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A B S T R A C T
The chorionic villi of human term placentae are a rich source of mesenchymal stem cells (PMSCs). The stem cell "niche" within the chorionic villi regulates how PMSCs participate in placental tissue generation, maintenance and repair, but the anatomic location of the niche has not been defined. A number of cell surface markers for phenotypic characterisation of mesenchymal stem cells (MSCs) were employed to identify the stem cell niche within the chorionic villi of first trimester and term human placenta. This included antibodies to pericyte cell surface markers STRO-1 and 3G5, which have been used to identify mesenchymal stem cells in other tissues, but have not been studied in placental tissues. PMSCs were isolated from term human placentae and shown to have stem cell properties by their ability to grow on untreated plastic culture ware, capacity for forming clones (i.e. clonogenicity) and their capability to differentiate into adipocytes, chondrocytes and osteocytes. Western analysis confirmed that STRO-1 and 3G5 are present in placental protein extracts and in PMSCs. Immunocytochemistry revealed STRO-1 antibody staining with that of endothelial cell marker vWF and found no significant overlap in staining. Immuno-histochemistry with antibodies to MSC cell surface markers on first trimester and term tissues revealed a vascular niche for PMSCs. Dual-label immunofluorescence analysis was used to compare STRO-1 antibody staining with that of endothelial cell marker vWF and found no significant overlap in staining. This indicated that some PMSCs have a pericyte-like phenotype. We propose that the vascular niche harbours a pool of PMSCs that can give rise to committed progenitors for tissue maintenance and repair, and that PMSCs contribute to vessel maturation and stabilization.

1. Introduction
The chorionic villi from human term placentae are a rich source of MSCs. The potential utility of PMSCs in therapeutic and regenerative medicine drives current research into their in vitro properties. The (trans)differentiation potential of PMSCs in vitro is now well known but there is scant knowledge of the natural distribution and biology of PMSCs in the chorionic villi of the placenta.

PMSCs are readily isolated from term placentae using a variety of methods. The most popular, routinely used method involves mechanical mincing of the chorionic placental tissue, followed by enzymatic digestion and seeding in stem cell-specific medium [1]. PMSCs selectively attach to the plastic cultureware, proliferate rapidly and are usually prepared without additional enrichment strategies [2]. PMSCs can be differentiated in vitro under specific stimulatory environments into derivatives of the mesenchymal cell lineage such as osteocytes, adipocytes, myocytes and chondrocytes [3,7–7]. In addition, there is evidence of differentiation in vivo into cell types characteristic of other lineages such as hepatocyte-like cells and neural-like cells [3,4,7–11], but in vivo evidence for such differentiation is very limited.

Various combinations of cell surface markers are used for the phenotypic characterisation of PMSC in vitro. Common positive markers for PMSCs are also used to identify MSCs from other sources and include CD90 (Thy-1), CD105 (endoglin), CD29 (beta 1 integrin), CD44 (hyaluronic acid), and CD73 (lymphocyte-vascular adhesion...
protein 2). PMSCs are consistently negative for specific endothelial cell surface markers [e.g. von Willebrand Factor (vWF), CD34 and CD45] and haematopoietic stem cell markers (CD34, CD117).

Despite the extensive phenotypic characterisation of PMSCs in vitro with cell surface markers, there have been no studies to exploit multiple cell surface markers to identify the microenvironment, or niche, of PMSCs in the chorionic villi of placental tissues.

The stem cell niche is a highly balanced microenvironment that allows stem cells to survive and remain quiescent and then respond by replicating, migrating and differentiating to replace or repair tissue when needed [12–14]. This balance between stem cell proliferation and differentiation is regulated through intracellular integration of a multitude of signals initiated by internal and external stimuli. The identification of the anatomic location of the stem cell niche within the placental chorionic villi not only provides clues as to the nature of these stimuli but can also reveal additional roles for stem cells in placental development and function.

Recent studies have employed a panel of cell surface markers associated with endothelial cells (vWF, CD34), pericytes and endothelial cells (CD146); smooth muscle cells (α-smooth muscle actin), and pericyte-specific antigens (STRO-1 and 3G5) to detect cells with MSC properties in bone marrow, periodontal ligament, dental pulp and human adipose tissue [15–18]. Importantly, these antibody markers were used to define the MSC niche in these tissues. Furthermore, in these studies the STRO-1, 3G5 and CD146 antibodies were able to enrich for subpopulations of progenitor MSCs [15,18–22] that have a high capacity to form fibroblastoid colonies in vitro (called the CFU-F population) [22], which is an important property of stem cells. CFU-F colonies derive from a single stem cell and have the ability to differentiate into many of the cells types that are characteristically derived from the niche [23].

Although STRO-1 and 3G5 antibodies have been used to identify in vitro cultured MSCs derived from the various sources described above, they have not been used for the characterisation of the PMSC niche in the chorionic villi. FACS analysis showed that freshly isolated cells from the placental chorionic villi express STRO-1 and that expression fell to undetectable levels after passage 3 of in vitro culture [3]. Another study however, could not detect STRO-1 expression in PMSCs [2]. 3G5 has not been tested as a cell surface marker for PMSCs and the expression patterns of STRO-1 and 3G5 in the placenta are not known.

Another marker which has been used to enrich the CFU-F population is CD49a [24]. Integrin alpha 1 (CD49a/VLA-1), which is the receptor for laminin and collagen, was also investigated. The CD49a cell surface marker has also been used to identify both human bone marrow mesenchymal stem cells [25] and PMSCs [1].

FACS analysis has been used routinely for the analysis of PMSC populations [1,3–7]. In this study, we used cell surface markers, including STRO-1, 3G5, CD146 and CD49a that enrich CFU-F populations, to characterise PMSC preparations by immunohistochemistry. PMSC properties were further demonstrated by CFU-F and differentiation assays. Finally, we used immunohistochemical and immunofluorescence studies to identify the anatomic location of the stem cell niche and to determine differences in the stem cell niche between early and late pregnancy.

2. Materials and methods

2.1. Patient details

There were 20 term human placentae collected for this study, with an average patient gestation of 38.5 ± 1.6 weeks (mean ± SD), maternal age of 32.9 ± 6.9 years, baby weight of 3419.0 ± 438.9 g and placental weight of 641.6 ± 90.4 g. Delivery mode was either elective Caesarean section (15/20) or vaginal delivery (5/20) and there were 12/20 males and 8/20 females. All samples were obtained from uncomplicated pregnancies and were therefore considered normal.

First trimester placental tissues were collected with the consent and the approval of the Federation of Medical Scientific Associations of the Netherlands. The Research and Ethics Committee of the Royal Women’s Hospital (Victoria, Australia) approved the use of term placenta tissue for this study. The study was performed in accordance with the National Health and Medical Research Council of Australia guidelines. All placental tissues were collected from healthy women who had medically uncomplicated pregnancies. Five first trimester samples were used in this study.

2.2. Antibodies

Monoclonal antibodies STRO-1 (mouse IgM anti-human stromal stem cells) [22] and 3G5 (IgM anti-human islet cells; ATCC) were used as undiluted tissue culture supernatants with total protein concentration of 4.7 mg/ml and 3.5 mg/ml, respectively. CD146 (used at 4 μg/ml), was a gift of S. Gronthos and A. Zannettino, Institute of Medical and Veterinary Science, Adelaide, Australia). Other antibodies included rabbit anti-integrin CD49a/VLA-1 (used at 8 μg/ml), mouse anti-human CD105 and CD106 (used at 1 μg/ml and 5 μg/ml respectively) (all from Millipore/Chemicon, Massachusetts, USA) and mouse anti-human α-smooth muscle actin (α-SMA, used at 4 μg/ml) (Sigma–Aldrich, St. Louis, USA). Endothelial cell surface markers were rabbit polyclonal anti-human vWF and mouse anti-human CD34 (QBEnd/10) (Labvision/Neomarkers, California, USA) (both used at 1 μg/ml), which have been previously used for immunohistochemical studies of the placenta [26]. The haematopoietic stem/progenitor cell surface marker used was mouse anti-human CD117 (used at 5 μg/ml) (Millipore/Chemicon, Massachusetts, USA). Control monoclonal antibody FDO66Q (Q66) (mouse IgG1 anti-human trophoblast cells) [27] was used at 1 μg/ml and obtained from Flinders Technologies, Pty. Ltd. Adelaide, Australia. Non-immune rabbit serum (NIRS, used at 5 μg/ml), control monoclonal antibody 1A6.12 (IgM), which was used as an undiluted tissue culture supernatant with a total protein concentration of 10 mg/ml (gift of S. Gronthos and A. Zannettino, Institute of Medical and Veterinary Science, Adelaide, Australia) was used as a negative control. The other negative control was X63 (mouse IgG1 anti-human), from the cell line P3X63.Ag8, which has no known cross-reactivity with human tissue, was used at 5 μg/ml [28] and was obtained from the Department of Immunology (Flinders Medical Centre, Adelaide, Australia).

2.3. First trimester placental sample collection

First trimester samples (10–12 weeks gestation) were collected by chorionic villus sampling by a transcervical approach, using a biopsy catheter (Cook, K-CMA-5000), at the University Hospital Groningen, The Netherlands. The samples collected were required for cytogenetic diagnosis and the surplus tissue was paraffin embedded and donated to this study.

2.4. Term placental sample collection

Term placentae were from either from normal vaginal deliveries or elective Caesarean sections and samples were processed within 20 min of delivery.

Following collection, the decidual surface of the placenta was removed and 2.5 cm cubes of placental tissue were taken from a central cotyledon of the villous vascular bed. The samples were
rinsed in 0.9% saline and either snap frozen in liquid nitrogen for protein extraction, formalin fixed for paraffin embedding, encased in Cryomatrix (Zeiss, Australia) and snap frozen for immunohistochemistry, or placed in warmed Hanks Balanced Salt Solution (Invitrogen/Gibco, California, USA) for stem cell isolation.

2.5. Isolation of stem cells from term placental tissue

Placental mesenchymal stem cells isolated from term placenta were prepared as previously described by [1] with the modifications described below. Placental sections of about 10 g (2.5 cm cubes) were extensively minced and then centrifuged at 550 g for 5 min. The pellet was then haemolysed in red blood cell lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 8.0), centrifuged for 5 min. The pellet was then haemolysed in red blood cell lysis buffer and then centrifuged at 550 g for 10 min at room temperature. The pellet was incubated at 37 °C for 0.05% Trypsin-EDTA solution (Invitrogen/Gibco, California, USA) for 10 minutes, resuspended twice in 50% FBS, subjected to centrifugation at 550 g for 5 min at room temperature. The pellet was incubated at 37 °C in 0.05% Trypsin-EDTA solution (Invitrogen/Gibco, California, USA) for 10 minutes, resuspended twice in 50% FBS, subjected to centrifugation at 550 g for 5 min at room temperature.

2.6. Western immunoblotting

Total protein from cultured stem cells was prepared using extraction buffer containing 50 mM glycine, 1 mM AEBSF and 0.5% Triton X-100. Cells were lysed by three freeze–thaw cycles and then gently homogenised using a 19-gauge needle before centrifugation at 3000 for 10 minutes, resuspended twice in 50% FBS, subjected to centrifugation at 550 g for 5 min at room temperature. The pellet was incubated at 37 °C in 0.05% Trypsin-EDTA solution (Invitrogen/Gibco, California, USA) for 10 minutes, resuspended twice in 50% FBS, subjected to centrifugation at 550 g for 5 min at room temperature. The pellet was incubated at 37 °C in 0.05% Trypsin-EDTA solution (Invitrogen/Gibco, California, USA) for 10 minutes, resuspended twice in 50% FBS, subjected to centrifugation at 550 g for 5 min at room temperature.

Immunoblotting was performed according to methods described previously under reducing conditions [26]. Briefly, 50 μg of snap-frozen placental tissue was added to 4 ml of extraction buffer [50 mM glycine, 1 mM AEBSF, 5 mM EDTA (pH 8) and 0.5% Triton X-100]. Samples were then homogenised on ice, using an Ultra Turrax T25 mechanical homogeniser for 1 min, followed by centrifugation at 2500 g for 10 min. Total protein concentration in the supernatants was determined by Pierce (Thermo Fisher/Pierce, MA, USA) protein assay reagent using bovine serum albumin (Sigma, Australia) as the standard.

2.7. Immunocytochemistry

Cells for immunocytochemical analysis were seeded into an 8-well chamber slides (BD, Australia) at a density of 50–100 cells/well, were maintained at 37 °C in 5%CO₂ for at least 48 h and then fixed in 10% formalin for 20 min. Antibody staining and detection was as described below.

2.8. Immunohistochemistry

Immunohistochemical analysis was carried out as described previously [26]. Briefly, five micron thick sections of paraffin, or Cryomatrix-embedded whole placental tissue samples, were cut for immunohistochemistry and placed onto coated slides was fractionated using 10%Tris–HCl polyacrylamide (BioRad, California, USA) gel electrophoresis and then transferred to nitrocellulose membranes. Membranes were incubated in blocking agent consisting of 5% skim milk powder in Tris-buffered saline (pH 7.4) for 1 h at room temperature, followed by incubation with primary antibodies to either STRO-1, 3G5 or CD49a overnight at 4 °C. Membranes were then incubated with goat anti-mouse or donkey anti-rabbit biotinylated secondary antibody (Dako, Glostrup, Denmark), followed by streptavidin-HRP (Dako, Glostrup, Denmark). Tyramide Signal Amplification (Perkin–Elmer, MA, USA) was used, following the manufacturer’s instructions, to amplify the immunoreactivity. Immunoreactivity was visualised by chemiluminescence using the ECL-Plus kit (GE Healthcare, Buckinghamshire, England) and autoradiography.

Table 1

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Staining intensity</th>
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<tr>
<td>X63</td>
<td>–</td>
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<tr>
<td>NIRS</td>
<td>–</td>
</tr>
<tr>
<td>FDO66Q</td>
<td>–</td>
</tr>
<tr>
<td>3G5</td>
<td>–/+</td>
</tr>
<tr>
<td>CD34</td>
<td>–</td>
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<tr>
<td>CD146</td>
<td>++</td>
</tr>
<tr>
<td>STRO-1</td>
<td>+</td>
</tr>
<tr>
<td>CD49a/VLA-1</td>
<td>++</td>
</tr>
<tr>
<td>vWF</td>
<td>–</td>
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<tr>
<td>CD105</td>
<td>+</td>
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<tr>
<td>CD117</td>
<td>–</td>
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<tr>
<td>CD106</td>
<td>+</td>
</tr>
<tr>
<td>α-SMA</td>
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*++*, strong staining; +, moderate staining; +/-, weak staining; –, no staining.
Fig. 2. Immunocytochemical staining of isolated PMSCs. Cells were negative for X63 (a), vWF (b) and NIRS, FD066Q, CD34, and CD117 (data not shown). Cells were positive for 3G5 (c), CD146 (d), CD49a/VLA-1 (e), α-smooth muscle actin (α-SMA; (f), STRO-1 (g) and CD106 (h) (magnification 200×). Cultured cells were clonogenic as shown by colony forming unit assay (Fig. 1i) (magnification 100×).
Paraffin embedded samples were de-waxed in Xylene and rehydrated in graded ethanol before use.

Immunostaining was performed using primary antibodies and a Histostain-Plus™ Broad Spectrum Kit (Invitrogen/Zymed, California, USA). A Tyramide Signal Amplification (TSA) kit (Perkin-Elmer, MA, USA) was used to amplify immunoreactivity from antibodies to 3G5 and STRO-1 according to the manufacturer’s instructions. Detection was either by 3-amino-9-ethyl-carazole (AEC) (Zymed, California, USA) staining with 0.05% methyl green counterstain or by 3,3-diaminobenzidine (DAB) staining with haematoxylin counterstain, depending on antibody compatibility. All slides were mounted in 80% glycerol, visualised using a Zeiss Axioscop microscope, and analysed with Axiovision software (Carl Zeiss Inc. New York, USA).

2.9. Dual-labelling immunofluorescence

Frozen placental sections were used for staining and were treated in the same manner as for immunohistochemistry described above with the following modifications. Polyclonal rabbit anti-human vWF antibody was incubated with mouse anti-human STRO-1. Donkey anti-rabbit Cy-3 conjugated secondary antibody and goat anti-mouse FITC-conjugated secondary antibody (Millipore/Chemicon, Massachusetts, USA) followed. A mountant containing DAPI nuclear counterstain (Cambio, Cambridge, UK) was used. Sections were visualised using a fluorescence microscope (Carl Zeiss, Inc., New York, USA). The staining for vWF (Cy3), STRO-1 (FITC), as well as DAPI nuclear staining, was detected with individual filters and was subsequently composited using the Adobe Photoshop Elements version 4.

2.10. Colony Forming Unit (CFU) assay

 Colony forming efficiency was assessed using methods described previously [29]. Isolated PMSCs at passage two were seeded into six well plates at a density of 100 cells/well in alpha-MEM medium supplemented with 20% FCS, 0.02% L-ascorbate-2-phosphate, 1% L-Glutamine, 0.5% penicillin/streptomycin, 0.5% Fungizone and 1% Tylosin. Unreplenished day 14 cultures were fixed with 10% formalin and then stained with haematoxylin. Aggregates of ≥50 cells were scored as colonies.

2.11. Differentiation of PMSCs

PMSCs were seeded into 8-well chamber slides at a density of 100 cells per well. Cells incubated with α-MEM medium (supplemented with 10% FCS, 0.02% L-ascorbate-2-phosphate, 1% L-Glutamine, 0.5% penicillin/streptomycin, 0.5% Fungizone and 1% Tylosin) were used as the control. PMSCs were incubated with adipogenic or chondrogenic or osteogenic differentiation medium. The medium in each well was replenished every 48 h. Differentiation media were used. Sections were visualised using a fluorescence microscope (Carl Zeiss, Inc., New York, USA). The staining for vWF (Cy3), STRO-1 (FITC), as well as DAPI nuclear staining, was detected with individual filters and was subsequently composited using the Adobe Photoshop Elements version 4.

![Fig. 3. Differentiation assays for cells in the mesenchymal lineage. Control undifferentiated PMSCs (a, c, e) were grown in α-MEM medium alone. Panels (b), (d), (f) show cells grown for the same time as the controls but in α-MEM medium containing differentiation factors. Staining was with Alcian Blue for chondrocytes (a, b), Oil red O for adipocytes (c, d) and Alizarin Red for osteocytes. Magnification for panels (a) and (b) was 100× and for panels (c)–(f) was 200×. The arrows in panel (d) show lipid droplets. Scale bars represent 50 μm.](image)

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purchased as bullet kits (Cambrex, New Jersey, USA) and differentiation was induced as per manufacturer’s instructions. At the end of experimental procedures, the cells were fixed in 10% formalin and stained. Cell stains for mesenchymal stem cells were carried out as described previously [10,30,31]. Cells were stained with Oil Red-O and haematoxylin counterstain to detect adipocytes and Alizarin red solution to detect osteocytes as recommended by the manufacturer. Alcian blue counterstain were used to detect chondrocytes using the monolayer staining method as described previously [44].

Each stain was performed in duplicate for each sample. Results were then visualised using a Zeiss Axioscop microscope and analysed with Axiovision software (Carl Zeiss Inc., New York, USA).

3. Results

3.1. Isolation of stem cells from term placental tissue

Placental stem cells were isolated from term human placentae and grown on untreated plastic cultureware. The mean cell number within a 25 cm² flask was $9 \times 10^5$. These cells were confluent for passaging about every 2 weeks. Stem cell cultures could be passaged up to five times (about 30 population doublings). All experiments in these studies were performed with cells at passage two and each experiment was repeated three or more times.
3.2. Western immunoblotting

Western analysis was used to show that STRO-1 and 3G5 were expressed in both PMSC protein extracts and in term placental tissue homogenates \((n = 5)\). CD49a/VLA-1 was used as a control as it is expressed in placental tissue and FACS analysis shows PMSCs are positive for this cell surface marker [1]. In placental tissue and PMSC extracts, the CD49a antibody revealed the expected 210 kDa and 80 kDa bands [32] and a minor 70 kDa band (Fig. 1A). The 3G5 antibody (Fig. 1B) detected a predominant, immunoreactive but diffuse band at 15 kDa, and minor bands at 10 kDa and 30 kDa, for both term placental tissue homogenate and PMSC protein extracts. The STRO-1 antibody detected a prominent 27 kDa band for both term placental tissue homogenate and PMSC protein extracts (Fig. 1C). Minor bands at 18 kDa and 50 kDa were also observed. Control blots with NIRS and the IA6.12 antibody showed no bands (Fig. 1A and B respectively).

3.3. Immunocytochemistry on cultured PMSC

Ten independent preparations of PMSCs were analysed qualitatively by immunocytochemistry. The panel of cell surface markers and the signal staining intensities are shown in Table 1. Representative staining on cultured cells is shown in Fig. 2. Cultured cells were negative for X63 (Fig. 2a), vWF (Fig. 2b), NIRS, trophoblast cell surface markers FD066Q, CD34 and CD117 (data not shown). Most of the cells (>90%) were positive for 3G5 (Fig. 2c), CD146 (Fig. 2d), CD49a/VLA-1 (Fig. 2e), α-SMA (Fig. 2f), CD105 (data not shown), STRO-1 (Fig. 3g) and CD106 (Fig. 3h).

3.4. Colony forming unit assay

Cultured cells were clonogenic as shown by colony forming unit assay (Fig. 2i). Circular clusters of five or more cells were counted as colonies. Seeding at 100 cells per well resulted in 14.3 ± 3.6%
(mean ± SEM) of the total cells per well forming colonies, with an average of 49.8 ± 8.2 cells/colony (n = 6, 24 colonies counted for each sample).

3.5. Differentiation of PMSCs into adipocytes, osteocytes and chondrocytes

Cells were also incubated with z-MEM medium supplemented with 10% FCS in each plate as a control (Fig. 3a). PMSCs were tested for their ability to differentiate into osteocytes (Fig. 3b), adipocytes (Fig. 3c) and chondrocytes (Fig. 3d) with incubation in either osteogenic, adipogenic or chondrogenic differentiation media, as shown by positive Alizarin Red, Oil red O and Alcian Blue staining respectively (n = 6). Control cells showed no morphological changes during culturing and did not stain with any of the cell-type specific stains described above (data not shown).

3.6. Immunohistochemical localisation of PMSCs in the first trimester and term placenta

Mesenchymal stem cell surface markers STRO-1, 3G5, CD146 and CD49a were chosen for further analysis by immunohistochemistry (n = 10). All antibodies, except STRO-1 and 3G5, gave strong signals on cultured PMSCs. STRO-1 and 3G5 antibodies required TSA amplification to visualise the signal. All tissues incubated with control NIRS and X63 showed no immunoreactivity in either first trimester or term placentals sections (X63 in Figs. 4a and 5a, NIRS data not shown). Control antibody FDO66Q showed specific immunoreactivity only in syncytiotrophoblast cells, in both first trimester (Fig. 4b) and term placentae (Fig. 5b). CD34 and vWF showed positive staining of endothelial cells in first trimester (Fig. 4g and h, respectively) and term placental villous vasculature (Fig. 5g and h, respectively). In first trimester placental tissue, STRO-1 (Fig. 4c), CD146 (Fig. 4d), CD49a (Fig. 4e) all showed immunoreactivity around the vessels. 3G5 (Fig. 4f) staining was specific to scattered cells around the vessels. In term placental tissue, the spatial distribution of antibody staining was not substantially different to first trimester with STRO-1 (Fig. 5c), CD146 (Fig. 5d), CD49a (Fig. 5e) and 3G5 (Fig. 5f) showing immunoreactivity around the vessels, and again 3G5 staining was specific to individual scattered cells around the vessels. In term placental sections, larger vessels also showed the same staining pattern with the cell surface markers (data not shown). No immunoreactivity was detected in the cytotrophoblast or syncytiotrophoblast cell types, which were revealed by the Q66 antibody (Figs. 4b, 5b and data not shown).

3.7. Dual-labelling immunofluorescence

Immunofluorescence staining was carried out with the endothelial cell surface marker vWF, and STRO-1 (n = 5). The endothelial cell surface marker, rabbit anti-human vWF antibody, was used with a donkey anti-rabbit Cy3 conjugated secondary antibody (Fig. 6a, d) combined with mouse STRO-1 anti-human antibody and a goat anti-mouse FITC-conjugated secondary antibody (Fig. 6b, e) and a blue DAPI counterstain to show cell nuclei (Fig. 6c, f). Fluorescence detection revealed discrete cells staining for the STRO-1 antibody around the vessels of various sizes (Fig. 6b and e). Composite images (Fig. 6c, f) showed immunoreactivity with STRO-1 was localised to the vascular regions of the term placental villi and did not overlap significantly with expression of vWF in endothelial cells that comprised the vessel walls (indicated by arrows in Fig. 6c, f). NIRS and X63 showed no specific staining (data not shown).

4. Discussion

The method of Fukuchi et al. [1], which is used routinely to prepare PMSCs, was employed to prepare cultured cells. Exposing cultured cells to specific media formulations that promote differentiation for 2–3 weeks produced cells that were positive for cell-type specific stains. Differentiation into cells with characteristics of adipocytes, osteocytes and chondrocytes was observed. These PMSCs preparations contained clonogenic cells as revealed by colony forming assays, with a variable frequency of colony forming units that was consistent with other studies [33].

Western analysis revealed that CD49a immunoreactive protein was detected in PMSC, as expected since this cell surface marker is

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**Fig. 6. Dual-label immunofluorescence staining with STRO-1/vWF (a–f) on term frozen placental sections; vWF antibody staining with Cy3 (red) (a), STRO-1 staining with fluorescein detection (green) (b), combined images of (a), (b) are shown in (c) with a DAPI nuclear counterstain. Staining from another placenta with vessels of various sizes is shown in panels (d)–(f); vWF antibody staining with Cy3 (d), STRO-1 staining with fluorescein (e), combined images of (d), (e) are shown in (f) with a DAPI nuclear counterstain. vWF antibody staining with Cy3 detection (f). NIRS and X63 controls showed low or no background staining (data not shown). Images are 400× magnification and the scale bar is 50 μm. Arrowheads in (c) and (f) show representative staining of STRO-1 in cells within the vascular region.**
used for phenotypic analysis of PMSC [1]. Western analyses revealed STR0-1 and 3G5 immunoreactive proteins were detected in PMSC and term placental extracts, but at low levels since it was necessary to amplify the signals.

Immunocytochemistry revealed the fibroblast-like cells produced by this method stained positively with antibodies to the cell surface markers (CD146, CD49a, CD105, CD106 and α-SMA STR0-1, 3G5, and CD49a), which have been used to identify MSCs and PMSCs using FACS analysis. This included antibodies to proteins that have been used to enrich CFU-F populations (CD146, STR0-1, 3G5, and CD49a). As expected, PMSCs were negative for the endothelial cell surface marker vWF and the haematopoietic cell surface marker CD34. Unlike FACS analysis, TSA amplification was necessary to detect 3G5 and STR0-1 staining by immunocytochemistry, and the staining intensity was not uniform in the population. These data are consistent with the notion that PMSCs prepared in this manner are heterogeneous populations. Since cultured cells stain positively for STR0-1 and 3G5 antibodies, and can differentiate along the mesenchymal stem cell lineage, we propose that STR0-1 and 3G5 are cell surface markers for cultured PMSCs. We successfully prepared PMSCs as revealed by the ability of the cells to differentiate into multiple cell types, CFU assays and the immunocytochemical detection of cell staining with a panel of MSC cell surface markers.

Immunohistochemistry on first trimester and term placental sections was used to identify the stem cell niche. Immunohistochemical staining with antibodies that have been used to enrich CFU-F populations in MSCs preparations and that are associated with mesenchymal and peripheral vessels (STR0-1, 3G5, CD146 and CD49a), produced consistent staining patterns around the blood vessels. The staining distribution between first trimester and term placentae was similar, suggesting that the spatial location of the niche does not change substantially after the first trimester. The vascular staining pattern of STR0-1, 3G5, CD146 is consistent with that seen for these antibodies in human adipose tissues [18] and in the human endometrium for CD146 [45] and these data were used to identify a vascular niche in these tissues. Recently, the frizzled-9 antibody (FZD-9) was shown to be a cell surface marker for PMSCs and can also be used to enrich the CFU-F population [10,34]. The FZD-9 antibody also detects cells in a vascular niche in the term placenta [10]. These data further support a vascular niche within the chorionic villi.

Dual-label fluorescence immunohistochemistry with STR0-1 and vWF further refined the niche to scattered cells around the vessels. There was no significant overlap in STR0-1 staining with vWF staining of the endothelial cells that line the vessels and this is consistent with previous findings by others [15]. These data are consistent with the proposed model of MSCs lying in, or attached to, the basement membrane opposed to endothelial cells [35]. Mesenchymal stem cells have been shown to exhibit a perivascular cell phenotype both in vitro and in vivo [18,35]. In response to intrinsic and extrinsic factors, the PMSCs would give rise to committed progenitors that incorporate into the surrounding stroma to effect maintenance and repair.

There may be multiple subpopulations of PMSCs in the vascular niche. STR0-1 and 3G5 are cell surface markers for pericytes [15,36–38] and therefore it is likely that one subpopulation of PMSCs detected by these antibodies in the chorionic villi are pericyte-like cells, or they are indeed pericytes. In vivo studies have provided evidence that pericytes might act as a source of undifferentiated cells during adipose and osseous tissue repair [39,40]. The possibility that MSCs are derived from pericyte cells has been raised in several studies [15,18,38,41,42]. Mouse gene knockout studies highlight the critical importance of pericytes in the maturation and stability of the placental vasculature [43]. We propose that the pericyte-like PMSCs contribute to the pool of recruited cells, which respond to growth factors and cytokines derived from the local villous microenvironment, and which subsequently regulate vessel maturation and stabilization within the chorionic villi of the human placenta.

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