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Conserved and divergent features of trophoblast stem cells

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Abstract

Trophoblast stem cells (TSC) are a proliferative multi-potent population derived from the trophectoderm of the blastocyst, which will give rise to all the functional cell types of the trophoblast compartment of the placenta. The isolation and culture of TSC *in vitro* represent a robust model to study mechanisms of trophoblast differentiation into mature cells both in successful and diseased pregnancy. Despite the highly conserved functions of the placenta, there is extreme variability in placental morphology, fetal/maternal interface, and development among eutherian mammals. This review aims to summarize the establishment and maintenance of TSC in mammals such as primates, including human, rodents, and non-traditional animal models with a primary emphasis on epigenetic regulation of their origin while defining gaps in the current literature and areas of further development. FGF signaling is critical for mouse TSC but dispensable for derivation of TSC in other species. Human, monkey and bovine TSC have much more complicated requirements of signaling pathways including activation of WNT and inhibition of TGFβ cascades. Epigenetic features such as DNA and histone methylation as well as histone acetylation are dynamic during development and are expressed in cell- and gestational age-specific pattern in placental trophoblasts. While TSC from different species seems to recapitulate some select epigenomic features, there is limitation in the comprehensive understanding of TSC and how well TSCs retain placental epigenetic marks. Therefore, future studies should be directed at investigating epigenomic features of global and placental-specific gene expression in primary trophoblasts and TSC.

Keywords

trophoblast stem cells; placenta; epigenomics; epigenetics; placenta

Introduction

Trophoblast stem cells (TSC) are defined as a proliferative population arising early in gestation from the trophectoderm (TE) layer, which further differentiates into all the

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different mature trophoblast cell types found in the fully developed placenta and that performs most of its major functions, including gas/nutrient exchange, hormone production, and interaction with the maternal immune system. Despite the highly conserved functions of the placenta, there is extreme variability in placental morphology, fetal/maternal interface, and development among eutherian mammals. Therefore, spatio-temporal rising of TSCs during placental development and their differentiation into mature trophoblast cell types has both conserved and divergent pathways. While initially only isolated in mouse, TSCs have now been derived in multiple species, including human, monkeys, other rodents, and bovine. The isolation and culture of TSC *in vitro* represent a robust model to study mechanisms of trophoblast differentiation into mature cells both in successful and diseased pregnancy.

Recent literature has covered the transcription factors networks sustaining trophoblast cell identity (Papuchova & Latos 2022). In this review, we will present literature about the *in vitro* isolation and maintenance of TSC in various species, focusing on the spatio-temporal localization of these cells, the signaling pathways required (Table 1), and their relationship to the *in vivo* counterpart. We will then cover the epigenetics and epigenomics features driving TSC specific gene expression, defining gaps in the current literature and areas of further development.

Traditional rodent TSC

Mouse TSCs (mTSCs) represent a proliferative population in the extraembryonic ectoderm (ExE), which develops from the polar trophectoderm (TE) after implantation. They can be isolated from E3.5 blastocyst, E6.5 extra-embryonic ectoderm (ExE), and up to E8.5 chorionic ectoderm (ChE) (Tanaka et al. 1998; Uy et al. 2002; Erlebacher et al. 2004). After E8.5, fusion of the ChE with the ectoplacental cone (EPC) causes the occlusion of the ectoplacental cavity, withdrawing important signaling for TSC proliferation, causing their disappearance. They require fibroblast growth factor 4 (FGF4), heparin (FGF signaling co-factor), and either a feeder layer of mouse embryonic fibroblasts (MEFs), MEF-conditioned-media, or TGFβ pathway activation (TGF-β1 or Activin A) for self-renewal/proliferation. FGF4 increases cell survival via the activation of the Src/Ras/ERK pathway (Yang et al. 2006) while blocking differentiation via the MKK4/JNK and p38 pathways (Abell et al. 2009). During development, the embryonic compartment provides both FGF4 and Nodal, a TGFβ ligand (Niswander & Martin 1992; Chai et al. 1998; Guzman-Ayala et al. 2004). *In vitro*, removal of FGF4, heparin, and Activin A causes differentiation into a mixed trophoblast population with bias toward TGCs, while maintenance of Activin A in the absence of FGF4 diverts differentiation towards the labyrinthine lineage (Natale et al. 2009). Recently both serum-free conditions (TGFβ1, FGF4 and heparin on Matrigel-coated plates) (Kubaczka et al. 2014) and chemically defined media (fibronectin-coated plates with FGF2, Activin A, XAV939 - a WNT inhibitor, and Y27263 – a ROCK inhibitor) were developed for mTSC (Ohinata & Tsukiyama 2014) (Please see Table 2 for details of small molecule inhibitors used for TSC derivation). While FGF and TGF-β pathways remain the main player in mTSC maintenance, both inhibition of the phosphoinositide 3-kinase (PI3K) pathway and autocrine bone morphogenetic protein (BMP) signaling play a role in maintenance of mTSC and inhibition of differentiation (Lee et al. 2019a; Au et al. 2021).

mTSCs have been a fundamental tool to identify genetic and epigenetic markers of trophoblast specification as well as TSC maintenance and differentiation (Simmons & Cross 2005; Roberts & Fisher 2011). An important advantage of studying trophoblast specification in mice is the ability to create chimeric mice, which allowed the ultimate validation of TSC potency, and the detailed dissection of the processes involved in the development of the different placental compartments (Tanaka et al. 1998; Erlebacher et al. 2004; Kubaczka et al. 2014; Ohinata & Tsukiyama 2014). One current limitation of this model is the inability to differentiate mTSC specifically into the labyrinthine, spongiotrophoblast, and TGC lineage *in vitro*, in order to investigate sub-lineage-specific mechanisms. However, this limitation of the *in vitro* model is compensated by the ability to perform chimeric studies and compartment-specific conditional knock-outs *in vivo* (Simmons et al. 2007; Wenzel & Leone 2007).

Rat TSCs have been isolated with similar maintenance requirements to that of mouse TSC. Rat TSCs have been used to study the invasive phenotype as rat placentas penetrate further into the maternal compartment than mouse (although not as much as human) (Asanoma et al. 2011).

Interestingly, both FGF-dependent and FGF-independent/LIF-dependent TSC lines have been derived from another small rodent, the common vole *M. levis* and *M. rossiaemeridionalis* (Grigor'eva et al. 2009; Vaskova et al. 2014). Of note, isolation of *bona fide* rabbit TSC was reported in 2019 with similar culture conditions to mTSC, including FGF1/FGF2 and TGFβ1 (Sanz et al. 2019). These cells have been mainly used to study the effect of fluid shear stress on TSC differentiation into syncytium showing increased microvilli formation, cell fusion, and lipid droplet accumulation compared to static culture conditions.

Human TSC

Conditions for the isolation of mouse TSC did not allow the derivation of a similar cell type from human blastocyst or early placental tissues, pointing to species-specific differences in signaling pathway required and/or spatio-temporal localization (Chang & Parast 2017).

Bona fide human TSC (hTSC) were finally derived in 2018 and required a much more complex media containing at minimum EGF (epidermal growth factor), canonical WNT pathway activation (CHIR 99021), ALK5/4/7 inhibitors (TGF-β/Activin receptors), a ROCK inhibitor, and valproic acid, a histone deacetylation (HDAC) inhibitor (Okae et al. 2018). This media allowed the isolation and maintenance of hTSCs from pre-implantation blastocysts and first trimester placental tissues, on collagen IV-coated plates. These cells showed a transcriptomic signature similar to *in vivo* villous cytotrophoblast (CTB), self-renewal, and differentiation into both villous syncytiotrophoblast (STB) and extra-villous trophoblasts (EVT). Later, these culture conditions were also used to derive hTSC from other sources, including primed and naïve pluripotent stem cells (PSC) (Castel et al. 2020; Dong et al. 2020; Wei et al. 2021; Soncin et al. 2022), direct re-programming of fibroblasts into induced TSC (iTSC) (Liu et al. 2020), and from triploid embryos (Kong et al. 2022). In our hands, while the minimal media identified by Okae et al. permits hTSC growth,

spontaneous differentiation is often occurring. An optimized culture conditions with the addition of FGF2, HGF (hepatocyte growth factor), and Noggin (the latter two factors also included in the initial screening in Okae et al. 2018) decreased cell heterogeneity and spontaneous differentiation into multinucleated cells (Bai et al. 2021; Soncin et al. 2022).

Other signaling pathways play a role in hTSC (Table 1). For example, Hippo pathway inhibition, with consequent nuclear localization of the co-factor YAP1 (yes-associated protein 1), as well as co-factor WWTR-1 (WW Domain Containing Transcription Regulator 1), are associated with blockage of STB differentiation, maintenance of the stem state, and improved EVT differentiation (Meinhardt et al. 2020; Saha et al. 2020; Ray et al. 2022). Moreover, (5Z)-7-Oxozeanol, a Smad-independent BMP inhibitor, was associated with accelerated differentiation of hTSC in culture, while maintenance of BMP signaling via exogenous BMP4 delayed EVT differentiation (Au et al. 2021).

Much less is known about the origin of these signals in human embryos during development. A transcriptional trajectory analysis by Chen and colleagues showed that, similar to mTSC derivation from blastocyst, the Okae media promote a TE to TSC transition in culture and revealed a pivotal role of MAPK (mitogen-activated protein kinase) signaling in trophoblast differentiation, specifically towards EVT (Chen et al. 2022). However, ethical considerations limit the direct study of early human embryo development.

Interestingly, a trophoblast stem state can also be captured *in vitro* in 3D organoid-like structures. Organoid culture conditions are very similar to 2D hTSC culture conditions and include all the factors in the original Okae hTSC media plus FGF2 and HGF (also present in our optimized hTSC media in Bai et al.) as well as R-Spondin (canonical WNT co-activator) and prostaglandin E2 (PGE2). This allows the maintenance of a CTB proliferative population on the outer layer of the organoid, while spontaneous STB differentiation occurs on the inside (similar to inside-out villi). In this context, EVT differentiation is induced by temporal modulation of the canonical WNT pathway (Haider et al. 2018).

Non-traditional animal TSC

Non-human primates:

The first proliferating trophoblast-like cells were derived by Vandevoort and colleagues (2007) from rhesus monkey blastocysts on rhesus embryonic fibroblasts and could be maintained in the absence of specific growth factors or fibroblast-conditioned media (Vandevoort et al. 2007). However, these cells showed spontaneous differentiation and co-expression of pluripotency markers and were not fully validated. Recently, two groups have derived *bona fide* cynomolgus (Cyn) macaque TSC using two distinct media. Matsumoto and colleagues based their media on mTSC and showed isolation and maintenance of cynTSC from primate blastocysts using FGF4, heparin, a pan-RAR inverse agonist (BMS493), and a ROCK inhibitor (Y-27632) (Matsumoto et al. 2023). Unlike mTSC, Activin A was not required for TSC maintenance and removal of FGF4/heparin did not cause TSC differentiation but decreased cell proliferation. A limitation of this TSC model is the differentiation into a mix of STB and EVT cells upon dibutyryl cAMP (a cAMP analog) treatment, precluding the study of trophoblast lineage specific mechanisms. On the other

hand, Schmidt and colleagues (2020) showed derivation of both rhesus and cynomolgus macaque TSC from placentas using the original Okae media for hTSC derivation, as well as the differentiation into STB (in 2D and 3D) and EVT (Schmidt et al. 2020). A recent paper submitted in BioRxiv showed the derivation of distinct populations of ESC (embryonic stem cells), XEN (extraembryonic endoderm stem cell), and TSC from mouse and cynomolgus monkey blastocyst using small variations of the same media containing FGF4, Activin A, and WNT activation (CHIR99021) (Wei et al. 2023), suggesting conserved pathway requirements for maintenance of stem cells from early embryo. Considering the variety of the media utilized for non-human primate TSC derivation, it would be interesting to investigate the signaling pathways present in the peri-implantation embryo to identify developmentally relevant culture conditions.

Bovine:

There has been a long-standing interest from the farm industry to establish models to study trophoblast specification and differentiation in ruminants, in particular bovine. High incidence of embryonic mortality is observed in ruminants during the peri-implantation period of pregnancy, which is a major factor limiting the reproductive efficiency and profitability of the livestock industry. Moreover, use of large animals in terminal studies for understanding the events of implantation is economically challenging. Development of *in vitro* models, such as the TSC from livestock species, would provide economically feasible alternative to study early embryonic development. Ruminants have epitheliochorial placenta where the conceptus elongates rapidly during the peri-implantation period of pregnancy and the placenta does not invade the maternal endometrium. Derivation of stem cells, both embryonic and trophoblast, from bovine embryos has been challenging. Proliferative TE-like cells were isolated from d7–11 blastocysts on various feeder cells or conditioned media (Pillai et al. 2019), including MEFS-coated plates and fetal bovine serum (FBS)-containing media (Talbot et al. 2000), porcine granulosa cells (Saadeldin et al. 2017) or on collagen-coated plates in bovine endometrial fibroblast-conditioned media (Nakano et al. 2002). While they did express typical trophoblast markers, including CDX2, placental lactogen (PL), and interferon tau (IFNT), their differentiation potential was never confirmed.

Huang and colleagues showed that a 2i media (MEK inhibitor PD0325901 and GSK3β inhibitor/WNT activator CHIR99021) sustained the expansion from d7 blastocyst of a cell population expressing both undifferentiated and differentiated trophoblast markers and formation of binucleated cells when injected into NOD-SCIID mice (Huang et al. 2014). Wang and colleagues showed that these cells required the activation of the non-canonical WNT-YAP/TAZ axis for the expression of CDX2 and proliferation maintenance (Wang et al. 2019). However, they also showed high expression of pluripotency markers, including OCT4 and NANOG, and embryonic-like differentiation (neuronal rosette). So, while they showed some characteristics of trophoblast stem cells, their exact nature remains unclear.

Recently, *bona fide* bovine TSC have been isolated and maintained from bovine blastocysts on MEFS (or MEF-conditioned media) with the addition of human LIF, CHIR99021 (WNT activator), dimethinedene maleate (DiM), and minocycline hydrochloride (MiH) (Wang et al. 2023). This media was originally designed for the derivation of expanded potential

stem cells (EPSCs) in mouse and human (Yang et al. 2017) but, in bovine blastocyst, allowed the expansion of cells expressing typical trophoblast but not pluripotency markers. Their differentiation into functional binucleated cells was demonstrated both *in vitro* with forskolin (cAMP activator) treatment and *in vivo* in teratoma assay in NOD-SCIID mice.

Transcription factor networks in TSC

TSC specification, like any cell type, is driven by a cell- type-specific network of transcription factors (TFs), whose expression is determined by the coordination of multiple signaling pathways in the environment. These TFs contribute to the trophoblast nature of the cells, their proliferative status, and/or prevent differentiation into mature cell types. As TSCs arise from the TE layer, some of these genes are expressed as early as the pre-implantation morula and blastocyst (i.e. Cdx2, Gata3), while others appear in the peri/post-implantation stages (i.e. Elf5, Ets2) (Roberts & Fisher 2011). Moreover, besides many conserved genes, species-specific TF are starting to be described, which account for the differences in spatio-temporal development of the placenta. Extensive work with loss/gain of function in mTSC in the 2000s has revealed the correlation between spatio-temporal expression of trophoblast genes and their role in mTSC. In mouse, Cdx2 and Gata3, downstream of Tead4/Yap1, as well as Tfap2c, and Eomes contribute to the specification of the TE and are required for mTSC derivation (Roberts & Fisher 2011). Other TF expressed later in the ExE, such as Elf5 and Ets2, are required for TSC self-renewal (Roberts & Fisher 2011). Interestingly, TF such as Sox2 and Esrrb are not specific to TSC and contribute to the proliferation of other stem cells, including pluripotent stem cells (Roberts & Fisher 2011; Adachi et al. 2013). While an apparent contradiction, these genes show cell type-specific cell signaling activation (Fgf in TSC, Lif in ESC), and partnering (i.e Sox2/Oct3/4 in ESC and Sox2/Tfap2c in TSC) with consequent differences in binding site occupancy (Adachi et al. 2013).

Studies of the role of TF in early TE specification and TSC derivation in other species have not been so detailed. A combination of lack of suitable models and species-to-species variability has contributed to this knowledge gap between the mouse model and other species, including human development. TF like GATA3, TFAP2C, TEAD4, SOX2, and ESSRB are conserved, at least partially, in early human embryos and in hTSC. Interestingly, Eomes is a mouse-specific trophoblast marker as EOMES is not expressed in early human embryos (Blakeley et al. 2015), human trophoblast cells across gestation (Soncin et al. 2018), nor in hTSC (Okae et al. 2018). While a key role for Cdx2 has been well-documented in mouse, CDX2 expression in human embryo/placenta is still under investigation. In pre-implantation human embryos, CDX2 is specifically expressed in the TE compartment; however, both single-cell RNA-seq and immunostaining data show heterogenous expression in the TE (Blakeley et al. 2015; Deglincerti et al. 2016). Similarly, in early gestation placenta, CDX2 is specific to the CTB compartment, but again in a heterogeneous manner, with higher expression in CTB near the chorionic plate compared to the basal plate (Soncin et al. 2018). *In vitro*, hTSC showed low/no expression of CDX2 so they cannot be used to study the role of this TF in human placenta (Okae et al. 2018). Efforts are currently under way to identify culture conditions for the expansion of the CDX2$^+$ CTB population. Human-specific CTB genes also expressed in hTSC include the epithelial marker TP63 (Li et al. 2013) and the TEAD co-factor VGLL1 (Soncin et al. 2018), which might

contribute to trophoblast specification independently from the other TEAD-binding factor YAP1 (unpublished data).

With the exception of VGLL1, all other TF found in TSCs are not specific to the trophoblast lineage (i.e. CDX2 in the intestinal crypt stem cells, Eomes is also a mesoderm marker). Therefore, cell identity cannot be determined simply by the sum of the single TF, but it must come from the concerted effort of a network of multiple TFs working together at cell type-specific binding regions, called enhancers and super-enhancers (Kidder & Palmer 2010). Moreover, not all TFs are created equal: some TFs, like GATA3 and TFAP2A/C, are defined as pioneer factors, as they can directly mediate chromatin remodeling and enhancer formation (Tanaka et al. 2020). Therefore, while the investigation of this TF network greatly contributes to our understanding of normal and diseased pregnancies, studies on the epigenetics landscape in which these TFs operate will better elucidate the mechanisms of normal and abnormal placentation. Moreover, while TF networks show some species-specificity, the regulation of chromatin structures and features to allow modulation of cell-specific gene expression shows a more conserved nature. Current knowledge of epigenetics and epigenomics features in the developing placenta and TSC are described in the next section.

Placenta and TSC epigenetics and epigenomics

Epigenetics are changes to the structure and function of chromatin, without any alternation to the DNA sequence, that regulates how and when certain genes are expressed (Figure 1). Such changes include modification of DNA nucleotide residues (methylation/demethylation) and histone proteins forming the nucleosomes (the chromatin functional unit), including methylation, acetylation, and ubiquitination. These modifications affect how tightly the DNA is wrapped around the nucleosomes, therefore affecting chromatin accessibility to transcription and other nuclear factors, leading to gene expression regulation. In general, DNA methylation downregulates gene expression, whereas demethylation of DNA upregulates expression of genes (Figure 1A). While this has been broadly documented in different tissues, the placenta often has shown poor correlation between methylation marks and corresponding gene expression levels (Avila et al. 2010; Gamage et al. 2018). While methylation of certain lysine residue of histones can either be active or repressive (Figure 1B), histone acetylation usually marks active enhancer regions and is associated with upregulated gene expression (Figure 1C) (See reviews by Klemm et al. 2019; Li 2021).

Such epigenetic modifications in the embryo regulate the expression of lineage-specific genes at very precise time during the developmental trajectory (Chen & Dent 2014). As such, epigenomics, the study of spatio-temporal changes in epigenetic features across the genome during development, provides valuable understanding of the state of chromatin during normal development. Abnormal epigenomic changes have been associated with placental disorders in diseased pregnancy such as pre-eclampsia (PE), pre-term birth, and fetal growth restriction (FGR) (Deshpande & Balasinor 2018; Shi et al. 2023; Vasconcelos et al. 2023). Therefore, investigating the epigenomics of the placenta across gestation can lead to identification of molecular biomarkers for the diagnosis of pregnancy-associated diseases.

Genomic imprinting and X chromosome inactivation (XCI) are two developmental phenomena regulated by inherited epigenetics marks and have profound effects on embryo development and placental function (Tucci et al. 2019; Patrat et al. 2020). Parent-specific expression of imprinted genes, usually organized in clusters, show parental allele-specific DNA methylation and chromatin modifications, acquired during germ cell formation. These germ-line marks are resistant to the extensive reprogramming of the genome that occurs in early embryo development (Tucci et al. 2019). Abnormal expression levels of some imprinted genes in the placenta, potentially associated with epigenetics mis-regulation, have been correlated with FGR and PE (Christians et al. 2017). Interestingly, XCI, required to compensate gene dosage in female (XX) vs male (XY) cells, shows tissues-specific and species-specific regulations. While in mice placental XCI is an imprinted feature, with preferential silencing of the paternal chromosome, in human evidence suggests a prevalence of random (or skewed) inactivation (Phung et al. 2022). Tissue-specific escape of XCI by certain genes could explain female resilience to some in utero-related stressor compared to males (Gong et al. 2018). Moreover, abnormal XCI has been associated with pregnancy losses (Sui et al. 2015) as well as FGR and PE (Deshpande & Balasinor 2018). Finally, environmental cues and stressors during pregnancy can affect placental chromatin status with consequences on pregnancy outcomes (Jaenisch & Bird 2003; Robinson & Price 2015; Vlahos et al. 2019).

The dynamics of epigenome during early embryonic development is important for the establishment of the trophoblast compartment and its sub-populations. Primary CTB, STB, and EVT have distinct epigenetics signatures, which change as gestation progresses (Grigoriu et al. 2011; Gamage et al. 2018; Zhang et al. 2021; Vasconcelos et al. 2023). Since the study of the epigenome of early human placenta is limited by technical and ethical issues, *in vitro* models like hTSC become an invaluable resource to investigate key developmental mechanisms such as epigenomics changes, providing they recapitulate the *in vivo* cellular development. In this section, we will review current knowledge on the epigenomics features of *in vitro* TSC and their differentiated cells in correlation with trophoblast populations in the placenta. General information on specific epigenetics marks in early embryo and placental development is summarized in the relative text boxes.

Moreover, protocols for the direct re-programming of somatic cells as well as trans-differentiation of embryonic stem cells into hTSC has provided novel insight in the dynamics of epigenetics changes required for trophoblast specification and will be reviewed in the last section.

DNA methylation

DNA methylome of mouse TSC

mTSC methylome show high similarities to *in vitro* cells (Supplementary Text Box 1): markers of TSC lineage identity and differentiation such as *Elf5* and *Plet1* are hypomethylated whereas pluripotency genes, such as *Nanog* and *Oct4,* as well as embryo-specific regulators, such as *Hoxb4,* are hypermethylated (Hattori et al. 2004; Senner et al. 2012; Kubaczka et al. 2014; Murray et al. 2016). In a comparative analysis between embryo-derived stem cells (ESC, Epiblast Stem cells (Epi), TSC, and extra-embyonic endoderm

(XEN) stem cells), each cell type showed unique DNA methylation profiles, with TSC featuring hypermethylation at embryo- and XEN-specific developmental regulators, and hypomethylation at TSC markers, including *Cdx2*, *Tfap2c*, *Elf5*, and *Eomes* (Senner et al. 2012). Substantial methylation reinforcement of pre-existing epigenetic repressive markers also occurs in TSC compared to *in vivo* TE, which has been associated with high Dnmt3b expression levels. Recently, Sun and colleagues showed that these changes are driven by FGF4 during TSC derivation and might be necessary to restrict embryonic development and ensure TSC formation (Sun et al. 2021). Interestingly, in contrast to mESC, mTSC differentiation is associated with globally increased chromatin accessibility and activated gene expression (Nelson et al. 2017).

As evidence that culture conditions affect DNA methylation pattern, Kubackza and colleagues showed that mTSC derived and cultured under serum-free defined conditions have a DNA methylation pattern more similar to the *in vivo* counterpart at E7.5 compared to the classic serum-rich culture conditions (Kubaczka et al. 2014). Recently, Weigert and colleagues observed that mTSCs show partially-methylated epigenome similar to human cytotrophoblast and that this state is highly flexible as it can return to this intermediate steady-state even after prolonged forced periods in either high or low methylation (Weigert et al. 2023).

DNA methylome of human TSC

Like placental CTB, hTSC derived from early placenta or blastocyst show 1) low global methylation, although at even lower level than the primary CTB counterpart, 2) hypermethylation of gene body of actively transcribed regions, and 3) transcriptional activation associated with hypomethylation at promoter/enhancers regions of key genes, including *ELF5* (E74 like ETS transcription factor 5) (Okae et al. 2018). The placental epigenome is also unique such that it has differentially methylated regions (DMRs), that are methylated based on maternal or paternal allele (Court et al. 2014). In agreement with primary CTB, hTSCs derived by Okae et al. also have intermediate methylation levels of those placental specific DMRs in imprinted genes. On the contrary, while Okae and coworkers confirmed the previously-reported intermediate methylation levels of PMDs in first trimester primary CTB (Schroeder et al. 2013), hTSC showed hypomethylation at PMD regions. Since genes in the PMDs are generally silenced, hypomethylation of the PMDs observed in hTSC may have aberrant expression of non-placenta-specific genes, the functional or physiological impact of which remains unknown.

DNA methylome of non-human primate TSC

Limited studies have investigated the DNA methylome and chromatin accessibility status in non-human primate TSC and placenta. Conserved methylation patterns have been observed between human and cynomolgus monkey TSC, including hypomethylation at the *ELF5* promoter and hypermethylation at the *POU5F1* promoter (Schmidt et al. 2020; Matsumoto et al. 2023). However, methylation at the imprinted C19MC locus showed high cell-to-cell line variability and warrants further investigation (Schmidt et al. 2020). Similar to humans, the placentas of both rhesus macaque and squirrel monkey are globally hypomethylated when compared to somatic brain tissue (Schroeder et al. 2015). However, while monkey

squirrel show the PMD/HMD bimodal methylation distribution observed in human, the rhesus macaque showed lower HMD (Schroeder et al. 2015).

DNA methylome of bovine TSC

The global methylation of bTSC is overall lower than that of EPSCs but much higher than that of trophectoderm, which is in concert with higher expression of *DNMTs* in bTSC (Wang et al. 2023). Analyses of DMRs between trophectoderm and TSC revealed that the hypermethylated regions in bTSC were enriched in junctional complexes chemokine signaling (pathways required for proliferation and migration of trophoblast cells during peri-implantation period) whereas the hypomethylated regions of bTSC were associated with cellular metabolism (Wang et al. 2023). Moreover, open chromatin regions in bTSC are enriched for trophoblast specific TFs such as the GATAs and TEADs family members (Wang et al. 2023). The authors investigated these epigenetic features of bTSC in comparison to the spherical/ovoid conceptus (Wang et al. 2023). However, the bovine placenta is highly dynamic and proliferative with distinct morphology (spherical, ovoid, tubular and filamentous) during the peri-implantation period. So, further studies comparing the epigenetics of the *in vivo* placenta and *in vitro* TSC is warranted to determine the developmental potential of the bTSC.

Histone methylation

Histone methylation in mouse TSC

Comparative studies between *in vitro* mESCs and mTSCs have demonstrated that these cell lines maintain most of the epigenetic marks and mechanisms observed *in vivo* (Supplementary Text Box 2), suggesting that similar models in other species might also serve the same purpose (Dahl et al. 2010; Senner et al. 2012) For example, embryonic genes are typically in a bivalent poised chromatin state (H3K4me3/H3K27me3) in the ICM/Epiblast as well as mESC cells but are silenced in extraembryonic tissues and mTSC through addition of H3K9me3 to create a repressive "trivalent" H3K4me3/H3K27me3/ H3K9me3 histone mark (Dahl et al. 2010; Senner et al. 2012).

Two separate groups observed overall low levels of the repressive H3K27me3 histone mark in mTSCs compared to mESC (Rugg-Gunn et al. 2010; Senner et al. 2012) although recent work showed the opposite trend with no clear explanation for the differences (Weigert et al. 2023). As post-implantation primary extraembryonic tissues show high gene-specific levels of H3K27me3, such difference could be explained two-fold: either rapid changes in histone post-translational modifications (PTMs) in the post-implantation tissues are not reflected in the *in vitro* model, or intrinsic adaptation during the establishment of the stem cell culture cause the divergence between *in vivo* and *in vitro* features (Rugg-Gunn et al. 2010). Instead of H3K27me3, mTSCs show high levels of the H3K9me3 mark associated with repressive state, representing evidence of lineage-specific mechanisms of epigenetic control. Therefore, either a bivalent H3K4me3/H3K9me3 or a trivalent H3K4me3/H3K27me3/H3K9me3, rather than the bivalent H3K4me3/H3K27me3 mark observed in mESC, control the repressive state in mTSC. H3K9me3 has also been implicated as a repressive histone modification in rat

TSC wherein it maintains trophoblast stem-state and prevents TSC differentiation (Wang et al. 2021).

Generally, the active H3K4me1 mark (with H3K27Ac) identifies enhancer regions (and super-enhancers, regions co-occupied by multiple cell identity-specific master transcription factors) controlling lineage-specific genes. Indeed, Lee and colleagues observed a high positive correlation between these marks and EP300 (a HAT enzyme) and mediator complex subunit 12 (Med12) occupancy around regions enriched in TSC-specific transcription factor binding sites in mTSC (Lee et al. 2019b). Moreover, dynamic changes in the super-enhancers regions and TF networks occurred upon TSC differentiation into more mature cells, revealing cell-type specific regulatory mechanisms.

The mechanisms of how PTMs regulate gene expression are still under investigation. One such mechanism is by affecting chromatin folding in a cell-type specific manner. For example, mESC are enriched in repressive chromatin interactions, mainly between gene promoters as well as Polycomb-driven H3K4me1/H3K27me3 interactions at enhancer regions to suppress TSC-specific gene expression (i.e. Cdx2, Eomes, and Dlx3). On the contrary, mTSCs are enriched in active enhancer-gene interaction involving TSC-specific transcription factors to promote expression of TSC genes (Schoenfelder et al. 2018).

Interestingly, as both histone methylation and DNA methylation mechanisms contribute to the stable repression of ESC-specific genes in TSC lineage independently, ablation of one of these two mechanisms alone is not sufficient to re-activate the expression of ESC markers in mTSC (Senner et al. 2012). More recently, Weigert and colleagues showed that these two epigenetic mechanisms, DNA methylation and Polycomb Repressive Complex (PRC)-mediated histone methylation, actually interact in an antagonistic fashion to regulate the highly dynamic epigenetic landscape observed in mTSC (Weigert et al. 2023).

Histone methylation in human TSC

Recently, work by Varberg and co-workers highlighted the potential of using hTSC for epigenetics studies (Varberg et al. 2023). They investigated the mechanisms underlying hTSC differentiation into EVT using multiple functional genomics approaches linked to next-generation sequencing (NGS), including RNA-sequencing (RNA-Seq), chromatin accessibility using Assay for Transposase-Accessible Chromatin-sequencing (ATAC-Seq), and high throughput chromosome conformation capture (Hi-C). with bioinformatics approaches they identified cell-type specific chromatin state and histone marks, including higher incidence of bivalent marks at promoter regions in hTSC (while EVT were enriched in active enhancer mark H3K27). Moreover, they identified long-range chromatin interactions associated with EVT specification, which were validated *in vitro* in hTSC and *in vivo* in first trimester placental samples.

Histone acetylation

Histone acetylation mouse TSC

Acetylation is a highly dynamic mechanism controlled by fine-tuned activity of both HATs and HDACs, which often binds simultaneously on actively transcribed genes around

acetylated histones (Supplementary Text Box 3) (Wang et al. 2009). For example, p300-mediated acetylation of H3K27 maintains mTSC self-renewal and prevents differentiation (Dou et al. 2023), while HDAC1 inhibition biases mTSC differentiation towards chorionic trophoblast vs TGC (Maltepe et al. 2005). Moreover, Sirt1 activity, a histone deacetylase, is required for correct mTSC differentiation (Arul Nambi Rajan et al. 2018) and its deficiency, associated with increased maternal age, causes abnormal placental development (Xiong et al. 2021). Interestingly, binding of HDAC1 has been observed at both active and repressed genes in mTSC including actively expressed TSC-specific genes such as Cdx2, Eomes, Elf5, and Sox2 (Kidder & Palmer 2012). Its presence at actively transcribed regions might be required to rapidly reset the histone acetylation status and prevent spurious transcription of unwanted genes (Chen & Dent 2014). On the contrary, H3 and H4 histones are highly hypoacetylated in regions upstream of pluripotency gene such as *Pou5f1* and *Nanog* in mouse TS cells compared to ES cells (Hattori et al. 2007).

To confirm its role as active marks, H3K27ac shows broader and stronger signature at regions overlapping with p300 and Med12 binding sites as well as associated with open chromatin. These regions, defined as super-enhancers, are rich in TSC-specific TF binding sites and are associated with greater gene activation of tissue-specific master regulator genes (Lee et al. 2019b).

While less studied, acetylation at other histone proteins might be important in trophoblast stem cell biology. For example, deacetylation of H2B histone at lysine 5 (H2BK5), via ubiquitination and degradation of HDAC6, promotes epithelial-mesenchymal transition in mTSC, and abnormal HDAC6 activity has been associate with failed implantation due to aberrant EMT in the extra-embryonic compartment (Abell et al. 2011; Mobley et al. 2017).

Histone acetylation in human TSC

Recently, a large number of studies have started to use hTSC to investigate the role of acetylation in hTSC maintenance ad differentiation. Varberg and co-workers found that the active enhancer H3K27ac was more abundant in EVT compared to hTSC (Varberg et al. 2023). In fact, Voorden and colleagues demonstrated that pharmacological inhibition of the histone acetyltransferase, EP300, during differentiation of hTSC prevented cell differentiation into both EVT or STB (Jantine van Voorden et al. 2023).

Recently, ChIP-seq data for H3K27Ac mark on primary CTB and *in vitro* hTSC have identified large families of primate-specific endogenous retroviruses as potential *bona fide* enhancers of genes with important roles in placentation (Frost et al. 2023). Similar to primary tissue, hTSC differentiation into STB also requires H3K27 acetylation at cell type-specific genes, including SDC1, SLC6A4, and TBX3 (Hornbachner et al. 2021).

Epigenetics of TSC: Knowledge from re-programmed and trans-differentiated TSC

Epigenomics in the re-programming process into TSC

Direct reprogramming of embryonic and adult fibroblasts into iTSCs was first reported in mouse by ectopic transient expression of Tfap2c, Gata3, Eomes, and Ets2 (Supplementary Text Box 4) (Kubaczka et al. 2015). Mouse iTSCs exhibit global DNA methylation of CGI highly similar to *bona fide* mTSC, with methylation of previously hypomethylated fibroblast-specific regions. Moreover, demethylation of key TSC loci, including *Elf5*, *Tead4*, and *Hand1*, correlated with higher gene expression.

Liu and colleagues have reported the direct re-programming of human dermal fibroblasts into iTSC using transient ectopic expression of the pluripotency TFs OKSM (OCT4, SOX2, KLF4 and MYC) followed by culture into Okae's TSC media (Liu et al. 2020). Similar to tissue-derived TSC, these iTSCs show open chromatin at the promoter and putative enhancer regions of the TSC-specific *ELF5* locus, also shown to be hypomethylated. However, recently, Naama and colleagues have identified GATA3, OCT4, KLF4 and MYC (GOKM) as a better combination for the direct reprogramming of fibroblast into iTSC (Naama et al. 2023). GOKM induce a much greater chromatin opening effect than OSKM at hTSC-specific loci (rather than loci shared by both hESC and hTSC). Moreover, GOKM deposited the histone mark H3K4me2 (active regions) more specifically in regions enriched in genes associated with trophoblast stem cells. GOKM-mediated reprogramming results in both *de novo* DNA methylation and de-methylation. Specifically, the promoter region of *ELF5* is hypomethylated whereas that for NANOG, a pluripotency marker, is methylated in iTSC, matching the methylation profile of *bona fide* hTSC. However, some of the parental fibroblast-specific hypomethylated regions that are hypermethylated in *bona fide* TSC fails to be methylated in iTSC suggesting that *de novo* methylation may be less rigorous during cellular reprogramming.

Interestingly, hTSC have also been derived from term-placenta villous CTB by transfection with the trophoblast factors s TFAP2C, TEAD4, CDX2, ELF5, and ETS2 (Bai et al. 2021). While term CTB are transcriptionally and epigenetically more similar to first trimester CTB, from which TSC are usually derived, compared to fibroblast cells used in the previous re-programming protocols, epigenetic differences observed between first trimester and term placenta suggest that some level of epigenetics re-programming needs to occur to reset these cells to a more progenitor state – a hypothesis that warrants further investigation (Vasconcelos et al. 2023).

Epigenomics in the trans-differentiation of PSC into TSC

hTSCs have been derived from both primed (post-implantation) (Wei et al. 2021; Soncin et al. 2022; Viukov et al. 2022) and naïve (pre-implantation) (Dong et al. 2020; Viukov et al. 2022) PSC by culture media and cell signaling manipulation. All groups have shown hypomethylation of the *ELF5* locus, considered as a robust mark for TSC. However, Wei and colleagues performed a thorough investigation of the epigenetics changes between primed hESC, the derived hTSC, and *bona fide* hTSC. Conversion of primed-PSC into

hTSC was associated with 1) more open chromatin, specifically at hTSC-specific regions, correlated with upregulation of genes involved in placental development and WNT signaling and enriched in binding sites for hTSC-specific TF TEAD4, GATA3 and GATA2; 2) global reduction of repressive H3K27me3 mark mostly on bivalent (H3K4me3/H3K27me3) genes in hESC, also associated with increased expression of placental and WNT signaling genes; and 3) loss of active H3K4me3 and gain of repressive H3K27me3 histone marks on hESC-specific genes such as *SOX2*, *NANOG*, *PRDM14* and *TDGF1*, suggesting that the repressive H3K27me3 has a critical role in derivation of hTSC. Indeed, induced loss of H3K27 methyltransferases during hTSC derivation from primed-hPSC enhanced the efficiency of hTSC derivation.

Conversion of naïve PSC into TSC seems to be more straightforward, probably because of lower epigenetics barriers between embryonic and extra-embryonic lineage at earlier developmental stages. Interestingly, Zijlmans and colleagues reported that inhibition of polycomb repressive complex 2 (PRC2)-mediated H3K27me3 mark in naïve hPSC does not affect expression of pluripotency markers but upregulates trophoblast-associated genes (*GATA2*, *GATA3*, *KRT7* and *VGLL1*), therefore suggesting that trimethylation of H3K27 restricts the induction of trophoblast fate (Zijlmans et al. 2022).

Mouse embryonic and extra-embryonic lineage seems to have a higher epigenetic barrier than human cells so that genetic manipulation is required to switch lineage fate in a process more similar to re-programming (Chen & Dent 2014; Hada et al. 2022). Initial protocols resulted in TS-like cells that, while showing some transcriptional and epigenetics changes towards *bona fide* TSC, failed to fully convert when analyzed wholistically. For example, transcriptional repression of pluripotency markers Oct4 and Nanog was correctly associated with a decrease in H3K9 and H3K14 acetylation as well as loss of HDAC1 and p300 binding, followed by dramatic changes in chromatin structure at core enhancers and increased CpG methylation (Carey et al. 2014). However, occupancy at key regulatory elements retained a distinct mESC epigenetic memory (Cambuli et al. 2014). In particular, a core of methylated lineage hallmark loci, including *ELF5*, seems refractory to demethylation and might function as safeguard from trans-differentiation in mouse.

Rhee and co-workers (2017) developed an improved protocol for conversion of mESC into TSC, modelled on the re-programming strategy from somatic cells, by ectopic expression of TSC-specific factors, Cdx2, Arid3a and Gata3 (CAG) (Rhee et al. 2017). In this process, CAG first binds open chromatin regions to repress pre-existing ESC-associated genes, followed by occupancy of closed chromatin regions to activate TSC genes. Both processes are associated with changes in H3K27ac histone mark within enhancers loci with deacetylation at ES-specific loci mediated by HDAC1.

Concluding remarks

Early embryo and placental development involve tight spatio-temporal regulation of transcriptomic signatures as well as epigenomics changes to allow proper trophoblast lineage specification and differentiation into mature cell types. Abnormalities in any of these mechanisms might result in placental defects and malfunction, with negative effects on

pregnancy outcomes. Moreover, these mechanisms show both conserved (epigenetics), and diverse (TF regulators), features so that species-specific models are required. *In vitro,* TSCs represent a robust species-specific tool to investigate such mechanisms in species where ethical concerns and financial challenges limit work on primary tissues. Studies in mTSC have provided evidence that *in vitro* cells retain most transcriptional and epigenomics marks of *in vivo* counterpart. This bodes well for similar TSC derived from other species, including human, non-human primates, and bovine. The diversity of culture conditions in which TSC have been derived from different species correlate with the highly diverse placental morphologies and architecture observed in eutherian mammals and with the different signals in the environment during development. However, as culture conditions can change the transcriptional and epigenetic landscape of cultured cells, it is important for early studies to validate findings in TSC with primary tissue, when possible, until the full characterization of this *in vitro* model has concluded. While effects of environmental, metabolic, and cellular stressors have been correlated with placental dysfunction and poor pregnancy outcomes, the exact causation remains unclear, especially in non-rodent models including humans. Derivation of TSC, providing they recapitulate the phenotypic, genotypic, transcriptomic, and epigenomic features of placenta, will facilitate the investigation of precise mechanisms of agents underlying placental diseases and help devise therapeutic or medical interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Due to space constraints, we apologize for any paper or studies that, while relevant to the topic, were left out of this review.

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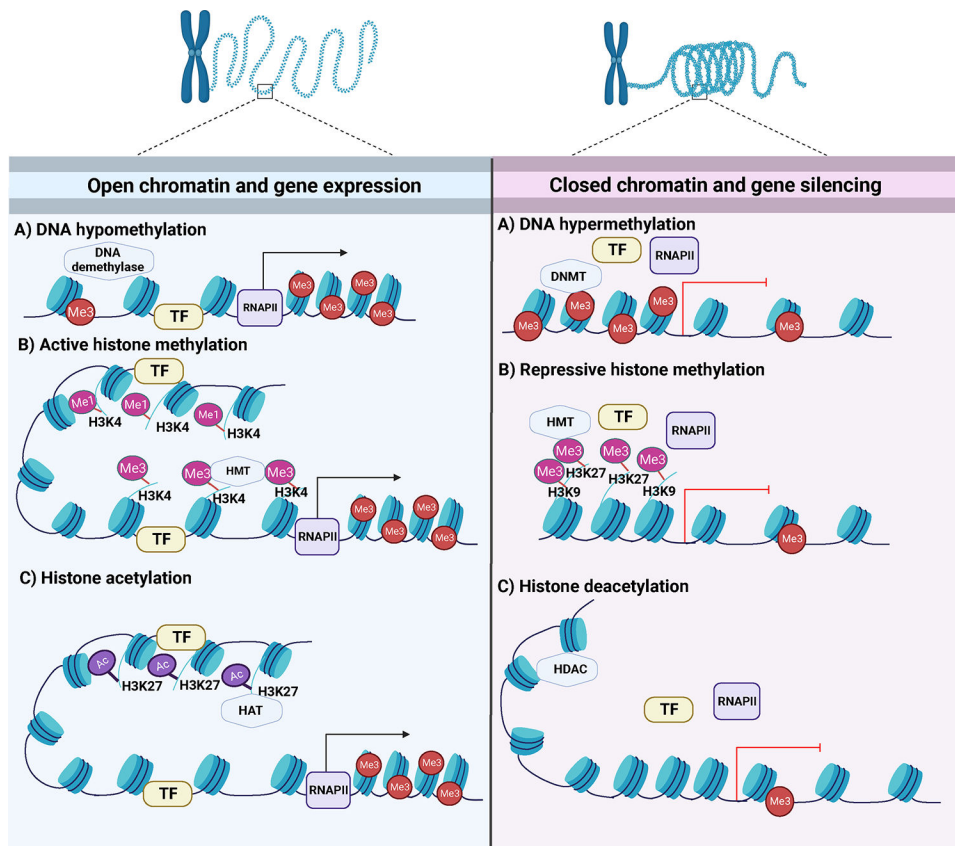
Figure 1:
Epigenetics features involved in chromatin remodeling and transcriptional activation or silencing. A) DNA demethylases remove, whereas DNA methyltransferases (DNMTs) add methyl groups to DNA cytosine residues to allow (or prevent) binding of transcription factors (TF) leading to activation (or silencing) of gene expression, respectively. B) Histone methyltransferases (HMTs) add methyl group to histone tails at promoters or enhancers and the site of methylation dictates active or repressive transcription. C) Histone acetyl transferases (HATs) actively add acetyl group to the histone tails and activates gene transcription whereas, histone deacetylases (HDACs) remove the activating acetyl modification from histones and inhibits transcriptional activity. RNAPII=RNA polymerase II, me1=mono-methylation, me3=tri-methylation, H3K4=histone 3 lysine 4 residue, H3K9=Histone 3 lysine 9 residue, and H3K27=Histone 3 lysine 27 residue.

Table 1:

Signaling pathway for TSC derivation across species.

Signaling pathways	hTSC	mTSC	bTSC	macTSC
EGF signaling	promotes proliferation; required	not required	ND	promotes proliferation; required
HGF signaling	maintains differentiation potential of TSC; dispensable	promotes TSC differentiation; not required	ND	maintains differentiation potential of TSC; dispensable
Wnt signaling	promotes proliferation; activation required	promotes early trophoblast lineage development; inhibition required	promotes proliferation; required	promotes proliferation; activation required
FGF signaling	not required	promotes proliferation, inhibits differentiation; FGF2 required	not required	not required
TGFb/activin signaling	TGFb inhibition promotes trophoblast proliferation; inhibition required	activin promotes proliferation, inhibits differentiation; required	activin A sourced from MEF; required	TGFb inhibition promotes trophoblast proliferation; inhibition required
BMP signaling	inhibition leads to maintenance of TSC; not required	inhibition leads to maintenance of TSC; dispensable	ND	ND
Hippo signaling	switched-off state promotes proliferation, inhibits differentiation; not required	switched-off state promotes proliferation, inhibits differentiation	ND	ND
Rho/ROCK pathway	inhibition required	ND	ND	inhibition required
JAK/STAT	ND	ND	inhibition required	ND
MAPK	ND	ND	inhibition required	ND
PARP1	ND	ND	inhibition required	ND
ECM	Collagen	fibronectin in 2D, matrigel in 3D	MEF as feeder layer or martigel with MEF conditioned medium	Collagen

*
ND= Not Determined

Table 2:

Small molecule inhibitors for TSC derivation

Inhibitors	Pathway inhibited	Receptors/Mechanism of action	Usage
Y27632	Rho-ROCK-myosin	competitive inhibition of phosphorylation of ROCK catalytic site	Essential for attachment of hTSCs, mTSC, and macTSC.
CHIR99021	GSK3b inhibitor; Wnt activator	activates Wnt-signaling by binding secreted Wnt-protein to its receptor, and disheveled (Dvl/Dsh) is recruited and inhibits the GSK3 located in the beta-catenin destruction complex.	Promotes proliferation and inhibits differentiation of hTSCs, bTSCs and macTSC.
LIF	JAK-STAT	LIFR/gp130	Prevents differentiation and promotes self-renewal of bTSC.
DiM	MAPK	G-protein coupled receptors (M2 muscarinic, and H1 histamine receptors)	Maintenance of stem cell developmental potential during bovine TSC derivation.
MiH	PARP1 (Poly [ADP-ribose] polymerase 1) axis	direction inhibition of PARP1 enzyme activity	Suppresses trophoblast differentiation and facilitate ES conversion to TS cells via regulating transcription and chromatin remodeling in human; used for derivation of bTSC.
VA	Histone deacetylation	binding to catalytic center of HDACs	Relieves HDAC-dependent transcriptional repression and is important for long-term maintenance of proliferative hTSC.
SB-431542	TGFb/Smad	ALK5	Induces conversion of hESC to hTSCs and enhances proliferation of hTSC.
XAV939	Wnt inhibitor	destruction of B-catenin by stabilizing axin-GSK3B complex via inhibition of ploy-ADP-ribosylation enzymes tankyrase 1 and 2	Derivation and maintenance of mouse TSC.
A83-01	TGF-beta/Smad	ALK5/4/7	Inhibits epithelial-to-mesenchymal transition and promotes proliferation of hTSC and macTSC.