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The role of chorionic cytotrophoblasts in the smooth chorion fusion with parietal decidua

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Abstract

Human placenta and chorion are rapidly growing transient embryonic organs built from diverse cell populations that are of either, ectodermal [placenta and chorion specific trophoblast (TB) cells], or mesodermal origin [villous core and chorionic mesenchyme]. The development of placenta and chorion is synchronized from the earliest phase of implantation. Little is known about the formative stages of the human chorion, in particular the steps between the formation of a smooth chorion and its fusion with the parietal decidua. Here, we provided evidence that the mechanism by which smooth chorion fuses with parietal decidua is the invasion of smooth chorionic cytotrophoblasts (schCTBs) into the uterine wall opposite to the implantation side. This process, which partially replicates some of the mechanisms of the blastocyst implantation, leads to the formation of a new zone of contacts between fetal and maternal cells. We further analyzed the characteristics of the recently established human self-renewing trophoblast progenitor cells (TBPC), derived from chorionic mesoderm, and we propose the mechanism by which they might *in vivo* contribute to the pool of the invasive schCTBs.

Keywords

Human pregnancy; Smooth chorion; Chorionic cytotrophoblast; Trophoblast progenitor cells; invasive CTBs

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Introduction

Fetal membranes, amnion and chorion, have been extensively studied. However, the main focus of these studies has been on mechanisms involved in the premature rupture of the fetal membranes [1], the pathology associated with the increased risk of pregnancy complications such as intrauterine infection, preterm delivery and neonatal morbidity and mortality (reviewed [2]). Consequently, most of the studies were done on term and preterm amnion and chorion. The subjects of these investigations have been the membrane extra cellular matrix (ECM) composition (reviewed [3]), the role of matrix metalloproteinases [4, 5] and of their inhibitors [6], cytokines involved in the regulation of metalloproteinases expression [7] and in apoptosis [8, 9].

Here, we examine the much less studied role of the second trimester schCTBs in the establishment of a new zone of contact between maternal and fetal cells. Our goal is to stimulate further investigation into the molecular mechanisms of this process and the ways in which its dis-regulation may contribute to the pregnancy complications such as preeclampsia, second trimester abortions and preterm labor.

Development of the chorion

The first trimester of pregnancy

The initial stages of the chorion development have been extensively reviewed [10]. Briefly, the formation of the chorion starts by day 14 post conception (pc) when network of mesenchymal cells, derived from the embryonic disc [11], spread underneath the inner surface of the cytotrophoblast layer of the implanted blastocyst and form the chorionic sac. By day15, blastocyst is completely embedded, the embryo is represented by the small bilaminar embryonic disc (bd) surrounded by multiple, cell column like, outgrowths of the mononucleated trophoblasts (Fig 1A). Branching, loosely connected, mesenchymal cells (mc) form a supportive layer underneath the trophoblastic outgrowths (Fig 1B). Trophoblast outgrowths (primary villi) cover the entire surface of the chorionic sac and mesenchymal cells gradually invade primary villi and transform them into the secondary villi.

By the 8th week of pregnancy, the chorionic sac segregates and forms two extra-embryonic tissues, placenta and chorion. Villi from the embryonic pole of the implanted blastocyst differentiate into the chorion frondosum and become placenta, whereas villi from the abembryonic pole, which face the uterine cavity, stop growing, degenerate and form the smooth chorion [12]. Depending on the spatial relationship between the chorionic sac and the uterine wall, decidua is subdivided into 3 segments: basal decidua between the implanted embryo and the uterine myometrium, capsular decidua that covers the implanted embryo and separates it from the uterine cavity, and parietal decidua that covers the remaining surface of the uterine cavity.

The second trimester of pregnancy

Between the 14th and approximately the 16th week of gestation the diameter of the chorionic sac increases and induces a focal degeneration of the capsular decidua so that the schCTBs come into close proximity of the uterine cavity. It is currently unknown what causes

degeneration of capsular decidua and what is the fate of the uterine epithelial cells, which face the uterine cavity. One possible mechanism is apoptosis of the epithelial cells of the capsular decidua induced by the mechanical stretch of the bulging chorionic sac. The association of mechanical distension with apoptosis has been reported in the pulmonary epithelial cells [13], but has not been, however, confirmed in amniotic epithelial (WISH) cells and human fetal membranes.

Pathologists that examine smooth chorion from different gestational ages cannot distinguish between cells of the capsular and parietal decidua [14]. A systematic analysis of second trimester smooth chorionic and decidual tissues, at the time when smooth chorion fuses with parietal decidua, has not been performed so far. Usually, only remnants of the capsular and parietal decidua, which remained attached to the chorion, are available for histological or immunohistochemical analysis. The thickness of these decidual remnants is variable and depends on gestational age and on procedures used to obtain specimens. Therefore, it is not surprising that very little is known about the cell populations in these tissues. As process of smooth chorion fusion is completed by 18th–20th weeks of gestation, the tissues needed to fully comprehend the sequence of events should be obtained from early to mid second trimester of pregnancies. The availability of this material is limited, which explains the paucity of information regarding the fate of the uterine epithelium, which covers capsular and parietal decidua, and stromal cells from both decidual tissues.

Degeneration of the capsular decidua is followed by fusion of smooth chorion with the parietal decidua, resulting in the almost complete obliteration of the entire surface of the uterine wall [10]. Consequently, the decidual layer that is attached to the term chorion after birth is stroma of parietal decidua [14]. We reasoned that to reach stroma of parietal decidua schCTBs should penetrate the capsular decidua (stroma and uterine epithelium) (Fig 2A) and uterine epithelial cells that cover parietal decidua (Fig 2B). To test the hypothesis that the mechanism of smooth chorion fusion with parietal decidua is achieved by schCTB invasion, we used the immunocytochemical approach to study schCTBs in chorionic tissues from 8 to 20 weeks of gestation. These experiments revealed that schCTBs in the capsular and parietal decidua (Fig 3A&C), and individual cells that migrated into the surrounding decidual stroma, express the same markers as iCTBs from basal plate (Fig 3B&D). The deepest schCTB invasion was observed in the smooth chorion from 20 weeks (Fig 4), at the time which probably coincides with the most active invasion of the parietal decidua. Consistent with the schCTBs invasive properties, collagenolytic enzymes and their inhibitors have been detected in the human amniochorionic membrane [4]. It has also been documented that the schCTBs and the placental cell column CTBs have similar composition of the extracellular matrix [15, 16], produce similar sets of cytokines [17, 18], and express prolactin receptors (Vicovac, unpublished results) and EGFR [19]. Both prolactin [20] and EGF [21] have been shown to stimulate invasion of the CTBs *in vitro*. At the functional level, as revealed in the standard invasion assay with Matrigel coated porous membranes, 16–18 weeks schCTBs were more invasive *in vitro* than placental CTBs from the same specimen (Genbacev, unpublished results). This finding is not surprising because the schCTBs have to breach 2 uterine epithelial layers before they reach the stroma of the parietal decidua.

In addition to the basal plate, the invasion of schCTBs into the stroma of parietal decidua creates a second huge area where maternal and fetal cells come into close contact. In most morphological descriptions of term chorion, parietal decidua is only peripherally mentioned [14]. The composition and characteristics of this additional maternal/fetal zone of contact is mostly unknown. For example, the rare analysis of the hysterectomy specimen at term revealed that in parietal decidua blood vessels are restricted to the deeper layers of the uterine wall, which remain in the uterus, and they are virtually absent in chorionic membranes obtained after birth [22]. It is therefore not surprising that there is no evidence that schCTB invade blood vessels of term parietal decidua. We did not detect any schCTB endovascular invasion in the examined chorionic tissue sections of 16–20 weeks of gestation chorion (Genbacev, unpublished results).

These findings are not totally unexpected. Capillary vessels containing fetal blood cells were present in specimens of chorionic mesenchyme up to 12 weeks of gestation and blood circulation could not be demonstrated in the material obtained after 6 months of pregnancy ([23]. As fetal blood vessels do not exist in smooth chorion after the sixth month of pregnancy, our assumption is that maternal blood does not reach fetal compartment after smooth chorion fuses with parietal decidua. In contrast to limited number of blood vessels, the stroma of second trimester and term decidua is rich in lymphatic vessels [24]. Most importantly, it has been shown that the region, adjacent to smooth chorionic membrane, had the greatest density of lymphatic vessels.

Furthermore, *in vivo* and *in vitro* experiments demonstrated that the development of these vessels in pregnant uterus is triggered by CTBs [25]. Interestingly, chorionic CTBs [26] stained positive for renin, the enzyme that participate in the control of the extracellular volume of lymph and interstitial fluids and regulates arterial pressure [27]. Much work has been done to determine the role of renin-angiotensin system (RAS) in PE [28]. Most studies were focused on the presence of RAS in placental tissues. Its role in the smooth chorion has not been explored so far. We, on the other hand, propose to test the hypothesis that dysfunction schCTBs may contribute to the onset of PE. To assess the validity of this hypothesis, we suggest to test, in control and PE chorionic tissues, three targets: schCTB invasion, schCTB renin production and development of the system of lymphatic vessels.

Progenitors of schCTBs

The mechanism by which the continuum of TB differentiation in human is maintained during pregnancy is poorly understood. In mouse, TB self renewal has been extensively studied using trophoblast stem cells derived from the outgrowth of, either blastocyst, or polar trophoctoderm after implantation [29]. Derivation of self-renewing progenitors from TB populations in the human has not been achieved so far. This may not come as a surprise since the expression of the pluripotency markers in TBs has not been reported and the gene expression analysis did not show the up-regulation of the corresponding genes [30]. Multiple *in vitro* models have been developed instead [31]. The most widely used are the cell lines established by conversion of pluripotent hESCs, or induced pluripotent stem cells (hiPSCs), to cells of TB lineage by treatment of hESCs with BMP4 [32] or activin/nodal inhibitor SB431542 [33].

We reasoned that self-renewing population of stem cells from chorionic mesoderm (CSC), which can differentiate into all 3 germ layers [34, 35], might be a source of TB progenitor cells (TBPC) [36]. We developed a method of isolation of the TBPCs from chorionic mesoderm and the culture conditions that supported their propagation *in vitro*. Molecular signature of these cells, defined by immunolocalization experiments and micro-array data [30], revealed that TBPCs co-express pluripotency markers and the trophoblast fate determinant/stage specific antigens. Using this system, we showed that these cells could differentiate into the various trophoblast cell types of the mature placenta [30]. In addition, immunostained chorionic tissues with TBPC markers confirmed the presence of this subpopulation in chorionic mesoderm. We have observed that in the first trimester chorion TBPCs are small and they are scattered among other populations of the mesenchyme cells [30]. During early second trimester, TBPCs become larger, and form clusters or a continuous cell layer underneath the basal membrane (Fig 5A–D).

Here we propose the following hypothetical scenario for the *in vivo* role of TBPCs. During the first trimester of pregnancy, low oxygen environment stimulates proliferation of CTBs [37, 24], which have a limited renewing capacity (Fig 6A). In contrast, a subpopulation of chorionic mesenchyme cells retains development potential of embryonic stem cells and divide asymmetrically to generate progenitors and differentiated cells [38]. With the increase of oxygen tension by the end of the first trimester of pregnancy (Fig 6B), CTBs gradually withdraw from the cell cycle so that the number of proliferative CTBs, in the later half of pregnancy becomes drastically reduced in placenta [39] and chorion. We hypothesize that in the chorionic mesenchyme, oxygen rich environment stimulates differentiation of TBPCs from chorionic stem cells (CSC) by the mechanism similar to TB differentiation from hESC [40]. It has been shown that the differentiation of TB cells from hESC is much more efficient in 20% than in 4% oxygen [40]. It has been proposed that the key molecule that controls this process is LEFTY 1/2, whose genes display 2–3 fold higher expression under 4% as compared to 20% oxygen [31]. Transient up-regulation of LEFTY in hypoxic environment antagonizes TB differentiation initiated by Nodal signaling [41, 31, 42]. Differentiating TBPCs in the chorionic mesoderm up-regulate markers of iCTBs, integrin $\alpha 4$ and HLAG (Fig 5B&C), invade through basal membrane [30] and contribute to the pool of the invasive schCTBs. Interestingly, it has been already proposed that in the first trimester placenta, in contrast to the accepted bi-potential model of CTB differentiation [43], two separate populations co-exist, one committed to iCTBs and the other to ST [44, 45]. Our hypothesis of the origin and the role of TBPCs remains to be tested. One possible direct proof of this concept might be obtained from the *in vitro* experiment in which the trajectory of GFP labeled hTPCs is traced in tissue explants of the smooth chorion in order to test whether they can reach, penetrate and incorporate into schCTB layer.

Concluding remarks

Smooth chorion fusion with parietal decidua entails specific cellular interactions and molecular mechanisms, which are poorly understood. At the cellular level, schCTBs invade into parietal decidua, anchor the gestational sac to the uterine wall opposite to the implantation site and create a new area of contacts between fetal and maternal cells in the parietal decidua. The expansion of the smooth chorion during the second half of pregnancy

is associated with the rapid increase in the number of schCTBs. Our hypothesis, still under investigation, is that mesenchymal cells from chorionic mesoderm and villous core are the source and provide a niche for, chorionic and placental progenitor cells. Consequently, the chorionic and villous mesenchymal cell dysfunction may affect the coordinated growth of the vital extraembryonic organs, placenta, and chorion, which may deregulate embryonic/fetal development. The exciting inference of such a view is that, as we come to understand the complexity of this system, we may find ways to modify and overcome some of the undesirable pregnancy outcomes. The successful derivation of the self renewing TB progenitors from chorionic mesoderm provided a model system which, with paralleled advancement in single cell based tools, might help to capture the initial stages of TB differentiation, a key event in pregnancy establishment and progression. Knowledge of the mechanisms that regulate schCTBs renewal, invasion, marker expression profiles in normal pregnancy will enable studies of the analogous processes in pregnancy complications such as PE and PTL and will be instrumental in devising early diagnostic tools and provide possible therapeutic strategies for preventing disease.

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Abbreviations

TB	trophoblast
schCTBs	smooth chorion cytotrophoblasts
TBPC	human trophoblast progenitor cells
PE	preeclampsia
PTL	preterm labor
mc	mesenchyme cells
iCTBs	invasive CTBs
CSCs	chorionic stem cells

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Highlights

1. Smooth chorionic cytotrophoblasts (schCTB) invade the parietal decidua
2. Invasion of schCTB forms secondary maternal fetal interface
3. Chorionic mesoderm is a niche and a source of trophoblast progenitors
4. Trophoblast progenitors co-express markers of hESC and of cytotrophoblasts
5. Dysfunction of schCTB and progenitors may contribute to pregnancy complications

Figure 1. Early human embryo development

Hematoxylin and eosin staining of day 15 pc implantation site shows the embedded blastocyst. *bd* bilaminar disk, *mc* mesenchyme, *ctbs* cytotrophoblast, *dec* decidua, *ue* uterine epithelium.

Tissue sections graciously provided and printed with the permission of Dr. Jelena Lazic from the Clinic of Obstetrics and Gynecology, University of Belgrade, Serbia.

Figure 2. Schematic illustration of the pregnant uterus

Depiction of the developing fetus and associated membranes between 16–18 weeks of pregnancy (A) and at term (B). After chorionic fusion with parietal decidua the uterine epithelium lining that covers the capsular and parietal decidua is indistinguishable and schCTBs are embedded in the stroma of the parietal decidua.

A

1 amniotic epithelium, 2 amniotic/chorionic mesoderm, 3 smooth chorion cytotrophoblasts, 4 capsular decidua, 5 uterine epithelium covering capsular decidua, 6 uterine cavity, 7 uterine epithelial lining of the parietal decidua, 8 parietal decidua

B

1 amniotic epithelium, 2a amniotic mesoderm, 2b chorionic mesoderm, 3 smooth chorion CTBs, 4 parietal decidua

Figure 3. Smooth chorion cytotrophoblast share the same trophoblast fate determinants with invasive cytotrophoblasts from the placental bed
Immunostaining of 20.3 weeks of gestation smooth chorion (A, C) and the basal plate (B, D) tissue sections, demonstrates that, during the invasion into decidual stroma, schCTBs up-regulate HLA-G (A, B) and integrin $\alpha 4$ (C, D) markers of iCTBs. Nuclei are stained with DAPI in blue. *ae* amniotic epithelium, *mc* mesenchyme, *iCTBs* invasive cytotrophoblast, *schCTBs* smooth chorion cytotrophoblasts, *dec* decidua, *ck7* cytokeratin 7.

Figure 4. Smooth chorion cytotrophoblast (schCTB) invasion

SchCTBs deeply invade the decidua stroma at 20 weeks of pregnancy. The arrow indicates invasion of schCTBs, identified by the expression of cytokeratin 7 (CK7), into parietal decidua. Nuclei are stained with DAPI in blue. *ae* amniotic epithelium, *mc* mesenchyme, *schCTBs* smooth chorion cytotrophoblasts, *p.dec* parietal decidua.

Figure 5. Trophoblast progenitor cell (TBPC) subpopulation reside in the mesoderm of the smooth chorion

Immunostaining of smooth chorion reveals, in the chorionic mesoderm, the presence of HMGA2, integrin $\alpha 4$ and HLA-G positive TBPCs (Fig A–D). A subpopulation of cytokeratin 7 (CK7) positive smooth chorion cytotrophoblasts co-express integrin $\alpha 4$ (A) and HLA-G (C). Panels B and D are enlarged images of the areas highlighted with rectangle in panels A and C respectively. Nuclei are stained with DAPI in blue.

Figure 6. Schematic representation of the model proposed for the *in vivo* role of trophoblast progenitor cells

Smooth chorion during first trimester of pregnancy (A) and second trimester of pregnancy (B). *CSC* chorionic stem cells, *TBPC* trophoblast progenitor cells, *iCTB* invasive cytotrophoblasts, *schCTB* smooth chorion cytotrophoblasts, *ischCTB* invasive smooth chorion cytotrophoblasts