertebrates, have two haemogenic sites equivalent to mammalians (Godin and Cumano, 2002; Hansen and Zapata, 1998; Matsunaga and Rahman, 1998). In spite of the fact, that haematopoietic ontogeny varies a great deal when closely related species are compared (Hansen and Zapata, 1998). That is to say, where different haematopoietic specific cell types come from and where they differentiate and migrate to, vary across species, analogies between mice and *Xenopus* are remarkable (Figure 5.2).

* p-Sp — para-aortic splanchnopleure, the embryonic region that will give rise to the AGM approximately one day later.

The yolk sac equivalent is the *Xenopus* Ventral Blood Island (VBI) and the AGM equivalent is the *Xenopus* Dorsal Lateral Plate (DLP). Like in mice, the first sign of primitive haematopoiesis is the development of primitive erythrocytes in the VBI, which are molecularly visible by the expression of *αT-globin*. The VBI is composed of an anterior and a posterior part, which are derived from different areas of the gastrula (Ciau-Uitz *et al.*, 2000; Lane and Sheets, 2002; Walmsley *et al.*, 2002). In *Xenopus,* two waves of haematopoiesis are also known. The independent and separate contribution of the VBI to primitive and of both the VBI and DLP to definitive haematopoiesis can be demonstrated as early as neurula (Turpen *et al.*, 1997). However, unlike mammals the VBI contains HSC's that can give rise to lymphocytes, as shown with transplantation experiments of cytogenetically different *Xenopus* embryos (Turpen, 1998; Zon, 2001).

To one's surprise, B cells that develop in the liver, the major and first haematopoietic organ during larval life (Hadji-Azimi *et al.*, 1982), do not originate from the VBI, but solely from the DLP (Chen and Turpen, 1995). Much later in adult life, B cell precursors are derived from both VBI and DLP. That is, during *Xenopus* larval development of B cells does not originate from a ventral location, the VBI, where *SpiB* is expressed (see below).

In contrast, primitive myelopoiesis that is uncharacterized in *Xenopus* is expected to occur in the analogous site to the yolk sac, the VBI. (Hansen and Zapata, 1998; Matsunaga and Rahman, 1998; Turpen, 1998; Turpen *et al.*, 1997; Zettergren, 2000; Zon, 2001).

Early Embryonic Macrophages

Adult macrophages are scavenger cells that eliminate apoptotic cells and engulf pathogens. Embryonic macrophage-like cells have been observed in many model organisms such as; *Drosophila,* zebrafish*, Xenopus* and mouse (Bennett *et al.*, 2001; Herbomel *et al.*, 1999; Holz *et al.*, 2003; Lichanska and Hume, 2000; Shepard and Zon, 2000; Smith *et al.*, 2002; Tepass *et al.*, 1994). Embryonic macrophages are myeloid but do not follow the monocytic pathway and express a set of different markers (Shepard and Zon, 2000) (Figure 5.2). In *Xenopus,* such a cell population is not characterized. Two molecular markers *XPOX2* and *XLURP-1* are expressed in migrating cells with phagocytic ability in *Xenopus* and have been described as amphibian embryonic macrophages. *XPOX2* and *XLURP1* are expressed in the VBI in a spotty pattern at tailbud stages (Smith *et al.*, 2002).

Embryonic macrophages originate in the yolk sac, and are expected to arise in the VBI of amphibian embryos. In zebrafish, where *SpiB* has not been described yet, *PU.1* is a marker for early embryonic myeloid cells (Lieschke *et al.*, 2002). Embryonic macrophages are migratory and may have several functions in the developing embryo. These functions range from

morphogenesis (removal of apoptotic cells in the interdigits), patterning and proper morphogenesis of the *Drosophila* CNS (hemocytes), participation in the immune response, and association with the developing vasculature where they might control angiogenesis as adult macrophages do (Herbomel *et al.*, 1999; Herbomel *et al.*, 2001; Holz *et al.*, 2003; Lichanska and Hume, 2000; Lieschke *et al.*, 2002; Shepard and Zon, 2000).

In summary, the site of B cell development in *Xenopus* is analogous to the mammalian. In frogs, B cells derive from the DLP and colonize the liver. In mammals, B cells derive from the AGM and also populate the liver. In addition, primitive myelopoiesis also happens in analogous regions of the embryo. In *Xenopus*, early myeloid markers have recently been identified (*XPOX2* and *XLURP1)*, and are expressed in the VBI, in an analogous manner to mammalian embryonic macrophages that develop in the yolk sac.

Having such analogous haematopoietic ontogeny, it was puzzling to observe the expression of a believed B cell specific gene (*SpiB*), in the VBI, where no B cells exist.

In oppostion to *SpiB* postulated function in amphibians, the development of B cells. *SpiB* expression in the VBI points to a function in primitive haematopoiesis, such is further supported by the fact that members of the SPI gene family have only described functions in haematopoiesis. Furthermore, no transcription factors have been described that are specific to embryonic macrophages in amphibians. I believe these were very strong reasons to investigate the expression and function of *SpiB* in *Xenopus.*

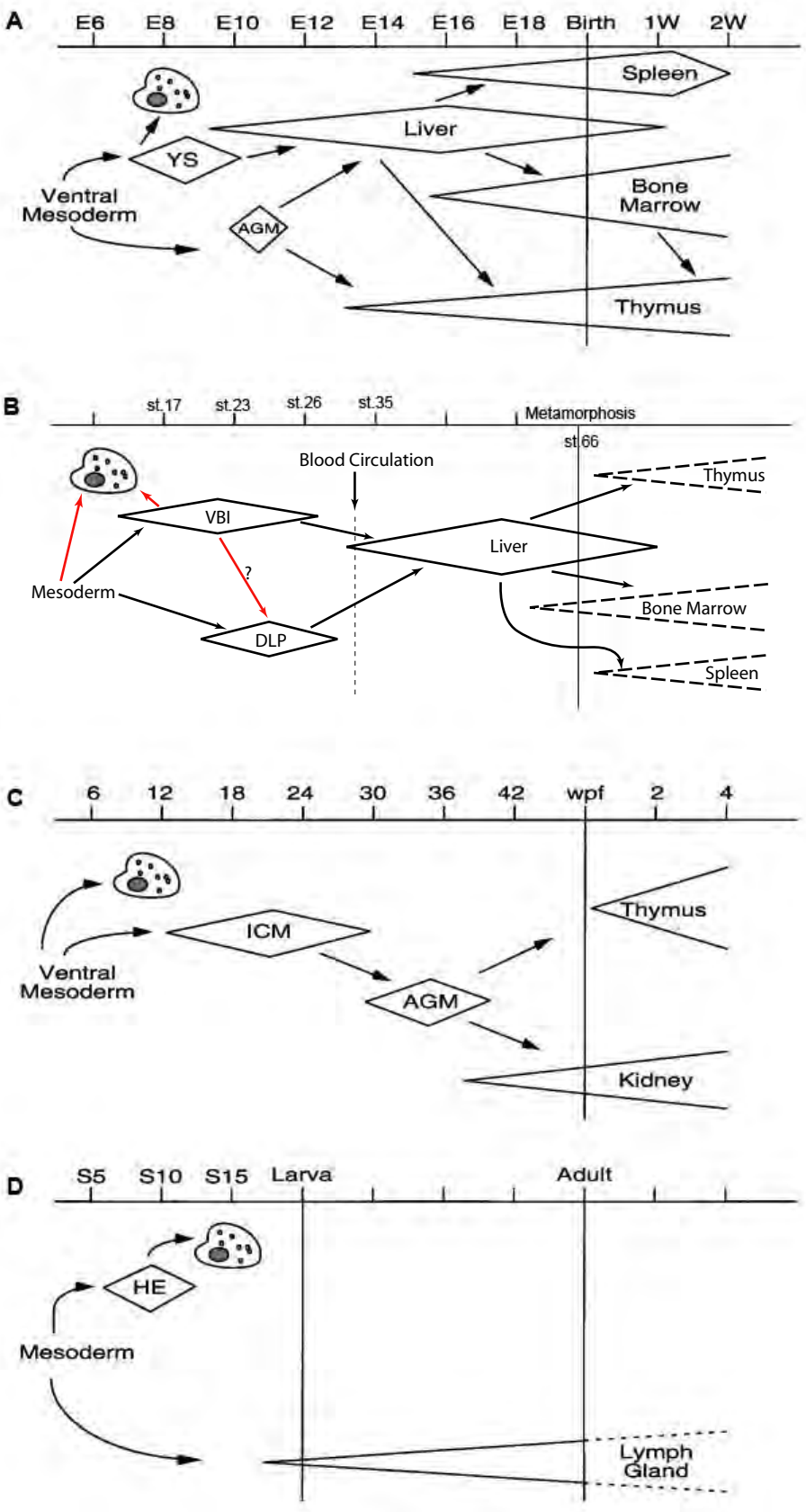
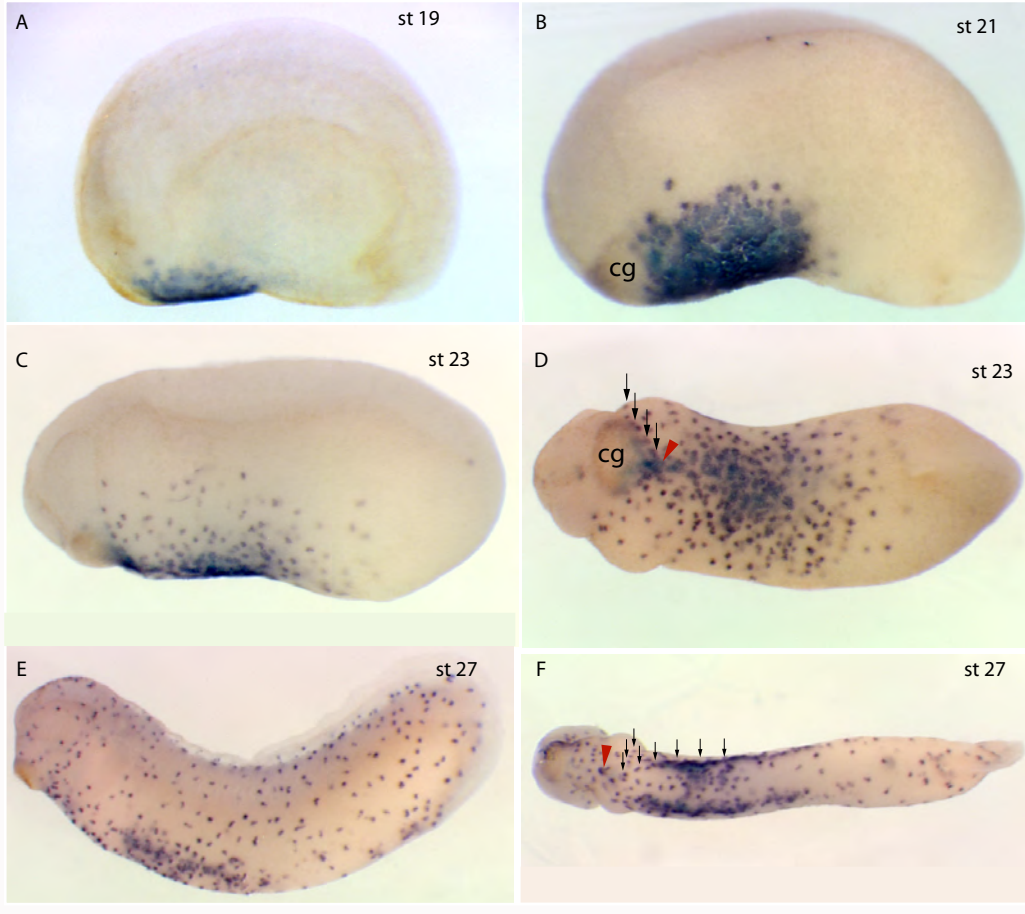


Figure 5.2 - Haematopoietic ontogeny in several model organisms. Adapted from Traver & Zon, 2002. **A**) mice, **B**) *Xenopus*, **C**) Zebrafish and **D**) *Drosophila*. The cell depicted in all panels is the early embryonic macrophage. Developmental time is shown in the horizontal bar with the corresponding stages on each model organism. Human and mice hematopoietic ontogeny are similar. But patterns of HSC migration and secondary lymphopoetic organs are very variable across phyla. *Xenopus* is not representative of all frogs and zebrafish of all fish. Black arrows indicate the migration and colonization paths known. Red arrows are emerging views on hematopoiesis of *Xenopus*. YS, Yolk-Sac; AGM, Aorta-Gonad Mesonephros; VBI, Ventral Blood Island; DLP, Dorsal Lateral Plate; ICM, Intermediate Cell Mass; HE, Hemocyte anlage.



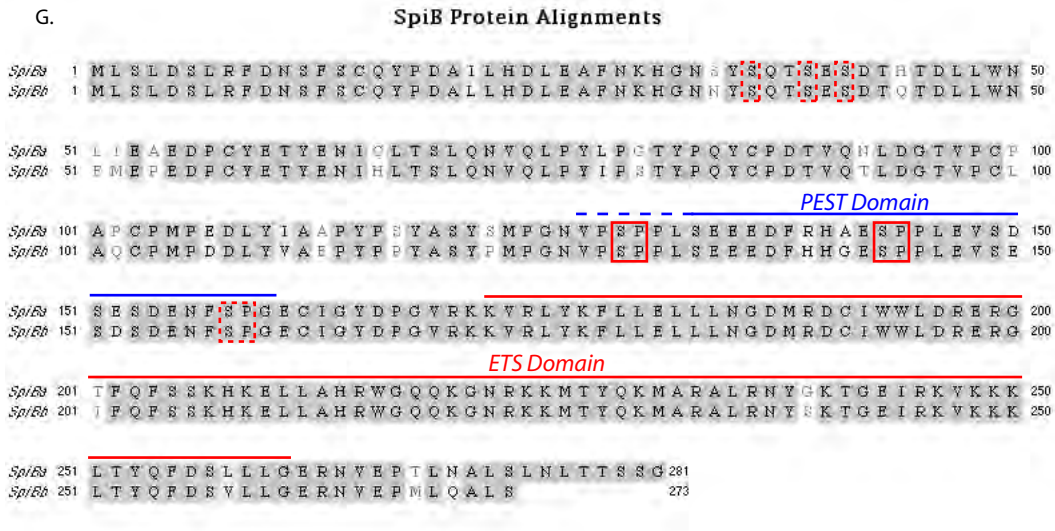


Figure 5.3 - *SpiB* Sequence and Embryonic Pattern of Expression. **A** to **F**) Pattern of expression by whole mount *in situ* hybridisation in tailbuds and tadpoles, anterior to the left, side views on A, B, C and E, ventral views on D and F. Black arrows point to the 'cluster streams' that appear to migrate away from the focal point, which is pointed by the red arrowheads. **G**) Homology between two *SpiB* paralogs (*SpiBa* and *SpiBb*) known in *Xenopus laevis*. Full red boxes show putative phosphorylation sites based on similarities with other mammalian ETS proteins. Dashed red boxes are also putative phosphorylation sites. Homologies among SpiB proteins can be found in *Shintani et al., 2000*. cg=cement gland

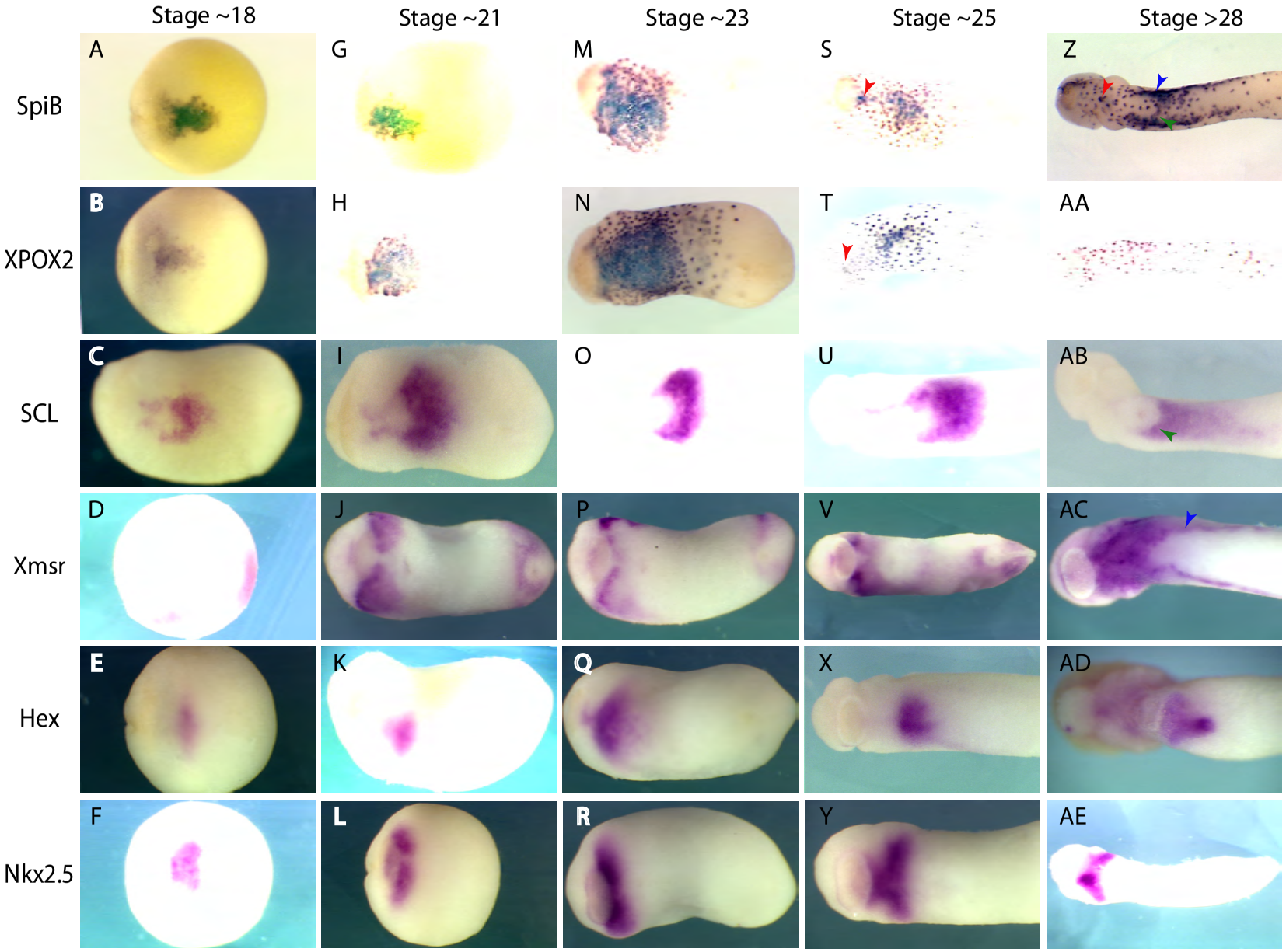


Figure 5.4 - Comparative analysis of *SpiB* pattern of expression. Patterns of expression by whole mount *in situ* hybridisation. Markers for the early embryonic macrophage (*SpiB, XPOX2)*, haemangioblast (blood, *SCL*), endothelial (*Xmsr, Hex*) and cardiac cell lineages (*Nkx2.5*). The expression of *SpiB* is identical to *XPOX2* for most of early embryonic development. The punctated expression of *SpiB* gives the impression of migrating streams of cell clusters. *SpiB* expression also overlaps with endothelial markers (blue arrowhead), with SCL in the posterior VBI where erythrocytes develop (green arrowhead). The red arrowhead indicates the focal point of migration between the cardiac fields described in *Smith et al. 2002*. Aproximate stages on top of the panels. All embryos on ventral views.

2.Results

2.1. Cloning of SpiB

Initially isolated as clone 176 (Table 3.1), *SpiB* is expressed in a salt and pepper pattern in the ventral mesoderm of the tailbud *Xenopus* embryo. Its cDNA sequence revealed an ETS transcription factor, containing a full-length coding sequence of 821 nucleotides. The translated protein sequence reveals a cDNA closely related to the previously reported *SpiB* (Shintani *et al.*, 2000). Since our sequence did not match exactly the previously reported *SpiB* sequence, similar clones from the EST database were selected and sequenced. In mice, two alternative translation start sites are used. I have looked for alternative start sites but found none any in all of the 5 full-length clones isolated (Chen *et al.*, 1998). Hereafter, I have renamed the previously reported *SpiB* as *SpiBa* and the sequence I isolated *SpiBb* (*SpiBa*-IMAGE: 5537169; *SpiBb*-IMAGE: 6957053).

SpiBa and *SpiBb* have different C-termini, and 3' UTR's which do not overlap in any extension and have different sizes, ~1Kb and ~2Kb, respectively (Figure 5.3). According with the EST database it seems that *SpiBa* is expressed at later developmental stages, only two ESTs for *SpiBa* are found in stage 66 *Xenopus* libraries. The alignment of all 5 sequences now available reveals that they cluster as two different *SpiB's.* Therefore, I isolated the paralog of the already known *SpiBa*, *SpiBa* and *SpiBb* are the two *SpiB* paralogs, 90% identical at the amino acid level (Figure 5.3).

The most similar proteins to *SpiB* in the database are *Raja eglanteria SpiD* (49%), *Paleosuchus palpebrosus SpiB* (49%) and both human and mouse *SpiB* (48%)(Accession numbers; AF320628, AF247364, AAH07921, XP_195649). It is known that the SPI sub-family contains the most divergent ETS transcription factors (Chen *et al.*, 1998) (pfam00178). In the SPI transcription factor family, the N-terminal transactivation domain is poorly conserved. However, over the ETS domain (169-262aa) the degree of identity conservation between mice and *Xenopus* is 78% (Figure 5.3). By homology to the known and biologically relevant used phosphorylation sites of mammalian *SpiB*, which control *SpiB* protein stability, I identified putative phosphorylation sites on *Xenopus SpiB* (128-30 and 143-5 aa). In particular, mouse *SpiB* turnover and transactivation properties are modulated by CKII through phosphorylation in serines 37 (N-termini), 129, 144 and 146 (PEST Domain) (Ray-Gallet and Moreau-Gachelin, 1999) (Figure 5.3 G). Other interesting motifs include the PEST domain (131-161aa). Conservation outside of the PEST and ETS domains is low.

2.2. SpiB Pattern of Expression

Expression of *SpiB* is first detectable by whole mount *in situ* hybridisation by stage 17 as a streak of cells on the ventral midline just posterior to the cement gland (Figure 5.1) *SCL* (Mead *et al.*, 1998), is a blood marker expressed at this stage. *SpiB* and *SCL* are expressed in the same area of the embryo, but with distinct patterns of expression (Figure 5.1 and 5.4). A few hours later such streak of cells resolves into an empty triangle pointing caudally. At this stage *SpiB* expression resembles the expression of *Xl-fli*. *Xl-fli* is another ETS transcription factor and a specific marker for endothelial cells (Meyer *et al.*, 1995). At stage 18, *SpiB* expression resembles more that of *XPOX2* a novel marker for early embryonic macrophages (Smith *et al.*, 2002). From stage 20 onwards, *SpiB* expression appears in a ventral square immediately posterior to the cement gland that starts to expand as punctuated expression. At stage 23/4, *SpiB* transcripts are detected in a more distinguishable spotty pattern extending from the immediate posterior of the cement gland to the rest of the embryo. Interestingly, the individual spots appear to be part of migrating cellular 'streams', originating from a focal point also described in *XPOX2* and *XLURP1* expressing cells (Smith *et al.*, 2002). Individual spots of expression reach the most dorsal parts of the embryo before stage 30 (Figure 5.3). I was not able to detect expression of *SpiB* in late tadpoles.

To test whether *SpiBa* and *SpiBb* had the same pattern of expression, anti-sense probes were constructed for the two paralogs and a double *in situ* hybridisation was performed with no visible differences in signal from the two probes. This means, that either the two probes cross hybridise to *SpiBb* sequences, or *SpiBa* and *SpiBb* do not show differences in their regulation, and that *SpiBa* is also expressed at early stages and just not found in the EST database.

From my *in situ* hybridisation analysis I cannot say that the *SpiB* cell clusters are migrating, or if we are in the presence of a dynamic pattern of expression. However, the *SpiB* expressing cell clusters appear to be migrating.

Smith *et al.* have recently described migrating cells expressing *XPOX2* and *XLURP1* for which the pattern of expression is very similar to *SpiB* (Smith *et al.*, 2002). In common, *SpiB* and *XPOX2* share the stage 19 ventral mesodermal triangular expressions, just posterior to the cement gland pointing caudally, and the expression there onwards. They also share the focal point of migration, described in stage 24 embryos located in between the two bilateral heart primordia. Moreover, expressing cells appear to be regularly spaced in imaginary lines from that focal point (Figure 5.3 black arrows). The spotty expression at stage 30 between *SpiB* and *XPOX2* is very similar (Figure 5.3 and 5.4). But differences do exist between *SpiB* and

XPOX2. At stage 28 and 30, *SpiB* expression appears in two very strong ventral 'streams', running from the focal point to the posterior on each side of the ventral blood island, that do not exist in *XPOX2* stained embryos. These 'streams', seen with *SpiB,* overlap with the staining obtained for endothelial markers, like *Xmsr* (Figure 5.4).

My results suggest that *SpiB* in amphibians is not a B cell marker, because of its location in the VBI, and is not even a definitive haematopoietic marker, as its expression does not overlap with *SCL* expression. Instead, *SpiB* is a marker of early embryonic macrophages, since its expression is identical to the expression of *XPOX2* and *XLURP-1*. In addition, *SpiB* expressing clusters might also be associated with endothelial development in analogy to the situation described in mouse, where macrophages, erythrocytes and endothelial cells develop in close association in structures called the blood islands.

To determine what was the nature of the punctuated expression, 40μm sections of WMISH *SpiB* embryos were analyzed at 3 different stages of development (stage 20, 26 and 30). At stage 20, expression is continuous over a few cell diameters. By stage 26, a continuous strip of expression still remains in the most ventral part of the embryo, however cell 'clusters' appear in the mesoderm at more lateral regions of the embryo. No hollow clusters were detected at stage 26. However, in stage 30 embryos, rare hollow 'clusters' of *SpiB* expressing cells can be observed (Figure 5.1C). At stage 30, even in the most ventral part of the embryo I can only find expression in cell clusters and not continuous over a several cell diameters. Although, at present I cannot identify these structures, which I name hollow cell clusters, they resemble the description of blood islands, or might be an intermidiate step in the formation of blood vessels.

To identify the cells expressing *SpiB,* I performed a comparative *in situ* hybridization analysis using markers for the haemangioblast, early myeloid, cardiac and endothelial lineages (Figure 5.4). I have used *SCL* (Mead *et al.*, 1998), *XPOX2* and *XLURP1* (Smith *et al.*, 2002), *Nkx2.5,* and both *Hex* (Newman *et al.*, 1997) and *Xmsr* (Devic *et al.*, 1996), respectively. At the end of neurulation (stage 18) and early tailbud stages (stage 21), the domain of *SpiB* expression overlaps with *Nkx2.5*, *Hex* and is very similar to the expression of *XPOX2*. However, at these stages the domain of *SpiB* expression is not similar to the domains of expression of either blood or endothelial markers, *SCL* or *Xmsr* (Figure 5.4 A-L). Later on (stage 23 to 25), the expression of *SpiB* is virtually indistinguishable from *XPOX2*, showing an expanding salt and pepper expression from the anterior ventral mesoderm to more lateral and posterior spots of expression (Figure 5.4 M, N, S, T). The core of *SpiB* expression is located within the 'empty' triangle of *SCL* expression, and considerably posterior to the presumptive liver and heart (Figure 5.4 O-R, U-Y). At stage 25, one can observe the focal

point described as the migration 'origin' of early myeloid cells (Figure 5.3 and Figure 5.4 S, T), between the heart fields (Smith *et al.*, 2002). One can also observe the 'streams' that appear to emerge from that focal point.

In tadpoles (stage 28, onwards), the expression of *SpiB* is substantially different from *XPOX2, SCL, Xmsr, Hex* or *Nkx2.5* (Figure 5.4 Z-AE) but shares several characteristics with many of them. With *XPOX2, SpiB* shares the punctuated expression that now reaches the most dorsal part of the embryo (Figure 5.4 AA). With *SCL* and *αT-globin*, *SpiB* cell clusters appear more condensed at the borders of the posterior VBI, delimited by *SCL* and *αT-globin* (Figure 5.4 AB, αT-globin not shown). Lastly, *SpiB* cell clusters overlap with *Xmsr* as it labels the endothelial cells on the viteline vessels flanking the VBI (Figure 5.4 AC & Figure 5.1B).

My comparison of *SpiB* expression with markers of blood, endothelial, heart, liver and myeloid cells confirms the expression of *SpiB* in a pattern identical to what has been found for the early myeloid lineage. However, *SpiB* expression is also associated with endothelial markers, which suggest that *SpiB* expressing cell clusters might be involved at some stage with endothelial development.

3.Discussion

Xenopus SpiB is the Homolog of Mice SpiB

The full-length amino acid sequence of *Xenopus SpiB* is 48% identical to mouse *SpiB* (Shintani *et al.*, 2000). The amino acid sequence outside of the C-terminal ETS DNA binding domain of ETS transcription factors present a high degree of divergence in all species analysed. Homologies in protein sequence alignments between *SpiB* homologs of several vertebrate species decay particularly fast with 'evolutionary' distance (for instance, identities between human/mouse are 83%, mouse/reptile (caiman) 52%, human/reptile 51% and human/amphibian 44%). Values for the closely related *PU.1/Spi-1* are not very different (Shintani *et al.*, 2000). In addition, within the SPI sub-family we find the most divergent ETS transcription factors (*PU.1/Spi-1*, *SpiB*, *SpiC* and *SpiD*) (Chen *et al.*, 1998). However, if we only compare the ETS domain of mouse and *Xenopus laevis SpiB,* conserved identities reach 78%. Furthermore, no other *Xenopus laevis* sequence on the database is closer to mouse *SpiB*. Importantly, *Xenopus SpiB* full-length protein sequence clearly clusters with other *SpiB* sequences and not with the closely related *PU.1/Spi-1* (Shintani *et al.*, 2000). For the above reasons, I am confident that I have isolated the *Xenopus laevis* homolog of the mouse transcription factor *SpiB.*

All 5 *Xenopus laevis SpiB* full-lenght sequences now available reveal two paralogs, *SpiBa* and *SpiBb*. Aminoacid identities over the coding region are of 90%. However, they present a different C-terminal and 3' UTR's (Figure 5.3 & data not shown). I have isolated *SpiBb* the paralog of the already known *Xenopus SpiBa.* Very little is known about the function of *SpiB* in amphibians and *SpiB* expression suggest an important embryonic function. Moreover, *Xenopus SpiB* embryonic expression indicates that its function in the embryo is not involved in B cell differentiation.

SpiB is Expressed in Embryonic Macrophages

I found that *SpiB* is a specific marker for a recently identified embryonic myeloid cell population, the early embryonic macrophages. Embryonic macrophages express *XPOX2* and *XLURP1* (Smith *et al.*, 2002). For most of early tailbud and tadpole development *SpiB* expression is identical to *XPOX2* (Smith *et al.*, 2002). No other early markers have been described for these cells in amphibians. However, two antibodies, XL-1 and XL-2, appear to recognise macrophage-like cells at later stages (Miyanaga *et al.*, 1998; Ohinata *et al.*, 1989; Ohinata *et al.*, 1990). Curiously, Smith *et al.* described the existence of four other molecules with an identical pattern of expression. In zebrafish, other markers such as plastin and draculin have been used (Herbomel *et al.*, 1999; Herbomel *et al.*, 2001). Such variety of

markers implies an advanced state of differentiation and is indicative of an important cellular type. Mammalian embryonic macrophages present different properties when compared with adult macrophages; these range from different proliferative abilities to the expression of different enzymes (Lichanska *et al.*, 1999; Lichanska and Hume, 2000). It would be interesting to evaluate these properties in the embryonic macrophages of the amphibian embryo, to further confirm the analogy of cell type in the different model organisms. Such, might provide clues in how the wave of primitive myelopoiesis evolved in vertebrates.

Embryonic macrophages or an equivalent cell type exist in a variety of organisms, from flies to mammals (Figure 5.2). They probably execute fundamental tasks during organogenesis either removing apoptotic cells, like in the interdigit space, or by participating in angiogenesis, like adult macrophages do (Bennett *et al.*, 2001; Lichanska *et al.*, 1999). It would be necessary to test these functions in the *Xenopus* embryo. Having found a transcription factor specific to embryonic macrophages, it will be valuable to understand primitive myelopoiesis and its role in early embryos. The onset of expression of *SpiB* on the VBI, the analogous region to the mammalian yolk sac, is another evidence for the equivalence between embryonic macrophages of *Xenopus* and mice, and most likely all the other embryonic macrophages in other organisms. *SpiB* embryonic expression has not been evaluated in other organisms, except mice, and could be a conserved marker for embryonic macrophages.

In zebrafish and *Xenopus* embryonic macrophages have been shown to be a migrating population of cells. *Drosophila* hemocytes are also migratory and participate in the morphogenesis of the embryo (Ribeiro *et al.*, 2003). Their path of migration is regulated by VEGF signalling (Cho *et al.*, 2002). Although I have not proven that *SpiB* expressing cells are motile, it would be interesting to study how embryonic macrophage are guided in their migration and how they form the 'streams' visible throughout the embryo at stage 24.

SpiB and the Haemangioblast

The *SpiB* pattern of expression indicated a close relationship between *SpiB* expressing cells and blood or endothelial cells (Figure 5.4). The haemangioblast is the precursor of both blood and endothelial cells, giving rise to the HSC and the angioblast. Perhaps, *SpiB* could be involved in cellular decisions the haemangioblast needs to make in order to differentiate. One reason to mention such speculation emerges from the morphological description of the haemangioblast, in ways resembling what I have observed in sections of *SpiB* expressing clusters. Haemangioblasts arise in mice YS at 7.5dpc, they form homogeneous clusters of cells, but shortly thereafter cells in the inner region differentiate into cells with haematopoietic fate (Zon, 2001, chapter 14). The *in situ* hybridisation analysis of *SpiB* in

sections reveals initially homogeneous clusters that later and on rare occasions appear hollow (data not shown). A more precise spatial correlation needs to be made in order to associate *SpiB* expressing cells with haemangioblast, but it remains plausible that *SpiB* cell clusters associate with the haemangioblast during development.

SpiB and Endothelial Cells

Xl-fli is another ETS transcription factor expressed at sites of important cellular migrations. At stage 17, *SpiB* is expressed in a pattern similar to *Xl-fli* (Meyer *et al.*, 1995). *SpiB* and *Xl-fli* are expressed near the heart primordia, posterior to the cement gland, in the mesoderm and as ventral triangle pointing to the posterior of the embryo. The domain of *SpiB* expression soon expands to the shape of a ventral rectangle (Figure 5.1 and 5.3A). *Xl-fli* is expressed in angioblasts and endothelial cells (Meyer *et al.*, 1995). Is *SpiB* expressed in cells that are or will become endothelial? *Xl-fli* and other ETS transcription factors regulate cellular motility and adhesiveness, but it remains to be tested if *SpiB* and *Xl-fli* are expressed in the same cells or cell clusters.

Other markers of the endothelial lineage are *Xmsr* and *Hex* (Devic *et al.*, 1996; Newman *et al.*, 1997). Curiously, *Hex* is also expressed in haematopoietic cell lines, in particular in myeloid and B-cells (Bedford *et al.*, 1993; Crompton *et al.*, 1992). But at the embryonic stages we are dealing with, *Hex* is only expressed in the developing liver, thyroid and weakly in the viteline veins (not visible in Figure 5.4) (Newman *et al.*, 1997). However, *Xmsr* is a marker of the developing vasculature, expressed in the endothelium associated with the developing heart and the viteline veins. At tadpole stage *SpiB* expression can be observed associated with *Xmsr* expression in the viteline veins flanking the blood islands (Figure 5.1 & 5.3T).

In summary, the expression of *SpiB* is highly dynamic and overlaps with several markers of endothelial development. The biological meaning of the association of *SpiB* expressing cells and endothelial cells is not clear at the moment.

SpiB and Blood

Mice *SpiB* is expressed only in B and T lymphocytes and believed to be functionally specific to the B cell lineage. Mice *SpiB* is expressed in the spleen at 19.5 dpc, but not detected in the embryonic liver (Su *et al.*, 1997; Su *et al.*, 1996). Furthermore, the authors have looked for the expression of *SpiB* within the embryo proper and not the analogous site to the VBI, the yolk sac, and therefore I consider that the expression data should be re-evaluated. The embryonic liver is the first and major B lymphopoietic organ during fetal life, where B

cell precursors can be detected at 14.5dpc. Moreover, B lineage committed cells can be detected slightly earlier in the AGM (de Andres *et al.*, 2002). This is to say that, *SpiB* in mice, is detected at relatively late stages of B cell development and late in mice embryonic life. Moreover, when compared with the amphibian expression, mice *SpiB* is not found in the equivalent anatomical sites of the embryo. This suggests, that either the expression of mammalian *SpiB* is not fully characterized, or mammalian *SpiB* is not the funtional homolog of amphibian *SpiB*.

However, B lymphopoiesis varies drastically among vertebrates (Hansen and Zapata, 1998; Zettergren, 2000). In vertebrates, the B lymphopoietic organs can be as different as the pronephros, mesonephros, kidney, thymus, spleen or the avian bursa of Fabricius, and follow different timings and paths of colonization.

Interestingly enough, *Xenopus* B cell development resembles the mammalian in many ways. B cells enter the liver at stage 39 (Hadji-Azimi *et al.*, 1982). More recent work, established that the presence of B cell in the peripheral layer of the liver at 3 dpf derive from HSCs in the DLP. At 26 dpf, *Xenopus* hepatic B cells are still largely derived from the DLP (Hansen and Zapata, 1998; Turpen, 1998; Turpen *et al.*, 1997). Therefore, the expression of *SpiB* at neurula, tailbud and early tadpole cannot represent expression in B cells or their immediate precursors. I have to assume that amphibian *SpiB* is not expressed in lymphocytes at the early stages of development described here. Hence, *SpiB* must have as earlier and different function in amphibian development when compared with mammalian *SpiB* function.

However, my data cannot exclude the possibility of expression of *SpiB* in *Xenopus* B cells that might appear later in development, either developing in the liver or in any other lymphopoietic organ. In those cells, if any, amphibian *SpiB* can have similar functions to mammalian *SpiB*.

SCL specifies haematopoietic mesoderm (Mead *et al.*, 1998). At stage 17, when *SpiB* staining is still weak, the domain of *SpiB* expression is well within the ventral domain of *SCL* expression (Ciau-Uitz *et al.*, 2000; Mead *et al.*, 2001; Mead *et al.*, 1998; Walmsley *et al.*, 2002). Interestingly, a few hours later (stage 18, Figure 5.1B & Figure 5.4) the expression of both genes appears to segregate into different domains and are mutually exclusive (Figure 5.1B). At this stage, *SCL* expression appears in two streams flanking the *SpiB* expression domain that run from the cement gland in a posterior direction, broadening to a ventro-lateral domain of expression. Later this ventro-lateral domain appears to arrange itself in a 'V' shape characteristic of the posterior blood island (SCL or αT-expression). Only at stage 25/26, the *SpiB* expression that appeared clustered within the flanking streams extends to a much

broader domain of punctuated expression through the ventral and ventro-lateral parts of the embryo (Figure 5.4 N&O). That is to say, although *SpiB* and *SCL* are expressed in the ventral haematopoietic region of the amphibian embryo, their expression can be sub-divided into distinct domains. The conclusion is that even in the earlier stages, *SpiB* expressing cells are not a subset of those expressing *SCL*, and either do not represent blood or represent a different subset of blood cells that do not express *SCL*.

In retrospect, *SpiB* expression marks early myeloid cells, the embryonic macrophages and not B cells as previously assumed. Furthermore, these *SpiB* cell clusters associate with markers of both blood and endothelial development, opening new lines of research at embryonic stages of haematopoietic development.

SpiB and the Evolution of the Adaptative Immune System

Nowadays, the evolution of the immune system is a matter of strong debate. The adaptative immune function, executed only by lymphocytes, is believed to have evolved with jawed vertebrates, predatory life styles and in close association with the digestive system (Matsunaga and Rahman, 1998). The case is that only jawed vertebrates produce antibodies in the way mammals do. One must realize that the amphibian immune response is substantially different from the mammalian, however parallels are expected. Interestingly, the SPI family of ETS transcription factors is only know in higher vertebrates. Taking advantage of the position of amphibians in the evolutionary scale it will be interesting to keep comparing not only the *SpiB* gene structure but also the *SpiB* gene expression in several model organisms to better understand the development of haematopoiesis and the evolution of primary waves of myelopoiesis.

Blood development occurs in two major waves, an embryonic (primitive) and a definitive one (Godin and Cumano, 2002). *SpiB* is a marker of primitive myelopoiesis, and might be associated with other cell types during development. Therefore, *SpiB* is not only a tool to explore the primitive wave of myelopoiesis, a poorly characterized phenomena. But perhaps, *SpiB* is also an important molecular player in the differentiation of important cell types such as the haemangioblast or endothelial cells.

Future Work

Many roads are unexplored at this stage, even the basic ones on the function of *SpiB*. Further studies on this project will have to start on the gain and loss-of-function, very likely using over expression, trying dominant negatives or morpholinos. Some markers are available for the analysis of the phenotype and there are many characteristics of the embryonic

macrophages yet unexplored. One other very informative experiment would be the lineage labelling of the *SpiB* cells. Do they just disappear? I would not expect it. If not, what will they become? This would perhaps be possible with the use of a *SpiB:GFP* transgenic line.

I would like to finish with two puzzling issues. The first issue has to do with the function of the embryonic macrophages. Are embryonic macrophages involved mostly on primary immune defence, or do they participate in organogenesis, or both? *Drosophila* hemocytes participate in the organogenesis of the CNS (Ribeiro *et al.*, 2003; Sears *et al.*, 2003). But the role of primary myelopoiesis in organogenesis is not characterized at all in vertebrates.

The second issue has to do with the migration of these cells. How are they guided, where do they stop? Again, *Drosophila* hemocytes are guided by VEGF signalling (Cho *et al.*, 2002). Indeed, ETS transcription factors are known to be downstream of receptor tyrosine kinase signalling cascades, similar to the VEGF signalling cascade.

Related with the issue of migrating embryonic macrophages, is the timing of their migration away from the VBI. Could these cell clusters migrate to the DLP? If one remembers the following facts; that the VBI is the equivalent site to the mouse yolk sac, and the DLP is equivalent to the mouse AGM; that no link in the form of motile cells has been made between the two locations that harbour HSC in the early mouse embryo; Such, added to the fact that, is not understood how HSC arise independently in the p-Sp/AGM; and that circulation in mice is established around 10.5 dpc (Godin and Cumano, 2002). Will easily realise the importance of the link between the two haemogenic sites in the embryo, either in mouse or *Xenopus.*

The reason to propose this hypothesis is only the timing of the migration. The migration away from the VBI occurs between stages 21 and 24 in frogs, which translate approximately into 8.5 and 9.5dpc in mice, the time at which HSC potential develops in the AGM. If embryonic macrophages reach the DLP they might represent a novel association between the VBI/ yolk sac and the DLP/AGM, independent from the colonization dependent on circulation, that in frogs starts later than stage 35. Such a link between the two haemogenic sites in the early embryo, if true, could change the way we look at blood development.

CONCLUDING STATEMENT

The work presented here has impact on our current understanding of two areas of embryonic development. The first area, and part of my initial goals, was to improve our knowledge on vertebrate endodermal patterning. The markers found in my screen reveal sub-domains of gene expression in the neurula and tailbud endoderm, where before, no markers were available. These will undoubtedly permit the study of patterning mechanisms that act in the endoderm, at a time where organ induction must occur, and inaccessible up to now. Surely, this will assist our understanding of endoderm development.

These endodermal gene expression domains also confirm that patterning is concomitant with endodermal specification. Furthermore, many of these genes were previously uncharacterised and their study might reveal important regulators of endoderm development. Ongoing preliminary loss-of-function experiments show phenotypes that correlate with the time and place of their endodermal expression.

The second area of embryonic development in which this work has impact is embryonic haematopoietic development. I found *SpiB,* a novel marker for early embryonic macrophages, a cell population only recently identified, and which is also an uncharacterised transcription factor in *Xenopus*. *SpiB* function poses many interesting questions yet to explore. *SpiB* might be an important transcription factor, which is specifically expressed on embryonic macrophages, cells that are postulated to participate in embryonic development through the removal of apoptotic cells, immune defence, and which development is closely associated with cell lineages essential to any organism, such as blood and endothelium.

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