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Human Embryonic Stem Cells as Models for Trophoblast Differentiation

L.C. Schulz [a], T. Ezashi [a], P. Das [a], S.D. Westfall [a], K.A. Livingston [a], R.M. Roberts [a,b,*]

[a] Division of Animal Sciences, University of Missouri-Columbia, Columbia, MO 65211, USA
[b] Division of Biochemistry, University of Missouri-Columbia, Columbia, MO 65211, USA

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Abstract

Trophectoderm is specified from pluripotent blastomeres at some time prior to blastocyst formation. Proliferating cytotrophoblast derived from trophectoderm is the forerunner of the entire trophoblast component of the mature human placenta, including extravillous cytotrophoblast and syncytiotrophoblast. Recently human embryonic stem cells (hESC) have been employed to study these events in an *in vitro* situation. Here we review some of the work in this emerging area of trophoblast biology. We concentrate primarily on a model in which colonies of hESC are exposed to BMP4 in stem cell growth medium lacking FGF2. Under both low (4%) and high (20%) O_2 conditions, differentiation proceeds unidirectionally towards trophoblast from the outside of the colonies inwards, with the progression fastest under high O_2. Immunohistochemical observations performed on whole colonies combined with microarray analysis of mRNA can be employed to track developmental transitions as they occur over time and in two-dimensional space as the cells respond to BMP4.

Keywords: Bone morphogenetic protein-4; Extravillous cytotrophoblast; Human embryonic stem cell; Oxygen; Syncytiotrophoblast

1. Introduction: the trophoblast lineage and its emergence

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

In the human, two main cell lineages arise from the trophectoderm. One, villous cytotrophoblast, layers the basement membrane that surrounds placental villi. At these sites villous cytotrophoblast gives rise through division and fusion to syncytiotrophoblast [6,7], a cell layer that makes direct contact with maternal blood and is characterized by the production of hCG and other placental hormones [8]. The second lineage derived from trophoblast stem cells is extravillous cytotrophoblast [9], which is non-polarized and multilayered, and the major invasive component of the placenta. These cells migrate in columns from the anchoring villi into the maternal endometrium by two routes: through the uterine stroma and via the lumen of maternal arteries [6] and play a variety of functions including support of the entire placental structure and modification of the spiral arteries [10]. Although the transcriptional programs that drive these events are beginning to be understood, experimental roadblocks exist to investigate how progenitor cells journey along these pathways of differentiation.

* Corresponding author. Division of Animal Sciences, University of Missouri-Columbia, 240b Bond Life Sciences Center, 1201 E. Rollins Street, Columbia, MO 65211, USA. Tel.: +1 573 882 0908; fax: +1 573 884 9676.
E-mail address: robertsrm@missouri.edu (R.M. Roberts).

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2. Models to study trophoblast development

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

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

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

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

3. hESC as models for trophoblast development

ESC from primates, including the human, has been reported to differentiate into trophoblast spontaneously during standard subculture, as evidenced by the production of hCG (measured as hCGβ) and progesterone [26–28]. The former is probably not the most reliable of markers, as it is expressed by many human tumors that have no relationship to trophoblast [29]. Indeed, Ezashi et al. [30] have shown that, in spontaneously differentiating hESC colonies, the cells that produce hCGβ are small and quite unlike syncytiotrophoblast. They are also found on the periphery of emerging areas of larger flattened cells within the body of the colonies. Moreover, the hCGβ-positive cells do not co-express the hCGα sub-unit, suggesting that they are not producing bioactive hCG and are probably not the trophoblast.

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

The ability to drive differentiation of hESC towards trophoblast by silencing genes essential for maintaining pluripotency was first demonstrated in murine cells after the knockdown of *Pou5F1 (Oct4)* [31–33]. Silencing of *NANOG* or *SOX2* also induces markers of trophoblast differentiation [34–36]. In hESC, siRNA silencing of *POU5F1* followed by culture in the absence of FGF2 leads to apparent trophectoderm differentiation, with expression of *BMP4*, *CDX2*, *CGA*, *CGB*, *EOMES*, *GATA2*, *GCM1*, and *ID2* [32,37,38]. A similar up-regulation of trophoblast markers and a transition in cell morphology were observed after *NANOG* silencing [39], although markers of extra-embryonic endoderm as well as those of trophoblast were increased. There is some evidence that mouse ESC can differentiate to trophoblast under certain conditions, but this process does not appear to occur as readily as in primate cells [40,41]. Studies in the mouse have largely concentrated on trophoblast stem cells [42], whose equivalent has as yet not been reported for the human.

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

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

A more recent approach to purify trophoblast cells from EBs has been based on the selection for adhesion rather than for hCG