0163-769X/09/$20.00/0
Printed in U.S.A.

Endocrine Reviews 30(3):228–240

doi: 10.1210/er.2009-0001

Trophoblast Stem Cells: Models for Investigating Trophectoderm Differentiation and Placental Development

Gordon C. Douglas, Catherine A. VandeVoort, Priyadarsini Kumar, Tien-Cheng Chang, and Thaddeus G. Golos

Department of Cell Biology and Human Anatomy, School of Medicine (G.C.D., P.K.), and California National Primate Research Center and Department of Obstetrics and Gynecology (C.A.V.), University of California-Davis, Davis California 95616; and Departments of Comparative Biosciences, School of Veterinary Medicine, and Obstetrics and Gynecology, School of Medicine and Public Health (T.G.G.), and the Wisconsin National Primate Research Center (T.-C.C., T.G.G.), University of Wisconsin-Madison, Madison Wisconsin 53715

The placenta is an ephemeral organ containing diverse populations of trophoblasts that are all derived from the embryonic trophectoderm but have morphological, functional, and molecular diversity within and across species. In hemochorial placentation, these cells play especially important roles, interfacing with and modifying the cells of the maternal decidua. Within the rapidly growing placenta, it has been shown that there are trophoblast stem cells well characterized in the mouse and postulated but not well understood in primates. This review will discuss the characteristics of candidates for human and nonhuman primate trophoblast stem cells, present the diverse methods of their generation, and propose future prospects for experimental systems in which they can shed light on developmental and pathophysiological processes in human pregnancy. (*Endocrine Reviews* 30: 228–240, 2009)

First Published Online March 18, 2009

Abbreviations: BMP, Bone morphogenetic protein; CG, chorionic gonadotropin; EOMES, eomesodermin; ESC, embryonic stem cell(s); FGF, fibroblast growth factor; hCG, human CG; HIF, hypoxia-inducible factor; ICM, inner cell mass; TSC, trophoblast stem cells.

***Endocrine Reviews* is published by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

I. Introduction

STUDIES USING MAMMALIAN embryos have provided considerable insight into the factors that regulate the formation and maintenance of trophectoderm—the outer epithelial layer of the blastocyst that gives rise to the trophoblasts of the placenta. These studies have demonstrated that a complex network of transcription factors regulates trophectoderm formation (see Ref. 1 for a recent review). Although there are similarities among species, it is clear that there are also differences in certain aspects of the regulation of trophectoderm formation and trophoblast differentiation in the mouse and higher primates (2–4). This is perhaps not surprising given the differences in the timing of implantation and in the morphology of placentation between the mouse and higher primates (5). For these reasons, understanding the mechanisms that regulate trophoblast differentiation and placental development across species presents a daunting challenge.

For several decades we and many other laboratories have successfully used cytotrophoblasts isolated from human and rhesus monkey placental villous tissue as model systems with which to study the regulation of villous trophoblast differentiation and function. Trophoblasts isolated from early gestation human and nonhuman primate placental/endometrial tissues include both villous and extravillous phenotypes. Cells with extravillous trophoblast characteristics have also been obtained from outgrowths of early gestation human placental villous explants. These primary cells have the disadvantage that they do not replicate extensively *in vitro* and must repeatedly be isolated from fresh tissue. Each of these placental tissue-derived cell systems takes ad-

vantage of the fact that some kind of trophoblast progenitor cell is present within the tissue and can be manipulated *in vitro* to undergo (or perhaps complete, if pathway selection has already occurred) differentiation along the villous and/or extravillous pathway. It is unclear whether trophoblast progenitors in early gestation villous tissue are similar to trophoblast progenitors in trophectoderm, but this seems unlikely given the dramatic morphological and biochemical transformations that take place from the time of implantation through the formation of the definitive placenta later in the first trimester (when human placental tissues become available for study).

In the past decade, using mouse genetic and stem cell-based paradigms, remarkable progress has been made in understanding the pathways that control the formation of placental trophoblasts. In this review, we will focus primarily on efforts to advance the study of primate (human and nonhuman) trophoblast biology, making reference to important principles revealed by mouse systems as appropriate. The reader is referred to other excellent reviews for further detailed discussion of progress in the mouse model (1, 6, 7).

II. Trophectoderm-Derived Trophoblast Stem Cells

Embryonic stem cells (ESC) classically derived from the inner cell mass of mouse embryos have been shown to be totipotent; that is, an individual mouse ESC can give rise to all tissues in the body as demonstrated through aggregation with mouse preimplantation embryos and analysis of the resultant offspring. Thus far, this signal characteristic has only been demonstrated for ESC derived from mouse embryos, or recently, induced pluripotent stem cells reprogrammed from adult mouse cells (8). Cells derived from the inner cell mass of human or rhesus monkey blastocysts also have extensive pluripotency, in that they can give rise *in vitro* to derivatives of all the embryonic germ layers, and these cells are conventionally referred to as ESC. However, primate ESC have not been demonstrated to be pluripotent by the classic criterion of aggregation and differentiation to tissues in live offspring. Although not ethically feasible to test pluripotency with human ESC and embryos, it remains possible with resources developed from nonhuman primates.

Trophoblast stem cells (TSC) are defined as pluripotent cells whose differentiated derivatives are restricted to the trophoblast lineages. Upon aggregation with mouse embryos, they can give rise to all trophoblast elements of the definitive mouse placenta (9), but they do not contribute to the embryonic germ layers giving rise to the tissues of the fetus proper. TSC derived directly from trophectoderm offer an appealing system with which to investigate the regulation of trophoblast differentiation because they putatively represent the earliest trophoblast progenitor population (10).

There have been efforts, where approved by local ethical oversight, to directly derive TSC from human embryos (11). The methods developed to successfully derive TSC from mouse embryos have not been reported to yielded comparable cells from human blastocysts. It is not clear why this should be the case. However, the growth factor requirements and regulatory pathways for ESC derivation and differentiation are diverse between mouse and human. For example, it was determined early on during work on nonhuman primate (12) and subsequently human (13) ESC derivation, that whereas leukemia inhibitory factor is critical for sustaining mouse ESC, it was ineffectual in the human system. The pathways that sustain undifferentiated growth in human ESC have subsequently been shown to rely primarily on fibroblast growth factor (FGF) 2 and TGF-β/activin signaling, directing suppression of bone morphogenetic protein (BMP) signaling, for undifferentiated growth of ESC (14). It seems reasonable to predict that TSC could eventually be derived from human embryos given sufficient material with which to evaluate a broad range of culture conditions; however, the ongoing scarcity of these resources for experimental purposes makes the development of alternative approaches necessary for progress beyond the mouse model.

A. Growth factor requirements of trophectoderm-derived stem cells

In the mouse, TSC are readily obtained by culturing cells from the extraembryonic ectoderm of implanting embryos or from outgrowths of cultured blastocysts (9, 11). A key feature of mouse TSC is their expression of transcription factors shown to be crucial in placental development, the homeobox transcription factor CDX2, the T box factor eomesodermin (EOMES), and the steroid receptor superfamily member estrogen receptor-related protein-b. This review will not provide a detailed account of the work relating to these transcription factors because the topic is well-covered elsewhere (1, 3, 15). Survival of mouse blastocyst-derived TSC requires FGF4 and embryonic fibroblast-conditioned medium (supplying TGF-β and activin-A) (16). In the presence of these factors, mouse TSC exhibit sustained undifferentiated proliferation, without significant expression of the phenotypic markers of placental trophoblasts, such as placental lactogen, or placental prolactin-related proteins. Removal of FGF4 results in rapid trophoblast giant cell formation and onset of hormone gene transcription (9).

Cells with TSC characteristics have also been obtained from outgrowths of bovine and porcine blastocysts (17, 18), although their analysis has been much more limited than in the mouse. Isolation of trophectoderm-derived stem cells from primate embryos proved more difficult, and it is only recently that cells with TSC characteristics were obtained from rhesus blastocyst outgrowths plated on collagen-coated dishes after initial expansion with rhesus embryonic feeder cells (19). These cells were maintained as continuously replicating cultures in the absence of growth factors or feeder layers, distinguishing blastocyst-derived TSC in the mouse and the rhesus monkey.

As with mouse and primate ESCs, the growth factor requirements for maintaining rhesus trophectoderm-derived stem cells also differ from those of mouse TSC. Bovine and porcine trophectoderm-derived stem cells also require feeder layers for maintenance, although in one report bovine TSC were maintained in the presence of fibroblast-conditioned medium alone (18). On the other hand, we found that rhesus trophectoderm-derived cells could be maintained in culture without feeder layers or the addition of BMP4 or FGFs (19).

It is likely important to note that whereas rhesus trophectoderm-derived trophoblasts do not require endogenous growth factor addition for their expansion, it may be that specific constituents of the culture medium, which contains 15% fetal bovine serum, specifically support growth.

Rhesus trophectoderm-derived trophoblasts express trophoblast markers CGβ, CD9, and CK7 and form multinucleated syncytiotrophoblast-like structures. Surprisingly, the cells do not express the mouse trophectoderm marker CDX2, although they do express EOMES. However, when these cells are cultured in the presence of activin-A, CDX2 expression (determined by Western blotting) is increased (P. Kumar and G. C. Douglas, unpublished observations). It is interesting to compare these findings with the effects of activin-A on human first trimester villous explants. In the latter case, activin appears to promote outgrowth of extravillous cells, suggesting that it may be involved in cytotrophoblast column formation *in vivo* (20, 21). Activin has also previously been shown to increase the secretion of human chorionic gonadotropin (hCG) by human villous syncytiotrophoblasts (22), suggesting that it may promote trophoblast differentiated function during pregnancy as well as trophoblast expansion in culture paradigms.

B. Transcription factors involved in trophoblast stem cell maintenance and differentiation

The maintenance of progenitor or stem cell pluripotency and initiation of differentiation are controlled by subtle changes in the expression levels of a network of transcription factors (23–25) as well as by interactions between them. As far as trophectoderm formation is concerned, key roles have been ascribed to the POU-family transcription factor POU5F1 (OCT4) and the homeobox domain transcription factor CDX2 (26, 27). With OCT4 as a key regulator maintaining pluripotency, studies of the effects of reduced expression have helped provide further insight into the important role of CDX2 in the mouse for trophectoderm differentiation. Studies with mouse ESC have demonstrated differentiation to the trophectoderm lineage upon conditional repression of OCT4 (27–29), and this differentiation relies on interactions of CDX2 with OCT4 in regulation of OCT4 target genes (26). CDX2 is essential for entry to the trophectoderm lineage, although it has recently been shown to be downstream of TEAD4 in trophoblast lineage specification in the mouse (30, 31).

The role of OCT4 and particularly CDX2 in human and nonhuman primate trophectoderm formation and in TSC differentiation is less clear than in the mouse, and it may be different. In human blastocysts, OCT4 is expressed by trophectoderm, although at lower levels than the inner cell mass (ICM) (32). Expression of OCT4 by both the ICM and trophectoderm has also been reported for porcine and bovine embryos (3, 33). OCT4 expression was found in the ICM and trophectoderm of early rhesus monkey blastocysts, although in hatched blastocysts expression it was only found in the ICM (34). Thus, unlike the mouse, OCT4 expression may not be restricted to pluripotent cells in all preimplantation embryos. OCT4 is expressed in undifferentiated human ESCs and in early differentiated ESCs (35) but is lost as differentiation proceeds (36). OCT4 is expressed by rhesus ESCs (12, 37, 38) but was still detected as the cells differentiated (39).

An important difference between OCT4 expression in mice and primates is the existence of splice variants in the human and the rhesus (40, 41), termed OCT3A and OCT3B, arising from alternative splicing (40). Two splice variants are also expressed in cynomolgus monkeys (*Macaca fascicularis*) (41). In the human blastocyst, OCT3A and OCT3B have different intracellular distributions suggesting possible differences in function (25). In cynomolgus monkey ESCs, transient overexpression of OCT3A caused ESC differentiation into endodermal and mesodermal lineages, whereas overexpression of OCT3B failed to induce ESC differentiation (41). The existence of these splice variants indicates that care should be used in the selection of antibodies used for expression studies. Similarly, the presence of OCT4 pseudogenes can confuse the interpretation of RT-PCR data unless primer design is carefully controlled (42).

Other studies with human or primate oocytes or embryos indicate that the intracellular dynamics of critical transcription factors warrant careful attention. For example, OCT4 transcripts are expressed by human oocytes and cleavage stage embryos, but OCT4 protein is found in the cytoplasm. In compacted embryos, OCT4 is found in the nucleus (25). Other results in the same study also suggest that the intracellular distribution of OCT4 variants (OCT3A and OCT3B) is different. Other transcription factors (including CDX2; see *Section C*) can also be found in the cytoplasm or nucleus (or both) depending on developmental stage or cell type (43).

C. CDX2 and trophectoderm determination

Microarray studies show that *CDX2* transcripts are expressed by trophectoderm isolated from human blastocysts (44). Interestingly, CDX2 expression was not discussed in a review of primate ESCs (45) or in recent reports dealing with rhesus ESC characterization (37, 38). Previously we reported that rhesus trophectoderm did not express CDX2 based on immunocytochemical staining of blastocysts (19). However, the antibody (ab22586; AbCam Inc., Cambridge, MA) used for characterization has since been withdrawn by the manufacturer due to concerns about specificity. Using a better-characterized anti-CDX2 antibody (clone CDX2–88; CellMarque, Rocklin, CA) we have been able to confirm that rhesus trophectoderm does express CDX2 (Fig. 1). Similar results have been reported in abstract form (46). We have so far not been able to confirm CDX2 expression by the initial cell outgrowths derived from rhesus blastocysts. After many passages in the absence of added growth factors or a feeder layer, rhesus blastocyst-derived TSC do not express CDX2 (measured by immunocytochemistry, Western blotting, and RT-PCR). However, CDX2 expression can be induced by culturing these cells with activin-A (see *Section A*).

To our knowledge, no forced CDX2 expression or silencing studies have been published using primate stem cells. In the cow, trophoblasts covering the embryonic disc express OCT4 and NANOG, but these trophoblasts show no expression of EOMES and only weak expression of CDX2 (47). It is therefore not clear whether CDX2 regulates trophectoderm formation and proliferation in primates (and ruminants) as it

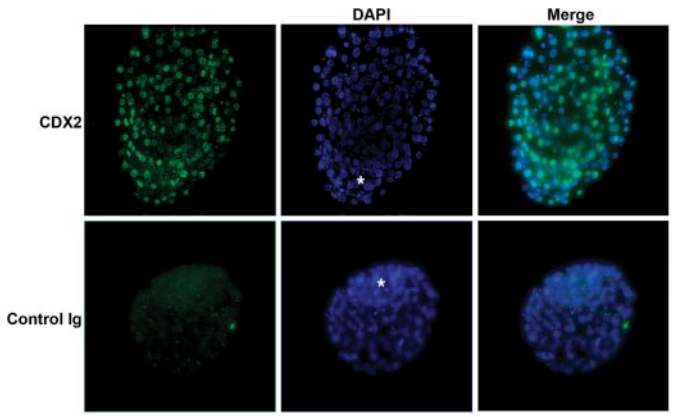

FIG. 1. Expression of CDX2 by the rhesus monkey blastocyst. Hatched rhesus blastocysts were fixed in paraformaldehyde, permeabilized with Triton X-100, and then incubated with mouse monoclonal anti-CDX2 antibody (CDX2–88, Cell-Marque, Rocklin, CA) followed by AlexaFluor488-conjugated rabbit antimouse secondary antibody (green fluorescence). Controls were incubated with isotype-matched mouse Ig instead of the anti-CDX2 antibody. Nuclei were stained with 4′,6-diamidino-2-phenylindole (*blue*). The location of the inner cell mass is indicated by an *asterisk*.

does in the mouse. Also, although TEAD4 appears to regulate CDX2 expression in the mouse (30, 31), its role in regulating CDX2 expression and trophectoderm lineage specification in primates has not been established.

D. Role of FOXD3 in trophoblast stem cell lineage determination

A transcription factor that has received relatively little attention with regard to trophoblast differentiation is the winged helix transcription factor FOXD3, which belongs to the Forkhead Box (Fox) family (48). All members share the highly conserved forkhead domain that is responsible for DNA binding and also for interaction with homeodomain transcription factors. FOXD3 and OCT4 have been shown to bind to identical regulatory DNA sequences, and OCT4 can bind to the FOXD3 DNA binding site, thereby repressing its activity (49).

Much of what is known about FOXD3 has come from studies of its role in neural crest determination in several species. However, FOXD3 also plays a crucial role in the maintenance of progenitor cells in both the epiblast and trophectoderm in the mouse (50, 51). Trophoblast progenitors in null Foxd3 mouse embryos are unable to self-renew and are not multipotent (51). The mutant cells give rise to an excess of trophoblast giant cells without development of the spongiotrophoblast and labyrinthine lineages. FOXD3 is expressed by trophoblast giant cells in the gastrula-stage mouse embryo, but expression is down-regulated at later stages of development. Recent studies using mouse ESCs harboring an inducible FOXD3 deletion mutation suggest that FOXD3 functions to maintain ESCs in an undifferentiated state by repressing differentiation toward extraembryonic and embryonic lineages (51).

Whereas FOXD3 is expressed by human and rhesus ESCs (37, 52) and its expression appears to be regulated by OCT4, virtually nothing is known of its role in trophectoderm/trophoblast differentiation in human and nonhuman primates. Indeed, a general assumption seems to be that FOXD3 expression is restricted to pluripotent stem cells and to multipotent neural crest cells.

Our RT-PCR, Western blotting, and immunocytochemical results (Fig. 2) show that FOXD3 is expressed by rhesus blastocyst-derived TSC as well as by primary cultures of early gestation rhesus trophoblasts. In some of these cells (Fig. 2C, *upper and middle rows*), FOXD3 is found only in the cytoplasm, whereas in other cells both a nuclear and cytoplasmic distribution is evident. We also found strong cytoplasmic FOXD3 expression in villous cytotrophoblasts and syncytiotrophoblasts (identified by positive cytokeratin staining) in sections of early gestation macaque placental/decidual tissue (Fig. 2C, *bottom row*). Trophoblasts in the cell columns (also identified by positive cytokeratin staining) of anchoring villi had low/absent FOXD3 expression, but expression was found in extravillous trophoblasts in the trophoblastic shell. The latter cells showed both nuclear and cytoplasmic FOXD3 expression. The persistence of FOXD3 expression in differentiated villous trophoblast cells and extravillous trophoblasts is clearly different from the pattern of expression in the mouse placenta and raises the obvious question as to its function in these cells. The exclusive cytoplasmic expression of FOXD3 in villous trophoblasts suggests that an extranuclear function predominates in these cells. Nonnuclear, nontranscriptional roles have been ascribed to other transcription factors (53–55), and so a cytoplasmic function for FOXD3 in villous trophoblasts cannot be discounted. The nuclear expression of FOXD3 in some extravillous trophoblasts in the trophoblastic shell region suggests that it could be active as a transcriptional regulator. Although the exact role of FOXD3 in extravillous trophoblasts is not currently known, we are investigating the idea that FOXD3 somehow regulates migratory behavior or the formation of migratory trophoblasts.

E. FOXD3 is expressed by migratory cells and regulates adhesion molecule expression

FOXD3 is generally considered to be a transcriptional repressor and to be involved in the maintenance of pluripotency. However, FOXD3 can also function as a transcriptional activator (23), and additional roles for FOXD3 are emerging particularly with regard to the differentiation of migratory cell phenotypes. FOXD3 is expressed in premigratory and migratory neural crest cells, and ectopic expression of FOXD3 was found to induce the expression of migratory neural crest markers and stimulate their migratory activity away from the neuroepithelium (56). During this epithelial-mesenchymal transformation, FOXD3 appears to regulate the expression of cell-cell adhesion molecules such as β1 integrin and cadherin 7 that are required for neural crest migration (57). While promoting the neural crest cell fate, FOXD3 blocks dorsal neuroepithelial cell differentiation into interneurons (56).

The formation of invasive trophoblasts has some features in common with epithelial-mesenchymal transformation (58). Changes in E-cadherin expression occur during embryogenesis and trophoblast invasion in the mouse and human

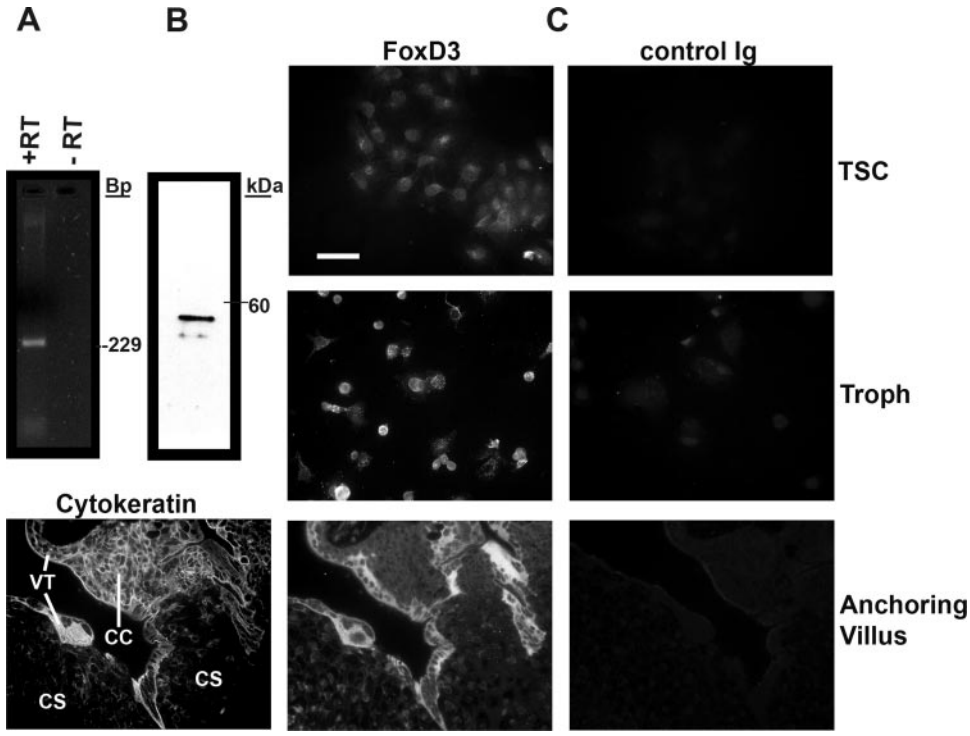

FIG. 2. Expression of FOXD3 by rhesus trophoblasts. A, RT-PCR analysis of rhesus trophoblast stem cells (1192T). RNA was incubated with or without reverse transcriptase (RT), and the cDNA was analyzed using primers against FOXD3 (forward, caccagcagcccctgacatt; reverse, gtgatgagcgcgatgtacga; product size 229 bp). B, Western blot analysis of trophoblast stem cells. Cells were lysed, heated in sample buffer, and subjected to Western blotting using rabbit anti-FOXD3 antibody (Ab64807; AbCam Inc., Cambridge, MA). C, Immunofluorescence analysis of FOXD3 expression in rhesus TSC, primary culture of early gestation trophoblasts (Troph), and paraffin-embedded placental/endometrial tissue (anchoring villus). Cultured cells were fixed and permeabilized using paraformaldehyde and Triton X-100. Tissue from a pregnant (GD35) rhesus implantation site was fixed in formalin and embedded in paraffin. Sections were subjected to antigen retrieval (Antigen retrieval tablets; GeneTex, Inc., San Antonio, TX). Fixed cells and tissue sections were stained using rabbit anti-FOXD3 antibody (AbCam), followed by AlexaFluor488-labeled goat anti-rabbit Ig. Tissue sections were double-stained with a mouse anti-pan-cytokeratin antibody (Biomeda Corp., Foster City, CA), followed by a AlexaFluor647-labeled goat antimouse secondary antibody. Controls were incubated with nonimmune rabbit Ig instead of the anti-FOXD3 antibody. CC, Cytotrophoblast column; CS, cytotrophoblast shell; VT, villous trophoblast. The *scale bar* represents 50 μm.

(59, 60). Changes in integrin expression and metalloproteinase expression also occur during the formation of invasive trophoblasts (61). It is tempting to speculate that FOXD3 may play a role in trophoblast lineage specification in primates either by promoting or suppressing differentiation of villous and/or extravillous trophoblasts. However, this hypothesis is essentially unexplored.

F. Additional considerations

Because canonical transcription factor function is dependent on nuclear localization, this implies that more attention should be given to understanding the regulation of transcription factor nucleocytoplasmic shuttling. Some information about the regulation of OCT4 nuclear import is already known for mouse ESCs (62). Phosphorylation of CDX2 regulates its nuclear translocation and degradation in human intestinal epithelial cells (63, 64). The mechanisms that regulate FOXD3 nucleocytoplasmic shuttling are not known. However, Akt-mediated phosphorylation of certain other forkhead family transcription factors causes their exit from the nucleus and retention within the cytoplasm (65, 66). Other studies have shown changes in the expression of importins and exportins during embryogenesis and stem cell differentiation (67, 68), but more work is needed in the context of trophectoderm maintenance and differentiation. Finally, transcription factor (including OCT4) expression/function may also be controlled by epigenetic factors (69, 70) and by exogenous factors such as hormones, growth factors, or oxygen levels (27, 71–73). These are all recognized as critical influences in preimplantation embryo development.

G. Summary

The availability of TSC offers real advantages to advancing our understanding of trophoblast differentiation and function. However, the approaches for deriving TSC in human and nonhuman primates from preimplantation embryos still require development. The ethical considerations and/or expense of conducting studies on periimplantation embryos and very early pregnancy implantation sites limit the studies that can be performed *in vivo* to elucidate trophoblast function and mechanisms of differentiation. Such *in vivo* studies in the murine model have provided the basis for validating TSC lines with specific gene expression profiles (10), but without similar guidance in primates the assumption cannot be made that *in vitro*-derived TSC function in an identical manner to their *in vivo* counterparts. The rhesus monkey

model is particularly important in determining appropriate markers for primate TSC function because the gene expression of embryos and early TSC outgrowths can be monitored without the ethical concerns of such work using human embryos. With improved methods for quantitative PCR and dot blot hybridization, it is possible to monitor gene expression in single primate embryos and in very small cell numbers such as those available during the initial outgrowth of the trophectoderm (74).

III. Embryonic Stem Cells as a Source for the Derivation of Trophoblast Stem Cells

The distinct advantage of deriving TSC from nonhuman primate embryos and those of other experimental and domestic species is the opportunity to directly study those cells with *in vivo* aggregation and microinjection studies. These studies will provide the best opportunities for testing hypotheses on maternal-placental signaling, transcriptional control of differentiation, and immune influences on endometrial biology, areas of placental biology with direct impact on pregnancy success and pathophysiological implantation. However, it is of course most desirable to be able to directly test, where possible, cellular and molecular hypotheses with human-derived cells to best consider possible therapeutic approaches for threatened or failing pregnancies. With the advent of embryonic stem-like cells from primates, the possibility of these cells serving as embryonic surrogates for the study of trophoblast development has been of interest. This section will summarize the current findings and future opportunities with emphasis on human and nonhuman primate cells.

A. Spontaneous differentiation of ESC to trophoblasts

With the first studies of rhesus monkey ESC, it became clear that in addition to growth factor dependence, there were other fundamental differences between mouse and primate ESC. Mouse ESC do not contribute to the trophoblast lineage in aggregated preimplantation embryos, and the *in vitro* differentiation of trophoblasts from mouse ESC only proceeds under certain experimental circumstances. As mentioned earlier, OCT4 repression allows CDX2 to promote formation of the trophectoderm lineage (26). Mouse ESC lacking poly (ADP-ribose) polymerase expression show enhanced spontaneous differentiation of trophoblast giant cells (75). Recently, it has also been reported that collagen type IV, a basement membrane constituent, can direct differentiation of mouse ESC toward the trophectoderm lineage (76).

Rhesus ESC allowed to spontaneously differentiate in culture led to the formation of trophoblasts, as revealed by the secretion of chorionic gonadotropin (CG) into the culture medium (12). The secretion of CG was confirmed by increased gene expression of both the α- and β-subunits of rhesus CG (12), which is a primate-specific marker of trophoblast differentiation. With the subsequent derivation of human ESC, the novel attribute of trophoblast differentiation seen with rhesus ESC was underscored in that human ESC likewise were able to spontaneously differentiate into trophoblasts, again, with the secretion of hCG as the signal hallmark of differentiation.

B. Directed trophoblast differentiation from human ESC

The use of human ESC as a model for studying early trophoblast differentiation was subsequently moved forward with a serendipitous observation made by Ren-He Xu in the Thomson laboratory (77). In the continuous presence of FGF2 and mouse embryonic fibroblast-conditioned medium (which typically sustain undifferentiated human ESC growth), addition of BMP-4 (or related ligands for BMP receptor IB, BMP-2, -7, or GDF-5) resulted in the differentiation of human ESC to cells of a uniform epithelial appearance. At low doses, morphological differentiation was detectable with 4-5 d and proceeded more rapidly at higher doses. Microarray analysis to define the phenotype of these cells revealed that the most highly differentially regulated genes included differentiated secretion products of trophoblasts, transcription factors, and growth factors associated with trophoblast differentiation (*e.g.*, CGβ, GCM1, HASH2, HLA-G). Confirmation of this trophoblast molecular phenotype was provided by the measurement of secreted hormones, including hCG, progesterone, and estradiol-17β. The cosecretion of gonadotropic and sex steroid hormones is most logically interpreted as indicating advanced trophoblast differentiation after BMP-4 treatment. An additional important morphological characteristic of these cells was the formation of occasional multinuclear syncytiotrophoblasts, the terminally differentiated cell of the chorionic villi. However, the mononuclear cell population did not exhibit unlimited growth and propagated poorly. It remains unclear whether this indicates senescence or dedifferentiation of the cells with prolonged culture, or whether culture/growth factor conditions can be identified that will sustain these cells for prolonged proliferation.

Other investigators have made additional use of this paradigm since it was first reported, utilizing both spontaneous differentiation (78) and the BMP treatment approach (79) to evaluate regional trophoblast differentiation within individual ESC colonies. A potentially significant role of cell-cell interactions was seen, with the cells located at the periphery of the ESC colonies undergoing more rapid differentiation and the more densely aggregated centrally located cells retaining OCT4 expression despite the presence of BMP-4. Whether this reflects differential expression of receptors or downstream signaling molecules has not been addressed. The time course of differentiation has been defined by further microarray studies, and the coordinate increase in the expression of genes associated with trophoblast differentiation and a decrease of genes associated with the maintenance of pluripotency (OCT4, NANOG, SOX2) supports the morphological observations (80). Some cells at the periphery of differentiation colonies also express HLA-G, typically an extravillous trophoblast marker. The apparent mixture of villous (hCG expression) and extravillous (HLA-G expression) phenotypes does suggest that multipotent trophoblast progenitors appear during ESC colony differentiation, which can give rise to both pathways of trophoblast development.

C. Oxygen tension and ESC differentiation

Numerous studies have demonstrated the crucial role of oxygen in the control of trophoblast differentiation across a spectrum of functional characteristics. A critical aspect of primate placental function is to provide extravillous trophoblasts that invade the endometrial stroma (interstitial invasion), as well as the maternal spiral arterioles of the decidua (endovascular trophoblasts). These latter cells play a critical role in the remodeling of these maternal vessels in implantation. Although all contributing mechanisms are not yet entirely understood (81, 82), it is clear that removal of the vascular endothelium with endovascular trophoblast migration and subsequent loss of the smooth muscle of the tunica media result in the formation of maternal vessels in the proximity of the placenta lacking vasoreactive smooth muscle and potentially proinflammatory endothelia. It is also clear that in pathological pregnancies characterized by poor placentation, including reduced extravillous trophoblast migration and deranged remodeling of the spiral arterioles, the trophoblasts in these placentas have an inappropriate phenotype, which suggest inadequate differentiation, and inappropriate responses or expression of factors important for oxygen sensing and the normal downstream responses to relative hypoxia (83). The poor outcome of these pregnancies, with reduced oxygenation at the maternal-fetal interface, thus may be due to inappropriate trophoblast differentiation. Thus, trophoblast function by oxygen tension is of fundamental clinical as well as biological importance.

The role of oxygen in trophoblast differentiation in mice has been creatively addressed with the derivation of TSC lacking the oxygen-sensing components HIF-1a, HIF-2a, and ARNT, interacting subunits of the hypoxia-inducible factor (HIF) transcriptional regulatory complex (84). The differentiation capacity of these cells is skewed toward formation of labyrinthine trophoblasts and a concomitant reduction in spongiotrophoblast and giant cell differentiation, likely at least in part due to changes in mash2 expression [a basic helix-loop-helix transcription factor important in trophoblast differentiation (85)]. Because in implantation low oxygen tension will typically be sensed by trophoblasts and other important cellular components of the developing placenta (particularly endothelial cells), it is logical for physiologically low oxygen to coordinately promote differentiated trophoblast function as well as angiogenesis and migration, "core processes of placentation" (86). Hypoxia-insensitive mouse TSC lacking HIF1a/HIF-2a/ARNT exhibit decreased migration, likely linked to decreased aVb3 integrin expression. In addition, OCT4 has been shown to be a direct target of HIF-2a, which up-regulates OCT4 expression in response to reduced oxygen tension, and thus may be crucial in the regulation of stem cell maintenance *vs.* proliferation.

The effects of oxygen on mouse TSC have parallels to results with human placental trophoblasts. Culture of human extravillous trophoblasts in "low" (physiological) oxygen tensions of 2.5–5% promotes proliferation of human placental cytotrophoblasts. Transition to higher O_2 (8%, as might be encountered in the maternal endometrial vascular bed) promoted invasive differentiation, and a broad spectrum of activities that collectively define the extravillous phenotype (87). These include expression of HLA-G, secretion of matrix metalloproteinases, and integrin switching to an extravillous phenotype (83). There have been excellent reviews of the regulation of trophoblasts by oxygen tension (88, 89), and the reader is referred to those for further discussion, which is beyond the scope of this current review.

Because both early embryo development and trophoblast differentiation proceed under relatively low intrauterine oxygen tensions, it could be suggested that culture of human ESC in conditions of relative hypoxia may either predispose them to differentiation toward the trophoblast lineage or sustain undifferentiated growth. Studies from the Roberts laboratory (78) have shown that the effects of reduced oxygen tension were to restrain differentiation (including BMP4-mediated trophoblast differentiation) and maintain the undifferentiated pluripotent state. Atmospheric oxygen tension accelerated trophoblast differentiation and the formation of multinuclear syncytiotrophoblasts (79). These results underscore the central importance of the transcriptional milieu in trophoblast differentiation, with other environmental cues such as oxygen tension having a variety of potential modulatory effects.

D. The embryoid body paradigm for trophoblast differentiation

Other paradigms for the differentiation of trophoblasts from human ESC have been explored as well. The formation of embryoid bodies is a long-standing approach for studying ES cell differentiation. With mouse ESC, embryoid bodies can be formed from small aggregates in hanging drops, in suppression culture in bacterial grade dishes, or in carboxymethyl cellulose gels (90). Embryoid bodies derived from human ESC also provide an environment for differentiation and have been used to initiate differentiation of endodermal, mesodermal, and ectodermal lineages (91). We considered the possibility that embryoid body formation represented an opportunity to provide spatial cues to the ESC that could promote formation of cells representing the trophectoderm lineage. Embryoid body formation was initiated by treatment with dispase and collagenase to liberate the human ESC colonies, and they were maintained in suspension to "reanneal" into spherical structures (92). Careful attention to growth of ESC colonies is important to provide consistent and homogeneous populations of human ESC embryoid bodies. Our prediction of trophoblast differentiation was borne out by endocrine and immunohistochemical criteria. We noted that within 48 h of initiation of embryoid body formation, there was a small but consistently detectable increase in the secretion of hCG, progesterone, and estradiol-17β into the culture medium (92). However, there was no further increase in hormone secretion from embryoid bodies maintained for up to 9 wk in continuous suspension culture. Thus, these data suggest that trophoblast differentiation may be initiated but does not progress in embryoid bodies in suspension culture to a phenotype with substantially increased endocrine differentiation.

We hypothesized that maintaining these embryoid bodies in suspension culture does not effectively mimic the process of embryo implantation. We have developed two paradigms

to further study the trophoblast population initiated by embryoid body formation. Reasoning that the rapid invasion of the human embryo into the decidua at the implantation site includes, at least in part, interactions of trophectoderm with the extracellular matrix of the endometrial stroma (83), a paradigm was developed based on studies utilizing chorionic villus explants placed onto droplets of solidified Matrigel extracellular matrix (93). Matrigel is soluble at 4 C but takes on a gel consistency at 37 C. Its principal extracellular matrix components of collagen IV, laminin, heparan sulfate proteoglycan (perlecan), and nidogen are similar to that described for the human first trimester decidual matrix (94). Embryoid bodies were formed in suspension culture and after 2–8 d were placed into extracellular matrix droplets, which in turn were maintained in suspension. Within 1–2 wk, cellular processes were seen to be extending outward from the surface of the embryoid bodies, and these processes continued to grow outward for at least several weeks. Intriguingly, as the outgrowths extended from the surface of the embryoid bodies, there was a concomitant and progressive rise in the secretion of hCG, progesterone, and estradiol-17β into the culture medium. Further studies confirmed that all these were undetectable in cultures without embryoid bodies, and that estradiol secretion relied on androgen substrate present in the fetal bovine serum, a component of the culture medium (92).

Although these results were intriguing, it is exceedingly tedious to work with this "three-dimensional" embryoid body culture paradigm, and we sought to simplify the system and allow direct access to the outgrowths from the embryoid bodies. Thus, we coated culture dishes with a thin layer of Matrigel and plated embryoid bodies directly onto the dish surface, or a "two-dimensional" culture paradigm (92). In this paradigm, we also noted substantial (and rapid) outgrowth of cells from the embryoid body culture surface. Within approximately 1 wk, there was detectable hCG, progesterone, and estradiol-17β secretion into the culture medium, presumably from the outgrowth cells. After 2 wk however, hormone secretion began to decrease and it was very low by 4 wk of culture, as cells became confluent. Further passage failed to reinitiate hormone secretion. Given these results, what are we able to conclude? First, with the H1 and H9 lines used in our studies, there is spontaneous formation of trophoblasts upon embryoid body formation. It is not known whether the phenotype of these trophoblasts is similar to those cells that are secreting hCG in spontaneously differentiating human ESC. In addition, it has not yet been feasible to isolate the outgrowths in three-dimensional culture, which is likely the primary source of hCG secretion, based on interpretation of immunohistochemical analyses of three-dimensional embryoid body cultures (92).

The two-dimensional paradigm provides a better opportunity for defining the outgrowth population. We have conducted preliminary studies of 2-wk outgrowths from embryoid bodies, demonstrating that the cells are essentially pure populations of hCG- and cytokeratin 8-postiive cells (G. Gerami-Naini, O.V. Dovzhenko, S.V. Dambaeva, M. Durning, M.A. Garthwaite, R.L. Grendell, T.G. Golos, in preparation). Examination of these cells by flow cytometry for cell surface markers and by microarray to better define their phenotype allows some potential conclusions. First, the cells are clearly within the trophoblast lineage. They express a broad spectrum of markers of trophoblast differentiated function (Table 1), as well as transcription factors associated with trophoblast differentiation in mouse placenta and also expressed in the human placenta. The cells are likely to represent cells relatively early in the pathway of placental development because they do not express certain markers of later differentiation (*e.g.*, chorionic somatomammotropin or placental GH). However, once again, they express genes for steroidogenic enzymes considered to be primarily indicative of villous syncytiotrophoblast differentiation. Thus the phenotype of these cells within the well-defined trophoblast compartments of the definitive placenta remains unclear.

Other investigators have used the embryoid body paradigm to study trophoblast differentiation. Harun *et al.* (95), utilizing H7 and H14 human ESCs, cultured individual embryoid bodies in microtiter wells, using mouse TS medium (9) to "screen" for cells with elevated hCG secretion as an indicator of trophoblast differentiation. This approach allowed the identification of a subset (approximately 4%) of embryoid bodies that were then disaggregated, passaged and expanded, and evaluated for evidence of further differentiation. Morphological differentiation was assessed in culture dishes on Matrigel, and it was observed that a subset of cells expressed HLA-G as evidenced by immunohistochemical and flow cytometric staining. These cells were also evaluated in cocultures with endometrial stromal and epithelial cells or in a migration assay for invasive activity, and it was determined that they were able to migrate through Matrigel, as well as displace endometrial cells from culture dishes. There was morphological evidence of syncytiotrophoblast differentiation. These authors concluded that the potential to form cells expressing HLA-G, as well as capable of morphologically forming syncytia, supports a cytotrophoblast stem cell-like identity.

Other human ESC lines may differ in their ability to differentiate into trophoblasts. Although it is clear that several cell lines tested from different laboratories respond to BMP treatment with trophoblast differentiation, there are also reports of different responses to BMP treatment. Treatment of

TABLE 1. Differential gene expression between embryoid body outgrowths and undifferentiated H1 human embryonic stem cells

Gene name	Fold-induction	Gene name	Fold-induction
CGα	8194	HAND1	356
GATA3	2431	CYP11A1	253
S100P	1269	GCM1	55
HSD3β	1130	CRH	33
CGβ	648	PSG1-9	9–80
AP2α	614	GnRH2	5.7
		BHLHB2 (stra13)	5

Five independent samples of undifferentiated human ESC RNA samples and five RNA samples from outgrowths of human ESC embryoid bodies were analyzed by Affymetrix microarray. Differential gene expression was examined to determine the fold-difference of mean intensity levels of each Affymetrix probe set. Values presented are the ratios of the mean intensity of five Affymetrix microarrays of two-dimensional trophoblastic embryoid body outgrowths to undifferentiated human ESC. Selected genes are presented, representing trophoblast hormones, steroidogenic enzymes, or transcription factors.

the HES-2 or HES-3 cell lines with BMP 2 resulted primarily in differentiation of extraembryonic endoderm (96), with the authors commenting that only occasionally were small foci seen that morphologically resembled trophoblasts. It is not understood what the intrinsic (cellular) or extrinsic (culture condition) differences may be that result in these differences.

E. Criteria for defining ESC-derived trophoblasts as TSC

The intriguing question that arises from studies with human ESC is whether the trophoblast-like cells that differentiate directly from these cells represent at least a first approximation of human TSC. To meet the definition of TSC identity, several critical criteria must be fulfilled. First, at a molecular level, extensive work with mouse TSC has established the salient expression of the critical transcription factors EOMES, ERRβ, and CDX2. As discussed above, there have been conflicting reports that these factors are not expressed in human or nonhuman primate embryos, placentas, or trophoblasts. In human ESC studies, we have failed to confirm induction of CDX2 expression in embryoid body-derived trophoblasts. There was no induction of CDX2 in BMP-4-induced trophoblasts (see Supplemental Table 2 in Ref. 73). On the other hand, some (97), but not all (98) studies have reported CDX2 up-regulation with OCT4 suppression in human ESC (97, 98), although in these latter cases specific trophoblast expression was not demonstrated. Similarly, we have failed to demonstrate EOMES or ERRβ mRNAs in embryoid body-derived trophoblasts, nor are they up-regulated in BMP-4-derived trophoblasts (77). It is possible that these different outcomes may arise in part from different reagents, methodological procedures, or genetic background of specimens analyzed, or that these canonical markers for mouse TSC may not be precisely recapitulated in human cells.

The second criterion would be the ability to give rise to all the trophoblast populations of the definitive placenta. On the one hand, investigators have shown human ESC differentiation to cells with the morphological demonstration of syncytium formation, indicating a villous cytotrophoblast phenotype, terminally differentiating to syncytiotrophoblasts. In addition, there is some indication of expression of HLA-G, an extravillous trophoblast marker (79, 95, 99), as well as demonstration of invasive activity as a marker of the movement of extravillous cells from the anchoring villus columns to interstitial and endovascular compartments (95). Syncytium formation and invasive activity are also noted in rhesus trophectoderm-derived trophoblasts (19). These observations support a judgment of a "stem" population arising from rhesus embryos and human ESC. Goals for future studies should be (based on hallmark mouse TSC studies) to define a transcriptional phenotype that defines the human/primate TSC population. In addition, defining genes up- and down-regulated during the differentiation toward the villous and extravillous pathways is needed.

From a cellular perspective, mouse TSC derived from blastocysts represent a stable cell population that can be expanded and maintained in an "undifferentiated" state with FGF4 and TFGβ/activin (16). Rhesus trophectoderm-derived cells (19) or human ESC-derived trophoblasts (95) can be maintained and expanded in culture; however, whether they represent a stable progenitor analogous to mouse TSC is not clear. Essentially all studies of trophoblast derivation from human ESC also use CG secretion or mRNA expression as a marker for the trophoblast phenotype. The question arises as to whether CG is a marker of differentiated trophoblasts or whether it could serve as a TSC marker for primates. Although CG secretion is generally considered a characteristic of differentiated syncytiotrophoblast function, the secretion of CG is in fact initiated in cultured rhesus (100), baboon (101), marmoset (102), and human (103) blastocysts in advance of implantation. That is, it is secreted by the blastocyst trophectoderm even before attachment to the endometrium and initiation of the differentiation steps that culminate in the formation of the villous and extravillous trophoblast compartments. Thus it is reasonable for CG expression to be considered a potential characteristic of human and nonhuman primate TSC.

In addition to previous studies with the R278.5 rhesus ESC (12), we have used other rhesus cell lines derived in the Thomson laboratory. Our approach has been to systematically employ the rhesus cells in our established paradigms for human ESC differentiation: BMP-4 treatment, embryoid body formation, and two-dimensional and three-dimensional culture in Matrigel environments. Analysis of these studies remains preliminary, but we have been surprised by apparent heterogeneity among the rhesus ESC lines. Embryoid body formation is consistent with all the lines, although the cell lines do exhibit different growth characteristics. Outgrowths from the embryoid bodies in both two-dimensional and three-dimensional culture is also reliably seen. Our ini-

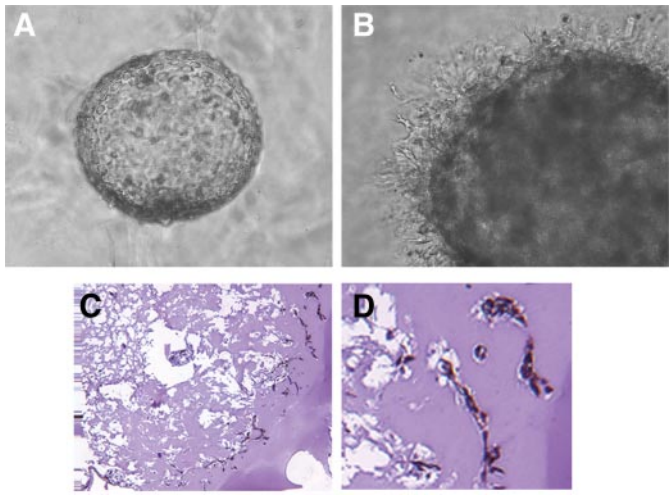

FIG. 3. Growth of rhesus monkey blastocysts in Matrigel explant culture. Rhesus monkey blastocysts were produced by *in vitro* fertilization and the day after hatching were placed into solidified Matrigel droplets. A, Day of placement into the Matrigel. B, Fourteen days after placement into Matrigel. Invasive outgrowths from the surface of the embryo are seen migrating into the Matrigel. C and D, Low-magnification and high-magnification appearance of histological sections of representative embryonic outgrowths from a Matrigel-cultured embryo embedded in paraffin, sectioned, and immunostained with an antibody against cytokeratin 7/8. The Matrigel beyond the outgrowths is undisturbed, whereas the Matrigel through which the outgrowths migrated has been substantially digested. Figure 4 presents a low-magnification image of the digestion of Matrigel at the periphery of an embryo cultured in Matrigel.

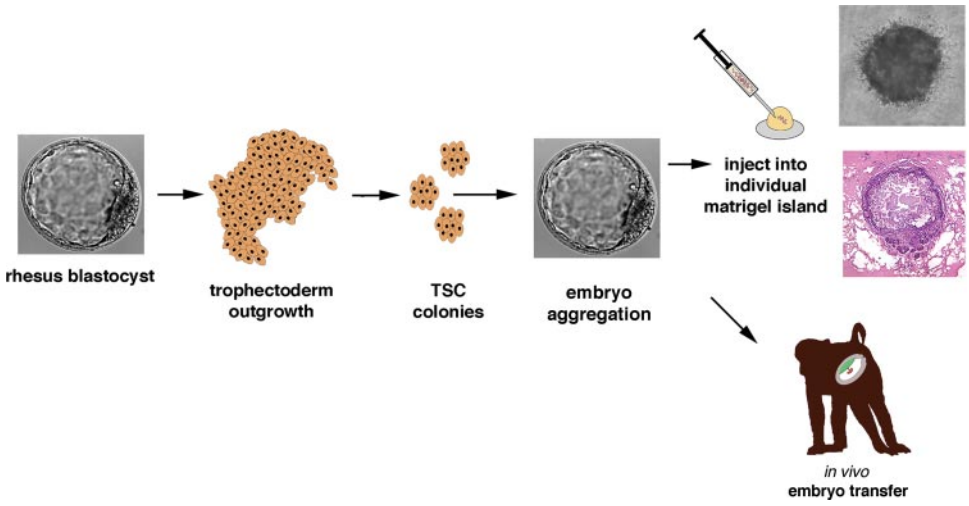

FIG. 4. Schematic diagram of opportunities for the use of primate embryos to derive TSC and test developmental potential in aggregated embryos and three-dimensional Matrigel explant culture. Rhesus blastocysts give rise *in vitro* to trophectodermal outgrowths and TSC colonies, which can be aggregated with rhesus embryos for *in vitro* (in Matrigel three-dimensional culture) and *in vivo* (with transfer to rhesus monkey transfer recipients) study of putative TSC allocation into differentiated trophoblasts.

tial studies with immunohistochemical analysis of suspension embryoid bodies indicates, however, that even the simple formation of a cytokeratin-positive outer surface of the embryoid bodies is not consistent among cell lines. The formation of this layer would seem to be essential for the initiation of trophoblast differentiation, and the R278.5 cell line used in initial studies of trophoblast differentiation remains the most reliable cell line for this step. For example, outgrowths from embryoid bodies in two-dimensional culture express the trophoblast-specific major histocompatibility complex-class I molecule Mamu-AG (M.A. Garthwaite, S.V. Dambaeva, T.G. Golos, unpublished data).

F. Future prospects for study of TSC differentiation potential

It will be important to have both *in vitro* and *in vivo* systems for defining the differentiation capacity of candidate TSC. Drawing on the embryoid body paradigm, we have been working to develop an *in vitro* system in which the development of rhesus embryos can be interrogated, much like the development of embryoid bodies with regard to outgrowth formation. We have piloted the placement of rhesus monkey blastocyst stage embryos obtained by *in vitro* fertilization into the three-dimensional Matrigel explant system. The hypothesis is that blastocyst trophectoderm will initiate extravillous trophoblast differentiation and invasion of the Matrigel. Differentiation of embryonic epiblast will give rise to hemangioblastic precursors and embryonic mesenchyme, which will become invested into the expanding trophoblastic shell, and establish cell-cell contact and interactions likely to be necessary for chorionic villus formation. Thus, an ultimate goal could be the formation *in vitro* of the fundamental components of a villous placenta. We have seen in our pilot studies outcomes of embryo placement into the three-dimensional Matrigel system that are supportive of the feasibility of this goal. In particular, embryos growing in Matrigel explants send invasive, cytokeratin-positive cells into the surrounding matrix (Fig. 3), which is degraded in the proximity of the embryo, likely as a result of matrix metalloproteinase secretion. At the same time, these embryos initiate the secretion of CG and steroid hormones into the culture medium, similar to previous results with embryoid bodies (T.-C. Chang, G.I. Bondarenko, B. Gerami-Naini, J.G. Drenzek, M. Durning, M.A. Garthwaite, T.G. Golos, manuscript in preparation). Thus the methods established with embryoid bodies will be applicable to embryo culture as well and illustrate the synergistic approaches feasible with the nonhuman primate model, using both embryonic and ESC models.

With a potential system available for *in vitro* embryo development, efforts must now be put into establishing aggregation approaches with ESC, TSC, and the three-dimensional culture system. It should be feasible to apply the experience available in the production of chimeric mouse embryos to optimize, *in vitro*, methods to promote incorporation of rhesus ESC or TSC candidates into developing embryos (Fig. 4). One of the significant drawbacks of the nonhuman primate model is the difficulty and cost of establishing successful pregnancies with *in vivo* transfer of manipulated embryos (104, 105). The three-dimensional culture system will hopefully allow progress with a more cost-effective paradigm of potential ways to improve ESC and TSC integration into developing embryos. ESC are advantageous for the derivation of TSC because they can (in theory) be genetically manipulated to explore the roles of specific factors in the function of TSC, in their contribution to differentiation events as cultured cells, in aggregated embryo paradigms, and ultimately, *in vivo*, with transfer of aggregated or manipulated embryos.

Acknowledgments

T.G.G. thanks all current and past members of the Golos laboratory for their contributions to the studies presented in this review and acknowledges ongoing collaborations with James Thomson and his laboratory in studies using rhesus monkey embryonic stem cells. In particular, we thank Gennadiy Bondarenko for immunohistochemistry staining and Rick Grendell for assistance with figure preparation. Members of the veterinary, animal care, and hormone assay staff of the Wisconsin National Primate Research Center were instrumental in obtaining gametes for *in vitro* fertilization studies, and Judith Peterson provided timely assistance with the preparation of this manuscript. G.C.D. and C.A.V. thank all colleagues and staff in the School of Medicine and California National Primate Research Center whose efforts made possible the studies reported here.

Received January 13, 2009. Accepted March 11, 2009.

Address all correspondence and requests for reprints to: Thaddeus G. Golos, Ph.D., Wisconsin National Primate Research Center, University of Wisconsin, 1223 Capitol Court, Madison, Wisconsin 53715-1299. E-mail: golos@primate.wisc.edu

This work was supported by National Institutes of Health (NIH) Grants R01 HD057114 (to G.C.D.), RR13439 (to C.A.V.), AA014173 (to C.A.V.), P51 RR00169 (to C.A.V.), R21 HD46919 (to T.G.G.), and P01 HD38843 (to T.G.G.); March of Dimes Grant 6-FY00-164 (to T.G.G.); and Children's Miracle Network Research Grant S-CMNGD06 (to G.C.D.). This publication was made possible in part by NIH Grant P51 RR000167 from the National Center for Research Resources (NCRR), a component of the NIH, to the Wisconsin National Primate Research Center, University of Wisconsin-Madison. This research was conducted in part at a facility constructed with support from Research Facilities Improvement Program Grants RR15459-01 and RR020141-01. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the NCRR or NIH.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Rossant J** 2008 Stem cells and early lineage development. Cell 132:527–531
2. **Koestenbauer S, Zech NH, Juch H, Vanderzwalmen P, Schoonjans L, Dohr G** 2006 Embryonic stem cells: similarities and differences between human and murine embryonic stem cells. Am J Reprod Immunol 55:169–180
3. **Kuijk EW, Du Puy L, Van Tol HT, Oei CH, Haagsman HP, Colenbrander B, Roelen BA** 2008 Differences in early lineage segregation between mammals. Dev Dyn 237:918–927
4. **Roberts RM, Ezashi T, Das P** 2004 Trophoblast gene expression: transcription factors in the specification of early trophoblast. Reprod Biol Endocrinol 2:47–56
5. **Carter AM** 2007 Animal models of human placentation—a review. Placenta 28:S41–S47
6. **Kunath T, Strumpf D, Rossant J** 2004 Early trophoblast determination and stem cell maintenance in the mouse—a review. Placenta 25(Suppl A):S32
7. **Oda M, Shiota K, Tanaka S** 2006 Trophoblast stem cells. Methods Enzymol 419:387–400
8. **Takahashi K, Yamanaka S** 2006 Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
9. **Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, Rossant J** 1998 Promotion of trophoblast stem cell proliferation by FGF4. Science 282:2072–2075
10. **Rielland M, Hue I, Renard JP, Alice J** 2008 Trophoblast stem cell derivation, cross-species comparison and use of nuclear transfer: new tools to study trophoblast growth and differentiation. Dev Biol 322:1–10
11. **Rossant J** 2001 Stem cells from the mammalian blastocyst. Stem Cells 19:477–482
12. **Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA, Hearn JP** 1995 Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci USA 92:7844–7848
13. **Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM** 1998 Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147
14. **Xu RH, Peck RM, Li DS, Feng X, Ludwig T, Thomson JA** 2005 Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods 2:185–190
15. **Ralston A, Rossant J** 2005 Genetic regulation of stem cell origins in the mouse embryo. Clin Genet 68:106–112
16. **Erlebacher A, Price KA, Glimcher LH** 2004 Maintenance of mouse trophoblast stem cell proliferation by TGF-[β]/activin. Dev Biol 275:158–169
17. **Fléchon JE, Laurie S, Notarianni E** 1995 Isolation and characterization of a feeder-dependent, porcine trophectoderm cell line obtained from a 9-day blastocyst. Placenta 16:643–658
18. **Hashizume K, Shimada A, Nakano H, Takahashi T** 2006 Bovine trophoblast cell culture systems: a technique to culture bovine trophoblast cells without feeder cells. Methods Mol Med 121:179–188
19. **Vandevoort CA, Thirkill TL, Douglas GC** 2007 Blastocyst-derived trophoblast stem cells from the rhesus monkey. Stem Cells Dev 16:779–788
20. **Caniggia I, Lye SJ, Cross JC** 1997 Activin is a local regulator of human cytotrophoblast cell differentiation. Endocrinology 138:3976–3986
21. **Chen VT, Peng C, Leung PC** 2003 Activin-A up-regulates type I activin receptor mRNA levels in human immortalized extravillous trophoblast cells. Reprod Biol Endocrinol 1:29–38
22. **Petraglia F, Vaughan J, Vale W** 1989 Inhibin and activin modulate the release of gonadotropin-releasing hormone, human chorionic gonadotropin, and progesterone from cultured human placental cells. Proc Natl Acad Sci USA 86:5114–5117
23. **Pan G, Li J, Zhou Y, Zheng H, Pei D** 2006 A negative feedback loop of transcription factors that controls stem cell pluripotency and self-renewal. FASEB J 20:1730–1732
24. **Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong KY, Sung KW, Lee CW, Zhao XD, Chiu KP, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei CL, Ruan Y, Lim B, Ng HH** 2006 The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet 38:431–440
25. **Cauffman G, Van de Velde H, Liebaers I, Van Steirteghem A** 2005 Oct-4 mRNA and protein expression during human preimplantation development. Mol Hum Reprod 11:173–181
26. **Niwa H, Toyooka Y, Shimosato D, Strumpf D, Takahashi K, Yagi R, Rossant J** 2005 Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. Cell 123:917–929
27. **Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Schöler H, Smith A** 1998 Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95:379–391
28. **Niwa H, Miyazaki J, Smith AG** 2000 Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 24:372–376
29. **Velkey JM, O'Shea KS** 2003 Oct4 RNA interference induces trophectoderm differentiation in mouse embryonic stem cells. Genesis 37:18–24
30. **Yagi R, Kohn MJ, Karavanova I, Kaneko KJ, Vullhorst D, DePamphilis ML, Buonanno A** 2007 Transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian development. Development 134:3827–3836
31. **Nishioka N, Yamamoto S, Kiyonari H, Sato H, Sawada A, Ota M, Nakao K, Sasaki H** 2008 Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. Mech Dev 125:270–283
32. **Hansis C, Grifo JA, Krey LC** 2000 Oct-4 expression in inner cell mass and trophectoderm of human blastocysts. Mol Hum Reprod 6:999–1004
33. **Kirchhof N, Carnwath JW, Lemme E, Anastassiadis K, Schöler H, Niemann H** 2000 Expression pattern of Oct-4 in preimplantation embryos of different species. Biol Reprod 63:1698–1705
34. **Mitalipov SM, Kuo HC, Hennebold JD, Wolf DP** 2003 Oct-4 expression in pluripotent cells of the rhesus monkey. Biol Reprod 69:1785–1792
35. **Henderson JK, Draper JS, Baillie HS, Fishel S, Thomson JA, Moore H, Andrews PW** 2002 Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem Cells 20:329–337
36. **Zwaka TP, Thomson JA** 2003 Homologous recombination in human embryonic stem cells. Nat Biotechnol 21:319–321
37. **Byrne JA, Mitalipov SM, Clepper L, Wolf DP** 2006 Transcriptional profiling of rhesus monkey embryonic stem cells. Biol Reprod 75:908–915
38. **Mitalipov S, Kuo HC, Byrne J, Clepper L, Meisner L, Johnson J, Zeier R, Wolf D** 2006 Isolation and characterization of novel rhesus monkey embryonic stem cell lines. Stem Cells 24:2177–2186
39. **Behr R, Heneweer C, Viebahn C, Denker HW, Thie M** 2005 Epithelial-mesenchymal transition in colonies of rhesus monkey embryonic stem cells: a model for processes involved in gastrulation. Stem Cells 23:805–816

40. **Takeda J, Seino S, Bell GI** 1992 Human Oct3 gene family: cDNA sequences, alternative splicing, gene organization, chromosomal location, and expression at low levels in adult tissues. Nucleic Acids Res 20:4613–4620
41. **Fujimoto Y, Hasegawa K, Suemori H, Ito J, Nakatsuji N** 2006 Molecular cloning and function of Oct-3 isoforms in cynomolgus monkey embryonic stem cells. Stem Cells Dev 15:566–574
42. **Liedtke S, Enczmann J, Waclawczyk S, Wernet P, Kögler G** 2007 Oct4 and its pseudogenes confuse stem cell research. Cell Stem Cell 1:364–366
43. **Strumpf D, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F, Rossant J** 2005 Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development 132:2093–2102
44. **Adjaye J, Huntriss J, Herwig R, BenKahla A, Brink TC, Wierling C, Hultschig C, Groth D, Yaspo ML, Picton HM, Gosden RG, Lehrach H** 2005 Primary differentiation in the human blastocyst: comparative molecular portraits of inner cell mass and trophectoderm cells. Stem Cells 23:1514–1525
45. **Pau KY, Wolf DP** 2004 Derivation and characterization of monkey embryonic stem cells. Reprod Biol Endocrinol 2:41
46. **Harvey AJ, Armant DR, Brenner CA** 2008 Stem cell markers of embryonic and trophoblast lineages in rhesus macaque blastocysts. Reprod Sci (Supp)15:611
47. **Degrelle SA, Campion E, Cabau C, Piumi F, Reinaud P, Richard C, Renard JP, Hue I** 2005 Molecular evidence for a critical period in mural trophoblast development in bovine blastocysts. Dev Biol 288:448–460
48. **Wijchers PJ, Burbach JP, Smidt MP** 2006 In control of biology: of mice, men and Foxes. Biochem J 397:233–246
49. **Guo Y, Costa R, Ramsey H, Starnes T, Vance G, Robertson K, Kelley M, Reinbold R, Scholer H, Hromas R** 2002 The embryonic stem cell transcription factors Oct-4 and FoxD3 interact to regulate endodermal-specific promoter expression. Proc Natl Acad Sci USA 99:3663–3667
50. **Tompers DM, Foreman RK, Wang Q, Kumanova M, Labosky PA** 2005 Foxd3 is required in the trophoblast progenitor cell lineage of the mouse embryo. Dev Biol 285:126–137
51. **Liu Y, Labosky PA** 2008 Regulation of embryonic stem cell self-renewal and pluripotency by Foxd3. Stem Cells 26:2475–2484
52. **Babaie Y, Herwig R, Greber B, Brink TC, Wruck W, Groth D, Lehrach H, Burdon T, Adjaye J** 2007 Analysis of OCT4-dependent transcriptional networks regulating self-renewal and pluripotency in human embryonic stem cells. Stem Cells 25:500–510
53. **Prescott JD, Koto KS, Singh M, Gutierrez-Hartmann A** 2004 The ETS transcription factor ESE-1 transforms MCF-12A human mammary epithelial cells via a novel cytoplasmic mechanism. Mol Cell Biol 24:5548–5564
54. **Ho KJ, Liao JK** 2002 Non-nuclear actions of estrogen: new targets for prevention and treatment of cardiovascular disease. Mol Interv 2:219–228
55. **Vousden KH** 2005 APOPTOSIS: p53 and PUMA: a deadly duo. Science 309:1685–1686
56. **Dottori M, Gross MK, Labosky P, Goulding M** 2001 The winged-helix transcription factor Foxd3 suppresses interneuron differentiation and promotes neural crest cell fate. Development 128:4127–4138
57. **Cheung M, Chaboissier MC, Mynett A, Hirst E, Schedl A, Briscoe J** 2005 The transcriptional control of trunk neural crest induction, survival, and delamination. Dev Cell 8:179–192
58. **Vicovac L, Aplin JD** 1996 Epithelial-mesenchymal transition during trophoblast differentiation. Acta Anat (Basel) 156:202–216
59. **Larue L, Antos C, Butz S, Huber O, Delmas V, Dominis M, Kemler R** 1996 A role for cadherins in tissue formation. Development 122:3185–3194
60. **Zhou Y, Fisher SJ, Janatpour M, Genbacev O, Dejana E, Wheelock M, Damsky CH** 1997 Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? J Clin Invest 99:2139–2151
61. **Pijnenborg R, Vercruysse L, Hanssens M, Van Asshe A** 2007 Trophoblast invasion in pre-eclampsia and other pregnancy disorders. In: Lyall F, Belfort M, eds. Pre-eclampsia: etiology and clinical practice. New York: Cambridge University Press; 3–19
62. **Pan G, Qin B, Liu N, Schöler HR, Pei D** 2004 Identification of a nuclear localization signal in OCT4 and generation of a dominant negative mutant by its ablation. J Biol Chem 279:37013–37020
63. **Boulanger J, Vézina A, Mongrain S, Boudreau F, Perreault N, Auclair BA, Lainé J, Asselin C, Rivard N** 2005 Cdk2-dependent phosphorylation of homeobox transcription factor CDX2 regulates its nuclear translocation and proteasome-mediated degradation in human intestinal epithelial cells. J Biol Chem 280:18095–18107
64. **Gross I, Lhermitte B, Domon-Dell C, Duluc I, Martin E, Gaiddon C, Kedinger M, Freund JN** 2005 Phosphorylation of the homeotic tumor suppressor Cdx2 mediates its ubiquitin-dependent proteasome degradation. Oncogene 24:7955–7963
65. **Jackson JG, Kreisberg JI, Koterba AP, Yee D, Brattain MG** 2000 Phosphorylation and nuclear exclusion of the forkhead transcription factor FKHR after epidermal growth factor treatment in human breast cancer cells. Oncogene 19:4574–4581
66. **Van Der Heide LP, Hoekman MF, Smidt MP** 2004 The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. Biochem J 380:297–309
67. **Sasai Y** 2007 A matter of some importins: nuclear transport factors in ES cell maintenance and differentiation. Dev Cell 12:172–174
68. **Okada N, Ishigami Y, Suzuki T, Kaneko A, Yasui K, Fukutomi R, Isemura M** 2008 Importins and exportins in cellular differentiation. J Cell Mol Med 12:1863–1871
69. **Hattori N, Nishino K, Ko YG, Hattori N, Ohgane J, Tanaka S, Shiota K** 2004 Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. J Biol Chem 279:17063–17069
70. **Tsuji-Takayama K, Inoue T, Ijiri Y, Otani T, Motoda R, Nakamura S, Orita K** 2004 Demethylating agent, 5-azacytidine, reverses differentiation of embryonic stem cells. Biochem Biophys Res Commun 323:86–90
71. **Chambers I** 2004 The molecular basis of pluripotency in mouse embryonic stem cells. Cloning Stem Cells 6:386–391
72. **Covello KL, Kehler J, Yu H, Gordan JD, Arsham AM, Hu CJ, Labosky PA, Simon MC, Keith B** 2006 HIF-2α regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. Genes Dev 20:557–570
73. **Westfall SD, Sachdev S, Das P, Hearne LB, Hannink M, Roberts RM, Ezashi T** 2008 Identification of oxygen-sensitive transcriptional programs in human embryonic stem cells. Stem Cells Dev 17:869–881
74. **Latham KE** 2006 The primate embryo gene expression resource in embryology and stem cell biology. Reprod Fertil Dev 18:807–810
75. **Hemberger M, Nozaki T, Winterhager E, Yamamoto H, Nakagama H, Kamada N, Suzuki H, Ohta T, Ohki M, Masutani M, Cross JC** 2003 Parp1-deficiency induces differentiation of ES cells into trophoblast derivatives. Dev Biol 257:371–381
76. **Schenke-Layland K, Angelis E, Rhodes KE, Heydarkhan-Hagvall S, Mikkola HK, Maclellan WR** 2007 Collagen IV induces trophoectoderm differentiation of mouse embryonic stem cells. Stem Cells 25:1529–1538
77. **Xu RH, Chen X, Li DS, Li R, Addicks GC, Glennon C, Zwaka TP, Thomson JA** 2002 BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat Biotechnol 20:1261–1264
78. **Ezashi T, Das P, Roberts RM** 2005 Low O2 tensions and the prevention of differentiation of hES cells. Proc Natl Acad Sci USA 102:4783–4788
79. **Das P, Ezashi T, Schulz LC, Westfall SD, Livingston KA, Roberts RM** 2007 Effects of FGF2 and oxygen in the BMP4-driven differentiation of trophoblast from human embryonic stem cells. Stem Cell Res 1:61–74
80. **Schulz LC, Ezashi T, Das P, Westfall SD, Livingston KA, Roberts RM** 2008 Human embryonic stem cells as models for trophoblast differentiation. Placenta 29(Suppl A):S10–S16
81. **Naruse K, Lash GE, Innes BA, Otun HA, Searle RF, Robson SC, Bulmer JN** 2009 Localization of matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitors for MMPs (TIMPs) in uterine natural killer cells in early human pregnancy. Hum Reprod 24:553–561
82. **Fons P, Chabot S, Cartwright JE, Lenfant F, L'Faqihi F, Giustiniani J, Herault JP, Gueguen G, Bono F, Savi P, Aguerre-Girr M, Fournel S, Malecaze F, Bensussan A, Plouët J, Le Bouteiller P** 2006 Soluble HLA-G1 inhibits angiogenesis through an apoptotic pathway and

by direct binding to CD160 receptor expressed by endothelial cells. Blood 108:2608–2615
83. **Red-Horse K, Zhou Y, Genbacev O, Prakobphol A, Foulk R, McMaster M, Fisher SJ** 2004 Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. J Clin Invest 114:744–754
84. **Fryer BH, Simon MC** 2006 Hypoxia, HIF and the placenta. Cell Cycle 5:495–498
85. **Adelman DM, Gertsenstein M, Nagy A, Simon MC, Maltepe E** 2000 Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. Genes Dev 14:3191–3203
86. **Simon MC, Keith B** 2008 The role of oxygen availability in embryonic development and stem cell function. Nat Rev Mol Cell Biol 9:285–296
87. **Genbacev O, Zhou Y, Ludlow JW, Fisher SJ** 1997 Regulation of human placental development by oxygen tension. Science 277: 1669–1672
88. **Caniggia I, Winter JL** 2002 Hypoxia inducible factor-1: oxygen regulation of trophoblast differentiation in normal and pre-eclamptic pregnancies—a review. Placenta 23(Suppl A):S47–S57
89. **Zhou Y, Genbacev O, Damsky CH, Fisher SJ** 1998 Oxygen regulates human cytotrophoblast differentiation and invasion: implications for endovascular invasion in normal pregnancy and in preeclampsia. J Reprod Immunol 39:197–213
90. **Kurosawa H** 2007 Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells. J Biosci Bioeng 103:389–398
91. **Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N** 2000 Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 6:88–95
92. **Gerami-Naini B, Dovzhenko OV, Durning M, Wegner FH, Thomson JA, Golos TG** 2004 Trophoblast differentiation in embryoid bodies derived from human embryonic stem cells. Endocrinology 145:1517–1524
93. **Bauer S, Pollheimer J, Hartmann J, Husslein P, Aplin JD, Knöfler M** 2004 Tumor necrosis factor-α inhibits trophoblast migration through elevation of plasminogen activator inhibitor-1 in first-trimester villous explant cultures. J Clin Endocrinol Metab 89:812–822
94. **Damsky CH, Fitzgerald ML, Fisher SJ** 1992 Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. J Clin Invest 89:210–222
95. **Harun R, Ruban L, Matin M, Draper J, Jenkins NM, Liew GC, Andrews PW, Li TC, Laird SM, Moore HD** 2006 Cytotrophoblast stem cell lines derived from human embryonic stem cells and their capacity to mimic invasive implantation events. Hum Reprod 21: 1349–1358
96. **Pera MF, Andrade J, Houssami S, Reubinoff B, Trounson A, Stanley EG, Ward-van Oostwaard D, Mummery C** 2004 Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. J Cell Sci 117:1269–1280
97. **Matin MM, Walsh JR, Gokhale PJ, Draper JS, Bahrami AR, Morton I, Moore HD, Andrews PW** 2004 Specific knockdown of Oct4 and β2-microglobulin expression by RNA interference in human embryonic stem cells and embryonic carcinoma cells. Stem Cells 22: 659–668
98. **Hay DC, Sutherland L, Clark J, Burdon T** 2004 Knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. Stem Cells 22:225–235
99. **Hunt JS** 2006 Stranger in a strange land. Immunol Rev 213:36–47
100. **Seshagiri PB, Terasawa E, Hearn JP** 1994 The secretion of gonadotrophin-releasing hormone by peri-implantation embryos of the rhesus monkey: comparison with the secretion of chorionic gonadotrophin. Hum Reprod 9:1300–1307
101. **Pope VZ, Pope CE, Beck LR** 1982 Gonadotropin production by baboon embryos *in vitro.* In: Hafey ESE, ed. In vitro fertilization and embryo transfer. Lancaster, UK: MTP Press, Ltd., pp 129–134
102. **Hearn JP, Hodges JK, Gems S** 1988 Early secretion of chorionic gonadotrophin by marmoset embryos *in vivo* and *in vitro.* J Endocrinol 119:249–255
103. **Fishel SB, Edwards RG, Evans CJ** 1984 Human chorionic gonadotropin secreted by preimplantation embryos cultured in vitro. Science 223:816–818
104. **Mitalipov SM, Yeoman RR, Kuo HC, Wolf DP** 2002 Monozygotic twinning in rhesus monkeys by manipulation of in vitro-derived embryos. Biol Reprod 66:1449–1455
105. **Chan AW, Dominko T, Luetjens CM, Neuber E, Martinovich C, Hewitson L, Simerly CR, Schatten GP** 2000 Clonal propagation of primate offspring by embryo splitting. Science 287:317–319

Endocrine Reviews **is published by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**