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Human Trophoblast Progenitors: Where Do They Reside?

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Abstract

In humans, very little is known about the factors that regulate trophoblast (TB) specification, expansion of the initial TB population, and formation of the cytotropho-blast (CTB) populations that populate the chorionic villi. The absence of human trophoblast progenitor cell (hTPC) lines that can be propagated in vitro has been a limiting factor. Because attempts to derive TB stem cells from the trophectoderm of the human blastocyst have so far failed, investigators use alternative systems as cell culture models including TBs derived from human embryonic stem cells (hESCs), immortalized CTBs, and cell lines established from TB tumors. Additionally, the characteristics of mature TBs have been extensively studied using primary cultures of CTBs and explants of placental chorionic villi. However, none of these models can be used to study TB progenitor self-renewal and differentiation. Furthermore, the propagation of human TB progenitors from villous CTBs (vCTBs) has not been achieved. The downregulation of key markers of cell cycle progression in vCTBs by the end of the first trimester of pregnancy may indicate that these cells are not a source of human TB progenitors later in pregnancy. In contrast, mesenchymal cells of the villi and chorion continue to proliferate until the end of pregnancy. We recently reported isolation of continuously self-renewing hTPCs from chorionic mesenchyme and showed that they differentiated into the mature TB cell types of the villi, evidence that they can function as TB progenitors. This new cell culture model enables a molecular analysis of the seminal steps in human TB differentiation that have yet to be studied in humans. In turn, this information can be used to trace the origins of pregnancy complications that are associated with faulty TB growth and differentiation.

Keywords

human placenta; trophoblast progenitors; chorion; placental stem cells; cytotrophoblast

Stem and progenitor cell culture models are powerful tools for analyzing developmental processes, with added value for human studies because in vivo experimentation is not possible. The placenta, a complex organ, contains multiple cell types that are of either

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ectodermal (placental-specific tro-phoblast [TB] cells) or mesodermal origin (villus core). Pregnancy begins when TB cells, differentiated from the trophectoderm (TE) of the blastocyst, attach the embryo to the uterus. They play essential roles during the peri-implantation period. However, their functions in humans have been impossible to study because we lack blastocyst-derived TB stem cells from our own species. Therefore, investigators use other approaches. One avenue is to use embryonic stem cells treated with bone morphogenetic protein 4, which produces cells with TB-like properties,[1] or TBs derived from human embryoid bodies.[2] However, both methods yield TB cells with limited or no proliferative capacity. Finally, in the absence of better models to study TB growth, virally transformed TB lines,[3,4] telomerase-immortalized TBs,[5] or choriocarcinoma cell lines[6] have been used in their place.

Human Villous Cytotrophoblast Differentiation

Very little is known about the formative early stages of human TB differentiation, in particular the steps between TE specification and formation of the TBs that populate the chorionic villi. The unique architecture of the human placenta is established as a result of villous cytotrophoblast (vCTB) differentiation. In turn, vCTB fate determines whether chorionic villi, the functional unit of the placenta, float in maternal blood or anchor the conceptus to the uterine wall. vCTBs form a polarized epithelium that is attached to the basement membrane surrounding the stromal cores of chorionic villi. In one pathway, vCTBs detach from this basement membrane and fuse to form multinucleated syncytiotrophoblasts (STBs), which transport substances to and from maternal blood that perfuses the intervillous space with which they are in direct contact. In the pathway that leads to invasion, vCTBs also leave this basement membrane, but they form columns of nonpolarized cells that attach to and then penetrate the uterine wall. The ends of the columns terminate within the superficial endometrium, where they give rise to invasive (extravillous) cytotrophoblasts (CTBs). During interstitial invasion, a subset of these cells, either individually or in small clusters, commingles with resident decidual, myometrial, and immune cells. During *endovascular* invasion, masses of CTBs breach and plug the vessels. Subsequently, these fetal cells replace the resident maternal endothelium and portions of the smooth muscle wall, a process that is much more aggressive on the arterial as compared with the venous side of the uterine circulation. The end result is patent hybrid vessels, made up of maternal and embryonic/fetal cells, which diverts uterine blood flow to the placenta and enables venous return.

Primary vCTB cultures[7,8] and chorionic villous explants[9] have been extensively used for many years to study the molecular mechanisms of human TB differentiation, invasion, and fusion. At a molecular level, the process whereby CTBs withdraw from the cell cycle and fuse to form multinucleated STBs involves the coordinated expression of several factors.[10–12] These include molecules with relevant functions (e.g., fusogenic human endogenous retroviruses). Other factors with potential roles in this process that are expressed in the human placenta include ADAM12 (in both membrane and soluble forms),[13] a disintegrin, and metalloproteinase. Caspase 8, which could contribute to cytoskeletal remodeling required for fusion, may also be involved.[10] There is strong evidence that other classes of molecules such as connexins are critical to STB formation.[14] Placental proteins also appear

to play a role. Placental protein 13, a galectin family member, inhibits CD98-induced fusion.[15] Hormones—including estradiol, glucocorticoids, and human chorionic gonadotropin—promote STB generation.[16] Interestingly, CD98, a putative amino acid transporter, may also play a role.[17–19] Finally, physiological factors are also key; for example, rising oxygen levels promote fusion.[20]

An interesting spectrum of factors regulates vCTB differentiation along the invasive pathway. CTB transformation of the uterine vasculature depends on the cells' ability to execute a unique epithelial-to-endothelial transition. In this regard, analysis of CTB invasion in situ and in vitro has established that this switch is a vital component of placental development.[21–25] Initially, vCTBs in floating villi express the epithelial type cell-cell adhesion molecule (E-cadherin) and α6β4 integrin. Upon differentiation/invasion, the cells down-regulate these molecules and upregulate the expression of vascular-endothelial cadherin, α5β1, αVβ3, PECAM-1, and vascular cell adhesion molecule 1 [24] as well as matrix metalloproteinase 9.[26,27] Recent data suggest that CTB differentiation/invasion also entails a switch from a venous to an arterial phenotype in terms of the cells' expression of Eph and ephrin bidirectional signaling molecules.[28] This change is accompanied by an upregulation of several growth factors and receptors (e.g., vascular endothelial growth factor and angiopoietin [Ang] family members) that function during conventional vasculogenesis and angiogenesis.[25] Oxygen tension also plays an important role, favoring CTB proliferation rather than differentiation/invasion.[29–31] In this context, it is not surprising that the hypoxia inducible factors are important regulators of CTB differentiation.[32]

One limitation of these models for studying TB progenitors is that cultured vCTBs, as isolated cells or as components of chorionic villi, exit the cell cycle. Therefore, they cannot be used to study factors that regulate TB proliferation, which is essential for understanding mechanisms of placental growth. One possible reason may be that the culture conditions that have been used to maintain these cells do not support the growth of the progenitor subpopulation. The other possibility is that CTBs isolated from chorionic villi are a differentiated population with a limited capacity to proliferate. To address these questions, we and others have used immunolocalization approaches to detect the expression of cell cycle regulators in tissue sections of the human placenta at different gestational ages. The mitotic index was the first parameter used to detect TB progenitors. The vCTBs population contains cells in S phase[33] as do the proximal portions of the cell columns associated with anchoring villi that attach the placenta to the uterine wall.[34,35] Immunostaining of first trimester placental bed biopsies with an antibody against the Ki67antigen, which is expressed by cells that are synthesizing DNA, shows that expression abruptly stops at sites where CTBs from anchoring villi invade the uterine wall. These data suggested that differentiation of invasive CTBs is coordinated with exit from the cell cycle. As antigen/antibody pairs for studying cell cycle regulation became available, we conducted the first systematic study that mapped expression of key regulators in placental tissue sections of the maternal-fetal interface (first trimester to term). We analyzed floating villi (where CTBs differentiate into STBs) and anchoring villi (where CTBs differentiate into invasive cells). In these experiments, we analyzed the expression of 16 different cell cycle regulators. The staining patterns changed during differentiation and as a function of gestational age.[36] The results of this study showed that >50% of vCTBs from first trimester specimens expressed

markers of cell cycle progression in terms of the G1-M transition, and they failed to stain for inhibitors of cyclin-CDK activity. This pool decreases abruptly as pregnancy progresses; <25% of second trimester vCTBs express markers of cell cycle progression with a further decrease to 1 to 5% at term. The major limitation of this in situ approach is the inability to distinguish differentiated cells with limited proliferative capacity from progenitors that self-renew. This fact may explain why isolated first trimester CTBs are unable to sustain proliferation in vitro. Additionally, the abrupt decrease in the percentage of cycling CTBs at the end of the first trimester of pregnancy suggested that subsequent placental growth is supported by progenitors that do not reside within the vCTB population. Accordingly, we tested the hypothesis that the mesenchymal compartment of villi and the chorion provide a niche for human trophoblast progenitor cells (hTPCs).

Placental Stem Cells

Stem cells that reside in the extraembryonic compartment have attracted a great deal of attention because they are derived from readily available tissues that are usually discarded after birth. Recently, pluripotent stem cells were isolated from the placenta (placental stem cells [PSCs]). Although their functions are poorly understood, PSCs very likely play an important role in regulating placental growth and TB differentiation. Extraembryonic in origin, they are classified as somatic stem cells, the placental equivalents of multipotent lines that have been isolated from tissues and organs of the body (soma). In contrast to human embryonic stem cells (hESCs), the differentiation capacity of the somatic stem is restricted to some, but not all, cell types of the body. They can either self-renew or form progenitors that advance to become fully differentiated progeny.

The extraembryonic tissues are composed of epithelial cells—TB cells of the placenta and chorion, and amniotic epithelial cells—and mesenchyme, which forms the core of chorionic villi and lines the chorion and amnion. Recent reports have demonstrated that the human term placenta is a rich source of mesenchyme-like stem cells (MSCs).[37–43] PSCs have been isolated from trypsin digests of term placentas. Clonal populations have been characterized with regard to their morphological characteristics, surface markers, gene expression patterns, and differentiation potential. They express stem cell markers OCT4, Rex 1,[37,39] SSEA-4, TRA-1–60, and TRA-1–80.[37,40,43] When expanded in culture, placenta-derived MSCs have multilineage differentiation capacity, giving rise to mesodermal, endodermal, and ectodermal derivatives.[37,43–45] Interestingly, in contrast to hESCs, these cells cannot be induced to differentiate into germ cells,[46] and initial attempts to differentiate them into TBs were not successful.[1,2,47] In vivo studies provided additional support regarding the differentiation potential of these cells. Upon transplantation, they integrated into multiple tissues and organ systems including bone marrow, heart, and liver.[48]

In contrast, the role of PSCs in placental growth and development remains relatively unexplored. For example, it is not known whether they can generate progenitors of the different cell types that make up chorionic villi, the functional units of the placenta, including TB cells. Addressing these questions is critical for understanding mechanisms of placental growth and its regulation in normal pregnancy as well as in pregnancy complications.

Human Trophoblast Progenitor Cells

In this context, the isolation of hTPCs is critical to filling this gap in our understanding of human placental development. To characterize the hTPC niche, we used samples of the placenta and chorion collected throughout gestation and an immunolocalization approach to identify cells that coexpressed pluripotency and TB markers. We hypothesized that this approach would enable localization of hTPCs in situ, the critical first step in their isolation.

As to study design, we characterized the expression of markers of pluripotency (OCT4, nanog, SSAE4), factors that specify a TB fate (CDX2, Eomes, GCM1), and cytokeratin (CK) 7, also a TB marker. Because new placental villi grow from the chorion, we included these samples in the analysis, theorizing that this compartment might be a niche for hTPCs. To test this hypothesis, we first examined the expression of Oct4, a transcription factor that determines whether hESCs either self-renew or differentiate. In mice, TB differentiation is accompanied by decreased OCT4 expression.[49,50] Conversely, forced repression of OCT4 expression in mESCs induces TB differentiation. The same results can be achieved by overexpression of CDX2[51,52] and Eomes.[51] In 6- to 8-week samples of the placenta and chorion, most of the vCTBs, CTBs in cell columns, and chorionic CTBs strongly reacted with anti-OCT4. Starting at 9 weeks, the number of OCT4-positive cells gradually decreased. By the second trimester, the expression of this transcription factor was restricted to cells in the villus core and chorionic mesenchyme. Detection of OCT4 expression by differentiated TBs and by cells in the mesenchymal compartments was unexpected. The other markers of pluri-potency, nanog and SSEA4, were detected only in early first trimester samples. In second trimester tissues, SSEA4-positive cells that coexpressed OCT4 resided in the villus core and chorionic mesenchyme.

Other investigators have demonstrated the expression of OCT4 in human placenta. The transcription activity of OCT4 is epigenetically regulated by methylation of its upstream regions.[49] Both methylated and unmethylated *OCT4* genes coexist in normal placentas.[53] Because DNA and RNA were isolated from intact tissue, the OCT4 methylation status of individual TB subtypes is not known. The decrease in the number of OCT4-positive CTBs in second trimester villi and chorion is coupled with cell cycle exit as evidenced by the low number of TBs expressing Ki67.

To address the important issue of whether OCT4-positive cells coexpressed molecules that confer a TB fate, we immunostained adjacent tissue sections with antibodies that recognized CDX2, GCM1, and Eomes. In 6- to 8-week specimens, small clusters of CDX2-positive cells that expressed CK7, but failed to react with anti-OCT4, were detected only in the vicinity of the basement membrane, beneath villous and chorionic CTBs, and as rare individual vCTBs. CDX2 expression was not detected in second and third trimester tissues. The same strict temporal and spatial restriction of CDX2 was reported by Hemberger et al.[54] These authors also showed that the transcription factor elf5 is necessary for TBSC selfrenewal, working in a network with CDX2 and Eomes to expand this population.[54] Furthermore, CDX2 colocalizes with high elf5 transcript levels in a subset of vCTBs only in the first trimester. Activation of this cascade may prime CDX2 for degradation, suggesting that vCTB self-renewal is limited to early gestation.

In the first trimester, immunolocalization of Eomes and GCM1 revealed that most villous and chorionic CTBs stained brightly. To our knowledge, this is the first such analysis of early gestation villi and chorion. GCM1 expression was previously detected in association with later gestation CTBs. Proposed functions include regulating transcription of placental growth factor,[55] maintaining a population of proliferative cells[56] by preventing TB terminal differentiation and controlling TB fusion.[57] Our results suggest that chorionic CTBs have a GCM1 expression pattern that is similar to vCTBs. In summary, we observed decreased CTB expression of stem cell markers and factors that specify a TB fate at the end of the first trimester.

Finally, we analyzed expression of these markers in villous and chorionic mesenchyme. OCT4/Ki67-positive, CK7-negative cells that coexpress Eomes and GCM1 are present from first trimester to term. Interestingly, these cells are more numerous in the second and third trimester chorionic mesenchyme than in the villous cores. Lack of CDX2 expression suggests that the CDX2/elf5 network may direct self-renewal of the vCTB population. Our findings also support the hypothesis that villous and chorionic mesenchyme might be the source of hTPCs.

Isolation of Trophoblast Progenitor Cells

Based on these results, we recently devised methods for isolating lines of continuously self-renewing trophoblast progenitor cells (TBPCs) from the chorion and showed that they differentiated into the mature TB populations of the villi.[58] Our experimental strategy exploited the observation that transforming growth factor (TGF)-β/activin/nodal signaling maintains hESC pluripotency; a chemical inhibitor that targets this pathway (SB431542) downregulates markers of the undifferentiated state,[59] suggesting the possibility that TB differentiation is initiated. We also took into consideration the fact that mouse tumor stem cells are derived and propagated in medium containing fibroblast growth factor (FGF).[60] Together, these results suggested that FGF signals in the absence of TGF-β/activin/nodal input might be key to the in vitro propagation of TBPCs in an undifferentiated state.

To test this theory, we separated the human amnion from the chorion and used a stepwise series of enzymatic digestions to isolate cells from the latter membrane. They were cultured in medium that contained FGF with or without SB431542. In the absence of this inhibitor, the cells grew as a dispersed monolayer with an elongated mesenchymal-like appearance. In the presence of this inhibitor, tightly packed colonies emerged that could be passaged 8 to 10 times. After they were dispersed by trypsin digestion and replated, they formed monolayers that could be continuously propagated in an undifferentiated state. These cells immunostained for transcriptional regulators that are required for TB fate specification and/or differentiation of the major lineages in mice including Eomes,[61,62] Geminin,[63] and GCM1.[64,65] When they were cultured under conditions that trigger TB differentiation, they formed the mature human TB populations— multinucleated STBs and invasive CTBs.

At a molecular level, the derivation conditions that we used suggested that TBPC self-renewal involves FGF pathways, as in the mouse, with the additional requirement of blocking activin/nodal signaling. Transcriptional profiling studies identified numerous

molecules that could play roles in specifying hTPC identity and/or enabling their self-renewal, a portion of which have been implicated in placental development and/or function. We are using this new cell culture model to analyze key early steps in human TB expansion and differentiation that are integral to formation of the mature CTB and STB populations. To our knowledge these processes, which regulate placental growth, have yet to be studied in humans. We anticipate that the information that emerges from this work will also enable the formation of novel testable hypotheses about the origins of pregnancy complications associated with faulty TB growth and differentiation including preeclampsia and growth restriction.

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