Epigenetic Features of Mouse Trophoblast

Peter J. Rugg-Gunn

Epigenetics Programme, The Babraham Institute

Babraham Research Campus, Cambridge, CB22 3AT, UK

Centre for Trophoblast Research, University of Cambridge

Downing Street, Cambridge, CB2 3EG, UK

Telephone: +44 1223 496385

Fax: +44 1223 496022

Email address: peter.rugg-gunn@babraham.ac.uk

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Abstract

Trophoblast cells are required for the growth and survival of the foetus during pregnancy, and failure to maintain appropriate trophoblast regulation is associated with placental insufficiencies and intrauterine growth restriction. Development of the trophoblast lineage is mediated by interactions between genetic and epigenetic factors. This review will focus on new insights that have been gained from analysis of mouse models into the epigenetic mechanisms that are required for the early establishment of the trophoblast lineage and for the development of specialised cell types of the foetal placenta. In particular, the importance of DNA methylation, 5-hydroxymethylcytosine and histone modifications in orchestrating trophoblast gene expression and functional outcome will be discussed. These insights are beginning to be extended towards human studies, and initial results suggest that the causes and consequences of a variety of placental pathologies are related to epigenetic processes. Furthermore, the epigenetic landscape that regulates trophoblast cells seems to be particularly vulnerable to perturbation during development. This has major implications for diet and other environmental factors during pregnancy.

Keywords:

Trophoblast, placenta, epigenetic, DNA methylation, 5-hydroxymethylcytosine, histone methylation

Introduction

Trophoblast cells are responsible for a remarkable array of highly specialised functions to ensure the healthy development of the foetus. Trophoblast cells mediate implantation of the embryo to the uterus, remodel the maternal environment to enable efficient gas and nutrient exchange, secrete gestational hormones, and alter the maternal immune system to tolerate the developing foetus (Cross et al., 2003, Maltepe et al., 2010). The importance of these functions are emphasised by the considerable time and resources that the conceptus devotes to the formation of trophoblast cells during the initial stages of development (Cockburn and Rossant, 2010, Cross 2005). The critical functions of trophoblast also mean that ~10% of human pregnancies suffer from defects in trophoblast function and placental insufficiency, including recurrent miscarriage, preeclampsia and intrauterine growth restriction (Benirschke and Baergen, 2006, Steegers et al., 2010). Furthermore, excessive trophoblast growth is associated with placental complications, including acreta (Nwagha, et al., 2005). These disorders demonstrate the importance of maintaining appropriate trophoblast regulation during pregnancy.

Formation of functional trophoblast cells during development is a result of complex interactions between genetic and epigenetic factors (Hemberger, 2010). Our understanding of human trophoblast has been gained largely through analysis of placental tissue following pregnancy termination or premature delivery. Although these studies are important for investigating human placental physiology, they do not tell us about the molecular mechanisms responsible for trophoblast formation or the very earliest events in establishing the trophoblast lineages. Animal models such as the mouse have proven invaluable for deciphering these questions. In particular, the embryonic lethality associated with many targeted mutations in mice has been shown to involve placental defects (Rossant and Cross, 2001, Watson and Cross, 2005). In addition to genetic factors and signaling molecules, this list of key

developmental regulators contains genes involved in a range of epigenetic processes and has therefore expanded greatly our understanding of epigenetic networks that function in trophoblast development. This review will focus on new insights that have been gained from analysis of mouse models into the epigenetic mechanisms that are required for the early establishment of the trophoblast lineage and for the development of specialised cell types of the foetal placenta.

Overview of mouse trophoblast development

In mice, the embryonic lethality associated with loss of epigenetic factors can be caused by defects in trophoblast formation and function at multiple stages of development (Figure 1). The trophoblast of the placenta arises from the first cell type to differentiate in the mammalian embryo – the outer trophectoderm (TE) layer of the blastocyst. Lineage tracing and chimaeric analyses have revealed that TE will contribute only to the placenta, whereas the inner cell mass (ICM) of the blastocyst will generate the fetus, yolk sac and placental vasculature (Gardner and Johnson, 1972, Gardner, et al., 1973, Papaioannou, 1982, Rossant and Croy, 1985, Rossant and Lis, 1979). TE cells that are not in contact with the ICM are called the mural TE. These cells stop proliferating soon after they are formed, but continue to endoreduplicate their DNA to generate polyploid trophoblast giant cells (TGC), which are critical for implantation, remodeling the maternal decidua and metabolic exchange between the embryo and mother during the initial yolk sac placental stage (Cross, et al., 1994). TE cells overlying the ICM are called the polar TE and they continue to proliferate, resulting in the formation of the extraembryonic ectoderm (ExE) and ectoplacental cone (EPC) of the early postimplantation conceptus (Copp, 1979, Gardner, Papaioannou and Barton, 1973). The ExE gives rise to the chorion, which will make contact with the extraembryonic mesoderm-derived allantois in a process called chorioallantoic attachment. This critical event occurs at approximately embryonic day E8.5 and is required to support the rapid growth of the foetus,

which can no longer rely on the yolk sac placenta for efficient nutrient exchange with the mother. Defects in the chorioallantoic placenta usually cause embryonic lethality between E9.5 and E13.5 of development (Rossant and Cross, 2001, Watson and Cross, 2005). The chorion-derived cells that form the trophoblast undergo extensive villous branching to create a densely packed structure called the labyrinth (Cross, et al., 2003). The allantoic-derived cells generate the foetal components of the placental vascular network. This tissue configuration creates highly efficient gas and nutrient exchange. The EPC gives rise to secondary TGC and the spongiotrophoblast, which consists of a compact layer of trophoblast cells that provides structural support to the placenta.

Although there are structural and functional differences between human and mouse placentas, considerable similarities also exist (Adamson, et al., 2002, Georgiades, et al., 2002). For instance, the labyrinth layer of the mouse placenta shares several functions with the chorionic villi of the human placenta and both tissues are responsible for exchange between the foetal and maternal blood supply. In humans and mice, the villi are covered by syncytiotrophoblasts that lie in direct contact with maternal blood (Coan et al., 2005, Kaufmann and Burton, 1994). Furthermore, recent studies have shown that human and mouse placentas are also similar at the molecular level, with over 70% co-expression of orthologous genes in both species (Cox, et al., 2009). These data suggest that the mouse placenta is an appropriate model to study the formation and function of the trophoblast lineages, results of which are highly relevant to understanding human physiology.

Another advantage of studying mouse development is the ability to derive stable self-renewing stem cells from trophoblast tissue, termed trophoblast stem (TS) cells (Figure 1) (Tanaka, et al., 1998). These cells retain the defining characteristics of TE and ExE cells, including gene expression patterns and growth characteristics. TS cells, therefore, can provide

a valuable and potentially limitless source of trophoblast cells for analysis. Importantly, when coaxed to differentiate *in vitro*, or transferred into a chimaeric host embryo, TS cells form only trophoblast-derived cell types and do not give rise to embryonic tissues (Tanaka, et al., 1998). TS cells have not been derived directly from human trophoblast tissue, perhaps due to differences in the developmental stage at which the highly proliferative trophoblast cells arise. Curiously, and in contrast to mouse embryonic stem (ES) cells, trophoblast-like cells can be differentiated from human ES cells *in vitro* and can be maintained in the short-term by continually selecting for cells with high chorionic gonadotrophin secretion (Harun, et al., 2006, Xu, et al., 2002). However, these cells proliferate poorly and display a number of molecular differences from trophoblast cells within the human placenta and from mouse TS cells (Hemberger, et al., 2010), leading to uncertainties surrounding their usefulness to study the regulation of human trophoblast. The mouse, therefore, continues to provide important and valuable *in vivo* and *in vitro* systems in which to study mammalian trophoblast development.

Epigenetic regulation of gene expression

Epigenetic regulation of gene expression is necessary for the correct establishment of developmental programs and for the maintenance of cell fates. Importantly, epigenetic processes are thought to be at the interface between gene regulation and the environment, such that external influences can have a major and potentially long-term impact on gene expression (Reik, 2007). New studies are making important links between alterations to epigenetic regulation in the placenta and diseases of gestation and early life. These findings have ~~major~~ critical implications for diet and other environmental factors during pregnancy (see article by Miguel Constancia in this issue).

The major epigenetic mechanisms in mammals include DNA methylation, covalent modifications to histone proteins, RNA-mediated processes (including small RNAs and long non-coding RNA) and higher-order chomatin organisation. The following sections will summarise recent studies on the role of DNA methylation and histone modifications in regulating trophoblast development and function. For information on the role of RNA-mediated process in placental regulation, readers are referred to the recent review by Maccani and Marsit, 2009.

DNA methylation is a critical regulator of trophoblast formation and function

The majority of DNA methylation in mammals occurs symmetrically on the cytosine base in CpG dinucleotides, termed 5-methylcytosine (5mC), and is generally associated with transcriptional repression. Mechanisms of establishment by *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b* and maintenance by DNA methyltransferase *Dnmt1* of DNA methylation are well characterized, thereby providing an ideal system in which to study epigenetic regulation of gene expression (Bird, 2002). DNA methylation has important roles during development and regulation of genomic imprinting, X-chromosome inactivation and preservation of chromosome stability (Bird, 2002, Goll and Bestor, 2005).

Genome-wide changes in DNA methylation occur during the initial stages of mammalian development, coinciding with the segregation of embryonic and trophoblast lineages (Hemberger, et al., 2009, Santos, et al., 2002). In mice and humans, fertilisation initiates a wave of epigenetic reprogramming that is characterised by extensive DNA demethylation (Dean, et al., 2003, Howlett and Reik, 1991, Mayer, et al., 2000, Monk, et al., 1987, Oswald, et al., 2000, Santos, et al., 2002, Santos, et al., 2010). Levels reach a nadir in morula stage embryos, followed by initiation of *de novo* DNA methylation before the blastocyst forms. Global DNA methylation levels are established to different levels in the embryonic and

extraembryonic lineages, thus creating a striking epigenetic asymmetry. Using an antibody that is highly specific for 5mC, the TE appears to be relatively hypomethylated compared to the ICM (Santos et al., 2002, Santos, et al., 2010). These global differences in DNA methylation levels are retained in later embryo and trophoblast tissues. Initial studies using methylation-sensitive Southern blotting revealed extensive undermethylation at repetitive regions and candidate genes in mouse EPC and ExE compared to embryonic tissues (Chapman, et al., 1984, Rossant, et al., 1986). More recently, genome-wide analysis of ES and TS cells confirmed the asymmetry in DNA methylation levels, but suggested that the majority of the differences are likely to be within intergenic regions, repeats sequences and centromeric heterochromatin, whereas promoter-associated regions are methylated at similar levels between embryonic and extraembryonic cells (Farthing, et al., 2008). Low global levels of DNA methylation are also present in human placental tissue and purified cytotrophoblasts, thus revealing similar epigenetic asymmetries (Fuke, et al., 2004, Gama-Sosa, et al., 1983, Novakovic, et al., 2010).

The acquisition of different DNA methylation levels between ICM and TE during development coincides with their restriction in cell fate to embryonic and extraembryonic lineages, offering the tantalising possibility that the two processes may be linked. However, there is no evidence to suggest that this epigenetic remodeling has a direct role in lineage establishment. For instance, mutant embryos with no detectable DNA methylation appear to form normal blastocysts, with appropriately segregated ICM and TE lineages (Sakaue, et al., 2010). Nevertheless, further characterisation of these early developmental processes would help reveal the molecular underpinnings of epigenetic programming. It is not known, for example, whether DNA methylation actually contributes to gene repression during the earliest stages of TE and ICM segregation, and therefore future studies to address this question using genome-wide DNA methylation and transcriptome profiling of embryo lineages should be

informative. Better understanding of the molecular mechanisms that orchestrate lineage-specific DNA methylation, and how they link with other developmental pathways, is also a priority for future research.

Although seemingly not involved in lineage establishment, it is possible that increased levels of DNA methylation in embryonic cells are required to prevent inappropriate conversion towards a trophoblast cell fate. Consistent with this idea, there is abundant evidence to support a role for DNA methylation in lineage maintenance. Genetic inactivation of *Dnmt1* results in ectopic expression of trophoblast genes in embryonic tissues at E9.5 (Ng, et al., 2008). Furthermore, ES cells with diminished DNA methylation levels, achieved either by removal of *Dnmt1* or treatment with the DNA methyltransferase inhibitor 5-azacytidine, readily differentiate into TGC in TS cell conditions and will also contribute to TE in aggregation chimeras (Ng, et al., 2008, Sakaue, et al., 2010). Similarly, *Dnmt3a*/*Dnmt3b* and *Dnmt1*/*Dnmt3a*/*Dnmt3b* mutant ES cells adopt a predominantly extraembryonic cell fate upon differentiation (Jackson, et al., 2004, Sakaue et al., 2010).

Could it be that only extraembryonic cell types can tolerate low DNA methylation levels, or is there a direct role for DNA methylation in preventing embryonic cells from switching to a trophoblast cell fate during development? Recent DNA methylation profiling studies provide strong evidence in support of a direct role. *Elf5* encodes an Ets family transcription factor that is essential for trophoblast development and appears to be epigenetically regulated (Ng, et al., 2008). The *Elf5* promoter is hypermethylated and transcriptionally repressed in embryonic tissues, and hypomethylated and expressed in trophoblast tissues. Importantly, Elf5 forms a regulatory loop with other key trophoblast factors. Therefore, when DNA methylation levels are experimentally reduced in embryonic cells, *Elf5* becomes ectopically activated and cells adopt a trophoblast fate. *Elf5* forms an important link between genetic and epigenetic

pathways during early mouse development and suggests that epigenetically regulated factors have important roles in lineage maintenance.

Additional biological reasons to explain lineage-specific DNA methylation levels have been proposed (Hemberger, 2010). Perhaps undermethylation may destabilise trophoblast cells so that they do not perdure after pregnancy and cause harm to the mother. Of course, one consequence might be that there is danger of activating repetitive elements within the trophoblast genome, but this may be an acceptable risk as the placenta is a short-lived organ. More provocatively, it is possible that transposon activation may have an instructive role in trophoblast establishment and regulation. In support of this idea, many genes known to be important for placental development are retrotransposon-related sequences (*Peg10*, *Rtl1*; Ono, et al., 2006, Sekita, et al., 2008), endogenous retrovirus envelope proteins (*ERVW-1*; Frendo, et al., 2003, Prudhomme, et al., 2005, Sekita, et al., 2008) or are controlled by retrovirally-derived regulatory elements (*Ptn*, *Ednrb*, *Mid1*, *Lep*, *Ipp*; Chang-Yeh, et al., 1993, Prudhomme, et al., 2005). Importantly, DNA methylation levels are critical for regulating transcription of these genes.

Despite low levels of global DNA methylation in trophoblast tissues, analysis of mouse mutants have shown that DNA methylation pathways are required for placental formation and function. Genetic inactivation of *Dnmt1* and *Dnmt3a*/*Dnmt3b* is lethal in mouse embryos and mutants have chorioallantoic defects (Li, et al., 1992, Okano, et al., 1999). *Dnmt1*-deficient placentas ectopically express the embryonic transcription factor *Pou5f1*, suggesting that a subset of genes need to be epigenetically repressed in trophoblast cells (Hattori, et al., 2004). In addition, *Dnmt3l*, which is highly expressed in the chorion, is required for the formation of the labyrinth layer of the placenta (Arima, et al., 2006). Mutants also reveal poor development of the spongiotrophoblast layer and excess TGC formation, demonstrating a critical role for

DNA methylation in regulating the specification of trophoblast lineages (Arima, et al., 2006). An opportune system in which to study these processes further are *Dnmt1*/*Dnmt3a*/*Dnmt3b* mutant TS cells, which have undetectable levels of DNA methylation at major satellite repeats (Sakaue, et al., 2010). These mutant TS cells apparently show similar growth properties to wild-type TS cells, raising the possibility that undifferentiated TS cells may not require DNA methylation for self-renewal or long-term proliferation. However, mutant TS cells do show differences in gene expression profiles compared to wild-type TS cells, including several genes encoding prolactin-like proteins and Rhox genes (Sakaue, et al., 2010). A number of imprinted genes are also affected, with elevated expression of *H19* and complete repression of *Igf2*, which encodes a growth factor required for placental growth (Constancia, et al., 2002). These changes may provide a molecular basis for certain trophoblast phenotypes associated with DNA methyltransferase mutants. TS cells devoid of DNA methylation are able to differentiate *in vitro* into a subset of trophoblast cell types including TGC and spongiotrophoblast and could provide important molecular insight into the role of DNA methylation in specialised trophoblast cells.

Changes in DNA methylation at individual gene promoters may provide one method of transcriptional regulation during trophoblast development. For example, *Ddah2* expression is higher in EPC and other differentiated trophoblast cells, as compared to ExE (Tomikawa, et al., 2006). These transcriptional changes are inversely correlated with DNA methylation levels at the *Ddah2* promoter. As DNA methylation inhibited a reporter plasmid carrying the *Ddah2* promoter sequence, these data imply that DNA methylation may regulate *Ddah2* expression during trophoblast differentiation. Similar effects on individual genes may also occur during human trophoblast development. For example, reduction of DNA methylation levels by treatment of human choriocarcinoma cell lines with 5-azacytidine is associated with increased expression of *E-Cadherin* and loss of invasive phenotype (Rahnama, et al., 2006).

Trophoblast migration and invasion during development, therefore, may be mediated partly through epigenetic regulation of cell adhesion molecules. Similarly, epigenetic control of the human growth hormone locus has been well characterised in human synciotrophoblasts (Kimura, et al., 2007). Further studies to expand our understanding of these processes to a genome-wide scale will lead to a better understanding of trophoblast regulation.

Thus overall, DNA methylation is a tightly regulated epigenetic process that is critical for trophoblast formation and function. DNA methylation can establish regulatory networks to maintain appropriate restriction of embryonic and trophoblast lineages, and can modulate gene transcription and cell differentiation during trophoblast development.

A role of 5-hydroxymethylcytosine in trophoblast regulation?

In addition to methylation of cytosine, other modifications to DNA may also have important implications for epigenetic regulation of trophoblast establishment and function. Found at low levels across the genome of diverse cell types, 5-hydroxymethylcytosine (5hmC) is a newly identified base that is generated by oxidation of 5mC by the Tet family of enzymes (Kriaucionis and Heintz, 2009, Tahiliani, et al., 2009). In turn, 5hmC can be excised by base excision and therefore may provide the long sought after pathway for active DNA demethylation (Guo, et al., 2011, He, et al., 2011). Although removal of 5mC is associated with alleviating gene repression, it is possible that 5hmC itself may also have an active, functional role in gene transcription.

Does 5hmC contribute to establishing the trophoblast lineage? Studies using highly specific antibodies detected 5hmC in all blastocyst cells, with slightly higher signal intensity in the ICM compared to the TE (Ruzov, et al., 2011). Tet1 protein is expressed at higher levels in ICM than TE, and this is consistent with elevated *Tet1* transcripts in ES cells compared with

TS cells (Ito, et al., 2010). Lineage-specific analysis of *Tet2* and *Tet3* expression has not been reported. Knockdown of *Tet1* in two-cell stage mouse embryos resulted in lineage skewing, such that cells with reduced *Tet1* appeared to contribute preferentially to the TE and not ICM (Ito, et al., 2010). However, mice lacking zygotic *Tet1* expression were found to be viable and fertile, arguing against a major role for *Tet1* in lineage specification (Dawlaty, et al., 2011). It is possible that other Tet proteins, Tet2 and Tet3, are compensating for the loss of Tet1, therefore double and triple knockout mice may need to be generated to establish the role of 5hmC in lineage segregation.

Genome-wide profiling experiments have recently explored the role of 5hmC in cell regulation. In ES cells, 5hmC and Tet1 are associated with promoters and gene bodies of both transcriptionally active and Polycomb-repressed genes (Ficz, et al., 2011, Koh, et al., 2011, Pastor, et al., 2011, Stroud, et al., 2011, Szulwach, et al., 2011, Williams, et al., 2011, Wu, et al., 2011a, Wu, et al., 2011b). Genes within the latter category include known trophoblast regulators, such as *Cdx2* and *Gata3*, therefore it is possible that 5hmC is contributing to lineage maintenance through transcriptional modulation of these key factors. Knockdown of *Tet1* in ES cells leads to activation of a subset of trophoblast genes including *Cdx2*, *Eomes* and *Elf5*, although transcript levels appear to be significantly lower than in TS cells suggesting a failure to fully adopt a trophoblast transcriptional program (Ficz, et al., 2011, Ito, et al., 2010, Koh, et al., 2011, Pastor, et al., 2011, Williams, et al., 2011, Wu, et al., 2011b). Consistent with increased expression of trophoblast factors, ES cells with reduced *Tet1* are able form trophoblast cells *in vitro* and TGC in teratoma assays, and in one study were able to contribute to the placenta at low frequency when injected into host blastocysts (Dawlaty, et al., 2011, Ficz, et al, 2011, Koh, et al., 2011). The effect is clearly context dependent though, as *Tet1* knockout ES cells do not contribute to TE or give rise to trophoblast cells when

combined with a chimaeric host embryo (Dawlaty, et al., 2011). The basis for the differences in experimental outcomes is currently not clear.

Interestingly, recent evidence may point towards a role for Tet1 in placental function (Dawlaty, et al., 2011). *Tet1* knockout mice are smaller at E12.5, at birth and at three weeks of age compared to controls. After weaning, mutant mice gain weight and catch up in size with their littermates. This phenotype is suggestive of placental insufficiency during development. Accordingly, wild-type trophoblast can rescue the growth deficiency of *Tet1*-null embryos. Further investigations will establish if there is a role for *Tet1* and 5hmC in trophoblast function.

Taken together, 5hmC presents a newly identified epigenetic pathway, with initial indications suggesting a potential role for 5hmC and the Tet family of proteins in regulating the development of trophoblast lineage and in preventing the upregulation of trophoblast regulatory networks in embryonic cells.

Histone modifications are crucial for trophoblast establishment and differentiation

Histones are a family of small, positively charged proteins that associate with DNA to form nucleosomes. Histone proteins can be marked by a range of posttranslational modifications, including acetylation, methylation, phosphorylation, ADP ribosylation and ubiquitination. The modifications can confer transcriptional information to the underlying DNA sequence, with activating and repressive signals. The writers, readers and erasers of histone modifications are extensive and fairly well characterised (Kouzarides, 2007).

A role for histone modifications in lineage segregation has been described for *Carm1*, which mediates histone H3 arginine methylation. Individual blastomeres with higher *Carm1* levels

contribute preferentially to the ICM (Torres-Padilla, et al., 2007). H3 arginine methylation confers active gene transcription and evidence suggests that Carm1 upregulates key embryonic factors, such as *Nanog*, which in turn could promote ICM skewing. Several other histone modifications have reported to be present in a lineage-specific manner. For example, H3 lysine 27 methylation (H3K27me), H3 lysine 9 acetylation (H3K9ac) and H4 lysine 16 acetylation (H4K16ac) levels are higher in ICM, as compared to TE (Erhardt, et al., 2003, Gupta, et al., 2008, Rugg-Gunn, et al., 2010, Sarmento, et al., 2004). However, there is currently no evidence that these asymmetrically distributed marks have a direct role in lineage establishment. For instance, blastocysts with maternal and zygotic deletion of *Ezh2* have no detectable H3K27me, yet the ICM and TE lineages are established appropriately (Terranova, et al., 2008).

Recent studies using mouse mutants and cell lines have identified an early role for histone methylation pathways in lineage maintenance. One example is *Setdb1*, which imposes transcriptional repression through methylation of H3 lysine 9 (H3K9me). Individual blastomeres with depleted levels of *Setdb1* have elevated *Cdx2* transcripts and contribute preferentially to the TE (Yuan, et al., 2009). Consistent with this finding, deletion of *Setdb1* results in embryonic lethality soon after the blastocyst forms due to defective ICM growth (Dodge, et al., 2004). Genome-wide studies provide further molecular insight by demonstrating that several important trophoblast regulators, including *Cdx2*, are targets of Setdb1 and H3K9me repression in ES cells (Bilodeau, et al., 2009, Yuan, et al., 2009). Knockdown of *Setdb1* in ES cells leads to upregulation of these factors and adoption of a trophoblast cell phenotype (Yeap, et al., 2009, Yuan, et al., 2009). When injected into host embryos, *Setdb1*-depleted ES cells contribute to the placenta, demonstrating successful cell conversion (Yuan, et al., 2009). Together, these data suggest that *Setdb1* restricts trophoblast cell fate during development through epigenetic repression of key trophoblast target genes.

H3K9me may also have a role in regulating embryonic gene expression in trophoblast cells (Alder, et al., 2010, Rugg-Gunn, et al., 2010). Analysis of candidate promoter regions identified the H3K9 methyltransferase *Suv39h1* as conferring epigenetic repression of embryo-specific genes in TS cells (Alder, et al., 2010). Initial characterisation of trophoblast cells *in vivo* suggests that similar pathways are functioning in the early embryo (Alder, et al., 2010, Rugg-Gunn, et al., 2010). Thus, H3K9me appears to have a prominent role in regulating lineage maintenance during the initial stages of development.

In addition to H3K9me, other histone modifications could be involved in transcriptional regulation during lineage restriction. Analysis of candidate gene promoters has revealed the expected localisation of histone marks in embryonic and trophoblast tissues from early stage mouse embryos (Alder, et al., 2010, Dahl, et al., 2010, O'Neill, et al., 2006, Rugg-Gunn, et al., 2010). For example, *Cdx2* and *Eomes* promoters in trophoblast cells are enriched in activating histone modifications such as H3 lysine 4 methylation (H3K4me) and H3K9ac, and have low levels of repressive modifications such as H3K27me. The same promoter regions tend to have higher levels of repressive marks in embryonic cells, which is consistent with differences in gene expression levels between the two cell lineages. Interestingly, chromatin immunoprecipitation analysis combined with DNA microarrays revealed that the ICM has approximately two-fold greater number of gene promoters marked by H3K27me than TE (Dahl, et al., 2010). Genome-wide examination of histone marks in cell lines revealed similar lineage-specific differences, whereby far fewer gene promoters were detected with H3K27me in TS cells, as compared to ES cells (Rugg-Gunn, et al., 2010). The low prevalence of H3K27me in TS cells also impacted the ability to recruit downstream mediators of H3K27me repression, thereby revealing major differences in epigenetic repression between embryonic and trophoblast cell types (Alder, et al., 2010, Rugg-Gunn, et al., 2010). Importantly, mutant

TS cells devoid of H3K27me show no dramatic differences in differentiation potential or gene expression profiles compared to wild-type TS cells, therefore the global absence of H3K27me does not affect trophoblast cell identity (Kalantry, et al., 2006). Biochemical analyses suggested that the low prevalence of H3K27me in wild-type TS cells may be due to lower levels of Eed, which is a Polycomb-group protein required for H3K27me (Rugg-Gunn, et al., 2010). These data are consistent with lower global levels of H3K27me and Eed in TE compared to ICM in the blastocyst, suggesting good concordance between stem cell lines and their tissues of origin within the embryo (Erhardt,et al., 2003, Kalantry and Magnuson, 2006, Rugg-Gunn, et al., 2010). Further research is needed to establish the molecular mechanism that determines lineage-specific levels of H3K27me during embryogenesis and how it links with other developmental processes.

Several histone modifications are required for trophoblast function at later stages of development. Detailed analysis of the mid-gestation embryos (E9.5 to E11.5) revealed a critical function for the Polycomb-group protein *Eed* in the formation of secondary TGC (Wang, et al., 2002). This finding was associated with gain of ectopic *Ascl2* expression, although deleting *Ascl2* in *Eed*-mutants did not rescue the TGC phenotype, thus the molecular basis for this effect remains unclear. Placental defects due to impaired chorion formation have also been reported for other Polycomb-group proteins *Ezh2, Suz12* and *Rnf2* (O'Carroll, et al., 2001, Pasini, et al., 2004, Voncken, et al., 2003). Deletion of H3K9 methyltransferase *G9a* resulted in mid-gestation lethality with defects in chorioallantoic attachment, thereby revealing a role for H3K9me repression in trophoblast function (Tachibana, et al., 2002). Although mice deficient in H3K9 methyltransferases *Suv39h1* / *Suv39h2* survive to term, fibroblasts derived from these mice show a propensity to become progressively polyploid in culture (Peters, et al., 2001) suggesting the methylation of heterochromatin regions may play a role in endoreduplication. Inactivation of the histone arginine methyltransferase, *Prmt1*, also

leads to trophoblast deficiencies during development with an inability to form the EPC (Pawlak, et al., 2000). In addition to histone methylation, histone acetylation may also have an important role in establishing the placenta. Mice deficient in the histone acetyltransferase *Myst2* die at approximately E10.5, partly due to a failure of chorioallantoic attachment (Kueh, et al., 2011). Conversely, deletion of *Hdac1*, a histone deacetylase that confers gene repression, also results in embryonic lethality between E9.5 and E10.5 due to the failure to form an allantois (Lagger, et al., 2002). Although the basis for these defects have not been fully characterised, chemical inhibition of histone deactylase function in TS cells promotes lineage skewing, with excess synctiotrophoblast formation (Maltepe, et al., 2005). These results suggest that histone acetylation may have an important role in regulating trophoblast differentiation. Similar mechanisms may also regulate trophoblast development in humans, as histone acteylation has been shown to have a crucial role in mediating *GCM1* expression during placental morphogenesis (Chuang, et al., 2006).

Overall, histone modifications are critical for multiple stages of trophoblast development, including establishment and maintenance of the TE lineage, formation of the functional chorioallantoic placenta, and regulation of differentiation pathways.

Discussion

Trophoblast development is critically dependent on numerous epigenetic processes, which ensure the appropriate deployment of transcriptional programs and maintenance of cell fates. Recent findings have demonstrated a clear role for DNA methylation and histone modification pathways in regulating trophoblast establishment and placental function. Epigenetic mechanisms may also enable fine-tuning of trophoblast regulation during development, perhaps in response to changing physiological or environment cues. This would provide the best possible start in life for the newborn. Of course, the flipside to this is that the

placenta may be particularly sensitive to epigenetic perturbations (Fortier, et al., 2008, Fowden, et al., 2011). Furthermore, aberrant epigenetic changes in foetal and placental tissues have been associated with several imprinting disorders that may result from assisted reproductive technology, raising concern about the safe use of infertility treatments (Grace and Sinclair, 2009, Wilkins-Haug, 2009). Therefore, there may be significant environmental impact on epigenetic regulation during development. We are only just beginning to understand the mechanisms of foetal programming, but this area of research has enormous implications for long-term health and disease risk (Barker, 1995, Barker, et al., 1995, McMillen and Robinson, 2005). Delineation of the epigenetic pathways that alter long-term gene expression programs in trophoblast cell types will be at the forefront of this research drive.

Much of our detailed understanding of the molecular mechanisms that regulate trophoblast development have arisen from careful analysis of animal models and there is continued need for their use in placental research. Importantly, these insights are beginning to be extended towards human studies, and initial results suggest that the causes and consequences of a variety of placental pathologies are related to epigenetic processes (Maccani and Marsit, 2009). Many epigenetic modifications are thought to be reversible, raising the exciting possibility of designing epigenetic therapies that could target specific epigenetic complexes in order to modulate gene transcription and potentially reverse any adverse environmental impact on the genome (Ellis, et al., 2009, Spannhoff, et al., 2009). Together, these insights will lead to better understanding of placental disorders and disease risk, develop therapies for treatment and also provide important insight into the development and regulation of the placenta, which has broad implications for other aspects of biology.

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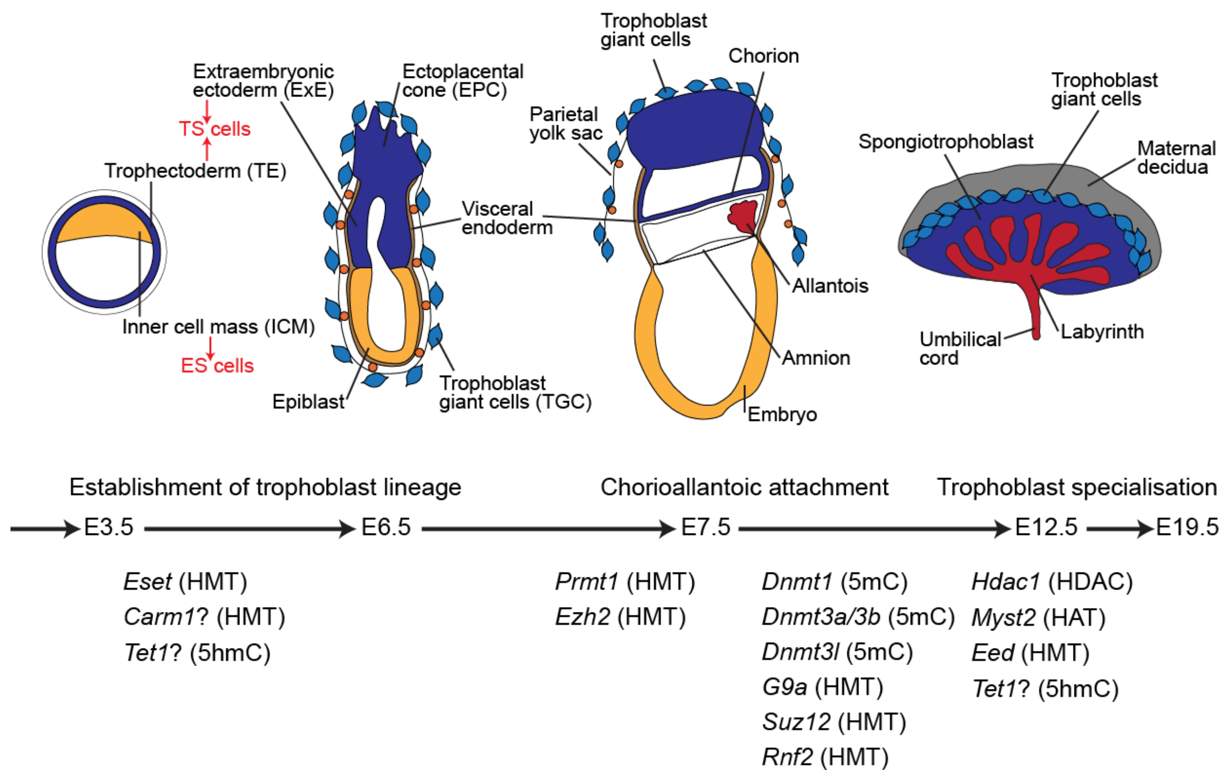

Figure 1

Critical roles of epigenetic modifiers during mouse trophoblast development. Development of the trophoblast lineage from the outer layer of E3.5 blastocysts to specialisation within the E12.5 foetal placenta. Tissue sources of TS cells and ES cells are indicated. Genes listed below the timeline provide examples of epigenetic modifiers that have important functions at different stages of trophoblast development. Question marks indicate the genes that require further functional data to confirm their roles in trophoblast regulation. HMT, histone methyltransferase; HDAC, histone deacetylase; HAT, histone acetyltransferase; 5mC, DNA methyltransferase; 5hmC, 5'hydroxymethylcytosine. This figure is adapted from Rossant and Cross, 2001 and Hemberger, 2007.