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Mechanisms Safeguarding the Trophoblast Multipotent State.

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Abstract The placenta is a highly specialized organ that is indispensable for intrauterine development to occur. Trophoblast cells are the major constituents of the developing placenta. They are the first cell type to arise very early in development, making up the trophectoderm, the outer layer of the blastocyst, segregating from the inner cell mass which gives rise to the embryo itself. The various functions of trophoblast cells early in development are vital for reproductive success, as they lay the foundations for a normal pregnancy and a healthy fetus. A better understanding of the mechanisms underlying these early events, including how the early trophoblast niche is regulated by transcription factors and specific epigenetic modifiers, is critical for understanding and eventually treating placental pathologies, which can inevitably cause pregnancy complications.
Keywords trophoblast, development, stem cells, placenta, epigenetics, transcription factors

The placenta is the defining organ of most mammals, providing a nutritive conduit that is crucial for embryonic development to occur. In addition to acting as a barrier protecting the fetus from any maternal immune attack and a bridge mediating the exchange of nutrients and waste products between maternal and fetal tissues, it is a factory of hormones (placental lactogens) (1), angiogenic factors (Vegf, Proliferin) (2) and tissue remodeling factors (Mmps, uPA) (3, 4) all required for a successful pregnancy. The mammalian placenta comprises several cell types of the trophoblast lineage. Specification of the trophoblast lineage occurs at the blastocyst stage, during which the first differentiation event takes place, resulting in two distinct cell groups: the inner cell mass (ICM) and the outer cell layer, known as the trophectoderm (TE). The developmental potential of these two cell groups is tightly restricted into the embryonic and extraembryonic lineages, respectively (5).

ICM and TE both harbour stem cell potential and can give rise to self-renewing stem cell lines *in vitro* following isolation from the mouse pre-implantation blastocyst: embryonic stem cells (ESCs) and trophoblast stem cells (TSCs), respectively. Upon blastocyst implantation the polar TE (in contact with the ICM) proliferates to form the extraembryonic ectoderm (ExE) and the ectoplacental cone (EPC), which together constitute the extraembryonic tissues of the post-implantation conceptus. TSC lines can also be derived from the ExE, which comprises a self-renewing trophoblast population, after implantation up to embryonic day (E) 8.5 of development (6). TSCs are considered multipotent, as they exhibit virtually the same developmental capacity as their progenitors in the blastocyst. In other words, they can differentiate into all of the distinct cell types of the placenta, namely trophoblast giant cells, spongiotrophoblasts and syncytiotrophoblasts. Importantly, they also contribute to the placental tissues in chimeras (6). Hence, TSCs are an invaluable model for studying the molecular mechanisms underpinning placental development, including trophoblast self-renewal and differentiation. Despite the integral role of the extraembryonic lineage in embryo implantation, development and long-term disease predisposition, TSCs have received comparatively less research attention than their developmental counterparts, the ESCs. However, focus appears to be shifting as recent ground-breaking research has considerably advanced our understanding of TSC regulation, including the definition of key transcription

networks, signaling pathways and epigenetic modifiers.

For instance, several studies have revealed the integral role specific transcription factors (TFs), such as Tead4, Cdx2 and Eomes, play in the establishment/maintenance of TS cell lines cultured *in vitro*, self-renewal and differentiation. Tead4 is one of the earliest-acting proteins in the specification of the murine TE, where it interacts with nuclear Yap1 to induce the expression of Cdx2. Mutation of *Tead4* results in a failure of blastocoel formation and thus embryonic lethality at the peri-implantation stage (7, 8), highlighting its importance in the initial stages of embryonic development. Cdx2 is one of the best studied TFs involved in trophoblast cell lineage establishment. Unlike Tead4, Cdx2 is not critical for TE specification but it plays an integral role in TE maintenance and proliferation. As such, *Cdx2* mutant embryos initially form blastocysts, but they subsequently collapse and cannot implant (9). Meanwhile, Eomes expression is first detected in the TE at the blastocyst stage and continues into ExE at the post-implantation stage. Eomes plays a role in further trophoblast expansion, as shown by *Eomes*$^{-/-}$ TE, which does not transition to trophoblast (10). Esrrb is another protein that is integral to the maintenance of the multipotent state. It is an orphan nuclear receptor acting as a TF, and its critical role in early mammalian development is evident from the embryonic lethality observed in *Esrrb*$^{-/-}$ mutant conceptuses, resulting from defects in the trophoblast compartment. Formation of ExE is unaffected in these mutants, but subsequent maintenance of trophoblast expansion in the chorionic ectoderm fails, resulting in aberrant trophoblast differentiation into giant cells (11). In the early post-implantation conceptus (E6.5), Eomes, Cdx2 and Esrrb exhibit a similar expression pattern in the distal portion of the ExE closest to the epiblast, a derivative of ICM. This expression profile, together with the inability to derive TSCs in the absence of any of these factors, supports the view of these TFs as TSC markers. Accordingly, they are rapidly downregulated upon trophoblast differentiation *in vivo* and *in vitro* (6).

A number of other TFs are known to have pivotal roles in the trophoblast compartment and in the maintenance of TSCs, although their expression profile also extends to trophoblast cells outside the immediate TSC niche. One of these, Elf5, is a TSC marker that is expressed from the late blastocyst stage onwards and is detected in most cells of the ExE. Thus, it initially acts downstream of Cdx2 and Eomes, but is subsequently essential to maintain *Cdx2* and *Eomes* expression in the ExE within the post-implantation conceptus (12, 13). The integral role of Elf5 in ExE formation is demonstrated by the embryonic lethality around E8.5 observed in Elf5 mutants, characterized by EPC differentiation in the absence of ExE formation (12, 14). AP-2γ, also known as Tfap2c, is another important TF in the regulation of TSC fate. AP-2γ cooperates with Sox2 in a TSC-specific manner to maintain the multipotent state of trophoblast stem or progenitor cells. AP-2γ can physically interact with Sox2 in TSCs, recruiting it to target sites where they co-occupy self-renewal gene loci (15). Interestingly, AP-2γ is expressed in all trophoblast cell types, including the TE of blastocyst-stage embryos, and after implantation in ExE, EPC and giant cells (16, 17). AP-2γ-null embryos exhibit several trophoblast defects, including diminished cell growth of the ExE and EPC, and a decreased giant cell population, eventually resulting in failure to form the terminally differentiated placental labyrinth (16). These defects lead to growth retardation of the embryo before mid-gestation and ultimately embryonic lethality around E9.5 (18).

The ability to grow stem cells *in vitro* and expand them indefinitely depends on our understanding of the external signals required for their self-renewal. In the case of TSCs, Fgf, heparin and mouse embryonic fibroblast conditioned medium (MEF-CM) were found to be necessary and sufficient for maintaining the proliferative and self-renewal capacity of TSCs *in vitro* (6). *In vivo*, Fgf4 is secreted by the ICM and,

subsequently, the epiblast (19). It acts in a paracrine manner on the TE and ExE, which express the cognate receptor, Fgfr2c. Activation of Fgf signaling mediates trophoblast self-renewal and proliferation (6, 20) and proximity to the Fgf source is, therefore, key to the maintenance of the trophoblast multipotent state. Accordingly, mural TE, which are not in contact with the ICM, are the first to differentiate into giant cells. Likewise, the EPC differentiates into spongiotrophoblasts and giant cells as it grows away from the epiblast. In TSCs cultured *in vitro,* Fgf4 withdrawal results in trophoblast differentiation, mainly towards the giant cell fate (6). Fgf signaling is indispensable for early embryonic development, particularly extraembryonic development, as indicated by Fgf-signaling-component mutants, which die peri- or early post-implantation. For example, Fgf4- and Fgfr2-depleted conceptuses die shortly after implantation (21-23), while mutation in *Frs2a*, a mediator of Fgf signaling, results in embryonic lethality by E7.5-8.0 (24, 25). Frs2a is integral to the maintenance of the TSC progenitor population in ExE, as *Frs2a*$^{-/-}$ embryos exhibit defective development and differentiation of the ExE due to impaired Fgf signaling (25). In response to the Fgf signal, Frs2a binds Shp2 and activates the Erk cascade. *Shp2* mutant embryos fail to expand the trophoblast lineage and die peri-implantation with diminished giant cell numbers and ICM death. Furthermore, TSCs cannot be derived from *Shp2* mutant blastocysts (26). These findings imply that Erk activation downstream of Fgf signaling is critical for the development of extraembryonic lineages. This conclusion is further supported by the phenotype of *Erk2*$^{-/-}$ embryos, which exhibit trophoblast proliferation defects, failure to form ExE and EPC, and post-implantation lethality (27). Collectively, these mutant studies highlight the pivotal role of Fgf signaling in TSC maintenance and normal expansion of the trophoblast compartment.

Regarding the essential, active component in MEF-CM, Erlebacher *et al.* reported the importance of Tgf-β superfamily members, namely either Tgf-β or Activin. These data corroborated earlier evidence from various Tgf-β family member knock-outs (KOs) that suggested Tgf-β signaling plays an important role in the maintenance of TSCs (28, 29). Tgf-β is the ligand of a heterotetrameric transmembrane receptor complex composed of type I (Tgfβr1) and type II (Tgfβr2) receptors, which are Ser-Thr kinases. Similarly, Activin binds type I receptor, Acvr1b, in complex with type II receptor, Acvr2 or Acvr2b (30). Upon Tgf-β or Activin binding, signal transduction involves the activation of Smad2/3 proteins via phosphorylation and nuclear translocation, which, in turn, regulate gene expression through interaction with transcriptional co-activators or co-repressors (30, 31). Tgf-β signaling in epithelial cells induces G1 cell-cycle arrest through Smad activation and altered expression of cell-cycle regulatory components, including upregulation of the cyclin-dependent kinase (Cdk) inhibitors, p15I^{nk4b} and p21^{Cip1}, and downregulation of Id1/2/3, which promote cell-cycle progression and inhibit cell differentiation (32, 33). Tgf-β signaling also downregulates c-myc: a TF essential for cell cycle progression (34). In TSCs, the cytostatic effect of Tgf-β is selectively inhibited by constitutively active Fgf signaling (28). The requirement of the Tgf-β signaling pathway for TSC maintenance and normal trophoblast differentiation is evident from mutations of the relevant receptors. Thus, deletion of the genes encoding the two Tgf-β receptors, Tgfβr1 and Tgfβr2, causes mid-gestational lethality with defective vasculogenesis in the yolk sac (35, 36). KOs of *Acvr1b* are affected even earlier, as they exhibit a disorganised ExE by 6.5 and die by E8.5 (37). Meanwhile, *Acvr2/2b* DKO embryos (Type II Activin receptors) exhibit severe growth restriction of both embryonic and extra-embryonic tissues, and they die by E8.5 (38). All three Activin receptors are expressed by the ExE, meaning that the effect of Activin is direct, i.e. via autocrine signaling (37, 39).

Nodal is another Tgf-β superfamily member that is involved in the maintenance of the proliferating TSC population *in vivo*. Nodal-depleted embryos exhibit failed TSC maintenance accompanied by TGC overgrowth

and absence of spongiotrophoblast and placental labyrinth formation (40). Nodal is expressed by the epiblast and binds to Activin receptors to activate Smad2/3, similar to Tgf-β and Activin (41). Nodal overexpression in TSCs can partially compensate for Fgf4 and MEF-CM removal, as it partially inhibits trophoblast differentiation (42). It is proposed that Tgf-β signaling is bi-functional, acting to maintain TSC pluripotency under Fgf control and, on the other hand, rapidly inducing TSC differentiation and cell-cycle arrest upon loss of Fgf4 signal (28). Overall, detailed molecular work both *in vivo* and *in vitro* has established the pivotal role of Fgf and Tgf-β signaling components for TSC maintenance.

The concerted actions of TFs and signaling cascades lead to the establishment and maintenance of the first cell lineages. These functions are influenced by epigenetic modifications that affect chromatin organisation to mediate cellular plasticity or to underpin cellular differentiation. The best-studied epigenetic modification is methylation of cytosine at the 5-carbon position of cytosine (5mC), which most commonly occurs at CpG dinucleotides (43). When present at gene promoters, this marker is frequently associated with gene repression (44). In general, it is believed that DNA methylation has a pivotal role in 'locking in' cell fate decisions (45). As such, the progressive restriction of cellular plasticity is accompanied by accumulation of DNA methylation marks that impose a cellular memory and, ultimately, ensure stable, terminal cellular differentiation. Indeed, methylation patterns can define and distinguish each cell lineage (46-48). For example, Senner *et al* identified developmentally regulated sequence elements, such as CpG islands and promoters, exhibiting methylation profiles that differ between embryonic and extraembryonic lineages. Strikingly the study also showed that methylation can distinguish between the different extraembryonic lineages, trophoblast and extraembryonic endoderm (XEN). For example, key TFs for trophoblast specification, such as Cdx2, Tfap2c and Elf5, are highly methylated, hence repressed, in XEN stem cells, while, conversely, XEN-TFs are hypermethylated in TSCs (49).

One key locus where the lineage-specific acquisition of DNA methylation has been shown to impose early cell fate restriction is the transcription factor, Elf5. *Elf5* is methylated and stably repressed in the embryonic lineage, but hypomethylated and expressed in the trophoblast lineage, where it upregulates the expression of the trophoblast stem cell genes, *Cdx2* and *Eomes* (50). Thus, after the first differentiation event, epigenetic modification of *Elf5* sets the barrier between the two cell lineages; Elf5, therefore, functions as a gatekeeper, maintaining cell fate by reinforcing commitment to the trophoblast lineage and preventing cells of the embryonic lineage from differentiating into trophoblasts (50).

Another intriguing aspect of TSC lineage restriction, maintenance and differentiation is the importance of protein abundance. For example, Latos *et al* showed that Elf5-mediated roles in TSCs are highly dependent on its protein expression level, which in turn affect its protein interactome. In TSCs, Elf5 interacts with Eomes, recruiting AP-2γ to trophoblast multipotency gene loci (triple-occupancy sites), inducing their expression and maintaining the trophoblast stem cell state. However, when Elf5 protein levels increase, it predominantly interacts with AP-2γ. As a result, this complex binds to double- and single-occupancy gene loci that contain the AP-2γ motif and are associated with trophoblast differentiation (51). In addition, Murray *et al* described how the dynamic expression of Plet1, another gene loci which is hypermethylated in ESCs, but hypomethylated in TSCs, affects trophoblast cell fate. In stem cell conditions Plet1 is highly expressed and facilitates the maintenance of the multipotent state through the induction of Elf5 expression. In contrast, Plet1 was shown to be also expressed in trophoblast giant cells, implying that this biphasic expression pattern is important in self-renewal and differentiation. High Plet1 levels induce trophoblast giant cell differentiation,

whereas Plet1 depletion favours differentiation towards the syncytiotrophoblasts lineage (52).

5-hydroxymethylcytosine (5hmC) is another important epigenetic modification. It derives from 5mC through the catalytic action of the Ten eleven translocation (Tet) enzymes (53). 5hmC has been associated with DNA demethylation, gene expression, open chromatin organisation and, generally, transcriptionally active genes (54-56). Both 5hmC and Tet proteins have been extensively studied for their role in maintaining the ES cell state, and their importance in epigenetic reprogramming during development has been well defined (57-60). Yet their role in the extra-embryonic lineage, and, in particular, in TSCs, had been widely overlooked until recently. Chrysanthou *et al* showed that Tet1/2 play a significant role in safeguarding the trophoblast multipotent state, as Tet1/2 depletion in TSCs resulted in trophoblast giant differentiation, accompanied by epithelial-to-mesenchymal transition (EMT) and a transition from the mitotic cell-cycle to the endocycle (61). The endocycle (also known as endoreduplication) is a highly specialized characteristic of trophoblast differentiation, specifically the giant cell lineage. It involves an exit from the mitotic cell cycle to undergo repeated rounds of endoreduplication, resulting in highly polyploid "giant" cells (62, 63). Despite extensive studies on endoreduplication, the exact mechanisms governing its regulation in trophoblast differentiation have yet to be fully elucidated. Much is known about the cell-cycle machinery involved in endocycle regulation, including; inhibition of Cyclin B1 translation, increased *Cyclin D1* expression (64) and oscillating Cyclin A/E expression (65). On the other hand, there is a limited understanding of how the epigenome is regulated during the mitotic cell cycle-to-endocycle transition (66, 67). The fact that the depletion of the epigenetic modifiers, Tet1/2, induces giant cell differentiation (61), indicates that apart from a direct association with the mitotic machinery, evident by the Tet1 interaction with Cyclin B1 (61), there could also be a general dynamic epigenetic signature facilitating cell cycle progression.

TSCs have tremendous biomedical relevance as ~30% of all human pregnancies are affected by placental-related defects (68), hence understanding placental development is paramount. A recent ground-breaking study highlighted the highly underestimated importance of proper placental development for embryo viability and growth (69). It was reported that 68% of KO lines that are lethal at or after mid-gestation exhibited placental dysmorphologies, whereas early lethality (E9.5–14.5) was almost 100% associated with severe placental malformations. This highlights the absolute necessity of including extraembryonic tissues in the analysis of mouse mutant strains in developmental studies (69). Such efforts complemented with *in vitro* TSC studies will further characterise molecular mechanisms and trophoblast-specific functions, paving the way to deciphering human placental development and human reproductive biology in general.

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