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Review

Epigenetic landscape required for placental development

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Abstract. Formation of extraembryonic tissues, and in particular the placenta, is an absolute necessity to ensure growth and survival of the embryo during intrauterine development in mammals. To date, an intriguing number of genes have been identified that are essential for development of extraembryonic structures. However, the underlying genetic information must be interpreted by a set of epigenetic instructions to both establish and maintain lineage- and cell type-specific expression profiles. Based on accumulating data in particular from studies in the mouse, this article is aimed at highlighting the epigenetic machinery required for differentiation of extraembryonic cell types and formation of the placenta. An overview of knockout models reveals key stages in extraembryonic development that are particularly sensitive to alterations in the chromatin environment. The article also summarizes the importance of complex epigenetically controlled mechanisms for placental development, such as imprinted gene expression and imprinted X chromosome inactivation. These investigations of the epigenetic regulation of transcriptional states will provide valuable insights into the dynamic chromatin environment that is specific to extraembryonic tissues and determines gene expression patterns required for normal trophoblast differentiation.

Keywords. Extraembryonic tissues, trophoblast, placenta, development, chromatin, epigenetics.

Introduction

The placenta is the hallmark organ of eutherian mammals that enables development of the embryo within the womb of the mother. From its earliest stages onwards until the end of gestation, mammalian fetal development depends on the proper function of extra-embryonic tissues, notably the placenta. Cells belonging to the extraembryonic (trophoblast) lineage are required to confer attachment of the blastocyst to the uterus. Subsequently, specialized trophoblast cell types gain access to the maternal blood supply and establish an intricate feto-maternal circulatory interface within the placenta that ensures adequate nutrition of the growing embryo. At the same time, the placenta is also essential to dispose of waste products from the embryo into the maternal circulation [1, 2].

Overview of murine extraembryonic development

Extraembryonic development begins at the blastocyst stage with formation of the outer trophectoderm layer (Fig. 1). In the mouse, trophectodermal cells that line the blastocoel cavity (mural trophectoderm) differentiate after implantation into enormously large, polyploid trophoblast giant cells. The angiogenic, vasodilatory and anti-coagulative properties of these primary trophoblast giant cells [3] result in the conceptus soon being surrounded by lacunae of maternal blood. From these blood pools, nutrients and oxygen can pass directly through the yolk sac to the embryo. In contrast to their mural counterparts, trophectodermal cells that overlie the inner cell mass (ICM) continue to proliferate after implantation and give rise to structures such as the chorionic ectoderm,

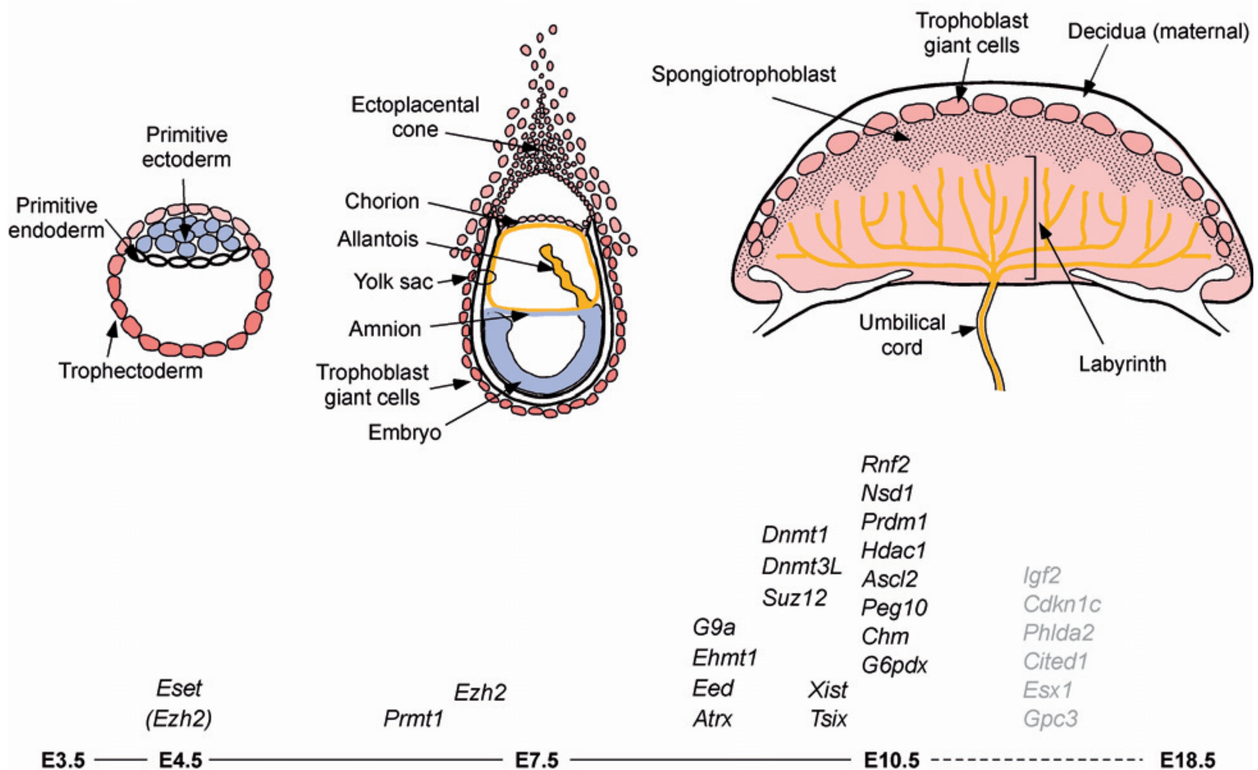

Figure 1. Overview of the main developmental stages of extraembryonic development. The trophoblast cell lineage (red) is established as the trophectoderm layer of the blastocyst. In the late blastocyst, the inner cell mass differentiates into the primitive ectoderm (blue), which gives rise to the embryo proper, and the primitive endoderm (black), which contributes to the yolk sac. After implantation, the main trophoblast subtypes consist of trophoblast giant cells that surround the implantation site (primary giant cells shown in dark red, secondary giant cells in lighter red), the chorionic ectoderm, and ectoplacental cone. The yolk sac membranes consist of the parietal yolk sac (trophoblast giant cells and parietal endoderm) and the visceral yolk sac (visceral endoderm and extraembryonic mesoderm). Extraembryonic mesoderm (yellow) also provides one cell layer of the amnion and the chorion, as well as the allantois. Chorioallantoic fusion is essential for formation of the mature placenta at mid-gestation. The allantoic mesoderm forms the endothelial cell lining of fetal blood vessels in the labyrinth zone. Distinct regions of the placenta include the labyrinth, the spongiotrophoblast and a discontinuous layer of trophoblast giant cells. Examples of epigenetic modifiers and epigenetically regulated genes that are important for extraembryonic, and consequently embryonic, development are given on a developmental time scale. Genes listed in black cause embryonic lethality when mutated; genes given in grey function in growth and proliferation control of the placenta.

the ectoplacental cone and secondary trophoblast giant cells [4–6]. It is these 'polar' trophectoderm cells that harbour a population of trophoblast stem cells. Trophoblast stem cells can be maintained *in vitro* where they retain the same differentiative potential as their progenitors in the blastocyst [7]. The physical connection of the fetus to the maternal vasculature is established by secondary trophoblast giant cells that differentiate at the margins of the ectoplacental cone. From here, they invade into the uterus and target maternal spiral arteries where they displace the endothelial cell lining and create entirely trophoblast-lined blood spaces. The sinusoidal canals formed by this process transport maternal blood towards the implantation site and into the future placenta.

After determination of the trophectoderm/trophoblast lineage, another important differentiation step takes place in the blastocyst: the primitive ectoderm, which gives rise to the embryo proper, separates from the primitive endoderm (Fig. 1). Like the trophectoderm, the primitive endoderm contributes exclusively to extraembryonic tissues and forms the parietal and visceral endoderm layers of the yolk sac. The importance of extraembryonic tissues for embryonic development is further emphasized by the 'investment' of yet a third cell lineage to the temporary structures that ensure intrauterine growth: at gastrulation, the extraembryonic mesoderm differentiates and forms the allantois and the mesodermal components of the visceral yolk sac, amnion and chorion (Fig. 1).

The mature mouse placenta is formed only at mid-gestation and is designed as a highly efficient nutrient and gas exchange organ. A crucial step for placental development is the fusion of the chorionic ectoderm layer with the allantois. The latter provides the extraembryonic mesoderm that invaginates into the flat sheet of chorionic trophoblast cells and branches

out to form the endothelial cell lining of a highly complex vascular network. These fetal vessels intermingle closely with trophoblast-lined sinusoids that carry maternal blood. This architecture ensures a minimal transfer distance between both circulations. In addition, the transfer capacity is further increased by a countercurrent-like orientation of fetal and maternal blood circulations. Because of its complex, mazelike structure, the region of the placenta where this anatomical structure develops is called the labyrinth. Other clearly distinguishable zones of the mature placenta comprise the spongiotrophoblast, which contains diploid trophoblasts and glycogen-storing cells, and a layer of trophoblast giant cells at the border to the maternal decidua (Fig. 1). The precise role of these other regions is much less clear but includes the production of vast amounts of pregnancy-associated hormones and the attraction of maternal blood flow towards to the implantation site [6, 8, 9].

Key events in differentiation of extraembryonic tissues: susceptibility to developmental disruptions

Development of extraembryonic tissues is characterized by several key events. Firstly, formation of the blastocyst's trophectoderm layer represents the earliest definitive cell commitment event to occur after fertilization. With this differentiation step, the trophoblast cell lineage is set aside. Pluri- or multipotency is maintained in trophoblast stem cells contained within this trophoblast population, but the differentiative potential of these stem cells is restricted to the various trophoblast subtypes [10]. Secondly, invasive trophoblast giant cells breach the uterine basement membrane and penetrate into the uterine stroma where they erode maternal arteries and adopt pseudo-endothelial cell functions. This step is crucial for the conceptus to become connected to the maternal vascular system. Thirdly, as judged by the number of mutants that fail at this stage, chorioallantoic fusion and establishment of a functional labyrinth represent a key step in placental development, a process for which the interplay of a multitude of factors is required that mediate induction events and a continuous cross-talk between the allantoic mesoderm and the chorionic ectoderm [11, 12].

Highly specialized trophoblast cell types

Trophoblast tissues are also characterized by many unusual features on the cellular level. Most noteworthy is the differentiation of polyploid trophoblast giant cells from diploid trophoblast precursors. This process requires a cell to exit the mitotic cell cycle and to undergo multiple rounds of DNA replication without intervening karyokinesis (a process called 'endoreduplication') resulting in the formation of enormously large cells and nuclei with a DNA content of up to 1000 N [13–15]. Another characteristic cell type is found in the labyrinth where trophoblast cells that line the maternal blood sinuses fuse to form a syncytiotrophoblast layer.

Genetic requirements and inheritability of gene expression profiles

Clearly, these complex and highly specialized differentiation processes require the fine-tuned expression of many transcription factors, cell cycle regulators, signalling molecules and surface receptors that have been summarized in several recent review articles [11, 12, 16, 17]. How is expression of such specific cohorts of genes regulated and stably inherited? As in all differentiation processes, expression of a cell type-specific repertoire of genes is epigenetically controlled to confer phenotypic stability to a cell. Thus, the expression profile that is essential to achieve trophoblast lineage separation and subsequent differentiation into various trophoblast subtypes is inextricably linked to the acquisition of epigenetic marks that define the lineage and the specific cell type. This article will focus on the epigenetic requirements that accompany the gene expression profiles necessary for extraembryonic development to proceed normally (Table 1).

DNA methylation

The symmetrical methylation of cytosine residues in CpG dinucleotides is the best-characterized epigenetic modification. It is commonly associated with transcriptional silencing when it occurs in gene promoter regions. DNA methylation levels are subject to a global remodelling process after fertilization, with phases of active and passive demethylation followed by *de novo* methylation. The extent of *de novo* methylation occurs more pronounced in cells of the ICM compared to the trophectoderm of the blastocyst, thereby establishing an inequality between both cell lineages [18]. This epigenetic difference is maintained throughout gestation since earlier investigations have shown that the embryo exhibits higher overall DNA methylation levels than the placenta [19, 20]. Despite the global hypomethylation of the trophoblast lineage, DNA methylation remains vitally

Table 1. Extraembryonic phenotypes of mutants for epigenetic modifiers and imprinted genes.

Gene	Name (Mouse Genome Informatics)	Function	Embryonic lethality	Phenotype	Reference
DNA methylation					
Dnmt1	DNA methyltransferase 1	Methylation of hemimethylated CpG dinucleotides	E9.5	chorioallantoic fusion defect	[22]
Dnmt3L	DNA (5-cytosine)-methyltransferase 3-like	Setting of maternal methylation imprints	E9.5 (offspring from mutant mothers)	no labyrinth formation, less spongiotrophoblast, more trophoblast giant cells	[26]
Histone lysine methylation					
G9a (= *Ehmt2*)	euchromatic histone lysine N-methyltransferase 2	H3K9 mono-/ dimethylation (euchromatic)	E8.5 – E9.5	chorioallantoic fusion defect	[31]
Ehmt1	euchromatic histone methyltransferase 1	H3K9 mono-/ dimethylation (euchromatic)	E8.5 – E9.5	not characterized	[32]
Eset = *Setdb1*	SET domain, bifurcated 1	H3K9 trimethylation (euchromatic)	E3.5 – E5.5	ICM defect	[33]
Suv39h1 / *Suv39h2*	suppressor of variegation 3–9 homologs 1 and 2	H3K9 methylation (pericentric heterochromatin)		impaired viability; genomic instability, polyploidy of fibroblasts	[34]
Polycomb group proteins					
Ezh2	enhancer of zeste homolog 2	H3K27 methylation	E7 – E7.5	amnion and chorion formation defect; ES cells not viable	[35]
Suz12	suppressor of zeste 12 homolog	H3K27 methylation as part of PRC2/3 complex	E10	lack of amnion and chorion; failure of amniotic, exocoelomic and ectoplacental cavity formation	[36]
Eed	embryonic ectoderm development	H3K27 methylation as part of PRC2/3 complex	E8.5 – E9.5	differentiation defect of secondary trophoblast giant cells	[38, 59]
Rnf2	ring finger protein 2	Component of PRC1 complex	E10.5	lack of amnion formation, chorion defects	[39]
Others					
Nsd1	nuclear receptor-binding SET-domain protein 1	H3K36 and H4K20 methylation	E10.5	lack of allantois	[46]
Histone arginine methylation					
Prmt1	protein arginine N-methyltransferase 1	arginine methylation	E6.5	lack of amnion; no proamniotic and ectoplacental cavity formation	[48]
Prdm1 (=*Blimp1*)	PR domain containing 1, with ZNF domain	Transcriptional repressor, associates with PRMT5 to methylate H2A R3 and H4R3	E10.5	branching morphogenesis defect in placental labyrinth	[50]
Histone acetylation					
Hdac1	histone deacetylase 1	deacetylation of histones	E9.5 – E10.5	lack of allatois formation	[52]
Autosomal imprinted genes					
Ascl2	achaete-scute complex homolog-like 2	bHLH transcription factor	E10.5	overabundance of trophoblast giant cells over spongiotrophoblasts; labyrinth formation defect	[66, 67]
Peg10	paternally expressed 10	retrotransposon-related gene	E10.5	lack of spongiotrophoblast; labyrinth formation defect	[69]
Igf2	insulin-like growth factor 2	mitogen, growth factor		fewer glycogen cells; thickened interhemal barrier in labyrinth	[71, 119]
Cdkn1c	cyclin-dependent kinase inhibitor 1C (P57)	cell cycle regulator		larger labyrinth and spongiotrophoblast layers	[72]
Phlda2 (=*Ipl*)	pleckstrin homology-like domain, family A, member 2	phosphatidylinositol lipid second messenger		larger spongiotrophoblast layer	[73]
Peg3	paternally expressed 3	Kruppel-like transcription factor		smaller placenta	[120]

Table 1 *(Continued)*

Gene	Name (Mouse Genome Informatics)	Function	Embryonic lethality	Phenotype	Reference
Peg1/Mest	mesoderm-specific transcript	putative hydrolase enzyme		smaller placenta, no gross difference in relative composition	[121]
Grb10	growth factor receptor-bound protein 10	signaling protein		larger placenta	[122]
Igf2r	insulin-like growth factor 2 receptor	non-transmitting receptor for Igf2		larger placenta	[123]
X-linked genes					
Xist	inactive X specific transcripts	non-coding RNA, coats inactive X chromosome	E10.5	less and smaller trophoblast giant cells	[96]
Tsix	X (inactive)-specific transcript, antisense	antisense transcript to *Xist*, non-coding	E9.5 – E10.5, variable	less trophoblast	[95]
Atrx	alpha thalassemia/mental retardation syndrome X-linked	member of the SNF2 family of ATPase/helicase proteins	E8.5 – E9.5	smaller ectoplacental cone; fewer trophoblast giant cells	[92]
Esx1	extraembryonic, spermatogenesis, homeobox 1	homeobox transcription factor		enlarged spongiotrophoblast with more glycogen cells; thickened interhemal barrier in labyrinth	[97]
Cited1	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	transcriptional cofactor		enlarged spongiotrophoblast; larger maternal sinusoids in labyrinth	[98]
Gpc3	glypican 3	cell-surface heparan sulfate proteoglycan		enlarged spongiotrophoblast	[99]
Chm	choroidermia	transport of Rab family GTPases to Rab geranyl-geranyl-transferase for prenylation	E10.5 – E11	overabundance of trophoblast giant cells over spongiotrophoblasts; labyrinth formation defect	[100]
G6pdx	glucose-6-phosphate dehydrogenase X-linked	enzyme of pentose phosphate pathway, housekeeping gene	E10.5	smaller spongiotrophoblast; labyrinth formation / vascularization defect	[101]
Nsdhl	NAD(P) dependent steroid dehydrogenase-like	cholesterol biosynthesis enzyme	~E9.5 – E13.5	fewer vessels in labyrinth; reduced labyrinth trophoblast proliferation induced by allantoic mesoderm	[102, 103]

Note: Only the major functions of gene products are listed; other potential or less prominent activities have been omitted.

important for development of extraembryonic tissues. Demethylation induced by systemic treatment of pregnant females with 5-azacytidine leads to the formation of smaller placentas, a severely reduced labyrinth layer and an excess of trophoblast giant cells in the rat [21]. More specific genetic approaches have shown that lack of the maintenance DNA methyltransferase *Dnmt1* causes mid-gestational lethality with defects including embryonic growth retardation, absence of yolk sac vasculature and an abnormal development of the allantois that does not fuse with the chorion [22]. Likewise, compound mutants of the *de novo* methyltransferases *Dnmt3a* and *Dnmt3b* are also embryonic lethal, displaying a very similar phenotype to *Dnmt1*$^{-/-}$ embryos [23], even though development of the extraembryonic tissues has not been investigated in depth. The most detailed insights into the role of DNA methylation in trophoblast differentiation comes from the knockout of the non-enzymatic member of *de novo* DNA methyltransferases *Dnmt3L*. DNMT3L co-localizes with DNMT3A and DNMT3B and has been shown to cooperate specifically with DNMT3A to establish germ line methylation imprints [24, 25]. *Dnmt3L* is extremely highly expressed in the chorion, which harbours the multipotent trophoblast stem cell population [24]. Offspring of mothers homozygous for a *Dnmt3L* mutation die before mid-gestation, and their placentas exhibit multiple defects, most prominently a lack of labyrinth formation [26]. This failure is a likely consequence of loss of *Gcm1* expression, a transcription factor essential to determine the invagination sites for allantoic blood vessels into the chorionic trophoblast [27]. In addition, the spongiotrophoblast layer of *Dnmt3L*-deficient placentas is reduced on the expense of overabundant trophoblast giant cells, and

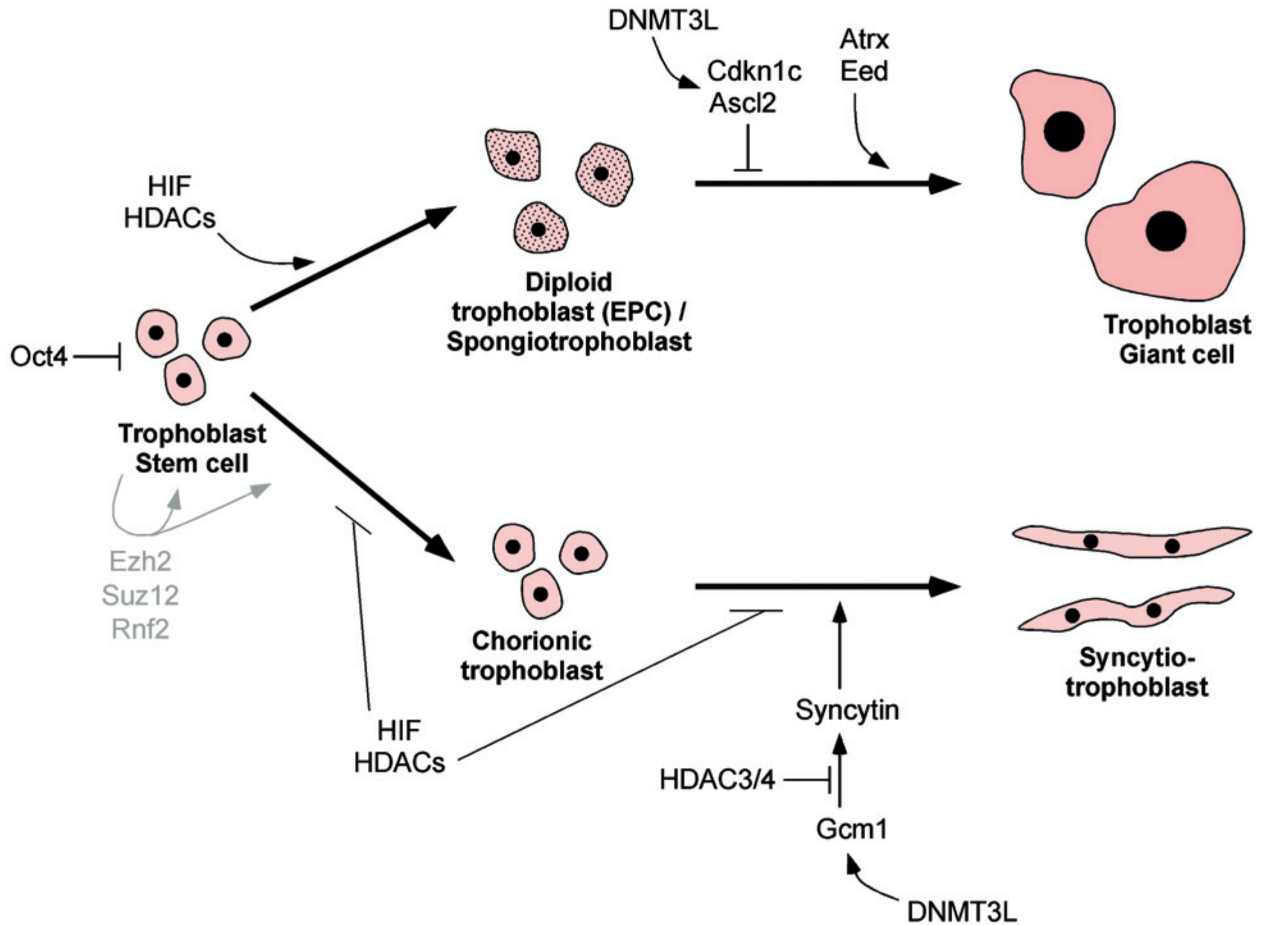

Figure 2. Main differentiation pathways of trophoblast stem cells into trophoblast giant cells or syncytiotrophoblasts. Epigenetic modifiers that regulate some key differentiation steps and their target genes are illustrated (where known). OCT4 is an embryonic lineage-determining transcription factor that is downregulated in the trophoblast lineage and transcriptionally silenced by DNA methylation. The precise roles of *Ezh2*, *Suz12* and *Rnf2* on trophoblast differentiation are not known; however, mutants exhibit severe defects in chorion formation, suggesting that these genes may play a role in trophoblast stem cell self-renewal and/or chorionic trophoblast formation. EPC, diploid trophoblast of the ectoplacental cone.

this phenotype may be caused by loss of expression of the imprinted genes *Ascl2* and *Cdkn1c* [26, 28]. These differentiation defects are recapitulated in *Dnmt3L*$^{-/-}$ trophoblast stem cells, demonstrating that DNA methylation exerts a cell autonomous control on trophoblast-specific gene expression and differentiation (Fig. 2). Taken together, these data demonstrate that DNA methylation is indispensable for normal extraembryonic development, with particular importance for chorioallantoic fusion, for the prevention of excessive trophoblast giant cell differentiation and for syncytiotrophoblast formation.

Histone modifications

The epigenetic marks set by DNA methylation are complemented by a variety of posttranslational histone tail modifications that can impose either repressive or transcriptionally permissive chromatin structures. Transcriptional repression mediated by histone marks is thought to be more easily reversible than DNA methylation and silences genes 'poised' for activation at later stages, thereby maintaining a cell's pluri- or multipotent state [29, 30]. Covalent histone modifications encompass methylation, acetylation, phosphorylation, ADP ribosylation and ubiquitination of individual amino acid residues of nucleosomal histones. From studies of these various modifications it is clear that histone marks play an instrumental role in development (Table 1).

Methylation of lysine residues

One of the best-studied histone modifications is the methylation of histone H3 at lysine residues 9 and 27 (H3K9 and H3K27). G9A is a histone methyltransferase that has been identified to modify mainly H3K9. Genetic ablation of this gene causes mid-gestational lethality

associated with growth retardation and defects in chorioallantoic fusion [31]. Deletion of a related H3K9 methyltransferase, *Ehmt1*, also leads to embryonic death at day (E) 8.5–E9.5 of gestation [32]. Since G9A and EHMT1 can heterodimerize and function cooperatively in H3K9 mono- and dimethylation, it can be speculated that *Ehmt1*$^{-/-}$ embryos exhibit extraembryonic defects similar to *G9a* mutants. In contrast to these phenotypes, lack of *Eset* (=*Setdb1*), an enzyme that catalyzes H3K9 di- to trimethylation in euchromatin, is lethal at peri-implantation stages with most severe effects on the ICM [33], indicating that H3K9me3-mediated gene silencing is essential for cell lineage segregation and lineage-specific proliferation. The phenotypes caused by genetic ablation of H3K9 methyltransferases that affect euchromatic regions are remarkably different from those that modify pericentric heterochromatin. Thus, *Suv39h1* and *Suv39h2* are not essential for embryonic survival, even though double-knockout pups are born at sub-Mendelian ratios [34]. Interestingly, however, *Suv39h1*$^{-/-}$/*Suv39h2*$^{-/-}$ fibroblasts become tetra- and polyploid, suggesting a potential role of pericentric heterochromatin organization in trophoblast giant cell formation.

H3K27 methylation is mediated by multimeric protein aggregates called Polycomb repressive complex (PRC) 2 and 3 that contain the Polycomb group (PcG) family members EED, EZH2 and SUZ12 as well as histone deacetylases. PcG proteins represent a conserved set of chromatin-modifying factors that are thought to maintain cellular transcriptional memory by stably, but reversibly, silencing gene expression. Although only EZH2 possesses intrinsic methyltransferase activity, deletion of other PRC2/3 components causes similar phenotypes with severe effects on development leading to embryonic lethality between E7 and E10 (Table 1). Notably, *Ezh2* and *Suz12* mutants are characterized by a failure of amnion and chorion formation. Consequently, the amniotic, exocoelomic and ectoplacental cavities are not distinguishable and placental development cannot proceed [35, 36]. *Eed* mutants have been analyzed most comprehensively for their development of extraembryonic tissues. They exhibit a distinct failure of secondary, invasive trophoblast giant cell differentiation. Instead, the region of diploid *Ascl2*-positive trophoblasts extends to the very tip of the ectoplacental cone [37, 38]. Importantly, the cell autonomous function of EED in trophoblast has been proven by tetraploid aggregation experiments in which wild-type trophoblast cells could rescue the mid-gestational embryonic lethality of *Eed* mutants [38].

Compared to PRC2/3, the PRC1 repressive complex consists of an even larger number of individual components and can regulate chromatin organization by multiple mechanisms. Most single-gene mutations of individual PRC1 proteins lead to perinatal lethality. Of note, however, is the Ring finger protein RNF2, a PRC1 component that can interact with several other PcG proteins and has a central role in PRC1 complex formation. *Rnf2*-deficient embryos exhibit similar defects to *Ezh2* and *Suz12* mutants in failing to develop an amniotic fold and chorion [39]. It is thus feasible that the mid-gestational lethality observed in some compound mutants of PRC1 components, such as *Phc1*/*Phc2* or *Rnf110*/*Bmi1* double knockouts [40, 41], is also caused by failure in amnion, chorion and/or chorioallantoic placenta formation.

In addition to H3K9 and H3K27 methylation, other histone lysine methylation marks are indispensable for embryogenesis as well. This is demonstrated by genetic ablation of several H3K4 methyltransferases or associated factors such as *Mll1*, *Mll2*, *Menin* and *Smyd1* [42–45] that are embryonic lethal. While placental failure has been ruled out for *Mll2*-deficient embryos [43], the other mutants remain to be investigated for possible defects in extraembryonic tissues. The necessity of a range of different histone lysine marks for extraembryonic development is emphasized by ablation of *Nsd1*, a SET domain containing protein that catalyzes H3K36 and H4K20 methylation (Table 1). *Nsd1*$^{-/-}$ embryos fail to form an allantois, thus chorioallantoic fusion and placental labyrinth development cannot proceed and the embryos die between E9.5 and E10.5 [46].

Methylation of arginine residues

Even though histone lysine modifications, particularly of H3, have been most extensively studied, other histone marks certainly contribute to determining transcriptional activity and overall chromatin organization of a given locus. Protein arginine methylation, for example, is also crucial for cell type-specific gene expression and, thus, differentiation. Importantly, cells of the ICM appear to require higher global levels of H3 arginine methylation, and overexpression of *Carm1*, an arginine methyltransferase catalyzing H3R2, H3R17 and H3R26 methylation, predetermines the fate of a two-cell blastomere towards the ICM [47]. Absence of the arginine methyltransferase *Prmt1* leads to intra-uterine death at E6.5 with embryos lacking the amniotic fold and being unable to form the proamniotic and ectoplacental cavities [48]. Further evidence for the importance of arginine methylation for extraembryonic development comes from conceptuses deficient in *Blimp1*, a zinc finger-containing transcriptional repressor that can associate with PRMT5 to direct methylation of arginine 3 on histones H2A and H4 [49]. *Blimp1* mutants suffer from placental defects with impaired vascular branching in the labyrinth layer that leads to embryonic death at E10.5 [50].

Acetylation of histones

The acetylation of core histones is generally associated with an open chromatin organization and potential transcriptional activity. Investigations into the role of histone acetylation have been facilitated by the availability of a general histone deacetylase (HDAC) inhibitor, Trichostatin A (TSA). Exposure of trophoblast stem cells to this compound has been shown to promote syncytiotrophoblast formation [51]. This is an intriguing finding because normally, only a very small fraction of trophoblast stem cells undergo syncytiotrophoblast differentiation compared to the dominant giant cell differentiation pathway. Although histone acetylation and deacetylation are mediated by a multitude of factors, direct genetic evidence for their precise role in extraembryonic development is evident only for *Hdac1*. This histone deacetylase is most abundantly expressed in trophoblast giant cells but its absence does not seem to impair giant cell function. However, *Hdac1*$^{-/-}$ embryos die at E9.5–E10.5 because of a failure in allantois formation and, therefore, a lack of chorioallantoic fusion [52].

This collection of mouse knockouts of epigenetic modifiers demonstrates that extraembryonic development depends on a specific chromatin environment. While trophoblast differentiation and placentation are key targets of epigenetic regulation, it is clear that our picture of the epigenetic landscape required for extraembryonic (and embryonic) development is still far from complete. The epigenome, with its dynamics and its ever-growing range of modifications, is a relatively new and fast-moving field that will unravel exciting mechanisms of gene regulation and chromatin organization [53]. Analysis of extraembryonic development in existing mouse mutants as well as generation of new knockouts is thus required to further our understanding of the epigenetic requirements specific to extraembryonic tissues.

Interdependence of epigenetic modifications

In light of the various modifications described and the phenotypes caused by their absence, it has to be highlighted that many epigenetic modifiers interact with each other and function in large chromatin-remodelling complexes. Recent evidence demonstrates that the acquisition of histone modifications and DNA methylation is interdependent. Histone H3K9 methylation, for example, directs DNA methylation to pericentric heterochromatin [54], a process that may be mediated by interaction between DNMT1 and SUV39H1 [55]. Furthermore, DNMT1 physically interacts with the histone methyltransferase G9A, and *Dnmt1* knockdown impairs H3K9 methylation on chromatin and ribosomal DNA repeats [55]. DNMT1 can also directly interact with EZH2, thereby mechanistically linking the two key epigenetic repression systems of DNA methylation and PcG-mediated gene silencing [56]. In addition to interacting with histone methyltransferases, DNA methylation is linked to histone acetylation as shown by TSA-induced down-regulation of *Dnmt1* mRNA and protein [57]. Also, HDACs (including HDAC1) have been found to associate with the EZH2/EED-containing PRC2/3 complex, thus combining repressive histone-modifying activities [58].

In this context of epigenetic interrelationships, it is interesting to consider whether the phenotypic similarity of some knockout models may be caused by a functional interaction between individual epigenetic modifiers (Table 2). This seems obvious for some factors such as the PRC2/3 components EZH2 and SUZ12. Since HDAC1 associates with this complex as well, it can be speculated that some of the phenotypic aspects are shared by these mutants. Likewise, the chorioallantoic fusion defects of *Dnmt1* and *G9a* mutants may well be based on the functional interdependence of DNA and histone methyltransferase activities. Further potential interactions could be suggested by the striking similarities of knockouts for *Ezh2*, *Suz12* and the PRC1 component *Rnf2*, as well as the histone arginine methyltransfrease *Prmt1*. At the same time, such a comparison may also reveal important differences between components of the same functional unit. In case of the PRC2/3 complex, for example, the amnion and chorion formation defects of *Ezh2* and *Suz12* mutants are not shared by *Eed*-deficient embryos [59], while the defect in secondary trophoblast giant cell differentiation seems to be specific to the *Eed* mutation. These phenotypic differences are unlikely to be caused merely by different genetic backgrounds, since the severity of the *Eed* mutant phenotype is retained also on an outbred strain background [37, 38]. It would thus be interesting to examine whether EED has a separate, cell cycle-specific function associated to endoreduplication.

Global epigenetic requirements for extraembryonic development

Several fundamental aspects on the epigenetic requirements of extraembryonic tissues emerge from the accumulating data. Firstly, on the global level, the trophectoderm/trophoblast lineage seems to be characterized by lower epigenetic modification levels than the embryonic lineage. This appears to be in particular the case for silencing modifications and has been

Table 2. Phenotypic comparison of knockout models.

Defects in formation of					
Amnion	Chorion	Allantois	Chorioallantoic fusion	Labyrinth formation	Trophoblast giant cell differentiaiton
			Dnmt1		
				Dnmt3L	Dnmt3L
			G9a		
			Ehmt1 (?)		
Ezh2	Ezh2				
Suz12	Suz12				
					Eed
Rnf2	Rnf2				
		Nsd1			
Prmt1					
				Prdm1	
		Hdac1			
				Ascl2	Ascl2
				Peg10	Peg10
					Atrx
				Chm	Chm
				G6pdx	

shown for overall DNA methylation levels [18–20] and for the repressive histone marks H3K27me1, H3K27me2, H3K27me3 at the blastocyst stage [60]. While mouse pre-implantation embryos have been analyzed most comprehensively, an epigenetic asymmetry between the embryonic and trophoblast lineage (with higher modification levels in the ICM) has also been described for epigenetic marks in other mammalian species like bovine, sheep and rabbit [61–63], and may thus be important for allocation or function of the lineages. It is important to note that despite their globally lower levels in trophoblast cells, the correct marking by these modifications is indispensable for extraembryonic development to occur. A reverse pattern with higher staining intensity in trophoectoderm has been described for H4/H2A serine 1 phosphorylation; however, this modification is also cell cycle regulated, which may interfere with a direct quantitative comparison between the two cell lineages [64].

Secondly, there is evidence for the importance of these modifications at very defined time points of development (Fig. 1): i) for establishment of the first cell lineages at the blastocyst stage (as revealed by *Eset* mutants and the non-viability of *Ezh2* ES cells [35]); ii) for specification and differentiation of extraembryonic mesoderm and trophoblasts as required for amnion, chorion, allantois and secondary trophoblast giant cell formation (*Ezh2*, *Suz12*, *Eed*, *Rnf2*, *Nsd1*, *Prmt1* and *Hdac1* mutants); and iii) for chorioallantoic fusion and development of a functional placental labyrinth (*Dnmt1*, *Dnmt3L*, *G9a*, *Prdm1*). The developmental stages associated with these processes represent the time points most vulnerable to alterations of the epigenetic landscape. It is thus compelling to correlate the mutant phenotypes of epigenetic modifiers to those of various genetic factors (Table 2, Fig. 2), and to examine whether the transcriptional regulation of these genes is known to be controlled by DNA methylation and/or histone modifications.

Imprinting control at autosomal loci

Genomic imprinting describes the allele-specific expression of a gene in a parent-of-origin-dependent manner. This epigenetic mechanism arose during mammalian evolution and may thus be associated with the evolution of intrauterine development that requires formation of a placenta [65]. This is reflected by the fact that most of the ~80 imprinted genes that have been identified to date are expressed in the placenta, many of them in the trophoblast component proper. The distinction of parental alleles is achieved by DNA methylation marks that are set in the germ line at very specific loci called differentially methylated regions (DMRs). Close links between maternal DNA methylation marks and imprinted gene expression have been highlighted by the *Dnmt3L* knockout where loss of imprinted *Ascl2* and *Cdkn1c* expression

may directly account for many of the observed trophoblast differentiation defects [26, 28]. The transcription factor *Ascl2* (*Mash2*) is one of the first imprinted genes that has been identified, by targeted deletion, to have a critical function in placental development [66, 67]. Upon maternal inheritance of a deleted allele, embryos die at E10.5 due to trophoblast defects. The fetal labyrinthine vasculature does not develop in mutants, and spongiotrophoblast cells are largely underrepresented or even missing, whereas trophoblast giant cells are overabundant. These defects can be rescued by providing mutant embryos with a wild-type placenta, indicating that *Ascl2* has cell-autonomous functions in the trophoblast lineage [68]. Interestingly, deletion of *Peg10*, a paternally expressed retrotransposon-related gene, causes an extremely similar phenotype that can also be rescued by wild-type trophoblasts [69]. Investigations of several other imprinted genes have further demonstrated their essential role in placental development and, consequently, their necessity for nutrient supply and growth of the embryo proper [70]. Many of these mutants display growth abnormalities in the placenta and/or embryo, but often survive the gestational period. Structures that are affected by individual gene mutations include the placental labyrinth where a thickened interhemal barrier between fetal and maternal blood circulations can lead to an impaired nutrient transfer capacity (*Igf2*, possibly *Cdkn1c*) [71, 72]. Also, differentiation and proliferation of individual cell types can be abnormal as shown by an increased number of labyrinth trophoblasts and spongiotrophoblasts in *Cdkn1c* and *Phlda2* mutants [72, 73]. These data demonstrate the importance of imprinted genes for extraembryonic and, specifically, trophoblast differentiation.

As to the epigenetic regulation of imprinted gene expression, substantial mechanistic insights have been gained over the last years that indicate a distinct role of DNA methylation and histone modifications in the embryo and placenta, respectively. Work on a specific locus of mouse distal chromosome 7 has shown that placenta-specific imprinting involves repressive histone modifications such as H3K9 di- and H3K27 trimethylation as well as non-coding RNA transcripts and the PcG proteins EZH2 and EED, but is largely independent of DNA methylation. This is in contrast to the embryo proper, where abrogation of the DNA methylation mark leads to biallelic gene expression [74–76]. Thus, epigenetic control of imprinted gene expression is remarkably different between the embryonic and extraembyonic compartment, with silencing in the trophoblast lineage sharing many aspects of imprinted X chromosome inactivation [77].

Imprinted X inactivation in extraembryonic tissues

An intriguing epigenetic feature of extraembryonic tissues in mice – as well as in marsupials and cattle – is the imprinted inactivation of the paternal X chromosome (X^P) [78–82]. As a consequence, trophoblast development is extremely susceptible to deviations from normal X inactivation and is impaired in situations of increased and reduced X^M/X^P chromosome dosage. In addition, mutations of X-linked genes important for placental development will have deleterious effects when inherited from the mother, even if the paternal allele is functional. This is of particular significance because the X chromosome seems to be proportionately enriched for genes expressed in the placenta [83]. X inactivation is achieved by expression of the non-coding RNA *Xist* from the inactive X chromosome that triggers silencing in *cis* (reviewed in [84]). The first signs of gene silencing on X^P can be detected from the eight-cell stage onwards in all blastomeres of the morula. At the blastocyst stage, inactivity of X^P is maintained in the trophectoderm lineage and in the primitive endoderm, while the X^P becomes reactivated in the ICM where random X inactivation ensues [85, 86]. Similar to the findings of imprinting control at autosomal loci, imprinted X^P inactivation is largely independent of DNA methylation [87]. Instead, maintenance of X^P silencing is ensured by recruitment of EED/EZH2 PcG complexes and repressive H3K27me3 and H3K9me2 histone marks [88]. Further epigenetic modifications that contribute to stable X^P inactivation are the global hypoacetylation of histones as facilitated by recruitment of HDACs in the PRC2 complex (see above) [38], and the ubiquitination of histone H2A [89, 90].

In *Eed* mutants, reactivation of X^P is observed in extraembryonic tissues of female conceptuses leading to a much more pronounced phenotype in females than in males [37]. Interestingly, this role of *Eed* is specific to imprinted X inactivation since random X inactivation in the embryo proper is not affected. EED is specifically required to propagate the silenced state of X^P during trophoblast differentiation into giant cells, while undifferentiated trophoblast stem cells retain imprinted X^P inactivation even in the absence of *Eed* [91]. Similar to the *Eed* mutation, trophoblast giant cell formation is also affected in embryos deficient in the SNF2 member of chromatin remodelling proteins *Atrx* [92], leading to embryonic lethality at E8.5–E9.5 of gestation. Surprisingly, however, some females that inherit the mutation from the mother can develop to term, indicating that the paternal *Atrx* allele can be reactivated and thus can partially overcome complete X^P inactivation. This finding might share similarities to the observation that the inactive X^P chromosome can also be reactivated in

monosomic X^PO and X^PY embryos and in androgenetic X^PX^P embryos even though these conceptuses remain developmentally retarded [93, 94].

Given the imprinted inactivation of X^P in extraembryonic tissues, expression of every X-linked gene is under tight epigenetic control. In addition to the direct regulators of X dosage *Tsix* and *Xist* [95, 96], placental defects are observed in mutations of individual X-linked genes such as *Esx1*, *Cited1*, *Gpc3, Chm*, *G6pdx* and *Nsdhl* [97–103]. Similar to several autosomal imprinted genes, maternal deletions of *Esx1*, *Cited1* and *Gpc3* lead to placental growth phenotypes and proliferation defects of specific placental subtypes, i.e. labyrinth trophoblasts, spongiotrophoblasts and glycogen cells. By contrast, genetic ablation of *Chm*, encoding an escort protein necessary for prenylation of small GTPases, results in much more severe defects, including mid-gestational lethality, lack of labyrinth vascularization and increased trophoblast giant cell numbers [100]. A similar phenotype is caused by deletion of the housekeeping gene *G6pdx*, illustrating that *G6pdx* deficiency is deleterious for placental development with trophoblast-induced defects on allantoic mesoderm development [101]. *Chm* and *G6pdx* may thus contribute to the phenotype of conceptuses that lack an active X^M such as X^PO and X^PY monosomic and X^PX^P androgenetic embryos and mutants for the non-coding RNA *Tsix* [95]. Interestingly, an opposite induction event to that of *G6pdx* is observed in mutants of the sterol dehydrogenase *Nsdhl* where signalling defects of the allantoic mesoderm lead to reduced labyrinth trophoblast proliferation [102, 103]. Thus, the *Nsdhl* mutation only affects males, while random X inactivation in the allantoic mesoderm ensures survival of heterozygous females.

Epigenetic regulation of autosomal, non-imprinted genes essential for extraembryonic development

The link between global chromatin signatures and transcriptional regulation of individual factors important for extraembryonic differentiation has been established for some autosomal, non-imprinted genes. The transcription factor OCT4, encoded by the *Pou5f1* gene, is essential for specification of the ICM lineage and for maintenance of pluripotency in derived ES cells. Transcription of OCT4 is epigenetically regulated in that the promoter region is acetylated at H3 and H4 in ES cells, whereas the gene is silenced by DNA methylation in trophoblast stem cells and in the placenta [104]. This silencing is achieved by a multi-step programme of inactivation where transcriptional repression is followed by G9A-mediated H3K9 methylation. Acquisition of these repressive histone modifications induces local heterochromatinization which in turn is required for subsequent *de novo* methylation at the promoter by the DNA methyltransferases Dnmt3A/B [105].

Several insights have also been gained into the epigenetic control of genes that are involved in development of the placental labyrinth. Branching morphogenesis of placental villi is initiated at sites of chorionic expression of the transcription factor *Gcm1*. This is followed by a differentiation process during which trophoblast cells fuse to form multinucleated syncytiotrophoblasts that line maternal blood sinusoids. Trophoblast cell fusion is mediated by Syncytins whose expression is directly controlled by GCM1 in humans and most likely in mice [106–108]. Epigenetic regulation of this system has been discovered on several levels. The *Dnmt3L* mutation has revealed that *Gcm1* expression depends on the presence of DNA methylation imprints [26]. *Syncytin* expression, by contrast, is repressed by DNA methylation in non-trophoblast cells that do not normally fuse [109]. In trophoblasts proper, fine-tuning of *Syncytin* expression is achieved, at least in part, by HDACs. HDAC3 can inhibit expression of (human) *Syncytin* by preventing GCM1-mediated transcriptional activation. Accordingly, HDAC3 is not associated with the *Syncytin* promoter in actively fusing trophoblasts [110]. This fairly detailed knowledge of epigenetic control of trophoblast fusion is further expanded by findings with trophoblast stem cells and placentas deficient for the hypoxia-inducible factor subunit HIF1β (ARNT). *Arnt*$^{-/-}$ trophoblast stem cells exhibit reduced global HDAC levels and differentiate predominantly into syncytiotrophoblasts. This altered differentiation preference can be recapitulated in wild-type trophoblast stem cells by treatment with the HDAC inhibitor TSA [51]. Thus, consistent with the results of *Syncytin* regulation, these data indicate that trophoblast fusion into syncytiotrophoblasts is favoured when HDAC activity levels are low. ARNT can control the function of HDACs by mediating their translocation into the nucleus [51]. The available data thus suggest that lack of (functional) ARNT in fusogenic trophoblasts prevents HDAC translocation to the nucleus and thereby enables GCM1-mediated *Syncytin* expression, resulting in cell fusion. ARNT function represents one of the possible mechanisms how hypoxia induces HDAC activity and, consequently, determines trophoblast differentiation. These findings demonstrate that the interplay between genetic and epigenetic factors is not only confined to direct transcriptional regulation, but can encompass their interaction and functional regulation by formation of protein-protein complexes.

Our knowledge of the epigenetic regulation of genes that are involved in the fine-tuned regulation of trophoblast differentiation and invasion is constantly expanding, mainly from studies in the mouse and in

human trophoblast cell lines [111–114]. Even if many of these genes are not absolutely essential for placental development, these insights contribute to the global picture of epigenetic control of extraembryonic development and the dynamic changes of chromatin organization during specific differentiation processes such as trophoblast stem cell to syncytiotrophoblast and trophoblast giant cell differentiation [113].

Conclusions

From the first steps of development onwards, a complex interplay between genetic and epigenetic factors determines expression profiles that characterize cell lineages and drive differentiation. Detailed knowledge of this cross-talk is critical to understand fundamental processes of development as well as the genetic and epigenetic requirements of pluripotency and reprogramming. Formation of extraembryonic tissues is an indispensable process for embryonic survival that represents an evolutionary acquisition to development *in utero*. Our knowledge of individual genes essential for extraembryonic development has grown substantially over the last years [11, 12]. The present survey of epigenetic modifiers emphasizes key steps during formation of extraembryonic tissues that appear to be particularly sensitive to alterations of chromatin organization. Linking these steps back to individual genes, it is clear that autosomal and X-linked imprinted genes play a major role in placental development. With accumulating insights into the epigenetic regulation of autosomal genes, valuable insights will also be gained into critical non-imprinted targets of epigenetic regulation. One important candidate that has emerged from these investigations is *Gcm1* whose epigenetic regulation may underlie the labyrinth defect observed in several mutants of chromatin modifiers [17].

The sensitivity of placental development to changes in chromatin structure is reflected by the placental malformations that are frequently associated with conceptuses generated by somatic cell nuclear transfer [115, 116]. An expanding knowledge of the epigenetic requirements for extraembryonic development is also vitally important to fully understand possible effects of subtle epigenetic alterations as they may be imposed by *in vitro* culture in assisted reproductive technology procedures [117, 118]. By studying epigenetic control mechanisms during differentiation of extraembryonic tissues, we will be able to define more precisely the constant interplay between genetic factors that regulate cell type-specific gene expression and the dynamics of the chromatin environment essential for placental development.

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