Focus on Stem Cells in Human Reproduction

Embryonic stem cells as models of trophoblast differentiation: progress, opportunities, and limitationsThaddeus G Golos^{1,2,3}, M Giakoumopoulos^{1,2} and M A Garthwaite^{1,2}¹Wisconsin National Primate Research Center, ²Department of Obstetrics and Gynecology and ³Department of Comparative Biosciences, University of Wisconsin, 1223 Capitol Court, Madison, Wisconsin 53715-1299, USACorrespondence should be addressed to T G Golos at Wisconsin National Primate Research Center, University of Wisconsin; Email: golos@primate.wisc.edu**Abstract**

While the determination of the trophoblast lineage and the facilitation of placental morphogenesis by trophoblast interactions with other cells of the placenta are crucial components for the establishment of pregnancy, these processes are not tractable at the time of human implantation. Embryonic stem cells (ESCs) provide an embryonic surrogate to derive insights into these processes. In this review, we will summarize current paradigms which promote trophoblast differentiation from ESCs, and potential opportunities for their use to further define signals directing morphogenesis of the placenta following implantation of the embryo into the endometrium.

Reproduction (2010) **140** 3–9**Introduction**

The earliest lineage commitment and differentiation event in mammalian preimplantation embryo development is the formation of the trophectoderm, heralded by embryo compaction at the morula stage of development 4–6 days following fertilization in human and nonhuman primates, and manifested by blastocyst formation within the next 1–2 days. These trophectodermal cells function as progenitor cells of the trophoblast lineage, giving rise in the primate placenta to cells of villous and extravillous phenotypes. Trophoblasts of the villous phenotype include the relatively undifferentiated villous cytotrophoblasts (CTBs) and the terminally differentiated villous syncytiotrophoblasts, which arise from fusion of the mononuclear CTBs. These villous trophoblasts are found within the chorionic villi which form beginning during the second week after implantation of the human or nonhuman primate embryo into the receptive endometrium (Kaufmann & Burton 1994). A third trophoblast population is found at the tips of the chorionic villi which anchor the placenta to the endometrium. These are CTBs of the cell column (CC).

While the CC anchors the placenta to the decidualized endometrium, an additional population of trophoblasts which expands from the column, the extravillous

trophoblasts (EVTs), invades into the maternal endometrium in the first weeks of gestation. These EVT cells play a key role in the responses to implantation of the maternal vasculature within the decidua. Histologically, two populations of EVT cells are recognizable within the decidua. Endovascular EVT cells are found in the lumen of the spiral arterioles from 6 weeks of human gestation onwards. These cells are likely to be present within maternal vessels earlier, since in the rhesus monkey, endovascular EVT cells are seen having substantially invaded spiral arterioles as early as 1 week after embryo attachment (Slukvin *et al.* 2000, Enders 2007). However, such very early human implantation sites are not readily available for detailed study. In addition to this endovascular location, EVT cells are seen within the decidualized stroma, outside of the blood vessels, and these cells are termed interstitial EVT cells.

Because of the limitations inherent in the study of static histological specimens, the pathway by which endovascular EVT cells arrive within the lumen of the spiral arteriole remains somewhat uncertain. It is possible that EVT cells arrive at an endovascular location primarily if not exclusively by breaching the vessel wall from an interstitial location. Alternatively, EVT cells may access the spiral arteriole lumen directly via erosion of the capillary microvasculature at the most internal region of the functional endometrium, and migrate down the walls of those vessels. Regardless of the route of invasion, vessels which are invaded by trophoblasts ultimately exhibit a loss of the endothelium, loss of vascular smooth muscle,

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and replacement of the tunica intima and tunica media with endovascular EVT (Harris & Aplin 2007). These trophoblasts form vascular channels which, in the absence of the vascular smooth muscle, now provide a nonvasoactive blood conduit to the intervillous space. Regardless of the route of migration, the remodeling of these vessels is a critical event in the first trimester of pregnancy. Poor EVT invasion and failure to appropriately remodel the decidual and distal myometrial vessels are widely associated with poor pregnancy outcomes, including uteroplacental insufficiency, intrauterine growth restriction, and preeclampsia (Redman & Sargent 2005, Pijnenborg *et al.* 2007). Overall placental growth and villous morphogenesis are widely recognized as crucial for pregnancy success. For these reasons, understanding early trophoblast differentiation, villous morphogenesis and placenta formation, and EVT differentiation and invasion are fundamental to considering potential therapeutic approaches for preventing or treating suboptimal implantation and pregnancy complications related to placental development.

Trophoblast differentiation from embryonic stem cells: importance of human and rhesus systems

Very early (<6 weeks of gestation) human implantation sites are not generally available for research or experimental purposes. Ideally, blastocyst-stage embryos should be an excellent starting point for defining the molecular events which underlie trophoblast differentiation, and the interaction of early invasive EVTs with stromal, immune and vascular elements of the decidua. The additional limitations on our understanding of the earliest events in human implantation are imposed by ethical restrictions on the use of human embryos for *in vitro* experimental purposes.

The derivation of human embryonic stem cells (hESCs) from IVF-produced blastocysts donated for research (Thomson *et al.* 1998) provided an unprecedented opportunity for new explorations in early development, of embryonic and fetal cell lineages, including the extraembryonic membranes. It has been known for many years that in general, mouse ESCs differentiate *in vitro* to the trophoblast lineage only poorly, if differentiation is at all noted, although some modifications (e.g. PARP1 deletion by homologous recombination) have been shown to increase the rate of mouse ESC differentiation into trophoblasts (Hemberger *et al.* 2003). When Thomson *et al.* (1998) reported the first derivation of hESCs, they noted that differentiation to trophoblasts was indicated in colonies undergoing spontaneous differentiation, as judged by the secretion of human chorionic gonadotropin (hCG), a generally trophoblast-specific marker essential for the establishment of human pregnancy. This result had in fact been foreshadowed by the previous observation in the Thomson laboratory

that a subset of rhesus monkey ESCs, derived from *in vivo* produced blastocysts obtained by flushing of the uterine cavity 24–48 h before the anticipated day of embryo implantation (Thomson *et al.* 1995), also spontaneously differentiated into trophoblasts, increasing the transcription of the CG- α and - β subunit mRNAs. While CG is typically thought of as a marker of differentiated trophoblasts, it has long been known (but not widely appreciated) that preimplantation human and nonhuman primate embryos already secrete detectable levels of CG *in vitro* (Pope *et al.* 1982, Fishel & Edwards 1984, Hearn *et al.* 1988, Seshagiri *et al.* 1994), most likely as a component of the program of gene expression activated during differentiation to the trophoblast lineage. Thus, CG secretion in spontaneously differentiating human and rhesus ESCs was reasonably interpreted as indicating that these ESCs could serve as a surrogate for blastocyst-stage embryos, at least from the perspective of trophoblast/trophoblast formation. Expression of α -fetoprotein also indicated differentiation of yolk sac (endoderm) in rhesus (Thomson *et al.* 1995) and, later, human (Reubinoff *et al.* 2000) ESCs. The formation of these extraembryonic lineages was exciting in that it allowed for the first time study of placenta formation, not from tissues which had already undergone morphogenesis and differentiation to villous and extravillous lineages (i.e. first trimester placental tissues, or term delivered placentas), but from the 'blank slate' represented by ESCs.

Modifiers of ESC culture conditions for trophoblast formation: bone morphogenetic protein, embryoid bodies, and extracellular matrix

Xu *et al.* (2002) reported that treatment of undifferentiated hESC lines developed at the University of Wisconsin with bone morphogenetic protein 4 (BMP4) or related ligands resulted in their uniform differentiation to cells of an epithelial morphology. Microarray analysis revealed that when compared with undifferentiated cells, many of the most highly up-regulated genes were associated with trophoblast function, particularly transcription factors (chiefly defined in mouse development), and endocrine markers of human trophoblast differentiation, including hCG, and enzymes important for steroid hormone synthesis (Gerami-Naini *et al.* 2004). Interestingly, it has also been reported that the effect of BMP or related ligands on hESCs is very cell line dependent. For example, treatment of the hESC lines also derived from IVF-derived preimplantation human blastocysts (Reubinoff *et al.* 2000, Pera *et al.* 2004) with BMPs results in only sporadic formation of small colonies of CG-expressing cells of epithelial morphology, and the more widespread outcome in this study was the formation of endoderm and yolk sac-like structures. It has not been determined what the specific

differences may be that result in diverse responses to these factors, but derivation and culture conditions, receptor populations, and signaling environments are all likely to contribute to the diversity of differentiation potential among hESC lines derived in different laboratories. While there seems little question that the cells derived from hESCs under various experimental conditions express many features of human placental trophoblasts, it seems prudent to recognize that as they are not yet clearly shown to represent a specific differentiated trophoblast cell type, and indeed, the peri-implantation trophoblast phenotype in human pregnancy remains a formidable challenge to define at a molecular level. Thus, it is reasonable to consider these hESC derivatives as 'trophoblast-like' cells, although for the sake of simplicity, we will refer to them as trophoblasts in this review.

A second approach with H1 and H9 hESCs for trophoblast differentiation from hESCs was identified with subsequent studies (Gerami-Naini *et al.* 2004). Reasoning that mimicking the spherical configuration of the preimplantation embryo with pluripotent hESCs may initiate signals parallel to those acting during preimplantation embryo development, we directed embryoid body (EB) formation by enzymatic release of undifferentiated ESC colonies from culture plates, and maintained them in suspension culture in bacterial grade culture dishes. Within 48 h, there was a significant elevation in the level of hCG secretion in suspension EB culture. Moreover, hCG secretion was accompanied by secretion of progesterone, as well as by secretion of estradiol-17 β (Gerami-Naini *et al.* 2004). Additional studies demonstrated that the endogenous androgens of the FCS were the substrate for cytochrome P450 aromatase, rather than androgens secreted by the EB itself (Gerami-Naini *et al.* 2004). Thus, these hormones represent a footprint of trophoblast differentiation, and recapitulated what was observed in BMP-treated cultures.

However, hormone secretion was not dramatically sustained in the suspension culture paradigm. Again, taking into consideration the *in vivo* environment that the preimplantation embryo is exposed to, we developed an approach, based on previous work done in the Aplin laboratory at the University of Manchester (Aplin *et al.* 1999), modeling EVT invasion of the extracellular matrix of the decidua during implantation. We developed an approach to embed hESC-derived EBs in pre-gelled Matrigel droplets, and transfer those Matrigel 'rafts' to suspension culture. In this situation, within several weeks, there were reliably discernable outgrowths from the EBs into the Matrigel scaffold. In parallel with this invasion, significant maintenance of differentiated function was observed, as manifested by sustained hormone secretion into the culture media (Gerami-Naini *et al.* 2004). Thus, signals conveyed either by extracellular

matrix or by the 3-dimensional environment provide support for sustained trophoblast differentiation.

The BMP- and EB-based approaches should be considered complementary, and each has its strengths and limitations. A significant strength of the BMP-treatment paradigm is that the relatively uniform response potentially allows the investigation of molecular mechanisms which promote the initiation of the trophoblast lineage. This is somewhat tempered by the recognition that the response appears to be divergent among hESC lines, and additionally, it remains unclear which *in vivo* trophoblast population is represented by BMP4-driven trophoblasts. Finally, the *in vivo* role of BMP4 (or related factor(s)) in placenta formation is not known.

It does not seem to have been widely explored if the EB-based approach is cell line dependent; current studies are largely restricted to the use of the original cell lines derived in the Thomson laboratory (Thomson *et al.* 1998). One advantage of the EB-based approach is that it could perhaps be reasoned that the spontaneous formation of trophoblasts may be more likely to represent *in vivo* trophoderm formation. However, this model is complicated by the heterogeneous nature of the EB itself, since all embryonic germ layers differentiate simultaneously during prolonged culture (Gerecht-Nir *et al.* 2004). Thus, the appropriate system should be chosen based on the question to be addressed.

The dynamics of differentiation of hESCs into trophoblast-like cells are influenced by spatial considerations with *in vitro* monolayer culture as well as in the 3-dimensional context of the EBs. In BMP-directed differentiation, Schulz *et al.* (2008) have demonstrated that expression of chorionic gonadotropin, as determined by immunohistochemistry, is more advanced at the outer edges of the adherent hESC colonies. We have also observed (B Gerami-Naini, OV Dovzhenko, MA Garthwaite, M Durning & TG Golos 2006, unpublished observations) that when EBs are cultured as adherent aggregates replated on Matrigel-coated culture dishes (Gerami-Naini *et al.* 2004), the morphology of the outermost cells of the outgrowing cells from the adherent EBs tends to be similar to that shown by Schulz *et al.* (2008). The influences or factors that promote this spatial variation in morphology and function are not well understood. It seems possible that variation in the deposition of extracellular matrix throughout the colony also influences ESC differentiation. Collagen IV has been reported to influence mouse trophoblast differentiation (Schenke-Layland *et al.* 2007). Although not well understood, it also seems likely that there are gradients of regulatory factors, both soluble and likely paracrine or juxtacrine, which influence the progress of differentiation within the ESC colonies as they respond to BMP stimulation. This may also influence the heterogeneity of morphology in BMP-directed differentiation (Schulz *et al.* 2008).

Important areas for further study: additional cell systems

A recent important development in the drive to understand pluripotency is the demonstration first in the mouse cells (Takahashi & Yamanaka 2006), and more recently in the human cells (Takahashi *et al.* 2007, Yu *et al.* 2007), that pluripotency is itself inducible and that differentiation is a reversible state. It has been shown that forced expression of a discrete subset of genes allows the reestablishment of a state of pluripotency in both human cells and mouse cells. The therapeutic potential for these induced pluripotent stem (iPS) cells is significant, with the possibility to overcome transplantation issues in regenerative therapies. The question arises as to whether the pluripotency of iPS cells extends to the ability to differentiate into trophoblasts. Mali *et al.* (2008) have recently reported that BMP4 treatment or EB formation leads to trophoblast differentiation; however, it should be noted that this conclusion was based on immunostaining with a TROMA-1 antibody, which recognizes cytokeratin-7, which is not a specific trophoblast marker. Thus, further study of this question is warranted.

Aggregation studies and morphogenesis models

One of the unique opportunities that hESCs (and primate ESCs) provide is the opportunity to develop models for early morphogenesis and development for areas where there are physiological or morphological limitations to rodent models. One of these areas is in the formation of the placenta. During early development, nontrophoblastic elements from the fetal embryonic germ layers (fibroblasts and endothelia) will become invested in the developing trophoblast during the formation of chorionic villi. With the hypothesis that these nontrophoblastic elements will influence placental morphogenesis, approaches will need to be developed in which they can become integrated into the EBs, and influence their morphology and function in either a suspension culture or a 3-dimensional environment. A platform for this approach, we have recently found, is the Aggrewell system (Stem Cell Technologies, Vancouver, BC, Canada), which utilizes a micropatterned culture surface and centrifugal forced aggregation to direct the formation of EBs of defined cell numbers (Ungrin *et al.* 2008). The differentially stained ESCs and fibroblasts shown in Fig. 1 illustrate the ability to consistently prepare aggregated EBs of experimentally determined heterogeneous composition (Fig. 1). Studies are underway to define trophoblast differentiation in these composite EBs.

ESC-derived trophoblasts: modeling endovascular invasion and trophoblast-endothelial interaction

Early in human pregnancy, the invasion and remodeling of the maternal spiral arterioles by EVT's provide the basis

Figure 1 Aggrewell-generated combination EBs. Aggrewell-generated EBs comprised 1000 cells (Ungrin *et al.* 2008). Term placental fibroblasts were stained for 1 h with 25 μ M Cell Tracker Red (Invitrogen), and ESCs were stained at the same concentration with Cell Tracker Green. Fibroblasts were then trypsinized, counted, and seeded at 600 000 cells per Aggrewell. ESCs were enzymatically dispersed with Accutase, counted, and also seeded at 600 000 cells per Aggrewell, bringing the total number of cells per Aggrewell to 1 200 000 (as per the manufacturer's recommendation for the production of 1000-cell EBs). The ROCK inhibitor (Y-27632) was added to each well at a concentration of 10 μ M, and cells were centrifuged into the Aggrewells and left overnight at 37 °C with CO₂ and 95% humidity to form EBs. On the following day, EBs were gently pipetted and passed through a 40- μ m cell strainer to remove excess cellular debris, and were imaged. The photomicrograph illustrates the presence of an internal core of red fibroblasts and an outer zone of green ESCs. Scale bar = 0.1 mm.

for the establishment of a low resistance, high blood flow environment allowing for greater nutrient and gas exchange to the developing fetus. Human umbilical vein endothelial cells (HUVECs) provide an established endothelial cell model for angiogenesis and endovascular remodeling. Studies have shown that the first trimester EVT cell line HTR8 will undergo spontaneous migration toward, and align with endothelial cells in contrast to term primary trophoblasts and immortalized third trimester EVT's (TCL1) that actually inhibit endothelial cell tube formation (Kalkunte *et al.* 2008). In addition, vascular endothelial growth factor C is secreted at higher levels from the first trimester EVT's (HTR8) compared with the third trimester and term trophoblasts, further establishing the differential function of the first trimester cells. Localization of angiogenic growth factors in the placental bed has further established the angiogenic potential of different trophoblast subtypes (Schiessl *et al.* 2009).

Although the use of first trimester placental tissue and cell lines provides useful information about pregnancy, most of the tissues and established cell lines originate from weeks 8 to 12 of gestation. We therefore have extended the use of the hESC-derived EB paradigm to study the HUVEC migratory potential in response to EB-derived trophoblastic outgrowths, and conversely, the EB-derived trophoblastic outgrowth migratory potential in response to HUVECs. The aims of these experiments are to better understand the bi-directional relationship between early pregnancy trophoblasts and endothelial cells *in vitro*. Pilot studies shown in Fig. 2 (M Giakoumopoulos & TG Golos 2010, unpublished observations) illustrate the time-dependent migration of trophoblastic outgrowths from EBs plated on Matrigel-coated invasion chambers. Demonstration that trophoblast-like cells derived from hESCs have biological characteristics of human placental cells will be important to define their trophoblast identity.

Our previous studies demonstrating close association of macrophages with decidual vessels in the first week of rhesus monkey gestation (Slukvin *et al.* 2004), and recent studies of decidual vessels in human pregnancy at 8–12 weeks of gestation (Smith *et al.* 2009), are in concordance with endothelial-leukocyte dialog in early gestation as well. Leukocyte-derived chemokines have been shown to influence trophoblast migration in a immunodeficient mouse transplant model (Hanna *et al.* 2006). Further definition of endothelial, leukocyte, and

chemokine influences on the migration of an EB-derived trophoblast can provide an opportunity to interrogate the mechanisms that provide the cues for the development of the human implantation site.

Future directions: further studies with rhesus ESC

The derivation of hESCs was preceded by the isolation of cells from rhesus monkey preimplantation blastocysts with ESC-like characteristics. These included the expression of markers of pluripotency including alkaline phosphatase, Tra-1-60, Tra-1-81, SSEA3, and SSEA4 (Thomson *et al.* 1995). In addition, there was morphological formation of derivatives of all three embryonic germ layers upon spontaneous differentiation, in the context of teratomas when injected into immunocompromised mice (Thomson *et al.* 1995), and spontaneous formation of trophoblast as evidenced by the transcription of CG- α and - β subunit genes. Based on the efficient differentiation of hESCs to trophoblasts with the EB- and BMP-regulated paradigms, we anticipated that these would as well be able to induce trophoblast differentiation in rhesus ESC lines. However, in ongoing studies, we have not found this to be the case. The differentiation of trophoblast populations from rhesus ESCs has proved to be very highly dependent on the cell line used (MA Garthwaite & TG Golos 2007, unpublished observations). A potentially important difference between the human and rhesus ESCs is the fact that the rhesus ESCs derived in the Thomson laboratory were from *in vivo* produced blastocysts, whereas all hESCs have been derived from IVF-produced embryos. Further studies are needed with rhesus ESCs derived from IVF-produced embryos (Mitalipov *et al.* 2006) or from rhesus nuclear transfer-generated blastocysts (Byrne *et al.* 2007).

Figure 2 Migration of EB-derived trophoblasts in Transwell invasion chambers. Left panel: schematic diagram of experimental setup, illustrating two possible treatments to identify effects on trophoblast invasion/migration. Right panel: time-dependent migration of EB-derived trophoblastic outgrowths through the chamber membrane. The mean of five photomicrographic fields from each of three independent chambers is shown.

Epigenetic alterations with ESC differentiation

Recent studies with mouse ESCs and mouse trophoblast stem cells (TSCs) have provided insight into the epigenetic mechanisms that influence trophoblast lineage specification (Ng *et al.* 2008). TSCs can be derived from the trophectoderm of the mouse blastocyst under selective culture conditions, and can give rise to all the differentiated trophoblasts of the mouse placenta (Tanaka *et al.* 1998). In early embryonic development, cells of the inner cell mass undergo global DNA methylation, whereas the cells of the trophectoderm remain in a hypomethylated state (Santos *et al.* 2002). In mouse ESCs in which the maintenance DNA methyltransferase *Dnmt1* has been deleted, culture conditions that support TSC growth dramatically increased the formation of trophoblast giant cells from ESCs *in vitro* (Ng *et al.* 2008). In these cells, a progression of transcription factor expression similar to that seen with TSC differentiation was also observed.

Ng *et al.* (2008) demonstrated that a similar increase in trophoblast differentiation was induced by treatment with the DNA methylation inhibitor 5-azacytidine. In particular, the Ets family transcription factor *Elf5* was shown to be differentially methylated in TSCs versus ESCs, and it formed a positive feedback loop with the transcription factors *Cdx2* and *Eomes*, which are critical for trophoblast lineage determination (Rossant 2007). This study may have significance for understanding the ability of human and nonhuman primate ESCs to form of trophoblast-like cells. It would seem to be imperative to define the methylation status of key genes in trophoblast differentiation with the BMP-directed or EB-initiated formation of trophoblast-like cells. Comparison with the methylation status of human placenta-derived trophoblasts will help clarify the status of derivatives of pluripotent ESCs originally derived from human and nonhuman primate embryos (as well as iPSC). Additionally, the methylation status of transcription factors critical for trophoblast differentiation may provide insight into the apparent differences in differentiation potential among hESCs derived from different laboratories (Xu *et al.* 2002, Pera *et al.* 2004) or between different rhesus ESC lines derived under similar conditions (MA Garthwaite, OV Dovzhenko & TG Golos 2007, unpublished observations).

Summary and future prospects

While the use of human ESCs to study trophoblast differentiation is of significant merit, progress needs to be made in several key areas in order to further refine the approaches currently available. First, it is very important to determine what *in vivo* trophoblast population(s) the hESC-derived trophoblasts represent. A better understanding of the human placental trophoblasts during the first weeks of pregnancy would be ideal, but it is not likely to be easily achieved, and nonhuman primates will likely be the surrogate for this information. There is a pressing need for this information. As trophoblast phenotypes are defined, new opportunities may arise to study trophoblast–leukocyte interactions. The differentiation of all leukocyte lineages from hESCs has been recently advanced dramatically (Vodyanik *et al.* 2005), and the ability to derive autologous leukocytes for hESC-derived trophoblast studies is highly attractive. Finally, while the *in vitro* hESC-derived trophoblast models are important, of equal importance is the need to develop *in vivo* opportunities to study ESC-derived trophoblast function. Again, this arena will likely be restricted to nonhuman primate models, where it is realistic to be able to conduct *in vivo* studies with ESC derivatives. As methods for the development of rhesus monkey or other primate iPSCs are refined, autologous sources of ESC-differentiated derivatives will be essential for developing *in vivo* models.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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