

Developmental cell biology of human villous trophoblast: current research problems

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ABSTRACT The common gestational pathologies are placental and developmental in origin. However, much remains to be learned about the key events of morphogenesis and growth in the placenta. Metabolic settings established early in both the fetus and placenta define their capacity to respond to later challenges, as well as the quality of the response. Placental growth is exponential in the first trimester and involves coordinated events in trophoblast and mesenchyme, with early cell segregation events having a strong influence on growth potential. One of these is the differentiation of progenitor cytotrophoblasts into villous intermediate cells programmed to fuse with the syncytium, or, alternatively, into extravillous migratory cells that transform the maternal vascular supply. In the latter case, contact with decidual extracellular matrix stimulates differentiation, and therefore this decision is influenced by the number of contact sites at the placental periphery, which in turn is a function of branching in the villous tree. The villous trophoblast bilayer is the primary barrier between maternal and fetal tissues. The maternal-facing layer is syncytial and post-mitotic: this limits traffic of pathogens to the fetus and chimaerism arising by shedding of (non-proliferative) syncytial elements into maternal circulation. Conventional cell culture models fail to replicate this critical vectorial relationship. Tissue explants can overcome the problem to some extent, and have been used to show that turnover of trophoblast in the villous environment is regulated by signals from both fetal and maternal tissues. Maternally delivered insulin-like growth factors stimulate growth and might be therapeutically useful when endogenous growth pathways falter.

KEY WORDS: *trophoblast, villus, proliferation, fusion, syncytium*

Introduction

Trophoblast progenitor cells, trophoblast-mesenchymal interaction and the early origins of gestational pathology

There is wide agreement that the major pregnancy pathologies of first trimester miscarriage, pre-eclampsia (PE) and fetal growth restriction (FGR) result from skewing of placental development. In order to understand pathogenesis, develop effective and accurate approaches to prediction and eventually produce treatments, key developmental processes require to be delineated.

The purpose of the present article is to point to some unsolved problems in villous trophoblast developmental cell biology. An important limitation is that the definitive events of placental morphogenesis that follow closely after implantation are inaccessible to study (Aplin and Jones, 2008). It is of interest that human trophoblast can be obtained from embryonic stem cells either by selection after spontaneous differentiation in embryoid bodies

(Harun *et al.*, 2006), or treatment with BMP4 (Chen *et al.*, 2008; Schulz *et al.*, 2008). These advances will make available cells representing earlier stages of placental development, and give rise to relevant lineages *in vitro* with greater fidelity than has hitherto been achieved.

It is important to recognise that early adjustments can be made to the blastocyst via altered cell allocation to trophectoderm or inner cell mass. In the early postimplantation stages, the extent of mesenchymal investment of the developing placenta is also likely to influence its final size. Global metabolic settings established early in placental development may also influence the later growth of the tissue; it is interesting in this regard that transcriptomic

Abbreviations used in this paper: HIF, hypoxia-inducible factor; IGF, insulin-like growth factor; EGF, epidermal growth factor; FGR, fetal growth restriction; PE, pre-eclampsia.

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data in a variety of species distinguishes subsets of genes that have been coopted during evolution in support of either earlier or later stages of placental development (Knox and Baker, 2008). The realisation that pathways that would hitherto have been understood as 'immunological' contribute strongly to the development of the maternal-fetal interface (see Hunt *et al.*, 2010; Bulmer *et al.*, 2010) represents a paradigm shift, though it is readily explicable in evolutionary terms.

The focus here is on defining and evaluating current understanding of events occurring in trophoblast in the first trimester villus that might be linked to later-manifesting pathologies. These include proliferation, differentiation, fusion, and selected syncytial functional outputs, and the control of trophoblast functions by the combined effect of paracrine signals from mesenchyme or soluble signals from the maternal circulation. Brief consideration is given to decisions in the villous cytotrophoblast progenitor population that specify a syncytial or invasive fate; the behaviour of the invasive lineage is discussed in detail elsewhere in this volume (see Knoefler *et al.*, 2010).

Improved insights, and testable hypotheses, should be achievable by studying developmental systems that, like the villous placenta, comprise epithelial and vascularised mesenchymal tissue. Thus, for example, one can recognise that the mechanisms giving rise to highly elaborated lung parenchyma (Metzger *et al.*, 2008) require a type of branching morphogenesis that has features in common with placenta. Indeed, members of the sprouty protein family of FGF antagonists, which control branching morphogenesis in the lung, are found in placental stromal macrophages in all three trimesters (Anteby *et al.*, 2005). The mechanisms whereby branching occurs during villous development, and the genes that control it, require experimental models that allow function to be tested (Cross *et al.*, 2006). Branching as well as growth abnormalities may contribute to the altered villous morphology seen in some types of FGR (Jackson *et al.*, 1995; Krebs *et al.*, 1996; Macara *et al.*, 1996; Mayhew *et al.*, 2004).

Conversely, villous cytotrophoblast expresses factors that probably stimulate the development of the fetal stroma and fetoplacental vasculature (Demir *et al.*, 2007). For example, these cells express VEGF from early gestation (Jackson *et al.*, 1994), indicating the possibility of a paracrine role in angiogenesis (Challier *et al.*, 2001). Thus bidirectional crosstalk between stroma and trophoblast is involved in placental development.

Control of proliferation and differentiation

Villous cytotrophoblast as a syncytial progenitor

The definitive human placenta has a continuous syncytium overlying a layer of cytotrophoblast that becomes progressively sparser as gestation advances (Jones and Fox, 1991). Villous cytotrophoblast contains a progenitor cell population that, all the way to term, continues to produce daughter cells that differentiate and fuse with the overlying syncytium (Jones and Fox, 1991), allowing expansion of syncytial surface area as well as replacement of material (membrane, cytoplasm and nuclei) that has been shed into the maternal circulation (Redman and Sargent, 2007). DNA synthesis occurs only in cytotrophoblasts; villous cytotrophoblast shows a mitotic index of approximately 1.5-2.9% in the period 6-9 weeks, and zero in the syncytium, which is exclusively post-mitotic (Chan *et al.*, 1999; Kar *et al.*, 2007). This arrange-

ment protects the mother from chimaerism (though incompletely; see Gammill and Nelson, 2010) as the vast majority of nuclear material that is shed is not mitotically competent. It is significant that this phenomenon of a post-mitotic maternally-exposed layer of trophoblast seems to be shared by hemochorial placentas in many species.

Villous cytotrophoblast must undergo asymmetric cell divisions, each of which gives rise to a progenitor cell that maintains the pool, and another cell that differentiates to form an intermediate cytotrophoblast, before fusing with the overlying syncytium. This may follow a series of up to four symmetrical transit-amplifying divisions (Mayhew, 2001). Divisions occur laterally, that is, the mitotic spindle forms in the plane of the villous basement membrane (Kar *et al.*, 2007) and suprabasal cytotrophoblast is not normally observed. To produce differentiated cells, a symmetry-breaking signal is required in advance of cytoplasmic polarisation, segregation of fate determinants and assembly of the mitotic spindle on the appropriate axis (Gonczy, 2008). The initiating signal remains unknown.

These considerations imply heterogeneity in the villous cytotrophoblast population at all stages of pregnancy. Indeed, TEM can clearly identify 'intermediate' or 'transitional' cells that are differentiated in preparation for fusion (Enders, 1965; Jones and Fox, 1991; Kar *et al.*, 2007), and that express molecular markers including the transcription factor GCM-1 (Baczyk *et al.*, 2004), caspases 8 and 10 (Black *et al.*, 2004; Huppertz *et al.*, 1999), FGFR2 (Baczyk *et al.*, 2006) and the α subunit of chorionic gonadotrophin (Hoshina *et al.*, 1982).

The cytotrophoblast pool increases with placental growth from 10-41 weeks but its volume fraction decreases, and, as growth of tissue surface area occurs, the cytotrophoblast layer alters from being relatively close-packed (indeed confluent in many places) in late first trimester to being rather sparse at term (Jones and Fox, 1991; Mayhew, 2001). The cells extend long thin processes laterally beneath the syncytium to create points of contact that can be seen in the electron microscope (Jones *et al.*, 2008). At the same time the syncytium becomes thinner, again reflecting increased surface area. This growth in surface area occurs alongside stromal growth, and it is possible that the driving force (as for villous branching) resides in the mesenchymal compartment.

Villous cytotrophoblast in vitro

Cytotrophoblasts may be isolated from the villus at any stage of pregnancy for primary culture, but they quickly exit the mitotic cycle, differentiate and fuse to form a kind of giant cell that degenerates within about 5 days (Aplin and Crocker, 2007; Handwerger and Aronow, 2003; Kliman *et al.*, 1986; Morrish *et al.*, 1997). Though it is often assumed that this replicates the differentiation pathway of villous trophoblast, it is not clear to what extent such end-stage cells may resemble villous syncytium, or the giant cells of the placental bed, or are a cell culture artifact.

It has not proved possible to maintain proliferation of human primary trophoblast isolates *in vitro* (Morrish *et al.*, 1997) though addition of EGF to highly purified term preparations can extend the proliferative lifespan of residual mononucleate cells beyond a week (Johnstone *et al.*, 2005). Some literature is misleading on this point, as isolates may be contaminated with mesenchymally-derived cells that proliferate rapidly *in vitro*. A small proportion of such cells can skew measurements of thymidine or BrdU uptake

if these are done without reliable phenotyping.

Oxygen, HIF1 and cytotrophoblast regulation

Factors in the three dimensional environment of the villus that are required to maintain progenitor cell characteristics in cytotrophoblast may include the subjacent mesenchyme and basement membrane, lateral adhesions to other cytotrophoblasts, and protection of the apical surface by syncytium. This last point is of interest in regard to the role of oxygen in regulating trophoblast proliferation. The discovery of a transition from low to higher oxygen concentration in the intervillous space at about 11 weeks (Foidart *et al.*, 1992; Jauniaux *et al.*, 2003) led to predictions that villous cytotrophoblast proliferation may be highly oxygen-dependent, but though there is a reduction in the mitotic index of cytotrophoblasts from 2.9 to 1.8 between 6 and 7 weeks of gestation (Kar *et al.*, 2007), evidence in support of a step-change in proliferation at the time of the oxygen transition is so far lacking, and mitosis in villous cytotrophoblast, though slowing down (Challier *et al.*, 2004), continues to term (Mayhew, 2001).

The oxygen-sensitive transcription factor HIF1 is an important regulator of placental development in mouse (Hickey and Simon, 2006) with direct effects on trophoblast fate (Cowden Dahl *et al.*, 2005). However, even after exposure to 24h of severe hypobaric hypoxia (6-7% oxygen), pregnant mice do not express HIF1 α in the placental labyrinth unless nitric oxide synthase is also inhibited (Schaffer *et al.*, 2006), though HIF is seen in less vascularised parts of the placenta. In tumour cell cultures, because of hydroxylation-dependent protein degradation, HIF1 α levels become elevated only when oxygen reaches levels below 1%, whereas in human placenta, HIF1 α is expressed in early gestation (Ietta *et al.*, 2006) when the ambient oxygen level in the intervillous space is estimated to be 2-3% (Jauniaux *et al.*, 2003) but also at term when it is higher (Rajakumar and Conrad, 2000). The homologue HIF2 α is also present (Rajakumar and Conrad, 2000). Thus it appears that HIF may regulate transcription throughout gestation. However, the levels observed in placental homogenates are variable, undermining studies that have attempted to compare concentrations at various gestational stages or in normal and diseased (eg pre-eclamptic) tissues. HIF is strongly dependent on factors other than oxygen; for example, the glucose metabolites pyruvate and oxaloacetate inhibit HIF1 α protein degradation (Lu *et al.*, 2005). The ambient oxygen concentration at the cytotrophoblast layer is unknown, though given the appreciable metabolic activity of the syncytial layer, it is clearly less than that found in the intervillous space. More precise, cell type-specific analysis of HIF levels and function are required.

Explant models of villous cytotrophoblast

The problems of loss of proliferation of primary cytotrophoblasts and their uncontrolled differentiation *in vitro* have been addressed by the development of methods for maintaining villous tissue as explants (Miller *et al.*, 2005). Here the normal vectorial relationship is preserved between mesenchyme, basement membrane and the trophoblast epithelium. In explanted first trimester tissue cytotrophoblast proliferation continues for at least several days at 6-20% oxygen even in the absence of serum (Forbes *et al.*, 2008). In term tissue, cytotrophoblast proliferation in explants is higher at 20% oxygen than 6%, dropping to very low values at 1% (Heazell *et al.*, 2008). Furthermore, blocking mitosis in cytotrophoblast in

term explants promotes cell differentiation so that cell cycle withdrawal may in itself be a stimulus to differentiation (Crocker *et al.*, 2007).

Proliferation in first trimester tissue can be increased at 4-5 weeks by supplying EGF (Maruo *et al.*, 1992b) or IGF (Maruo *et al.*, 1995), and from 8-12 weeks IGF1 or IGF2 (Forbes *et al.*, 2008; Maruo *et al.*, 1995) to the syncytial surface (ie from the 'maternal' side). IGF activates the MAP kinase pathway via the receptor IGFR1. Cytotrophoblast survival is also enhanced by IGF in a PI3 kinase-dependent process (Forbes *et al.*, 2008).

This in turn raises questions about how signals delivered from maternal circulation might cross the mitotically inactive syncytium to stimulate villous cytotrophoblasts. If syncytium is removed using trypsin, basal proliferation ceases, indicating the importance of the overlying cell layer in regulating proliferation of progenitor cells. Exogenous IGF acting via IGFR1 (Forbes *et al.*, 2008), or FGF4 acting via FGFR2 (Baczyk *et al.*, 2006), can directly stimulate proliferation of the exposed cytotrophoblasts. Thus it is possible that growth factors may be delivered from the syncytium to the underlying cytotrophoblast layer.

These observations suggest the possibility of rescuing undergrown placentas, and hence possibly fetuses, by means of growth factors supplied from maternal circulation. Such an approach should be targeted to avoid side effects in other maternal organs.

Establishment of the extravillous cytotrophoblast lineage

First steps

The villous cytotrophoblast population is also precursor to invasive extravillous cells. At sites of contact with the decidua, these proliferate to form anchoring sites containing cell columns in a plane perpendicular to the villous basement membrane, that eventually break away to infiltrate maternal tissues (Aplin *et al.*, 1998). These extravillous precursor cells divide more than once as the cell column forms as indicated by the recognition of mitotic figures in suprabasal layers (Vicovac *et al.*, 1995). The orientation of the mitotic spindle seems to be altered relative to the villous cytotrophoblast progenitor cells that differentiate to fuse with the syncytium. Cells entering the extravillous pathway lose contact with the villous basement membrane, a step that removes them from the progenitor cell niche and is likely to be of key importance in specifying this pathway. Thus it will be of interest to identify the factors that determine the orientation of the mitotic spindle and asymmetric segregation of cytoplasmic constituents prior to division.

Modelling anchoring sites

In explant culture these extravillous cells stay in cycle somewhat longer in 2% than 6% oxygen (Genbacev *et al.*, 1997), although the effects on the numbers of cells populating the lineage are at best modest (Newby *et al.*, 2005; Seeho *et al.*, 2008). Bearing in mind the aforementioned data on villous cytotrophoblast proliferation, and the quite distinct 3D environments occupied by the villous and invasive cell populations, it is possible that oxygen effects are different in the two lineages. Exposure of pregnant rats to hypobaric hypoxia during early pregnancy (days 8.5-9.5) programs a later increase in the size of the junctional

zone and increased endovascular trophoblast invasion in mid gestation (Seeho *et al.*, 2008), perhaps indicating a lineage-specific effect.

Potency of cytotrophoblast progenitors

There remains the question of whether all villous cytotrophoblasts are bipotent, that is, retain the potential to differentiate both in the extravillous and villous lineages. When explants are set up in which villous trophoblast makes contact with a 3D extracellular matrix such as matrigel or collagen, extravillous-type outgrowths that resemble columns of extravillous cells arise from the villous tips (Aplin *et al.*, 1999; Aplin *et al.*, 1998; Bauer *et al.*, 2004; Vicovac and Aplin, 1996). Furthermore, this activity is retained in first trimester villous tissue after culture under conditions in which the subsyncytial, non-tip cytotrophoblasts lose viability (James *et al.*, 2005), and the resulting cells do not fuse (James *et al.*, 2007). This might suggest that cytotrophoblasts at the tips are pre-determined extravillous cell precursors. However, villous tips arise by branching, and so as long as the placenta continues to undergo branching morphogenesis, cells at tips are derived from precursor cells previously situated at a non-branching site. Thus, since the columns act as feeder sites for migratory extravillous trophoblast, the ultimate size of this population is a function of branching in the villous tree.

The molecular basis of fusion

Progression of villous cytotrophoblast to intermediate status is required to acquire fusion competence

One clear prediction arising from the hypothesis of asymmetric division in the villous cytotrophoblast is that one daughter cell should begin to express gene products that mediate fusion. Fusion occurs in an unregulated fashion in term trophoblast primary cultures, which advance spontaneously *in vitro* to fusion competence, while in first trimester primary cultures some fusion is observed. This suggests a progressive shift with gestation from a higher to a lower proportion of cells committed to the syncytial fate or, perhaps, that have advanced to intermediate status. Achieving full control of this transition *in vitro* remains an important research objective.

Numerous cell adhesion molecules expressed at the cytotrophoblast-syncytial interface are classically associated with epithelial junctions (cadherins, tight junction proteins, and others; Aplin *et al.*, 2009). However there is so far only limited evidence for alterations in their distribution or abundance to coincide with differentiation or fusion. Several gene products that are expressed in villous trophoblast have been shown to be capable of mediating cell-cell fusion in cell lines. These include ADAM12 and the endogenous retroviral envelope proteins syncytin 1 and 2 (Huppertz *et al.*, 2006a). Syncytin 2 is in villous cytotrophoblast (Kudaka *et al.*, 2008; Malassine *et al.*, 2007) while syncytin 1 is regulated by GCM-1 (Lin *et al.*, 2005) and probably arises in intermediate cytotrophoblasts in advance of fusion, as well as being expressed in the syncytium (Huppertz *et al.*, 2006a; Mi *et al.*, 2000; Smallwood *et al.*, 2003). Ultrastructural observations have implicated lysosomal enzymes in fusion (Contractor *et al.*, 1977).

Once this has been accomplished there is a need to dispose of excess membrane. Fragments of membrane are visible in the

syncytioplasm near sites of fusion (Contractor *et al.*, 1977) and it is not clear whether this material is then broken down in lysosomes, recycled or transferred to the apical syncytial microvillous membrane (MVM) prior to shedding. Curiously, numerous adhesion molecules (cadherins, tight junction proteins, integrins; Aplin *et al.*, 2009) thought to function in cytotrophoblast-cytotrophoblast adhesion appear in the MVM where their function is unclear.

Syncytial function

Post-fusion maturation, nuclear condensation, aggregation, apoptosis and particle shedding

The syncytium is a barrier to pathogens and maternal cells, a glycoprotein factory and secretory compartment, and a transporting epithelium (see Desforges and Sibley, 2010). Estimates suggest that cytotrophoblast nuclei that become incorporated into the syncytium spend some 3 weeks therein before becoming sloughed into maternal circulation, where fetal DNA comprises as much as 5% of the total. Their morphology ranges from large and rich in euchromatin (similar to intermediate cytotrophoblast), to highly condensed and fragmenting structures reminiscent of apoptotic bodies (Huppertz *et al.*, 2006b; Jones and Fox, 1991). Therefore, during their time in the syncytium, nuclei probably undergo a change from active to inactive and eventually degenerative forms. Later in pregnancy, lateral redistribution of nuclei leads to clustering at thicker areas known as syncytial knots, with sparse ER and an apical membrane with relatively few microvilli. Other areas of syncytium exhibit an apical microvillous membrane where there is probably active transport across a thinner syncytioplasm with sparser nuclei and more synthetic and secretory apparatus. Cytoskeletal activity is likely to be required for this process. Crosslinking of actin by tissue transglutaminase prevents actin from depolymerising, and may play a role in stabilising domains within the syncytium in preparation for shedding (Robinson *et al.*, 2007).

The observation that caspase 8 is activated in pre-fusion cytotrophoblasts (Gauster *et al.*, 2009) has led to a hypothesis that apoptotic machinery is delivered at fusion into syncytium (Huppertz *et al.*, 2006b). There are clear lateral variations in the abundance of apoptotic markers such as the cytokeratin 8 cleavage product known as M13 in the syncytioplasm, and evidence that syncytial knots harbour both M13 and fragmenting nuclei. However nuclei have been estimated to reside in the syncytium for 3-4 weeks before shedding, implying that condensation and degradation are arrested after fusion, presumably allowing these nuclei a period of transcriptional function, before the nuclear apoptotic cascade is reactivated. Rough endoplasmic reticulum, polysomes, and mitochondria are relatively less abundant in syncytial knots (Jones and Fox, 1991).

Transcription and translation in syncytium

Glycoproteins produced in large quantities in the syncytium include chorionic gonadotrophin (CG), chorionic somatomammotropin (CS, also known as placental lactogen) and pregnancy-specific glycoprotein (PSG). *In situ* hybridisation studies show that mRNA encoding the CG α subunit is expressed in intermediate cytotrophoblasts as well as in syncytium, while CS is mostly in syncytium (Hoshina *et al.*, 1982). A detailed analysis of the CG α promoter has suggested that transcriptional control

may utilise different factors in the syncytium than in cytotrophoblast (Knofler *et al.*, 2000). Thus phosphorylated CREB-1 and ATF-1 (dependent on protein kinase A), which bind cAMP response elements, and the coactivator CBP, are primarily located in syncytial nuclei, suggesting the presence therein of transcriptional complexes (Knofler *et al.*, 2000). AP-2 α levels also increase in differentiating cytotrophoblasts and there is *in vitro* evidence to show that it regulates a programme of gene expression that does not involve fusion but rather other aspects of syncytial function including the production of PSG and CG (Cheng *et al.*, 2004). Cytotrophoblasts in placenta prior to 6 weeks express both CS and CG (Maruo *et al.*, 1992a), including a hyperglycosylated form of CG that is postulated to have distinct autocrine functions (Cole and Khanlian, 2007).

PSG polypeptide is exclusively syncytial in late first trimester, and ultrastructural immunolocalisation places it in rough ER, Golgi and secretory vesicles (Zhou *et al.*, 1997). Indeed large amounts of these organelles must be produced in the syncytium to mediate protein production, glycosylation and transit. By analogy with other secretory cells, this may rely on the unfolded protein response (UPR) by which feedback from the ER reaches the nucleus to activate the gene network that produces an expanded protein biosynthetic and secretory compartment (Malhotra and Kaufman, 2007).

The syncytium also produces growth factors; mRNA encoding a soluble splice variant of the VEGF receptor known as sFlt1-14 has surprisingly been localised to syncytial knots at term (Sela *et al.*, 2008), suggesting that some specific transcriptional activity may be needed at these loci.

Though it has been suggested that transcriptional rates are low in syncytium (Huppertz *et al.*, 2006a), and it may very well be that mRNA delivered from intermediate cells at fusion contributes to the total template, it is clear that transcription also occurs within the syncytium (Ellery *et al.*, 2009). Other transcription factors that have been identified in this compartment include Hash-2, I-mfa and Alf-1/HEB (Meinhardt *et al.*, 2005). There is likely to be a role for microRNA (miRNA) in trophoblast differentiation but this remains to be unravelled (Donker *et al.*, 2007).

Conclusions

Trophoblast development and function are dependent on both maternal and fetal signals

Syncytiotrophoblast is a challenging tissue to interrogate. Mouse models are limited; though the murine placenta exhibits a transporting epithelium that contains two layers of syncytiotrophoblast, current evidence indicates that these originate as independent founder cell populations, fusing laterally as villous development proceeds in the labyrinth (Simmons *et al.*, 2008). In contrast, the human villous epithelium differentiates 'vertically' with progenitor cells that are distributed throughout the villous tree producing daughters that populate the overlying syncytium. *In vitro*, the giant multinucleated masses that result from fusion of primary human cytotrophoblast cultures are of limited lifespan, do not form a confluent layer that is amenable to permeability or transport studies, and lack the stromal support to create a polarised epithelium. The cell cycle exit and unregulated differentiation seen in primary cytotrophoblast cultures may be the result of the loss of an overlying adherent, metabolically active syncytium.

Human villous explant cultures in which cytotrophoblast progenitor cells continue to proliferate and fuse offer an alternative model (Forbes *et al.*, 2008) in which the vectorial relationships are retained between cytotrophoblast, the underlying basement membrane and mesenchyme, and the overlying syncytium. This has revealed that turnover of trophoblast in the villous environment is regulated by signals from both fetal and maternal tissues.

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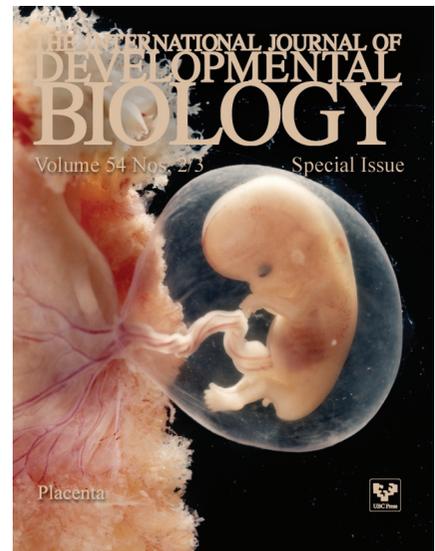
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