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# Three-dimensional cultures of trophoblast stem cells autonomously develop vascular-like spaces lined by trophoblast giant cells

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ABSTRACT

The maternal blood space in the mouse placenta is lined not by endothelial cells but rather by various subtypes of trophoblast giant cells (TGCs), defined by their location and different patterns of gene expression. While TGCs invade the spiral arteries to displace the maternal endothelium, the rest of the vascular space is created de novo but the mechanisms are not well understood. We cultured mouse trophoblast stem (TS) cells in suspension and found that they readily form spheroids (trophospheres). Compared to cells grown in monolayer, differentiating trophospheres showed accelerated expression of TGC-specific genes. Morphological and gene expression studies showed that cavities form within the trophospheres that are primarily lined by *Prl3d1/Pl1*$\alpha$-positive cells analogous to parietal-TGCs (P-TGCs) which line the maternal venous blood within the placenta. Lumen formation in trophospheres and in vivo was associated with cell polarization including CD34 sialomucin deposition on the apical side and cytoskeletal rearrangement. While P-TGCs preferentially formed in trophospheres at atmospheric oxygen levels (19%), decreasing oxygen to 3% shifted differentiation towards *Ctsq*-positive sinusoidal and/or channel TGCs. These studies show that trophoblast cells have the intrinsic ability to form vascular channels in ways analogous to endothelial cells. The trophosphere system will be valuable for assessing mechanisms that regulate specification of different TGC subtypes and their morphogenesis into vascular spaces.



## Introduction

The ancestral type of placenta among mammals is hemochorial, in which maternal blood is lined not by endothelial cells but rather by conceptus-derived cells of the trophoblast cell lineage (Wildman et al., 2006), and is observed in humans and rodents. On the arterial side, specialized trophoblast cells invade into maternal arteries to replace the endothelial cells, a process called vascular invasion and mimicry (Rai and Cross, 2014). However, within the placenta itself, trophoblast cells undergo morphogenesis to create the maternal blood compartment (vasculogenic mimicry) (Rai and Cross, 2014). On the venous side, trophoblast cells must connect with maternal endothelial cells to deliver the maternal blood back into maternal vessels. Given the complexity of these interactions, it is not surprising that pregnancy-related complications occur in humans with defects in the maternal vascular space but associated with the trophoblast compartment including preeclampsia and placenta abruption (Kaufmann et al., 2003; Rossant and Cross, 2001). Likewise, there are several examples of mouse mutants with defects in the maternal blood compartment (Gasperowicz et al., 2013b; Guzman-Ayala et al., 2004; Scott et al., 2000), as recently reviewed (Rai and Cross, 2014).

The maternal vascular network in the placenta is best described in mice and is lined by five subtypes of trophoblast giant cells (TGCs) which share properties of being large, polyploid cells arising from endoreduplication and expression of a complex array of hormones (Gasperowicz et al., 2013b; Simmons et al., 2007). Blood enters the placenta through radial arteries that then branch into several spiral arteries within the decidua (Adamson et al., 2002). Spiral artery TGCs (SpA-TGCs) invade the spiral arteries, replacing the endothelial cells and hence taking over the vasculature. The arteries also lose their smooth muscle and become greatly dilated, a process known as spiral artery remodeling (Adamson et al., 2002). Cell ablation experiments have shown that SpA-TGCs are critical to initiate the remodeling (Hu and Cross, 2011), though the remodeling occurs well upstream of even the deepest invading SpA-TGCs (Adamson et al., 2002), and is thought to involve interactions with maternal uterine NK cells (Matson and Caron, 2014; Soares et al., 2014). The spiral arteries converge to

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form a large canal that carries oxygenated blood to the base of the placenta where it then enters the small sinusoid spaces within the labyrinth spaces lined by canal TGCs (C-TGC) and sinusoidal TGCs (S-TGC), respectively. After exchange of gases and nutrients, blood then moves out through channels lined by channel TGCs (Ch-TGCS) into large lacunae lined by parietal TGC (P-TGC) that connect with endothelial cells in the uterine veins. The subtypes of TGCs differ not just in location, but also in cell lineage origin and expression of hormones (Simmons et al., 2007) implying that they have different functions.

While the bHLH transcription factor H*and1* is crucial for differentiation of all subtypes of TGCs (Hu and Cross, 2011; Simmons et al., 2007), there are only a few insights into mechanisms that specify different TGC subtypes. In culture, retinoic acid promotes development of P-TGCs but suppresses formation of S-TGCs, Ch-TGCs and C-TGCs (Simmons et al., 2007; Yan et al., 2001). Deletion of transcription factor Tle3 leads to reduction in number of *Prl2c2/Plf*-positive P-TGCs and Ch-TGCs (Gasperowicz et al., 2013b). Notch2 promotes differentiation of TGCs though more on the arterial side of the maternal vascular space (Gasperowicz et al., 2013b); Hamada et al., 1999; Hunkapiller et al., 2011). Tissue oxygen levels regulate trophoblast differentiation and there are suggestions that it may differentially impact different TGC subtypes. The oxygen tension in the human placenta changes during various stages of gestation from less than 20 to more than 50 mm Hg (Jauniaux et al., 2000). It is hypothesized that initial placental development in mice also occurs at low oxygen levels (Adelman et al., 2000; Dunwoodie, 2009), the time when the first TGC subtype, P-TGCs, differentiate. In the mature mouse placenta, the subtypes of TGCs on the venous side, Ch-TGCs and P-TGCs, are exposed to deoxygenated blood whereas SpA-TGCs and C-TGCs are in contact with oxygenated blood, and S-TGCs are bathed in diminishing concentrations of oxygen depending on the level within the labyrinth. Previous studies with trophoblast stem (TS) cells grown as adherent monolayers, showed that culturing in 3% oxygen, compared to the atmospheric levels (~20%), increased expression of a P-TGC-specific gene (*Prl3d1/Pl1α*) but had no effect on other TGC-specific genes (Koch et al., 2012). The cellular response to low oxygen levels is regulated by a family of transcription factors, known as hypoxia inducible factors (Hifs). Knockout mice for *Hif1α*, *Hif2α* and *Arnt* have shown that these factors also regulate trophoblast differentiation (Cowden Dahl et al., 2005). TS cells derived from Hif mutants also show defects in differentiation (Choi et al., 2013). *Hif1α/2α* double mutants and Arnt mutants show reduced expression of the P-TGC-specific gene *Prl3d1* (Adelman et al., 2000; Cowden Dahl et al., 2005), though at the time of the analysis, markers of other TGC subtypes were not assessed. Mutant TS cells lacking the gap junction protein Cx31 are unable to stabilize the *Hif1a* transcription factor under hypoxic conditions, fail to increase *Prl3d1* mRNA expression in response to hypoxia, but instead increase expression of other TGC-specific genes including *Ctsq* which is specific to S- and Ch-TGCs (Koch et al., 2012).

All cells in vivo develop in a three-dimensional (3D) space and there are several examples in which the microenvironment is crucial for determining their characteristics, which cannot be provided in monolayer culture. Many 3D culture systems have been established including mammospheres, endothelial cell spheroids, and neurospheres (Fennema et al., 2013). Human cytotrophoblast cells have been grown as spheroids (Dokras et al., 2001; Korff et al., 2004) but whether this influences patterns of differentiation is not entirely clear. The extracellular microenvironment of cultured mouse TS cells has been shown to affect the differentiation of syncytiotrophoblast versus TGCs. (Choi et al., 2013). The five TGC subtypes form in specific locations within the mouse placenta, suggesting that there may be location-specific cues. Our goal here was to develop a 3D culture system to determine if differentiating TS cells have an intrinsic ability to form vascular-like structures or if external signals from the uterus or maternal blood are required.

## Materials and methods

### *Mice*

CD1 strain pregnant female mice were dissected at embryonic days (E) 7.5 through 12.5 (noon on the day of the vaginal plug was designated as E0.5). All animals were housed under normal light conditions (12 h light/12 h dark) with free access to food and water. All animal procedures were carried out in accordance with the University of Calgary Animal Care Committee.

### *Trophoblast stem (TS) cell culture*

The TgRS26 line of TS cells was cultured as previously described (Tanaka et al., 1998). Proliferation medium contained RPMI1640 (Invitrogen) with 20% fetal bovine serum (FBS), 1 mM sodium pyruvate, 50 μg/ml penicillin/streptomycin, $5.5 \times 10^{-5}$ M β-mercaptoethanol, 25 ng/ml basic fibroblast growth factor (bFGF, Sigma Chemical Company, St Louis, USA), 1 μg/ml heparin (Invitrogen) and 70% of media preconditioned by embryonic fibroblast cells for two days. Differentiation media simply lacked bFGF, heparin and embryonic fibroblast conditioned medium. For consistency of results all experiments used cells between passages 45 and 55.

To generate spheres of defined size, $10^3$ and $10^4$ cells were first suspended in 5 or 10 ml of proliferation medium in Ultra Low Attachment dishes (Corning, USA), or 50–500 cells were implanted in hanging drops (20–30 μl), for up to two days. Then the nascent trophospheres were collected, washed in 1X phosphate buffer saline (PBS) and transferred to Ultra Low Attachment dishes with differentiation medium for another 6–8 days. The cell suspension was cultured in 100 mm, 60 mm or 96 well Ultra Low Attachment dishes at 37 °C under 95% humidity, 5% $CO_2$ and atmospheric $O_2$ (19%) and nitrogen in a Forma Scientific Incubator (Thermo Fisher Scientific, USA). To achieve low oxygen conditions, cultures were maintained at 37 °C in a Heracell 150i incubator (Thermo Fisher Scientific, USA) with 5% $CO_2$ and nitrogen to balance the oxygen levels that were fixed to 1%, 3% or 8%.

### *Histology*

For histological analysis, placentas were dissected and fixed at 4 °C in 4% paraformaldehyde in 1 × PBS overnight, washed 3 times with 1 × PBS, dehydrated through ethanol gradients and xylene, paraffin embedded, sectioned (10 μm thick), deparaffinized and stained with hematoxylin and eosin or treated for in situ hybridization or immunostaining. Placentas for cryosection were dissected, fixed at 4 °C in 4% paraformaldehyde (in 1X PBS) overnight, washed three times with 1X PBS, incubated in 15% and 30% sucrose/1X PBS solutions and embedded in OCT compound (Sakura Finetek, Torrence, CA USA) on dry ice and frozen at −80 °C.

### *In situ hybridization*

In situ hybridization was performed as described previously (Simmons et al., 2007) with some modifications. Briefly, 10 m paraffin sections were adhered to Super Frost Plus slides (VWR International, West Chester, PA, USA), deparaffinized in xylene and rehydrated through ethanol gradients, post-fixed in 4% PFA, treated with proteinase K (30 g/ml for 20 min at room temperature; Roche, Laval, Quebec, Canada), acetylated for 10 min (acetic anhydride, 0.25%; Sigma-

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