

Review

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Trophoblast gene expression: Transcription factors in the specification of early trophoblast

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Abstract

A zone of trophoblast specification is established when the embryo is a morula, presumably reflecting a unique combination of transcription factors in that zone of cells and the influence of various environmental cues and growth factors on them. A key first step in this process of specification is the down-regulation of Oct4, a transcription factor that acts as a negative regulator of trophoblast specification and of genes normally up-regulated as the trophectoderm first forms. The transcription factors believed to have a positive association with trophectoderm specification have been inferred primarily in two ways: by their expression patterns in embryos, ES cells and TS cells and by the consequences of gene disruption on embryonic development. Many of these transcription factors also control the expression of genes characteristically expressed in trophoblast but not in the epiblast, primitive endoderm and their derivatives. ES and TS cells from the mouse and other species are beginning to provide insights into the changes in gene expression that accompany lineage specification and the subsequent post-specification events that lead to functional trophoblast derivatives.

Introduction

Trophectoderm is the progenitor tissue of the entire outer epithelial component of the placenta, known as trophoblast, and provides the functional bridge between the fetus and the mother. Trophoblast, which ultimately consists of a range of terminally differentiated cell types, performs the majority of the absorptive, immunoprotective and endocrinological functions of the placenta. Trophectoderm differentiates as a simple epithelium, enclosing the fluid-filled blastocoel cavity and the pluripotent inner cell mass (i.c.m.). In the mouse, the best studied of all species, the transition from the morula stage to the blastocyst occurs at about day 3.5 post fertilization, whereas in humans and farm species, the process begins a few days later. Shortly after the formation of the blastocoel, an additional cell layer, known as either primitive or extrae-

mbryonic endoderm, the precursor of the visceral and parietal yolk sac, grows out from the blastocoelic surface of the i.c.m. and along the inner surface of the trophectoderm. Although not discussed further in this review, there is recent evidence from the mouse that the precursors of primitive endoderm can be identified on blastocysts between day 3.5 and 4.5 embryos as a group of cells segregating from the rest of the i.c.m. that express the transcription factor GATA6 [1]. Some days after this simple placenta of two cell layers forms, derivatives of the i.c.m. provide extraembryonic mesoderm and the precursors of the allantoic and amniotic membranes.

At about the time that the primitive endoderm emerges as an identifiable lineage of cells, human and mouse blastocysts are beginning to expand to "hatch" from the

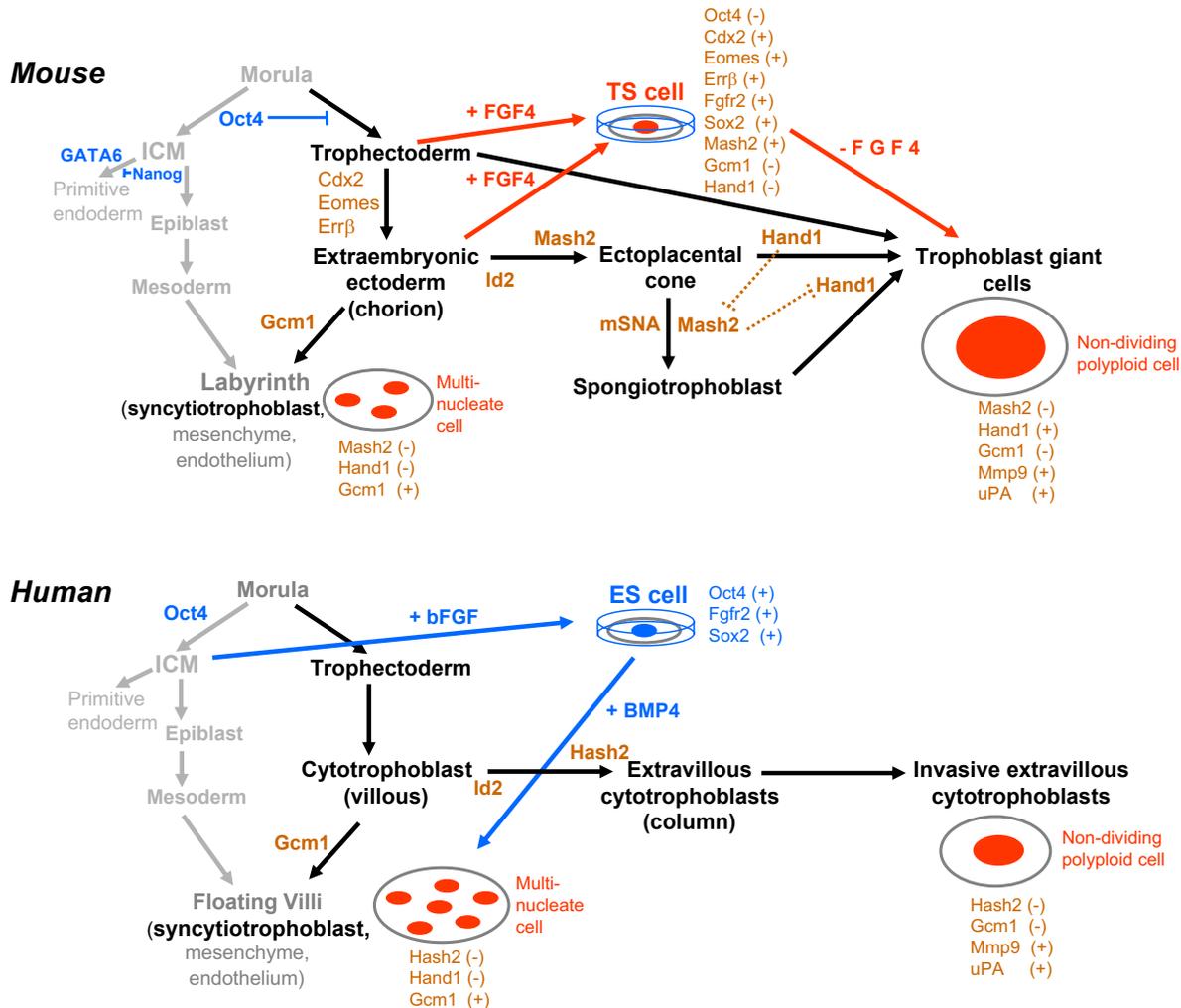


Figure 1

A comparison of trophoblast lineage derivation in the mouse (upper) and human (lower). This diagram is based on that of Cross et al. [4]. Trophoblast lineage cell types are illustrated in black letters, with the direction of differentiation shown in black arrows; other lineages are shown in gray. Key transcription factors that either support or drive differentiation are shown adjacent to the arrows. Most of these are discussed in the text. Attempts have been made to illustrate possible homology between the two species. *Oct4* is expressed in the ICM both species. Mouse trophoctoderm is only specified when *Oct4* becomes down-regulated. As discussed in the text, *Cdx2* and *Eomes* are required early in the mouse trophoctoderm development. Mouse TS cells can be derived from blastocysts and early postimplantation trophoblasts and grow in the presence of FGF4. Removal of FGF4 from the TS cell culture causes them to differentiate into trophoblast giant cells and other trophoblast subtypes. Human ES cells are derived from blastocyst and maintained in the presence of bFGF. They are able to give rise to all cell types of the embryo but can also differentiate into trophoblast cells either spontaneously or in a directed manner when provided with BMP4. *Id2* is expressed in both mouse chorionic trophoblast and human villous cytotrophoblast [11, 79], while *Mash2* is expressed in murine spongiotrophoblast and in the cytotrophoblast columns of the human placenta [77, 80]. *Hand1*, is necessary for the formation of mouse giant cells but not for the specification of spongiotrophoblast and syncytiotrophoblast. By contrast, *Mash2* (or *Hash2* in the human), has the opposite effect to *Hand1*. In the giant cell lineage, *Hand1* must be down-regulated for giant cells to form. The gene *mSNA*, which represses the transcription of genes that promote the transition from mitotic to endoreplicative cell cycles in mouse trophoblast [74], becomes down regulated during giant cell differentiation, but has not been studied in other species. Human extravillous cytotrophoblast may be the functional homolog of the rodent trophoblast giant cells, although expression of *Hand1* has not been detected in human placental villi [75]. On the other hand, expression of *Gcm1* in mouse labyrinth [76] and human chorionic villi [77, 78] is consistent with structural homology of the tissues.

enclosing zona pellucida. They then quickly attach and start to implant. In contrast, implantation in many other species is delayed. In pigs for example, the conceptuses first expand and then elongate end to end along the villous folds of the uterus. Even more surprising, the trophoblast never erodes the uterine epithelium of the mother and never gains direct access to the maternal blood stream [2]. The situation is only slightly different in cattle, sheep and other ruminant species, where the invasive component is restricted to a subpopulation of so-called binucleate cells that fuse with maternal uterine epithelial cells but do not breach the underlying basement membrane [3]. Not surprisingly, the "end" cells of trophoblast differentiation (Fig. 1) are functionally various and their relative homologies across species not entirely clear. For example, there is no obvious equivalent of the murine spongiotrophoblast in cattle, pigs and their relatives. This structure in the mouse may be equivalent to the supportive cytotrophoblast cell columns seen in the invading human trophoblast, but has no obvious homolog in species where the trophoblast does not invade [see [4]]. It is possible that the rodent trophoblast giant cell is the functional homolog of the invasive extravillous cytotrophoblast cells of humans and the invasive binucleate cells of ruminants and horses, but, since homologies are not altogether clear, this review concentrates on the specification of the early trophoblast. We focus largely on the transcription factors that play a part in these early events. Much of the genetic and developmental information has been derived from the mouse where most information exists (Fig. 1), but wherever possible we have ventured into comparative placentation, particularly where some obvious threads of connection exist across species.

Specification of trophoblast in the mouse

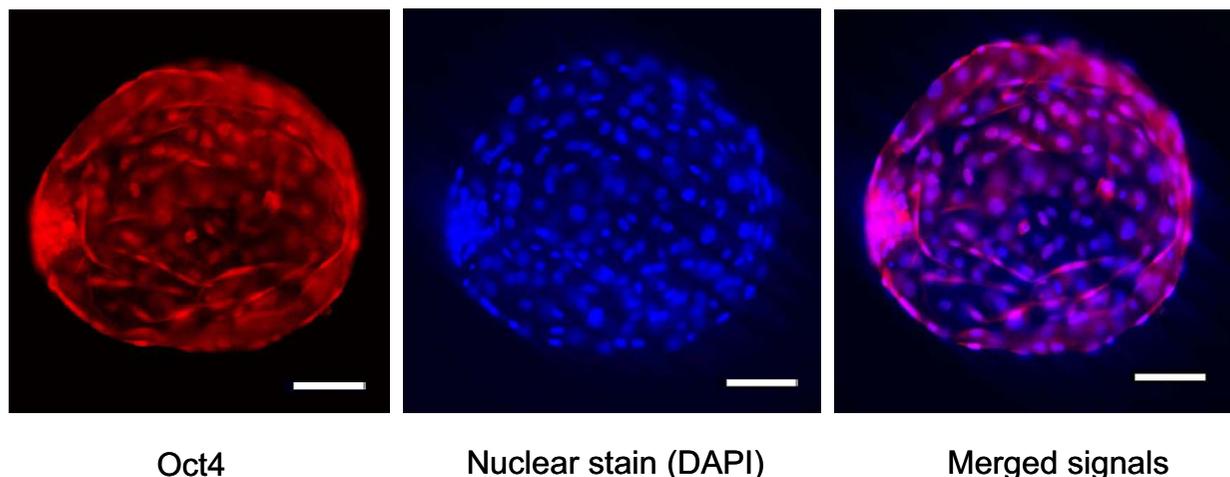
At the time the mouse blastocyst implants, it contains three types of cell: epiblast (or i.c.m.), primitive endoderm, and trophoblast. The segregation of the lineages appears to occur according to the positions that the cells occupy in the compacted morula, so that by the time the blastocyst is ready to implant, the three lineages are no longer interconvertible [1], although they continue to exhibit interdependence during subsequent post-implantation development. In modern parlance, each lineage has its own population of stem cells and expresses its own characteristic set of genes by the time the embryo consists of 32 to 64 cells. This patterning is achieved by the action of transcription factors, whose combinatorial expression, most probably directed by growth factors and fine-tuned by environmental inputs such as oxygen and nutrient availability, establishes boundaries of cell lineage specification within the early embryo.

Transcription factors involved in trophoblast lineage specification

Knockout experiments in the mouse have revealed many genes that when deleted cause embryonic lethality. Sometimes these defects arise from failure within the epiblast lineage, but a significant number of such mutations cause the trophoblast to develop improperly and the conceptus to die before a fully functional placenta is formed [1,4-8]. The majority of such mutations affecting the trophoblast have been discovered by chance when the investigators were in reality hoping to observe a phenotype in the adult animal, but were instead confronted by a failure to obtain homozygous mutants that died well before birth.

Other knockouts have been intentionally engineered after noting an association in expression of a particular gene with a specific placental cell type or after cloning out expressed cDNA from placental tissues. Examples of the latter have included knockouts for several genes encoding transcription factors that have been associated with some of the developmental transitions shown in Fig. 1. Thus, the basic helix-loop-helix (bHLH) gene, *Hand1*, is necessary for the formation of mouse giant cells but not for the specification of spongiotrophoblast and syncytiotrophoblast. By contrast, *Mash2* (or *Hash2* in the human) another bHLH gene, seems to have an opposing effect to *Hand1* in the giant cell lineage and must be down-regulated for giant cells to form. Likewise, the gene, *Gcm1*, must be active for syncytiotrophoblast to develop from its precursors. This ability to limit a particular developmental transition in the trophoblast does not imply that these factors are expressed exclusively in that lineage of cells. For example, the *Hand1* gene is expressed in heart and several other tissues derived from the epiblast [9]. Presumably, it is not the presence of either single transactivators or silencing factors that drive specification events, but a combination of such factors in the correct proportions within the context of the precursor cell (Fig. 1). For example, several additional positive and negative regulators have been implicated in the formation of trophoblast giant cells from their mitotic precursors. These include mSNA (a zinc finger transcription factor) [74], SOCS3 (a suppressor of cytokine signaling) [10], Id2, which is a dominant-negative antagonist of bHLH transcription factors [11], and the orphan nuclear receptor *Errβ* [12]. As the products identified in genetic screens for trophoblast-expressed genes increase and are further analyzed, the list of transcription factors involved in lineage specification will undoubtedly grow too [13].

Some transcription factors that are widely expressed in adult and fetal tissues have been demonstrated in knockout studies to be required for placental development. Good examples are *Ets2* [14], *AP-2γ* [15,16], and some of the subunit genes for AP-1 [17,18]. In each case,

**Figure 2**

Oct-4 expression in an *in vitro*-derived d10 bovine embryo. Strong Oct-4 nuclear immunofluorescence (left panel; red) is detected in the i.c.m. at the left pole of the embryo, while weaker, more diffuse immunofluorescence is present over trophoblast. DAPI nuclear staining (blue) is shown in the center panel. In the right panel, Oct-4 (red) and DAPI (blue) signals have been merged. The merged image indicates that, whereas all nuclei of the i.c.m. are strongly Oct-4 positive, Oct-4 signals are weaker and more variable over trophoblast, with some cells apparently Oct-4 negative. Controls performed with a non-relevant IgG failed to show nuclear staining (not shown). Positive controls (also not shown) with the anti-Oct-4 IgG provided nuclear staining in undifferentiated F9 embryonic carcinoma cells but not in JAr choriocarcinoma cells (data not shown). The bovine embryos were fixed with 2% paraformaldehyde-PBS for 30 min at room temperature, permeabilized with 1% Triton X-100 for 30 min, and incubated overnight at 4 C with primary antibody (affinity purified rabbit anti-Oct-4 IgG in PBS; T.E., R.M.R. unpublished) at a concentration of 4 ng/ μ l. After washing, the blastocysts were exposed to secondary antibody (goat anti-rabbit IgG conjugated with Alexa Fluor 568; Molecular Probes, Eugene, OR) diluted 1:1000. Nuclear staining was performed with DAPI (Sigma, St. Louis, MO) at a concentration of 5 ng/ μ l. Bars represent 100 μ m. Images were captured by using a Nikon Eclipse 800 microscope equipped with a CoolSnap HQ RTE/CCD 1217 digital camera operated by MetaMorph 4.6 software (Universal Imaging Corp., Downingtown, PA) and edited by Adobe Photoshop 6.0.

blastocysts form and implant, but the trophoblast exhibits early abnormalities, and the conceptus soon dies. The role of these familiar transcription factors in lineage-specific events is of particular interest as they also regulate production of characteristic secretory products of trophoblast (discussed later).

Genes required for initial trophoblast specification

Some murine genes, when deleted, cause developmental failure before or at implantation. A good example is the POU-domain transcription factor, Oct4 (encoded by *Pou5f1*, we refer here as *Oct4*). Normally, *Oct4* is expressed in the nuclei of all cells of the cleavage stage embryo, but by the time the blastocyst forms, its expression is restricted to the i.c.m. [19]. When the gene is knocked out, however, no i.c.m. forms and all cells of the embryo default to trophoblast [20]. Accordingly, trophoblast is only specified in the mouse when *Oct4* becomes down-regu-

lated. This generality does not apply across all species, however. In bovine embryos, Oct4 can be detected in trophoblast until day 10, two to three days after the blastocyst first forms [21,22], although its expression there is clearly lower than in the i.c.m. (Fig. 2). Indeed it may be that the mouse is the exception rather than the rule, since Oct4 is also expressed in early human trophoblast [23]. These observations indicate that Oct4 is not a binary off-on switch but that its dosage is critical.

The Sox2 transcription factor exhibits a similar expression pattern to Oct4 during early development of the mouse except that it remains active in trophoblast. Its deletion causes early post implantation failure, but does not prevent blastocyst formation and the differentiation of trophoblast. Sox2 is, however, important in lineage specification because it and Oct4 together regulate the production of FGF4 by the epiblast, a necessary growth

factor for trophoblast proliferation [[1,8,24], and references therein]. Accordingly, Oct4 has a dual role. Its down-regulation is permissive for trophoblast to be specified, but its expression with Sox2 in the i.c.m. leads to the production of a required trophoblast growth factor.

As pointed out by Rossant et al. [1], the formation of trophoblast is not simply a default pathway initiated by the down-regulation of *Oct4*, but probably requires specific transcription factors. One of these is the homeodomain protein, *Cdx2*, and another is the T-box gene *Eomes*. Both show a reciprocal pattern of expression to *Oct4* at the blastocyst stage [25,26], i.e. they are absent from the i.c.m. but expressed in trophoblast. *Cdx2* [27] and *Eomes* [28] knockout embryos fail to implant, although they develop to the early blastocyst stage but generally advance no further, and do not form trophoblast outgrowths when cultured. Finally, *Cdx2*^{-/-} embryos cannot be coaxed to produce trophoblast stem cells [8]. It seems fair to conclude that *Cdx2* and *Eomes* are required early in the development of trophoblast, but possibly not in its earliest specification. At a minimum, murine trophoblast requires the down-regulation of *Oct4* followed by the up-regulation of *Cdx2*, *Eomes*. For the lineage to expand, the i.c.m. must produce the Sox2/Oct4-regulated FGF4 growth factor (Fig. 1). Whether these general rules apply universally across species remains unclear.

ES cells, trophoblast stem (TS) cells, and their interrelationship

Embryonic stem (ES) cells can now be routinely derived from the isolated i.c.m. of mouse blastocysts and maintained in a continuously dividing, undifferentiated mode as long as the cytokine leukemia inhibitory factor (LIF) is provided in the medium [[1,8] and references therein]. These cell lines are generally pluripotent and able to give rise to a variety of differentiated cell types when LIF is withdrawn.

ES cell lines have been derived from two species of monkey [29] and from human blastocysts [30]. Paradoxically, the primate cell lines fail to show any dependence on LIF for continued proliferation in the undifferentiated state. Instead of LIF, these cells need basic FGF (bFGF; Fig. 1) and the support of additional factors that are best supplied by a "feeder" layer of embryonic fibroblasts [31]. ES cells from these primate species can be induced during *in vitro* culture to progress to a wide range of differentiated cell types and are considered, like those from mouse, to be pluripotent. This pluripotency endows ES cells with exciting potential for use in tissue repair and replacement. In the case of the mouse, pluripotency has been demonstrated conclusively by introducing the cells into blastocysts, where they colonize the i.c.m. rather than trophoblast and ultimately contribute to the entire

embryo, including the germ cells [see [1,8]]. The failure of mouse ES cells to form trophoblast in such chimeras and not to differentiate into trophoblast cells in culture is puzzling since the ES cells derived from primate embryos spontaneously form trophoblast over time and can be induced to do so rapidly if supplied with the growth factor BMP4 [32]. Whether BMP4 is the signal for trophoblast differentiation *in vivo* remains to be determined. Such a determination will require either a genetic knock out approach, which can only ethically be performed on primates, or the use of RNAi or other silencing technologies whose outcome is often equivocal.

There are, however, circumstances when mouse ES cells can be directed towards trophoblast. The effects of mutating both copies of the *Oct4* gene on embryo development has been noted earlier [20], and conditional knock out of the same gene in cultured ES cells causes cells expressing trophoblast markers to appear in culture [33]. On the other hand, removal of LIF, which effectively causes *Oct4* to be silenced, does not lead to directed or widespread trophoblast differentiation of ES cell colonies. Instead, other differentiated cell types appear [34]. To explain this paradox, Hemberger et al. [35] have suggested that mouse ES cells contain some trophoblast precursor cells at a low frequency. They have gone on to show that disruption of the gene for the enzyme poly(ADP-ribose) polymerase (*Parp1*) increases the likelihood of mouse ES cells differentiating spontaneously towards trophoblast. It seems conceivable that the difference between the primate ES cell, which tends to form trophoblast readily, and the mouse ES cell, which does not, may not be as profound as previously thought. Slight differences in responses to external signals, possibly the result of differences in receptor numbers or intracellular signaling pathways, and in the expression levels of a few key genes may be sufficient to favor one pathway of "spontaneous" differentiation over another. It should be recalled that mouse and human stem cells require different growth conditions to continue dividing and to remain pluripotent. Mouse ES cells need LIF, whereas LIF seems unimportant to human ES cells, which have a requirement for bFGF instead.

Cells with some features of ES cells have been derived from domestic species, such as sheep, cattle and swine [36-40], but there has been little follow-up since. It remains unclear whether or not these cells can transform into trophoblast, although they can form an array of differentiated derivatives suggestive of an epiblast origin.

Trophoblast stem (TS) cells, seemingly capable of forming only trophoblast derivatives, i.e. restricted in their developmental potential, have been isolated for mouse but not yet from primate embryos (unless primate ES cells are regarded also as TS cells). Two approaches have been

used to derive murine TS cells. The first has been to culture trypsin-dissociated cells from the ectoplacental cone of implanting day 6.5 mouse embryos [41]. The second has been to use primary outgrowths from cultured blastocysts [8]. In both, FGF4 and a growth medium conditioned by embryonic fibroblasts were critical requirements for the cells to proliferate. Removal of FGF4 led to spontaneous differentiation into trophoblast giant cells and the expression of a range of genes characteristic of end-stage trophoblast. A requirement for FGF4 was inferred from expression patterns in early embryos where it is expressed only by the epiblast and its precursor, the i.c.m. [1,8,42]. In contrast, its receptor first appears in outer blastomeres of morulae and then becomes localized on trophoblast [43,44]. Moreover, embryos lacking either a functional gene for FGF4 [45] or for FGFR2 [46], its receptor, fail to grow after implantation.

Various bovine, ovine, porcine and caprine trophoblast cell lines have been described [47-52], although, as discussed below, their status as continuously proliferating stem cells is unclear. One reasonably well characterized bovine cell line, CT-1, which was derived from a blastocyst outgrowth, has special growth requirements, best provided by a feeder layer of fibroblasts, and expresses genes, such as those for interferon (IFN)- τ [53], pregnancy-associated glycoproteins and trophoblast Kunitz-domain proteins characteristic of the bovine trophoblast lineage (J. MacLean, J. Green, & R.M. Roberts, unpublished results). Whether CT-1 or any of the other trophoblast lines are truly stem cells, representing the very earliest stages of lineage specification, is unclear, since they clearly express post-specification genes. They may be progenitor cells, one step along the committed lineage. They have not been well studied with regard to either gene expression or their capacity to differentiate in response to external cues.

There are, in addition, to the cell lines described above a number of trophoblast lines derived from tumors. These include the rat Rcho cell line [54], and an analogous human embryonal carcinoma line HT-H [55]. In both lines, dividing cells easily detach from substratum, but differentiate spontaneously under crowded culture conditions to larger, adherent cells with a more differentiated phenotype. Rcho cultures convert to giant cells with endoduplicated DNA in their nuclei and produce placental lactogen (PL)-I and -II, while HT-H cells form what resembles syncytial trophoblast that are active in steroid synthesis and hCG production. Such cell lines are proving to be valuable in following changes in gene expression that accompany differentiation. There are, in addition, many cell lines that have been derived from human choriocarcinomas, the best known of which are JA_r, BeWo, and Jeg-3. Exactly where these cells fit in the human trophoblast lineage is unclear, but like Rcho and HT-H

cells, they are probably transformed derivatives of early stage trophoblast progenitor cells. Human choriocarcinoma cell lines have been invaluable in determining the processes that control expression of trophoblast products such as hCG, placental lactogens, and steroid hormones [56].

Transcriptional profiling of differentiating TS cells

Attempts are being made to use various types of transcriptional profiling to define the phenotype of differentiating of TS cells, and the changes that occur over time as such cells differentiate [41,57-59]. Oct4, for example, is not detectable in murine TS cells, while the transcription factors *Err β* , *Cdx2*, and *mEomes* are strongly expressed when the cells are proliferating, but down-regulated as differentiation begins and when markers such as PL-I begin to appear.

Microarrays have been employed to follow the BMP4-induced transition of human ES cells to cells that actively produce hCG, HLA-G, estradiol and progesterone [32]. After 7 days of BMP4 treatment, the mRNA for two genes characteristic of pluripotent ES cells, *Oct4* and telomerase reverse transcriptase (*TERT*), had significantly decreased. In contrast, BMP4 rapidly up-regulated some transcription factors, including *Cdx2*, *GATA2*, *GATA3*, and AP-2 genes (*TFAP2*), which had been previously implicated in either placental development or in regulating genes characteristically expressed in differentiating trophoblast. Curiously, the human orthologs of two other transcription factors, *Err β* and *Mash2*, which have been discussed earlier in relation to their likely central role in specifying mouse trophoblast (Fig. 1), were expressed in the original ES cells and showed no significant change in response to BMP4. Attempts to make sense of this microarray information and to make valid comparisons across species are confounded by the sheer volume of the data and by the fact that the cultures undoubtedly contain a range of cell types. Resolving the inconsistencies will not be easy.

Transcription factors that control expression of trophoblast products

As trophoblast emerges and then progresses to form the end-stage cells of mature trophoblast, a series of characteristic products, including several hormones, are released that play important roles in maintaining the pregnancy. For example, IFN- τ , the luteoprotective hormone of ruminant species, is first produced by the early bovine blastocyst and is massively up-regulated a few days later [60,61]. A somewhat similar situation has been observed for hCG production in the human [62] and PL-I production by the mouse [63]. Analysis of the promoters regions of such genes has revealed that they are under the control of many of the same transcription factors, that, when knocked out in the mouse, cause trophoblast devel-

opment to fail. A good example is *Ets2*, a gene essential for placenta formation in the mouse [14]. *Ets2* is also a key transcriptional regulator of the *IFN- τ* genes (*IFNT*) in cattle and sheep [64], the *hCG α* and *- β* subunit genes [65,66], and *CYP11* (P-450 side-chain cleavage enzyme) genes in human [67], and the *PL-II* gene in the mouse [68]. *Ets2* also regulates the urokinase-type plasminogen activator and several metalloproteinases, including MMP9, likely to be involved in invasive implantation [69,70]. Cross et al. [4] discusses additional examples of transcription factors that regulate both trophoblast development and placental hormone production. It seems reasonable to conclude that many of the same genes that control cell fate in the trophoblast lineage also control the transcription of genes characteristically expressed in differentiated, end-stage trophoblast cells.

Several, possibly the majority, of the genes that are transcriptionally up-regulated as trophoblast first forms and differentiates are silenced by Oct4 [see [71]]. We have suggested that one way that Oct4 acts in pluripotent ES cells and in epiblast is to silence the expression of genes that are lineage-specific for trophoblast, such as those encoding the *hCG α* and *- β* subunits [72,73], and *IFNT* [71]. Once *Oct4* itself is down-regulated, this restraint is relieved and the genes can come under the control of transcriptional activators.

Conclusions

A zone of trophoblast specification is established when the embryo is a morula, presumably reflecting a unique combination of transcription factors in that zone of cells and the influence of various environmental cues and growth factors on them. A key first step in this process of specification is the down-regulation of *Oct4*, a transcription factor that acts as a negative regulator of trophoblast specification and of genes normally up-regulated as the trophoblast first forms. The transcription factors believed to have a positive association with trophoblast specification have been inferred primarily in two ways: by their expression patterns in embryos, ES cells and TS cells and by the consequences of gene disruption on embryonic development. Many of these transcription factors also control the expression of genes characteristically expressed in trophoblast but not in the epiblast, primitive endoderm and their derivatives. ES and TS cells from the mouse and other species are beginning to provide insights into the changes in gene expression that accompany lineage specification and the subsequent post-specification events that lead to functional trophoblast derivatives.

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