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Identifying human perivascular stem cell subsets

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Declaration

I declare that the work in this thesis is my own, unless otherwise stated. This thesis has not been previously submitted for any other degree or professional qualification and has been composed by myself.

Shuaishuai Hu

July 15th 2015

Scientific Abstract

Perivascular stem cells (PSCs) include pericytes and adventitial cells. PSCs present multiple properties. PSCs are involved in angiogenesis, immunoregulation, and haematopoiesis support and are multi-lineage progenitor cells. Therefore, PSCs are a heterogeneous group of cells. We investigated PSC subsets based on novel markers: CD10 and CD107a. We analysed whether the expression of CD10 or CD107a on PSCs from foetal muscle correlates with the capability of differentiation and fibroblast-colony forming unit (CFU-f) content. The CD10-positive or CD107a-positive PSCs were separated from CD10-negative or CD107a-negative PSCs by cell sorting. CFU-f was quantified. The differentiation of PSC subsets in culture was documented by cytochemistry. We confirmed that CD10 and CD107a PSCs subsets exist in multiple human tissues. CD10-positive and CD10-negative PSC subsets show similar ability for both CFU-f potential and osteogenesis in vitro. CD107a-negative cells show higher CFU-f potential. However, CD107a-positive PSCs were associated with a higher osteogenic differentiation potential in human foetal muscle in vitro. Our study provides early evidence that CD107a-positive adventitial cells present a subset that is prone to differentiate into osteoblasts.

Key words: perivascular stem cell; CD10; CD107a; pericyte; adventitial cell; mesenchymal stem cell

Lay Summary

In all organs, stem cells exist, attached to blood vessels. Recently, these stem cells have been isolated and shown to have the ability to regenerate tissue damaged by injury or disease, including bone, muscle, and even the heart. These blood vessel-associated stem cells are not, however, all the same: some – probably a minority – have a much stronger potential for organ repair. One of the main projects of our laboratory – and the principal aim of my master’s thesis – is to identify and purify these high-potential stem cells, which will be much more efficient to regenerate organs in patients.

To achieve this goal, I have 1) identified different subsets of blood vessel-associated stem cells with respect to the presence, at their surface, of a particular molecule produced by the cell itself; 2) purified these stem cell subsets based on the presence of these specific molecules. To this end, we use a complex machine called a fluorescence activated cell sorter (FACS); and 3) tested the ability of these different subsets of purified stem cells to regenerate tissues. I have worked principally on two of these molecules present at the cell surface and shown that blood vessel-associated stem cells, whether or not they possess these molecules, exhibit distinct potentials, notably for bone repair.

These results support the validity of our approach and represent a step toward the characterisation of stem cells of optimal potential for organ repair.

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Chapter I: Introduction

1.1 Definition of perivascular stem cells

Perivascular stem cells (PSCs) consist of pericytes and adventitial stem cells. Pericytes ensheath capillaries and microvessels, while adventitial stem cells surround larger arteries and veins. Both cells can be reprogrammed into regenerative cells. In culture, PSCs give rise to mesenchymal stem cells (MSCs).

1.2 Pericytes and adventitial cells: an introduction

Pericytes were originally discovered by Charles-Marie Benjamin Rouget and were known as Rouget cells [1]. They were also referred to as mural cells because of their contractile fibres. In 1923, Zimmermann discovered the variation in the distribution of pericytes around blood vessels [2]. Zimmermann renamed them as pericytes. They are located abluminal to the endothelial cells and luminal to the parenchymal cells. They also play a vital role in the post capillary venules and cellular constituents of the capillaries. Pericytes are located in the capillaries, precapillary arterioles and postcapillary venules of the brain. Elements of the basal lamina are deposited and vascular components surround them completely [2,43].

Pericytes play an important role in the stabilisation of blood vessels. They are also involved in the vascular development and maturation [3-8]. Pericytes are derived from multiple organs, involved in homeostasis, and implicated in several diseases. Recent evidence suggests that PSCs display multiple properties in terms of tissue development and regeneration.

Pericytes are present in the basement membrane, they use direct as well as physical contact to interact with the blood vessels of the body. Pericytes help the brain in

performing homeostatic functions. They play an important role in the neurovascular unit. The main functions of the pericyte is to regulate blood flow in the capillaries, clear the debris present in the cells and the permeability of the blood–brain barrier. The density of the pericytes varies with respect to the blood vessels and organs in which they are found. They can be found abundantly in the arterioles and small venules but are rarely found on the capillaries. The pericytes are most dense in vessels of the neural tissues of the body like the brain and the retina [3,42]. This is because the endothelial cells in the brain form a continuous endothelium with complex, tight junctions. They also interact with various pericytes and astrocytic pedicels to create the blood-brain barrier. The blood-brain barrier protects the brain cells from toxic blood-derived factors. The blood-brain barrier regulates the flow of essential components, nutrients, proteins, chemical substances, and other microscopic organisms between the bloodstream and the parenchymal tissue[6,42]

The functioning of the pericytes is not clear because of different definitions of pericytes . The deficiency of pericytes leads to the breakdown of the blood-brain barrier of the central nervous system [39]. Pericytes mainly work on the stabilization and hemodynamic processes of blood vessels. They can sense angiogenic stimuli, guide sprouting tubes, elicit endothelial survival functions, and even exhibit macrophage-like activities[6,40].

All the members of our group have documented that PSCs are multi-lineage progenitor cells [3, 5]. Moreover, subsets of PSCs have been identified as ancestors of mesenchymal stem cells (MSCs), and these subsets express MSC markers (CD105, CD90, CD73, CD44) and differentiate into adipocytes, osteoblasts, and chondrocytes. Reports from the study conducted in this field show that PSCs could support hematopoietic stem cells as “niche cells” with endothelial cells [9-10].

Chien-wen Chen et al. also pointed out that PSCs can regenerate the ischemic heart [11]. The result of another study has shown that perivascular stem cells are involved in the process of myofibroblast development in the liver [12]. Therefore, it is essential to identify and document the functions of pericytes.

Adventitial cells located in the tunica adventitial of blood vessels can be differentiated into smooth muscle cells, endothelial-like cells, and mesodermal cells [13-14]. Adventitial progenitor cells express Sca1+ and CD34 but not CD31 [15]. Bruno Péault et al. purified and identified adventitial cells by adipose tissue cell sorting [16]; the study also showed that adventitial stromal cells can give rise to mesenchymal stem cells in vitro. The typical phenotype of adventitial stromal cells is CD34+CD31-CD146-CD45-, which is distinct from pericytes (CD146+CD31-CD34-CD45-). Bruno Péault et al identified fresh adventitial cells derived from adipose express typical mesenchymal stem cells marker CD90, CD105, CD44 and CD73 and differentiated into mesodermal cells [16]. On the basis of complicated roles of adventitial cells and pericytes in the process of diseases. Therefore, it is essential to clarify the different functions of adventitial cells and pericytes to be used for distinct clinical applications.

1.3 “Nature” of mesenchymal stem cells (MSCs)

The mesenchymal stem cell can be applied to the regeneration field, considering that MSCs show multiple properties, including repairing damaged organs such as the heart, muscles, and bones; immunomodulatory activity; and proliferation ability [17-18]. Mesenchymal stem cells (MSC) are stem cells present in many tissues. They are present in the bone marrow, fat tissue and in the umbilical cord. They renew on their own into multiple tissues like fat cells, bone, muscle and fat cells and connective

tissues. However, because MSCs are heterogeneous group, it is necessary to understand their native nature and anatomical location in the body. Recently, a study has shown that subsets of perivascular stem cells derived from multiple human tissues give rise to typical mesenchymal stem cell markers (CD44, CD73, CD105, CD90, and CD10) and present MSC multipotency, including osteogenesis, adipogenesis, and chondrogenesis[5], suggesting that subsets of perivascular stem cells are the progenitors of MSCs. Mesenchymal stem cells can produce more than one type of specialized cell in the body. They are a subset of multipotent precursors present in the stromal fraction of adult tissues. They multiply in long-term cultures of total cells from the bone marrow or other organs.

1.4 CD10 and CD107a

CD10 is known as membrane metallo-endopeptidase (MME), neutral endopeptidase, or neprilysin. The two main functions of CD10 are extracellular enzymatic activity and intracellular signalling pathways [19]. It is widely expressed in many human tissues, lungs, intestine, uterus, as well as many other organs [19]. CD10 is a biomarker of cancer with stem cell features and is involved in the progression of prostate, breast, and cancers to other organs of the body[20-22]. CD10 is well known as a marker of hematopoietic progenitor cells [23]. A study has also shown that CD10 plays a niche role to maintain the progenitor and stem cell pools in the mammary lineage [24]. CD10 also plays a significant role in haematological diagnosis. Recently, Sanna et al. reported that CD10 is an immunochemical marker of MSCs in the development of the kidneys [25]. Furthermore, there are evidences that CD10 expression is significantly increased during MSC differentiation into osteocytes and adipocytes [26]. However, another study has shown that there is no obvious difference in the level of CD10 expression during synovial-derived MSC

differentiation into chondrocytes [27]. Neprilysin is allied with many biochemical processes and it can be highly expressed in lung and kidney tissues. There are observations that Neprilysin is associated with cancers to many organs of the body.

CD107a is also known as lysosomal-associated membrane protein-1 (LAMP). It is widely expressed in the endosome-lysosome membranes of cells. It is a protein in the body that is encoded by the LAMP-1 gene. It resides mainly on the membranes of the lysosomes. CD107a has a variety of cellular functions in the immune response and in cancer metastasis [28]. Paper shows the level of LAMP was reduced by increasing cysteine cathepsin expression and activity. Furthermore, the paper also shows LAMP can protect cells from lysosomal cell death pathways [41]. CD107a is expressed in tumour stem cells [29]. The proliferation of human bone marrow mesenchymal stem cells was reduced by blocking the LAMP-1 receptor, suggesting that LAMP-1 is involved in MAPK-ERK signalling to influence the proliferation of MSCs [30]. LAMP-1 is also associated with the stemness of neural stem cells [31]. On the basis of above functions of LAMP-1, it is necessary to further study the functions of CD107a in PSC.

1.5 Aim and hypothesis of this project

This project focuses on subsets of perivascular stem cells identified with two novel markers: CD10 and CD107a, detected with the Lyoplate® array system.

In view of the above studies, the hypothesis of this project is that there are distinct functional subsets of PSCs. The aim of the study is to perform the following: 1) Documentation of the project and quantification of marker expression on PSCs by flow cytometry and immunohistochemistry; 2) Purification of the subsets of PSCs by cell

sorting and culturing cells; and 3) Documentation and assessment of the functions of
PSC subsets.

Chapter II: Methodology and Materials

2.1 Human tissue

Human foetal tissue samples were procured after elective surgical abortion (PMI 4-48 h). The human foetal tissue samples and adult fat samples were permitted to be studied with the full permission of the NHS Lothian Research Ethics Committee (REC 08/S1101/1).

The following specimens were obtained for research purposes: 11 foetal skeletal muscle specimens, one foetal brain specimen, one placenta specimen, and one adult fat tissue specimen. All specimens were analysed by flow cytometry. Each experiment included seven foetal skeletal muscle specimens, four used in CD10 PSC subset experiments and three used in CD107a PSC subset experiments.

2.2 Flow cytometry and cell sorting

Fresh skeletal muscle tissues were cut into small pieces in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 20% foetal calf serum (FCS, GIBCO), 1% penicillin-streptomycin (PS, GIBCO), and collagenases I, II, and IV (1 mg/mL, Sigma) and then incubated at 37°C and 120rpm for 60 minutes in a water bath shaker. Cells were centrifuged and re-suspended in PBS with 2% FCS and then passed through 100µm and 70µm cell strainers, centrifuged, and re-suspended in erythrocyte lysis buffer and incubated for 10 minutes at room temperature. The cells were then centrifuged again and passed through a 40µm cell strainer to achieve single cell suspension. After this process, the cells were ready for cell sorting.

The cells were re-suspended in PBS containing 2% FCS and incubated with APC-Cy7 conjugated mouse anti-human CD45 (557833, BD, 1:100), FITC conjugated mouse

anti-human CD34 (555821, BD, 1:100), BV711 conjugated mouse anti-human CD146 (563186, BD, 1:100), PE-Cy7 conjugated mouse anti-human CD56 (557747, BD, 1:100), Percp-Cy5.5 conjugated mouse anti-human CD144 (561566, BD, 1:100), APC conjugated mouse anti-human CD10 (655404, BD, 1:100), or CD107a (560664, BD, 1:100) antibodies on ice for 20 minutes in the dark. Fluorescence Minus One (FMO) and isotype-matched antibodies were used as negative controls. The cells were incubated with 4', 6-diamidino-2-phenylindole (DAPI, BD, 1:1000) for dead cell exclusion, and cell sorting was performed on a BDFACS Fusion flow cytometer. Compensation was calculated using single stain bead controls.

The sorted cells were cultured and reanalysed by flow cytometry at passage 2, passage 3, and passage 4 with the same combination of antibodies used for cell sorting. Mesenchymal stem cell markers were also assessed by flow analysis. Cultured cells were labelled at passage 3 and passage 4 with the following antibody: PE-CF 594 conjugated mouse anti-human CD105 (562380, BD, 1:100), FITC conjugated mouse anti-human CD90 (555595, BD, 1:100), AlexaFluor700 conjugated mouse anti-human CD44 (561289, BD, 1:100), and Brilliant Violet 421 conjugated mouse anti-human CD73 (562430, BD, 1:100). Isotype-matched antibodies were used.

Cultured cells were used to purify pure populations of CD107a-positive and CD107-negative when 3xT75 flasks were 70% confluent. The cells were detached by 0.25% trypsin-EDTA (GIBCO) for 10 minutes at 37°C, re-suspended in PBS/2% FCS, and then labelled with CD45-APC-Cy7, CD34-FITC, CD146-BV711, CD56-PE-Cy7, CD107a-APC, CD144-Perpcy5.5, CD105-PECF594, and CD73-Brilliant Violet 421. Cells were incubated with 7-amino-actinomycin D (7-AAD, 1:100, BD) for 10 minutes to exclude dead cells. FMO and isotype-matched antibodies were as negative controls.

2.3 Long-term cell culture and cell cryopreservation

FACS-sorted perivascular cells were seeded at 5×10^3 cells per well (96 wells, Costar®) coated with 0.2% gelatin (Calbiochem) in Endothelial Cell Growth Medium 2 (EGM2, Cambrex BioScience) and cultured at 37°C for one week. When the cells were confluent, they were detached using 0.25% trypsin-EDTA (GIBCO) for 10 minutes at 37°C. The cells were then seeded in two wells of 12-well plates (Costar®). They were fed by DMEM with 20% FCS and 1%P/S (penicillin/streptomycin solution). The culture medium was refreshed twice a week. On the first passage, the cells were split into two groups; one half was treated with TRIzol® reagent for PCR analysis and the other half was seeded into six-well plates (Costar®). At confluence, one well of the six-well plate was harvested for flow analysis, one well was used for colony-forming unit–fibroblast (CFU-f) assay, and one well was passaged into two T75 flasks (Corning®). The remaining cells were frozen and stored at -80°C. The freezing medium contained 10% dimethyl sulfoxide in FCS. On the fourth passage, the cells were used for cell counting, differentiation, and flow analysis.

2.4 Cell growth curves and doubling time

At the third passage, when the cells reached 80% to 90% confluent, the cells were detached by 0.25% trypsin-EDTA and plated at a density of 5×10^4 cells/well in six-well plates. After three, five, and seven days, cells from two wells were counted using a cell counting chamber using trypan blue solution (CORNING) for dead cell exclusion. The doubling time of the cells was calculated using a doubling time online calculator (<http://www.doubling-time.com/compute.php>).

2.5 Colony-forming unit–fibroblast (CFU-f) assays

For an in vitro perivascular stem cell colony assay, CFU-f assay was performed by plating 100 cells/well (cultured cells) or 500 cells/well (cell sorting) in culture medium in a six-well plate pre-coated with 0.1% gelatin. After 14 days, cells were fixed in methanol and stained with 0.5% Crystal Violet Solution. Colonies were defined as groups of more than 50 cells as detected using an imaging cytometer (Celigo).

2.6 Adipogenic differentiation assay

For in vitro adipogenic differentiation, 2×10^4 cells/well were seeded into 24-well plates and fed DMEM culture medium with 10% FCS and 1% P/S. When the cells reached 70% confluence, 10 wells of the 24-well plate were treated with differentiation medium DMEM with 10% FCS, 1 μ M dexamethasone, 0.5 μ M isobutylmethylxanthine, 60 μ M indomethacin, and 170 μ M insulin (all reagents from Sigma-Aldrich), and 10 wells were kept on the original medium as a control. For both conditions, the medium was changed twice weekly. After 14 days, five wells of cells from each condition were fixed using 4% PFA at room temperature, washed in 60% isopropanol, and incubated with 2ml/well Oil Red O (Sigma) working solution for five minutes to detect lipids. The remaining wells for each condition were treated with TRIzol® for PCR analysis.

2.7 Osteogenic differentiation assay

For in vitro osteogenic differentiation, 2×10^4 cells/well were seeded into 24-well plates, cells were fed DMEM culture medium with 10% FCS and 1% P/S. When the cells reached 70% confluence, 10 wells of the 24-well plate were treated with differentiation medium DMEM with 10% FCS, 0.1 μ M dexamethasone, 10mM β -

glycerophosphate, and 50 µg/ml ascorbic acid (all reagents from Sigma-Aldrich), and 10 wells were kept on the original medium as a control. For both conditions, the medium was changed twice weekly. After 14 days, 10 wells cells were fixed in 4% PFA at room temperature. Mineralisation by osteoblasts was detected by Von Kossa staining [0.5% (w/v) silver nitrate, Sigma; 5% (w/v) sodium thiosulfate, Sigma]. Ten wells of cells were treated with TRIzol® for PCR analysis.

2.8 Immunohistochemistry

Fresh tissue samples were embedded in gelatin/sucrose and then gradually frozen by immersion in liquid nitrogen, after which the tissues were frozen at -80°C.

For immunostaining, sections (7 µm) were cut on a cryostat and subsequently fixed in acetone and methanol (1:1) on ice for 10 minutes before drying for 10 minutes at room temperature. Double staining was achieved by combining alkaline phosphatase (AP) (Fast Red Coloring) with 3, 3' diaminobenzidine (DAB) staining (Diaminobenzidine Coloring).

For Fast Red Coloring: to block endogenous peroxidase/alkaline phosphatase, sections were blocked for 10 minutes in the block solution (Bloxall™, Vector), followed by blocking with normal 2.5% horse serum (Vector) for one hour at room temperature. The sections were then incubated with the primary antibody CD107a (36291401, Sigma) or CD10 (NBP1-47356, Novus) at 4°C overnight. Primary antibodies were diluted 1:300 in phosphate-buffered saline (PBS) supplemented with 1% (w/v) bovine serum albumin (BSA). The next day, the sections were washed three times in PBS and then incubated with AP-conjugated secondary antibody (Vector, Anti-Rabbit Ig or Anti-Mouse Ig based on the primary antibody) for 30 minutes at room temperature. The sections were then washed once in PBS for five minutes and then in alkaline

phosphatase substrate (alkaline phosphatase substrate kit) until the sections presented a red colour. For DAB staining, the processes are the same as for alkaline phosphatase staining. After sections were blocked with normal 2.5% horse serum (Vector) for one hour at room temperature, the sections were incubated with primary antibody CD31 (ab28364, Abcam or M0823, Dako) or CD146 (ab75769, Abcam) at 4°C overnight. CD31 or CD146 was diluted in 1:100 in PBS supplemented with 1% BSA. The next day, the sections were washed three times in PBS and then incubated with hydrogen peroxide (HRP)-conjugated secondary antibody (Vector, Anti-Rabbit Ig or Anti-Mouse Ig based on the primary antibody) for 30 minutes at room temperature. The sections were then washed once in PBS for five minutes and in then peroxidase substrate (peroxidase substrate kit) until the sections presented a brown colour. Finally, the sections were dehydrated for mounting onto slides. The sections were then ready for observing by microscopy (Zeiss or Olympus).

2.9 Statistical analysis

The statistical analyses were performed using the SPSS Statistics 21.0 software package to calculate the independent t-test for normally distributed data. Numbers or percentages were presented as mean \pm standard deviation. $P < 0.05$ was considered a significant difference.

Chapter III: Results

CD10 on perivascular stem cells

3.1.1 Flow analysis and sorting

CD10[±] subsets of PSCs were analysed in human placenta, human foetal muscle, human foetal brain, and adult adipose tissue. Table 1 shows that foetal muscle contained the highest percentage of CD10-positive adventitial cells, so we mainly focused on adventitial cells derived from human foetal muscle.

Table 1: Distribution of CD10 subsets of perivascular stem cells in different human tissues.

	Brain (n=1)	Muscle (n=1)	Placenta (n=1)	Fat tissue (n=1)
Adventitial CD10- positive	6.8%	84.9%	66.5%	81.5%
Adventitial CD10- negative	86.8%	10.7%	30.5%	10.8%
Pericyte CD10- positive	27.7%	15.2%	34.6%	10.2%
Pericyte CD10- negative	62.4%	45%	59.8%	74.3%

CD10 subsets of PSCs were sorted by FACS (Fig.1 & 2). Four foetal muscle samples were used (13-16 weeks), and the following observations were made: CD10-positive and negative adventitial stem cells expressed CD34 but not CD144 (excluding endothelial cells), CD56 (excluding myogenic cells), CD45 (excluding hematopoietic cells), and CD146. CD10-positive or -negative pericytes expressed CD146 but not CD144, CD56, CD45, and CD34.

The population of CD10+, CD34+, CD56-, CD144-, and CD45- cells accounted for 54.93% \pm 18.48% of the total adventitial cells. CD10-, CD34+, CD146-, CD56-, CD144-, and CD45- cells amounted to 33.2% \pm 15.97% of the total adventitial cells. CD10+, CD146+, CD56-, CD144-, and CD45- pericytes accounted for 8.3% \pm 3.21% of the total pericytes, and CD10-, CD146+, CD34-, CD56-, CD144-, and CD45- cells for 85.0% \pm 7.21% (Fig. 3). In summary, the percentage of CD10-positive adventitial cells is higher than that of CD10-negative adventitial cells. In contrast, CD10-negative pericytes are much more abundant than CD10-positive pericytes.

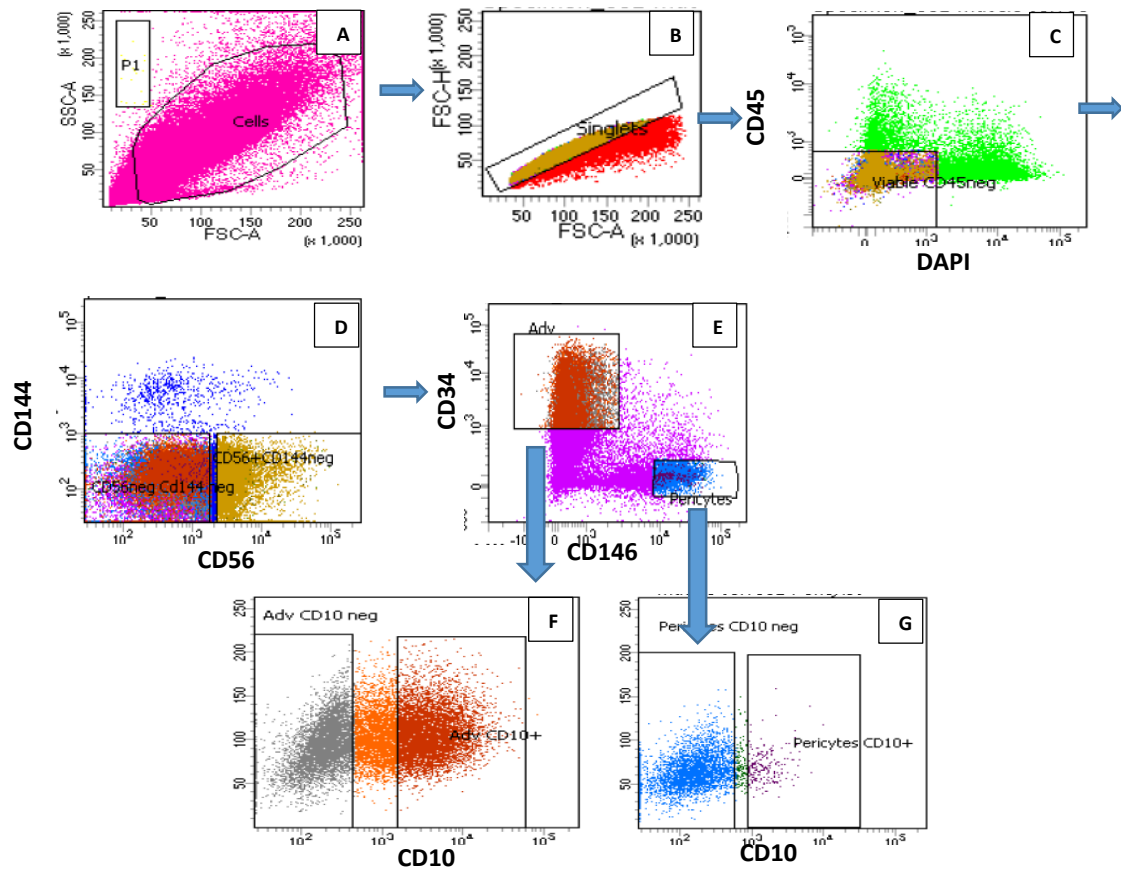


Figure 1: Isolation of CD10 subsets of perivascular stem cells by FACS.

Human foetal muscle cells were processed to purify CD10 subsets of PSCs. **A & B:** Forward-scattered light (FSC) and side-scattered light (SSC) were used. **C:** DAPI was used to exclude dead cells and CD45 to exclude hematopoietic cells. **D:** CD56 was used to exclude myogenic cells; CD144 was used to exclude endothelial cells. **E:** CD34 was used to identify adventitial cells (CD34+, CD146-, CD56-, CD144-, CD45-). CD146 was used to identify pericytes (CD34-, CD146+, CD56-, CD144-, CD45-). **F&G:** CD10 subsets of adventitial cells (CD10+, CD34+, CD146-, CD45-, CD144-, CD56- and CD10-, CD34+, CD56-, CD45-, CD144-, CD56-) and CD10 subsets of pericytes (CD10+, CD146+, CD34-, CD56-, CD144- and CD10-, CD146+, CD34 CD56-, CD144-).

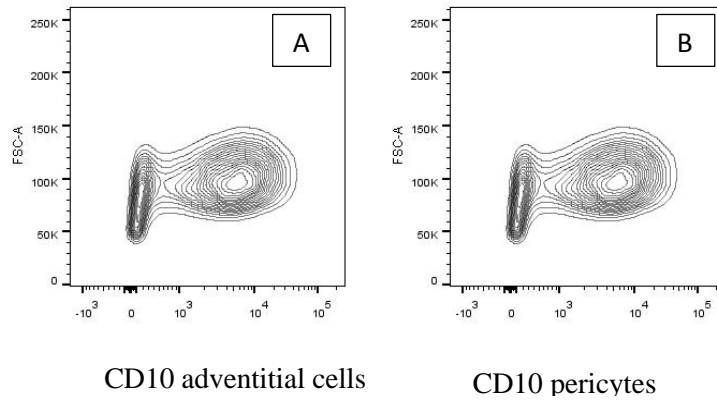


Figure 2: Cell sorting shows distinct CD10 subsets of PSCs

A: CD10 subsets of adventitial cells. **B:** CD10 subsets of pericytes

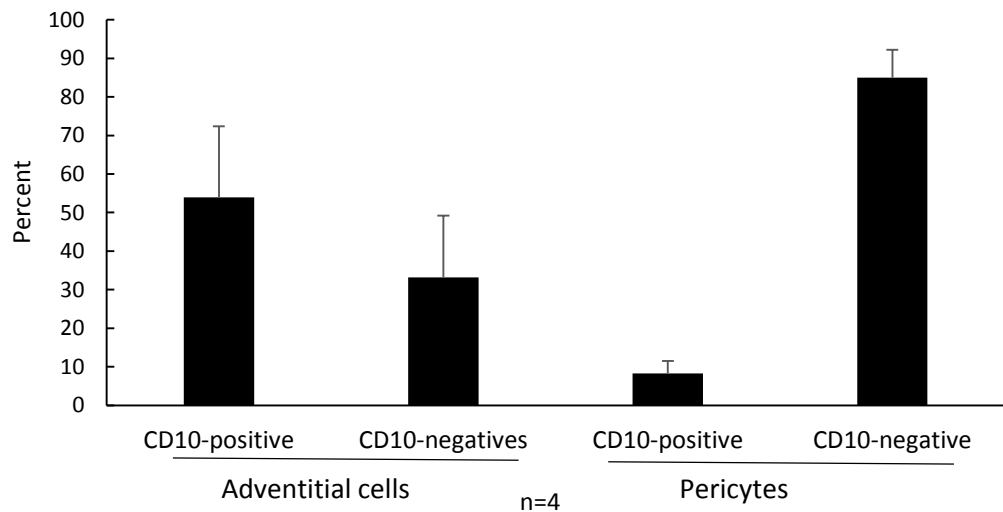


Figure 3: Distribution of CD10 subsets of perivascular stem cells in human foetal muscle.

3.1.2 Cell culture

Cultured CD10[±] PSCs are polygonal, and when cells become more than 80% confluent, they become elongated and spindle-shaped. There was no morphologic difference between CD10-positive and CD10-negative PSCs (Fig. 4).

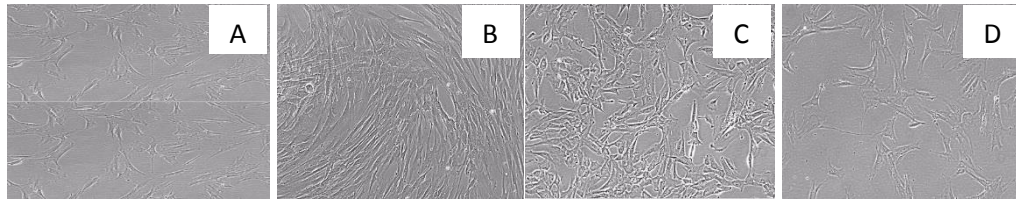
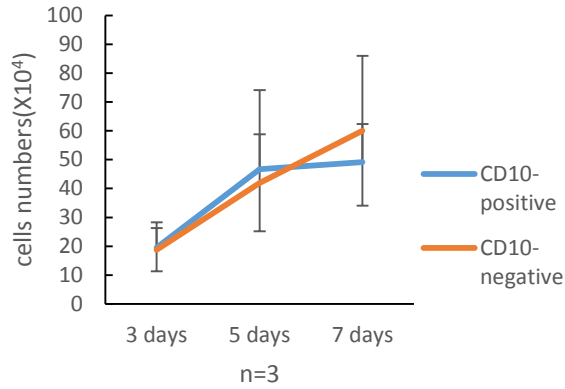


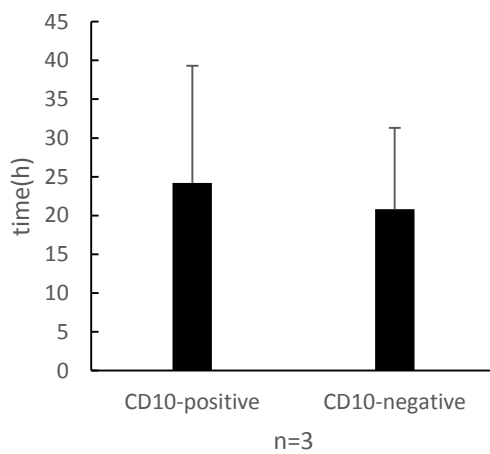
Figure 4: Morphology of perivascular stem cells in culture.

A: CD10-positive adventitial cells. **B:** CD10-negative adventitial cells. **C:** CD10-positive pericytes. **D:** CD10-negative pericytes (5×10^4 cells were seeded; cells were cultured around day 5, 80% confluent).

Growth curves and doubling times were described. Fig. 5 (A-B) shows that there was no obvious difference between CD10-positive and CD10-negative subsets of adventitial cells.



A



B

Figure 5 A-B: The proliferation of CD10 subsets of PSCs.

A: Growth curve of CD10 subsets of adventitial cells. There was no great difference regarding proliferation potential. **B:** The doubling time was assessed online during days 3 to 5. There was no obvious difference between them. (Doubling time assessed using <http://www.doubling-time.com/compute.php>.)

3.1.3 Cultured CD10-negative adventitial cells give rise to CD10-positive cells

Culture cells (passage 2-4) were reanalysed by flow analysis using three human foetal muscle specimens. Neither CD10-positive nor CD10-negative adventitial cells express CD45 (hematopoietic cells), nor did they express CD144 or CD34 (endothelial cells)

or CD56 (myogenic cells). Cells expressed CD146 (Fig. 6-7). CD10-negative adventitial cells that gave rise to CD10-positive cells from passage 2 to passage 4 (Fig. 8).

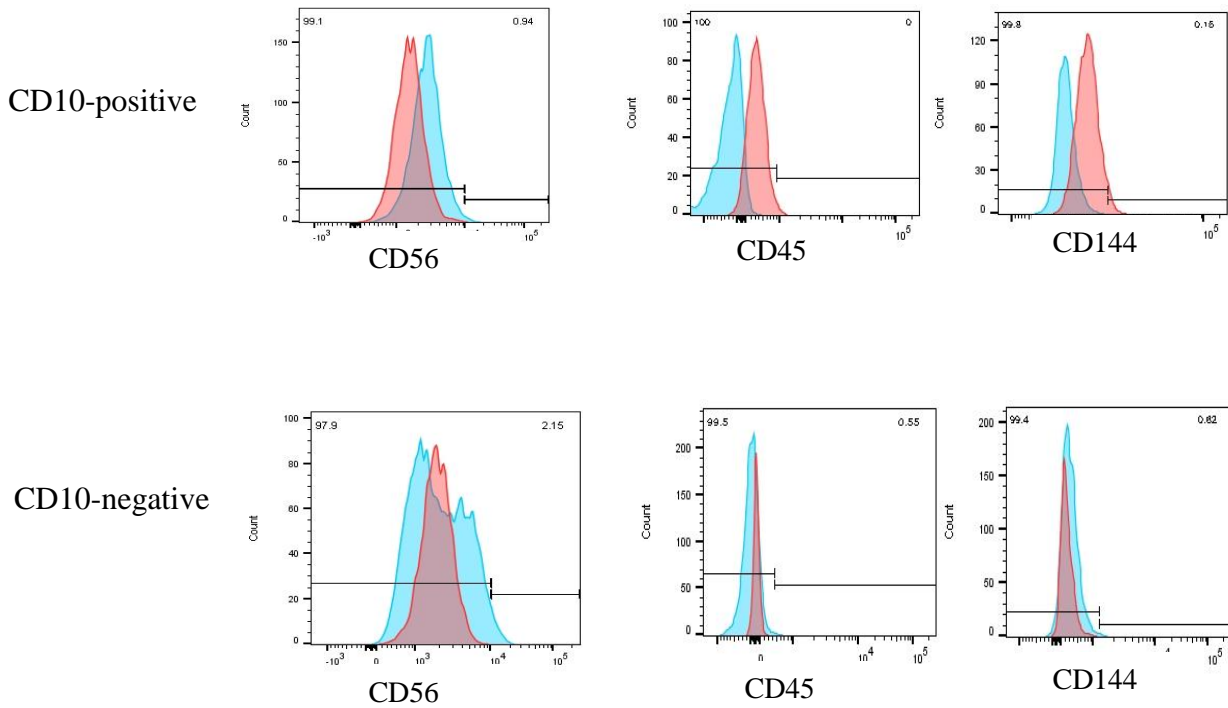


Figure 6: Adventitial cells do not express CD56, CD45, and CD144 in culture.

In culture, CD10-positive and CD10-negative adventitial cells remain negative for CD56, CD45, and CD144. Red represents isotype control staining, and blue represents antibody staining.

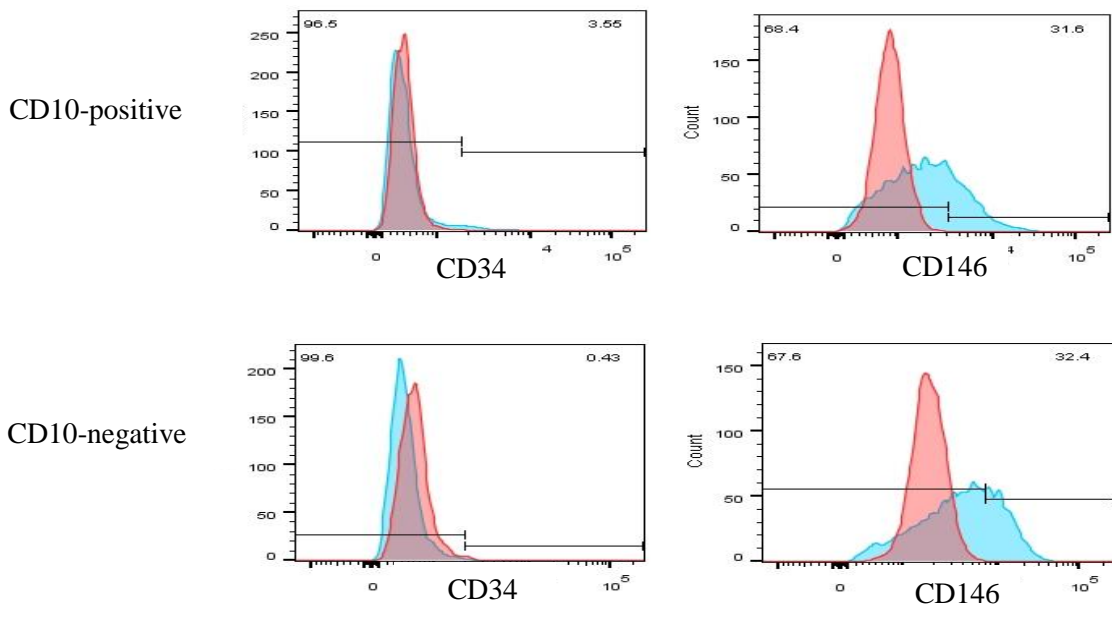


Figure 7: Both CD10-positive and CD10-negative adventitial cells were negative for CD34 expression but expressed CD146 in culture.

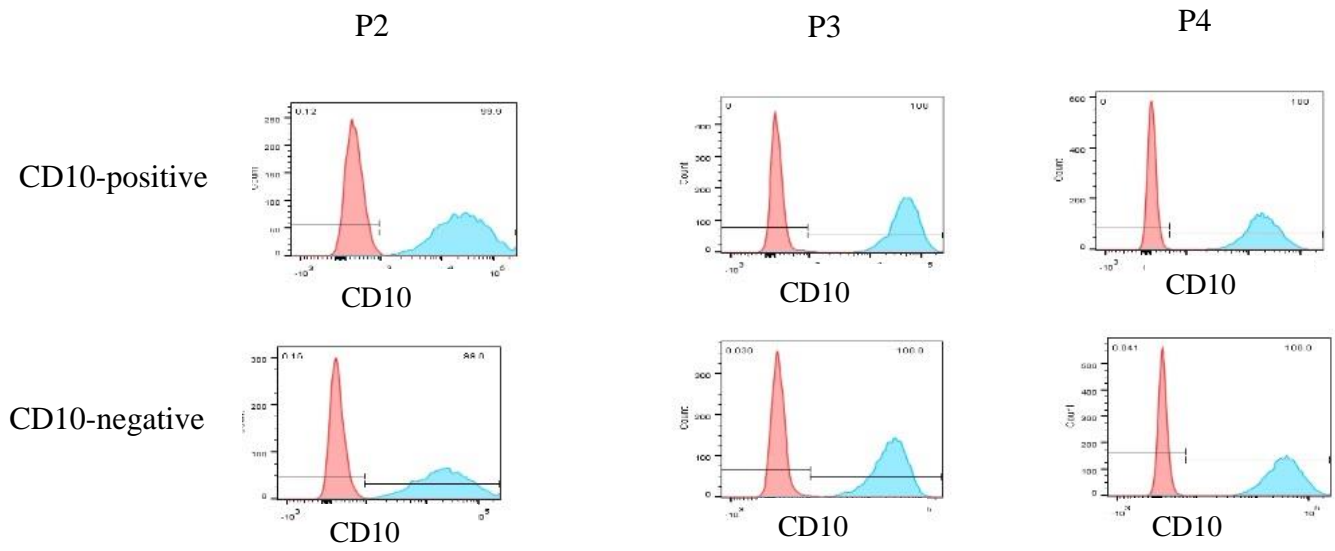


Figure 8: 100% CD10-positive and CD10-negative adventitial cells expressed CD10 in culture.

3.1.4 Cultured CD10-negative and CD10-positive adventitial cells express mesenchymal stem cell markers

At passage 3 (Fig. 9), both CD10-positive and CD10-negative adventitial cells expressed typical MSC markers: CD73, CD105, CD90, and CD44. Specifically, more than 90% of both CD10-negative and CD10-positive adventitial cells were shown to express CD44, CD90, CD73, and CD105. No difference was found between CD10-positive and CD10-negative adventitial cells in culture. This result further confirms that MSCs are derived from adventitial stem cells. Both CD10-positive and CD10-negative adventitial stem cells showed osteogenesis differentiation potential in vitro. This also suggests that CD10 is a marker of mesenchymal stem cells.

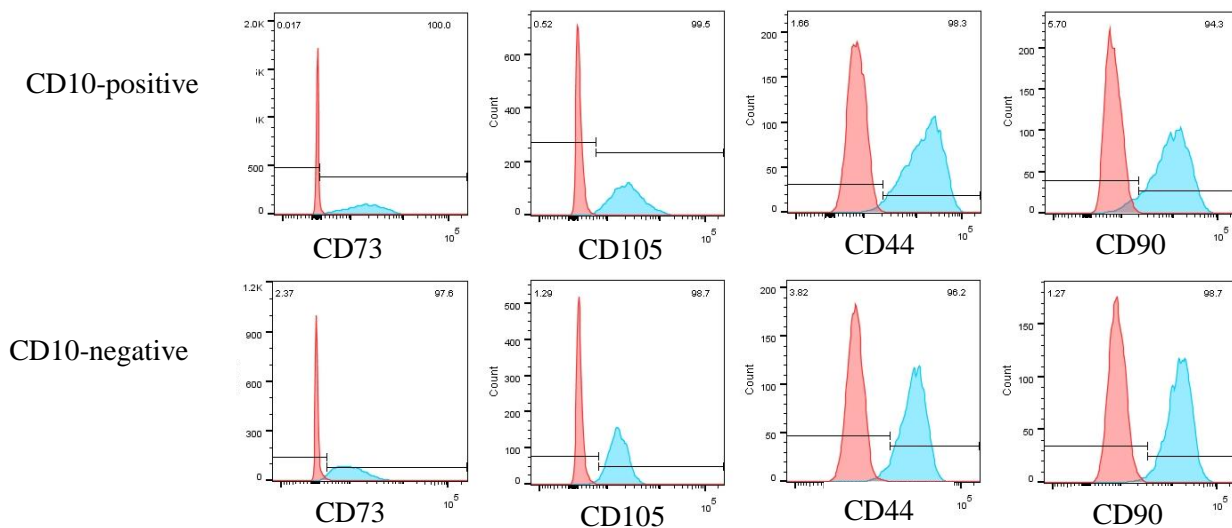
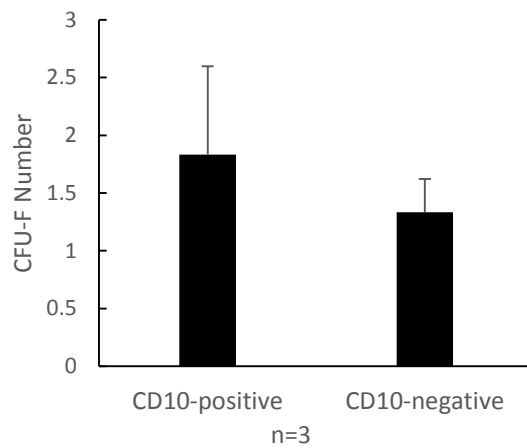


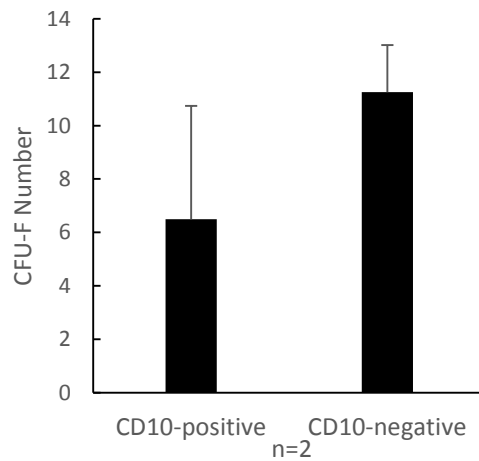
Figure 9: Both CD10-positive and CD10-negative adventitial cells express mesenchymal stem cell markers in culture (CD44, CD105, CD73, and CD90).

3.1.5 CFU-f assay

Cultured CD10-positive and CD10-negative adventitial cells did not show a significant difference in the CFU-f assay (Fig. 10A-B). CD10-negative adventitial cells produced more colonies than CD10-positive adventitial cells.



A



B

Figure 10 A-B: CD10 subsets of adventitial cells demonstrate CFU-f potential.

A: For cultured cells, 100 cells were seeded in 2 wells of 6-well plates (duplicates). After 14 days, cells were stained using 0.5% Crystal Violet Solution. A colony contains more than 50 cells. Cultured CD10-positive adventitial cells showed CFU-f potential similar to CD10-negative adventitial cells in 3 human foetal muscle tissue specimens. **B:** For fresh cells, after cell sorting, 500 cells directly were seeded in 2

wells of 6-well plates (duplicates); CD10-negative adventitial cells formed more colonies in 2 human foetal muscle tissue specimens. Another foetal sample was excluded because there was no cells after 14 days.

3.1.6 Both CD10-positive and CD10-negative adventitial cells show osteogenesis potential

To assess the differentiation potential of CD10 subsets of adventitial cells, both CD10-positive and CD10-negative subsets underwent osteogenic differentiation in culture (Fig. 11). There was no distinct difference between CD10-positive and CD10-negative adventitial cells.

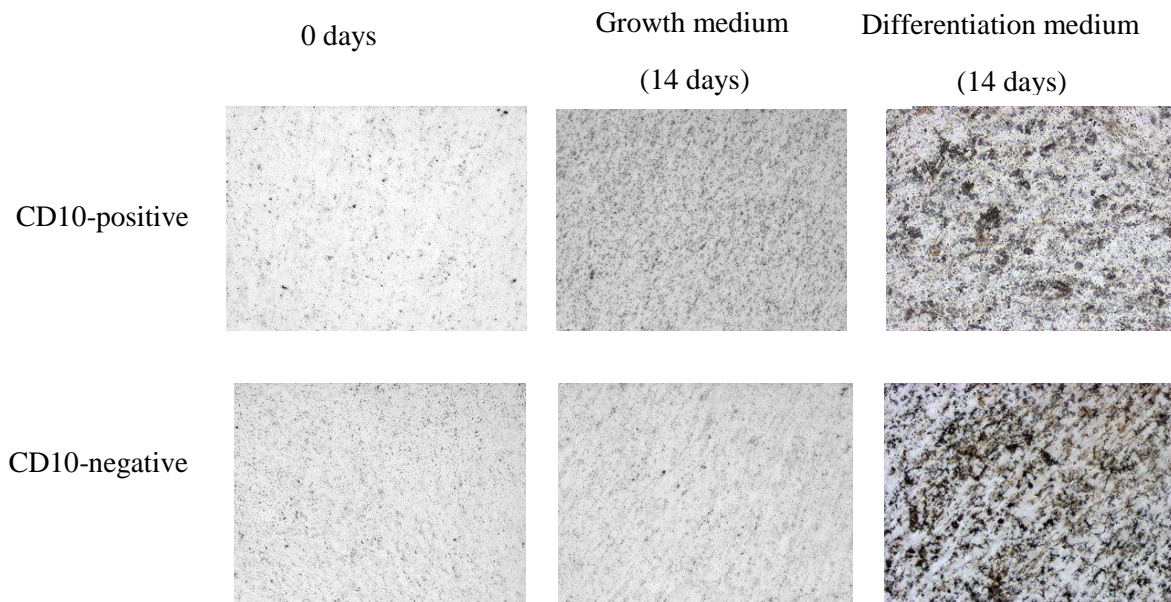
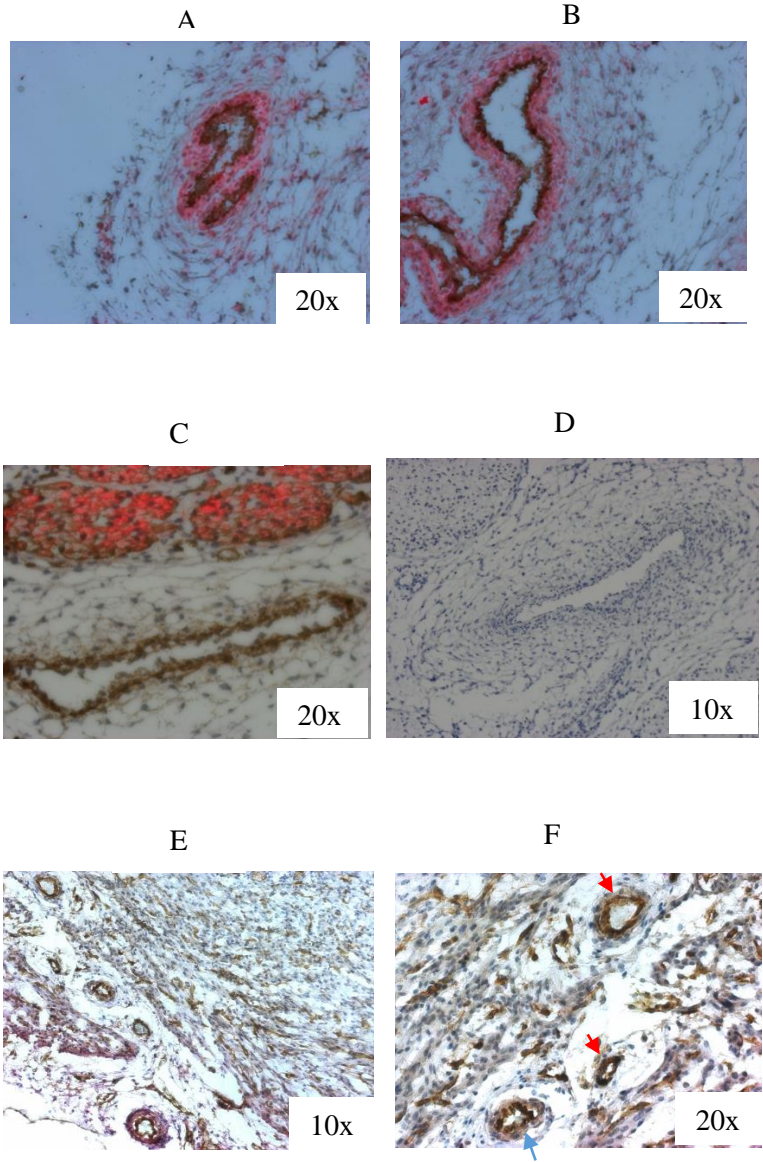


Figure 11: CD10 subsets of adventitial cells in osteogenic differentiation.

CD10-positive and negative adventitial cells cultivated in osteogenic medium for 14 days were fixed and probed for alkaline phosphatase before being incubated in a 0.5% silver nitrate solution [von Kossa staining; mineral deposits appear black (20x)]; cells cultivated in growth medium for 14 days and 0 days as controls.

3.1.7 Immuno-histochemical detection of CD10 in foetal muscle and heart

To further confirm that CD10 PSC subsets exist in vivo, we investigated foetal heart and muscle tissues. We found that not all vessels express CD10 but that CD10 expression is present in large and small vessels (Fig. 12).



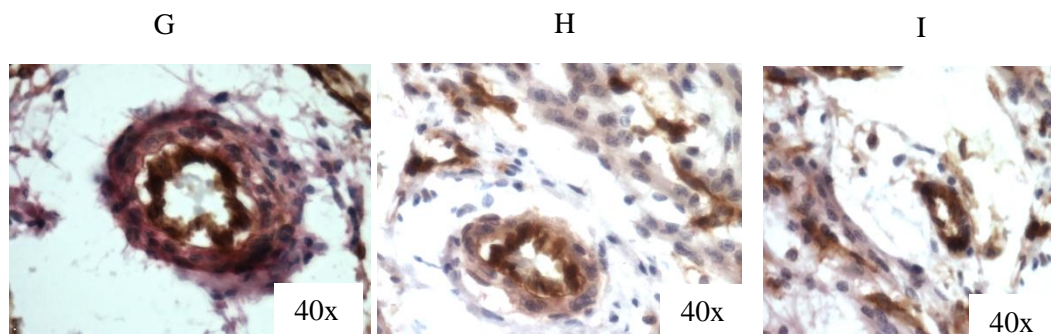


Figure 12: Detection of CD10 in human foetal muscle and heart.

A & B: CD10 is expressed in large and small vessels in human foetal muscle. CD31 = brown staining; CD31 is expressed on endothelial cells. CD10 = red staining. **C:** CD10 is expressed in muscle fibres; large vessels are negative for CD10 in human foetal muscle. CD146 is shown in brown staining; CD10 is shown in red staining. **D:** Isotype control in human foetal muscle. **E–I:** CD10 is expressed in small vessels. CD31 is shown in brown staining, CD31 is labelled on endothelial cells, and CD10 is shown in red staining. Blue arrow indicates CD10 staining around the vessel; red arrow indicates CD10-negative vessels.

3.2 CD107a on perivascular stem cells

3.2.1 Cell sorting

CD107a subsets of perivascular stem cells were sorted by FACS; six foetal muscle samples were used. CD107a positive and negative adventitial stem cells expressed CD34 and were negative for CD144 (excluding endothelial cells), CD56 (excluding myogenic cells), CD45 (excluding hematopoietic cells), and CD146. CD107a positive or negative pericytes expressed CD146 but did not express CD144, CD56, CD45, or CD34. The population of CD107a+, CD34+, CD56-, CD144-, and CD45- cells accounted for 32.67% ± 29.25% of the total adventitial cells, whereas CD107a-, CD34+, CD146-, CD56-, CD144-, and CD45- cells amounted to 52.93%±30.91% of total adventitial cells. The population of CD107a+, CD146+, CD56-, CD144-, and CD45- cells accounted for 14.78% ± 15.91% of the total pericytes. CD107a-, CD146+,

CD34-, CD56-, CD144-, and CD45- amounted to $77.73\% \pm 20.91\%$ of the total pericytes (Fig. 13).

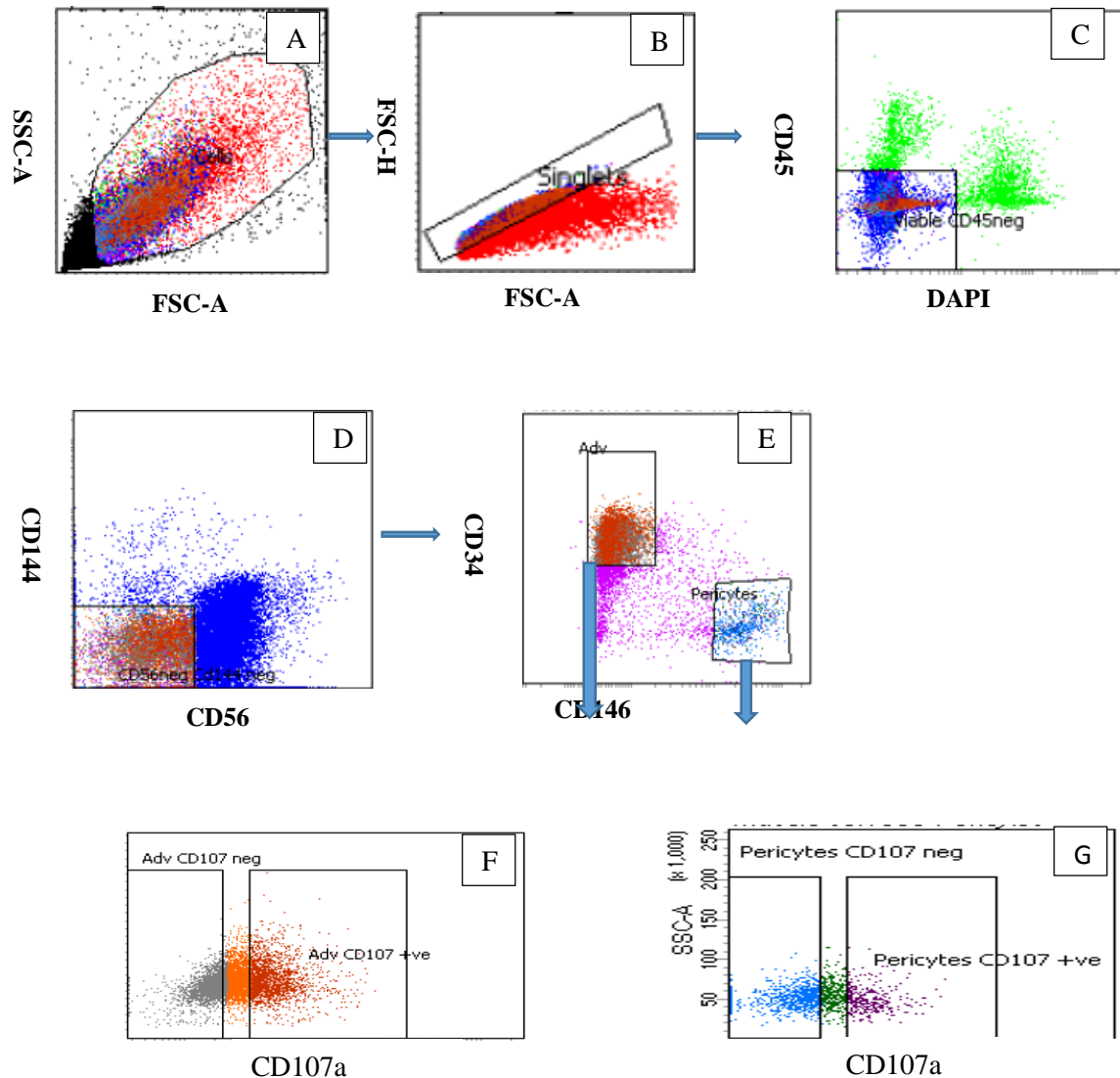


Figure 13: Isolating CD107a subsets of perivascular stem cells from human foetal muscle.

Human foetal muscles were processed to purify CD107a PSC subsets. **A & B:** Forward-scattered light (FSC) and side-scattered light (SSC) were used to find viable single cells. **C:** DAPI was used to exclude dead cells and CD45 to exclude hematopoietic cells. **D:** CD56 was used to exclude myogenic cells and CD144 to exclude endothelial cells. **E:** CD34 was used to identify adventitial cells (CD34+, CD146-, CD56-, CD144-, CD45-). CD146 was used to identify pericytes (CD34-, CD146+, CD56-, CD144-, CD45-). **F & G:** CD107a subsets of adventitial cells CD107a+, CD34+, CD146-, CD45-, CD144-, CD56- and CD107a-, CD34+, CD56-, CD45-, CD144-, CD56-) and CD107a subsets of pericytes (CD107a+, CD146+, CD34-, CD56-, CD144- and CD107a-, CD146+, CD34-, D56-, CD144-).

3.2.2 Cell culture

Cultured CD107a subsets of perivascular cells were observed at passage 1-2 under microscopy (Zeiss) phase contrast. CD107a subsets of perivascular stem cells displayed polygonal morphology with short arms at 30% confluency. At 90% confluence, cells were spindle-shaped (Fig. 14). There was no significant morphological difference between CD107 subsets of PSCs.

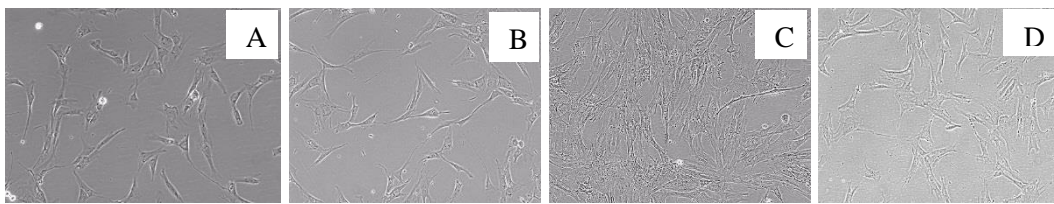
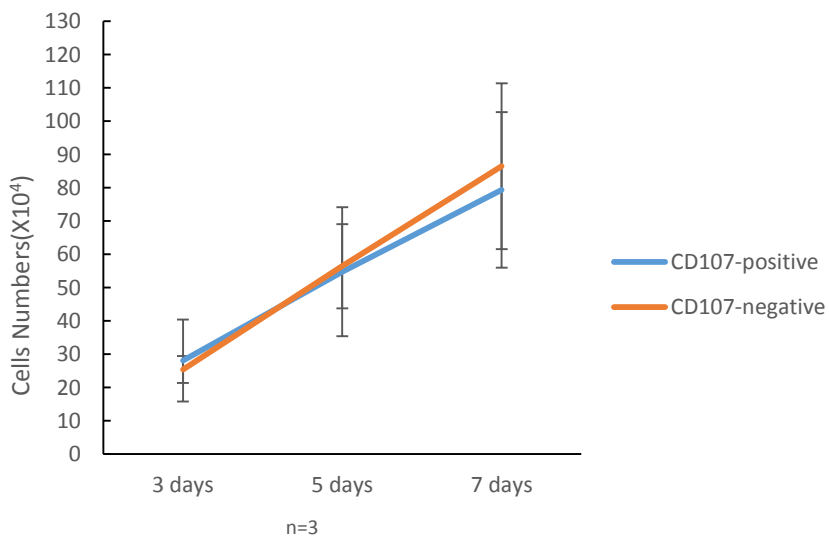


Figure 14: Morphology of CD107a perivascular stem cells.

A: CD107a-positive adventitial cells. **B:** CD107a-negative adventitial cells (5×10^4 cells were seeded; pictures were taken after 3 days). **C:** CD107a-positive pericytes. **D:** CD107a-negative pericytes (5×10^4 cells were seeded; pictures were taken after 5 days).

Cell proliferation was compared using cell growth curves and doubling times (Fig. 15A-B). There was no significant difference regarding proliferation between CD107a-positive and CD107a-negative adventitial cells.



A

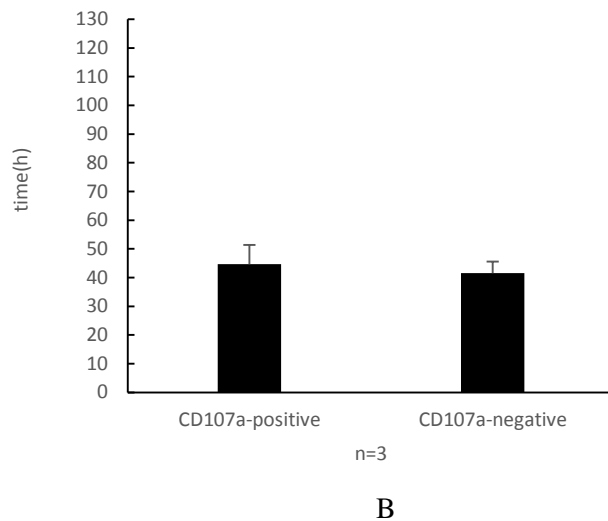


Figure 15: Proliferation of CD107a subsets of adventitial cells (passage 3).

A: There was no great difference in growth between CD107a-positive and negative adventitial cells (cells were counted at 3, 5, and 7 days in duplicate). **B:** Doubling time of CD107a-positive and CD107a-negative adventitial cells, CD107a-negative adventitial cells showed shorter doubling time, but there was no great difference (doubling time assessed using <http://www.doubling-time.com/compute.php>).

3.2.3 Flow cytometry analysis of cultured cells

Cultured CD107a subsets of adventitial cells were reassessed using the same markers as used for cell sorting. No cells in culture expressed endothelial cell markers (CD144 and CD31), hematopoietic cell markers (CD45), or myogenic cell markers (CD56) (Fig. 16-17). In culture, CD107a subsets of adventitial cells were negative for CD34

but expressed CD146 (Fig. 18). CD107a subsets of adventitial stem cells gave rise to CD107a-positive and CD107a-negative cells in culture (Fig. 19).

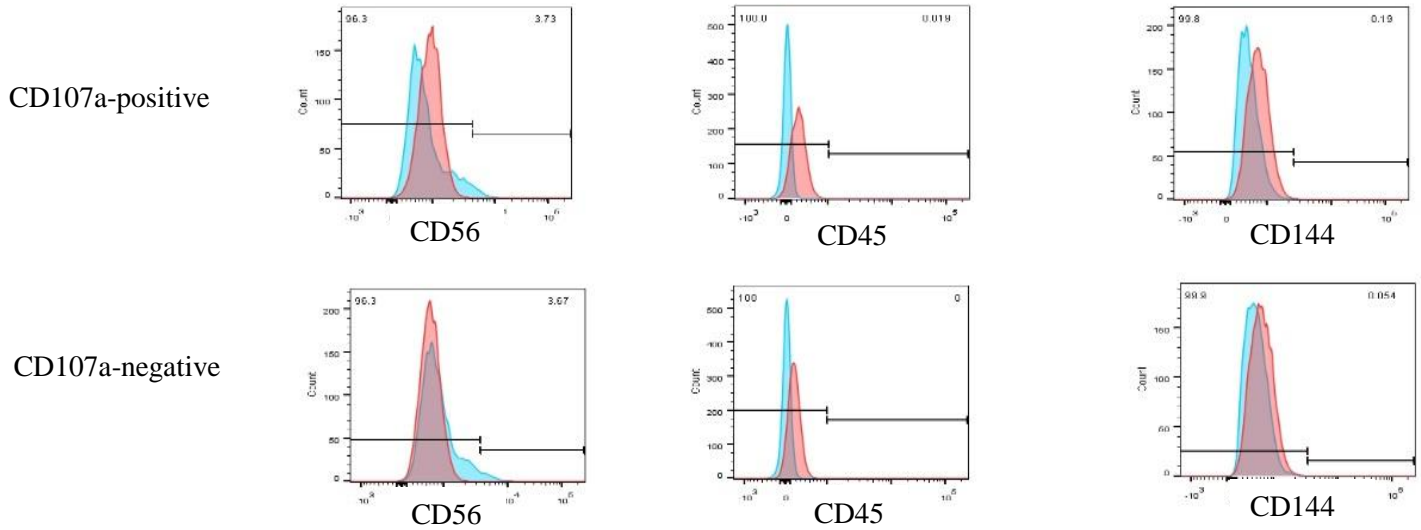


Figure 16: Both CD107a-positive and CD107a-negative adventitial cells were negative for CD56, CD45, and CD144 in culture.

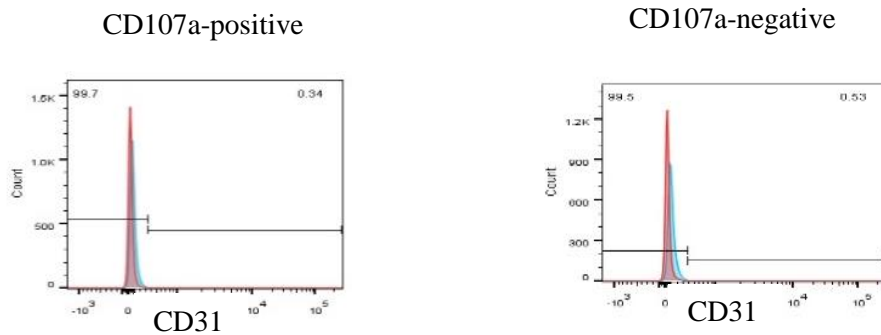


Figure 17: Both CD107a-positive and CD107a-negative adventitial cells were negative for CD31 (excluding endothelial cells).

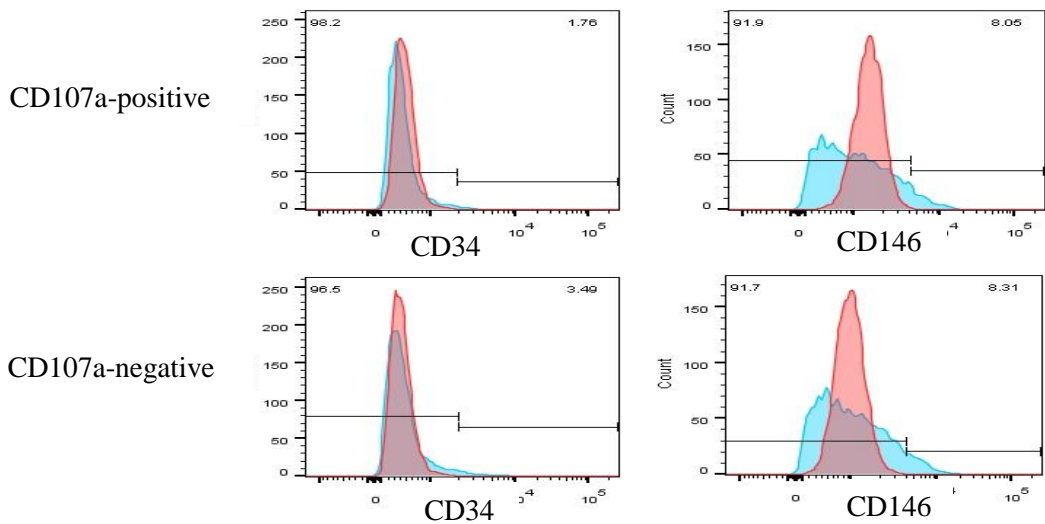


Figure 18: CD107a-positive and CD107a-negative adventitial cells were negative for CD34 but expressed CD146 in culture.

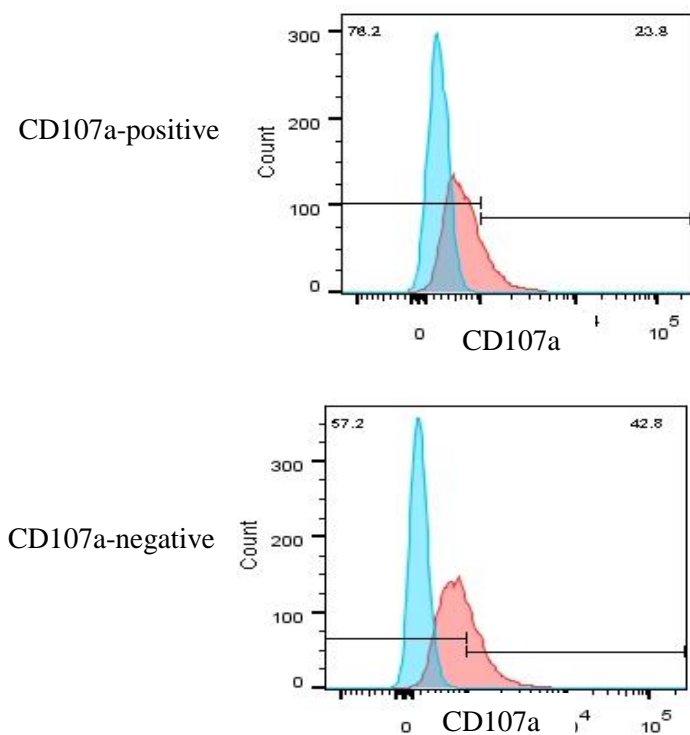


Figure 19: CD107a subsets of adventitial cells gave rise to CD107a-positive and CD107a-negative cells (n=3).

Three human foetal muscle specimens were analysed for mesenchymal stem cell markers by flow cytometry (Fig. 20). We found that both CD107a-positive and

CD107a-negative adventitial cells express CD105, CD44, CD73, and CD90 in culture. Additionally, CD107a-positive and CD107a-negative adventitial cells expressed the mesenchymal stem cell marker CD10 (Fig. 21)

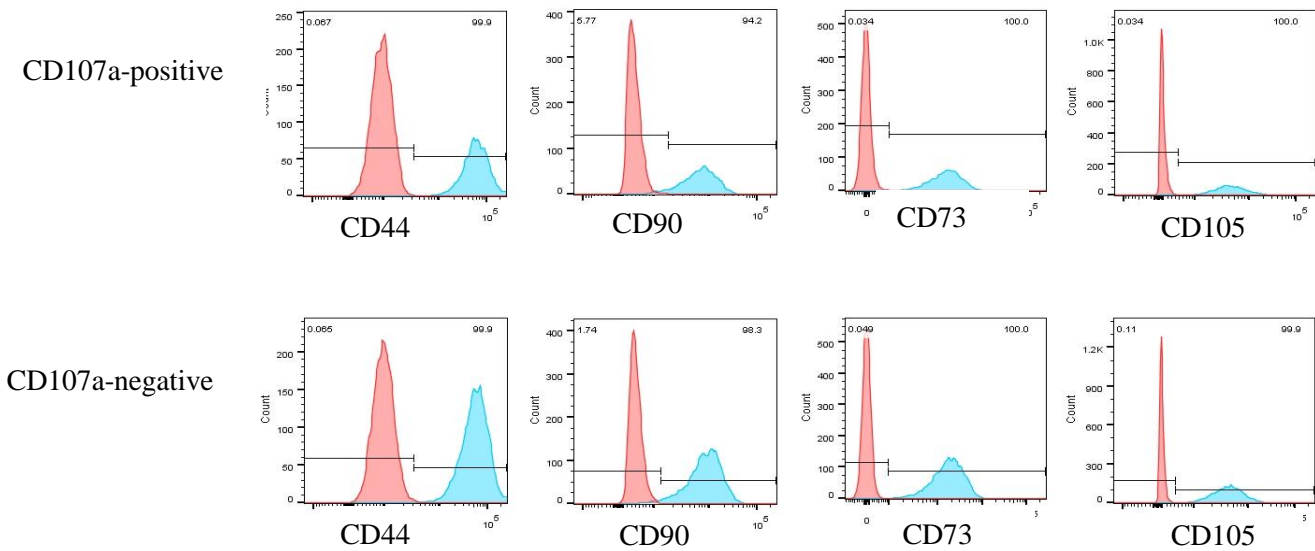


Figure 20: In culture, CD107a-positive and CD107a-negative adventitial cells express mesenchymal stem cell markers (CD44, CD90, CD73, & CD105).

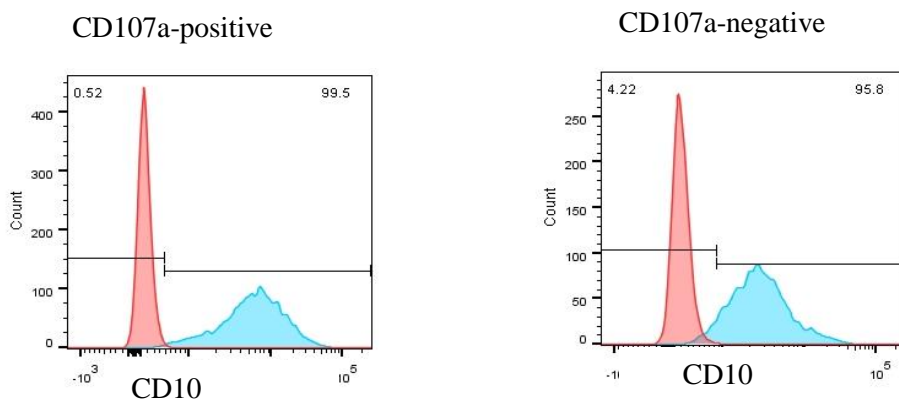


Figure 21: In culture, CD107a-positive and CD107a-negative adventitial cells expressed the mesenchymal stem cell marker CD10.

3.2.4 Clonogenic potential of CD107a subsets

The CFU-f assay was applied to show the potential of forming colonies in vitro. For cultured cells, the results show that CD107a-negative adventitial cells have a higher potential to form colonies in culture than CD107a-positive adventitial cells ($p < 0.05$). For freshly sorted cells, there is no significant difference between CD107a-positive and CD107a-negative adventitial cells (Fig22A-B).

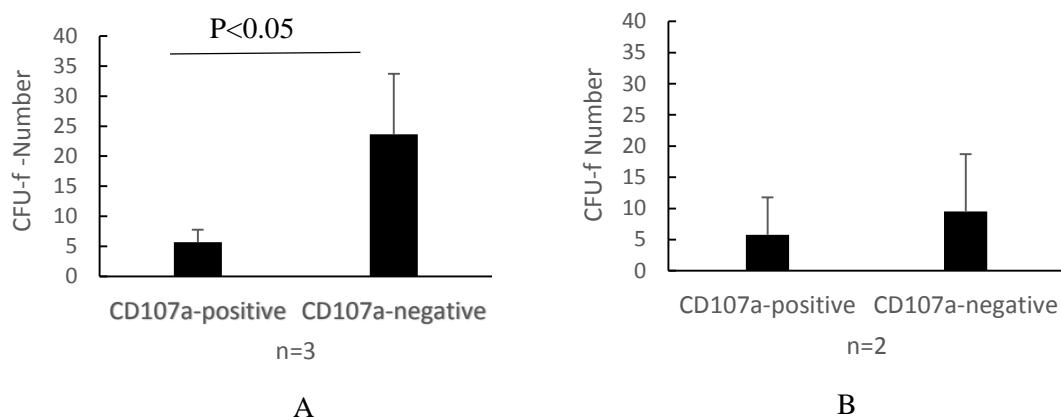


Figure 22: CD107a-negative adventitial cells show high colony forming efficiency.

A: Cultured CD107a-negative and CD107a-positive adventitial cells were assessed using the CFU-f test; CD107a-negative adventitial cells formed higher colony numbers compared to CD107a-positive adventitial cells (3 human foetal muscle specimens were investigated). **B:** for fresh cells, after cell sorting, CD107a-negative adventitial cells formed colony numbers (2 human foetal muscle specimens were assessed).

3.2.5 Both CD107a-positive and CD107a-negative subsets of adventitial cells can differentiate into osteocytes and adipocytes

To assess the differentiation potential of CD107a subsets of adventitial cells, both CD107a-positive and CD107a-negative adventitial cells were cultured in osteogenic differentiation medium (Fig. 23) CD107a-positive adventitial cells presented higher

osteogenic potential ($p=0.049$) compared to CD107a-negative adventitial cells, which showed poor capability for osteogenesis.

Both CD107a-positive and CD107a-negative adventitial cells had the potential to form adipocytes (Fig. 24). CD107a-positive adventitial cells were prone to differentiate into adipocytes.

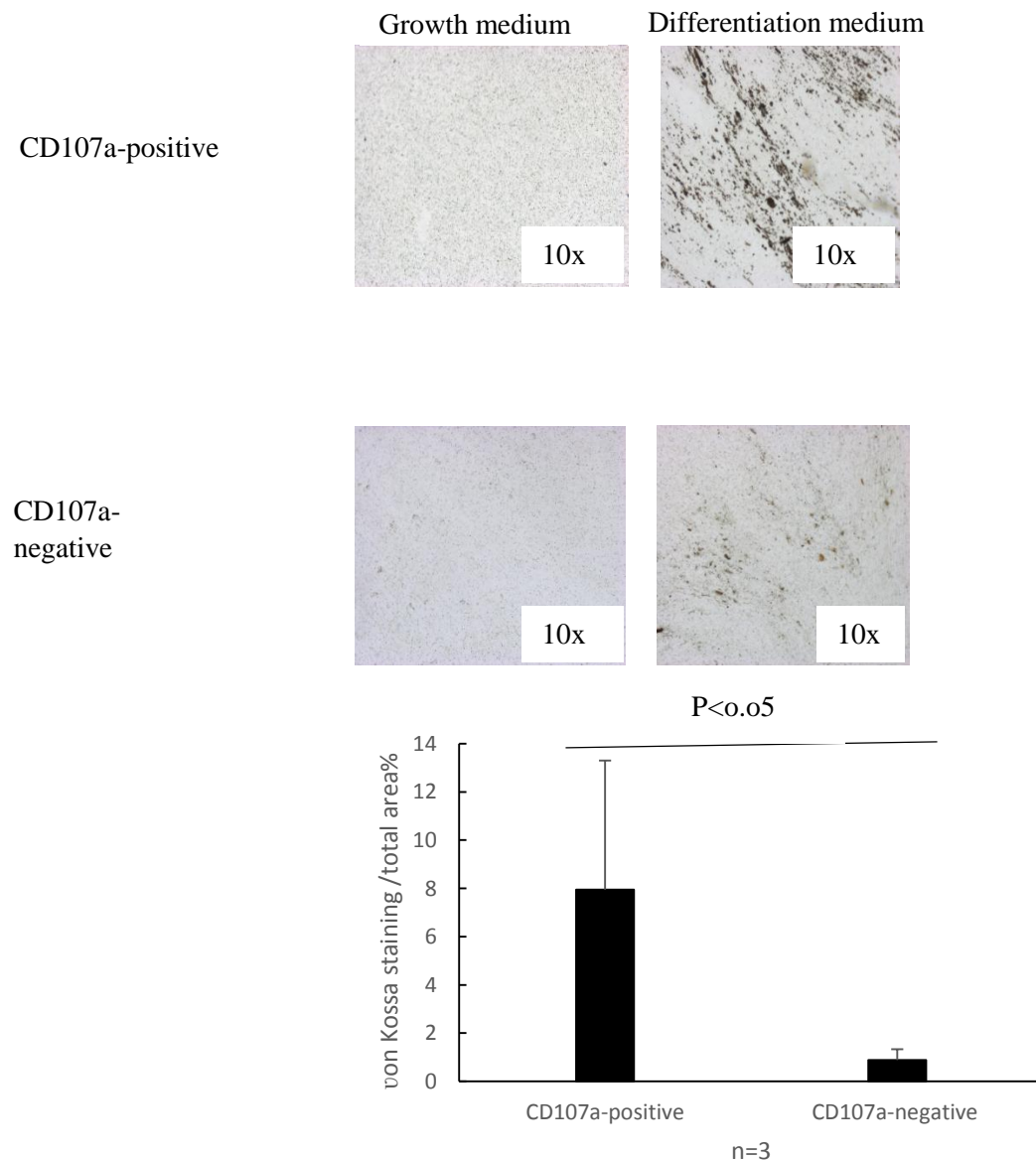


Figure 23: CD107a-positive adventitial cells show robust osteogenesis potential in vitro (3 human foetal muscle specimens).

CD107a-positive adventitial cells formed more mineral deposits (black 10x; cells were cultured in growth medium as a control).

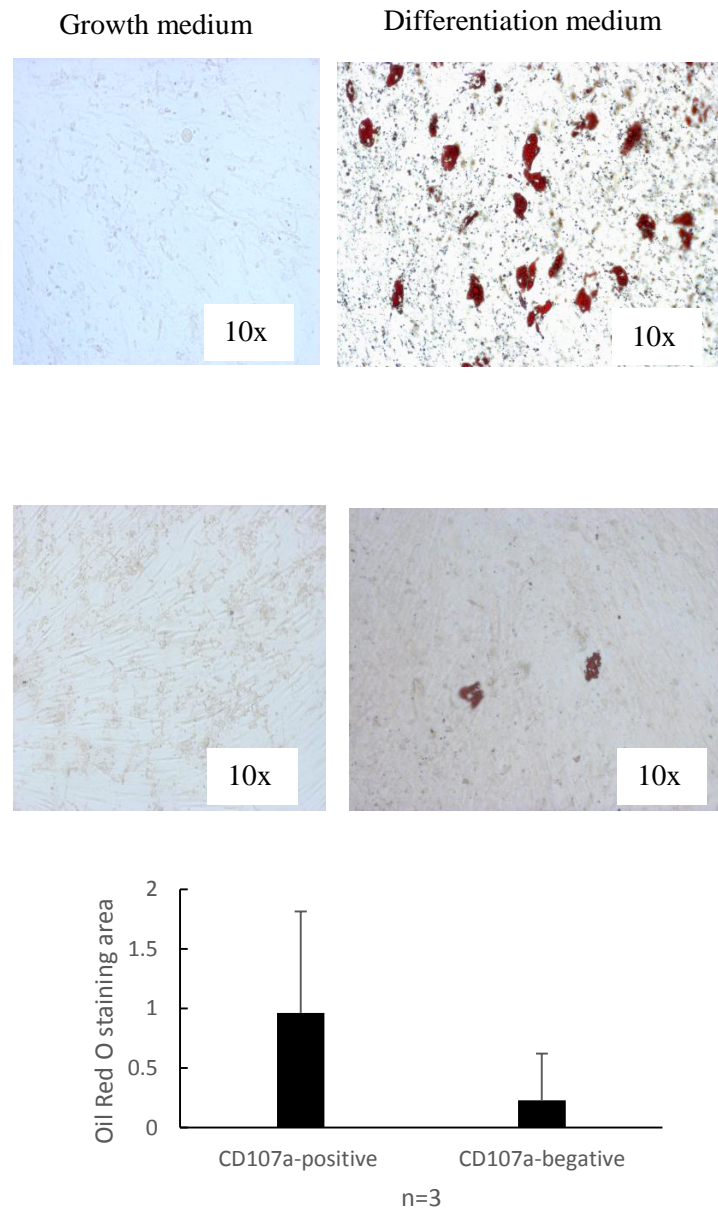


Figure 24: CD107a-positive adventitial cells show a high capability of adipogenic differentiation.

Lipids are presented in red (10x). Cultured cells were further cultured in adipogenic medium for 14 days; cells were then fixed and incubated with Oil Red O to detect lipids.

3.2.6 Osteogenic potential of CD107a subsets re-sorted from cultured cells

CD107a-positive and CD107a-negative adventitial cells were purified from foetal muscle. After 3-4 weeks, cultured cells were detached and CD107a-positive and CD107a-negative cells were re-sorted from each cultured cell population.

The results show that CD107a-positive cells re-sorted from cultured CD107a-positive cells remained osteogenic differentiation, while, CD107a-negative cells re-sorted from cultured CD107a-negative cells remained resistant to osteogenesis (Fig. 25).

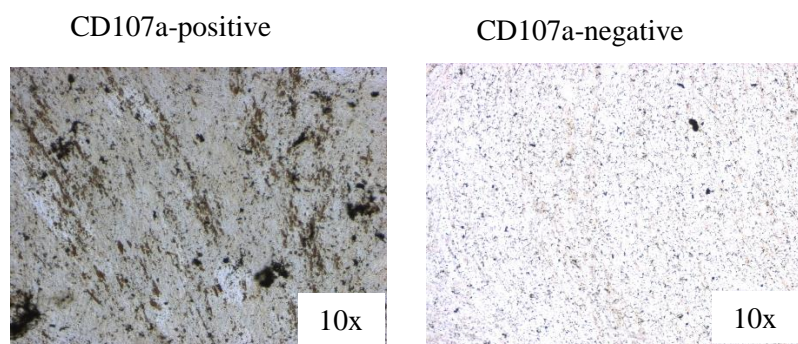


Figure 25: CD107a-positive adventitial cells show robust osteogenic differentiation potential in vitro.

After cell sorting, CD107a-positive cells and CD107a-negative cells were isolated from cultured CD107a-positive and negative adventitial cells, respectively. CD107a-positive adventitial cells differentiated into osteocytes, but CD107a negative cells failed to do so (3 human foetal tissues were assessed).

3.2.7 Detection of CD107a in human tissues in situ

To confirm the location of CD107a subsets of perivascular cells in situ, immunohistochemistry was applied to different tissues, including heart and muscle. As Fig. 26 shows, CD107a is expressed in most big vessels and some small vessels.

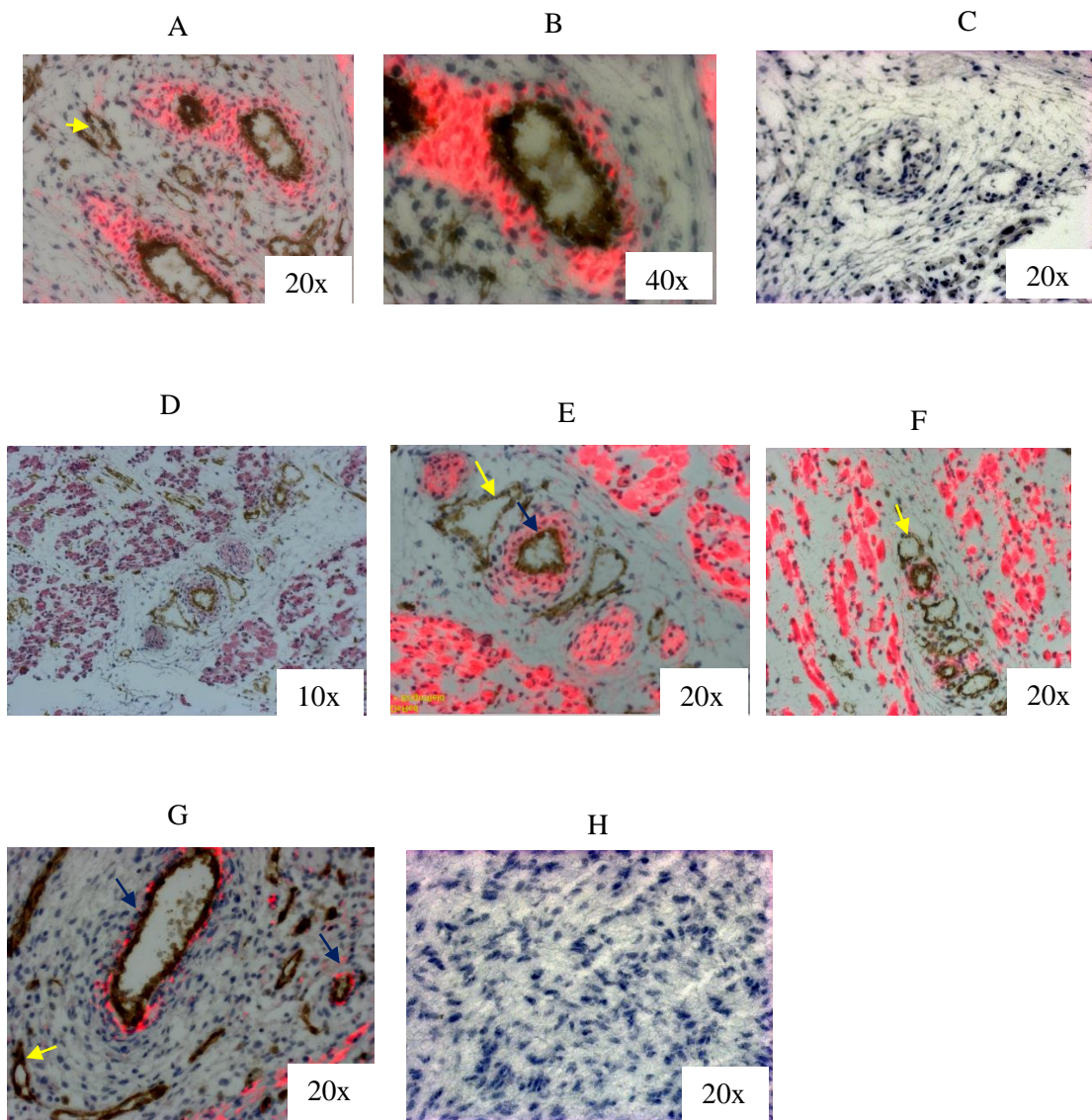


Figure 26: Detection CD107a in vessels in situ.

A–F: CD107a is expressed in the vessels of foetal muscles. CD107a is shown in red, CD31 is shown in brown (endothelial cell staining). Yellow arrows represent CD107a-negative vessels. **C:** Isotype control staining. **G–H:** CD107a is expressed in large and small vessels in the foetal heart. CD107a-negative vessels are indicated by yellow arrows; CD107a-positive vessels are indicated by blue arrows. **H:** Isotype control staining.

Chapter IV: Discussion

The current study provides evidence that distinct PSC subsets exist *in vivo* and can be isolated from human foetal muscle based on CD10 or CD107a surface marker expression. The multiple properties of PSCs highlight the need for new markers to identify and purify PSC subsets. This is the first study investigating different subsets of perivascular stem cells based on the novel markers CD10 and CD107a.

The study focuses on foetal muscle rather than other tissues (brain, placenta and adult fat). The main reason is that foetal muscle contains high percentage of CD10-positive PSC. Additionally, although fat contains high percentage of CD10-positive PSC, we found that the growth speed of CD10PSC was much slower than CD10 derived from foetal muscle in culture. Because time was limited, foetal muscle was studied. The study focuses on adventitial cells, and PSC was applied into the thesis because PSC includes adventitial cells and pericytes; therefore, the PSC in the thesis points to adventitial cells.

CD10 was originally discovered and isolated from the kidney and has an enzymatic function to regulate differentiation [19]. CD10 is a potential biomarker for the diagnosis and prognosis of various tumours [32]. Furthermore, CD10 is a surface marker of tumour stem cells in multiple tissue types. CD10 is mainly involved in tumour proliferation, differentiation, and metastasis [33]. Recently, several studies have identified CD10-expressing mesenchymal stem cells derived from multiple human tissues, such as bone marrow, kidney, embryonic cartilage, and adipose tissue [19, 25, 34-35]. The current study provides evidence of CD10 expression on perivascular stem cells from human foetal muscle. Previous studies have found human umbilical cord mesenchymal stem cells in the perivascular area and that

100% of these cells express CD10 and possess contractile properties in culture [36]. Our group has demonstrated that mesenchymal stem cells are derived from perivascular stem cells [5]. The present study has provided evidence that CD10-positive and CD10-negative perivascular stem cells exist in situ in the human foetal heart and muscle tissues. Furthermore, we confirmed that not all vessels expressed CD10 in situ; however, the study showed that CD10-negative cells can give rise to CD10-positive cells in culture, the precise reason for which is still unclear. Furthermore, we also tried to isolate CD10-positive and CD10-negative single cells, wherein CD10-negative cells still expressed CD10-positive in culture. We strongly suggest that the culture medium influences the phenotype of CD10 cells. Hagmann et al. report that different culture mediums significantly influence CD10 marker expression [38]. It requires further experiments to test how different mediums influence surface marker CD10 expression. Human bone marrow-derived mesenchymal stem cells express high amounts of CD10 during osteogenic and adipogenic differentiation [26]. Our results further confirmed that CD10-positive cells showed the potential of osteogenic differentiation. Both CD10-positive and CD10-negative cells expressed typical mesenchymal stem cell markers CD44, CD90, CD105 and CD73. It is further suggested that the progenitor of mesenchymal stem cells is a subset of PSC. Owing to CD10-negative cells giving rise to CD10-positive, we did not find any difference between CFU-f and osteogenic differentiation between CD10-positive and CD10-negative adventitial cells. Evidence shows that CD10 is involved in the differentiation of stem cells and cells signalling pathway [19], and it is essential to study the precise role of CD10 in PSC to be applied into clinical practise.

Overall, our results support the hypothesis that distinct functional perivascular stem cell subsets exist in vivo. We also confirm that progenitors of mesenchymal stem cells are located in the perivascular area and belong to PSCs. More work is needed to investigate the different functions of CD10-positive and CD10-negative perivascular stem cells. If the different functions of CD10-positive and CD10-negative PSC are explored, it will definitely indicate whether CD10-positive or CD10-negative PSC can be applied in treatment based on different situations.

CD107a is widely used as a marker to identify natural killer cell activity [37]. Jensen et al. reported that CD107a and CD133 are co-expressed in tumour stem cells [30]. CD107a is also expressed in mesenchymal stem cells derived from bone marrow [30]. The authors also reported that full-length amelogenin can connect to the CD107a receptor to regulate the proliferation of mesenchymal stem cells. However, no studies have reported the function of CD107a in terms of CFU-f and differentiation potentials. We confirm that CD107a-positive and CD107-negative PSC subsets exist in situ in multiple human tissues by immunohistochemistry. Specifically, while CD107a-positive is expressed in big vessels and some small vessels, CD107a-negative is mainly expressed in small vessels. CD107a-positive and CD107a-negative PSC were cell-sorted from human foetal muscle. From CFU-f assays, we found that CD107a-negative adventitial cells showed higher CFU-f potential in culture. It suggested that CD107a-negative adventitial cells show high proliferation ability. Both CD107a-positive and CD107a negative adventitial cells expressed typical mesenchymal stem cells markers CD90, CD105, CD44 and CD73. However, cultured CD107a-positive adventitial cells demonstrated higher osteogenesis potential. CD107a adventitial cells gave rise to CD107a-positive and CD107a-negative cells in culture. We further purified CD107a subsets of adventitial

cells and investigated osteogenic differentiation potential, and found that CD107a-positive adventitial cells show robust osteogenic differentiation potential. In contrast, CD107a-negative adventitial cells failed to do so. Our study demonstrated that there are distinct functional CD107a PSC subsets in vivo, CD107a-positive showed robust osteogenic and adipogenic differentiation. It requires further study to investigate the gene level of CD107a and explore if the level of CD107a increases during the osteogenic and adipogenic differentiation to understand the functions of CD107a. Given that there are few papers reporting the role of CD107a in mesenchymal stem cells, our results also provide evidence that it is important to further explore the role of CD107a in stem cells.

Overall, this work suggests that CD107a-positive cells present mesenchymal stem cell properties.

In conclusion, we support the hypothesis that there are distinct functional perivascular stem cell subsets in vivo. This is the first study of perivascular stem cells subsets based on novel markers CD10 and CD107a. Our study showed the promising resource of CD10 PSC or CD107a PSC for regeneration medicine. The current study focused on CD10 or CD107a adventitial cells. However, future studies will be required to focus on CD10 or CD107a pericytes.

Chapter V: Conclusion & Limitations

5.1 Conclusion

The findings of the current research provide insight into PSC subsets typified using the novel markers CD10 and CD107a. We have demonstrated that CD10 or CD107a PSC subsets exist in human foetal tissues. We also purified and identified the subsets of perivascular stem cells by flow cytometry. CD10 subsets of perivascular stem cells presented the characteristics of mesenchymal stem cells in vitro. For CD107a subsets of perivascular stem cells, we provide evidence that CD107a-positive and CD107a-negative PSCs exist in vivo. In vitro, CD107a-positive adventitial cells have robust osteogenic differentiation potential. Therefore, CD107a-positive and CD107a-negative cells represent two subsets of perivascular stem cells. This is the first time that it has been reported that CD107a positive subsets of perivascular stem cells showed robust osteogenic differentiation potential. Therefore, it may be necessary to separate distinct functional subsets to optimize the application of perivascular stem cells in the field of regenerative medicine. Overall, this study supports our hypothesis that distinct functional subsets exist in vivo.

5.2 Limitations

There are two main limitations to this study: first, more samples are needed to confirm current results. Second, currently, our studies are performed in vitro. In future studies, we need to test whether subsets of perivascular stem cells show alternative differentiation potentials in vivo.

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