

Regulation of Primate Trophoblast Lineage Differentiation—Insights Learned from Human Embryonic Stem Cells

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Abstract: Trophoctoderm formation is the first cell lineage segregation during early embryo development. Further differentiation of trophoctoderm to various types of trophoblasts is the key event of placentation process, which is crucial for embryonic implantation and successful pregnancy. The anatomic structure of human placenta is different from that of rodents which have provided the best model to study molecular and cellular mechanisms of trophoblast development. However, due to ethical and practical restrictions, the regulatory mechanisms of human trophoblast development are poorly explored and understood. Recent evidences demonstrate that human embryonic stem cells (ESCs) are capable of differentiation towards trophoblasts in culture, after induction by extracellular signals or specific genetic manipulation of master regulatory factors. This review summarizes recent advances in deriving human trophoblasts from human ESCs, and suggests novel mechanisms that regulate the human trophoctoderm commitment.

Keywords: trophoblast commitment, embryonic stem cells, BMP signaling, activin/Nodal signaling, primate

Introduction

One of the prominent innovations in early mammalian embryogenesis is the formation of the placenta, the specialized tissue that subsequently forms trophic interface between the embryo and the mother. The trophoctoderm (TE, the outer epithelial layer of the blastocyst) is the first cell lineage exhibiting highly differentiated function during embryo development. TE goes on to develop into various subsets of trophoblasts and forms fetal part of placenta (Rossant and Cross, 2001).

The differentiation of trophoblast cells is a highly regulated process. In human, the trophoblast cells are tumor-like in their aspect of invasion. However, their invasiveness is precisely controlled in a way that spatially the cells stop penetration at the inner third of the myometrium, and temporally, the invasion occurs only at the early stage of pregnancy (Graham and Lala, 1992). Any failure in the controlled trophoblast differentiation results in serious pregnant complications, such as abortion and pre-eclampsia due to deficient implantation, hydatidiform moles or even choriocarcinoma due to over invasion (Fisher, 2004; Shih and Kurman, 2002).

Due to ethical consideration, experiments performed with human embryos or feto-maternal interface at early pregnancy are limited. Much of our understanding on preimplantation and implantation stages of human embryonic development is inferred mainly from comparisons to animal development. However, the mechanisms underlying the processes of implantation and placentation are much different among various species, especially between human and commonly-used mammalian model animals such as mouse and rat (Malassiné et al. 2003). Therefore, the knowledge of cellular and molecular mechanisms involved in the formation of human trophoblast and placenta are largely restricted. Recent advances revealed that human embryonic stem cells (ESCs) can differentiate toward trophoblast given the correct extracellular signals or specific genetic manipulation of master regulatory factors, which strongly indicate the usage of ESC as an alternative *in vitro* model to study the regulatory mechanism of trophoblast lineage differentiation.

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Embryonic implantation and trophoblast cell lineage

The fertilized egg (zygote) divides to form morula when transiting the Fallopian tube and reaching the uterine cavity 2–3 days after fertilization. The embryo further develops to blastocyst which is composed of compact inner cell mass (ICM) and the out-layered trophectoderm. The ICM gives rise to the three embryonic layers and extraembryonic endoderm and mesoderm, while the trophectoderm is the origin of the placenta trophoblasts. Embryonic implantation occurs around 6–7 days postconception (pc) in human, 9–10 days pc in rhesus monkey, and 4–5 days pc in mouse after the blastocyst emerges from the zona pellucida. The blastocyst adheres to and further invades into the uterine wall through an interaction between trophoblasts and the uterine luminal epithelium. In human, the initial invasion is characterized by the penetration of uterine epithelium by the multinucleated syncytiotrophoblast. The blastocyst is completely embedded in uterine stromal tissue by day 10 pc (Kaufmann and Burton, 1994).

During embryo development, the trophectoderm of the blastocyst is the first cell lineage exhibiting highly differentiated function. In human, the highly proliferative, undifferentiated primitive cytotrophoblast cells deriving from the trophectoderm act as the progenitor cells that give rise to all types of differentiated trophoblast cells. The human trophoblast cells follow two separate pathways of differentiation and result in two types of highly specialized chorionic villi, the floating villi and the anchoring villi. In the floating villi, the mononucleated cytotrophoblast (CTB) cells fuse and form the multinucleated syncytiotrophoblast (STB) cells, which are characterized by high level of human chorionic gonadotropin (hCG) production. The STB layer floats in endometrial sinusoid and contacts with maternal blood directly, mediating gas and nutrient exchanges between the developing fetus and the mother. In the anchoring villi, the CTB cells stream out of the trophoblastic shell and differentiate along the invasive pathway. Some of them (interstitial trophoblasts) migrate into the deep layer of the maternal endometrium and even the inner third of the myometrium, thus anchoring the fetus to the mother. Meanwhile, some others (endovascular trophoblasts) penetrate the uterine spiral arteries and remodel them into low-resistance, high-capacity utero-placental arteries to ensure the placental blood supply and fetal nutrition. These highly invasive

trophoblasts are also termed as extravillous cytotrophoblast (Loke and King, 1996).

The differentiation process of rhesus monkey trophoblast is similar to that of human, except that the interstitial trophoblast cells do not migrate to the deep layer of the maternal endometrium (Ramsey et al. 1976; Qin et al. 2003). In contrast, the mouse embryo implants at the mesometrial pole of the uterine endometrium at day 4.5 pc. The trophectoderm cells overlying the ICM (polar trophectoderm) first attach to and invade into endometrium. They keep on proliferate and give rise to the diploid extra-embryonic ectoderm and ectoplacental cone. The outer region of the ectoplacental cone differentiates to form multinucleated trophoblast giant cells. The extra-embryonic ectoderm gives rise to the various layers of labyrinthine trophoblast cells, which are in association with fetal components of placental vascular network, and create a densely packed structure called the labyrinth. The trophectoderm cells away from the ICM (mural trophectoderm) continue to replicate their DNA without mitosis and form trophoblast giant cells, and they begin to invade uterine endometrium at the antimesometrial pole at around day 8 pc. The labyrinth is structurally supported by the spongiotrophoblast which is largely believed to be derived from the cells of the ectoplacental cone. The spongiotrophoblast is a compact layer of non-syncytial cells between the labyrinth and the trophoblast giant cells (Cross, 2000; Rossant and Cross, 2001). Therefore, mouse trophectoderm and placenta development is spatially and temporally different from that of the human (Fig. 1).

Regulation of human ESCs differentiation toward trophoblasts

One of the main obstacles in research of human trophoblast development is the lack of an adequate experimental model. Primary cytotrophoblast cells can be isolated from placenta tissues at either early or late gestational stages. The cultured cells represent some characteristics of normal trophoblasts *in vivo*. For example, the trophoblast cells derived from late pregnancy can spontaneously fuse and form syncytiotrophoblast cells in the presence of fetal calf serum (Kliman et al. 1986), and those isolated from the first-trimester placenta tissue maintain the ability of active proliferation and invasion (Yagel et al. 1988; Xu et al. 2000). However, these short-lived cells are difficult to be maintained stably and shared among laboratories. Meanwhile, several trophoblast cell lines

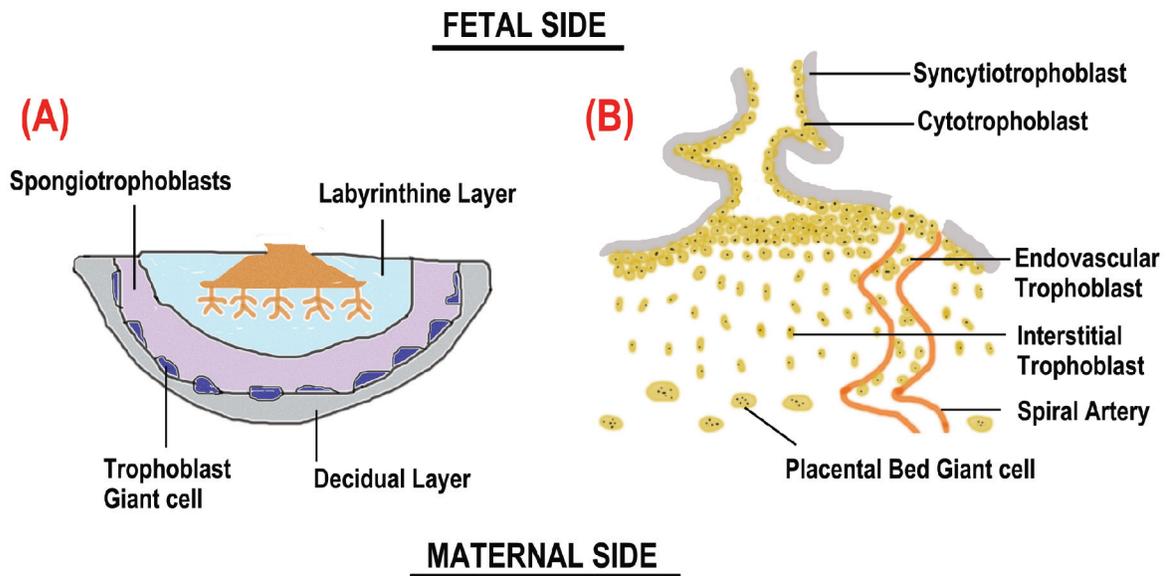


Figure 1. Sketch map showing the placenta structure in mouse (A) and human (B).

have been established with exogenous expression of either oncogenes (Lei et al. 1992; Graham et al. 1993) or telomeric reverse transcriptase (TERT) gene by one of us (Wang et al. 2006). These cells are derived from cultures of human normal placenta tissues, and represent some properties of differentiated trophoblast cells. They, however, are not suitable to study the early commitment stage of trophoblast lineage. Since the establishment of human ESCs a decade ago (Thomson et al. 1998), increasing data provide evidence of the promising usage of human ESC as an alternative *in vitro* model to examine the emergence and differentiation of trophoblasts.

Spontaneous trophoblast differentiation of human ESCs

Thomson et al. (1998) first found the spontaneous differentiation of human ESC to trophoblast lineage, as evidenced by the production of human chorionic gonadotropin (hCG). This is consistent with the observation that nonhuman primate ESCs derived several years earlier also exhibited similar spontaneous differentiation property (Thomson et al. 1995, 1996). However, the event rarely occurred in mouse ESCs without certain genetic or transcriptional modification (Niwa et al. 2000; Velkey et al. 2003; Hay et al. 2004; Hough et al. 2006; Ivanova et al. 2006; Loh et al. 2006). It is later confirmed that primate ESCs are more readily directed to trophoblast lineage especially after directed differentiation (see below).

Differentiation of human trophoblasts using embryonic body (EB) cultures

EB formation has been widely used as a 3D model to investigate the differentiation of ESCs towards the three embryonic germ layers, which mimics some of the features of early embryonic development. In human EBs cultured in suspension with a medium lacking growth factors that support pluripotency, some peripheral cells are proved to be spontaneously differentiated trophoblasts (Gerami-Naini et al. 2004). The differentiated trophoblast cells could be isolated by either selection for high hCG production (Harun et al. 2006), or by selection for adhesion molecule PECAM-1 (Peiffer et al. 2007). The former cells represent mainly villous trophoblasts and a minority of invasive extravillous trophoblasts, and the later ones exhibit properties of extravillous trophoblasts with a small portion of syncytiotrophoblasts.

However, spontaneous differentiation of primate ESCs to trophoblasts remains an inefficient process, which hinders its usage as a model to study primate trophoblast formation and differentiation.

BMP signaling is critical to human trophoblast cell differentiation

In 2002, Xu et al. first reported their striking findings on induction of human ESC differentiation to trophoblast. In the presence of mouse embryonic fibroblasts as feeder cells (or derived conditioned

medium) and basic fibroblast growth factor (bFGF), treatment of human ESC lines in a monolayer culture with bone morphogenetic protein 4 (BMP4) led to synchronously morphological and endocrine change of human ESCs toward trophoblasts. By DNA microarray and RT-PCR, they demonstrated that treatment of BMP4 caused a dramatic increase in mRNA expression of genes that have been reported to be expressed in human trophoblasts, including those encoding hCG α and hCG β (two subunits of hCG), placental growth factor, etc. Meanwhile, the transcription of genes for pluripotency maintenance, such as POU domain, class 5, transcription factor (POU5F1, also known as OCT4 or OCT3/4) and TERT were significantly decreased. Measurement of trophoblast-associated hormones production (including hCG, progesterone and estradiol) further demonstrated the trophoblastic characteristics of the BMP4-induced cells. The syncytial formation in these cells was observed when seeding at low density. They also showed that BMP4-related factors, including BMP2, BMP7, and GDF5, have similar effects on differentiation. The work was later confirmed by several independent laboratories using human ESC lines H1 with or without stable GFP expression (Liu et al. 2004; Ezashi et al. 2005; Chen et al. 2008; Wu et al. 2008).

Our recent report (Chen et al. 2008) in H1 line further supported the indispensable role of BMP signaling in directing human ESCs differentiation to trophoblasts. Two stable human ESC clones (AR1-c1 and AR2-c1) lacking glycosylphosphatidyl-inositol-anchored proteins (GPI-APs) were generated by suppressing the expression of phosphatidyl-inositol-glycan class A (PIG-A) gene which is required for the first step of GPI synthesis. AR1-c1 and AR2-c1 cells exhibited normal human ESC properties, and were capable of forming embryoid bodies and initiating cell differentiation into the three embryonic germ layers. Notably, these GPI-AP deficient human ESCs lost their ability to form trophoblasts in response to BMP4. A group of GPI-APs, DRAGON and related proteins RGM, were recently identified as co-receptor (or receptor III) for BMP2 and BMP4. Interestingly, overexpression of DRAGON in AR1-c1 and AR2-c1 cells restored their differentiation ability to trophoblasts with induction of BMP4 as did an activated type I BMP receptor (ALK3 or ALK6) gene vector. Conversely, knock-down of DRAGON gene in H9 human ESC cells

phenocopied the observation with AR1-c1 cells. Based on these data, it is concluded that that GPI-anchored coreceptors such as DRAGON are critical to the full activation of BMP signaling and in turn, human trophoblast formation. This hypothesis is also supported by gene array data that BMP ligands (such as BMP4) and downstream target genes (such as human ID2 and eHAND/Hand1) are expressed significantly higher in human TE tissues versus inner cell mass (Adjaye et al. 2005).

The fact that BMP4 at a high level together with bFGF and feeder-derived factors promotes trophoblast formation does not exclude the possibility that BMP signaling is also required for other lineages at this or later stages of development. Pera et al. (2004) demonstrated that BMP2 treatment promoted human ESCs (line HES-2 and HES-3) differentiation to extra-embryonic endoderm lineage as evidenced by the upregulation of a range of markers characteristic of endoderm, including transcription factors HNF3 α , HNF4, GATA4 and GATA6. Only small portion of colonies (less than 5%) exhibited morphology resembling the trophoblasts. They showed that BMP4, BMP7 or BMP2/BMP7 heterodimers had the same effect as BMP2. The BMP antagonist noggin could block this form of differentiation and induced the appearance of neural precursor cells. Recently Boyd et al. (2007) reported that BMP4 treatment in two human ESC lines (BG02 and WA09/H9) significantly promoted the formation of primitive vascular networks when the cells were cultured on a 3D-substrate of Matrigel in endothelial cell growth medium-2. Gene expression analysis revealed a general upregulation of endoderm, mesoderm and endothelial markers. Similar effect of BMP4 to induce efficient hematopoietic differentiation has also been previously reported in rhesus monkey embryonic stem cells (Li et al. 2001). This is largely consistent with previously studies in *Xenopus* and mouse systems where BMP inhibits neuronal differentiation (Munoz-Sanjuan and Brivanlou, 2002). Ying et al. and Qi et al. also demonstrated that BMPs played critical role in maintaining mouse ESC self-renewal (Ying et al. 2003; Qi et al. 2004). This is in sharp contrast with many studies in primate (human and monkey) ESCs that BMP signaling is a dominating factor to prompt differentiation to trophoblast (and mesoendoderm), while related ligands such as Nodal and Activin act to

help the maintaining of undifferentiation state in human ESCs (Beattie et al. 2005; James et al. 2005; Xiao et al. 2006).

Inhibition of Activin/Nodal signaling initiates human trophoblast differentiation

Activin, Nodal and BMPs belong to the TGF- β super-family. They signal through serine/threonine receptor complexes comprised of type I and type II receptors. The type I receptors are phosphorylated by the type II receptors and in turn, activate Smads proteins. Five type II and seven type I receptors (also known as activin receptor-like kinase, ALK) have been characterized in mammals. Activin, Nodal as

well as TGF- β use one set of receptors (ALK4/5/7) and downstream signal molecules (Smad 2/3), while BMPs such as BMP4 utilize a different set of receptors (ALK1/2/3/6) and activate different Smad transducers (Smad1/5/8) (Attisano et al. 2002; Graham and Peng, 2006). Nodal shares the same receptor as Activin, but also requires Cripto (a GPI-AP) as a co-receptor for its signaling though Smad2/3. Activated Smad2/3 (by Activin, Nodal or TGF- β) or Smad1/5/8 (by BMP) competes for the common Smad4 to regulate specific target genes. Therefore, the signaling of Activin/Nodal and BMPs naturally antagonizes each other (Fig. 2).

Activin/Nodal signaling has been shown to play important role in maintaining undifferentiated

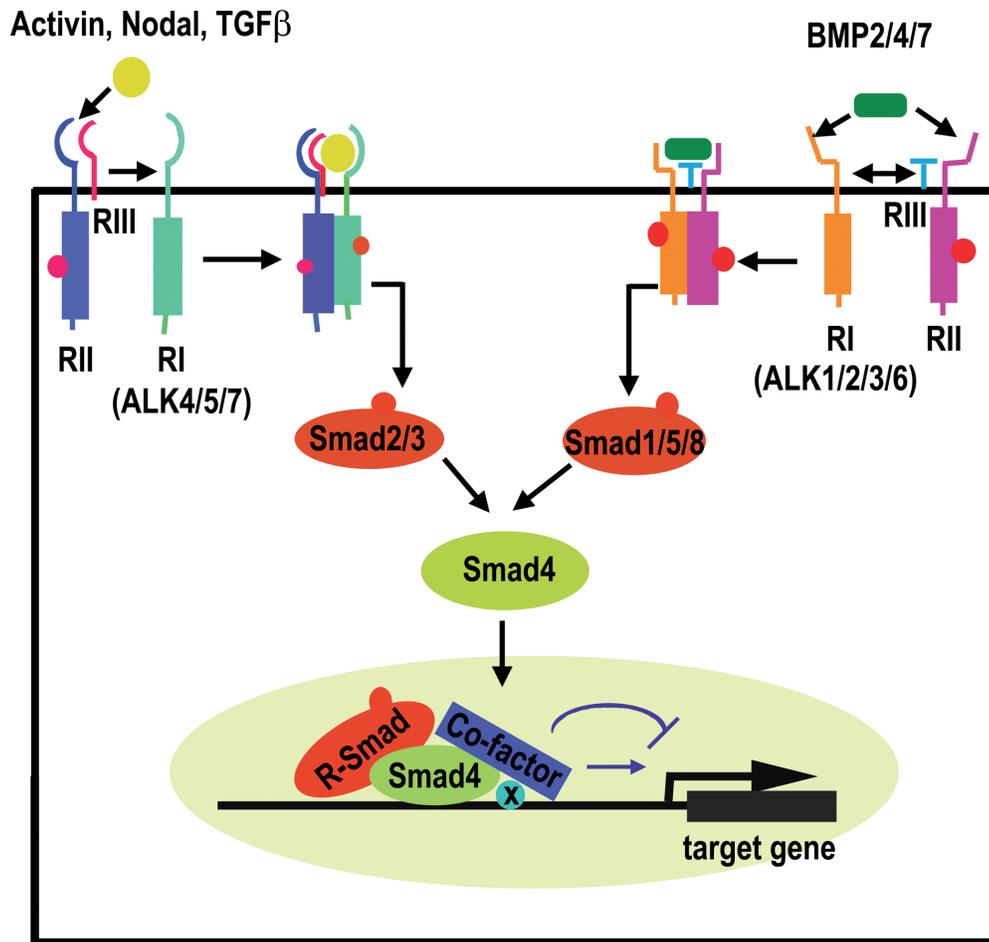


Figure 2. A schematic illustration of key signal transduction pathways of the TGF- β growth factor superfamily that includes Activin, Nodal (left) and BMPs (right). Ligands form complexes with type I and type II receptors (RI and RII) with intrinsic kinase activities. A non-kinase cell surface molecule also involved as a co-receptor (or RIII) to facilitate ligand binding to RII. For example, Cripto is a co-receptor for Nodal, while DRAGON or RGM is a co-receptor for BMP2 and 4. The type I receptors (ALK), upon phosphorylation by their type II receptors, activate receptor-regulated Smads (R-Smads; including Smad2/3 and Smad1/5/8) which interact with a common factor Smad4 to transduce the signals to the nucleus. Within the nucleus, the R-Smad/Smad4 complex interacts with other DNA-binding factors (X) and transcription co-activators or co-repressors (co-factors) to regulate target gene expression. The activated RI by the left or right classes of ligands may have other targets and antagonize each other. See more details in reviews (Attisano L and Wrana JL. 2002. *Science*, 296:1646–7. Graham, H and Peng C. 2006. *Endo. Metab. Immune. Disord. Drug Targets*, 6:45–58).

human ESCs (Beattie et al. 2005; James et al. 2005; Xiao et al. 2006). A very recent study by Xiao and colleagues (Wu et al. 2008) further revealed the crucial virtue of Activin/Nodal signaling in initiating trophoblast differentiation. They demonstrate that inhibition of Activin/Nodal signaling effectively causes the loss of human ESC pluripotency and initiation of trophoblast differentiation, similar to what BMP4 does. However, in a human ESC clone (AR1-c1) that lacks GPI-APs resulting in reduced BMP signaling and lack of Cripto (an GPI-AP serving as a co-receptor for Nodal), inhibition of Activin/Nodal signaling does not give rise to trophoblast differentiation. What's more, BMP4 is sufficient to represses Activin/Nodal signaling, while inhibition of Activin/Nodal causes upregulation of BMP4 expression. The data indicate that Activin/Nodal and BMP form a reciprocal negative feedback loop. This probably explains why exogenous BMP4-induced human ESC differentiation to trophoblasts can be reversed by adding of Activin A. Therefore, both BMP activation and Activin/Nodal inhibition are critical to human ESC differentiation to trophoblasts.

Interestingly, Smith et al. (2008) found that inhibition of the Activin/Nodal signaling in human ESCs (line H9 and hSF-6) led to differentiation toward neuroectoderm. Noticeably, the human ESCs they used were cultured in Chemically Defined Medium (CDM) where BMP signaling is quiescent. In the report of Wu et al. (2008), they did not demonstrate the status of neuroectoderm differentiation after repression of Activin/Nodal signaling. However, the two reports are compatible in showing the indispensability of Activin/Nodal signaling in maintaining pluripotency of human ESC, and indicating the effect of BMP on directing human ESC differentiation. A lately reported work (Xu et al. 2008) provides further mechanistic insights of human ESC fate regulation by the competition of TGF- β /Activin/Nodal and BMP signaling. It is revealed that Smads can bind with the NANOG proximal promoter, and NANOG promoter activity is enhanced by TGF- β /Activin but is decreased by BMP signaling. The data are strongly suggesting the direct involvement of the two related but antagonizing signals by TGF- β /Activin/Nodal and BMP in lineage commitment of human ESCs *in vitro*.

Transcriptional manipulation of human ESCs differentiation to trophoblasts

In addition to NANOG, master transcription factors including OCT4 and SOX2 play indispensable role

in maintaining ESCs pluripotency and preventing differentiation to various committed lineages. Differentiation of ESCs has been accompanied by the diminished expression of these molecules. The possibility to drive mouse ESCs differentiation towards trophoblasts by reducing OCT4 gene expression has been reported by several laboratories (Niwa et al. 2000; Velkey et al. 2003; Hay et al. 2004). Silencing of NANOG or SOX2 gene expression in mouse ESCs has similar effects to induce expression of trophoblast markers (Hough et al. 2006; Ivanova et al. 2006; Loh et al. 2006). In human ESCs, siRNA silencing of OCT4 gene also resulted in trophoblast differentiation (Hay et al. 2004; Matin et al. 2004; Babaie et al. 2007). However, unlike what happened in mouse ESCs, the evident induction of trophoblast markers in human ESCs was dependent on the culture condition, say, culturing the cells in the medium that lacks serum supplement and FGF2 (Hay et al. 2004). Gene expression analysis revealed significant suppression in pluripotency genes including NANOG and SOX2, as well as increase in those which are critical for trophoblast differentiation, such as BMP4, Caudal related transcription factor 2 (CDX2), hCG, EOMES (Babaie et al. 2007). The gene expression profile overlapped with that observed in BMP4-treated human ESCs reported by Xu et al. (2002). Similarly, NANOG silencing in human ESCs led to a transition in cell morphology and up-regulation of trophoblast markers such as CDX2, hCG α , hCG β , and GATA2 (Hyslop et al. 2005).

Generally, the approaches that have been attempted to induce trophoblast differentiation in human ESCs are summarized in Table 1.

Different trophoblast commitment differentiation among species

As a matter of fact, it has been well accepted that there exists dramatic differences in the processes of embryonic development and placentation at the morphological and molecular levels among various species. Thus far the divergent properties that have been found in ESC lines originating from rodents and primates (including human) may reflect their distinct regulatory mechanisms underlying embryonic development.

Distinct differentiation potential of ESCs

to trophoblasts between rodents and primates Studies revealed that human and mouse ESCs differ significantly in several ways, such as certain

Table 1. Summary of the approaches for the differentiation of hESCs into trophoblast lineage.

Approach	Signaling pathways	Cell lines	Yield	References
EB culture		H1 H7, H14 hES3	~70%	Gerami-Naini et al. 2004 Harun et al. 2006 Peiffer et al. 2007
Stimulation with BMP4/ BMP2/BMP7/GDF5	BMP-ALK1/2/3/6- Smad1/5/8	H1, H7, H9, H14 H1 HES-2 and HES-3	uniformly uniformly <5%	Xu et al. 2002 Liu et al. 2004; Ezashi et al. 2005; Chen et al. 2008; Wu et al. 2008 Pera et al. 2004
Inhibition of Activin/ Nodal signaling	Activin/Nodal- ALK4/7-Smad2/3; Forming a reciprocal negative feedback loop with BMP4 signaling	H1, HUES-17	>70%	Wu et al. 2008
siRNA for Oct4	Microarray analysis indicated differential regulation of more than 1000 genes	H1, H9 H7, H14 H1		Hay et al. 2004 Matin et al. 2004 Babaie et al. 2007
siRNA for Nanog		hES-NCL1, H1		Hyslop et al. 2005

genes expression, growth properties, and signaling pathways maintaining self-renewal and regulating differentiation (Ginis et al. 2004). For instance, LIF/STAT3 signaling is critical for mouse ESC self-renewal, but is dispensable for human ESCs to maintain the undifferentiated state (Daheron et al. 2004; Humphrey et al. 2004). BMP4 (in substituting serum factors) together with LIF is sufficient to maintain undifferentiated mouse ESCs, which lack the natural ability to differentiate to trophoblasts without a genetic manipulation.

The major difference between primate and rodent ESCs is their distinct potential to give rise to trophoblast lineages. As mentioned above, human ESCs can differentiate spontaneously to trophoblasts (Xu et al. 2002), and this seems a common cell event in the primates, as reported by Thomson et al. in rhesus monkey and common marmoset (Thomson et al. 1995, 1996). But the phenomenon has not been commonly observed in mouse ESC lines. *In vivo* chimeric experiment demonstrates that mouse ESCs contribute exclusively to embryonic tissues, but rarely to the trophoblast layers of the placenta. Activation of BMP4 signaling and repression of Nodal/Activin signaling largely induces human ESCs differentiation to cells

of trophoblast lineage (Xu et al. 2002; Xiao et al. 2008). Conversely, BMP4 together with LIF supports expansion of undifferentiated mouse ESCs (Qi et al. 2004; Ying et al. 2003). By far the most efficient way to trigger mouse ESCs differentiation to trophoblast-like cells has been the genetic manipulation of some master regulatory factors including OCT4, NANOG, SOX2 or CDX2 (Niwa et al. 2000; Velkey et al. 2003; Hay et al. 2004; Hough et al. 2006; Ivanova et al. 2006; Loh et al. 2006; Niwa et al. 2005).

One possible explanation for such divergent properties of rodent and primate ESCs may be that these cells represent different stages of embryonic development. Studies in mouse embryos indicated that the cell polarization in late morula would determine the eventually differential specification of ICM and trophectoderm cells of the early blastocyst (Kunath et al. 2004). The timing of commitment to ICM or trophectoderm has not been established for any primate species, but it is likely differing among species. The potential of human and non-human primate ESCs to derive trophoblasts indicate that they may derived from an earlier developmental stage than mouse ESCs when the embryonic cells are totipotent, or that the ability of ICM cells to form

trophoblast persists longer in primates. Indeed, comparison of gene expression pattern between mouse and human ESCs has revealed fundamental distinctions apart from simple species differences, indicating that these cell populations might be different classes of ES cells (Sato et al. 2003).

The concept of diverse temporal origins of human and mouse ESCs was strongly supported by the striking work of Brons et al. (2007), but they suggest that the unique properties of human ESCs might reflect a late epiblast origin. They efficiently develop epiblast stem cells (EpiSC) from the late epiblast layer of post-implantation embryos in mouse and rat. Intriguingly, they found that mouse EpiSCs share with human ESCs not only morphology and a strict dependence on Activin/Nodal (but not LIF) signaling to maintain their pluripotent status, but also the potential to differentiate into trophoblast in the presence of BMP4. Therefore, EpiSC might be a valuable model to determine the diverse temporal development between human and mouse ESCs.

Noticeably, a few recent reports are indicating the capability of mouse ESC commitment to trophoblast. In mouse ESC lines D3 and R1, Schenke-Layland et al. (2007) showed that collagen type IV (Col IV) could induce the expression of a panel of trophoblast-restricted markers apart from genes specific to hematopoietic, endothelial and smooth muscle cells. They also observed that differentiation to trophoblastic lineages was only for mESCs maintained on mouse embryonic feeder (MEF) layers that may be providing additional signaling factors. Another interesting observation is that the trophoblast differentiation is CDX2-dependent. This is in concert with the latest study of He et al. (2008) that reveals the potential of Wnt3a, in the absence of LIF, in initiating trophoblast lineage differentiation by triggering CDX2 expression in mouse ESCs. These data indicate that CDX2 induction is the critical cue forcing mouse ESCs to differentiate into trophoblast lineage. Different from Schenke-Layland et al. (2007), He and colleagues maintained the mouse ESCs in feeder-free system, however, they also demonstrated that the Wnt3a-stimulated cells need to be placed in TS medium to provide favorable extracellular conditions for trophoblast derivation. It is therefore believed that mouse ESCs are potent in trophoblast differentiation given the appropriate extracellular milieu.

Trophoblast stem cell lines

Although ESCs can be induced to trophoblasts, the cells are always mixed populations with various

characteristics, or are of limited proliferation. A more reliable model to study the control of placenta development should be trophoblast stem (TS) cells. Evidences also demonstrate great distinctions in TS cell properties among species.

In mouse, TS cell lines have been successfully established from blastocyst or extraembryonic ectoderm (ExE) at embryonic day 6.5. A feeder layer of MEF and supplement of FGF4 and heparin were crucial to keep self-renewal of TS cells, because the removal of FGF4, heparin, or the MEF cells resulted in a rapid decline in proliferation, with a subsequent differentiation into cells with a giant cell-like phenotype. The TS cells contribute to the tissues of the trophoblast lineage including ExE, ectoplacental cone (EPC) and giant cells, but never in the embryonic tissues in chimeras *in vivo* (Tanaka et al. 1998). It has been shown that FGF/MAPK signaling is the most essential for early trophoblast maintenance, and some other transcription factors such as CDX2, Eomes, Sox2, ERR β also play important roles in trophoblast development in mouse (Kunath et al. 2004).

Cells with trophoblast characteristics have been obtained from outgrowths of bovine and porcine blastocysts (Flechon et al. 1995; Talbot et al. 2000; Shimada et al. 2001; Hashizume et al. 2006). Recently, trophoblast stem cell lines were also established directly from rhesus monkey blastocyst outgrowths (Vandevoort et al. 2007). The cells were maintained in human placenta collagen-coated surface for multiple passages in the absence of feeder layers or growth factors. They kept the abilities to form syncytial-like structures during prolonged culturing period, and also exhibited invasive capability similar to extravillous trophoblasts. Of note, these TS cells do not express CDX2, being different from the characteristics of mouse TS cells as well as human ES-derived or EB-derived trophoblasts. Human trophoblast stem cell lines have as yet not been derived using any of the reported strategies from early human embryos. The investigation will remain difficult due to the ethical and practical restrictions. We and others have tried the approach of deriving TS cells by knocking down OCT4 or over-expressing CDX2 in human ESCs. So far we have not been able to establish human TS cell lines that retain the ability to generate trophoblasts but not the derivatives of the three embryonic germ layers (Cheng lab, unpublished results, 2007–2008).

However, over-expression of an inducible version of CDX2 transgene seems to enhance BMP4-induced trophoblast differentiation (unpublished results). From the knowledge of human ESC differentiation, correlated Activin/Nodal and BMP4 signaling might be the essential pathways to initiate the first differentiation event of human embryonic development, namely the trophoblast lineage commitment (Xu et al. 2002; Chen, et al. 2008; Wu et al. 2008). In the human morula, the cells that receive active Activin/Nodal signals may form the inner cell mass; other cells that receive repressed Activin/Nodal signals but active BMP signals may develop into the trophoblast (Wu et al. 2008). However, even BMP4 or antagonist of Activin/Nodal signaling can sufficiently induce human ESC differentiation to trophoblast *in vitro*, the cells propagate poorly and tend to form syncytial cells that represent the terminally differentiated trophoblasts. We noticed that gene expression profile comparison between human ICM and TE cells has shown that several signaling pathways may interact to control the emergence of pluripotent ICM and TE cell lineages from the morula. These include WNT, MAPK, TGF- β , NOTCH, integrin, PI3K, and apoptosis signal molecules (Adjaye et al. 2005). Therefore, the critical factors to maintain the “stemness” of human trophoblast stem cells remain to be further explored.

Future Prospective

By far, there exist some discrepancies in trophoblast commitment among different ESC lines used. ESC lines are derived from blastocyst non-clonally, it is reasonable to hypothesize that ESC culture is a heterogeneous population of cells with various characteristics and potential (Allegrucci et al. 2007). Therefore, the intrinsic properties of different ESC lines may determine their divergent responses to experimental conditions. More investigations are yet to be carried out to identify ideal lines which exhibit marked propensities to differentiate into specific trophoblast lineages.

With the advent of other methods to generate pluripotent or even totipotent primate stem cells by techniques such as somatic cell nuclear transfer, parthenogenesis and direct reprogramming by genetic manipulation, strategies using primate pluripotent stem cell lines as starting materials will be more expanded and more powerful. It is of interest to determine whether these stem cell lines

can form trophoblast-derived cells as the conventional human ESCs do. Our preliminary results with induced pluripotent stem (iPS) cells suggest it is the case (Mali et al. 2008). In the future, it is of value to learn more on the mechanisms beyond the transformation of the somatic cells to pluripotent cells and their potential to differentiate to trophoblast phenotypes. Consequently, it will provide a new way to identify key factors directing trophoblast commitment and placenta development by using disease-specific iPS system. However, to what extent the *in vitro* study can represent the physiological condition *in vivo* remains to be evaluated, and non-human primate may provide a critical bridge between *in vivo* and *in vitro* studies in human. Undoubtedly, human as well as non-human primate ESCs, iPS and/or human TS cell lines will provide unprecedented and much-needed research tools for exploring the development and function of human placenta in normal and pathological processes.

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Disclosure

The authors report no conflicts of interest.

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