1 The origins of placental mesenchymal stromal cells: Full spectrum flow cytometry reveals
2 mesenchymal heterogeneity in first trimester placentae, and phenotypic convergence in culture

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Abstract

Single-cell technologies (RNA-sequencing, flow cytometry) are critical tools to reveal how cell heterogeneity impacts developmental pathways. The placenta is a fetal exchange organ, containing a heterogeneous mix of mesenchymal cells (fibroblasts, myofibroblasts, perivascular, and progenitor cells). Placental mesenchymal stromal cells (pMSC) are also routinely isolated, for therapeutic and research purposes. However, our understanding of the diverse phenotypes of placental mesenchymal lineages, and their relationships remain unclear. We designed a 23-colour flow cytometry panel to assess mesenchymal heterogeneity in first-trimester human placentae. Four distinct mesenchymal subsets were identified; CD73⁺CD90⁺ mesenchymal cells, CD146⁺CD271⁺ perivascular cells, podoplanin⁺CD36⁺ stromal cells, and CD26⁺CD90⁺ myofibroblasts. CD73⁺CD90⁺ and podoplanin⁺CD36⁺ cells expressed markers consistent with cultured pMSCs, and were explored further. Despite their distinct ex-vivo phenotype, in culture CD73⁺CD90⁺ cells and podoplanin⁺CD36⁺ cells underwent phenotypic convergence, losing CD271 or CD36 expression respectively, and homogeneously exhibiting a basic MSC phenotype (CD73⁺CD90⁺CD31⁻CD144⁻CD45⁻). However, some markers (CD26, CD146) were not impacted, or differentially impacted by culture in different populations. Comparisons of cultured phenotypes to pMSCs further suggested cultured pMSCs originate from podoplanin⁺CD36⁺ cells. This highlights the importance of detailed cell phenotyping to optimise therapeutic capacity, and ensure use of relevant cells in functional assays.

37 Introduction

38 Mesenchymal stromal cells (MSCs) are a heterogeneous population of cells with secretory,
 39 immunomodulatory, and homing properties (Viswanathan *et al.*, 2019). This population
 40 encompasses fibroblasts, myofibroblasts, pericytes, and tissue-specific progenitor populations.
 41 Traditionally researchers defined *in vitro* expanded MSCs by 1) plastic adherence, 2) expression of
 42 CD73, CD90, and CD105, lack of hematopoietic and endothelial marker expression (CD31, CD34,
 43 CD45, HLA-DR), and 3) the capacity to differentiate into adipocyte, chondrocyte and osteoblast
 44 lineages *in vitro* (Dominici *et al.*, 2006). However, despite MSCs from different tissue sources (i.e.
 45 bone-marrow, adipose, umbilical cord, placenta) sharing expression of the minimal criteria antigens
 46 after *in vitro* culture, they functionally differ in a tissue-specific manner (Du, Chi, *et al.*, 2016;
 47 Kozłowska *et al.*, 2019). This has been more recently acknowledged in a refined ISCT position
 48 statement that requires the tissue origin of MSCs to be provided along with evidence for their *in*
 49 *vitro* and *in vivo* functionality (Viswanathan *et al.*, 2019).

50 It has become clear that *in vitro* culture may have significant impact on MSC phenotype, meaning
 51 that cultured MSCs may have a different phenotype from their *in vivo*/freshly isolated counterparts.
 52 For example, prior to culture adipose-derived MSCs are CD34+, whilst *in vitro* culture induces the
 53 loss of CD34 expression (Lin *et al.*, 2012). The MSC-associated marker CD271 is also lost with plastic
 54 culture (Brooks *et al.*, 2020), and MSC α SMA expression can be heavily impacted by culture media
 55 (Boss *et al.*, 2020). Thus, the culture conditions used to expand MSCs can mask their true *in vivo*
 56 phenotype and heterogeneity. Furthermore, in any set of culture conditions the most competitive
 57 stromal cells will thrive and take over the culture, whilst other functionally diverse cells may be
 58 diluted out or lost. Understanding the true *in vivo* phenotype, and optimising culture conditions to
 59 minimize phenotypic ‘drift’ *in vitro* may enable researchers to better take advantage of the tissue
 60 specific properties of MSCs, and enable more accurate study of their roles *in vivo*.

61 Placental MSCs (pMSCs) are routinely isolated from the core of placental villi and propagated in
 62 culture (Abumaree *et al.*, 2013; Castrechini *et al.*, 2010; Kusuma *et al.*, 2018). The placenta is a highly
 63 vascularised organ that develops rapidly over gestation in order to act as the conduit for nutrient
 64 and gas exchange between mother and fetus. pMSCs have been reported to reside in a perivascular
 65 niche where they can influence vascular development and function (Castrechini *et al.*, 2010). In line
 66 with this, cultured pMSCs have well established angiogenic and haematopoietic paracrine effects *in*
 67 *vitro* and *in vivo* (Chen *et al.*, 2009; Du, Li, *et al.*, 2016). pMSCs are typically isolated by gross tissue
 68 culture methods (explant outgrowths or enzymatic digestion followed by plastic adherence), rather
 69 than by expression of specific cell surface markers (Abumaree *et al.*, 2013; Pelekanos *et al.*, 2016).

70 However, using gross digestion techniques it is impossible to determine the niche in which pMSCs
71 reside. Previous work has assumed that *in vitro* pMSC expression of CD146, a marker with
72 established perivascular expression *in vivo*, provides evidence of perivascular origins (Castrechini *et al.*,
73 2010). However, work with bone marrow MSCs suggest CD146 expression is a tissue culture
74 artefact (Blocki *et al.*, 2013), and chipcymetry has localised *in vitro* MSC markers CD73, CD105 and
75 CD90 outside of the perivascular niche in term placentae (Consentius *et al.*, 2018). Together this
76 sheds doubt on the perivascular origins of pMSCs, and highlights the need to better characterise
77 their *ex vivo* phenotype understand their functional role *in vivo*.

78 Our understanding of mesenchymal heterogeneity is expanding exponentially, and advances in
79 single-cell technologies have uncovered a wide spectrum of mesenchymal and endothelial
80 phenotypes with specific functions and spatial distributions (Suryawanshi *et al.*, 2018; Takeda *et al.*,
81 2019; Vijay *et al.*, 2019). Mesenchymal heterogeneity can be used to spatially localise cells, identify
82 cells involved in morphogenesis, angiogenesis or quiescence, and characterise inflammatory, anti-
83 inflammatory, or invasive phenotypes (Cimini and Kishore, 2021; Mezawa *et al.*, 2019; Mezheyski
84 *et al.*, 2020; Nazari *et al.*, 2016; Quintanilla *et al.*, 2019). Dramatic advances in flow cytometry over
85 the past few decades have enabled the simultaneous quantification of upwards of 30 antigens,
86 allowing detection of in depth cell heterogeneity. Whilst initiated in the haematopoietic field, the
87 wealth of data produced using multicolour flow cytometry has driven the use of this technique for
88 analysis of other cell types, including the mesenchymal lineages. To date the largest panel used to
89 assess mesenchymal cells (16-colours) was designed for analysis with conventional flow cytometry
90 (Brooks *et al.*, 2020). However, spectral flow cytometry has significant potential to further improve
91 analysis of mesenchymal cells. The ability of spectral flow cytometry to measure a larger portion of
92 the spectral signature of each fluorophore, and thus enable small differences between fluorophores
93 to be discriminated, combined with the ability to remove autofluorescence (a prominent feature of
94 mesenchymal cells) paves the way for assessing cells previously regarded as challenging for
95 multicolour flow cytometry.

96 Here, we developed what to our knowledge is the largest multicolour spectral flow cytometry panel
97 employed to date, in order to characterise the mesenchymal heterogeneity in first trimester
98 placental tissue, and to ascertain the impact of *in vitro* MSC culture conditions on the subsequent
99 phenotype of these populations.

Results

Design of a 23-colour flow cytometry panel to characterise the placental villous core

In order to investigate mesenchymal populations of the placental core a 23-colour flow cytometry panel (Panel One) was developed by starting from a 16-colour panel designed to investigate adipose-derived stromal vascular fraction on a conventional flow cytometer (BD FACS Aria II) (Brooks *et al.*, 2020). The antigens included in this panel are based on the current literature surrounding the placenta, pMSCs, stromal/mesenchymal populations, endothelial progenitors and haematopoietic cells (Table 1). All antibodies were titrated on placental villous digests (Supplementary Figure 1-3).

Table 1: Antigens included in Panel One and their functional roles with respect to the villous core. Antigens are grouped by cell type (grey and white boxes).

Antigen	Full name/s	Functional capacity/relevance	References
ITGβ4/ CD104	β4 integrin	Cell adhesion molecule that uniquely identifies cytotrophoblasts in the placenta.	(James <i>et al.</i> , 2015)
CD45	Protein tyrosine phosphatase, receptor type, C. lymphocyte common antigen	Expressed on all nucleated haematopoietic cells.	(Hermiston <i>et al.</i> , 2003)
HLA-DR	Human Leukocyte Antigen – DR isotype	Expressed by antigen presenting cells i.e. macrophages, MHC class II cell surface receptor, involved in antigen presentation and adaptive immunity,	(Cruz-Tapias <i>et al.</i> , 2013)
CD235a/GPA	Glycophorin A	Red blood cell marker, identifies early emerging RBCs/erythroblasts from haemogenic endothelium	(Garcia-Alegria <i>et al.</i> , 2018; Mao <i>et al.</i> , 2016)
CD41	Integrin alpha-IIb	Platelet marker. Expressed by earliest emerging haematopoietic cells from haemogenic endothelium	(Garcia-Alegria <i>et al.</i> , 2018; Li <i>et al.</i> , 2005)
CD117/cKIT	Mast/stem cell growth factor receptor	Receptor expressed on haematopoietic stem cells and involved in their differentiation	(Rönstrand, 2004)
CD144/ VE-cadherin	Vascular endothelial cadherin	Endothelial cell-cell adherens junctional marker, stabilises vessels, inhibits vascular growth, regulates vascular permeability.	(Giannotta <i>et al.</i> , 2013)

CD34	Haematopoietic Progenitor Cell Antigen	Expressed by haematopoietic and vascular progenitors and adipose-derived MSCs. Transmembrane phosphoglycoprotein, thought to identify early placental progenitors	(Brooks <i>et al.</i> , 2020; Sidney <i>et al.</i> , 2014; Yoder, 2009)
CD31/PECAM1	Platelet endothelial cell adhesion molecule	Expressed by endothelial cells, platelets, macrophages and Kupffer cells, granulocytes, lymphocytes, megakaryocytes. Adhesion molecule found at endothelial intercellular junctions.	(Marelli-Berg <i>et al.</i> , 2013)
VEGFR2/KDR	Vascular Endothelial Growth Factor Receptor 2/Kinase Insert Domain Receptor	Expressed by endothelial cells and thought to identify placental core progenitors. Receptor for angiogenic VEGF, involved in vasculogenesis and angiogenesis.	(Demir <i>et al.</i> , 2007)
CD54/ICAM1	Intercellular Adhesion Molecule 1	Expressed at low levels on endothelial cells, monocytes and lymphocytes. Increased expression in response to inflammatory cytokines.	(Hubbard and Rothlein, 2000)
CD36/FAT	Platelet glycoprotein 4/fatty acid translocase	Expressed by microvascular endothelial cells, and fibroblasts. Has an anti-angiogenic effect via binding Thrombospondin 1. Involved in fatty acid uptake.	(Dye <i>et al.</i> , 2001; Heinzelmann <i>et al.</i> , 2018; Silverstein and Febbraio, 2009)
CD90/Thy-1	Thymocyte differentiation antigen 1	Expressed by MSCs, haematopoietic stem cells, fibroblasts, myofibroblasts.	(Viswanathan <i>et al.</i> , 2019)
CD73/NT5E	Ecto-5'-nucleotidase	Expressed by MSCs and endothelial cells. Works with CD39 to convert extracellular ATP to adenosine to create immunosuppressive effect.	(Roh <i>et al.</i> , 2020; Viswanathan <i>et al.</i> , 2019)
CD39/NTPDase	Ectonucleoside triphosphate diphosphohydrolase-1	Upregulated by MSCs to suppress lymphocyte activation. Immunosuppressive actions via the conversion of extracellular ATP (inflammatory) into adenosine (anti-inflammatory)	(Saldanha-Araujo <i>et al.</i> , 2011; Zhao <i>et al.</i> , 2017)
CD55/DAF	Decay-accelerating factor	Expressed by MSCs. Complement regulatory protein, inhibits C3 convertases thereby creating a threshold for complement activation, increased expression correlated	(Ruiz-Argüelles and Llorente, 2007; Soland <i>et al.</i> , 2013)

		with evasion of innate immune system	
CD271/ NGFR/ p75NTR	Low-affinity Nerve Growth Factor Receptor	Used in MSC and pericyte identification. Hypothesised to identify “stem-cell” or progenitor populations with superior differentiation and colony forming capacity.	(Barilani <i>et al.</i> , 2018; Kumar <i>et al.</i> , 2017)
CD146/MCAM	Melanoma cell adhesion molecule	Adhesion molecule expressed by pericytes, endothelial cells and smooth muscle cells. Involved in the regulation of angiogenesis and vessel permeability.	(Crisan <i>et al.</i> , 2009; Leroy <i>et al.</i> , 2019)
CD248	Endosialin/ tumor endothelial marker 1	Pericyte and stromal cell marker. Involved in cell–cell adhesion, and host defence.	(Lax <i>et al.</i> , 2010; Tomkowicz <i>et al.</i> , 2010)
CD142/TF	Tissue Factor/ thromboplastin	Expression correlated with pericytes, smooth muscle and fibroblasts. Activates blood clotting after injury, located outside the vasculature,	(Abe <i>et al.</i> , 1999; Morrissey, 2004)
CD26/DDP4	Dipeptidyl peptidase-4, adenosine deaminase complexing protein 2	Expressed by many tissues; T-cells, epithelial cells, ESCs, progenitor cells, placental myofibroblasts. Serine protease that cleaves a range of chemokines. Downregulation is correlated with increased stromal/myofibroblast proliferation.	(Kohnen <i>et al.</i> , 1996; Mezawa <i>et al.</i> , 2019; Ou <i>et al.</i> , 2013)
PDPN	Podoplanin	Lymphatic vascular marker. Expression is correlated with increased fibroblast migration. Binds to CLEC-2 receptor on platelets.	(Astarita <i>et al.</i> , 2012; Suchanski <i>et al.</i> , 2017)

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111 Placental explants were dissected from the villous tissue and enzymatically digested to obtain a
112 single cell suspension for flow cytometry (Figure 6). To enable us to focus on the mesenchymal core
113 cells of interest, we needed to exclude trophoblasts (epithelial cells found in a bilayer on the outer
114 edge of placental villi), endothelial cells, and hematopoietic cells. Whilst the initial enzymatic
115 digestion removed the outer syncytiotrophoblast layer, inclusion of the cytotrophoblast marker $\beta 4$
116 integrin in Panel One ensured any potential contaminating cytotrophoblasts directly beneath this

were also excluded from analysis (Table 1, Figure 1B, C). Endothelial cells were identified by expression of CD31 and CD144, specific expression of which is seen in blood vessels and early endothelial cell cords in first trimester placental villi (**Error! Reference source not found.**D). The hematopoietic marker CD45 was used to exclude all cells of this lineage, while CD235a was used to identify and exclude red blood cells (Table 1). All remaining cells were considered to constitute the stromal fraction that makes up the mesenchymal core of the villi.

Full spectrum flow cytometry uncovers heterogeneous mesenchymal populations in the core of first trimester placental villi

First trimester placental villous explants were enzymatically digested to obtain a single-cell suspension that was stained with a master mix for Panel One and analysed on a Cytex Aurora. Due to the complexity of data obtained from a 23 colour panel, unbiased high-dimensional clustering of the data was undertaken using viSNE (Cytobank). viSNE enables visualization of high-dimensional single-cell data and is based on the t-Distributed Stochastic Neighbour Embedding (t-SNE) algorithm (Amir *et al.*, 2013). To run the algorithm, debris, doublets, dead cells, $\beta 4$ integrin+ cytotrophoblasts and CD45+/CD235a hematopoietic cells were excluded by manual gating, then an equal number of cells from each villous core digest was selected using down-sampling (n=5) (**Figure 2**). Combining viSNE with known population marker expression, five distinct subsets were uncovered (**Figure 2**). Endothelial cells co-expressed CD31 and CD34 (Subset One) and made up $3.9 \pm 2.5\%$ of villous core cells (**Figure 3C, D**). CD73⁺CD90⁺ mesenchymal cells (Subset Two) made up $6.8 \pm 9.7\%$ of villous core cells on average, but decreased in abundance with increasing gestational age within the first trimester (**Figure 2C**). This gestational difference was confirmed by the inclusion of data from 22 additional first trimester villous core digests, analysed using a smaller panel targeted at cell sorting but containing the markers required to identify this population (**Figure 2D**). Together this data showed that at <10 weeks of gestation CD73⁺CD90⁺ cells constituted $14.02 \pm 8.84\%$ of villous core cells, but significantly decreased to $0.77 \pm 0.83\%$ of villous core cells at ≥ 10 weeks of gestation ($p < 0.001$, n=27, placentae= 7-13.1 weeks of gestation) (**Figure 3C**). Perivascular cells (Subset Three)

were identified by their expression of CD146⁺CD271⁺, and constituted 10.3±4.7% of villous core cells (Figure 3C). Subset Four made up the largest proportion of mesenchymal cells in the core (47.8±17.0%) and were identified by co-expression of podoplanin⁺CD36⁺ (Figure 3C). The remaining cells, expressing only the myofibroblast-associated markers CD26⁺CD90⁺, were classified as Subset Five and constituted 31.3±12.6% of villous core cells. Subset Five were negative for all other markers investigated, suggesting they are more differentiated myofibroblast-like cells (Figure 3C).

Mesenchymal subsets have distinct phenotypes indicating functionally diverse roles in the villus core

In order to consider what the different subsets functionally represent within the core of placental villi, we further interrogated their phenotype and proportional contribution to the villus core, with consideration to which subset phenotypes pMSCs may originate from. CD73⁺CD90⁺ cells (Subset Two) were the only villous population to co-express CD90 and CD73, both MSC-associated markers. This subset also expressed podoplanin (correlated with increased migratory capacity), low levels of CD142 (involved in blood coagulation (Morrissey, 2004)), and had heterogeneous expression of CD146 and CD271 (perivascular associated markers) (Figure 3B). A lack of CD26 (associated with myofibroblasts and cell proliferation) made this population distinct from the other perivascular and mesenchymal cell populations identified in the villous core using Panel One.

Perivascular cells (Subset Three), identified by expression of CD271 or CD146, did not co-express CD73, CD142 or podoplanin. However, they did express CD26 and CD90, and in line with this they co-localised with Subset Five (also CD26⁺CD90⁺) on the viSNE plot (Figure 3B). The phenotype of CD271⁺CD146⁺ cells was relatively conserved between placentae with some inter-placental variance in CD271, CD36 and CD90 expression (Figure 3B).

Subset Four was identified by expression of podoplanin and CD36, and co-expressed CD26, CD90, and CD142, but was negative for the MSC-associated marker CD73 (Figure 3B). As the largest population within the villous core and expressing proliferative/migratory associated markers (CD26,

podoplanin (Suchanski *et al.*, 2017)) this subset was considered the most likely *in vivo* candidate to contribute to pMSCs that are derived in explant derived cultures.

Phenotypically divergent mesenchymal stromal cell populations converge after *in vitro* culture

In order to investigate the origins of *in vitro* cultured pMSCs in relation to the subsets identified with Panel One we used fluorescence activated cell sorting (FACS) to isolate subsets of interest from first trimester villous digests and assessed their phenotype in relation to cultured pMSCs obtained using an explant outgrowth method. Here we focussed on two *ex vivo* cell populations; CD73⁺CD90⁺ cells (Subset Two) as the only population to phenotypically align with the ISCT marker criteria for MSCs (Dominici *et al.*, 2006), and podoplanin⁺CD36⁺ cells (Subset Four), which expressed markers of proliferative/migratory cells (characteristics associated with pMSCs). Following sorting, cells were cultured for 7 days in EGM-2 (previously demonstrated to sustain fetal-derived pMSCs (Boss *et al.*, 2020)) (**Figure 4**). In culture, both populations underwent phenotypic convergence, forming cells with a mesenchymal-like morphology, that were CD90⁺CD73⁺CD142⁺CD271⁺CD36⁻ at day 7 of culture (**Figure 4B and C**).

Despite this overall convergence, differences between the two populations did remain. The proliferation/myofibroblast associated marker CD26 was only expressed by a small proportion of CD73⁺CD90⁺ cells at the time of isolation and although the percentage of CD26⁺ cells increased over time in culture, most cells remained negative suggesting this marker was not upregulated with the culture conditions used here. Conversely, all podoplanin⁺CD36⁺ cells were CD26⁺ at isolation and remained CD26⁺ after culture. Although CD146, was used to identify perivascular cells in the placenta (Castrechini *et al.*, 2010), was not assessed on cells during isolation (this marker was not contained in Panel Two), phenotyping experiments with Panel One demonstrated that a subpopulation of CD73⁺CD90⁺ cells expressed CD146 (5.89±5.82%) and therefore, could be perivascular. Whereas, podoplanin⁺CD36⁺ cells were negative for CD146. After 7 days culture this

193 CD146⁺ subpopulation was still present in CD73⁺CD90⁺ cells (15.55±6.87%). However, all
194 podoplanin⁺CD36⁺ cells expressed CD146 after seven days in culture, demonstrating differential
195 response of these subsets to the *in vitro* culture conditions (Figure 3Figure 4D and E, and
196 Supplementary Figure 4).

197 In order to determine whether cultured pMSCs are likely to be made up of CD73⁺CD90⁺ or
198 podoplanin⁺CD36⁺ populations, we simultaneously characterised explant isolated pMSCs that had
199 been generated and cultured in the same medium (EGM-2) (n=3, p2-6). This demonstrated that
200 pMSCs were CD90^{low/neg}CD73⁺CD26⁺CD142⁺CD146⁺podo⁺CD34⁻CD271⁻CD36⁻ (Figure 4F), more closely
201 aligning with the phenotype of cultured podoplanin⁺CD36⁺ cells (Subset Four).

202 In previous work we have demonstrated that pMSCs upregulate the contractile markers αSMA and
203 calponin when transferred from EGM-2 to standard MSC medium (advanced-DMEM/F12) (Boss *et*
204 *al.*, 2020). In order to investigate whether CD73⁺CD90⁺ cells (Subset Two) or podoplanin⁺CD36⁺ cells
205 (Subset Four) could similarly upregulate contractile markers, after 7 days in EGM-2 the medium was
206 changed to advanced-DMEM/F12. Whilst in EGM-2 very few cells in either population expressed
207 αSMA and no cells expressed calponin, in advanced-DMEM/F12, both populations upregulated both
208 of these markers (Figure 5). Expression of the more mature smooth muscle marker MYH-11 was not
209 observed in either CD73⁺CD90⁺ or podoplanin⁺CD36⁺ cells cultured in either EGM-2 or advanced
210 DMEM/F12 (Figure 5).

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Discussion

Common sources of MSCs used in clinical trials include; uncharacterised adipose, bone-marrow, and placental stromat. Identifying functionally different mesenchymal cells and defining the origins for tissue-specific MSCs could improve these applications. Here, we used a high-dimensional flow cytometry panel to uncover at least four different mesenchymal populations with distinct expression profiles in the villous core of first trimester placentae, indicating the presence of functionally specialised cells. This mesenchymal heterogeneity could translate to different physiological capabilities important for clinical therapies and aid understanding of the role of these cells *in vivo*.

Characterising the villous core populations allows us to better understand how the placenta functions and develops, and enables targeting of populations for functional analysis. From the four mesenchymal populations classified in this work three populations, perivascular cells (Subset Three), podoplanin⁺CD36⁺ cells (Subset Four), and CD26⁺CD90⁺ myofibroblasts (Subset Five) all closely aligned with populations described in prior single-cell RNA sequencing data from first trimester placentae (Suryawanshi *et al.*, 2018). However, this single-cell RNA sequencing dataset did not report a population that aligned with CD73⁺CD90⁺ cells (Subset Two), instead reporting that only endothelial cells expressed CD73 in the placental core. The ability to assess larger cell numbers by flow cytometry in our work, and the low abundance of CD73⁺CD90⁺ cells after 9 weeks of gestation, means that this population may not have been detected as a distinct population by single cell RNA-seq. Furthermore, RNA expression does not always accurately reflect protein expression (Reimegård *et al.*, 2021). This highlights the importance of protein level characterisation of cells from fresh tissue, and the ability of multicolour flow cytometry to undertake this at single cell resolution complements and builds on single cell gene expression datasets.

The first major aspect of this work to consider is what each subset could represent and what its phenotype reflects. CD73⁺CD90⁺ cells were unique from the other mesenchymal populations and could be identified by their expression of CD73, which was shared only with endothelial cells. The

abundance of this population at <10 weeks of gestation hints at their involvement in processes that occur early in gestation such as vasculogenesis or haematopoiesis. CD73⁺CD90⁺ cells co-expressed podoplanin, but were distinct from other mesenchymal subsets in that they did not express CD26. This combined expression pattern alludes to two potential functional roles of these cells in the placenta. First, expression of podoplanin and low expression of CD26 are both associated with a migratory/invasive phenotype (Mezawa *et al.*, 2019; Ward *et al.*, 2019). As the decline in abundance of these cells after 9 weeks of gestation corresponds to the regression of villi and blood vessels from the distal side of the gestational sac (Burton and Jauniaux, 2018), this raises the possibility that CD73⁺CD90⁺ cells could be involved in this regression of the villi, or they arise transiently as the result of an early endothelial to mesenchymal transition in the placenta whereby, endothelial cells involved in vascular regression lose their junctional markers and gain a mesenchymal/migratory phenotype (Pinto *et al.*, 2016). Secondly, expression of podoplanin and a lack of CD26 expression on CD73⁺CD90⁺ cells indicates potential roles in haematopoiesis. Several studies have demonstrated that inhibiting CD26 enhances homing, engraftment and differentiation of hematopoietic stem cells (Christopherson *et al.*, 2004; Kawai *et al.*, 2007), and thus a lack of CD26 expression on CD73⁺CD90⁺ cells could suggest they are involved in blood lineage migration/differentiation as both Hofbauer cells (placental macrophages) and nucleated red blood cells are found in the villous stroma in the first trimester (Van Handel *et al.*, 2007). Furthermore, podoplanin identifies connective stroma in lymphoid organs that produces a network of collagen-rich fibres to enable movement of lymphocytes via interactions with leukocyte CLEC-2 receptors (Link *et al.*, 2011; Nazari *et al.*, 2016; Onak Kandemir *et al.*, 2019; Wang *et al.*, 2011). The stroma of early placental villi is poorly vascularised therefore podoplanin⁺ cells could enable blood lineage cells (including Hofbauer cells) to migrate throughout the villous core (Ingman *et al.*, 2010). Indeed, Hofbauer cells have been co-localised with podoplanin expressing stroma in term placentae (Onak Kandemir *et al.*, 2019). Together this suggests CD73⁺CD90⁺ cells may represent a functionally divergent population, that is abundant in the early first trimester placenta which is specialised to facilitate/promote

266 hematopoietic migration and differentiation, and morphogenesis of the placenta during regression
267 of the distal villi.

268 The largest subset identified, podoplanin⁺CD36⁺ cells (Subset Four) was hypothesised to constitute
269 the majority of connective villous stroma and play an important role in villous stroma expansion.
270 Similar to CD73⁺CD90⁺ cells, the expression of podoplanin in this population suggests they may play a
271 role in facilitating hematopoietic transport throughout the poorly vascularised first trimester stroma,
272 and may have a migratory phenotype. However, podoplanin⁺CD36⁺ cells had a high expression of
273 CD26 which identifies fibroblasts involved in wound healing/fibrosis and the associated extracellular
274 matrix deposition (Soare *et al.*, 2020; Worthen *et al.*, 2020) suggesting an alternative or additional
275 role in villous expansion and growth. The corresponding podoplanin⁺CD36⁺ population observed in
276 the prior single cell RNA-seq dataset, expressed genes associated with angiogenesis (*IL6*, *ANGPTL-4*,
277 *TNFAIP6*), suggesting they may also provide a paracrine stimulus for placental vascular development
278 which is undergoing vasculogenesis and angiogenesis over first trimester (Chang *et al.*, 2012; Hato *et*
279 *al.*, 2008; Suryawanshi *et al.*, 2018).

280 Perivascular cells (Subset Three) were identified by their expression of either CD271 or CD146, both
281 markers that localise in a perivascular niche (Castrechini *et al.*, 2010; Lv *et al.*, 2014). CD26⁺CD90⁺
282 myofibroblasts (Subset Five) were negative for all other markers in Panel One and therefore, were
283 hypothesised to represent contractile/less proliferative myofibroblasts. Indeed, prior single-cell RNA
284 sequencing work identified that populations aligning with perivascular cells and CD26⁺CD90⁺
285 myofibroblasts reported here had a respectively low or high expression of markers associated with
286 contractile myofibroblasts and mature perivascular cells (*ACTA2* (α SMA) and *CNN1* (calponin))
287 (Kumar *et al.*, 2017; Suryawanshi *et al.*, 2018). The lower expression of contractile markers in
288 perivascular cells in comparison to CD26⁺CD90⁺ myofibroblasts may initially appear unexpected, but
289 as the first trimester placental vasculature is relatively immature it would not necessarily be
290 expected that perivascular cells had high expression of contractile markers at this stage of gestation

(Zhang *et al.*, 2002). Interestingly, our *in vitro* experiments demonstrated that both CD73⁺CD90⁺ cells (Subset Two) and podoplanin⁺CD36⁺ cells (Subset Four) upregulated α SMA and calponin when transferred from EGM-2 to advanced-DMEM/F12, paralleling the upregulation observed in explant-isolated first trimester pMSCs when cultured in this medium (Boss *et al.*, 2020). The *in vivo* niche that CD73⁺CD90⁺ cells and podoplanin⁺CD36⁺ cells reside in could determine whether differentiated cells contribute to perivascular or myofibroblast contractile cells and more specific markers may be required to tease apart these differences. Indeed, in term placentae contractile perivascular cells express both α SMA and calponin, but extravascular α SMA⁺ cells are also present and thought to allow stem villi to contract and expand in volume (Dellschaft *et al.*, 2020; Farley *et al.*, 2004).

Cultured MSCs are often described as homogeneous. However, there is strong evidence that MSCs upregulate/downregulate markers in response to *in vitro* culture conditions, and underlying functional and phenotypic heterogeneity could be missed (Blocki *et al.*, 2013; Boss *et al.*, 2020; Da Silva Meirelles *et al.*, 2015). In the MSC field, CD73 is thought to be ubiquitously expressed by MSCs (Dominici *et al.*, 2006). However, that podoplanin⁺CD36⁺ cells upregulate CD73 expression after culture demonstrates expression of this marker may also be a culture artefact. The phenotypic convergence of different mesenchymal populations was further demonstrated by the loss of CD36 and CD271 expression, from podoplanin⁺CD36⁺ and CD73⁺CD90⁺ cells respectively, additional markers that made these populations distinct *ex vivo*. However, expression of CD26 was unaffected by culture, and CD146 was differentiability affected by culture in the two populations. This demonstrates that the nuances of culture adaptation are cell-specific as much as they may be media-specific.

Changes to “MSC” phenotype do not necessarily reflect gain/loss in function. For example bone-marrow MSCs isolated by their expression of CD146 stabilize endothelial tube formation *in vitro*, whereas, CD146⁻ MSCs, that subsequently upregulate CD146 *in vitro*, are unable to stabilize tube formation (Blocki *et al.*, 2013). Thus, *ex vivo* phenotype may be a more accurate indicator of a cells’

functional capacity and highlights the importance of characterizing mesenchymal cells prior to culture. The four placental mesenchymal populations identified here are likely to play different roles in the placenta, and therefore have different functional and therapeutic capacities. pMSCs are often acquired by macerating the villous tissue in order to obtain a single cell suspension that is plated in plastic tissue culture flasks (Papait *et al.*, 2020; Pelekanos *et al.*, 2016). Thus all of the populations identified in this work could be present within such single cell suspensions. However, the population/s with a competitive advantage in culture, either as a result of the culture conditions employed, or as a result of the isolation process itself (enzymes involved, explant-based, plated suspension, enriched for expression of a marker), are likely to outcompete and become the dominant cell in culture. Indeed, our group has demonstrated that under different media conditions fetal pMSCs rather than maternal cells are enriched in culture in EGM-2 (Boss *et al.*, 2020). Characterising the explant isolated pMSCs in more depth in this work demonstrated they express podoplanin, CD26, and CD142, making their origins more likely to be podoplanin⁺CD36⁺ (Subset Four) rather than CD73⁺CD90⁺ cells (Subset Two), perivascular cells (Subset Three) or CD26⁺CD90⁺ myofibroblasts (Subset Five). The heterogeneity identified in this work creates an opportunity to select subsets of these mesenchymal cells more suited to specific uses i.e. with anti-inflammatory/immunomodulatory or angiogenic capacity, and to target media conditions that maintain or transform their *in vivo* phenotype and desired properties. Furthermore, targeting and separately culturing these different subsets will allow us to better to understand how they contribute to placental development.

In conclusion, this work has demonstrated how simplistic post-culture MSC phenotyping will fail to detect the abundant mesenchymal heterogeneity that exists within the placenta. The phenotypic heterogeneity uncovered in first trimester placental mesenchymal cells indicates their potentially diverse functional roles and highlights the crucial need to thoroughly characterise mesenchymal cells that are used in functional assays or in a therapeutic capacity. Indeed, it is important to consider that within the first trimester a placental mesenchymal progenitor capable of wide differentiation

potential (including endothelial and mesenchymal lineages found in the placenta) is likely to exist, and further functional experiments are required to understand the specific capacity of each subset identified in this work. The success of this multicolour flow cytometry panel in uncovering mesenchymal cell heterogeneity paves the way for other panels assessing mesenchymal and vascularised tissues. Panels like this could be used to assess cellular dysfunction in pathologies such as cancer, cardiovascular disease and infection, as well as to interrogate different tissues in order to better understand cell function. Indeed, identifying the pathological phenotype of mesenchymal/vascular cells in pathologies could lead to therapeutic targets. This flow cytometry antibody panel, optimised for placental cells, could also provide an important tool to better understand normal and abnormal placental development and function across gestation.

Methods

Immunohistochemistry

First trimester placental villous tissue (7-10 weeks of gestation) was snap-frozen in OCT compound (VWR) and 5µm cryosections of tissue were cut and fixed in ice-cold acetone for 5 minutes. Sections were blocked with Fc Blocking Reagent (Miltenyi Biotech) for 30 minutes, then incubated with primary antibodies for 1 hour (Table 2). β4 integrin and CD31 were visualised by their directly conjugated fluorophores (Table 2). CD144-BV421 was visualised by the addition of Biotin-SP AffiniPure Goat Anti-Mouse IgG (1µg/mL) (Jackson Lab, JI115065166) for 1 hr, washing in PBS-tween, and then the addition of Streptavidin Alexa Fluor™ 594 (0.5µg/mL, Invitrogen) for 30 minutes. Nuclei were visualised by counterstaining with DAPI (2 µg/ml) for 5 minutes. Sections were mounted using Citifluor AF1 (Agar Scientific), and imaged on an inverted Zeiss Axioplan 2 fluorescent microscope (Zeiss).

Table 2: Primary conjugated antibodies used to confirm specificity of cytotrophoblast or endothelial markers.

Antigen	Fluorophore	Clone	Dilution	Supplier
β4 integrin	FITC/APC	450-9D	1:200	Thermofisher
CD144	BV421™	55-7H1	1:200	BD
CD31	BV480™	WM59	1:200	BD

Isolation of placental villous core cells for *ex vivo* phenotyping

Placentae were collected following informed consent with approval from the Northern X Health and Disability Ethics Committee (NTX/12/06/057/AM09). In order to assess placental villous core cells we modified the protocol developed by (Pelekanos *et al.*, 2016) (Figure 6). In brief, villous tissue was carefully dissected away from the fetal membranes and washed thoroughly in PBS to remove maternal blood and debris. Placental explants (~1cm²) were denuded of trophoblasts (the epithelial cells that are present around the outside of placental villi) by digestion in 10mL/1g tissue of Enzyme Digest Solution (1mg/mL Dispase II, 0.5mg/mL Collagenase A and 1.5mg/mL DNase I (Sigma-Aldrich, USA)) in advanced-DMEM/F12 (Thermofisher) for ten minutes at 37°C as previously described (Boss *et al.*, 2020). Explants were washed repeatedly in PBS and then placed in a 90mm Falcon® petri dish (Corning, US) and finely macerated with two sterile disposable scalpels (Swann Morton No.20,

England). The tissue was transferred to a 50mL Falcon® tube (Corning, China) with 10mL of Enzyme Digest Solution. The Falcon® tube was briefly vortexed and then placed onto a rocker at room temperature overnight. The next morning the Falcon® tube containing the digested villous tissue and enzyme solution was diluted with 40mL PBS and filtered through a 70µm filter. The filtrate containing villous core cells was centrifuged at 220xg for five minutes, and the supernatant was removed. 10mL of 1x RBC Lysis Buffer (Biolegend, USA) was incubated with the cell suspension for ten minutes to remove unwanted RBCs. Remaining cells were centrifuged at 220xg for five minutes and supernatant removed prior to flow cytometry.

Multicolour panel design and optimisation

Panel One was designed for analysis on a three laser Cytex Aurora and aimed to characterise mesenchymal lineages but with specific design considerations for first trimester placental villous core cells (Table 3). First, antigens specific for cytotrophoblasts (an epithelial cell that is present around the outside of placental villi, identified by $\beta 4$ integrin) or haematopoietic cells (CD235a, CD45) were incorporated to allow their exclusion from analysis. Then endothelial, mesenchymal, myofibroblast, and progenitor antigens were selected based on reported literature in the placental and mesenchymal fields, or included in an exploratory role based on reported antigen expression in other tissues or stem cell models (Table 1 and Table 3).

The online Cytex Full Spectrum Viewer was used to identify fluorophores with distinct signatures that could be included in each panel. The panel was designed to spectrally spread out antigens with high density expression in order to prevent spectral overlap or interference. Where possible the brightest fluorophores were combined with the weakest antigens or where antigen was of special interest (ie PE) (Ferrer-Font *et al.*, 2020). Conversely, where the targets of the antigen were highly expressed dim fluorophores were selected (ie CD34 PerCP).

Each antibody was titrated on first trimester placental villous core cells (Supplementary Figure 1), and where required the specificity of the antibody was confirmed using immunofluorescence (**Error! Reference source not found.**). Biological controls included stromal vascular fraction and peripheral blood mononuclear cells, as have been previously characterised (Brooks *et al.*, 2020).

417 Table 3: Panel One designed to assess villous core cells on a 3L Cytex Aurora. Beads= Anti Ms Ig CompBead Plus
418 Set (BD, 560497).

Antibody	Fluorophore	Clone	Flow cytometry Dose (μL)	Reference control	Supplier
CD55	BB515	IA10	0.6	Villous core cells	Beckman Coulter
β4 integrin	FITC	450-9D	0.6	Villous core cells	Thermofisher
CD34	PerCP	581	0.3	Villous core cells	BD
CD36	PerCpVio700	REA760	0.6	Villous core cells	Miltenyi Biotec
VEGFR2	PE	7D4-6	0.6	Villous core cells	Biolegend
CD271	PE-CF594	C40-1457	0.6	Stromal vascular fraction	Biolegend
CD142	BB700	HTF-1	1.25	Villous core cells	BD
CD26	PE/Cy5	BA5b	0.15	Villous core cells	BD
PDPN	PE/Cy7	NC-08	0.3	Villous core cells	BD
CD248	Alexa Fluor® 647	B1/35	0.6	Stromal vascular fraction	BD
CD41	APC	HIP8	0.3	Beads	BD
CD90	Alexa700	5E10	0.6	Villous core cells	Biolegend
CD39	BUV737	TU66	1.25	Beads	BD
ICAM1	APC/Fire750	HA58	2.5	Stromal vascular fraction	Biolegend
CD144	BV421™	55-7H1	1.25	Villous core cells	BD
CD235a	Pacific Blue™	H1264	0.3	Villous core cells	BD
CD31	BV480™	WM59	0.3	Villous core cells	BD
CD45	Krome-Orange	B61840	0.3	Villous core cells	Beckman Coulter
CD146	BV605™	PIH12	0.3	Villous core cells	BD
CD117	BV650™	104D2	0.3	Beads	BD
HLADR	BV750™	L243	0.3	Villous core cells	BD
CD73	BV785™	AD2	0.3	Villous core cells	BD

Spectral flow cytometry

Freshly isolated cells were blocked with 5µL of Human TruStain FcX™ (Biolegend, USA) and 5µL of True-Stain Monocyte Blocker™ (Biolegend, USA) on ice for 30 minutes. An antibody master mix containing antibodies listed in Table 3Error! Reference source not found. was prepared in 5µl of Brilliant Stain Buffer (Biosciences, US) in order to block BD Horizon Brilliant fluorescent polymer dyes from interacting with each other. The master mix was added to the cells in blocking solution to make a final volume of 50µl and incubated on ice for 30 minutes in the dark. Stained cells were washed with 1mL of ice cold FACS buffer (PBS, 2mM EDTA (Thermofisher, US) and 1% FBS (Thermofisher, NZ)) and centrifuged at 220xg for five minutes at 4°C. The supernatant was decanted and the wash was repeated. Cells were then resuspended in 100µl of FACS buffer and kept at 4°C. DAPI (1:5000, Akoya Biosciences) was spiked into FACS tubes directly prior to analysis on a Cytex Aurora in order to detect live/dead cells. Spectral data was processed using the Cytex SpectroFlo® Software Package.

Fluorescence minus one (FMO) experiments were performed to accurately gate cell populations and to assess fluorophore spreading error where required. This was completed by creating a master mix containing all the antibodies in the panel minus the antibody of interest. The FMO was then compared to the full panel stain to identify whether the expression detected by that antibody is only present in the full stain, and therefore represents true positive expression.

Data analysis

Spectrally unmixed FCS files were exported from SpectroFlo® and either manually analysed using 2 dimensional dot plots with FCS Express (v7) or uploaded onto Cytobank, a platform enabling datasets to be analysed by algorithms designed to assess high dimensional flow cytometry datasets <https://cytobank.org/> (Kotecha N, Krutzik PO, 2010). In Cytobank, debris, doublets, and dead cells were excluded by manual gating then equal sampling (200,000 villous cells from each placenta were selected, n=5). VisNE enables visualisation of high dimensional data in two dimensions, each cell is displayed as a point on a scatter-like plot where cells with similar antigen expression will group closely and dissimilar cells will be further apart.

For further analysis of subsets of core populations, cytotrophoblasts (identified by their expression of $\beta 4$ integrin) and hematopoietic cells (identified by their expression of CD45) were excluded by gating. Villus core subsets were then interrogated using expression heatmaps (Cytobank) and flow cytometry plots (FCS Express7). The gating strategy employed to identify populations is displayed in **Figure 3**.

450 FACS sorting and *in vitro* culture of mesenchymal populations

451 In order to determine *in vitro* characteristics of Subset Two (CD73⁺CD90⁺) and Subset Four
 452 (podoplanin⁺CD36⁺) these populations were isolated using FACS for *in vitro* culture. To do this,
 453 placental villous core cells were isolated and stained with Panel Two master mix (Table 4) using the
 454 same methods as above. Cells were resuspended in 500μL of Sort Buffer (10% FBS, 2mM EDTA in
 455 PBS) and incubated on ice until immediately prior to sorting. Subset Two (CD73⁺CD90⁺) and Subset
 456 Four (podoplanin⁺CD36⁺) were sorted into 5mL FACS tubes containing endothelial basal media (EGM-
 457 2 medium without supplements added, Lonza, USA) supplemented with 10% FBS at 4°C degrees. To
 458 sort cells of interest, the gating strategy displayed in **Figure 3** was used to exclude debris, doublets,
 459 dead cells, trophoblasts, and endothelial cells, before gating the populations of interest. For each
 460 population 3000 cells/cm² were seeded onto 24-well plates in EGM-2. After 7 days, the *in vitro*
 461 phenotype of cells was determined using Panel Three (Table 5). In brief, TryPLE express was used to
 462 detach cells from flasks. Cells were blocked and then stained with a master mix for Panel Three
 463 (Table 5) as described above. All antibodies were titrated prior to the experiment (Supplementary
 464 Figure 2).

465 Placental MSCs were isolated in EGM-2 using an explant method as described in our previous work
 466 (Boss *et al.*, 2020) were assessed in parallel by flow cytometry.

467 Table 4: Composition of Panel Two. This panel was developed to sort CD73+CD90+ and podoplanin+CD36+
 468 cells from placental villous core using a BD FACS Aria II.

Antibody	Fluorophore	Clone	Dose (μL)	Supplier
CD45	FITC	HI30	0.3	BD
PDPN	PE	NC-08	0.3	Biolegend
CD26	PE/Cy7	BA5b	0.6	BD
CD271	PE/Dazzle™ 594	C40-1457	0.6	BD
CD144	PerCP-5.5	55-7H1	0.6	BD
CD90	Alexa700	5E10	0.6	Biolegend
CD36	APC-Cy7	5-271	0.6	Biolegend
β4 integrin	APC	450-9D	0.6	Thermofisher
CD31	BV480™	WM59	0.3	BD
CD73	BV785™	AD2	0.3	BD
CD34	BUV395™	581	1.25	BD

469

470 Table 5: Composition of Panel Three. This panel was designed to assess the phenotype of placental
471 populations after culture *in vitro* using a three laser Cytex Aurora.

Antibody	Fluorophore	Clone	Dose	Supplier
CD34	PerCP	581	0.3	BD
CD36	PerCpVio700	REA760	0.6	Miltenyi Biotec
VEGFR2	PE	7D4-6	0.6	Biolegend
CD271	PE/Dazzle™ 594	C40-1457	0.6	Biolegend
CD142	BB700	HTF-1	1.25	BD
CD26	PE/Cy5	BA5b	0.15	BD
PDPN	PE/Cy7	NC-08	0.3	BD
CD90	Alexa700	5E10	0.6	Biolegend
CD144	BV421™	55-7H1	1.25	BD
CD31	BV480™	WM59	0.3	BD
CD45	Krome-Orange	B61840	0.3	Beckman Coulter
CD146	BV605™	PIH12	0.3	BD
HLADR	BV750™	L243	0.3	BD
CD73	BV785™	AD2	0.3	BD

472

473 Immunocytochemistry

474 In order to determine the capacity of Subset Two (CD73⁺CD90⁺) and Subset Four (podoplanin⁺CD36⁺)
475 to upregulate contractile markers, in a similar way to pMSCs (Boss *et al.*, 2020), cells were FACS
476 sorted (as above) and seeded into 96 well plates at 2000cells/cm² in EGM-2, with medium replaced
477 every 2-3 days. At day 7 medium was switched to advanced-DMEM/F12 or kept as EGM-2 for a
478 further 7 days. At day 14 of culture, cells were fixed with methanol for 10min, washed in PBS, then
479 incubated in 10% normal goat serum in PBS-tween for 1h at room temperature. Cells were
480 incubated with primary antibodies (either 5µg/ml of anti-Calponin (Abcam-ab216651), 1µg/ml anti-
481 α-smooth muscle actin (αSMA) (Thermofisher Scientific, 14-9760-82) or 1µg/ml anti-smooth muscle
482 heavy chain 11 (Abcam-ab53219) or the relevant control antibodies rabbit IgG (Abacus,

483 JI111036144) or mouse IgG (Abacus, JI115036146), for 1h at room temperature. Endogenous
484 peroxidase activity was quenched by addition of 3% H₂O₂ in methanol for 5 minutes. A Histostain
485 Plus Bulk Kit (Invitrogen) with AEC chromogen (Invitrogen) was used as per the manufacturer's
486 instructions to visualise antibody binding. Nuclei were counterstained with Gills II Haematoxylin
487 (Sigma-Aldrich).

488 Figure legends

489 **Figure 1: Placental villus structure and specificity of markers used to exclude unwanted cell populations.** A)
490 Placental villous morphology and plane of section, B) haematoxylin and eosin staining of a thin section through
491 a placental villus (7.1 weeks), localisation of C) β 4 integrin (red) to cytotrophoblasts and E) CD144 (red) and
492 CD31 (green) to blood vessels (white arrows) in placental villus sections confirmed antibody specificity. No
493 fluorescence is seen in negative IgG controls (D, F) run simultaneously. Nuclei are counterstained with DAPI
494 (blue). Scale bar= 100 μ m. Rendered images in A) have been acquired from Biorender.com.

495 **Figure 2: Categorisation of placental villus core subsets using Panel One markers.** A) Samples were gated to
496 exclude debris, doublets, dead cells, hematopoietic cells (CD45⁺ and CD235a⁺), and cytotrophoblasts (β 4
497 integrin). B) Marker expression was used to categorise five subsets that were overlaid onto viSNE plots
498 generated in Cytobank. C) The average percentage contribution of each subset is presented as a pie chart. D)
499 A scatter plot with bars depicting the mean percentage of CD73⁺CD90⁺ cells from villous core cells across first
500 trimester analysed on an Aria II (n= 24, black) or an Aurora spectral analyser (n=5, red). Error bars represent
501 the standard deviation of the mean.

502 **Figure 3: Phenotypic characterisation of villous core subsets.** A) The gating strategy used to identify subsets
503 (CD31⁺CD34⁺, CD73⁺CD90⁺, perivascular cells, podoplanin⁺CD36⁺ and CD26⁺CD90⁺), and B) heat maps
504 comparing the expression of specific antigens between subsets (n=5).

505 **Figure 4: *In vitro* culture of CD73⁺CD90⁺ and podoplanin⁺CD36⁺ cells.** A) FACS sorting was used to isolate
506 CD73⁺CD90⁺ and podoplanin⁺CD36⁺ cells from placental villous core cells (n=3). B) Morphology and phenotype
507 of CD73⁺CD90⁺ cells after 7 days in culture. C) Phenotype of podoplanin⁺CD36⁺ cells after 7 days in culture. D)
508 CD146 and CD142 expression on CD73⁺CD90⁺ analysed with Panel One (day 0, n=5) or at 7 days after culture
509 (n=3). E) CD146 and CD142 expression on podoplanin⁺CD36⁺ cells analysed with Panel One (day 0, n=5) or at 7
510 days after culture (n=3). F) Isolation of explant-derived pMSCs, and morphology and phenotype of passaged
511 pMSCs (n=3). Error bars = standard error of the mean and scale bar=100 μ m.

512 **Figure 5: CD73⁺CD90⁺ and podoplanin⁺CD36⁺ cells upregulate markers consistent with contractile cells.** A)
513 FACS sorting was used to isolate CD73⁺CD90⁺ and podoplanin⁺CD36⁺ cells from placental villous core digests
514 (n=3). CD73⁺CD90⁺ (B) and podoplanin⁺CD36⁺ (I) cell expression of α SMA (C, F, J, M), calponin (D, G, K, H) or
515 MYH-11 (E, H, L, O) following 7 days of culture in advanced-DMEM/F12 or EGM-2. Irrelevant mouse IgG (P) and

rabbit IgG (Q) were used as negative controls. Decidual sections containing spiral arteries with intact smooth muscle layers were used as positive controls for staining (R-T).

Figure 6: Schematic diagram demonstrating the enzymatic digestion process used to obtain a single-cell suspension of placental villous core cells for flow cytometry analysis.

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Competing interests

The authors declare that no competing interest exist

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