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BMP4 regulation of human trophoblast development

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ABSTRACT Since the derivation of human embryonic stem cells, and the subsequent generation of induced pluripotent stem cells, there has been much excitement about the ability to model and evaluate human organ development *in vitro*. The finding that these cells, when treated with BMP4, are able to generate the extraembryonic cell type, trophoblast, which is the predominant functional epithelium in the placenta, has not been widely accepted. This review evaluates this model, providing comparison to early known events during placentation in both human and mouse and addresses specific challenges. Keeping in mind the ultimate goal of understanding human placental development and pregnancy disorders, our aim here is two-fold: to distinguish gaps in our knowledge arising from mis- or over-interpretation of data, and to recognize the limitations of both mouse and human models, but to work within those limitations towards the ultimate goal.

KEY WORDS: *trophoblast, placenta, BMP4, stem cells*

Introduction

Early development of the human placenta is mostly a black box. Most of what we know about the early stages in formation of this important organ comes from images from the limited number of samples in the Boyd and Carnegie Collections (recently reviewed in James *et al.,* 2012a,b). While intriguing, these images fall short of conclusive evidence which can be gathered from both *in vitro* and *in vivo* manipulation of embryos in animal models. For this reason, much of the scientific knowledge about early embryonic (and placental) development comes from intricate studies in rodent models; at times, based on little more than assumption, these data are taken to be fully representative of early human development.

Over the past decade, the establishment and widespread availability of human pluripotent stem cell (hPSC) lines—both derived from embryos (human embryonic stem cells/hESCs) and subsequently by reprogramming of somatic cells (human induced pluripotent stem cells/hiPSCs)—has opened a new chapter in the study of early human development. The early observation of trophoblast differentiation following BMP4 treatment of human embryonic stem cells by Jamie Thomson's group extended this opening to placental biologists (Xu *et al.,* 2002). Since then, multiple groups have used this model to probe the mechanisms of human trophoblast lineage specification and differentiation. Nevertheless, this model remains under-appreciated by most reproductive biologists and the wider stem cell research community.

The goal of this review is to highlight the knowledge gained about trophoblast differentiation through the use of hPSCs, including hESCs and hiPSCs. Comparison to mouse embryonic (mESC) and mouse trophoblast stem cell (mTSC) differentiation and placental development will be made to highlight similarities as well as differences. Ultimately, however, as with differentiation of hPSCs to any other cell type, comparison to primary human cells and tissues is required to determine the true identity of *in vitro*-derived cells. Hence, we will begin with a brief overview of human trophoblast differentiation, with a focus on early placentation.

Early human placental development

Early following attachment to the endometrium, the human blastocyst implants and becomes fully surrounded by decidualized endometrial stroma. At this stage, two trophoblast populations are present in the primitive placenta: a mononuclear "cytotrophoblast" (CTB) and a multinucleated "syncytium" (James *et al.,* 2012a). Aside from morphology, little is known about these particular cell types, their markers and function. While the Ki67$^+$ CTB is taken to be the proliferative "stem" cell type, the primitive syncytium, which

Abbreviations used in this paper: BMP4, Bone morphogenetic protein 4; CDX2, caudal-type homeobox-2; CTB, cytotrophoblast; ESC, embryonic stem cell; EVT, extravillous trophoblast; ExE, extraembryonic ectoderm; FCM, feeder-conditioned media; FGF, fibroblast growth factor; hCG, human chorionic gonadotropin; ICM, inner cell mass; iPSC, induced pluripotent stem cell; PSC, pluripotent stem cell; STB, syncytiotrophoblast; TE, trophectoderm; TSC, trophoblast stem cell.

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appears to digest away the decidua at the leading edge of the implanting embryo, potentially acts in an "invasive" capacity. Such multinucleated "invasive" trophoblast, present in early implantation sites, are sometimes also referred to as "giant" cells (James *et al.,* 2012a) (see Fig. 1B below). For this reason, it is at times mistakenly assumed that these cells are equivalent to invasive mouse trophoblast of the same name, which arise by endoreduplication (John and Hemberger, 2012). The primitive syncytium forms fluid-filled spaces, called lacunae, which breach maternal sinusoids, thereby allowing flow of maternal blood directly to the growing embryo (James *et al.,* 2012a).

The exact nature of the primitive syncytium, the mechanism of its formation, and its functional capacity to invade and/or erode tissue has not been determined. In addition, its relationship to the definitive syncytium, the multinucleated cell layer abutting maternal sinusoids, and later, the intervillous space, is also unclear. The definitive syncytiotrophoblast (STB), first noted at day 15 of gestation (Fig. 1A), is thought to arise by fusion of CTB to line the maternal sinusoids. Thereafter, the STB grows by continuous fusion of underlying CTB (James *et al.,* 2012a).

True invasive extravillous trophoblasts (EVTs) appear to arise from anchoring villi, differentiating from cytotrophoblast cell columns (Fig. 1B). These cells invade through the uterine wall (interstitial EVT) and remodel maternal spiral arterioles (endovascular EVT) (Knofler, 2010; James *et al.,* 2012a; Soares *et al.,* 2012). The regulation of differentiation of this lineage as well as their invasive capacity are pivotal to understanding pregnancy loss and complications, including the pregnancy-induced hypertensive syndrome, preeclampsia (Knofler, 2010; Soares *et al.,* 2012). These cells are known to lose proliferative capacity as they gain invasive potential (Knofler, 2010; James *et al.,* 2012a; Soares *et al.,* 2012). Intricate communication with surrounding cells, particularly decidua and the immune-modulatory cells infiltrating this tissue, is central to establishment of the "proper" level of uterine invasion (Gellersen *et al.,* 2007; Bulmer *et al.,* 2010). In fact, poor decidualization (as seen, for example, in cases of implantation in the lower uterine segment—"previa") is associated with abnormally adherent placentation ("creta"), which often requires removal of the in-situ placenta by hysterectomy (Wortman and Alexander, 2013).

Outside the implantation site, EVT also exist in fetal membranes as well as in the trophoblast "islands" in the placental disc proper (Lee *et al.,* 2007) (Fig. 1C-D). How these EVT subtypes differ from their basal plate counterparts remains to be elucidated.

Characteristics of different trophoblast sub-lineages

Multiple markers are routinely used to highlight different trophoblast subtypes. While cytokeratin 7 (KRT7) is used as a pan-trophoblast marker starting from the blastocyst stage of human development (Niakan and Eggan, 2013), EGFR and CAD1 (E-cadherin) are often used to highlight CTB (Ferretti *et al.,* 2007), and in fact can be used to sort out this cell population out of human placental cell preps with high specificity. EVT are specifically characterized by surface expression of HLA-G, although soluble forms are also noted in CTB (Rizzo *et al.,* 2011). CTB and EVT have been further defined based on integrin expression, with CTB expressing $\alpha_6\beta_4$, proximal column EVT expressing $\alpha_5\beta_1$ and distal column and invasive EVT expressing $\alpha_1\beta_1$ integrins (Damsky *et al.,* 1992). The STB compartment is defined by secretion of the pregnancy hormones hCG and hPL (Fujimoto *et al.,* 1986; Cole, 2012); however, two caveats must be considered here. First, hCG is secreted in multiple forms, including a hyperglycosylated form (H-hCG) which was determined to be secreted primarily by early invasive trophoblast (Handschuh *et al.,* 2007; Guibourdenche *et al.,* 2010; Cole, 2010, 2012); whether this includes the primitive syncytium is unknown, while villous STB are known to be negative for this marker (Handschuh *et al.,* 2007). Second, placental lactogen (hPL, also known as chorionic somatomammotrophin/CSH), also a characteristic marker of STB, is secreted by more mature STB, with expression increasing with increasing gestational age (Fujimoto *et al.,* 1986).

Few lineage-specific transcription factors which can differentiate between villous and extravillous trophoblast subtypes have been described in the human placenta. One such factor, CTB-specific p63, is described below. *ID2* has also been identified as CTB specific (Janatpour *et al.,* 2000), while *ASCL2* is enriched in cell column trophoblast and EVT (Meinhardt *et al.,* 2005; Murthi *et al.,* 2013). Other transcription factors, including the homeobox genes *DLX3* and *DLX4* have been identified in both villous and extravillous trophoblast (Murthi *et al.,* 2013). We recently identified the pluripotency factor, *KLF4*, to be expressed in villous STB (Li *et al.,* 2013). Wnt-activated TCF4 has been suggested to play a role in formation of EVT (Knofler and Pollheimer, 2013); however, TCF4 has also been identified to play a role in fusion of human

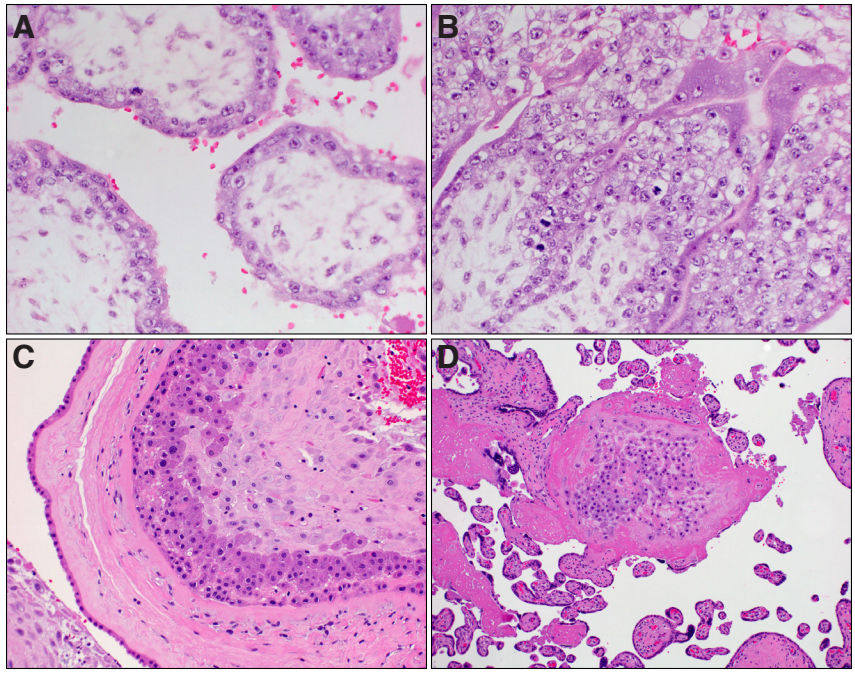

Fig. 1. H&E staining of early placenta tissue, from a rare archived 16-day human blastocyst specimen. (A) *Continuous STB layer covering underlying CTB layer.* **(B)** *Column CTB and a few multinucleated "giant cells." Some EVT also exist in chorionic (fetal) membranes* **(C)** *and intraplacental trophoblastic islands* **(D)**.

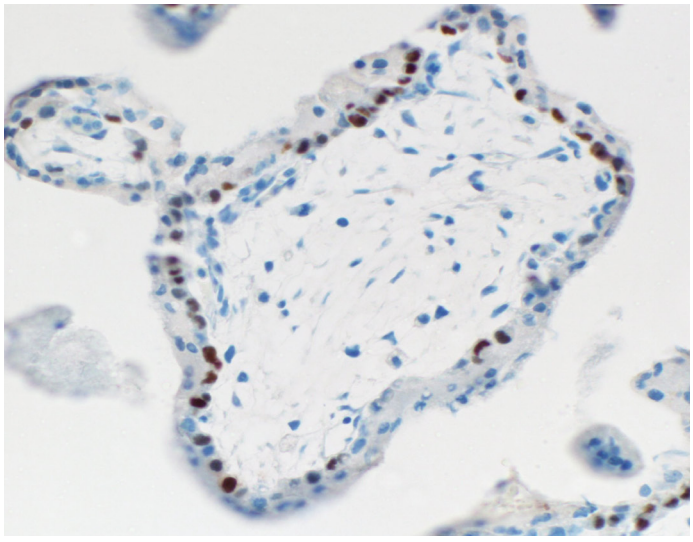

Fig. 2. Immunohistochemical staining for CDX2 in early (7-week) placental tissue. *CDX2 (brown) is expressed only in a subset of CTBs. The tissue section is counterstained with hematoxylin (blue).*

choriocarcinoma cells (Matsuura *et al.,* 2011), so its specificity to the EVT lineage remains to be confirmed. Other transcription factors, such as PPARγ, are expressed in all trophoblast, although activation of PPARγ has been associated specifically with inhibition of trophoblast invasion (Fournier *et al.,* 2011). Overall, the majority of studies are based on detection of RNA, either by RT-PCR or in-situ hybridization; detection of the corresponding proteins are more difficult due to lack of high-quality reagents. For example, *GCM1*, which is thought to regulate villous branching and CTB fusion, is thought to be confined to a subgroup of CTB (Baczyk *et al.,* 2009), although different antibodies and staining methodologies show different staining patterns (Genbacev *et al.,* 2011; compare to STB staining on the Human Protein Atlas website).

In order to determine whether an *in vitro* model is representative of human trophoblast differentiation *in vivo*, detailed analysis of the various trophoblast subtypes, at various times during gestation, is required. In one such study (Lee *et al.,* 2007), we showed that p63, a member of the p53 family of nuclear proteins, marks all proliferative CTB at all gestational ages in the human placenta; this marker is lost when CTB differentiate, either into EVT or multinucleated STB. In addition, by immunohistochemistry, we determined the exact pattern of expression of several trophoblast-associated markers, including MelCAM and inhibin: while the former is confined to mature EVT, the latter is expressed in both EVT and STB (Lee *et al.,* 2007). More such studies are needed to probe differential marker expression between trophoblast cell types in-situ.

The trophoblast stem cell niche

Mouse trophoblast stem cells are typically derived from E3.5 blastocysts, using a combination of feeders and FGF4 (Tanaka *et al.,* 1998). These cells are characterized by expression of multiple transcription factors, including *Cdx2*, *Elf5*, and *Eomes* (Roberts and Fisher, 2011; John and Hemberger, 2012). When plated in the absence of feeders (or feeder-conditioned medium) and FGF4, these cells lose the above stem cell markers, and begin to express markers of both labyrinthine (villous) trophoblast and trophoblast giant cell (EVT) lineages (Tanaka *et al.,* 1998). When injected into blastocysts, mouse TS cells are able to contribute to all trophoblast sublineages *in vivo* (Tanaka *et al.,* 1998).

Whether a similar "TS" population exists in the human placenta is unknown. While human blastocysts do express CDX2 specifically in the trophectoderm (Niakan and Eggan, 2013), derivation of TS cells from human embryos has so far been unsuccessful (Rossant, 2007; Genbacev *et al.,* 2011). ELF5 has been identified in a subset of first trimester CTB (Hemberger *et al.,* 2010), suggesting that the post-implantation placenta may harbor such TS cells. We have recently confirmed the presence of CDX2 in a subset of first trimester CTB (Fig. 2), providing more evidence for the latter hypothesis. However, whether these cells would be truly bipotential, or whether EVT and STB each arise from their own independent precursor cell types, remains controversial (Knofler 2010). Finally, the non-trophoblast (mesoderm-derived) portion of the human chorion has also been proposed as a TS cell niche, with cell lines derived using a combination of FGF and activin/nodal inhibitors (Genbacev *et al.,* 2011); however, the true nature of these cells remains to be independently confirmed.

While defining the human TS cell niche remains an important issue, the more difficult question may be how to maintain such cells *in vitro*. Mouse TS cells require a combination of FGF and activin signaling in order to maintain their multipotent state (Tanaka *et al.,* 1998; Erlebacher *et al.,* 2004). The majority of studies, however, use feeder-conditioned media (FCM), in combination with FGF4, to derive and maintain these cells. The requirement of 20% fetal bovine serum has made it difficult to develop defined media for these cells. Interestingly, undifferentiated mouse TS cells also secrete BMP4 (Murohashi *et al.,* 2010) and express BMP receptors, including *Bmpr1a* and *Bmpr2* (Kishigami and Mishina, 2005); however, the role of this signaling in maintenance and/or differentiation of mouse TS cells remains entirely unknown (see below). Culture on tissue culture plastic in low oxygen inhibits differentiation of mouse TS cells (Tache *et al.,* 2013), while culture on a fibronectin-rich matrix in hypoxia promotes formation of giant cells (Choi *et al.,* 2013).

Turning to the human placenta, isolated CTB rapidly lose their proliferative capacity *in vitro* (Bilban *et al.,* 2010; Knofler 2010). For this reason, STB-denuded first trimester placental explants have been used at times for evaluation of CTB proliferation, confirming the importance of the underlying stroma in this process (Baczyk *et al.,* 2006; Forbes *et al.,* 2008; Knofler 2010). Both models are suboptimal for evaluation of CTB proliferation; nevertheless, studies with the latter model have suggested a role for IGF and FGF signaling, through the MAPK pathway, in promoting CTB proliferation (Baczyk *et al.,* 2006; Forbes *et al.,* 2008). Conversely, TGFβ signaling has been shown to inhibit CTB proliferation (Graham *et al.,* 1992; Pollheimer and Knofler 2005). BMP signaling has not been explored in human CTB, although we have confirmed the expression of both ligands and receptors in first trimester placental explants and CTB (Parast *et al.,* unpublished data). The maintenance of CTB proliferation *in vivo* is likely much more complex, and dependent on crosstalk between these and other signaling pathways, with concomitant regulation of cell survival, differentiation, and invasion (Knofler 2010). It is clear, however, that the number of proliferative CTB is drastically diminished in the latter half of pregnancy, and also relatively well-accepted that later gestation CTB have a reduced ability to differentiate into EVT. This seriously limits the study of both the human "TS" cell niche

and the disease-relevant EVT to those researchers with access to first trimester placental tissues.

BMP4 and induction of the trophoblast lineage in human pluripotent stem cells

Human ESCs were first found to differentiate into trophoblast following BMP4 treatment over ten years ago (Xu *et al.,* 2002). In this original study, BMP4 was added to hESCs in the presence of feeder-conditioned media (FCM) as well as bFGF, and cells were found to flatten, form multinucleated cells, express trophoblast-associated genes based on microarray analysis, and secrete hCG, estradiol, and progesterone (Xu *et al.,* 2002). Similar differentiation was said to occur in the absence of FCM/bFGF; however, it was more asynchronous (Xu *et al.,* 2002). This study was followed by two others, by independent groups, the first showing induction of EVT-like cells (based on HLA-G expression), in addition to multinucleated syncytiotrophoblast-like cells, following similar BMP4 treatment (Das *et al.,* 2007), and the second showing a similar differentiation pattern following treatment, in presence of FCM, with an inhibitor of activin/nodal signaling, instead of BMP4 (Wu *et al.,* 2008).

Since then, additional studies have refined culture conditions for trophoblast differentiation, finding that BMP4-induced differentiation into the trophoblast lineage is in fact more uniform in the absence of bFGF (Das *et al.,* 2007, Yu *et al.,* 2011), and even in minimal media without FCM (Erb *et al.,* 2011). Most recently, it has been shown that additional inhibitors of FGF (Sudheer *et al.,* 2012) and activin/nodal signaling (Amita *et al.,* 2013) during BMP4 treatment unidirectionally differentiate hESCs into trophoblast. That signaling through the BMP4 receptors is involved in differentiation of this lineage is supported by a study showing that hESC lines with a deficiency in the phosphatidyl-inositol-glycan class A (*PIG-A*) gene cannot differentiate into trophoblast (Chen *et al.,* 2008). PIG-A is required for the synthesis of glycosyl-phosphatidyl-inositol (GPI)-anchored proteins, including BMP coreceptors.

Multiple other groups have used the BMP4 model to further evaluate the early steps in human trophoblast differentiation. Drukker *et al.,* (2012) were able to isolate APA^+ syncytiotrophoblast precursors from BMP4-treated hESC cultures, while Marchand *et al.,* (2011) applied transcriptomic analysis of these cells to identify novel genes involved in trophoblast differentiation. In fact, by comparative analysis of human trophectoderm (TE) samples, Bai *et al.,* (2011) have found that BMP4-treated hESCs indeed express TE-specific transcription factors, confirming the utility of this model for study of early lineage specification. In addition, Chen *et al.,* (2013), among others, have shown that hiPSCs behave similarly to hESCs when treated with BMP4. Finally, as hinted in the original study of Xu *et al.,* (2002), Lichtner *et al.,* (2013) have now shown that other BMP family members can also induce the trophoblast lineage, with BMP10, being most resistant to inhibition by NOGGIN, as the most potent.

Although the focus of this review is on BMP4 signaling, it should be mentioned here that induction of trophoblast from hESCs has also been noted following culture of hESC-derived embryoid bodies on Matrigel, with slightly different gene expression profile, but essentially the same final conclusion: that these cells retain the ability to differentiate into this extraembryonic cell type (Giakoumopoulos and Golos, 2013).

Challenges to the BMP4 Model

The trophoblast differentiation potential of hESCs was at first surprising, since LIF-dependent mouse ES cells rarely contribute to the trophectoderm compartment when injected into blastocysts (Beddington and Robertson, 1989; Nagy *et al.,* 1990). In fact, BMP4 signaling is required for maintenance of pluripotency in mESCs (Ying *et al.,* 2003). Nevertheless, mESCs have been shown to differentiate into trophoblast *in vitro*, when cultured either on laminin in serum-free media containing BMP4 (Hayashi *et al.,* 2010) or when differentiated on collagen IV (Schenke-Layland *et al.,* 2007). The latter study found the trophoblast differentiation potential to be confined to feeder-dependent mESC lines and to be dependent on *Cdx2*. More recently, mouse epiblast stem cells (mEpiSCs) were derived from post-implantation embryos. These cells require FGF and activin signaling to maintain their pluripotency and have the unusual property of expressing *Cdx2* and forming "giant cells" following BMP4 treatment (Brons *et al.,* 2007; Tesar *et al.,* 2007). This was deemed consistent with trophoblast differentiation potential, which came to be accepted as a characteristic which distinguishes these cells from mESCs. However, the exact nature of BMP4-treated mEpiSCs is not clear. First, a "TS" cell state has not been identified. Second, aside from *Cdx2*, other more specific mouse trophoblast-associated markers such as *Tpbpa*, have yet to be demonstrated in these cells.

More recently, based partially on the above data, Bernardo *et al.,* (2011) challenged the premise of BMP4-induction of the trophoblast lineage from hESCs, stating that BMP4 is known mainly as an inducer of the mesoderm lineage. In fact, short term BMP4 treatment of hESCs has been shown to induce mesoderm (Zhang *et al.,* 2008), and authors of the above studies on BMP4-induced trophoblast differentiation have noted induction of some mesoderm markers (including *T/BRACHYURY*) alongside trophoblast markers, particularly at early timepoints following BMP4 treatment (Xu *et al.,* 2002; Yu *et al.,* 2011). Using CDX2 expression as the main marker of the trophoblast lineage, Bernardo *et al.,* (2011) showed that knockdown of BRACHYURY abrogated CDX2 expression following BMP4 treatment. They concluded that this result is consistent with the BMP4-treated cells being in fact of mesodermal, and not trophectodermal, origin. They correctly pointed to the fact that, in the mouse embryo, *Cdx2* is also expressed in early mesoderm tissue and that, by itself, is not specific to trophectoderm (Chawengsaksophak *et al.,* 2004). They also challenged the nature of BMP4-treated mEpiSCs, again pointing to *Cdx2* being used as the sole indicator for trophoblast differentiation. They reason that mEpiSCs, being derived from the post-implantation embryo, should not be able to "go backward" and differentiate into trophoblast. Since hESCs show an epigenetic profile more similar to "primed" mEpiSCs than "ground state" mESCs (Nichols and Smith, 2012; De Los Angeles *et al.,* 2012), Bernardo *et al.,* thus extended the above reasoning to hESCs, emphasizing that these cells also should not have the capacity to differentiate into trophoblast.

However intriguing, the major conclusions of this study are based on several assumptions. The first is the specificity of the marker BRACHYURY. This gene is in fact first expressed in extraembryonic ectoderm (ExE), the compartment which houses trophoblast stem cells in the mouse, at E5.5, before gastrulation even begins (Rivera-Perez and Magnuson, 2005). In fact, both BRACHYURY RNA and protein are expressed in the ExE in the

early post-implantation period (Rivera-Perez and Magnuson, 2005; Inman and Downs, 2006). However, to date, no studies have been done to determine the role of BRACHYURY in the ExE or mouse TS cells. We have found that *Brachyury* RNA is in fact expressed in undifferentiated mouse TS cells and decreases with differentiation *in vitro* (Parast *et al.,* unpublished data). Given this expression pattern, it remains plausible that *Brachyury* would be involved in maintenance of the undifferentiated TS cell state, perhaps by regulating *Cdx2*.

Second is the assumption that BMP4 signaling does not play a role in the ExE compartment. In fact, as mentioned above, several BMP receptor genes, including *Bmpr1a*, are expressed in the ExE (Kishigami and Mishina, 2005); the ExE is also known to secrete BMP4 which initiates formation of the primitive streak in the early post-implantation embryo (Murohashi *et al.,* 2010). Embryos deficient in *Bmpr1a* do implant; however, they die in the early post-implantation period, with several abnormalities (Mishina *et al.,* 1995). While it is assumed that this phenotype is due only to abnormalities in the embryonic compartment, it should be noted that the extraembryonic compartment in these embryos is also abnormal, missing both *Brachyury* and *Eomes* expression from the ExE (Di-Gregorio *et al.,* 2007). While the function of Brachyury in the ExE is yet to be investigated, it is known that *Eomes* plays a major role in the maintenance of this compartment (Russ *et al.,* 2000), pointing to the plausible explanation that signaling through Bmpr1a may be required in ExE maintenance. Based on these examples of *Brachyury* and *Bmpr1a*, it is apparent that further studies are needed in order to probe the roles of these genes in the ExE, and that lack of data in this area should not be taken as negative data.

Third is the overarching assumption that early mouse and human development are highly similar. In fact, despite the known similarities, when it comes to trophoblast lineage specification and placental development, the rule is one of divergence. While the trophoblast lineage is specified early in the mouse preimplantation embryo, with reciprocal expression of POU5F1/OCT4 and CDX2 in the inner cell mass and TE, respectively, the human embryo shows a lag in trophoblast lineage segregation, with a period of time during which OCT4 and CDX2 are coexpressed in the TE (Niakan and Eggan, 2013). In fact, the human OCT4 gene regulatory elements are more similar to those in rabbit and bovine, and divergent from mouse (Berg *et al.,* 2011): bovine *Oct4* constructs were not repressed when put into mouse TE, consistent with the observation that TE lineage commitment does not occur until later stages in cattle. Interestingly, similar to human, BMP4 has also been shown to induce the trophoblast lineage in rabbit ESCs (Tan *et al.,* 2011) and used to establish trophoblast cell lines from bovine embryos (Suzuki *et al.,* 2011). Similarly, X-chromosome inactivation in female embryos has recently been found to be divergent in rabbits and humans, compared to mice: while mice show inactivation of the paternal X in early embryogenesis, with reversal of this process in the ICM followed by random inactivation in the ICM-derived epiblast, X inactivation begins later in rabbit and human embryos and occurs in a high proportion of ICM cells in the blastocyst (Okamoto *et al.,* 2011). This study casts doubt on statements of equivalency of mEpiSCs and hESCs based solely on their epigenetic profile and growth factor requirements.

Finally, in challenging the conclusions of Bernardo *et al.,* (2011), a word regarding their culture conditions is required. Although this has been extensively discussed elsewhere (Ezashi *et al.,* 2012) and comparative studies completed (Amita *et al.,* 2013), the fact that the authors used chemically defined media, typically used for culture of mouse EpiSCs, has to be addressed here. These conditions are vastly different from any of the studies involving BMP4 and human ESCs published before or since. The addition of a PI3-kinase inhibitor is also curious; while it is justified as a factor which "enhances hESC differentiation," the cited manuscript describes a protocol for enhancing mesendoderm differentiation (McLean *et al.,* 2007). The use of this inhibitor also ignores data from studies of primary human trophoblast, showing that the PI3-kinase pathway is in fact involved in differentiation of the EVT lineage (Knofler 2010; Soares *et al.,* 2012).

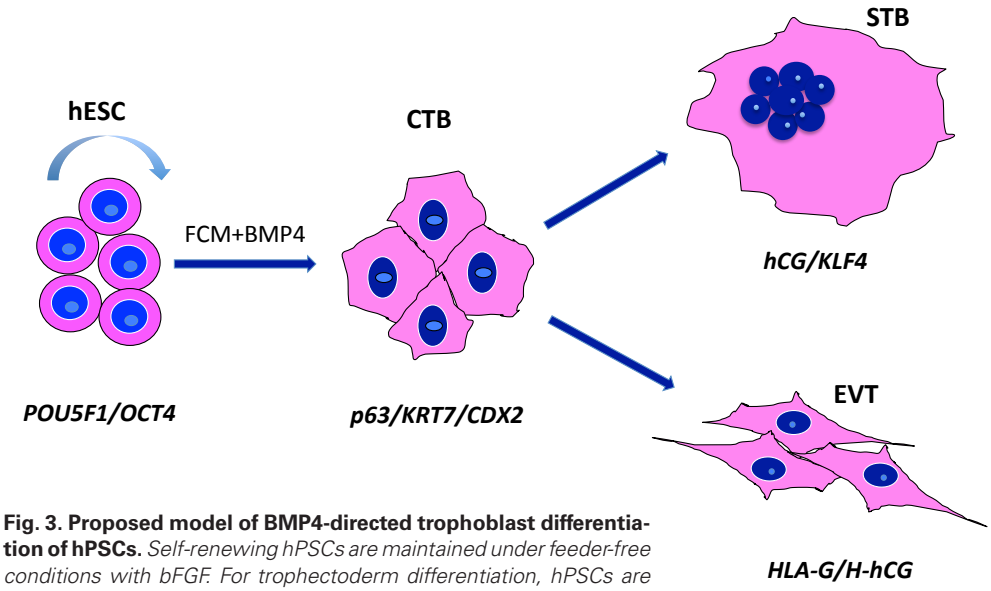

Fig. 3. Proposed model of BMP4-directed trophoblast differentiation of hPSCs. *Self-renewing hPSCs are maintained under feeder-free conditions with bFGF. For trophectoderm differentiation, hPSCs are cultured in feeder-conditioned medium supplemented with 10 ng/ml BMP4 for up to 8 days. BMP4-treated hPSCs undergo a p63/KRT7/CDX2 triple-positive CTB "stem cell" state prior to terminal differentiation into HLA-G/H-hCG positive EVT and hCG/KLF4 positive STB.*

Looking ahead: to BMP4 or not to BMP4?

In order to determine whether the BMP4 model—or in fact any *in vitro* differentiation protocol applied to hESCs/iPSCs—is useful, it needs to be compared to human cells and tissues *in vivo*. We recently completed such a study of BMP4-treated hESCs, comparing them to a series of human fetal and placental cells and tissues (Li *et al.,* 2013). Genome-wide expression analysis showed that the BMP4-treated cells are most closely related to isolated CTB from first trimester placental tissues, clustering away from mesoderm-derived amnion and placental stroma (Li *et al.,* 2013). It should be pointed out that differ-

ences remained in gene expression profile between the primary and *in vitro*-generated cells, likely due to suboptimal "2D" culture conditions. We are currently working to enhance the *in vitro* conditions, using human placenta-derived extracellular matrix as well as screening for other useful growth factors and small molecules.

Whether the BMP4-hESC model can generate a bona-fide human "TS" cell also remains an open question. Given our limited understanding of this cell type and its niche, care should be taken to avoid use of a single marker to define this compartment, particularly markers which have yet to be validated in the human placenta. In our recently-published study of BMP4-treated hESC (Li *et al.,* 2013), we used p63 as a marker of proliferative CTB, based on our previous study of human placental tissues at varying gestational ages (Lee *et al.,* 2007). We showed that BMP4-treated hESCs in fact undergo a $p63^{+/}KRT7^{+}$ CTB "stem cell" state, prior to differentiating into surface $HLAG^{+}$ EVT and hCG-secreting STB (Fig. 3), and that knockdown of p63 inhibited differentiation into these terminal lineages (Li *et al.,* 2013). A subset of these $p63^{+}$ cells were also positive for CDX2, again showing similarities to CTB in first trimester placenta (Li *et al.,* 2013). Our conclusion—that these hESC-derived cells are bona-fide trophoblast—was based, not just on p63 and CDX2 expression, but on the entirety of the experimental evidence, including comparison to primary CTB, based on genome-wide expression profiles, and functional assays (Li *et al.,* 2013).

Going forward, much remains to be elucidated regarding human placental development, including the role of BMP4 signaling, trophoblast lineage specification and subtype-specific differentiation, and comparison to mouse and other animal models. The placenta overall remains a poorly-understood organ, even in the mouse, where the necessity of this compartment for embryonic growth and development remains under-appreciated; routine phenotyping of many transgenic mouse models, including those with embryonic lethality, are done with only a fleeting glance at the extraembryonic compartment. More thorough examination, including TE-specific knockdown in addition to wild-type tetraploid rescue, should be done to determine or exclude a role for an individual gene/protein in placental development and trophoblast differentiation. While gene-specific knockdown in the mouse remains the gold standard for evaluating the role of individual genes during embryonic and placental development, expression studies in primary human placental tissues and isolated CTBs should be performed to document similar expression and potential regulation in human tissues, prior to drawing conclusions about limitations of an hESC-based model. Careful gene expression profiling studies of human CTB, EVT, and STB are required in order to identify lineage-specific transcription factors, as well as specific signaling pathways involved in their maintenance and differentiation. Finally, continued research using both mouse and human pluripotent stem cells is required. Specifically, in the mouse, where such experimentation is possible, studies are needed to delineate the differences between *in vitro* and *in vivo* differentiation potential of mESCs with respect to contribution to the trophectoderm. Several recent publications point to meta-stable "totipotent" states in mESC, which can be stabilized by alterations in culture conditions (Macfarlan *et al.,* 2012; Morgani *et al.,* 2013); similarly, a recent study has verified totipotency of *in vivo* derived mouse iPSCs, with the ability to contribute to trophectoderm-derived tissues (Abad *et al.,* 2013). These studies should focus our attention on the still-present gaps in knowledge about toti- and pluripotency in general, and trophectoderm specification in particular. It is imperative that reproductive, stem cell, and developmental biologists forge ahead with these investigations, working together and always keeping an open mind.

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