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HUMAN EMBRYONIC STEM CELLS AS MODELS FOR TROPHOBLAST DIFFERENTIATION

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Abstract

Trophectoderm is specified from pluripotent blastomeres at some time prior to blastocyst formation. Proliferating cytotrophoblast derived from trophoctoderm is the forerunner of the entire trophoblast component of the mature human placenta, including extra-villous cytotrophoblast and syncytiotrophoblast. Recently human embryonic stem cells (hESC) have been employed to study these events in an *in vitro* situation. Here we review some of the work in this emerging area of trophoblast biology. We concentrate primarily on a model in which colonies of hESC are exposed to BMP4 in stem cell growth medium lacking FGF2. Under both low (4 %) and high (20 %) O₂ conditions, differentiation proceeds unidirectionally towards trophoblast from the outside of the colonies inwards, with progression fastest under high O₂. Immunohistochemical observations performed on whole colonies combined with microarray analysis of mRNA can be employed to track developmental transitions as they occur over time and in two-dimensional space as the cells respond to BMP4.

Introduction: the trophoblast lineage and its emergence

Trophectoderm, the precursor of placental trophoblast, becomes apparent as the blastocoel cavity forms and expands and provides the first visual evidence of separation of cell lineages during the development of the mammalian conceptus and goes on to form the majority of the components of the mature fetal placenta. Considerable controversy exists about what drives the specification of the trophoblast lineage, whether there is some degree of pre-patterning in the early cleavage stage conceptus or even the oocyte, and about how early in development the separation between the trophoctoderm and embryonic lineages occurs [1-5]. Whichever view prevails, it is clear that that trophoctoderm precursor cells arise from pluripotent antecedents early in conceptus development.

In the human, two main cell lineages arise from trophoctoderm. One, villous cytotrophoblast, layers the basement membrane that surrounds placental villi. At these sites villous cytotrophoblast gives rise through division and fusion to syncytiotrophoblast [6,7], a cell layer that makes direct contact with maternal blood and is characterized by production of hCG and other placental hormones [8]. The second lineage derived from trophoblast stem cells is

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extravillous cytotrophoblast [9], which is non-polarized and multilayered, and the major invasive component of the placenta. These cells migrate in columns from the anchoring villi into the maternal endometrium by two routes: through the uterine stroma and via the lumen of maternal arteries [6] and play a variety of functions including support of the entire placental structure and modification of the spiral arteries [10]. Although the transcriptional programs that drive these events are beginning to be understood, experimental roadblocks exist to investigate how progenitor cells journey along these pathways of differentiation.

Models to study trophoblast development

Although many animal models, especially the mouse, can be used to follow trophoblast development from the blastocyst stage to the fully functional placenta, all show differences from the human [11]. In addition, only limited morphological information is available on the initial stages of trophoblast invasion, mainly from archived specimens [12]. Until recently the best *in vitro* models to study the development of human placenta have been choriocarcinoma-derived trophoblast cell lines, such as JAr or JEG3, and primary trophoblast cultures derived from placenta either at term or earlier in pregnancy [13]. There are shortcomings and advantages to each of these models. Importantly, both choriocarcinoma cell lines and primary cytotrophoblast are already committed to trophoblast so that early lineage decisions cannot be addressed.

Many choriocarcinoma cell lines are available [14,15]. These lines are easy to maintain, and share many characteristics with primary trophoblast, including an ability to up-regulate hCG production in response to external stimuli, form a syncytium by cell fusion, and invade through an artificial connective tissue matrix. On the other hand, their behavior is often quite different from that of the primary trophoblast they are intended to mimic [16-18]. These occasional anomalies are probably due in part to their tumor cell origins. For example, primary trophoblasts transformed with SV40 show increased proliferative and invasive behavior compared to normal control cells [19-22].

Primary human trophoblast cells, in contrast, better represent the behavior of normal human trophoblasts *in vivo* [23]. They have been used very effectively to elucidate key aspects of syncytiotrophoblast formation [24]. However, only primary cells derived from first trimester placentas, will spontaneously form extravillous trophoblast [25], and such samples are not widely available for study.

Thus, the search continues for a model which, like choriocarcinoma cells, is readily available, easily maintained in the laboratory, and at the same time closely represents normal trophoblast, particularly in its early commitment stages. Fortunately, human embryonic stem cells (hESC) have recently emerged as a useful alternative to other models and provide the potential to examine both the emergence of trophoblast from a precursor stem population and the subsequent differentiation of these cells. Among their advantages are: 1) They can be maintained indefinitely in the laboratory. 2) They do not suffer the disadvantages of being transformed. 3) They provide the ability to study the initial embryonic/trophoblast transition.

hESC as models for trophoblast development

ESC from primates, including the human, has been reported to differentiate into trophoblast spontaneously during standard subculture, as evidenced by the production of hCG (measured as hCG β) and progesterone [26-28]. The former is probably not the most reliable of markers, as it is expressed by many human tumors that have no relationship to trophoblast [29]. Indeed, Ezashi et al. [30] have shown that, in spontaneously differentiating hESC colonies, the cells that produce hCG β are small and quite unlike syncytiotrophoblast. They are also found on the

periphery of emerging areas of larger flattened cells within the body of the colonies. Moreover, the hCG β -positive cells do not co-express the hCG α sub-unit, suggesting that they are not producing bioactive hCG and are probably not trophoblast.

On the other hand, hESC can be prompted to form trophoblast. One approach is by the transgenic knockdown of genes associated with pluripotency. The second is culture of embryoid bodies (EB) in solid or semi-solid medium in the absence of factors that normally maintain pluripotency. The third is BMP4 treatment of stem cells cultured on Matrigel™.

The ability to drive differentiation of hESC towards trophoblast by silencing genes essential for maintaining pluripotency was first demonstrated in murine cells after knock down of *OCT4* (*POU5F1*) [31-33]. Silencing of *NANOG* or *SOX2* also induces markers of trophoblast differentiation [34-36]. In hESC, siRNA silencing of *POU5F1* followed by culture in the absence of FGF2 leads to apparent trophoblast differentiation, with expression of *BMP4*, *CDX2*, *CGA*, *CGB*, *EOMES*, *GATA2*, *GCM1*, and *ID2* [32,37,38]. A similar up-regulation of trophoblast markers and a transition in cell morphology was observed after *NANOG* silencing [39], although markers of extra-embryonic endoderm as well as those of trophoblast were increased. There is some evidence that mouse ESC can differentiate to trophoblast under certain conditions, but this process does not appear to occur as readily as in primate cells [40,41]. Studies in the mouse have largely concentrated on trophoblast stem cells [42], whose equivalent has as yet not been reported for the human.

As an alternative to genetic manipulation, some investigators have attempted to collect spontaneously differentiating trophoblast from EBs, embryo-like structures formed from disaggregated hESC cultured in medium lacking growth factors that support pluripotency. EBs contain cells representative of all three embryonic lineages plus some peripheral cells with the properties of trophoblast [43,44]. This model system has the advantage of developing in three dimensions and mimicking some of the features of blastocyst and trophoblast formation and even the early stages of implantation.

Two strategies have been developed to purify trophoblast cells from EBs. In the first, EBs were disaggregated and clonal cell lines processed through several rounds of selection for high hCG production [45]. By this method, lines were developed that were able to proliferate indefinitely in culture but that did not express the “classical” embryonic stem cell markers Nanog and OCT4. The majority (although not all) of these cells expressed cytokeratin 7 and a minority the extravillous trophoblast markers HLA-G and CD9. If not passaged frequently, these cells spontaneously formed syncytia and began to secrete hCG. They could also form “trophoblast bodies”, structures morphologically similar to EBs and were capable of invasion through artificial matrices *in vitro* [45].

A more recent approach to purify trophoblast cells from EBs has been based on selection for adhesion rather than for hCG production [46]. HESC were cultured in a semi-solid, methylcellulose-containing medium. Over time in culture, they formed EBs. A PECAM-1 positive cell layer adhered firmly to the plastic and remained after EBs were removed by micro dissection. The adherent cells were almost all cytokeratin 7 and VE-cadherin positive, suggesting that they were extravillous trophoblast. Cells at the periphery of the outgrowths secreted hCG and stained positively with GB25, an antibody that recognizes syncytiotrophoblast and cytotrophoblast. Real-time PCR confirmed the down regulation of pluripotent marker genes and the presence of transcripts for several additional trophoblast markers, including *CDX2* and *GATA2* [46].

A more directed conversion to trophoblast can be achieved in two-dimensional cultures when hESC are grown in the presence of growth factors belonging to the BMP family, especially BMP4, the most effective compound tested [47]. Addition of BMP4 between 10 and 100 ng/

ml led to the appearance of larger, more flattened cells at the periphery of the colonies. Over time a “wave of differentiation” progressed inwards towards the center of the colonies at a rate that was dependent on the BMP4 concentration [47,48]. A parallel microarray analysis, performed at fixed times after addition of BMP4 to H1 hESC, indicated an immediate up-regulation of genes encoding a group of transcription factors, some of which have been linked to trophoblast differentiation, e.g. *GATA2*, *GATA3*, *MSX2*, *ID2*. By five days the colonies strongly expressed a range of genes associated with differentiated trophoblast, e.g. *CGA*, *CGB*, *MMP9*, *KRT7*, and *IGFBP3*. Finally, when dissociated cells were plated at a low density in presence of BMP4, some cells fused to form multinucleated patches of cells with a continuous cytoplasm that were positive for hCG. These areas of fused cells resembled syncytiotrophoblast [47]. It remained unclear whether differentiation induced by BMP4 was unidirectional, i.e., directed entirely towards trophoblast, or whether other lineages arose within the colonies. In particular, by 24h there was an up-regulation of genes more typical of endoderm and placental yolk sac than of trophoblast, including ones encoding alpha-fetoprotein (*AFP*), fibrinogen chains (*FGA*; *FGG*), apolipoprotein A4 (*APOA4*), transthyretin (*TTR*) and plasma retinol binding protein [47]. Thus, although hESC may serve as a model for studying extra-embryonic tissue development, there was a likelihood that BMP4 treatment led to the appearance of more than one lineage of cells.

BMP4-driven differentiation of hESC in absence of FGF2 at atmospheric and physiological O₂ concentrations

In the case of hESC, FGF2 is the crucial factor for sustaining proliferation and pluripotency, possibly through its ability to antagonize BMP signaling [49], although some recent evidence suggests that FGF2 up-regulates IGF2 and TGF β production in FGFR-positive cells on the periphery of the colony and that these proteins are the “true” pluripotency factors required by the ES cells [50]. Whatever the role of FGF2 in maintaining “stemness”, its presence in the medium is likely to confound a study of BMP4-driven trophoblast differentiation. Accordingly our laboratory now omits FGF2 when testing BMP4 effects and has confirmed that 10 ng/ml BMP4 can be used successfully to initiate trophoblast formation in H1 and H9 cells [51]. Since O₂ tension is believed to influence both proliferation and differentiation of both villous and extra-villous trophoblast [52-54], we are also examining whether a reduction in O₂ concentration from 20 % to 4 % influences the progressive differentiation of the hESC in response to BMP4. We have employed morphological criteria, hormone assays (hCG, progesterone), immunostaining for specific trophoblast lineage markers [51], and microarray analysis (unpublished data) to follow trophoblast differentiation in H1 and H9 colonies and made the following observations:

1. In absence of FGF2, BMP drives differentiation unidirectionally towards trophoblast, unlike what is observed in EBs. A major difference from the study of Xu et al [47], was that markers of endoderm formation were not up-regulated in our microarray studies (unpublished data).
2. During the initial 1-3 days of exposure to BMP4, cells in the center of colonies begin to pile up, possibly because they continue to divide in space that is progressively restricted in area. The cells that ring this core demonstrate overt signs of morphological differentiation, taking on a flattened appearance (Fig. 1). However, they remain mitotically active and Oct4 positive, and they lack markers for differentiated trophoblast, such as cytokeratin 7 and -8 (Fig. 2B).

As noted by Xu et al. [47], we observed a decline in expression of genes associated with pluripotency, e.g. *POU5F1*, *NANOG*, *SOX2*, and *LEFTY2* over time, although the down-regulation was by no means immediate (Figure 3A). Interestingly, the expression of one gene, *LEFTY2*, was strongly influenced by the oxygen atmosphere under which the cells were cultured.

3. The acquisition of trophoblast differentiation markers, beginning on day 3, proceeds from the periphery towards the interior and is accelerated by about 24 h in cultures under 20 % O₂ relative to those under 4 % O₂. By day 5, most of the cells outside the central core are positive for general trophoblast markers (Fig. 2B). Genes for these markers are also upregulated (Fig. 3B).

4. By day 5, regions contain sub-populations of cells strongly positive for markers of specialized trophoblast cell types (Fig. 2A). The hCG positive cells are syncytial, with multiple nuclei in a continuous cytoplasm [51]. At day 5, hCG is secreted abundantly into the medium of hESC cultured under 20% but is almost undetectable under 4% [51]. Similar results were seen for expression of the CGA and CGB5 genes by microarray analysis (Fig. 3B). Other colony regions stain brightly for HLA-G, generally considered a marker of extravillous cytotrophoblast [51]. Transcription factors such as GATA2 also show heterogeneous staining within the colonies, suggesting complex patterns of [51].

Conclusions

Together the above published and unpublished data indicate the potential usefulness of hESC grown in presence of BMP4 and absence of FGF2 for studying both the emergence of trophoblast from hESC precursors and for its subsequent differentiation over time and space. A number of intriguing questions have arisen during the course of the studies so far. First, are the visibly differentiated, yet OCT4-positive, cells that immediately emerge in the periphery of the colonies treated with BMP4 equivalent to trophoblast stem cell population? Are the strongly OCT4-positive cells remaining within the apparently undifferentiated “core” of the colonies still pluripotent, even after they have been exposed to BMP4 for several days? Why does differentiation proceed from the periphery towards the center of these colonies even though the entire colony is immersed in BMP4-containing medium? In this regard each colony appears to demonstrate “quorum sensing”, a phenomenon well known in colonies of microorganisms [55,56] that must involve some form of chemical communication between individual cells within the colony. A final question is whether the transcription factors up-regulated immediately following BMP4 exposure are key to trophoblast lineage-decision making. These and other questions are currently being addressed in this laboratory.

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Figure 1.

Phase contrast images showing morphology of H1 hESC colonies cultured in the absence (**a**) or presence of 10ng/ml BMP4 for 3 (**b, c**) days. Cells were cultured in low (**a, b**) or atmospheric (**c**) oxygen, and in the presence (**a**) or absence (**b, c**) of FGF2. An area of morphologically differentiated cells, characterized by a cobblestone appearance becomes visible at the periphery of the colony, and progresses inwards under cultures added BMP4 (**b, c**). Central areas in **b** and **c** contain small, presumably undifferentiated cells, appear quite similar to cells in **a**. BMP4 driven differentiation was slower under low oxygen. The scale bar shown in panel **c** represents 1mm.

Figure 2.

Differentiation in BMP4 treated H9 h ESC colonies proceeds from the periphery inward. **(A)** On day 5, cells at the periphery express syncytiotrophoblast marker hCGα (green) and -β (red). Nuclei are counterstained with TO-PRO 3 (blue). **(B)** At day 6, cells in the core stain positively for pluripotency marker Oct4 (red), whereas the rest of the colony expresses the trophoblast marker cytokeratin 7 (green). Scale bars represent 500 μm.

Figure 3.

Changes in microarray expression (with values normalized to the median intensity of the array) for four genes associated with a pluripotent phenotype (*POU5F1*, *NANOG*, *SOX2*, and *LEFTY2*) and four genes that are regularly used as trophoblast markers (*CGA*, *CGB5*, *KRT7*, and *KRT8*). H1 hESC were exposed to BMP4 (10 ng/ml) in either 4% (gray bar) or 20% (black bar) oxygen and collected after 0, 3, 12, 24, 72 and 120 h. These data were obtained on Agilent Whole Human Genome Oligo microarrays. Genes that were changing over time within each oxygen condition were identified by ANOVA. The genes shown here were selected from 33,814 genes of which 5,194 changed expression more than 2-fold at $P < 0.01$ in at least one

time point. Changes in gene expression have been verified by real-time PCR with cDNA from H1 and H9 cells for POU5F1, NANOG, SOX2, LEFTY2, CGA and CGB.