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Embryonic stem cell-derived trophoblast differentiation: A comparative review of the biology, function, and signaling mechanisms

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Abstract

The development of the placenta is imperative for successful pregnancy establishment, yet the earliest differentiation events of the blastocyst-derived trophectoderm that forms the placenta remains difficult to study in humans. Human embryonic stem cells (hESC) display a unique ability to form trophoblast cells when induced to differentiate either by the addition of exogenous BMP4 or by the formation of cellular aggregates called embryoid bodies (EBs). While mouse trophoblast stem cells have been isolated from blastocyst outgrowths, mouse ESC do not spontaneously differentiate into trophoblast cells

In this review, we focus on addressing the similarities and differences between mouse trophoblast stem cell differentiation and human ESC-derived trophoblast differentiation. We discuss the functional and mechanistic diversity that is found in different species models. Of central importance are the unique signaling events that trigger downstream gene expression that create specific cellular fate decisions. We support the idea that we must understand the nuances that hESC differentiation models display so that investigators can choose the appropriate model system to fit experimental needs.

Introduction

Theories of embryological development date back to Aritstotle's time (382-322 B.C.) with the concept of epigenesis, where it was thought that the embryo developed from an amorphous mass derived from the mother. Aristotle believed that the male contribution of sperm was what gave the soul to this mass and helped guide development (Aristotle, translated by Peck 1943). Other early thinkers believed in the preformationist theory where a mini-individual (homunculus) existed within the germ cell and initiated embryonic development (Magner, 2002). While current knowledge has advanced beyond these early hypotheses, a deeper understanding of the events in early embryogenesis and the key regulators involved in the establishment of a healthy pregnancy remains a goal only incompletely realized. Early pregnancy loss is thought to occur in 10- 25% of all clinically recognized pregnancies, and preeclampsia and other hypertensive disorders that can be linked to placental biology affect 5-8% of pregnancies in the US (http://

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Declaration of interest:

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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www.americanpregnancy.org/pregnancycomplications/miscarriage.html/; http://www.preeclampsia.org/health-information/faq). Thus, the basic developmental mechanisms that direct placentation are of high clinical relevance.

The first differentiation event in the preimplantation mammalian embryo is the formation of the trophectoderm that will contribute the trophoblast compartment of the placenta. The responsibilities of the trophoblasts include signaling the presence of the conceptus to the maternal reproductive and immune systems, and acquiring the vital nutrition necessary for fetal growth during pregnancy. Since placentation is the earliest morphogenetic event in pregnancy, animal models and embryos have contributed significantly to studies of placental development, with mouse trophoblast stem cells providing an important research tool while a fully equivalent cell line has not been isolated in primates. The isolation of human embryonic stem cells (hESC) from blastocyst stage embryos has provided a unique and powerful embryonic surrogate to begin understanding human development, and overcoming the obvious ethical limitations of working with human embryos (Thomson, et al. 1998). These hESC have been used to identify approaches that induce trophoblast differentiation, aimed to provide an understanding of the mechanisms, which support a commitment to the trophoblast lineage in embryonic development. Herein we will review the similarities and differences, where known, in mouse and human trophoblast differentiation and placental development. The differentiation of trophoblast cells from human embryonic stem cells will be highlighted on a functional and mechanistic level, presenting current thinking on the signaling events necessary to achieve trophoblast differentiation.

Trophoblast Development

Mouse placental development

During the initial stages of placental development, both mouse and human pregnancy presents a deep interstitial implantation, and the development of a hemochorial placenta where the trophoblasts are in direct contact with the maternal blood (Pijnenborg, et al. 1981). Although both are hemochorial, organization that allows the placental trophoblast to interface with maternal blood differs between the two. In the mouse, the fetal blood vessels within the placenta are interconnected to form complex capillary networks among which maternal blood vessels intertwine and thus form a placental labyrinthe (Cross 2005; Rossant and Cross 2001). The trophoblasts line channels through which the maternal blood circulates within the labyrinth, forming the exchange surface between fetal and maternal blood. In distinction, in the human (as well as in old world nonhuman primates), a villous placenta forms in which the trophoblasts develop villi that arborize into terminal branches that have few interconnections (Kingdom, et al. 2000). Within these villi, the fetal vasculature develops, and since the villi have a trophoblast surface and display extensive branching, a large surface area is created for gas and nutrient exchange between the mother and fetus. Thus, the organization of the maternal-fetal exchange surface is distinct between these two placentas.

Differences between the human and mouse placenta can also be seen in the morphology and phenotype of the trophoblasts that arise during development. In the mouse, at the time of implantation, the trophectoderm cells that lie away from the inner cell mass (ICM) halt division but undergo endoreduplication, thus forming the trophoblast giant cells. These cells eventually form the outer regions of the ectoplacental cone surrounding the conceptus (Cross 2005; Rossant and Cross 2001). The ectoplacental cone is also composed of diploid trophoblast cells that give rise to the spongiotrophoblast that forms the outer structural layer of the placenta (Cross 2005; Rossant and Cross 2001). The syncytiotrophoblasts within the mouse placenta are multinucleate cells that lie within the labyrinthe and are the direct interface for gas and nutrient exchange between the maternal and fetal vasculatures.

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Both the trophoblast giant cells and the spongiotrophoblast secrete many factors that support the establishment and maintenance of pregnancy. These factors include hormones, angiogenic and tissue remodeling factors like placental lactogens, proliferin, vascular endothelial growth factor (VEGF), matrix metalloproteinases, and urokinase type plasminogen activator (uPA) (Achen, et al. 1997; Cross 2005; Groskopf, et al. 1997; Rossant and Cross 2001; Soares, et al. 1996; Teesalu, et al. 1998; Teesalu, et al. 1999; Vuorela, et al. 1997). We will not discuss mouse placental physiology in detail here, and the reader is referred to other excellent reviews for further detailed discussion (Cross 2005; Rossant and Cross 2001).

Human placental development

As with the mouse blastocyst, the human blastocyst upon apposition and adhesion to the uterine luminal epithelium rapidly penetrates to the endometrial stroma, where the formation of a multinuclear syncytium, and proliferating cytotrophoblasts advance embryonic remodeling of the superficial endometrium to become surrounded by maternal tissues (Carter and Pijnenborg 2011). As the human placenta continues its development, the cytotrophoblasts are the main proliferating trophoblasts that give rise to the cytotrophoblast columns. The cytotrophoblasts fuse to form the syncytiotrophoblasts that cover the branch-like protrusions (villi) that erupt from the cell columns. The syncytiotrophoblasts is the primary endocrine and transport interface, directly exposed to maternal blood in the intervillous space that forms from the erosion of maternal decidual vessels during implantation and early placental growth (Benirschke, et al. 2006). Growth factors such as vascular endothelial growth factor (VEGF), angiopoietins, angiostatins, and placental growth factor (PlGF) act within the human villi to control placental villous vasculogenesis and angiogenesis (Kingdom et al. 2000). Also arising from the cytotrophoblasts in the human placenta are the extravillous cytotrophoblasts that leave the cell column and migrate into the maternal decidua. Further discussion of these cells appears below.

Human and mouse placentation: remodeling of maternal tissues

A notable similarity between mouse and human placental development is the migration of the trophoblastic cells into the maternal decidua and their remodeling of the spiral arterioles (Pijnenborg et al. 1981). In the mouse, the trophoblast giant cells invade the maternal arterioles during the early postimplantation period, followed by the glycogen trophoblasts that carry out later interstitial migration and invasion resulting in dilated arteries that lack elastic lamina and smooth muscle cells but are lined with trophoblasts that form canals that carry maternal blood to the base of the placenta (Adamson, et al. 2002; Cross 2005). The maternal blood then percolates back through the labyrinthe space providing nutrients to fetus via the syncytiotrophoblast transport (Adamson et al. 2002; Cross 2005). In the human, the extravillous trophoblasts reside in the trophoblast cell columns and undergo an epithelial-mesenchymal transition, where they migrate, invade, and remodel the maternal spiral arterioles. More specifically, the interstitial extravillous trophoblasts will migrate into the stroma of the maternal decidua and come into close contact with uterine immune cells that aid in vessel remodeling (Kaufmann, et al. 2003). The endovascular extravillous trophoblasts will appear within the lumen of maternal vessels but there is still controversy on the mode of invasion of the extravillous trophoblasts in the human placenta (Kaufmann et al. 2003). One possibility is that trophoblasts from the decidua come into close contact with the outside of the vessels (intravasation), remove the smooth muscle, and eventually line the vessels.

Alternatively, trophoblasts presumably enter vessels proximal to the placenta, arrive in the lumen, move opposite to the maternal blood flow, and proceed to remodel and line the maternal vessels (extravasation) (Kaufmann et al. 2003; Pijnenborg, et al. 2006). Regardless

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of the pathway that they take, a significant outcome of the initial invasion in the human is to create vascular plugs in the maternal vessels thereby decreasing the arterial pressure towards the implantation site (Hamilton and Boyd 1966). The extended remodeling creates dilated maternal vessels resulting in a low pressure, high output environment providing ample nutrition to the developing fetus (Kingdom et al. 2000; Pijnenborg, et al. 1980; Pijnenborg, et al. 1982). Much work still remains to uncover the details regarding trophoblast differentiation and function during formation of the placental-decidual interface, therefore the development of tractable model systems is imperative for the field.

Trophoblast Lineage and Stem Cell Differentiation

Trophoblast stem cells (TSC) were first isolated from the polar trophectoderm and from the extraembryonic ectoderm of mouse blastocysts (Tanaka, et al. 1998). Maintenance of the undifferentiated state and self-renewal in mouse TSC are controlled by a combination of fibroblast growth factor 4 (FGF4), heparin, and secreted factors from fetal fibroblasts (Rielland, et al. 2008; Tanaka et al. 1998). Activin A or transforming growth factor beta1 (TGFB1) can replace conditioned medium from fetal fibroblasts to maintain mouse TSC (Erlebacher, et al. 2004; Roberts and Fisher 2011). It was subsequently demonstrated that TSC could also be directly derived from mouse embryonic stem cells through repression of the Pit1-Octamer-Unc86 (POU) transcription factor *Oct4* and in the presence of FGF4 and feeder cells (Niwa, et al. 2000).

Transcriptional Control

Mouse molecular genetics has provided extensive information regarding the molecular mechanisms that drive differentiation of trophoblast stem cells and placental development. Of central importance are the genes involved in trophoblast lineage development. Table 1 lists the factors discussed in this review, and gives a brief description of the significance of the genes discussed in more detail below. Although the caudal-type homeobox gene *Cdx2* has been classified as a key regulator in determining trophoblast fate, it is itself regulated by the transcription factor *Tead4. Tead4* mutants are capable of producing embryonic stem cells, but fail to produce the trophectoderm lineage or TSC (Yagi, et al. 2007). Moreover, disruption of the *Tead4* gene after implantation results in viable offspring and conversely, over-expression of *Tead4* results in TSC-like cell lines, pinpointing the necessity of *Tead4* expression for trophoblast lineage commitment in the developing embryo (Nishioka, et al. 2009; Senner and Hemberger 2010; Yagi et al. 2007). Interestingly, Hippo signaling that is associated with the epithelial-to-mesenchymal transition in mammals is also linked to TEAD4 transcriptional activity. Inactive Hippo signaling in the outside cells of the developing embryo allows the Hippo co-activator Yap to remain unphosphorylated and thus translocate to the nucleus where it assists in *Tead4* activation (Figure 1A) (Nishioka et al. 2009; Senner and Hemberger 2010). Recent studies have shown that in an evolutionarily conserved mechanism, the nuclear localization of *Tead4* in the outer blastomeres of the embryo leads to a trophoblast-specific transcriptional program that is selectively impaired in the inner cells of the embryo and thus allows the inner cells to become the ICM (Figure 1A) (Home, et al. 2012).

Paracrine signaling initiated by factors including FGF and EGF from the mouse ICM is also necessary to support trophoblast and TSC self-renewal. During later developmental stages, the Smad4 inhibitor, ectodermin (*Trim*) determines the appropriate amount of *Nodal* activity derived from the epiblast, thus titrating the balance between the trophoblast undifferentiated and differentiated state (Morsut, et al. 2010; Roberts and Fisher 2011). FGF4-stimulated expression of *Cdx2* in TSC, in turn lead to *Cdx2* binding to an FGF4-responsive enhancer element in the promoter region of BMP4 that results in growth factors that maintain the ICM (Murohashi, et al. 2010; Roberts and Fisher 2011).

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Once induction of the trophoblast fate has been initiated, *Cdx2* maintains trophoblast/TSC function (Figure 1B). *Tead4* also regulates the transcription factor *Gata3* to induce trophoblast differentiation from ESC but *Gata3* functions independently of *Cdx2* (Ralston, et al. 2010). Interestingly, transcription factor binding sites for *Tcfap2* found in the mouse that mediate *Cdx2*-independent repression of the pluripotency marker *Oct4* are not found in humans and cattle, suggesting a crucial difference between the molecular cues that initiate trophoblast development in different species (Berg, et al. 2011; James, et al. 2012). The homeobox transcription factor Eomesodermin (*Eomes*) is expressed after blastocyst development in response to *Cdx2* expression but also enhances *Cdx2* and aids in extraembryonic ectoderm expansion (Rossant and Cross 2001; Russ, et al. 2000; Senner and Hemberger 2010). Similarly, the nuclear hormone receptor *Esrrb*, and the transcription factors *Ets2, Tcfap2c,* and *Elf5* all enhance *Cdx2* expression, and direct extraembryonic ectoderm expansion. *Essrb* deletion results in trophoblast defects where TSC isolation is impaired (Chawengsaksophak, et al. 1997; Donnison, et al. 2005; Luo, et al. 1997; Rossant and Cross 2001; Russ et al. 2000; Senner and Hemberger 2010; Werling and Schorle 2002; Yamamoto, et al. 1998). More specifically, trophoblasts can be induced by over-expression of *Cdx2* or by *Tcfap2c* in ESC independently whereas *Elf5* activation requires *Cdx2* and *Tcfap2c* co-expression in order to derive trophoblasts from ESC (Kuckenberg, et al. 2010; Senner and Hemberger 2010). Mutations in the genes that directly support the differentiation of the trophoblast giant cells (*Hand1* and *Mdfi*) and the maintenance of the spongiotrophoblast (*Mash2* and *Egfr*) also result in impaired development of each respective trophoblast subtype thus illustrating coordinated regulation necessary for trophoblast differentiation (Guillemot, et al. 1994; Kraut, et al. 1998; Riley, et al. 1998; Rossant and Cross 2001; Sibilia and Wagner 1995; Threadgill, et al. 1995). Similar to the mouse spongiotrophoblasts that offer structural support to the placenta, the human cytotrophoblasts offer support and express the basic helix-loop-helix (bHLH) transcription factor *HASH2*, a human homologue of the murine *Ascl2* (*Mash2*) (Benirschke et al. 2006; Hamilton and Boyd 1960; Janatpour, et al. 1999; Roberts, et al. 2004). The syncytiotrophoblasts express the transcription factor glial cell missing 1 (*GCM1*) as do their functional counterpart in the mouse labyrinth, also termed syncytioptrophoblasts (Anson-Cartwright, et al. 2000; Janatpour et al. 1999; Roberts et al. 2004).

Epigenetic regulation of trophoblast lineage-specific transcription factors

Recently, studies on the epigenetic regulation of lineage-specific transcription factors in mouse have highlighted methylation patterns associated with expressing/repressing embryo-specific genes in the trophoblast lineage. More specifically, the trivalent histone/lysine footprint H3K9me3, H3K4me2/3, and H3K27me3 or the bivalent H3K4me3 and H3K9me3 footprint have been shown to silence embryonic genes in cells developing into trophoblasts (Azuara, et al. 2006; Bernstein, et al. 2006; Boyer, et al. 2006; Senner and Hemberger 2010). Conversely, the methyltransferase *Eset* is recruited by *Oct4* to silence trophoblast genes via histone H3K9 methylation in the ICM and ESC in a selective way that is still not well understood but may involve SUMOylation of *Oct4* (Senner and Hemberger 2010; Yeap, et al. 2009). At center stage of epigenetic regulation in regards to trophoblast development is the transcription factor *Elf5. Elf5* seems to be a key regulator in cell fate decisions for trophoblast development in the embryo by maintaining *Cdx2* and *Eomes* gene expression (Donnison et al. 2005; Ng, et al. 2008). *Elf5* is hypomethylated and therefore expressed in TSC, but methylated, and therefore silenced, in ESC (Ng et al. 2008). Subsequent studies have identified a “TSC-like” compartment in the villous cytotrophoblasts of the human placenta where *ELF5*$^{+}$/*CDX2*$^{+}$ cells reside (Hemberger, et al. 2010). In addition, these authors determined that the human ESC lines that they derived expressed negligible amounts of *ELF5* compared to trophoblast cell lines and an 8-week human placental sample, and moreover, the ESC-derived trophoblasts did not express *ELF5*

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at all leading to the conclusion that hESC-derived cells should not be considered to be authentic trophoblasts (Hemberger et al. 2010).

These results may depend on the ESC-derived trophoblast preparations used for study. The trophoblast cell preparations used were established cultures having undergone selective expansion based on hCG expression following embryoid body (EB) formation (Hemberger et al. 2010). However, using the H1 ESC line, we have recently found *ELF5* mRNA differentially expressed through the initial 1-4 weeks during trophoblast outgrowth culture derived from EBs of various sizes and (Gerami-Naini, Giakoumopoulos, et al., in preparation). This underscores that trophoblast derivation methods might result in the formation of different trophoblast subtypes depending on the state of the parental hESC line, and the differentiation paradigm employed. It is clear that heterogeneity of hESC can profoundly influence trophoblast differentiation potential (Pera, et al. 2004; Xu, et al. 2002). Thus, as discussed further below, the field will benefit from complementary approaches being taken to define models for trophoblast differentiation during embryonic development.

Cell Signaling and Trophoblast Differentiation

Mouse, Human, and Non-Human Primates TSC

TSC have offered many insights into mouse placental development but recapitulating the characteristics of their mouse counterparts have yet to be isolated and established from many other relevant species. Rhesus macaque and human TSC-like lines have been established but do not express the same transcriptional repertoire found in the mouse-derived TSC (Douglas, et al. 2009; Harun, et al. 2006; Vandevoort, et al. 2007). The rhesus-derived lines lack expression of *CDX2* but express many trophoblast markers such as chorionic gonadotrophin, *CD9, KRT7, POU5F1*, and *EOMES* (Kamei, et al. 2002; Roberts and Fisher 2011; Vandevoort et al. 2007). Also, of central importance is that although isolated by blastocyst outgrowth similarly to mouse TSC, the rhesus cell lines are able to proliferate in the absence of feeder layers or bFGF (Roberts and Fisher 2011; Vandevoort et al. 2007).

In the human study, EBs were generated from ESC and multiple rounds of cellular aggregation and disaggregation were used in combination with human chorionic gonadotrophin (hCG) as a marker, to isolate their TSC lines (Harun et al. 2006). Similarly to the mouse, human TSC lines were maintained in “TSC” medium as described by Tanaka et al. 1998 in the presence of FGF4 thus variations exist in line derivation and maintenance that make comparisons challenging between mice and primates (Harun et al. 2006). The human-derived lines express *CDX2, HLAG, CD9* and *KRT7* but do not express *EOMES* and after some time in culture, these lines fuse to form syncytium (Harun et al. 2006). More recently, a trophoblast progenitor cell niche has been identified from the chorion of the human placenta providing yet another renewable source of multipotent cells reported to be capable of differentiating into all three trophoblast subtypes (Genbacev, et al. 2011). That these human and other cell lines express differentiation markers such as hCG suggests that they are more highly differentiated than mouse TSC, which lack expression of endocrine markers such as mouse placental lactogens (Douglas et al. 2009; Tanaka et al. 1998).

The naïve vs. primed cellular state

Species differences during cell fate decisions may be due to the signaling differences that dictate the potency of the cells within their cellular compartments *in vivo*. These differences are apparent when comparing human ESC (hESC) to mouse ESC (mESC) and mouse epiblast stem cells (mEpiSC). Mouse EpiSC can be isolated from both pre- and post-implantation embryos and it has been established that hESC have similar gene expression and cell signaling profiles to mEpiSC compared to mESC, suggesting that hESC represent a more advanced embryonic stage than mESC (Najm, et al. 2011; Tesar, et al. 2007). Since a

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given ESC line provides a “snapshot” in time of a transient developmental state defining the expression and functional profiles associated with each line are critical. These differences may illuminate the proposed “naïve” vs. “primed” pluripotent state in which established stem cell lines exist *in vitro* (De Los Angeles, et al. 2012; Nichols and Smith 2009). Mouse ESC derived from female embryos exist in a naïve pluripotent state consisting of two active X chromosomes; the expression of pluripotency markers *Oct4, Sox2*, and *Nanog*; and are able to form chimeras that result in germline transmission (De Los Angeles et al. 2012). Human ESC and mEpiSC exist in a primed state *in vitro*, where cells display one active and one inactive X chromosome, express the above mentioned pluripotency markers, but are not able to form chimeras or incorporate into the germline (De Los Angeles et al. 2012). Although differences do exist, important similarities are also found between hESC and mESC. Briefly, hESC and mESC both 1.) express the ICM marker *REX1*; 2.) do not express the epiblast marker *FGF5*; and 3.) express *KLF4*, which is also used to reprogram somatic cells into an ESC-like state (Adjaye, et al. 2005; Darr, et al. 2006; Greber, et al. 2010; Pelton, et al. 2002). Interestingly, *KLF4* can also be used to revert primed mEpiSC into an ESC-like naïve state (Guo, et al. 2009; Takahashi, et al. 2007; Yu, et al. 2007), similar to the reprogramming of “terminally-differentiated” somatic cells such as fibroblasts to induced pluripotent stem cells (iPSC) (Yu, et al. 2007; Takahashi, et al. 2007). Interestingly, a recent study has shown that derivation of hESC from a female embryo in 5% oxygen compared to 20% oxygen results in ESC with two active X chromosomes, therefore the potential to obtain developmental equivalents to the naïve pluripotent state found in mESC exists (De Los Angeles et al. 2012; Lengner, et al. 2010).

FGF at the top of the signaling cascade

Of central importance in providing the signaling cues that dictate mESC, mEpiSC, and hESC self-renewal and differentiation are fibroblast growth factors 2 and 4 (FGF2,-4). In the mouse, Leukemia Inhibitory Factor (LIF) is required for ESC self-renewal and FGF/ERK signaling drives differentiation of ESC (Burdon, et al. 1999). When inhibitors block FGF receptor tyrosine kinases, Map Kinase ERK Kinase (MEK), and Glycogen Synthase Kinase (GSK) signaling (termed 3i), or MEK and GSK alone (termed 2i), naïve pluripotent cells can be derived from mouse embryos (Nichols and Smith 2009; Silva and Smith 2008; Ying, et al. 2008). Conversely, hESC require FGF2/ERK to self-renew and can be induced to differentiate by the canonical WNT/beta-catenin signaling pathway (Sumi, et al. 2008). Activin/Nodal signaling also contribute to hESC self-renewal, and BMP-induced trophoblast differentiation is dependent on inhibition of Activin/Nodal signaling (Wu, et al. 2008). Transforming Growth Factor beta (TGFb) and FGF2 work together to sustain the hESC in an undifferentiated state by maintaining *NANOG, OCT4*, and *SOX2* in addition to inhibiting BMP signaling expression (Xu, et al. 2008). More specifically, TGFb signaling through SMAD 2/3 promotes enhanced *NANOG* expression in hESC thus maintaining an undifferentiated state whereas TGFb signaling through SMAD 1/5/8, which is generally found to be repressed in undifferentiated hESC, results in decreased *NANOG* expression leading to ESC differentiation (Xu et al. 2008). In addition, it has been shown that ERK2 phosphorylates *OCT4* at multiple sites outside the *OCT4* DNA binding domains, possibly due to FGF2 acting directly on *OCT4* thus resulting in self-renewal of hESC (Brumbaugh, et al. 2012).

Similarly to hESC, culturing mEpiSC in the presence of BMP and Activin A will also maintain an undifferentiated cellular state, but FGF signaling appears to play different roles in each cell type. In mEpiSC, FGF2 stabilizes the epiblast state by inhibiting differentiation to neuroectoderm and inhibiting the reversion to a mESC-like state but in hESC, FGF synergizes with SMAD 2/3 signaling resulting in *NANOG* gene expression and maintenance of self-renewal (Greber et al. 2010). Thus, each cell line and the subsequent data generated

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from each cell line provide unique and specific results to the individual line. Moreover, caution should be taken when directly translating results from one species to another.

BMP-induced differentiation

One of the early breakthroughs in the use of hESC to model trophoblast differentiation was the discovery that treatment of hESC with BMP4 or other similar ligands (BMP2, BMP7, GDF5) induced uniform trophoblast differentiation, in a time- and dose-dependent manner (Xu et al. 2002). Since this seminal observation, the role of BMP also as a regulator of somatic and extraembryonic lineages becomes more apparent (Greber 2011; Xu et al. 2002). On the other hand, Pera, et al., (2004) found that BMP2 resulted in cells displaying extra-embryonic endoderm characteristics and further showed that treatment of hESC with BMP-antagonist noggin resulted in neural precursors (Pera et al. 2004). In addition, along with WNT/b-catenin signaling, BMP establishes posterior primitive streak and mesoderm progenitors from hESC, but delaying BMP signals results in the differentiation of anterior primitive streak and endoderm progenitors (Sumi et al. 2008). Yu, et al., (2011) showed that in the presence of high concentrations of FGF2, BMP4-induced differentiation of hESC results in mesendoderm (an epiblast-derived progenitor of mesoderm or endoderm) rather than extraembryonic trophoblast differentiation (Yu, et al. 2011). More specifically, it was shown that FGF2, acting through MEK/ERK signaling and in the presence of BMP4, results in the prolonged expression of *NANOG* that results in FGF-independent, BMP4-induction of mesendoderm (Greber 2011; Yu et al. 2011). Complementary to this study, it has also been shown that the induction of mesoderm from BMP4/FGF2-treated hESC also involves the well-established extraembryonic marker *CDX2* and that the mesoderm marker Brachyury (*T*) precedes *CDX2* induction (Bernardo, et al. 2011; Greber 2011). These studies have prompted the conclusion that the cells differentiated in the presence of BMP4 are not trophoblasts but indeed cells of the extraembryonic mesoderm (Bernardo et al. 2011; Greber 2011; Yu et al. 2011).

This conclusion has been challenged by others, who suggest that the choice of using *T* as a marker for mesoderm may not be valid because *T* expression has been found in teratocarcinoma cells and in trophoblast cell lines (Ezashi, et al. 2012; Gokhale, et al. 2000). In addition, Ezashi, et al., (2012) have reported that the gene expression profiles indicating embryonic and extra-embryonic endoderm (ie. *FLK1, VCAM1*, and *TBX4*) are expressed differentially between the H9 hESC line used by Bernardo, et al., 2011 and the H1 ESC line that they use before and after BMP4-induced differentiation (Ezashi et al. 2012). Moreover, the *ELF5* gene was found to be inactive by methylation status in the BMP4-treated cells by Bernardo, et al., (2011) in opposition to what is seen in mTSC, suggesting that trophoblast was not being faithfully differentiated. However, a small subset of the cells actually expressed the ELF5 protein but the methylation status of the *ELF5* promoter was not determined therefore indicating a potential early differentiation state of a trophoblast subpopulation (Ezashi et al. 2012). Finally, Bernardo, et al., (2011) did not detect the non-polymorphic surface class I molecule, *HLA-G* on BMP-derived trophoblasts, which is most often used as a placental marker. In contrast, the H1 hESC line was reported to express HLA-G mRNA when induced to form trophoblasts with BMP4 at even low doses of 10 ng/mL (Das, et al. 2007; Ezashi et al. 2012; Xu et al. 2002). Thus, it remains controversial as to whether BMP4-treated hESC truly differentiate into trophoblast cells.

A recent study helping to clarify the differences in gene expression that may arise when ESC cells are primed for differentiation has defined putative progenitor signatures for mesoderm, vascular endothelium, and trophoblast from hESC (Drukker, et al. 2012). Interestingly, when hESC were treated with 100 ng/mL of BMP4 for three days, screened with a monoclonal antibody library using flow cytometry, and subsequently FACS sorted, three distinct progenitor populations were derived (Drukker et al. 2012). Global gene expression

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analysis indicated trophoblast-specific gene induction, and cell cultures of these progenitors were able to form syncytium. They did not form teratomas when engrafted into immunodeficient mice, but rather formed mesenchymal tissues and epithelial structures (Drukker et al. 2012). Therefore the point is made that BMP4 differentiation induction may be bi-directional and that potentially a small subset of cells truly undergo differentiation toward the trophoblast lineage and are not an artifact of differentiation toward the mesoderm lineage (Figure 2).

Moreover, comparative transcriptome analysis of hESC-derived trophoblasts by BMP4 treatment and mural trophectoderm cells isolated from human blastocysts revealed 138 genes in common between the groups with similarities in major canonical pathways and proteins secreted that are involved in the implantation process (Aghajanova, et al. 2012). Additionally, it was determined that trophoblast cells derived on days 8, 10, and 12 of BMP4 treatment were more consistent on a transcriptional level to trophectoderm cells than trophoblasts derived on days 0, 2, 4, and 6 of BMP4 treatment thus further supporting BMP4 treatment of hESC as a viable model to study trophoblast differentiation and development (Aghajanova et al. 2012).

The embryoid-body model for trophoblast differentiation

An alternative approach for the formation of trophoblasts from hESC was established which entails forming suspension cultures of aggregates or EBs from undifferentiated hESC (Gerami-Naini, et al. 2004). This system has proven amenable for studies investigating the spatial interactions between cells and the surrounding extracellular matrix (Gerami-Naini et al. 2004; Giakoumopoulos, et al. 2010). Differentiation in this paradigm is achieved by EB formation, the simultaneous withdrawal of FGF2 from the culture medium, and the addition of fetal bovine serum (FBS). When EBs were maintained in suspension culture hCG, progesterone, and estradiol-17b secretion was initiated compared to unconditioned culture medium alone (Gerami-Naini et al. 2004). Adherent cultures of these EBs led to outgrowths of cells with epithelial morphology that maintained hCG, progesterone, and steroid hormone secretion (Figure 3). Moreover, when these suspended EBs were in 3-dimensional culture with Matrigel "rafts", cellular protrusions appeared and placental hormone secretion was significantly higher compared to EBs maintained in suspension culture or cells in 2-dimensional adherent culture (Gerami-Naini et al. 2004).

We have attempted to elucidate the specific extracellular matrix component providing the cues to support EB-derived trophoblast hormone secretion by allowing EBs to adhere to various extracellular matrices in 2-dimensions. Regardless of the matrix utilized, hormone secretion was similar under all conditions, suggesting that adhesion per se, rather than a specific interaction, is adequate for maintaining hormone secretion (Gerami-Naini, et al., in preparation). EB-derived trophoblast outgrowths are able to display migratory characteristics, as is seen in extravillous cytotrophoblasts, when placed in a migration chamber and display enhanced migration in the presence of secreted factors from endothelial cells (Golos, et al. 2010). In addition, incorporating placental fibroblasts into EBs with an aggregation protocol followed by suspension culture provides cellular cues that enhanced placental hormone secretion (Giakoumopoulos et al. 2010). Moreover, Harun, et al., 2006 utilized the EB model system to derive their TSC lines (Harun et al. 2006). Thus, the EB system has provided a useful and adaptable model for trophoblast derivation.

Recent efforts in our lab have been to utilize a system that aggregates EBs of uniform size and shape thus alleviating some of the heterogeneity that results from traditional methods where EBs were formed from undifferentiated colonies of heterogeneous size by light enzymatic digestion. Improving the consistency among EBs will be a valuable refinement of the alternative to BMP4 treatment for trophoblast differentiation.

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One of the more problematic issues in the use of hESC to derive trophoblasts remains the uncertainty as to the heterogeneity of the "trophoblast" population obtained. The outgrowths that result from adherent EB culture (Figure 3), display morphologically distinct cells as cells grow away from the EB "core". Similar heterogeneity of differentiation was demonstrated by Ezashi, et al., (2012) and spatial heterogeneity in functional individual markers such as hCG expression was dependent on cellular location within the hESC colony when differentiation was induced by BMP4 treatment.

Thus, an important goal for refining the use of hESC models for the formation of trophoblasts is a better definition of cell heterogeneity, and the formulation of approaches to achieve more highly purified populations of cells. This quest to isolate a pure differentiated population is ongoing and Drukker, et al., (2012) clearly stated the necessity to do so by saying, "Without purifying true pluripotent cells and differentiating cells, it is difficult to determine, for example, whether low mRNA levels of differentiating genes detected in cultures of mESCs (or hESCs) reflect priming of lineage specification programs in undifferentiated cells or the presence of small populations of differentiating cells." By using selective surface markers for subpopulation isolation, highly purified differentiated cells can be obtained and thus genomic/proteomic libraries can be created for universal reference before embarking on a new experimental protocol or manipulation.

Afterword

Extrapolating in vitro studies with hESC to the in vivo process of embryo implantation and placenta formation is a major challenge for the field. One important consideration is that the hESC lines that are used may actually give different results due to differences in isolation methods, passage number, maintenance of pluripotency, culture density, and specific culture conditions, such as extracellular matrix or soluble factors used for differentiation induction. Thus, standardized protocols and careful descriptions of cellular detail is important. In addition, emerging evidence indicates that the time course of differentiation is crucial and current studies suggest that BMP4-induced differentiation of hESC results in differential lineage induction at different time points and under different culture conditions (e.g., in the presence of FGF2). Finally, careful selection of markers used for lineage identification, and attention to the specificity of the expression profile generated by spatial and temporal expression patterns produced *in vivo* is a critical area for further study. It is hoped that in the future, defining the signaling nuances that exist between the pluripotent, primed, and differentiated state of ESC, will lead toward the development of robust protocols for human TSC derivation that will aid in understanding human embryo implantation and subsequent development of the placenta, the organ that, is a prerequisite of our own existence.

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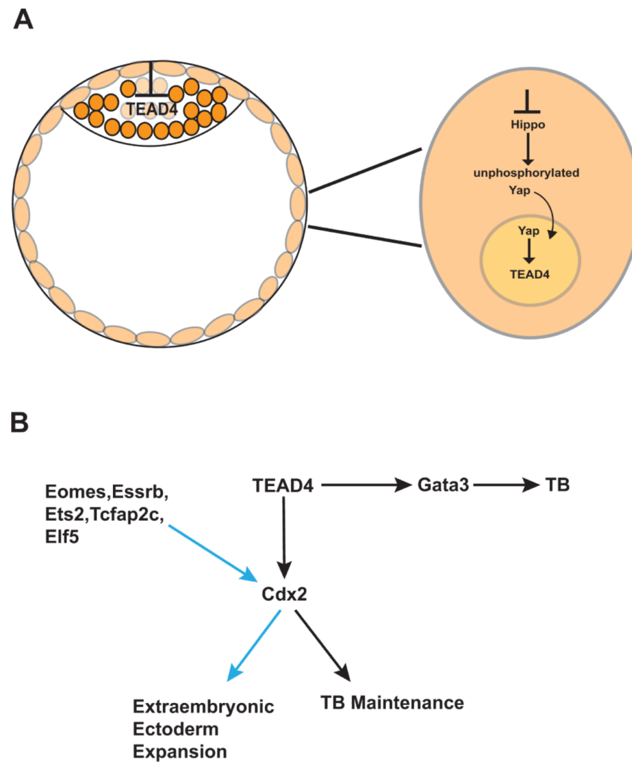

Figure 1.
Schematic diagram of the cell signaling that dictates trophoblast (TB) differentiation in the mouse. A. The nuclear localization of TEAD4 in the outer blastomeres of the embryo leads to a trophoblast-specific transcriptional program, which is selectively impaired in the inner cells of the embryo (ie. the ICM). Inactive Hippo signaling maintains Yap in an unphosphorylated state, resulting in translocation of Yap to the nucleus and thus induction of the TEAD4 transcriptional program in the outer cells of the embryo. B. Trophoblast differentiation is not limited to Cdx2 induction (Gata3 induction results in trophoblast differentiation) and likewise Cdx2 induction is not only limited to trophoblast differentiation but also results in extraembryonic ectoderm expansion. Black arrows indicate positive induction pathways. Blue arrows indicated enhancement of Cdx2 leading to extraembryonic ectoderm expansion.

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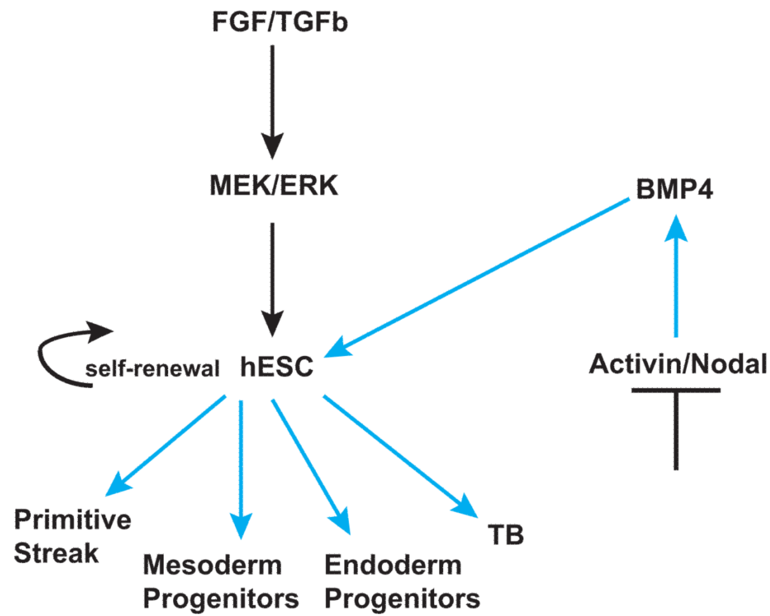

Figure 2.

Schematic of the cell signaling that dictates hESC self-renewal and differentiation induction by BMP4. Controversy exists as to whether BMP4 signaling results in trophoblast (TB) lineage differentiation or whether BMP4 along with the continuous presence of FGF result in primitive streak and mesendoderm derivitives and not trophoblast. Black arrows indicate inductive pathways for hESC self-renewal. Blue arrows indicate differentiation induction of hESC.

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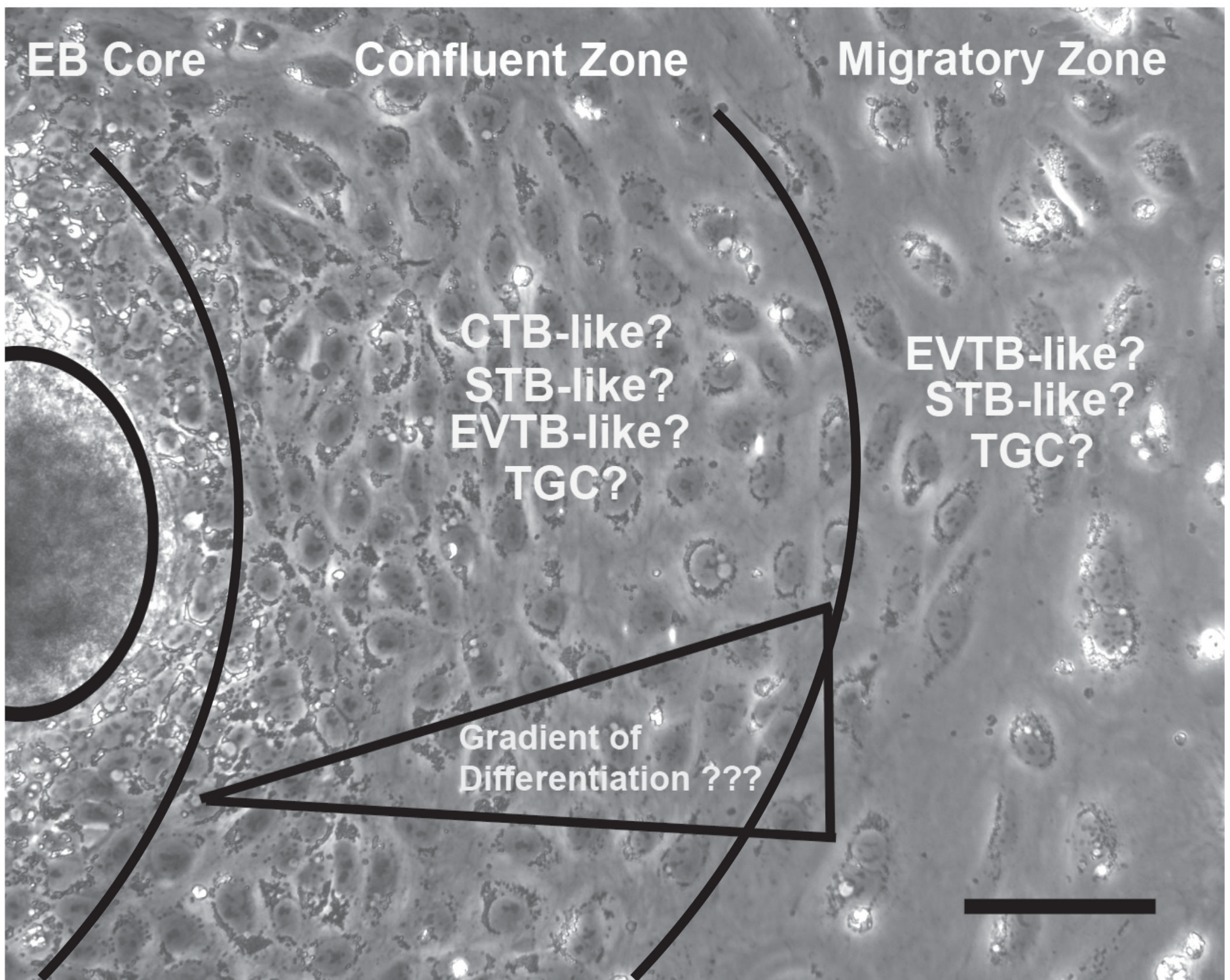

Figure 3.
Photomicrograph depicts EB-derived trophoblast outgrowths. Once the EB has adhered to the surface, outgrowths grow and move away from the EB core. The undifferentiated EB mass is circled left. With the EB core still maintaining the potential to differentiate into other tissue derivatives, interests lie in isolating cells near to the core, which make up a confluent cellular zone and isolating the cells at the farthest edge away from the core, which constitute the migratory zone of cells. Studies still remain to elucidate the potential gradient of differentiation that might exist resulting in various trophoblast subtypes. CTB=cytotrophoblasts, STB=syncytiotrophoblasts, EVTB=extravillous cytotrophoblasts, TGC=trophoblast giant cells. Scale bar=100μm.

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Table 1

Genes with significant relevance to trophoblast differentiation and placental development.

Gene Symbol	Summary of significance (see text for details)
Fgf4	Maintenance of mouse TSC proliferation
Tgfb1	Maintenance of mouse TSC proliferation
Oct4 (Pou5f1)	ESC pluripotency transcription factor
Cdx2	Directs mouse trophoblast lineage selection and proliferation
Tead4	Trophoblast lineage selection upstream of Cdx2
Hippo	Indirect Tead4 regulator
Yap	Tead4 coactivator
*Trim (*ectodermin*)*	Nodal expression regulation
Nodal	TGFB1 regulator, trophoblast differentiation
Bmp4	Inner cell mass maintenance (mouse)
Gata3	Promotes trophoblast differentiation
Eomes	Mouse TSC maintenance
Esrrb	Mouse TSC maintenance
Ets2	Enhances Cdx2 action
Tcfap2c (tfap2c)	Trophoblast differentiation, enhances Cdx2 action
Elf5	Trophoblast lineage determination, Cdx2, Eomes interactions
Hand1	Trophoblast giant cell differentiation (mouse)
Mdfi	Trophoblast giant cell differentiation (mouse)
Mash2/Ascl2	Maintenance of mouse spongiotrophoblast
HASH2	Expressed in human cytotrophoblasts
Egfr	Maintenance of mouse spongiotrophoblast
Gcm1	Mouse syncytiotrophoblast differentiation
Eset	Methylation site-related silencing of trophoblast genes
Sox2	Pluripotency factor for ESC
Nanog	Pluripotency factor for ESC
Rex1	Inner cell mass marker
Fgf5	Epiblast marker
Klf4	Pluripotency factor for ESC
Lif	Maintenance of mouse ESC self-renewal