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Assessing the Functional Heterogeneity of Human Perivascular Cells

Mario Armando Gomez Salazar

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DECLARATION

This dissertation is my own work and includes nothing, which is the outcome of work done in collaboration, except where specifically indicated in the text.

The data included in this text has not been submitted for any other degree or professional qualification, nor does it exceed the word limit of 100,000 words set by the College of Medicine and Veterinary Medicine.

Mario Armando Gomez Salazar

ABSTRACT

Perivascular cells found in multiple organs give rise to mesenchymal stromal/stem cells or MSCs *in vitro*. These include adventitial cells ($CD34^+$ $CD146^-$ $CD31^-$ $CD45^-$) isolated from large vessels and pericytes ($CD34^-$ $CD146^+$ $CD31^-CD45^-$) found in small vessels and capillaries. Both populations play a key role in tissue remodelling during injury and disease and display phenotypic and functional heterogeneity. However, specific stem cell properties of both populations are unknown. During my PhD, I have identified aldehyde dehydrogenase (ALDH) activity as a marker of stem cell-like perivascular cells. We performed RNA sequencing on perivascular cells isolated based upon high- or low ALDH activity. I found transcriptional differences that suggest different functions *in vivo* including progenitor capacity and interaction with the immune system. Interestingly, I found that ALDH1A1, which has been associated with stem cell properties, is the main isoform present in ALDH high adventitial cells suggesting their involvement in the regeneration process post-tissue injury. Whether these cells change in culture remains unclear. I found that ALDH subsets isolated from adventitial cells become similar both transcriptionally and functionally upon expansion in MSC culture. Conversely, pericytes seem to maintain a different identity compared to adventitial cells *in vitro*. I next analysed ALDH1A1 expression in pathological tissues. In human glioblastoma, I found that ALDH1A1 expression in perivascular cells changes suggesting their involvement in angiogenesis and tumour growth. Compared to control, skeletal muscle in mice post-injury

showed changes in ALDH1A1 expression distribution, suggesting their contribution to the regeneration process.

In conclusion, my results show that ALDH high adventitial cells in large vessels mark a population of MSC progenitors expressing genes involved in processes such as angiogenesis and, matrix remodelling, amongst others. Importantly, I documented how culture conditions change these perivascular cells once they become MSCs *in vitro.* Finally, I showed that ALDH1A1 expression and cell distribution in disease or after injury is altered.

LAY SUMMARY

Stem cells named “mesenchymal stem cells” that reside along blood vessels can be cultured and expanded in plastic dishes for clinical use for numerous diseases. In the tissues, some of these cells surround the large vessels and are called adventitial cells and some are found around small blood vessels, in which case they are called pericytes. They were shown to be directly involved in the healing process by either contributing to scarring or replenishing damaged tissue. However, the mode of action of these stem cells is not well understood, despite their high therapeutic value. They are heterogeneous and thus, it is crucial to investigate whether perivascular stem cell subsets play distinct functions. This will help us to better understand the treatment and to design better strategies to treat patients according to the particular disease in which these subsets are involved. In my thesis, I describe a novel population of adventitial cells with high activity of the enzyme family named ALDH. These enzymes are involved in cell detoxification, but are also indicative of stem cell properties. I successfully isolated adventitial cells with high ALDH activity from human fat and characterised them *in vitro*. Furthermore, I found that ALDH1A1 is almost exclusively expressed in adventitial cells in different tissues. This was translated into mouse tissues, where we saw ALDH1A1 being expressed after injury in skeletal muscle in immature muscle fibres suggesting a role in proliferation. Overall, this research provides evidence of the existence of a novel subset of perivascular cells which may repair organs after injury.

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PUBLICATIONS RELATED TO THIS WORK

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Gomez-Salazar, M., Gonzalez, Z., Casamitjana, J., James, A., Crisan, M., Péault, B. (2019). Three decades later, are mesenchymal stem cells facing senescence or immortality. Frontiers in Tissue Engineering and Regenerative Medicine (In revision)

Vezzani, B., **Gomez-Salazar, M**., Casamitjana, J., Tremolada, C., Péault, B. (2019)Human Adipose Tissue Micro-fragmentation for Cell Phenotyping and Secretome Characterization. J. Vis. Exp. (152), e60117, doi:10.3791/60117.

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Gomez-Salazar, Hardy, R., M.,Holler, F., Alexandre, M., Medici, D., Crisan, M., Péault, B. Assessment of the molecular transition from perivascular cells to mesenchymal stem cells. (in preparation)

Contents

TABLES

FIGURES

ABBREVIATIONS

ADV- adventitial cells

ALDH- aldehyde dehydrogenase

BAA - BODIPY-aminoacetate

BAAA - BODIPY-aminoacetaldehyde

CCL4 - carbon tetrachloride

CD- cluster of differentiation

COX-2 - cyclooxygenase 2

DEAB - diethylaminobenzaldehyde

DMEM – dulbecco's modified eagle medium

EGFRII - human epidermal growth factor receptor type 2

ER - estrogen receptor

EV- extracellular vesicles

FACS- fluorescence activated cell sorting

FCS - foetal calf serum

GBM – glioblastoma multiforme

Gli1- GLI Family Zinc Finger 1

HER2 - human epidermal growth factor receptor 2

HSC - Hematopoietic stem cells

IDO - indoleamine-pyrrole 2,3- dioxygenase

IL – interleukin

MHC - Major histocompability complex

MSCs- mesenchymal stem/stromal cells

NOS - nitric oxide synthase

PBS - phosphate-buffered saline

PC- pericytes

PD-1 - programmed death 1

PDGFRa- platelet derived growth factor receptor alpha

PDGFRb- platelet derived growth factor receptor beta

PGE2 - prostaglandin E2

RA- retinoic acid

RNAseq - ribonucleic acid sequencing

Tbx18 - T-Box transcription factor 18

TGFB - transforming growth factor beta

VEGF - vascular endothelial growth factor

Chapter 1 GENERAL INTRODUCTION

1.1 Mesenchymal stem cells

1.1.1 Definition and discovery of mesenchymal stem cells

The presence of cells with osteogenic potential in the bone marrow was first described by Tavassoli using entire fragments of bone-free bone marrow (Tavassoli and Crosby, 1968). However, these cells were first described in detail by the soviet scientist Alexander Friedenstein when he found that a population of adherent cells from the human bone marrow gives rise to clones, could give rise to fibroblasts in culture. He observed that these cells do not belong to the hematopoietic cell lineage and could give rise to bone and cartilage-forming cells. (Friedenstein *et al*., 1966; Friedenstein *et al.*, 1974).

Owen and Friedenstein (Owen and Friedenstein, 1988) and Piersma (Piersma *et al.*, 1985) further characterized these cells and along with other investigations (Friedenstein *et al.,* 1987; Wakitani *et al.*, 2002) and established that these cells, isolated by plastic adherence, can form osteoblasts, chondrocytes, adipocytes and myoblasts. Hence, multipotent progenitors cultured from total mouse bone marrow were shown to exhibit developmental plasticity, giving rise to diverse mesodermal cell lineages. The term “mesenchymal stem cells” or MSCs was first used by Arnold Caplan (Caplan, 1991). He proposed a parallel with the stem cells at the origin of mesodermal tissues in the embryo, and was also the first one to grow these cells from human tissues (Caplan, 1991). They were described as non-hematopoietic multipotent progenitors that can differentiate into cell types of the mesodermal

lineages such as adipocytes, chondrocytes and osteoblasts (Chamberlain *et al.*, 2007) (Figure 1).

Haynesworth and colleagues (Haynesworth *et al.*, 1992b) cultured and expanded bone marrow MSCs from the iliac crest of human donors showing for the first time that human bone marrow also contains cells with osteogenic potential. The same group created antibodies identifying SH-2 and SH-3 as unique cell surface antigens on MSCs (Haynesworth *et al.,* 1992a). Later Barry and colleagues described the ligands of the SH-2 and SH-3 antibodies as CD105 and CD73, respectively (Barry *et al.*, 1999, 2001). MSCs could be now selected on ability to adhere and proliferate in culture, expression of cell surface markers: CD73, CD90, CD105, CD44, CD124 (Haynesworth *et al.,* 1992a; Barry *et al.*, 1999, 2001) and potential to give rise to mesodermal cell lineages *in vitro*. Then Pittenger isolated presumptive MSCs from over 50 donors that were expanded, all responding positively to osteogenic, adipogenic and chondrogenic inductions. Cells displayed normal karyotype and telomerase activity (Pittenger *et al.*, 1999).

Bone marrow was the first organ to be studied as a source of MSCs. Later, cells isolated from adult adipose tissue, which remains a major provider of MSCs, demonstrated similar multipotency *ex vivo* (Zuk *et al.*, 2002; Rodriguez *et al.*, 2005; Xu *et al.*, 2005; Rodeheffer *et al.,* 2008). These findings were extended to multiple other organs, concluding that MSCs can be isolated from

all vascularized tissues (Gronthos *et al.*, 2000; Arai *et al.*, 2002; Romanov *et al.,* 2003; Mansilla *et al.*, 2006; Zheng *et al.*, 2007; Crisan *et al.*, 2008)

Human MSCs were first isolated from the bone marrow as mentioned before (Caplan, 1991) and since then, they have been isolated from the stroma of practically all post-natal tissues (Zuk *et al.*, 2002; Meirelles *et al*., 2006; Sagar *et al.*, 2018). However, MSCs are a mixed population of cells. The International Society for Cellular Therapy (ISCT) set the following minimal criteria to identify an MSC (Dominici *et al.*, 2006):

- Be adherent to plastic
- Express the cell surface markers CD105, CD90, CD44 and CD73
- Lack expression of the surface markers CD45, CD19, CD14, CD11b, CD34, CD79α, and HLA-DR
- Be able to differentiate into osteocytes, chondrocytes and adipocytes.

In addition to their differentiation properties *in vitro*, mesenchymal stem cells can also provide *in vitro* support to hematopoietic stem cells (Majumdar *et al.*, 1998), and are considered of high interest for cell therapy due to their regenerative and immunomodulatory properties. Indeed, so far, MSCs have been used in almost 1000 clinical trials (see ClinicalTrials.Gov).

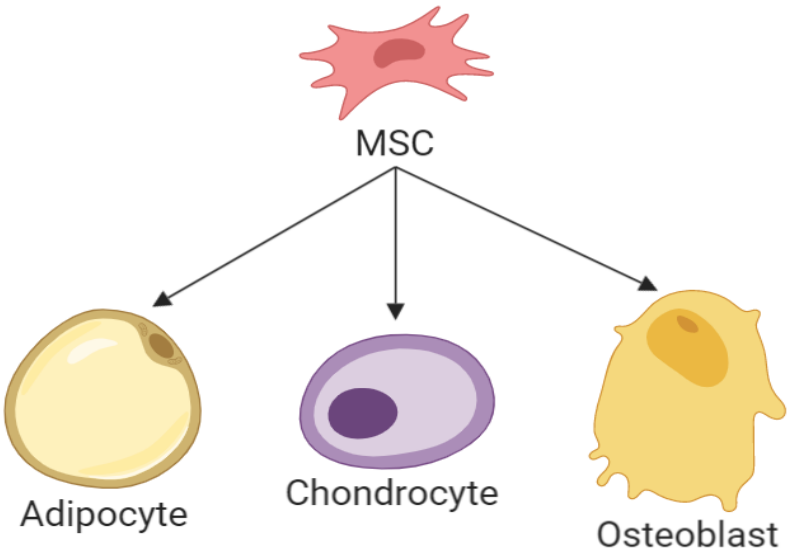


Figure 1 Mesenchymal stem cell tri-differentiation potential.

MSCs can differentiate into cell types of the mesodermal lineage such as adipocytes, chondrocytes and osteoblasts. Image created using Biorender.

1.1.2 Immunomodulatory properties of MSCs

Mesenchymal stem/stromal cells have regenerative and immunomodulatory properties that make them highly valuable for cell therapy (Kode *et al.*, 2009). The first medical use of MSCs was in 1995 using bone marrow cells from patients with hematologic malignancies in complete remission, which were subsequently expanded and reinfused to patients (autologous transplantation). MSCs have also been used to treat patients with breast cancer (Koç *et al.*, 2000) and since then these experiments have been used in clinical trials. It is now well documented that MSC benefits are due mainly to the release of growth factors and cytokines along with extracellular vesicles to activate cell proliferation, prevent apoptosis, and ultimately improve regenerative responses (Shigemoto-Kuroda *et al.*, 2017; Yang *et al.*, 2017). Furthermore, MSCs can also modulate responses by interacting with different immune cells either by direct contact or the release of soluble factors (Figure 2). However, the mechanisms by which MSCs prevent inflammation and promote healing are not fully understood.

The interaction of MSCs with immune cells including T cells and natural killer cells require direct contact through different mechanisms. For example, engagement of PD-1 (programmed death 1) and its ligands PD-L1 and PD-L2 is required for inhibition of proliferation and subsequent signalling by cytokines (Augello *et al.*, 2005). Likewise, the release of antibodies by B cells is also inhibited by MSC administration. However, interactions between MSCs and B

cells are not well understood, and probably require other intermediates (Fan *et al.*, 2016). Indeed, CD3+ T cells are needed for B-cell inhibition (Rosado *et al.*, 2014).

There are multiple reasons why MSCs do not activate the immune system. These include the lack of expression of co-stimulatory molecules CD80 and CD86, required for immune activation, the absence of major histocompatibility complex (MHC) class II antigens and low expression of MHC class I (Krampera *et al.*, 2003; Le Blanc *et al.*, 2003; Nauta and Fibbe, 2007). On the other hand, soluble factors are also required for immune modulation by MSCs. The main mediators of MSC-driven immune modulation are indoleamine-pyrrole 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2) and cyclooxygenase 2 (COX-2) in the presence of pro-inflammatory cytokines (Ryan *et al.*, 2007). For example, PEG2 is involved in the production of anti-inflammatory IL-10 by macrophages (Németh *et al.*, 2008) and blocks differentiation of monocytes into dendritic cells (DCs) (Spaggiari *et al.*, 2009).

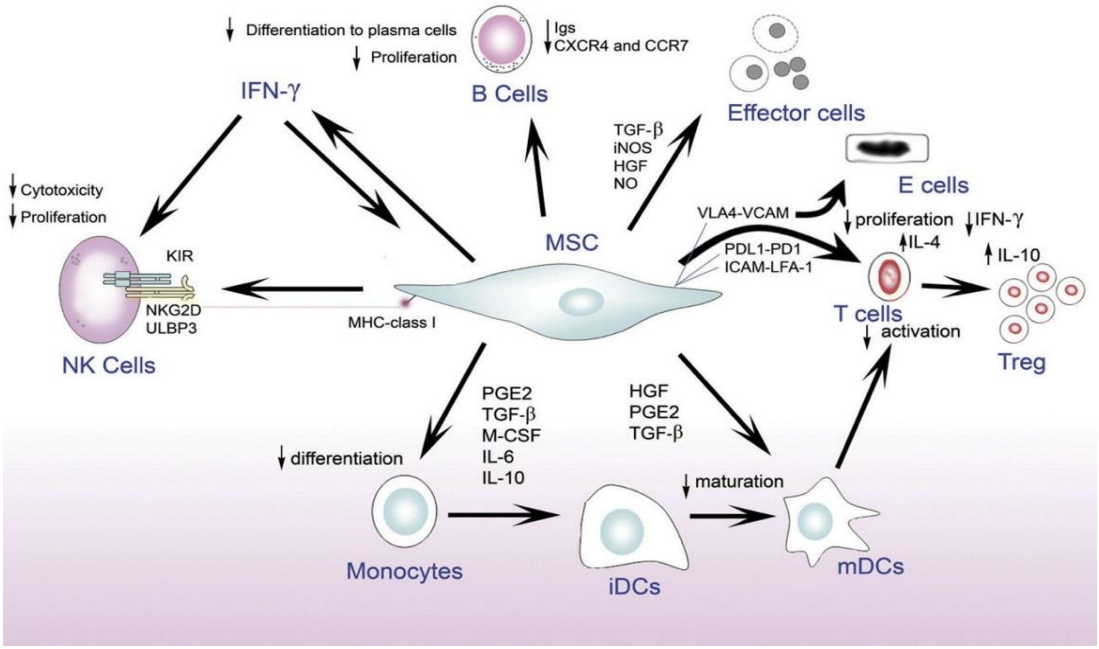


Figure 2 Immunomodulatory properties of MSCs.

Regulation of the immune response by MSCs is a complex mechanism. MSCs can supress naïve and memory T cells and inhibit proliferation of B cells. MSCs reduce IFN-γ production and prevents cytotoxicity of Natural Killer (NK) cells through soluble factors. Moreover, MSC can inhibit the differentiation of monocytes into mature dendritic cells (DC). Adapted from Kode et al., 2009

1.1.3 Regeneration mechanisms of MSCs

In addition to their immunomodulatory properties, MSCs also promote regeneration by direct differentiation (although variable) and by promoting the proliferation of host endogenous cells by the release of soluble factors. One of the best known mechanisms of MSC-driven regeneration is promotion of angiogenesis. Direct injection of perivascular MSC-progenitors into ischemic hearts resulted in improvement of vascularization in the cardiac muscle (Chen *et al.*, 2015), and moreover, direct delivery of bone-marrow-derived MSCs improved heart regeneration after myocardial infarction (Amado *et al.*, 2005). This improvement after MSC treatment is due to the release of different factors. For example, MSC secrete vascular endothelial growth factor (VEGF) which triggers angiogenesis (Sorrell *et al*., 2009), and nitric oxide synthase (NOS) that prevents fibrosis (Ferrini *et al.*, 2002).

Lastly, MSCs release exosomes/microvesicles that are non-cellular transporters of regulatory RNAs, proteins and lipids. These extracellular vesicles (EV) can induce proliferation of progenitors and prevent scar formation (Lai *et al.*, 2010; Liu *et al.*, 2018). For example, in a model of carbon tetrachloride (CCL4)-induced liver fibrosis, MSC-derived exosomes alleviated tissue damage (Jiang *et al.*, 2018).

Although the use of MSC-derived EVs for cell therapy seems promising, more research is needed to completely understand the mechanisms of regeneration.

For example, EVs are themselves highly heterogeneous, and evolving criteria for their isolation and characterization represent important guidelines for standardization in the field (Théry *et al.*, 2018).

1.1.4 MSCs in clinical trials and alternative approaches to improve clinical outcome

MSCs have been used clinically for more than two decades, and over 980 registered MSC trials are listed by the FDA (www.clinicaltrials.gov). Results have often fallen short of expectations though. In a phase III trial using MSCs (Prochymal) for treatment of steroid-refractory graft-versus-host disease (GVHD), MSC treatment showed no significant difference after 28 days compared to the placebo control (Martin *et al.*, 2010). However, the stratification of the data showed thatchildren responded better to MSC treatment, leading to approval of Prochymal in Canada (Reicin *et al.,* 2012). With similar results, cardiopoietic primed bone-marrow derived MSCs were used to treat ischemic heart failure by the Belgium based company Celyad with good preliminary results. However, further trials showed no significant differences between the MSC treatment and placebo (Bartunek *et al.*, 2013, 2016).

Despite many setbacks in clinical trials, MSC therapy has been approved in different countries. In Japan, the use of MSCs was approved after the Act on the Safety of Regenerative Medicine and the Pharmaceuticals, Medical Devices and Other Therapeutic Products Act were introduced (Sipp, 2015). More recently, the European Medicines Agency (EMA) recommended the approval of Alofisel to treat Crohn's disease (Sheridan, 2018). Overall, the use of MSCs for cell therapy is becoming a reality.

One of the main problems regarding MSC therapy is the variable nature of the results. Factors like tissue of origin, donor gender and medical history can influence clinical outcomes. Moreover processing of the tissue, culture conditions, freezing and thawing of cells along with the administration routes are other factors to be taken into consideration (reviewed by Galipeau and Sensébé, 2018). Lastly, MSCs are expanded for long periods of time to obtain enough cells for therapeutics, which induces changes in gene expression and allows clonal selection, therefore affecting biological properties and the heterogeneity of the cell population.

The heterogeneity of MSCs along with the diversity of the tissues they are isolated from adds to this complexity in the nature of MSCs. The most commonly used tissue to isolate MSCs are bone marrow, adipose tissue and cord blood (Gao *et al.*, 2016), although MSCs can be obtained from all vascularized organs including skeletal muscle (Crisan *et al.*, 2008; Corselli *et*

al., 2012) and brain (Lojewski *et al.*, 2015). It has been documented that the tissue of origin can influence the secretome of MSCs (Kalinina *et al.*, 2015). Furthermore, MSCs derived from diseased donors have shown negative results (Dzhoyashvili *et al.*, 2014). Finally, the age of the donor is a key factor affecting MSC efficacy. For example, MSCs isolated from neonatal tissues show a longer lifespan, higher proliferation rate and differentiation potential when compared to adult tissues (Donders *et al.*, 2017).

The ideal conditions for MSCs to be used for cell therapy include the use of medium with no animal products. One of the most common additives for cell culture is fetal calf serum which is not well characterized, and properties vary between batches. To obtain high number of cells MSCs need to be expanded *in vitro*. This cell expansion requires the passaging of cells in which proteolytic enzymes are used, thus inducing cell damage (Penna *et al.*, 2015). Furthermore, during culture conditions MSCs are cultured at high oxygen levels that may compromise the therapeutic benefits of MSCs. Native perivascular cells (MSC progenitors) tissue environments range between 1 and 7% O2; during culture cells sense an oxygen concentration of 20%, which may cause oxidative stress affecting viability, and eventually leading to senescence. Indeed, hypoxia has been shown to increase proliferation of MSCs (Zhu *et al.*, 2016). Moreover, cells under hypoxic conditions maintain their undifferentiated status and multipotency (Basciano *et al.*, 2011). Hypoxia also improves angiogenesis (Bader *et al.*, 2015) and migration toward the site of injury (Vertelov *et al.*, 2013).

Contrasting results using MSCs is likely due to the combination of variables such as donor demographics, cell processing technical conditions and organ source. Therefore, cell therapy using MSCs need to be tailored to every specific type of injury or disease which may include the pre-conditioning of cell to enhance clinical outcome and screening of the host's immune response (reviewed in Pittenger *et al.*, 2019).

Despite intense use of these cells for cell therapy, the fate of transplanted MSCs is not well documented (autologous, allogeneic, or xenogeneic transplantation). Injected MSC direct contribution to new tissue formation is minimal, with only a small proportion of xenogeneic (human) cells engrafting mouse tissues (Chen *et al*., 2015b), and those cells not engrafted cleared from the tissue 72 h post administration (Lee *et al.*, 2009; Gholamrezanezhad *et al.*, 2011; von Bahr *et al.*, 2012) .Nonetheless, this clearing of MSCs may be of clinical interest. It has been shown that dying transplanted MSCs engulfed by recipient macrophages release immunosuppressive soluble factors (Galleu *et al.*, 2017), suggesting that death within host tissues contributes directly to the beneficial effects of MSCs.

Different approaches to improve the clinical benefit of MSC therapy have been used. For example, a suitable option to improve the benefit of MSCs is the use of scaffolds populated that, when engrafted, provide higher regeneration. MSCs have been a useful tool in tissue engineering for regenerative medicine.

Tissue engineering can be used to mimic organ microenvironment for organoid culture (Fatehullah *et al.,* 2016) and may rely on 3-dimensional printing (Poldervaart *et al.*, 2017). Tissue engineering can also be used to improve MSC residence after administration, to which aim MSC-based scaffolds have been used, using either biodegradable or non-degradable polymers to form hydrogel matrices (Park *et al.*, 2018), which can be supplemented with growth factors. MSC based scaffolds systems have been used for bone and cartilage regeneration (Kim *et al.*, 2019), as well as for the reproduction of blood vessels (Pinnock *et al.*, 2016), cardiac tissue (Rashedi *et al.*, 2017; Ichihara *et al.*, 2018), and skeletal muscle (Witt *et al.*, 2017). Optimal tissue replacement efficiency relies on the physical characteristics of the scaffolds (Alakpa *et al.*, 2017; Jeon *et al.*, 2017; Mouser *et al.*, 2018), as each mechanical property can modify the fate of the transplanted cells. For instance, stiff matrices can be determinant to drive MSC differentiation into chondrogenic or osteogenic cell lineages (Alakpa *et al.*, 2016), whereas softer substrates can favour myogenic development (Gilbert *et al.*, 2010). In addition to stiffness, dimensionality and degradability of the matrix can regulate mechanisms critical for cell differentiation (Caliari *et al.*, 2016).

An alternative method to improve the delivery, engraftment and therefore clinical benefit of MSCs, is the use of sorted perivascular presumptive MSCs for direct transplantation instead of cultured cells (Murray and Péault, 2015; James and Péault, 2019), the latter being susceptible to modifications hindering regenerative potentials (Zaitseva *et al.*, 2006).

Other aspect to consider is that the expansion of MSCs selects the fastest growing clones and after enough passages, the whole cell population becomes homogeneous (Selich *et al.*, 2016). Furthermore, MSC clones have different mesodermal differentiation potentials (Muraglia *et al.,* 2000). Following this rationale, the use of freshly sorted stromal cells with High ALDH activity in a model of hind limb ischemia has yielded striking results (Capoccia *et al.*, 2009), documenting another dramatic difference between subpopulations of MSC ancestors that may be lost after culture.

Another alternative for the use of uncultured cells relies on the administration of microfragmented adipose tissue, in which the microenvironment of perivascular progenitors is maintained (Vezzani *et al.*, 2019). This preservation of the niche sustains higher secretory activity, releasing abundant cytokines and growth factors (Vezzani *et al.*, 2018). In general, transplantation of uncultured cells may be ideal to improve clinical outcome, although numbers of cells obtained are lower than in culture conditions and may not be enough for proper treatment in some indications.

1.2 Blood Vessels as a Source of Mesenchymal Stem Cells

Although mesenchymal stem/stromal cells have been used in numerous studies and clinical trials, the origin of these cells was not known. MSCs are typically isolated by adherence to plastic and selection by consecutive passaging. However, the true identity of MSCs remained unknown. The idea of a common precursor in the vasculature was proposed due to the presence of blood vessels in most organs (Crisan *et al.*, 2008). One of the important observations to identify the MSC progenitor *in vivo* was the relationship between vascular density and MSC yield. Indeed, the higher the number of blood vessels in the tissue, the higher the yield of MSCs (da Silva Meirelles *et al.*, 2008). The attention for the possible MSC progenitors was drawn to blood vessels. Indeed, pericytes in capillaries and adventitial cells in larger vessels are MSC progenitors (Figure 3) (Crisan *et al.*, 2008; Corselli *et al.*, 2012).

1.2.1 Pericytes as MSC progenitors

Pericytes (PCs), also called mural or Rouget cells, are cells embedded in the basement membrane of microvessels. These cells were first described by Eberth and Charles-Marie Benjamin Rouget (Attwell *et al.*, 2015). However, Zimmermann was the one who called these "Rouget cells" pericytes due to the proximity of these cells to endothelial cells (Zimmermann, 1923). Pericytes interact with endothelial cells through different molecules and activate different pathways including: PDGFRβ/PDGF-b, angiopoietin-1/Tie-2 and TGFβ among

others (Armulik *et al*., 2011). One of the main functions of pericytes, similar to smooth muscle cells of large vessels, are vasoconstriction and vasodilation to regulate vascular diameter and the blood flow in capillaries (Rucker *et al.,* 2000). The expression of contractile proteins such as α-SMA, tropomyosin, and myosin in pericytes provides evidence of their function. These proteins are also produced in smooth muscle cells which is the source of confusion between the definition of a pericytes and a smooth muscle cell. Pericyte contractile function is regulated by many molecules. Pericytes respond to β-adrenergic stimuli leading to relaxation, whereas α-adrenergic signals produce contraction (Rucker *et al.,* 2000). Oxygen levels also regulate pericyte contraction. For example, hypoxia increases the contraction of pericytes in vitro and CO2 induces relaxation.

The function of pericytes *in vivo* has shown that they response to vasoactive substances in skeletal muscle (Hirschi and D'Amore, 1996). Lastly, pericytes have also been described to act as progenitor during injury (Dellavalle *et al.*, 2011).

MSC progenitors were described to be in the perivascular niche. However, identifying pericytes is not easy since there are no specific markers for this cell type. Crisan and colleagues identified pericytes as MSC progenitors expressing canonical MSC markers, and a set of surface markers including CD146, NG2 and PDGFRβ by comparing both *in situ* and *in vitro* traits of

pericytes and MSCs. A combination of markers (CD146+ CD31- CD45- CD34-), common between all organs has been used to isolate pericytes from all blood vessels (Crisan *et al.*, 2008).

Pericytes are MSC precursors, and their function as progenitors *in vivo* has been described. For example, tissue resident pericytes in skeletal muscle can differentiate into muscle fibres (Dellavalle *et al.*, 2011) and pericytes in adipose tissue are adipocyte progenitors(Tang *et al.*, 2008). In addition some pericytes expressing gli1 differentiate into myofibroblasts (Kramann *et al*.2015). However, the contribution of pericytes in the *in vivo* setting has been questioned. Indeed, Guimarães-Camboa showed that pericytes in different organs are at the origin of MSCs in culture but do not contribute to other lineages in aging and injury of different tissues *in vivo* (Guimarães-Camboa *et al.*, 2017). However, Tbx18, the marker used to trace pericytes, is questionable since not all pericytes express it. This suggests that not all pericytes contribute to regeneration. Moreover, this work was performed in mice and thus there is no clear evidence that this conclusion applies to human. Our own studies found that pericytes are less primitive compared to other perivascular cells and express genes more related to their function such as blood flow regulation than to “stemness” (Hardy *et al.*, 2017). However, as mentioned, some pericytes act as progenitors.

1.2.2 Are adventitial cells the true MSC progenitors *in vivo*?

Large blood vessels, arteries and veins, consist of three layers: tunica intima, tunica media and tunica adventitia (in veins there is less smooth muscle and connective tissue). The innermost layer, the tunica intima, is comprised of the endothelium, connective tissue (mainly collagen, fibronectin and other extracellular matrix molecules). The middle layer or tunica media is formed primarily by smooth muscle cells and is the thickest layer. This layer is especially important since it provides support and can change diameter and regulate blood flow and pressure. The tunica adventitia is the outermost layer and consists of connective tissue, fibroblasts, inflammatory cells, vasa vasorum and other cells (Moreno *et al.*, 2006).

For a long time, the adventitia layer was regarded as a support connective tissue containing collagen and fibroblasts only. However, in the last years many studies have identified this layer as a dynamic compartment for cell trafficking to and from the vessel, and showed that it also participates in growth and repair (Majesky *et al.*, 2011). The adventitial layer plays key roles in biological processes such as the retrieval, integration, storage and release of cellular regulators of vessel wall function. It is the most complex layer of the large vessel and is comprised of a variety of cells including progenitor cells, fibroblasts, immunomodulatory cells, vasa vasorum endothelial cells and pericytes, and adrenergic nerves (Stenmark *et al.*, 2006, 2013).

Upon injury, cells in the adventitia layer have been proposed to differentiate into myofibroblasts, and to migrate to the inner layers of the vessel wall to regulate vascular remodelling (Siow *et al*. 2003; Stenmark *et al*. 2006). Cells in the adventitia have also been described as mediators of remodelling by regulating reactive oxygen species (Haurani and Pagano, 2007). Furthermore, the adventitia layer harbours a population of cells ($CD34^+$ $CD31^-$) able to differentiate into endothelial cells and participate in vessel formation (Zengin *et al.*, 2006; Hu and Xu, 2011). Interestingly, a similar population of cells in the adventitia layer called adventitial cells with the following immunophenotype: $CD34^+$ $CD31^-$ $CD146^-$ show characteristics of stem cells, and give rise to MSCs *in vitro* when purified by FACS *(Figure 3)* (Corselli *et al.*, 2010, 2012). Cells in the adventitia were shown to act as progenitors *in vivo*. For example, resident gli1+ adventitial cells are able to differentiate into myofibroblasts after injury in different organs (Kramann *et al.*, 2015) and during kidney disease can contribute to calcification (Kramann *et al.*, 2016). Similarly, $PDGFR\beta^+$ cells contribute to fibrosis after injury in the skeletal and cardiac muscle (Murray *et al.*, 2017) . Furthermore, a subset of PDGFRβ+ cells that co-express PDGFRα is highly fibrotic and contributes to fatty degeneration in a model of rotator cuff injury (Jensen *et al.*, 2018).

Overall, these studies suggest that the adventitia layer acts as a reservoir of progenitors showing heterogeneity of unknown significance. The identification of novel markers for adventitial cells is crucial to understand the mechanisms that drive different processes during and after injury.

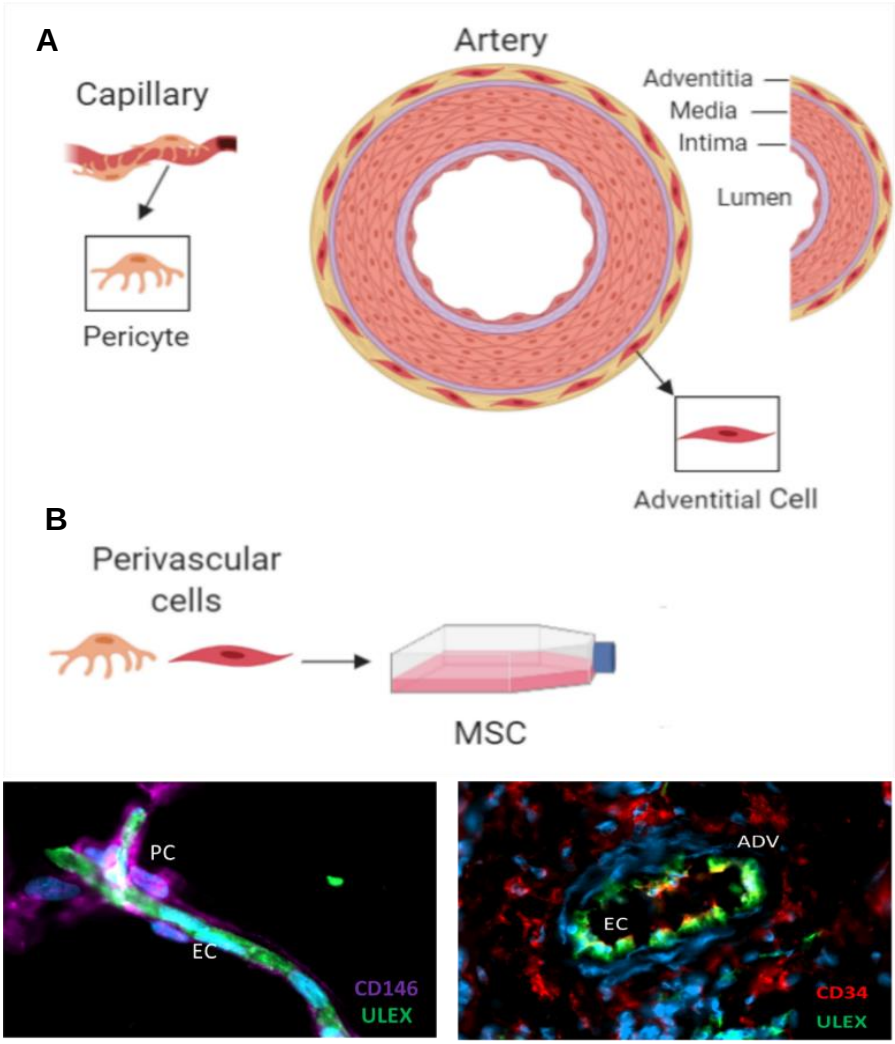


Figure 3 MSC progenitors are located in capillaries and large vessels.

Schematic of MSC progenitors in blood vessels (A) and immunofluorescence staining of adipose tissue (B) showing pericytes expressing CD146 in close contact with the endothelium stained with the Ulex europaeus lectin. Adventitial progenitor cells are located in the outermost layer of veins and arteries, and express CD34. DAPI staining of cell nuclei is blue. Endothelial cells appear yellow/green, because they express both CD34 and the receptor for Ulex. Figure was made using BioRender.

1.2.3 Heterogeneity of perivascular progenitors and derived MSCs

MSCs are heterogenous, as can be expected from cultures of total cell suspensions, although this high heterogeneity is reduced over time *in vitro*, allowing better protocol standardization. Indeed, clonal analysis MSC cultures for long periods of time has shown that diversity is dramatically decreased after multiple passages (Selich *et al.*, 2016). Moreover, MSC clones exhibit diverse differentiation potentials (Muraglia *et al.,* 2000)

The heterogeneity of conventional culture derived MSCs also reflects the diversity of their native progenitors (pericytes and adventitial cells). Analysis of perivascular MSCs has revealed that these cells are phenotypically and functionally diverse which also applies to different organs and tissues. A developmental hierarchy of pericytes and adventitial perivascular cells has been established in human adipose tissue (Hardy *et al.*, 2017). These two cell types which both contribute to conventional cultured MSCs play distinct roles in osteogenesis *in vivo* (Wang *et al.*, 2019) and probably to other cell lineages. Capoccia *et al*. (2009) found that bone marrow MSC like cells with high aldehyde dehydrogenase (ALDH) activity sustain better improvement of the ischaemic hind limb, as compared to the whole stromal cell population at low cell numbers. It was recently confirmed that ALDH high perivascular progenitors are developmentally more primitive than their ALDH low counterparts (Hardy *et al.,* 2017). Perivascular cells and derived MSCs

expressing CD10 are considerably enriched in osteogenic progenitors (Ding *et al.*, 2019).

1.2.4 ALDH activity as a marker of stem cells

1.2.4.1 Aldehyde dehydrogenases family of enzymes

Aldehyde dehydrogenases (ALDHs) are a group of enzymes that contain 19 isoforms in the human genome (Table 1). ALDHs catalyse the oxidation of aldehyde substrates to their corresponding carboxylic acids. Endogenous aldehydes are formed through normal metabolism of amino acids, lipids, alcohols and vitamins, whereas exogenous ones are derived from environmental agents and drugs such as cigarette smoke and cytotoxic drugs that are metabolized (Reviewed in Tomita *et al*. 2016).

Among the different functions of ALDH enzymes, there is cellular detoxification but also gene regulation (Duester *et al*. 1995). There are 19 functional ALDH isoforms, although the ALDH1 family of enzymes is the most relevant for stem-cell like properties. Furthermore, expression of ALDH1 family members is associated with drug resistance of cancer cells (Yoshida *et al*., 1993). ALDH1 enzymes are located in the cytosol and are required for the biosynthesis of retinoic acid (RA) (Vassalli, 2019). ALDH1A1 is highly involved in stem cell-like properties due to its high affinity to oxidase retinaldehyde (retinal) to retinoic acid which is involved in proliferation and differentiation, although ALDH1A2 and ALDH1A3 also metabolise retinal. RA functions as a ligand for

nuclear RA receptors (RARs) and retinoid X receptors (RXRs), regulating gene expression; consequently, RA synthesis is essential for differentiation, development, maintenance of organs and tissues in vertebrate animals.

Table 1 ALDH Isoforms

Isoform	Preferred aldehyde	Localisation
ALDH1A1	Retinal	Cytosol
ALDH1A2	Retinal	Cytosol
ALDH1A3	Retinal	Cytosol
ALDH1B1	Acetaldehyde	Mitochondria
ALDH1L1	10-Formyltetrahydrofolate	Cytosol
ALDH1L2	Unknown	Unknown
ALDH2	Acetaldehyde	Mitochondria
ALDH3A1	Aromatic, aliphatic aldehydes	Cytosol/nucleus
ALDH3A2	Fatty aldehydes	Microsomes, Peroxisomes
ALDH3B1	Unknown	Cytosol
ALDH3B2	Unknown	Unknown
ALDH4A1	Glutamate γ-semialdehyde	Mitochondria
ALDH5A1	Succinatesemialdehyde	Mitochondria
ALDH6A1	Malonate semialdehyde	Mitochondria
ALDH7A1	α-Aminoadipic semialdehyde	Cytosol, Nucleus, Mitochondria
ALDH8A1	Retinal	Cytosol
ALDH9A1	γ-Aminobutyraldehyde	Cytosol
ALDH16A1	Unknown	Unknown
ALDH18A1	Glutamicγ-semialdehyde	Mitochondria

Adapted from Marchitti *et al*. 2008; Tomita *et al*. 2016

The retinoid signalling pathway plays important roles in normal and cancer stem cells (*Figure 4*). The regulation and function of ALDH in vitamin A (retinol) metabolism (Duester *et al*. 2003; Tomita *et al*. 2016) starts with retinol being oxidized by retinol dehydrogenases to retinal. Subsequently, retinal is oxidize by the ALDH1 family of enzymes, and ALDH8A1. RA can induce transcription of downstream effectors by entering into the nucleus to activate heterodimers of RAR and RXR (Tomita *et al*., 2016; Vassalli 2019). The ALDH1 family of proteins is highly active in cancer and normal stem cells making it an attractive marker of stem cells along with the fact that it plays functional roles such as self-protection, differentiation, and expansion of stem cells (Levi *et al.*, 2009). However, the isolation of cells using antibodies involves the permeabilization of the cell membrane to allow antibodies entering the cytosol, therefore killing the cells. The development of the ALDEFLUOR flow cytometry assay enabled the isolation of cells based on ALDH activity, which allowed the study of live cells.

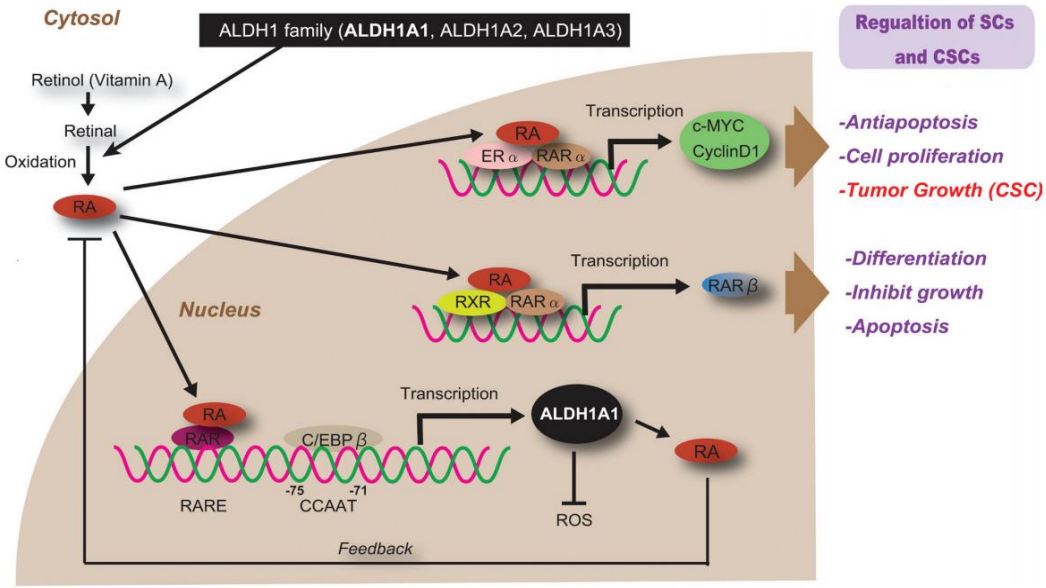


Figure 4 Metabolism of retinol by ALDHs.

Retinol in the cytosol is oxidize by retino dehydrogenases into retinal. Then ALDH1 family of enzymes oxidase retinal into retinoic acid (RA). RA enters the nucleus and interacts with intracellular receptors RARs and RXRs regulating cell proliferation, differentiation and apoptosis. [adapted from Tomita et al., 2016)

1.2.4.2 Aldehyde dehydrogenases in normal and cancer stem cells

ALDH activity has been used as a marker of normal stem cells. Indeed, hematopoietic and neural stem and progenitor cells have high ALDH activity (Armstrong *et al.*, 2004; Corti *et al.*, 2006). High ALDH activity has also been reported in adipose tissue (Estes *et al.*, 2006) and myogenic progenitors (Jean *et al.*, 2011). However, the ALDH enzymatic activity, and the expression of specific isoforms have been characterized mainly in cancer stem cells.

High ALDH activity has been linked, in various types of cancers, to stem cell-like features such as tumour initiating capacity, clonogenic growth, self-renewal and drug resistance (Awad *et al.*, 2010; van den Hoogen *et al.*, 2010). Indeed, there is clinical evidence that the remaining tumour tissue which survived chemotherapy contains a higher number of cancer stem cells, including high ALDH cells, in comparison to the original tumour tissue (Dylla *et al.*, 2008; Li *et al.*, 2008; Tanei *et al.*, 2009). Tumour-initiating cells with high ALDH activity have been described in liver (Ma *et al.*, 2008; Dollé *et al.*, 2012), ovary (Li *et al.*, 2018), stomach (Wakamatsu *et al.*, 2012), skin (Luo et al. 2018), prostate (van den Hoogen *et al.*, 2010), breast (Ginestier *et al.*, 2007), brain (Choi *et al.*, 2014), lung (Moreb *et al.*, 2007), and bone marrow (Giordano *et al.*, 2013). The presence of cells with high ALDH activity is associated with poor clinical outcomes (Ginestier *et al.*, 2007; Charafe-Jauffret *et al.*, 2010; Li *et al.*, 2010; Kuroda *et al.*, 2013). These ALDH high cells are also more clonogenic and tumorigenic than ALDH low cells (Januchowski et al. 2013).

Cancer stem cells resistant to cytotoxic drugs such as cisplatin, dacarbazine and doxorubicin express ALDH. These cancer cells develop resistance through the oxidation of specific aldehyde groups of the drug (Magni *et al.*, 1996).

The use of chemical inhibitors of ALDH activity sensitizes drug-resistant ALDH high ovarian cancer stem cells to chemotherapy (Landen *et al.*, 2010). However, ALDH-dependent resistance not only affects cancer stem cells. Normal stem cells such as hematopoietic stem cells (HSCs) are affected by inhibitors of ALDH, making them more sensitive to, for example, mafosfamide, which is used to clear remnant cancer cells (Kaizer *et al.*, 1985). ALDH1+ cells seem to contribute to resistance against chemotherapy. Moreover, ALDH1+ breast cancers are negative for ER (oestrogen receptor), positive for Ki-67 and positive for human epidermal growth factor receptor type 2 (EGFRII), and have lower response to adjuvant chemotherapy compared to ALDH1- breast cancer types (Morimoto *et al.*, 2009). Interestingly, ALDH activity is involved in resistance against radiation. In particular, breast cancers with high ALDH activity have been shown to be HER2+ CD44+ and CD24-/low, demonstrating tumour forming potential and radioresistance (Duru *et al.*, 2012).

1.2.4.3 The ALDEFLUOR assay

The ALDEFLUOR assay was first developed by Storms *et al*. to isolate HSCs with high ALDH activity (Storms *et al.*, 1999). In this assay, cells with high ALDH activity metabolize an aldehyde (BODIPY-aminoacetaldehyde, BAAA) into the fluorescent product (BODIPY-aminoacetate, BAA). This BAA product is retained inside the cells, and its accumulation can be detected by flow cytometry, allowing the sorting of live cells (*Figure 5).* Originally, the ALDEFLUOR assay was developed to detect the activity of the ALDH1A1 isoform (Jones *et al.*, 1995; Storms *et al.*, 1999). However, other isoforms such as ALDH1A2, ALDH1A3 and ALDH2 have shown activity in the ALDEFLUOR assay (Marcato *et al.*, 2011; Moreb *et al.*, 2012).

Many ALDH isoforms have been described to be able to oxidase the ALDEFLUOR substrate making it more difficult to identify specific enzymes in different cell types. Zhou and colleagues analysed the contribution of the 19 ALDH isoforms to the ALDH high compartment of different cancer cell lines. The findings revealed that 9 ALDH isoforms are active in the ALDEFLUOR assay (Zhou *et al.*, 2019). Therefore, it is essential to identify the specific ALDH isoform present in the ALDH high compartment of a given cell population.

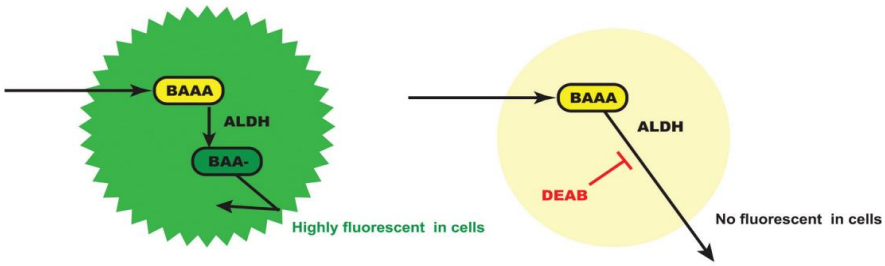


Figure 5 ALDEFLUOR assay principle.
Cells with high ALDH activity convert (BODIPY-aminoacetaldehyde, BAAA) into the fluorescent product (BODIPY-aminoacetate, BAA). The ALDH inhibitor, diethylaminobenzaldehyde (DEAB) is used to stop the oxidation of the substrate, allowing the detection of cells with high ALDH activity

1.2.5 Hierarchy of perivascular cells based on their ALDH activity

Perivascular cells, pericytes and adventitial cells, are involved in different processes such as tissue turnover, and are progenitors of MSCs *in vitro.* However, specific *in vivo* functions of these cell types are still unknown due to heterogeneity and lack of a common marker that can identify all perivascular cells in the human body. Adventitial cells have been described to be pericyte progenitors *in vitro* and *ex vivo* (Howson *et al.*, 2005; Corselli *et al.*, 2012). Furthermore, MSC-derived adventitial cells proliferate at higher rates than MSC-derived pericytes and have a different phenotype (Corselli *et al.*, 2012) .

Also, it has been suggested that pericytes do not behave as MSC progenitors *in vivo* in mice (Guimarães-Camboa *et al.*, 2017) whereas it is well documented that adventitial progenitors can proliferate and contribute to fibrosis and calcification, and probably to muscle cells (Kramann *et al.*, 2015, 2016; Murray *et al.*, 2017). More recently, adventitial cells have been described to be more primitive than pericytes (Hardy *et al.*, 2017). Both types of perivascular cells were isolated based on their high or low ALDH activity and network analysis of single cells was performed. The resulting analysis of 500 genes in every population of cells revealed a hierarchy of perivascular cells (Figure 6): ALDH high ADV (most primitive); ALDH low ADV; ALDH high PC; ALDH low PC (least primitive). Moreover, adventitial cells showed an evenly distribution of cells extending from low to high ALDH, whereas pericytes mostly showed low ALDH activity. Enzymatic activity reflected by the ALDEFLUOR staining intensity is

correlated with stem cell properties, suggesting that a higher percentage of adventitial cells show stem cell-like phenotypes as compared to pericytes (Figure 6). Therefore, adventitial cells with high ALDH activity may contain cells with stem cell properties.

However, a comprehensive characterisation of ALDH subsets of perivascular cells *in vitro* and *in vivo* has not been done. In this thesis I aimed to characterise the ALDH high subset of adventitial cells to assess their *in vitro* and *in vivo* functions.

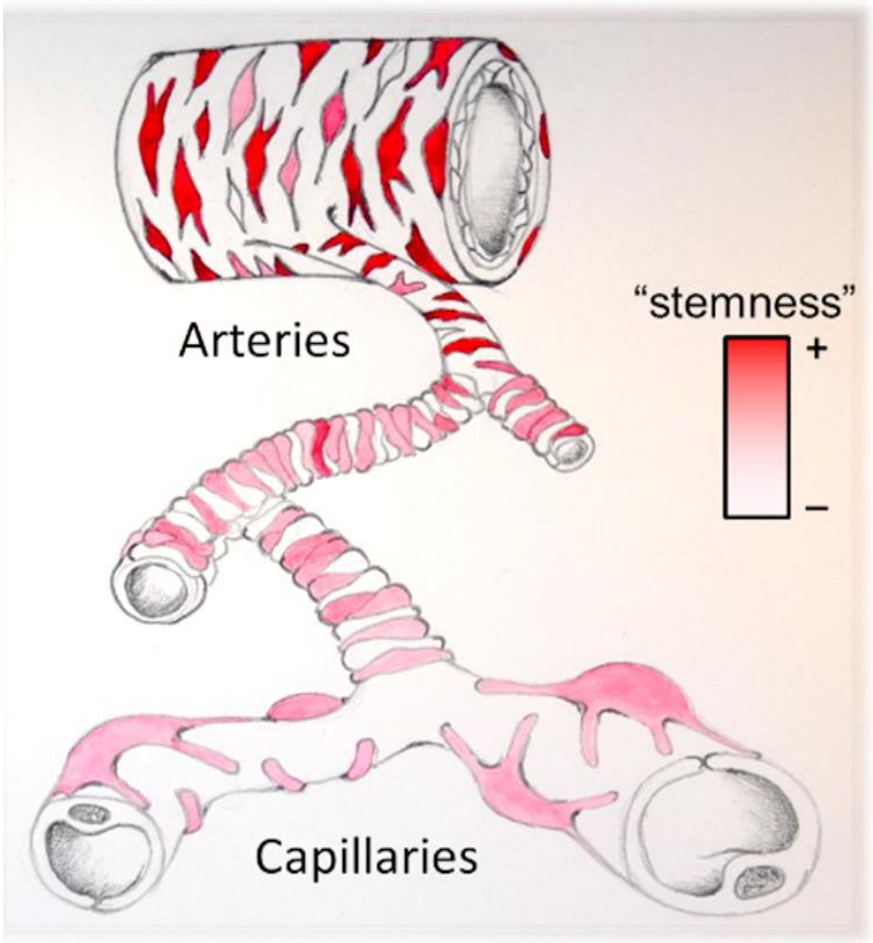


Figure 6. Proposed hierarchy of perivascular cells.

Proposed model of hierarchy of perivascular cells where adventitial cells harbour a population of stem-cell like cells. [Created by Bruno Péault]

Chapter 2 METHODS

2.1 Tissue Procurement

2.1.1 Human adipose tissue

Adipose tissue was collected from human female volunteers undergoing cosmetic liposuction. Informed consent from the donor was signed, and ethical permission was granted by the South East Scotland Research Ethics committee (Ref number: 16/SS/0103)

2.1.2 Human brain tissue

Brain tissue (glioblastoma) was kindly donated by Dr. Paul Brennan from the University of Edinburgh. The ethical permission number is: 15/ES/0094

2.1.3 Human foetal heart and skeletal muscle

These samples were obtained by our laboratory member Joan Casamitjana from the Center for Vascular Sciences

2.1.4 Human uterus

These samples were kindly donated by Professor Hilary Critchley from the Center for Reproductive Health (CRH) of the University of Edinburgh.

2.2 Mouse models

2.2.1 Rotator cuff injury

2.2.1.1 C7BL/6 background mice

The tissue sections of injured rotator cuff muscle in wild type mice were kindly provided by Dr. Ayelet Dar from UCLA.

2.2.1.2 Surgical procedure (performed by collaborators at UCLA)

A massive rotator cuff tear was done by making a 1-cm skin incision over the right glenohumeral joint. Subsequently, the deltoid fibers were split directly posterior to the deltoid tuberosity longitudinally. The supraspinatus and infraspinatus tendons were dissected, and the distal was resected 5 mm of each tendon to prevent scar formation to the humerus. Then, the suprascapular nerve was identified and cut. Last, the deltoid muscle was closed.

2.2.2 Mouse heart tissue

Mouse cardiac tissue was kindly provided by Dr. Gillian Gray from the Center for Vascular Science (CVS) of the University of Edinburgh,.

2.3 Tissue processing for cell isolation

2.3.1 Human white adipose tissue dissociation and flow cytometry

Human adipose tissue was collected from surgical procedures in sterile containers and stored at 4°C in PBS supplemented with 5% (v/v) fetal calf serum (FCS). The tissue was next treated according to our established protocol (Crisan *et al.*, 2008; Corselli *et al.*, 2012). In short, I minced the tissue prior to digestion; mixed with PBS supplemented with 2% (v/v) FCS and

centrifuged at 448 Relative Centrifugal Force (RCF) for 10 min at room temperature (RT). This allowed the separation of cells into 3 phases, oil, stromal vascular fraction (SVF) containing my cells of interest and debris. I next aspirated the oil and debris, and suspended the cell pellet in PBS supplemented with 2% (v/v) FCS . Approximately, 25ml of human adipose tissue was mixed with 25ml of FACS buffer and spun down at 2000 rpm for 10 min at RT. The supernatant was removed and 25ml of digestion solution (1mg/ml - collagenase II-S in DMEM) was added to the tube; I next transferred the tubes into a shaking water bath at 37°C, 4 RCF for 45 min.

The digested tissue was filtered using strainers (400 µm,100 µm, 70 µm and 40 µm pore size) and washed with PBS/2% FCS (v/v), then incubated with red blood cell lysis buffer (ammonium chloride/Tris base (9:1 ratio), pH 7.65) for 10 min at RT. After incubation, the cells were washed and stained with the ALDEFLUOR kit for 45 min at 37°C according to manufacturer's instructions (StemCell technologies). Cells were then resuspended in ALDEFLUOR buffer and stained with fluorescent antibodies (Table *2*) for 20 min on ice. Pericytes and adventitial cells were sorted using a BD FACS FUSION sorter based on the marker combinations previously described (Crisan *et al.*, 2008; Corselli *et al.*, 2012; Hardy *et al.*, 2017).

Table 2 Antibodies used for cell sorting of perivascular cells based on ALDH activity

Marker	Fluorochrome	Supplier	Dilution
CD146	BV711	BD Horizon 563186	1:100
CD34	PE	BD Horizon 555822	1:100
CD31	V450	BD Horizon 561653	1:400
CD45	V450	BD Horizon 560367	1:400
CD56	V450	BD Horizon 560360	1:400
ALDH activity (no antibody)	488/green channel	StemCell Technologies	_ _ _ _ _ _ _ _ _

2.3.2 Human brain tissue preparation and flow cytometry

Human malignant brain tissue (glioblastoma) was collected from surgical procedures in sterile containers and stored at 4°C in PBS supplemented with 5% (v/v) FCS. The tissue was next treated according to our established protocol (Crisan *et al.*, 2008; Corselli *et al.*, 2012). Cells were washed and stained with the ALDEFLUOR kit for 45 min at 37°C according to manufacturer's instructions (StemCell technologies). Cells were then resuspended in ALDEFLUOR buffer and stained with fluorescent antibodies (Table *3*) for 20 min on ice. Pericytes and adventitial cells were analysed using a BD Fortessa cell analyser based on marker combinations previously described (Crisan *et al.*, 2008; Corselli *et al.*, 2012; Hardy *et al.*, 2017). CD56 antibody was added to the combination of antibodies to discard neuron which express this marker.

Table 3 Antibodies used to analyse brain tissue (GBM)

Marker	Fluorochrome	Supplier	Dilution
CD146	BV711	BD Horizon 563186	1:100
CD34	PE	BD Horizon 555822	1:100
CD31	V450	BD Horizon 561653	1:400
CD45	V450	BD Horizon 560367	1:400
CD56	PECy7	BD Horizon 557747	1:100
PDGFRb	APC	BD Biosciences 323608	1:100
ALDH activity (no antibody)	488/green channel	StemCell Technologies	_ _ _ _ _ _ _ _ _

2.3.3 Quantification of ALDH activity in flow cytometry

In order to quantify ALDH activity, the mean fluorescent intensity (MFI) of the ALDH high compartment (channel 488 filter) was measured in each type of perivascular cells. Subsequently, the value of the ALDH low remaining compartment was subtracted to obtain a comparable value between the two cell types.

2.4 Cell culture

2.4.1 Standard cell culture

Sorted perivascular cells were seeded into culture plates at a density of 20000 cells/cm^2 and cultured (37 °C, 5% CO_2) in Endothelial Cell Growth Medium (EGM-2 Bulletkit- Lonza) in plates pre-coated with 0.1% gelatin. Once cells were confluent, we passaged them into DMEM, high glucose, GlutaMAX™ supplement (Sigma Aldrich), 20% FCS. On subsequent passages, the cells were re-plated in larger wells with the appropriate volume of medium (Table *4*). Once confluent in a T75 flask the cells were passaged at a ratio of 1:4.

Table 4 Culture plates and flasks used for perivascular cells

Well plate	Surface Area (cm^2)	Working Volume of Medium (ml)
96-well plate	0.32	0.370
48-well plate	0.75	0.5-0.8
24-well plate	2	0.8-1
12-well plate	3.8	1.5-2.2
6-well plate	9.6	2.5-3
25 cm^2 flask (T25)	25	5
75 cm^2 flask (T75)	75	10

2.4.2 Differentiation into mesodermal cell lineages

For adipogenic differentiation, cells were seeded at a density of 20000 cells/cm^2 and incubated at 37°C overnight to allow cells to attach. Next day, media was switched to DMEM, 10% FSC,1 µM dexamethasone, 0.5 mM isobutylmethylxanthine, 200 µM indomethacine, and 10 µM insulin (all from Sigma-Aldrich). Cells were fixed with 4% PFA (paraformaldehyde) at RT at different time points (14, 21 and 28 days), washed with 70% ethanol and incubated with Oil red O (Sigma- Aldrich) for 15 min at RT for detection of lipids.

For osteogenic differentiation, cells were seeded at a density of 20000 cells/cm^2 and incubated at 37°C overnight to allow cells to attach. Next day, media were switched to DMEM,10% FSC, 0.1 µM dexamethasone, 50 µM ascorbate-2-phosphate, and 10 mM β-glycerophosphate (all from Sigma-Aldrich). Cells were fixed with 4% PFA at RT at different time points (14, 21 and 28 days), washed with milliQ water and incubated in alizarin red (Sigma-Aldrich) solution for 15 min at RT for detection of calcium.

2.4.3 Quantification of differentiation

For osteogenesis, 10% acetic acid was added to alizarin red-stained cells and incubated with shaking for 30 min. Next, the acid was taken along with the cells by scraping the bottom of the well and transferred into a 1.5 ml Eppendorf tube, the tubes were heated for 10 min at 85°C, and put on ice to cool down for 5

min. The tubes were centrifuged for 10 min at 21952 RCF, next 500ul were taken into a new tube to which 200ul of ammonium hydroxide was added. After mixing the tubes, 50ul were transferred into a well of a 96 well plate, then absorbance at 405nm was measured in a FLUOstar Omega Microplate Reader.

2.4.4 Angiogenesis assay *in vitro*

Subsets of ALDH adventitial cells and human umbilical vein endothelial cells (HUVEC) were used to analyse angiogenesis support. 10ul of matrigel (BD Membrane Matrix, BD Biosciences) were seeded into 96-well plates (Ibidi) and allowed to set for at least 20 min at 37°C. After gelation, cells were suspended in EGM-2 and then seeded into the wells with matrigel. The ratio used was 10:1 HUVEC to perivascular cells. Images were taken after 6 h, once the tubes were formed. Tube formation was analysed using angiogenesis plugin of ImageJ, Fiji.

2.4.5 Cell proliferation assay

Cells were seeded at a density of 4210 cells/cm^2 with 3 technical repeats per cell type. Cells were detached using 0.25% trypsin in EDTA and counted.

2.4.6 AlamarBlue assay of cell viability

Cells were plated at 5000 cells per well in clear flat bottomed 96 well plates (Corning) and allowed to adhere. After administration of treatments, AlamarBlue cell viability dye (Invitrogen) was added to culture medium at a ratio of 1:10. Fluorescence was measured on a GloMax Explorer spectrometer (Promega, Southampton, UK) at 1-hr intervals to ensure a linear rate of change, and the three h time point used for analysis.

Fluorescence values for cell containing wells were normalized to zero based on readings from cell free wells containing AlamarBlue reagent in medium.

2.5 RNA sequencing

RNA sequencing was performed in University of California Los Angeles (UCLA) by Dr Reef Hardy. Sub-populations of ALDH low and high perivascular cells from 3 healthy donors (age range 35-63 years; BMI range:21.1 – 32.0) were sorted into cell lysis solution and purified using the Qiagen RNeasy Micro kit (Qiagen). cDNA for each subpopulation was prepared using the NuGen's Ovation RNA-Seq System V2 kit from approximately 100 ng of RNA pooled from 2 or 3 donors. High throughput sequencing was conducted on an Illumina Hiseq 2000 platform. Samples were normalized by RPMK (reads per kilobase of exon per million mapped reads) which takes into account both library size and gene length in within-sample comparisons. Similar numbers of reads between libraries (i.e., depths of coverage) made cross-library, individual gene comparisons possible.

RNA Sequencing for cultured cells was done after 3 passages. Adventitial cells and pericytes were stained with fluorescent antibodies to CD31, CD45, CD146 and CD34. All adventitial cells were collected as they showed no expression of the markers; in the case of pericytes, only those expressing CD146 were isolated for RNA sequencing.

I performed principal component analysis (PCA) and volcano plot using PARTEK software with the raw FASTQ files. Heatmaps and further analysis ere performed using the package “pheatmap” on Rstudio.

2.6 Histology

2.6.1 Immunofluorescence

Frozen sections were fixed in acetone: methanol (1:1) for 10 min prior staining.

Paraffin sections were dewaxed by sequential incubations with xylene for 10 min, 100% ethanol for 1 min, 95% ethanol for 1 min, 80% ethanol for 1 min and 70% ethanol for 1 min. After dewaxing, slides went through an antigen retrieval step by boiling the slides in citrate buffer (0.05% Tween, pH 6) for 5 min.

After fixation/dewaxing, slides were washed 3 times with PBS for 10 min then blocked with 10% goat serum for 1 h at RT. Sections were then incubated with primary antibodies (Table *5*) overnight at 4°C. Next day, slides were washed 3 times with PBS for 1 min, then secondary antibodies (Table *6*) were added and incubated at RT for 1 h after incubation slides were washed once more 3

times with PBS for 10 min and mounted with mounting medium containing DAPI for nucleus staining.

For intracellular staining, tissue sections were incubated with 0.5% triton-X-100 in PBS for 10 min and washed 2X with PBS for 10 min prior to blocking with goat serum.

If the primary antibody was biotinylated, a step of blocking with avidin and biotin was performed prior to blocking with serum. Tissue sections were analysed on a Zeiss Observer inverted microscope

Table 5 Primary antibodies and reagents used for detection of perivascular cells in human tissues

Marker	Species Raised	Reactivity	Cells Recognized	Company	dilution
CD146	Mouse	Human	Pericytes, endothelial cells	Abcam ab75769	1:100
NG2	Mouse	Human	Pericytes	Millipore AB5320	1:100
CD34	Mouse	Human	Adventitial cells	Abcam ab139551	1:100
PDGFRb	Rabbit	Human	Pericytes	Abcam ab32570	1:50
Biotinylated (UEA I)	_ _ _ _ _	Human	Endothelial cells	Vector B-1065	1:300
α-SMA	Mouse	Human	Pericytes, smooth muscle cells	Sigma A2547	1:100
ALDH1A1	rabbit	Human/mouse	_ _ _ _ _ _	Proteintech 22109-1-AP	1:100

Table 6 Secondary antibodies used to detect primary antibodies

Colour	Species raised	Reactivity	Supplier	Dilution
Alexa Fluor 488 Streptavidin		Biotin	Life technologies	1:300
Alexa Fluor 555	Goat	Mouse	Life technologies	1:300
Alexa Fluor 647	Goat	Rabbit	Life technologies	1:300
Alexa Fluor 488	Goat	Mouse	Life technologies	1:300

2.6.2 Flow cytometry analysis of cultured cells

Cells were detached with 0.25% trypsin EDTA and washed with PBS supplemented with 2% FCS. Then cells were stained with conjugated antibodies for mesenchymal stromal/stem cell markers: CD44,CD105 ,CD90 and CD73 (Table 7).

Cells were analysed using flow cytometry by passing the cells through a 5 laser BD Fortessa cell analyser. Next the data were analysed on Flowjo software.

Table 7 Antibodies used for analysis of MSC markers

Marker	Fluorochrome	Supplier	Dilution
CD73	PerCP Cy5.5	BD Pharmingen 561260	1:100
CD44	Alexa Fluor 700	BD Pharmingen 561289	1:1000
CD90	APC	BD Pharmingen 559869	1:1000
CD105	PE	BD Pharmingen 560839	1:100

2.6.3 Imaging

Images were taken using a wide field Zeiss Observer microscope with a Colibri7 LED light source (Zeiss). A Hamamatsu Flash 4.0 v3 camera (Hamamatsu Photonics) was used for monochromatic fluorescent and brightfield images and an inverted widefield Live Imaging Nikon TiE for angiogenesis assays. Magnifications of 10X, 20X and 63X were used to take images.

2.6.4 Statistical analysis

Data were analysed using GraphPad Prism software. Number of biological replicates is indicated as (n). Every biological replicate has 3 technical replicate. Shapiro- Wilk normality test was performed in all data sets. I used unpaired T-test with Welch's correction, and when appropriate, paired T-test.

For more than 2 groups analysed, I used One-way Anova with Tukey post-test for multiple comparisons (parametric), or Kruskal-Wallis test with Dunn’s post test (non-parametric).

Chapter 3 CHARACTERISATION OF MESENCHYMAL STEM CELL IDENTITY OF ALDH SUBSET OF ADVENTITIAL CELLS

3.1 Introduction

In the last decade, blood vessels were identified to harbour progenitors of mesenchymal stem/stromal cells (MSCs), which reflects the isolation of MSCs from every vascularized organ (Nombela-Arrieta *et al*., 2011) . Two populations of perivascular cells give rise to MSCs: pericytes (CD146+ CD34- CD31- CD45-) embedded in the basement membrane in close contact with endothelial cells, and adventitial cells (CD146- CD34+ CD31- CD45-), found in the adventitial layer of large vessels give rise to MSCs in vitro (Crisan *et al*., 2008; Corselli *et al*., 2012).

Subsets of perivascular cells have been characterised *in vitro* once they transition into MSCs. The study of subsets is of therapeutic importance as specific subsets of perivascular cells have different mesodermal differentiation potential. For example, CD10+ and PDGFRα+ subsets of perivascular cells show higher osteogenic potential (Ding *et al.*, 2019; Wang *et al.*, 2019) which may be beneficial for bone repair. PDGFRβ+ PDGFRα- perivascular cells are myogenic *in vivo* and *in vitro* (Jensen *et al.*, 2018) suggesting higher regeneration potential for skeletal muscle injury. MSCs with high ALDH activity have been previously described (Sherman *et al.*, 2017; Najar *et al.*, 2018). However, the studies were performed after *in vitro* expanded MSCs. Here, I characterised the MSC identity and the differentiation potential into osteogenic and adipogenic cell lineages of native adventitial cells with high ALDH activity once they transition into MSCs.

3.2 Hypothesis and aims

3.2.1 Hypothesis

"High ALDH activity identifies subsets of adventitial cells that give rise to MSCs with higher osteogenic, adipogenic and angiogenic support potential"

3.2.2 Aims

- To sort and expand subsets of adventitial cells based on ALDH activity
- To characterise differentiation potential (osteogenesis and adipogenesis) *in vitro* of ALDH subsets
- Characterisation of expression of MSC markers on ALDH subsets
- To determine the proliferation capacity and the angiogenesis support capacity of perivascular ALDH subsets *in vitro*

3.3 Results

3.3.1 The stromal vascular fraction is enriched in adventitial cells

Perivascular cells, pericytes and adventitial cells, are present in human tissues in different frequencies depending on the types of vessels present. Adipose tissue is highly vascularized and is a great source of mesenchymal stem cells. However, the contribution of each type of perivascular cell to the stromal vascular fraction is not known.

In order to assess percentages of perivascular cells in adipose tissue, I used flow cytometry and quantified these cell types in the live compartment. Since the stromal vascular fraction is comprised of several types of cells including hematopoietic cells, endothelial cells, fibroblasts, perivascular cells and other cell types, we discarded these cells using negative selection of CD31, CD45 and CD56 positive cells (*Figure 7*,A) to remove endothelial cells and hematopoietic cells. Pericytes and adventitial cells were then identified by the expression of CD146 and CD34, respectively. Adventitial cells comprised around 80% of the live cells (after negative selection) whereas pericytes between 15-20% (*Figure 7*,B).

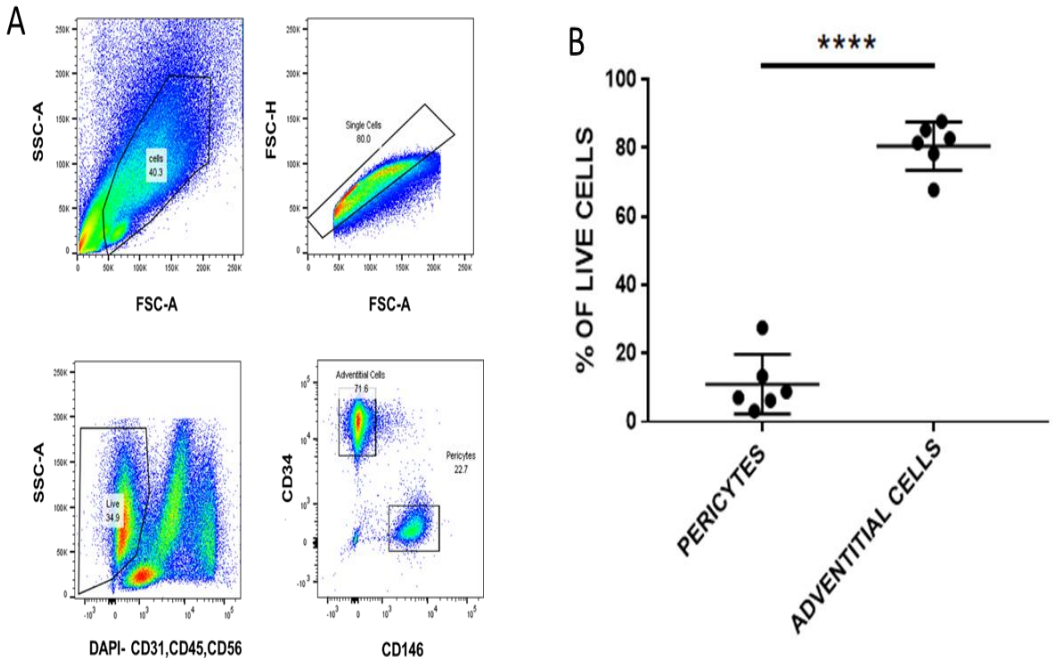


Figure 7 Quantification of the perivascular cell compartment in the stromal vascular fraction.

A. Perivascular cells are sorted as live single cells, negative for CD31,CD45,CD56. Pericytes are CD146+CD34- and adventitial cells CD146-CD34+. **B.** Numbers of pericytes and adventitial cells in the stromal vascular fraction show a higher percentage of adventitial cells than pericytes. Unpaired t-test with Welch's correction was used. Data are shown as mean ± SD. n=6.****P <0.0001

3.3.2 Perivascular cells can be sorted according to ALDH activity

In order to sort cells with high ALDH activity from each type of perivascular cells, we performed the ALDEFLUOR assay. This involved the use of a reagent that is metabolised by ALDH enzymes and DEAB (N,N-diethylaminobenzaldehyde) inhibitor to gate the ALDH high population (*Figure 8*,B&C). The low ALDH population was gated at the other extreme of the plot to avoid contamination by cells with higher ALDH activity.

I found that 16.5 % (± 3.076, n=6) and 14.53 %(± 5.418, n=6) of adventitial cells and pericytes respectively were ALDH high (*Figure 8*). Therefore, the percentages of ALDH high cells in both types of perivascular cells are similar. However, adventitial cells with high ALDH activity are in higher numbers in the SVF since adventitial cells comprise 80% of the cells. In addition, ALDH activity is correlated with a primitive phenotype, therefore it is important to investigate the enzymatic activity in each cell type.

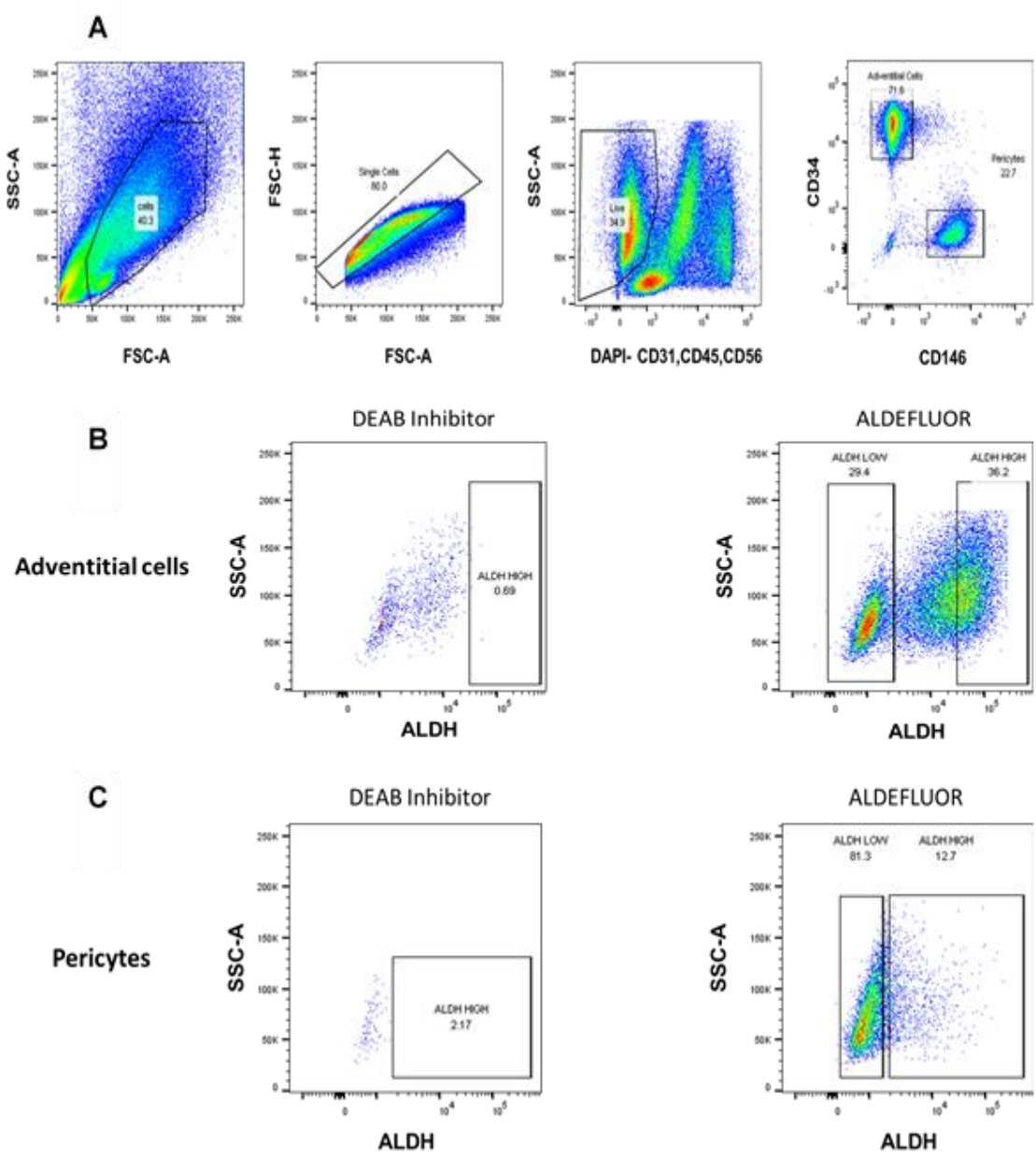


Figure 8 Human perivascular cell isolation from white adipose tissue according to their ALDH activity

A. Perivascular cells are identified as live single cells, negative for CD31,CD45,CD56. Adventitial cells and pericytes were gated based on the expression of CD34 and CD146. **B&C.** ALDH high adventitial cells and pericytes were then gated based on ALDH activity and the inhibitor control. n=6

3.3.3 ALDH subpopulations of adventitial cells express MSC markers and can differentiate into adipocytes and osteocytes

Once cells were expanded in culture and transitioned into mesenchymal stem/stromal cells (MSCs)I performed standard experiments to characterise MSCs in ALDH subsets *in vitro* (Chamberlain *et al.*, 2007) that include MSC marker expression analysis by flow cytometry (CD73,CD105,CD90, CD44) and functional *in vitro* assays to test their potential to differentiate into mesodermal cell lineages. Since it is also well documented that some subpopulations of MSCs in culture have different potentials to differentiate into osteogenic or adipogenic lineages, differentiation capacity was also quantified.

MSC markers

Cells expanded in culture were stained with fluorescent antibodies and analysed for the expression of typical MSC markers. Data were analysed using FlowJo software. ALDH low and high subsets of adventitial cells expressed CD73, CD105, CD90 and CD44 at the same levels as typical MSCs isolated from adipose tissue (Figure 9).

Osteogenic differentiation

Cells undergoing osteogenic differentiation accumulate calcium deposits that are stainable with alizarin red. Osteogenic capacity was quantified by

extraction of alizarin from the calcium deposits with acetic acid and subsequent analysis using spectrophotometry (Figure 10,B).

Adipose differentiation

Cells undergoing adipose differentiation change their morphology and form intra-cytoplasmic lipid droplets. These lipid deposits are stained with Oil red O . Since accumulation of intra-cytoplasmic lipids is proportional to differentiation capacity, we quantified differentiation by measuring the area covered by lipid droplets stained with Oil Red O (*Figure 11*,B).

In 4 independent experiments, I found no significant differences between ALDH high and low adventitial cells with respect to capacity to differentiate towards adipocytes or osteocytes, although there seems to be a trend suggesting that ALDH high adventitial cells are more osteogenic at day 21 and less adipogenic at day 28.

In conclusion, these studies confirmed the MSC identity of the ALDH subsets. However, quantification of cell differentiation shows that ALDH subsets of adventitial cells are similar.

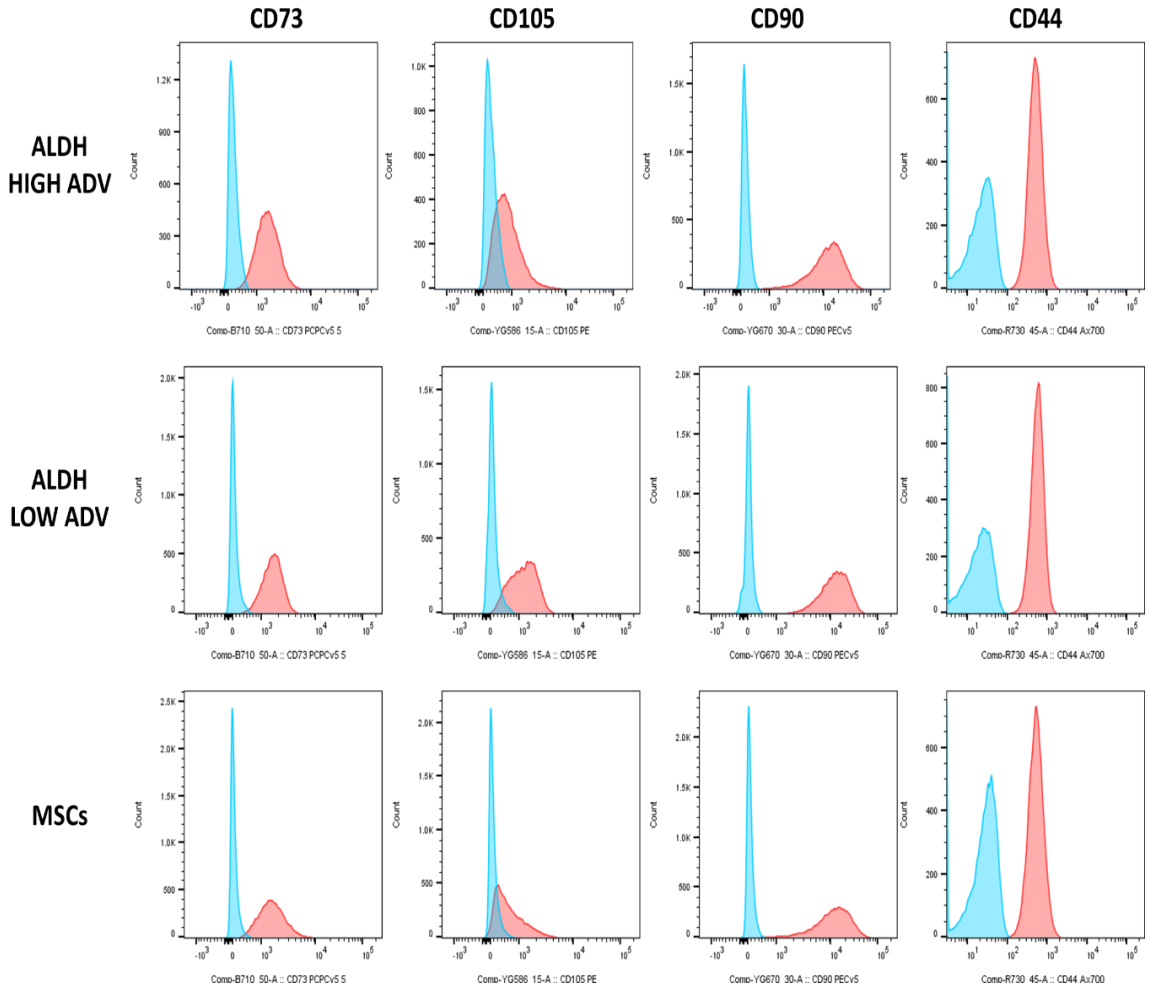


Figure 9 ALDH sub-populations of adventitial cells express MSC markers in vitro.

Sub-populations of adventitial cells were sorted according to their ALDH activity and put in culture for 3 weeks. Cells were then stained with antibodies to the canonical MSC markers CD73,CD105,CD90 and CD44. Typical MSCs isolated from adipose tissue were used as control. n=3

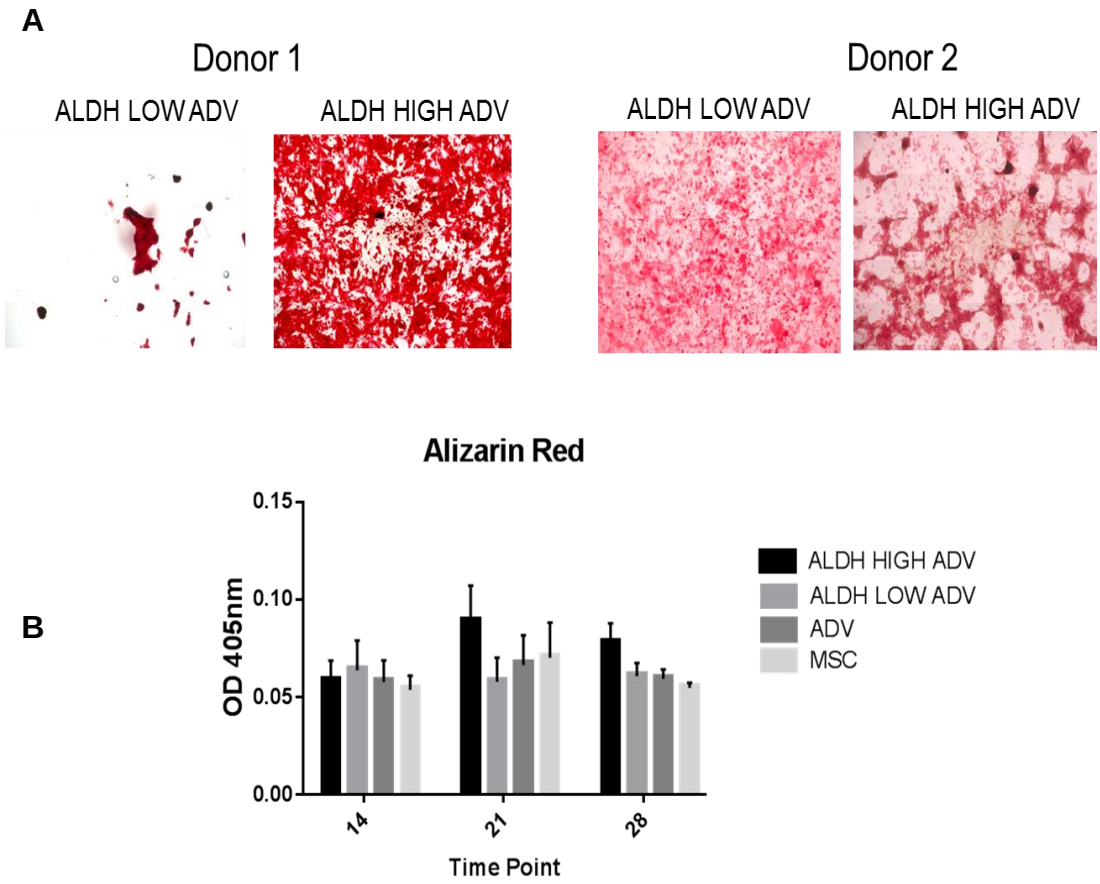


Figure 10 Osteogenic differentiation of subsets of perivascular cells.

Cultured perivascular cells were put under osteogenic differentiation conditions, stained and quantified at different time points. **A.** Differentiation capacity seems to be donor dependent. **B**. Quantification of osteogenesis at different time points for ALDH subsets and whole population of adventitial cells and MSCs as control. Time points represent days post-induction. Two-Way ANOVA with Tukey post test. Data are shown as mean ± SD. n=4

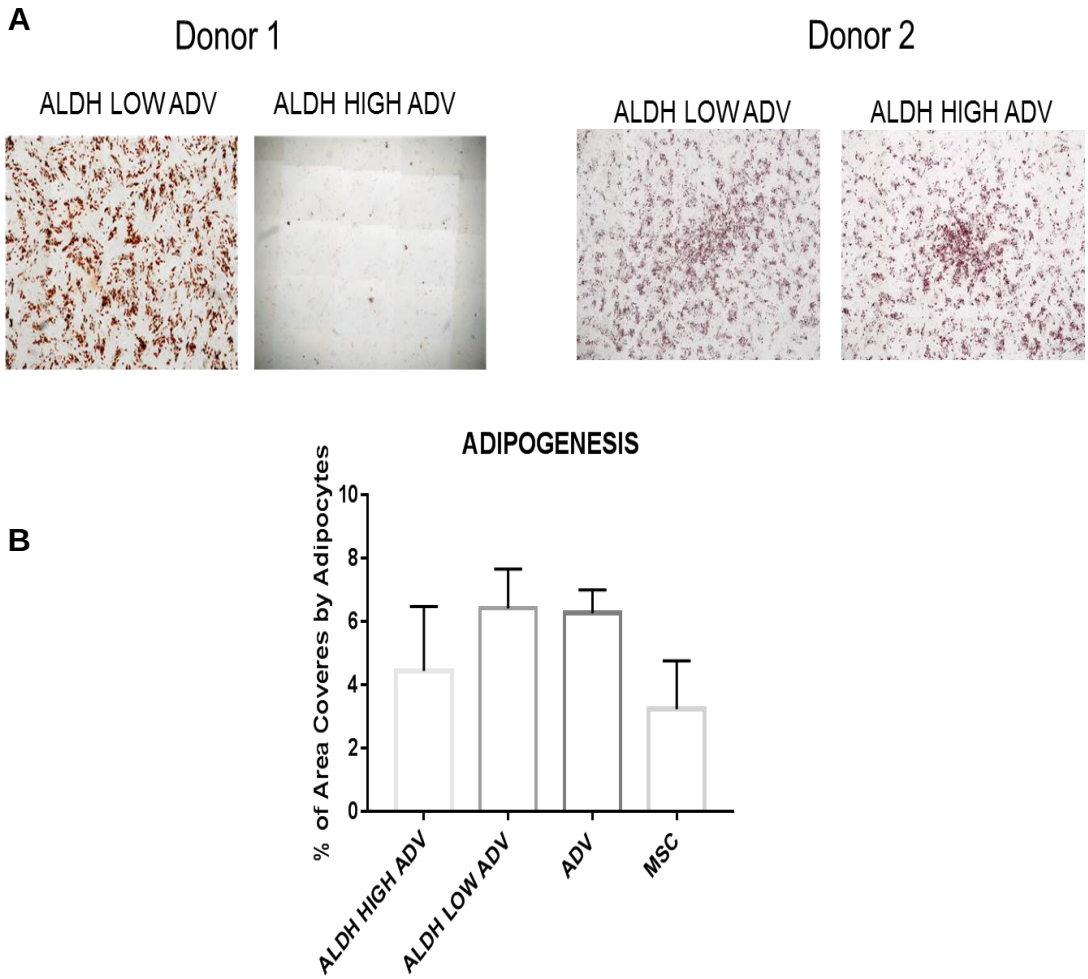


Figure 11 Adipogenic differentiation of subsets of perivascular cells.

Cultured perivascular cells were put under adipogenic differentiation conditions, stained and the area covered by adipocytes was quantified after 28 days. **A.** Differentiation capacity seems to be donor dependent. **B**. Quantification of adipogenesis for ALDH subsets and whole population of adventitial cells and MSCs as control. One-Way ANOVA with Tukey post test. Data are shown as mean ± SD.n=4

3.3.4 There is no difference in proliferation and metabolic activity between ALDH subsets of adventitial cells

The proliferation rate of a given cell type may give us information about their behaviour in their niche. Stem cells are considered to be quiescent and divide at low rate. However, once in culture, this may change since their environment has been drastically modified.

In order to assess the proliferation rate of our subsets of adventitial cells, I used two methods. The first one by seeding a given number of cells and counting every 3 days to calculate differences in cell numbers. The other method was by using AlamarBlue, a reagent that is oxidized by the cell and is correlated with metabolic health and indirectly with cell proliferation.

There was no significant difference in proliferation rate between the different ALDH subsets, and also when compared to the whole population of adventitial cells and MSCs (*Figure* 12). Similar results were found using AlamarBlue This means that cells, after *in vitro* expansion, become similar. However, in the initial culture, ALDH low adventitial cells took longer to start proliferating.

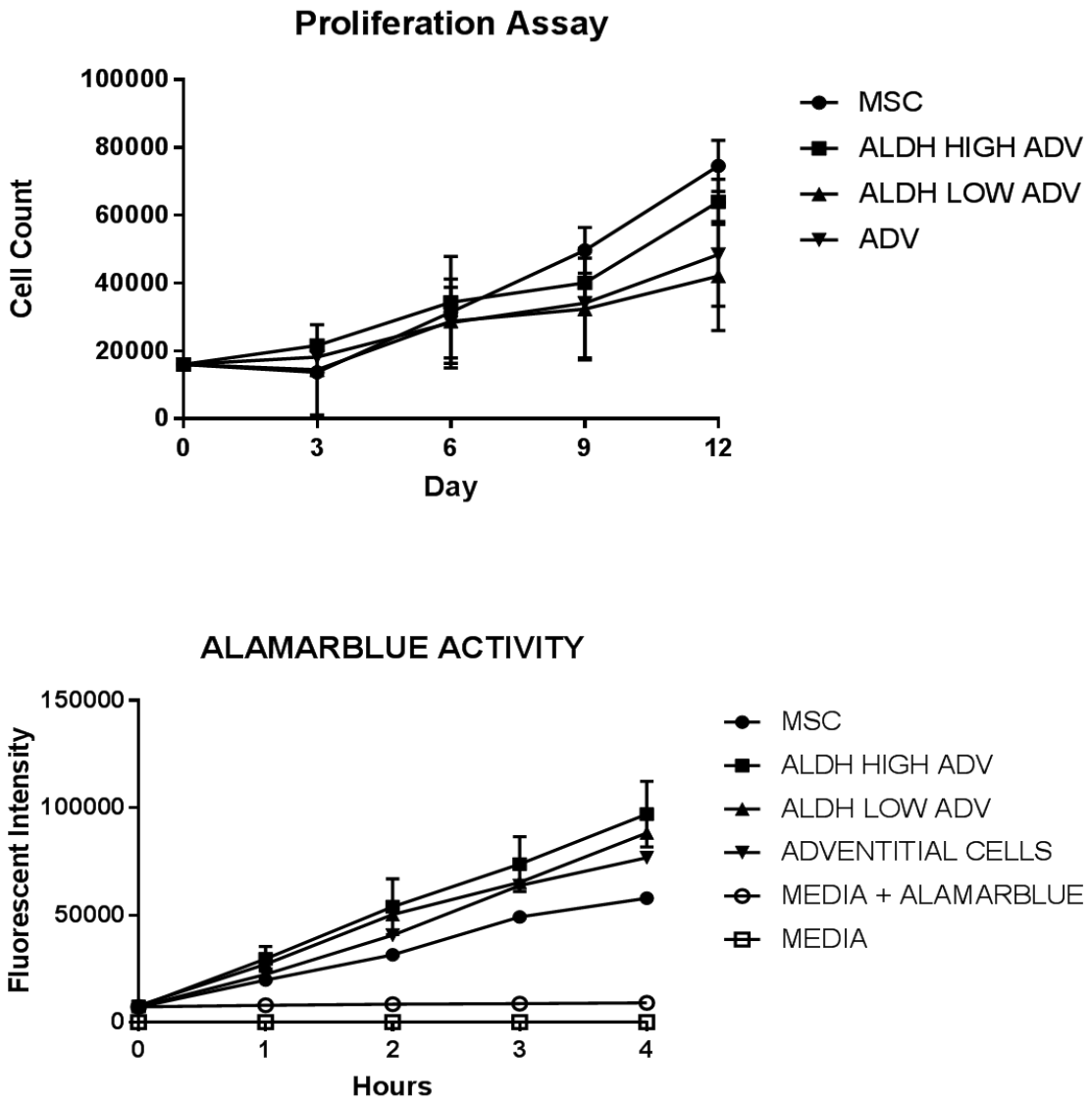


Figure 12 Proliferation assay and AlamarBlue activity.

A. Cells were seeded at a known density and counted every 3 days to analyse the proliferation rate of subsets of adventitial cells, and also common MSCs as control. **B.** AlamarBlue is used to indirectly analyse the proliferation and metabolic health of the cells. The different subsets were seeded with alamarblue reagent and the fluorescence intensity was measured every hour. Two-Way ANOVA with Tukey for multiple comparisons. Data are shown as mean ± (SD).n=4

3.3.5 ALDH subsets of adventitial cells modify the vascular network formation by HUVEC *in vitro*

Angiogenesis takes place during normal tissue development and also after injury during wound healing. This process is associated with different pathological conditions including autoimmune disorders, atherosclerosis, and cancer, among others. Due to the involvement of angiogenesis in various diseases, better understanding of this process is crucial to design therapeutics.

Perivascular cells are involved in angiogenesis *in vivo*, and MSCs derived from perivascular cells have been already used to promote angiogenesis in different disease models. Hence, we investigated whether ALDH high adventitial cells are different than ALDH low counterparts when co-cultured with human umbilical vein endothelial cells (HUVEC).

To determine the angiogenesis support by ALDH subsets of adventitial cells, I co-cultured ALDH subsets with HUVEC for a period of six hours once tubes were formed (4 biological replicates with 3 technical replicates each).

To quantify angiogenic support by ALDH subsets, pictures of the co-cultures after tubes were formed were taken and analysed using the angiogenesis plugin of ImageJ. This plugin extracts a binary image of the tubes which is then used to count nodes, length, branches, junctions, extremities, among others (*Figure* 13).

We found that 2 out of the 8 features analysed were different between the ALDH subsets and HUVEC alone (*Figure* 14). The number of nodes was higher in ALDH low adventitial cells when compared to ALDH high adventitial cells and HUVEC. On the other hand, the number of extremities was lower in co-cultures with subsets of ALDH adventitial cells than HUVEC.

In conclusion, I found that ALDH subsets of adventitial cells are similar in regard to angiogenic support *in vitro*. However, to a certain extent both perivascular cells modified the vascular network. Moreover, whether the cells have different angiogenic properties *in vivo* is yet to be tested.

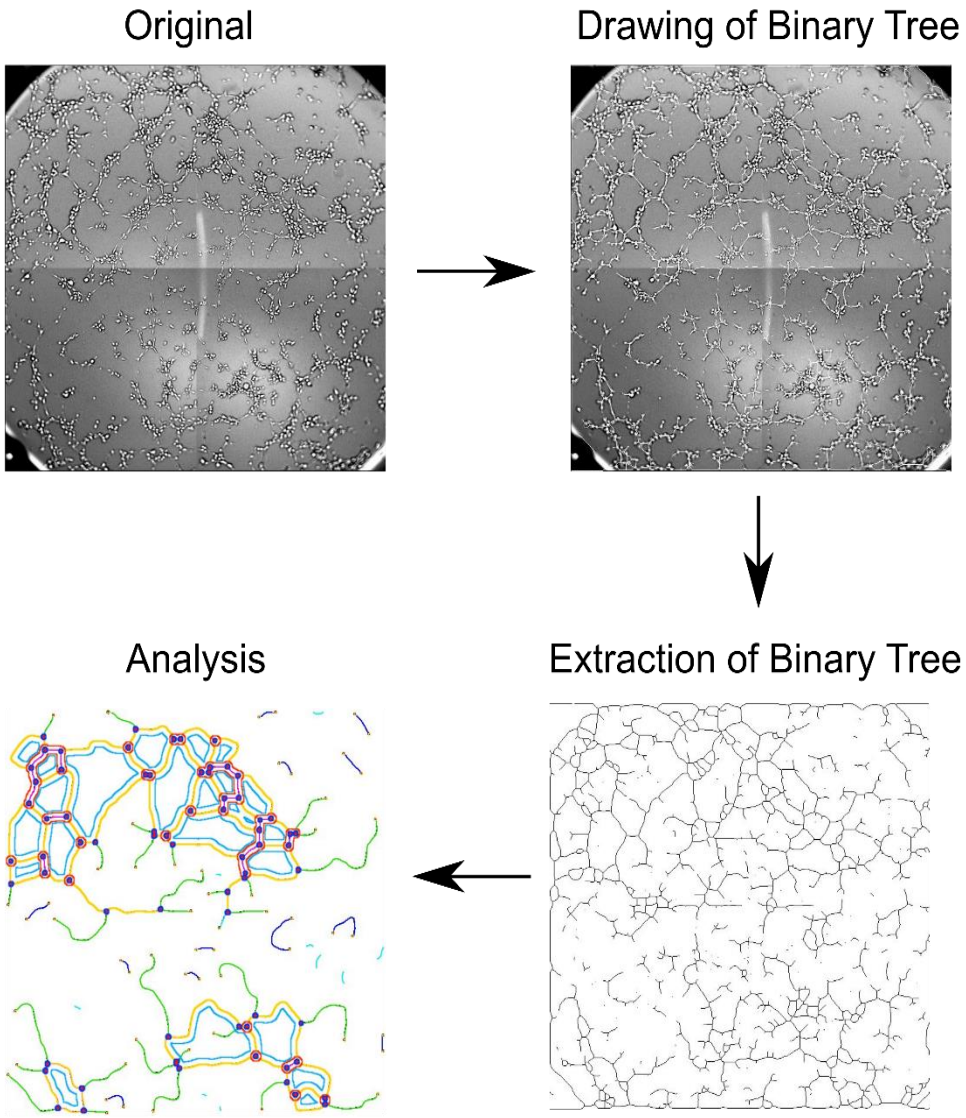


Figure 13 Analysis of tube formation on ImageJ with the angiogenesis function

After 6 hours of co-culture of perivascular cells with HUVEC, images of the vascular network were taken and analysed. Then, images are processed on ImageJ with the angiogenesis plugin. The vascular network is analysed and a binary tree is created, and analysed with the software to quantify nodes, branching and junctions.

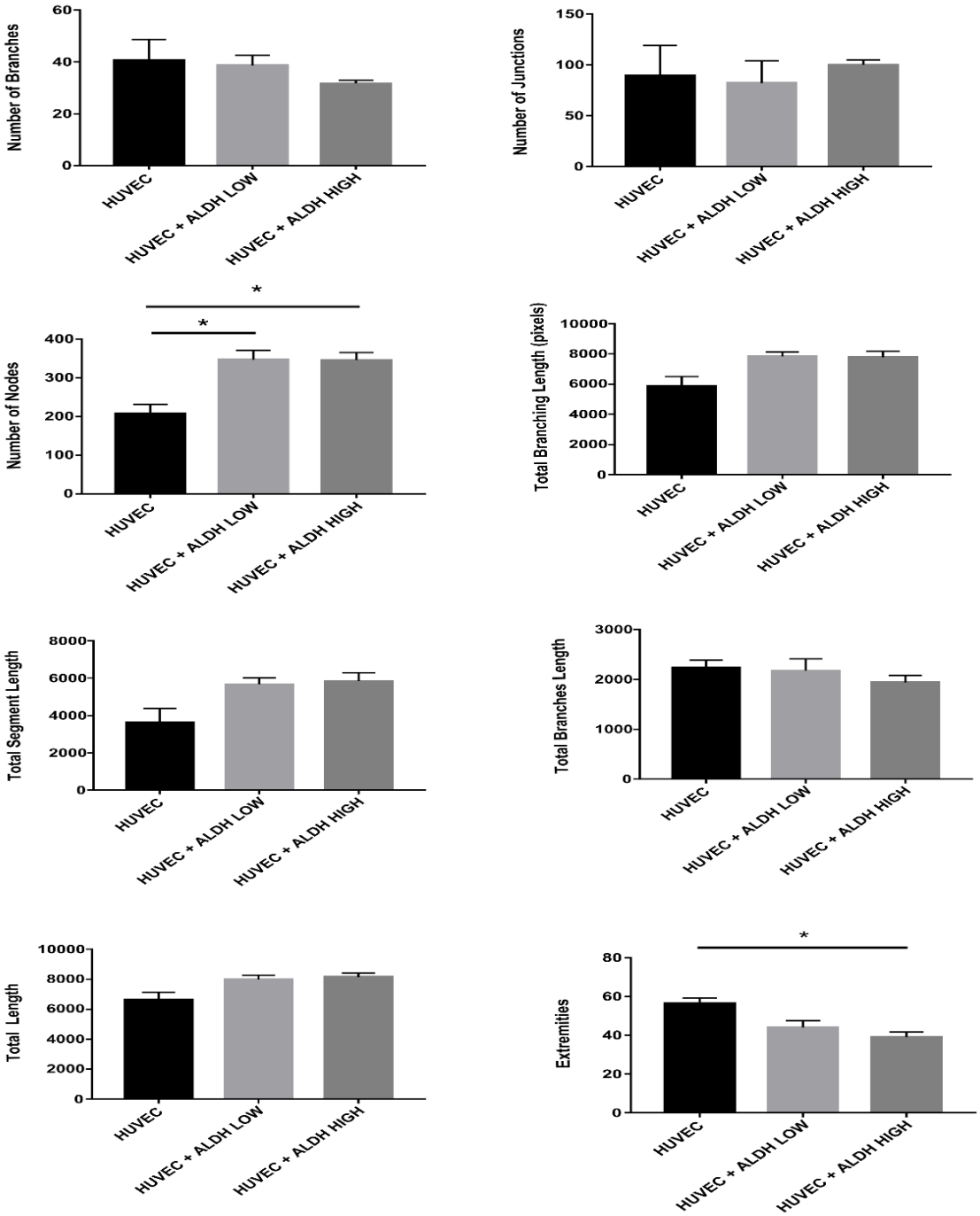


Figure 14 Results of tube formation on ImageJ with angiogenesis function

After 6 hours of co-culture of perivascular cells with HUVEC, images of the vascular network were taken and analysed. Images were are processed on ImageJ and a binary tree is created, and analyse with the angiogenesis function of ImageJ to quantify, nodes, branching and junctions. One way Anova with Tukey for multiple comparisons. Data are shown as mean ± (SD).n=4. *P=0.0167

3.4 Discussion

3.4.1 Mesenchymal stem cell identity and functionality analysis: differentiation potential and angiogenesis support

Once the characterization of the ALDH activity on our fresh and cultured subsets of adventitial cells was performed, I characterized the subsets with the typical assays that define an MSC.

Mesenchymal stem cells or MSCs are well known to express CD44,CD73, CD90, and CD105 (Chamberlain *et al.*, 2007). These canonical MSC markers are a hallmark to identify the MSC phenotype. Indeed, ALDH high and low adventitial cells express the canonical MSC markers. However, in order to properly identify our cells as MSCs, differentiation assays into mesodermal cell lineages were required.

Two mesodermal cell lineages were analysed in the ALDH subsets of adventitial cells: bone and fat. These two lineages are of high interest for our laboratories since the group is involved in the study of the normal and pathologic musculoskeletal system. ALDH high MSCs were previously described to be more osteogenic, adipogenic, and chondrogenic than the ALDH low compartment (Sherman *et al.*, 2017). However, this was only studied on cultured MSCs.

Quantification of osteogenesis and adipogenesis in our ALDH subsets of adventitial cells showed no statistical difference. However, donor variation was a main factor in this result. ALDH high derived cells from two out of four donors were highly osteogenic, whereas the other two donors show less differentiation potential. The medical history of the patients is not accessible, thus unknown factors may affect the differentiation capacity of these cells. Indeed, it has been reported that smoking and drug use affects MSC differentiation potential (Greenberg *et al*., 2017). Therefore, stratification of donors by age and medical history would greatly improve the study.

Cell therapy requires high number of cells, usually between 1-2 million per kg of body weight in humans, with optimal metabolic fitness (Galipeau and Sensébé 2018; Katarzyna *et al*. 2019) Therefore, the proliferation rate and metabolic health of ALDH subsets of adventitial cells were analysed. The results showed that the subsets, including the controls (MSC and ADV), are similar after *in vitro* expansion in terms of proliferation. Taken together, all the *in vitro* analysis suggests that after expansion ALDH subsets of adventitial cells are similar in phenotype and differentiation potential. It appears that after a certain number of passages primary cells become a homogenous population. Indeed, it has been reported that after 11 passages the MSC population is derived from just a few clones from the initial heterogeneous population, and this selection occurs at early passages (Selich *et al.*, 2016). However, other subsets of *in vitro* expanded perivascular cells have shown statistical differences in differentiation potential *in vitro*. For example, subsets

of perivascular cells expressing PDGFRα and CD10 perivascular cells show a higher osteogenic differentiation potential *in vitro* compared to their negative counterpart (Ding *et al*. 2019; Wang *et al*. 2019). These contrasting results may be because the ALDH high compartment of cells contains other subsets including PDGFRα+ and CD10+ cells. Indeed, mRNA levels of CD10, PDGFRα (osteogenic markers) and CD107a (novel adipogenic marker) are similar in both ALDH subsets of adventitial cells (*Figure 15*).

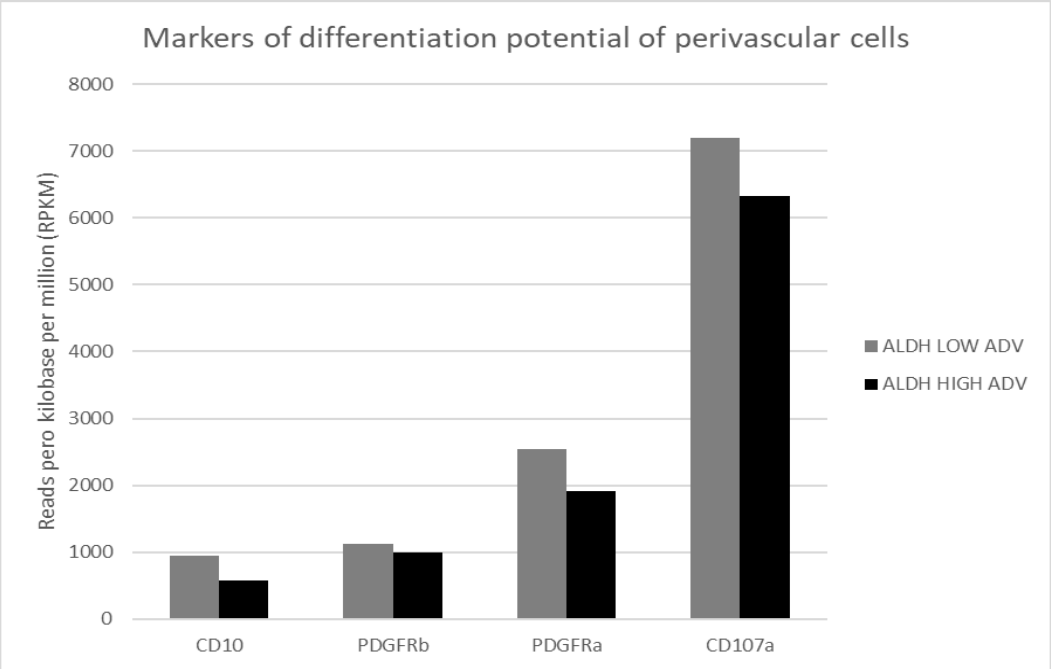


Figure 15 Markers of differentiation potential in perivascular cells

RNA sequencing performed in ALDH low and high compartments of adventitial cells reveals the expression of different cellular markers involved in differentiation potential.

Finally, MSCs have been used to prevent fibrosis and re-establish blood flow in models of myocardial infarction (Amado *et al.*, 2005; Jeong *et al.*, 2018; Madigan and Atoui, 2018) and hind limb ischemia (Capoccia *et al.*, 2009). These studies showed that MSCs increase angiogenesis by the release of factors such as VEGF (Sorrell *et al*., 2009). However, this was performed using expanded MSCs that originate from the heterogeneous stroma. Moreover, data from our laboratories (*Gonzalez et al., in preparation)* showed that pericytes and adventitial cells have different abilities to induce angiogenesis. Following this rationale, I performed *in vitro* angiogenesis using HUVEC co-culture with ALDH subsets of adventitial cells. It was found that these cells do not change the vascular network after tubes are formed. However, the number of nodes and extremities significantly changed compared to the control HUVEC alone culture. (*Figure* 14). A possible reason for this similarity between the ALDH subsets is the loss of heterogeneity during *in vitro* expansion. ALDH high cells have been used in a model of hind limb ischemia with positive results. However, this study was performed using freshly sorted cells from bone marrow (Capoccia *et al.*, 2009). As it was shown in this thesis, our ALDH subsets of cells upon *in vitro* expansion become phenotypically similar which may explain why ALDH high cells are not as osteogenic, adipogenic and angiogenic as previously reported (Capoccia *et al.*, 2009; Sherman *et al.*, 2017; Najar *et al.*, 2018). Najar *et al* and Sherman *et a*l used ALDH high cultured MSCs. However, they performed mRNA analysis right after isolation and the angiogenesis assay was performed *in vivo making it difficult to link* our data. Moreover, Capoccia *et al* used freshly sorted bone marrow derived MSC

progenitors showing that even at low number ALDH high cells were better at inducing vascularisation compared to either the ALDH low subset or the whole population of bone marrow cells at high numbers (*Figure 16*). Indeed the use of uncultured cells has been proposed (James and Péault 2019; Murray and Péault 2015). The use of cultured cells is time consuming and increases the likelihood of tumorigenicity and genetic instability. Moreover, MSCs after consecutive passages decrease the capacity to differentiate into mesodermal lineages, undergo senescence and the expression of adhesion molecules and chemokines, along with the ability to respond to soluble factors is reduced (Muraglia *et al*., 2000; Zuk *et al.*, 2002; Baxter *et al.*, 2004; Dahl *et al.*, 2008; Røsland *et al.*, 2009; Ren *et al.*, 2011)

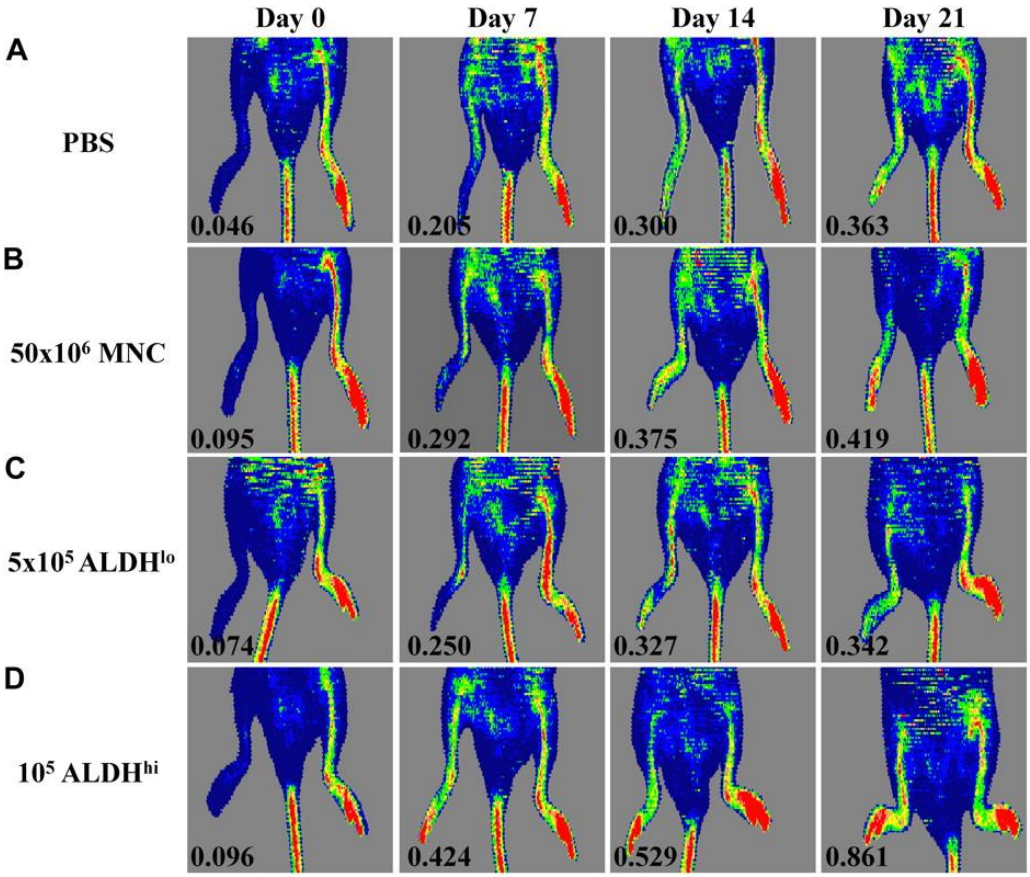


Figure 16 Capillary density after ALDH high cell treatment in mouse hind limb ischemia

Tail vein injection of different groups of uncultured cells after right femoral artery ligation was performed to analyse the recovery induced by different subsets of cells derived from bone marrow. PBS, MNC=Mononuclear cells, ALDH low cells, ALDH high cells were injected. ALDH high cells showed an increase of perfusion compared to the other cell types even at much lower numbers. **A**. mice injected with PBS. **B**. Mice treated with mononuclear cells (whole population). **C**. Mice treated with ALDH low cells. **D**. Mice treated with ALDH high cells. Adapted from Capoccia *et al*., 2009.

To our knowledge a study comparing the use of uncultured MSC progenitor cells and their *in vitro* counterpart has not been performed. Nevertheless, our data and other reports suggest that uncultured cells are a better option for cell therapy. Another variation of uncultured cell therapy is the use of micro-fragmented fat containing MSC progenitors that is already on the market under the name of Lipogems. It was shown that mechanically processed fat conserves the perivascular niche that may help improve regeneration compared to enzymatically digested tissue by the increased release of soluble factors (Vezzani *et al.*, 2018).

In conclusion, based on our data along with previous reports, the use of uncultured cells would be a better approach to improve the clinical outcome, although the number of cells obtained is much lower than in culture conditions. Therefore, not applicable to all protocols of cell therapy. The dosage of cells is still under study and is dependent on condition being treated. For example, Schuleri *et al*. directly injected autologous MSCs into pig hearts with reduction of the infarct size with a high dose (200 million cells) compared to low dose (20 million cells) (Schuleri *et al.*, 2009). In contrast to this result, Hashemi *et al*. showed that lower dose of MSCs (24 and 240 million cells) exhibited a significant decrease in infarct size, whereas the higher dose group of MSCs (400 million cells) did not (Hashemi *et al.*, 2007). Thus, high or low cell dose efficacy remains unknown.

Chapter 4 CHARACTERISATION OF ALDH ACTIVITY IN WHITE ADIPOSE TISSUE DERIVED HUMAN PERIVASCULAR CELLS

4.1 Introduction

Perivascular cells display high heterogeneity. Indeed, various groups have identified sub-populations of perivascular cells involved in different pathologies such as vessel remodelling and organ fibrosis (Stenmark *et al.*, 2006; Di Carlo and Peduto, 2018) . Expression of Gli1 marks profibrotic perivascular cells in the kidney and heart (Kramann *et al*. 2015), bone marrow (Schneider *et al*., 2017). Lineage tracing experiments showed that Gli1+ cells differentiate into smooth muscle cells, migrate to the intima and contribute to vessel calcification in chronic kidney disease (Kramann *et al.*, 2016). Members of our laboratory have also identified MSC-like cells expressing PDGFRβ that contribute to fibrosis in the skeletal muscle and heart (Murray *et al.*, 2017). In addition, a subset of PDGFRβ+ cells co-expressing PDGFRα was identified as a driver of fibrosis (Jensen *et al.*, 2018). Since perivascular cells are heterogeneous, it remains unclear whether specific subsets play distinct functions, and if these are tissue-dependent and how they are developmentally linked. For example pericytes in the developing kidney express renin and produce it *in vitro* (Stefanska *et al.*, 2016), and it has been suggested that expression of VEGFR2 in adventitial cells identifies a population that give rise to cardiomyocytes (Mekala *et al.*, 2018).

ALDH activity is described as a stem cell marker in different types of cancer and normal tissues (Burger *et al.*, 2009; Tomita *et al.*, 2016). Hardy *et al*. isolated both pericytes and adventitial cells from adipose tissue according to

ALDH activity and performed network analysis of gene expression by single cells (Hardy *et al.*, 2017). The results showed that adventitial cells have a more primitive phenotype than pericytes, and that adventitial cells with high ALDH activity are the most primitive of perivascular cells. ALDH activity has been widely used to isolate normal and cancer stem cells from different tissues using the ALDEFLUOR assay (Burger *et al.*, 2009; Schuurhuis *et al.*, 2013; Ohmura-Kakutani *et al.*, 2014; Raha *et al.*, 2014; Luo *et al.*, 2018). However, there has been no characterisation of perivascular cells with differential ALDH activity. Therefore, I sought to study the ALDH high compartment of adventitial cells. High ALDH activity in adventitial cells indicates the existence of a subsets of primitive progenitors subset of adventitial cells that may play a role in regeneration and vessel remodelling. In order to characterise this subset of adventitial cells, *in vitro* studies as well as transcriptional analysis were performed. Moreover, I link the findings in normal tissue to cancer, and finally I translate our findings on human to mouse tissue.

4.2 Hypothesis and aims

4.2.1 Hypothesis

“High ALDH activity marks a stem-like cell population in the adventitial layer of large vessels”

ALDH activity is a stem cell marker in different types of tissues and cancers, and it has been shown that adventitial cells contain a population with high activity of ALDH enzymes. However, this ALDH high cell compartment has not been characterized in the native niche, neither its potential as mesenchymal progenitors *in vitro.*

4.2.2 Aims

- To sequence RNA on freshly FACS sorted ALDH perivascular cell subsets
- To analyse expression of known markers for fibrosis, the PDGF receptors, in the ALDH cell compartments using flow cytometry
- To identify subpopulations of adventitial cells and pericytes using immunofluorescence in different human tissues based on novel markers found by RNAseq.
- To investigate by RNA sequencing transcriptomic changes in ALDH perivascular cell subsets in culture.

4.3 Results

4.3.1 Adventitial cells have higher ALDH activity than pericytes

In order to quantify ALDH activity, the mean fluorescence intensity (MFI) of the ALDH channel (488 nm filter) was measured in each type of perivascular cells. Subsequently, the fluorescence intensity of the remaining ALDH low compartment was subtracted to obtain a comparable value between the two cell types. Adventitial cells show higher ALDH activity compared to pericytes (pericytes, 4648 ± 448.6 and adventitial cells, 60639 ± 8130, n=5) (*Figure 17*, B) providing more evidence that adventitial cells have a more stem cell-like phenotype than pericytes. Nevertheless, the percentage of ALDH high cells is similar (*Figure 17*,A).

As perivascular cells become MSCs *in vitro*, we then investigated how culture conditions changed the ALDH activity profile of each sub-population of adventitial cells. Subsets of ALDH (low/high) adventitial cells were expanded in normal culture conditions for 3 to 4 passages to obtain enough cells to perform the experiments. Cells were detached with trypsin, counted and the ALDEFLUOR assay was performed following manufacturer instructions.

ALDH subsets of pericytes were not sorted due to the low number of ALDH high cells that were not able to expand *in vitro*. However, since adventitial cells have the most primitive phenotype, I focused on the characterisation of ALDH subsets of adventitial cells.

ALDH activity in both subsets of adventitial cells was similar (*Figure 17,*C), in the same manner percentages of ALDH high cells in both sub populations were similar (*Figure 17*,C). Moreover, cultured ALDH low adventitial cells now include an ALDH high compartment, which further suggests that ALDH activity in cultured perivascular cells is not relevant to distinguish the ALDH subsets of adventitial cells.

In conclusion, adventitial cells have higher ALDH activity than pericytes. However, the transition into MSCs after *in vitro* expansion makes both subsets similar in terms of ALDH activity. This may indicate that ALDH activity only serves as a marker of adventitial cells in uncultured cells, making the correlation between *in vitro* and *in vivo* studies difficult to establish. In addition, it is not clear whether ALDH low cells became high or if the opposite happened to the ALDH high subset of adventitial cells.

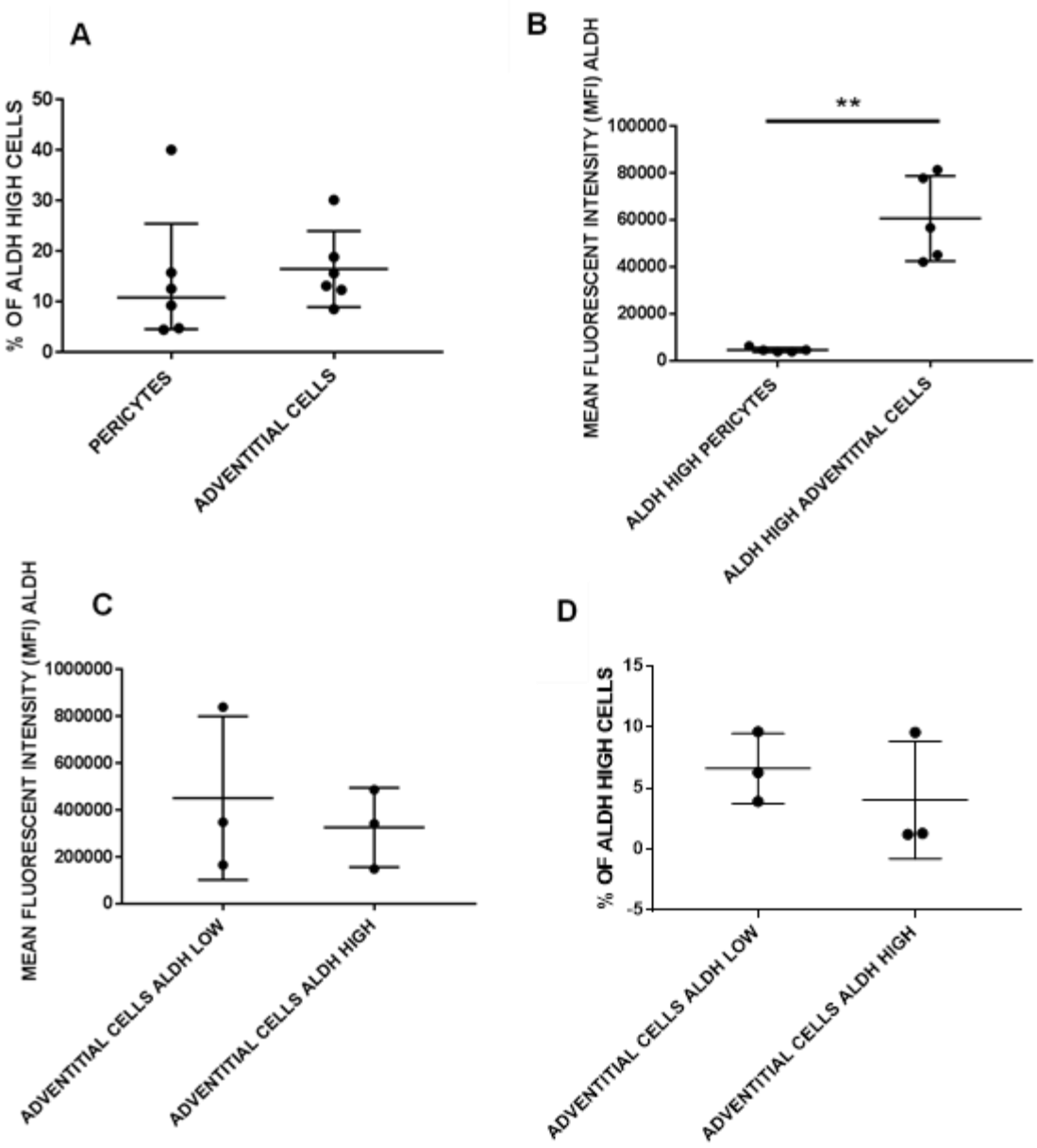


Figure 17 Quantification of ALDH activity in fresh and cultured perivascular cells

A. Percentage of ALDH high cells within pericytes and adventitial cells. **B** Mean ALDH fluorescence intensity (MFI) of percytes and adventitial cells. **C**. After culture of ALDH high and low adventitial cells, differences in ALDH MFI disappear.**D.** After culture,ALDH high and low adventitial cells have similar percentages of ALDH high cells. Unpaired t-test with Welch's correction was used. Data are shown as mean ± SD.n=3. ** P=0.0023

4.3.2 ALDH activity is correlated with CD34 expression in adventitial cells, and association and interactions network analysis suggests functional roles in vessel remodelling

The surface receptors that identify pericytes and adventitial cells have been previously described to be markers of stem cells in different organs. Since ALDH activity is well characterized as a stem cell marker, I decided to analyse the expression of perivascular cell markers in the different ALDH compartments.

In order to assess the relation of the two markers that distinguish pericytes (CD146) from adventitial cells (CD34), I measured the mean fluorescence intensity of CD34 and CD146 in the high and low ALDH compartments. It was found that higher ALDH activity is related with higher CD34 expression, whereas CD146 expression remains the same in both compartments (Figure 18,A&B).

To further investigate the relationship of CD34 with ALDH, network analysis using GeneMania.org was performed. GeneMania finds genes that are related to the set of input genes, using a large set of functional association. These data include protein and genetic interactions, co-expression, pathways, protein domain similarity and co-localisation which helps predict the functions of your data set.

I queried the two genes of interest (Figure 19) revealing interactions with genes involved in vessel remodelling such as *PDGFRb, KDR, TIE1*, among others. I decided to use the ALDH1 family since it is the one described as present in the ALDH high compartment. However, whether a specific ALDH1 isoform is the one metabolising the ALDEFLUOR reagent is yet to be confirmed.

In conclusion, I here show that ALDH and CD34 expressions are correlated in processes involved in blood vessel growth and development. Among the genes in the network I found well known markers involved with perivascular cells such as: *PDGFRb, COL15A1, KDR, TIE1* and *ENG*. This suggests that ALDH1A1 expression/activity is correlated with different processes such as: vasculogenesis, endothelial cell development, extracellular matrix organization, integrin binding and VEGF pathways among others. However, this requires further validation by immunohistochemistry for example that allows to assess the co-expression of these markers *in situ*.

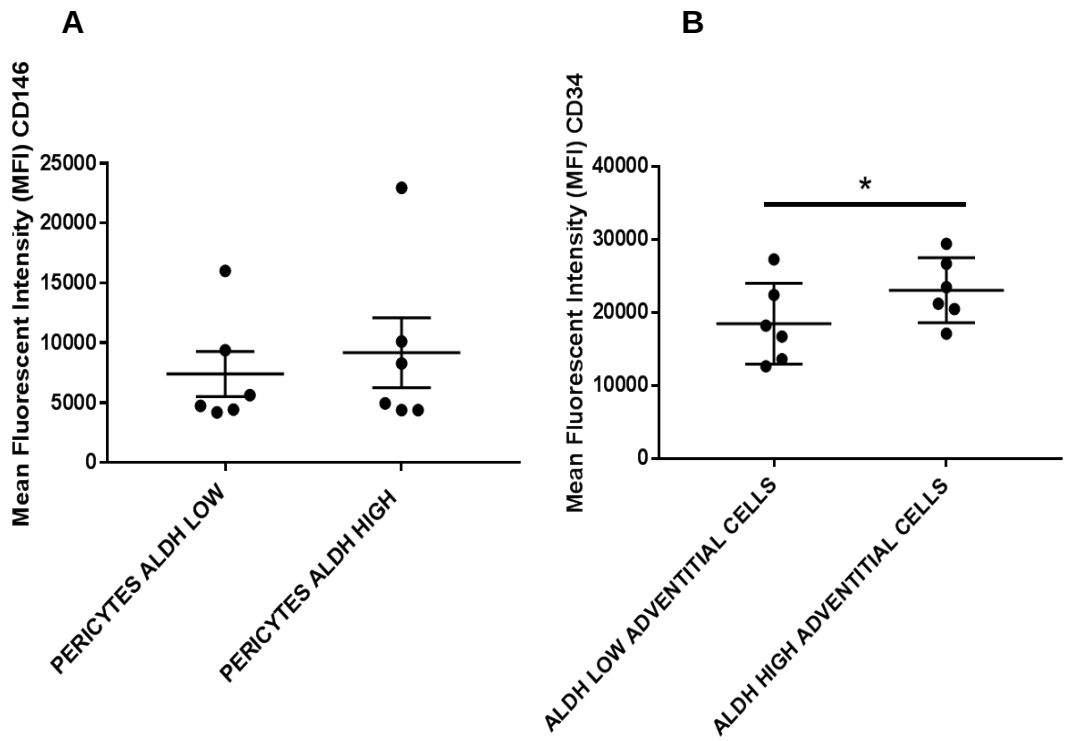


Figure 18 Mean fluorescence intensity of perivascular cell markers reveals a relation with ALDH activity.

The mean fluorescence intensites of CD146 in pericytes (**A)** and CD34 in adventitial cells **(B)** were measured in the low and high ALDH compartments showing that expression of CD34, but not CD146, is related with ALDH activity. Paired t-test was used for all data sets. Data are shown as mean ± SD. n=6. *P=0.0171

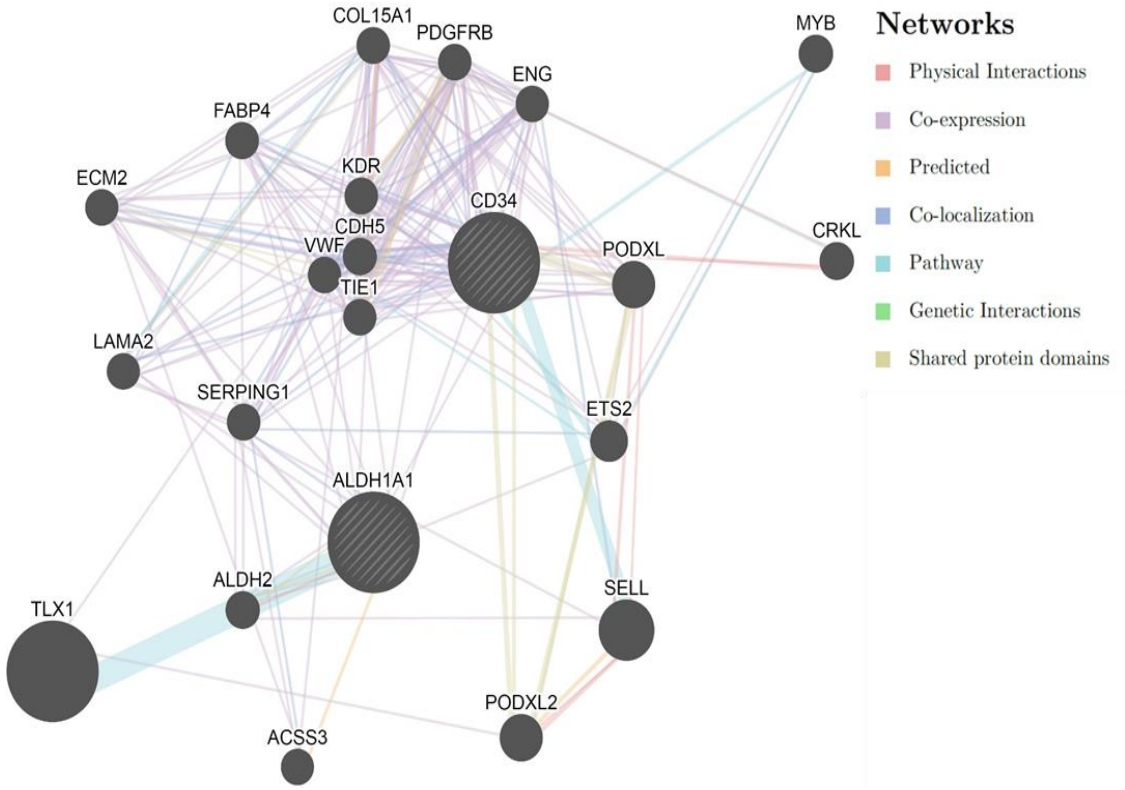


Figure 19 Protein interaction analysis of CD34 and ALDH1A1.

Network analysis of CD34 and ALDH1A1 was performed using Genemania (https://genemania.org). This shows the different types of networks where CD34 and ALDH1A1 are involved. Several of the genes in the network are involved in endothelial cell proliferation, vasculogenesis, regulation of vasculogenesis and extracellular matrix organization among others.

4.3.3 The ALDH high compartment is dependent on cell density in culture

Due to the fact that ALDH activity is similar in ALDH low and high adventitial cells once they are expanded *in vitro* (*Figure 17*), I decided to study the ALDEFLUOR assay again to document the changes of ALDH activity in culture. It is well known that confluence of cells affects proliferation *in vitro*. Therefore, I hypothesized that in culture ALDH activity is dependent on cell confluence.

In order to assess how cell density affects results of the ALDEFLUOR assay, ALDH low adventitial cells were seeded at different densities. Only ALDH low cells were used since ALDH high cells were similar in previous analysis. Cells at a density of 5000 cells/cm^2 showed a higher number of ALDH high cells compared to those seeded at a 10,000 cells/cm^2 (Figure 20). This indicates that in culture, ALDH activity is only present in proliferative cells that maintain the population growing.

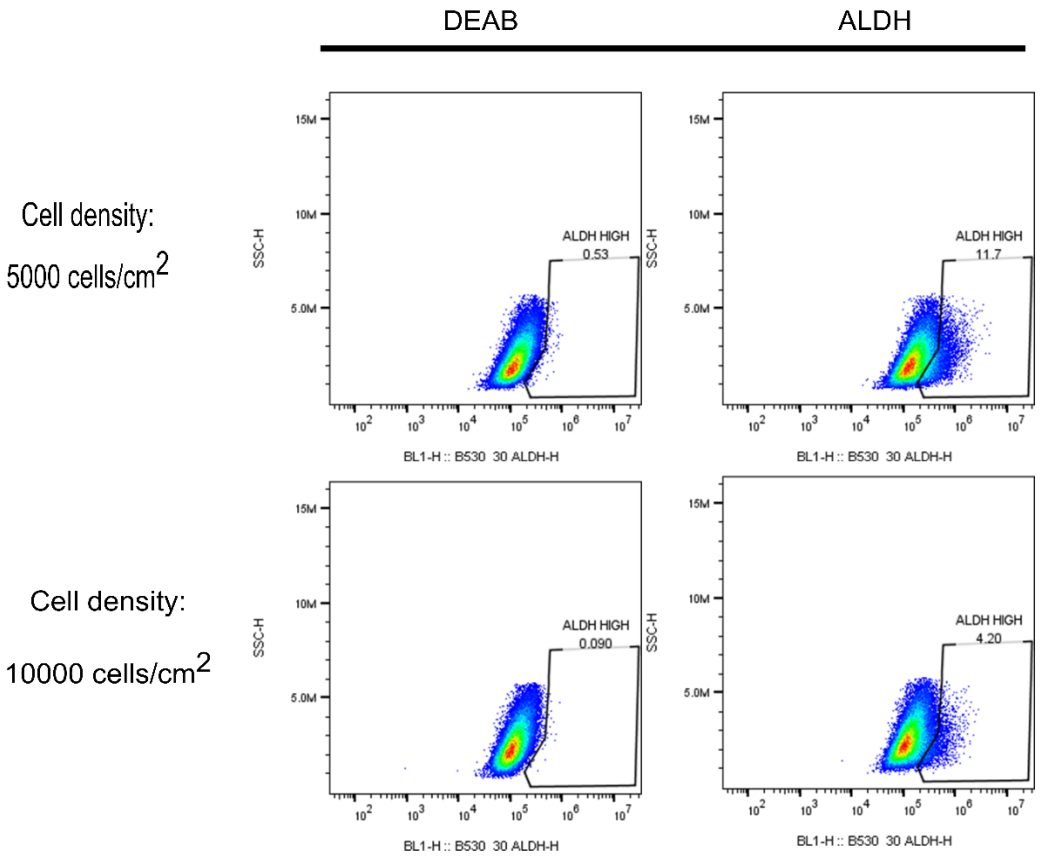


Figure 20 The ALDEFLUOR assay is affected by confluence of cells.

ALDH low adventitial cells were seeded at 5000 and 10000 cells/cm2 and ALDEFLUOR assay was performed to assess the effect on the ALDH high compartment. Cells cultured at low density have a higher percentage of ALDH high cells. n=2

4.3.4 PDGFRα and -β do not show differential expression in perivascular cells

Platelet derived growth factor receptors (PDGFRs) α and β are expressed in subsets of perivascular cells (Chen *et al.*, 2015; Murray *et al.*, 2017). The expression of these receptors has been linked to the production of collagen and fibrosis as well as new muscle cells (Jensen *et al.*, 2018). Studies in our laboratory have previously shown that a subset of PDGFRβ+ perivascular cells co-expressing PDGFRα are highly adipo-fibrogenic (Jensen *et al.*, 2018).

Due to the high importance of PDGF receptors in fibrosis and regeneration, we analysed the expression of the receptors in pericytes and adventitial cells (Figure 21, A&B), and in the ALDH subsets of adventitial cells (Figure 21, C&D). Comparing adventitial cells and pericytes we found no differences in the percentage of cells expressing only PDGFRβ, or in double positive cells expressing PDGFRβ and α. Published and unpublished data from our laboratory have shown that adventitial cells are the main perivascular cell type expressing PDGFRα. It was hypothesised that ALDH high adventitial cells have a different percentage of double positive cells compared to ALDH low cells. However, we found no differences in either single positives for PDGFRβ or double positives expressing also PDGFRα (Figure 21, C&D). Interestingly, cells expressing only PDGFRα were absent in all perivascular cells.

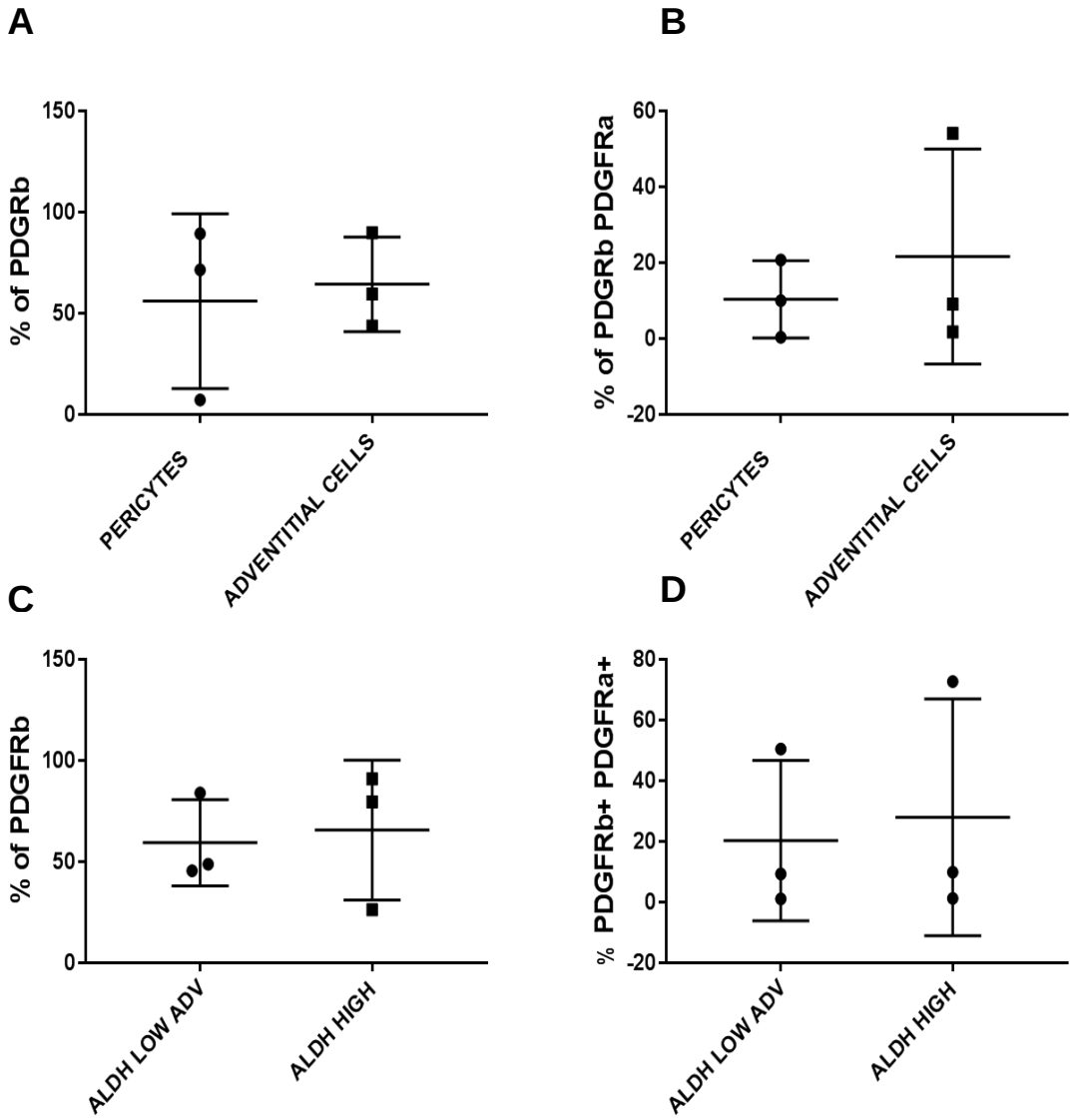


Figure 21 Analysis of PDGF receptors in perivascular cells derived from adipose tissue.

Adipose tissue was processed to get a single cell suspension, stained with antibodies and run through a flow cytometer to analyse percentages of cells expressing PDGF receptors. **A.** Analysis of pericytes and adventitial cells expressing PDGFRβ, but not α**. B.** Perivascular cells expressing both PDGFRβ and PDGFRα**. C.** Expression of single positives for **PDGFRβ** in ALDH subsets of adventitial cells. **D**. Expression of double positive cells in ALDH subsets of adventitial cells. Unpaired t-test with Welch's correction was used. Data are shown as mean ± (SD).n=3

4.3.5 ALDH high adventitial cells, but not ALDH low adventitial cells, are clonogenic

In order to assess the heterogeneity of adventitial cells, we performed single cell index sorting by FACS to expand clones further experiments.

It was found that only ALDH high adventitial cells are clonogenic (n=3), whereas cells from the ALDH low compartment are not able to proliferate in clonal conditions. The clonal efficiency was between 10 to 20% of ALDH high adventitial cells, and it was donor dependent. From the clones that were expanded only 35% were able to differentiate into mesodermal cell lineages.

Osteogenesis potential of the expanded clones was quantified by extracting alizarin red taken up by osteoblasts showing a small subset of clones with high osteogenic potential (Figure 22). This gives more evidence of the heterogeneity of adventitial cells.

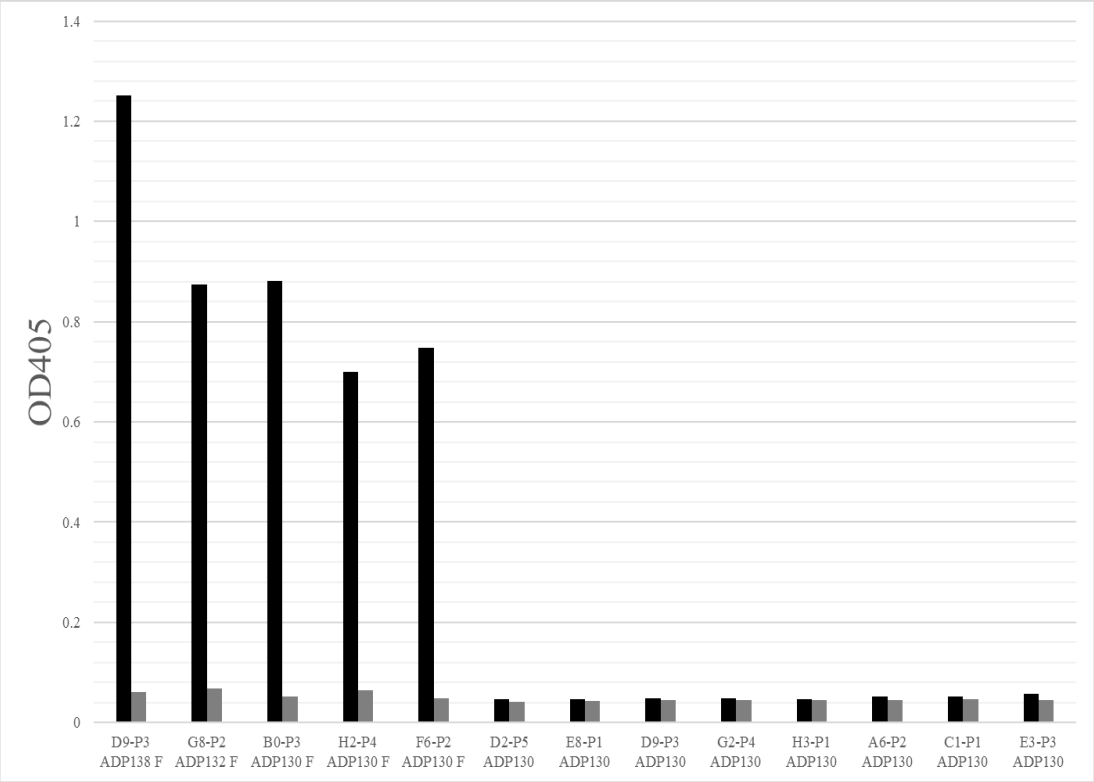


Figure 22 Osteogenic potential of ALDH high adventitial cell clones.

Single cell index sorting was performed on adventitial cells with high ALDH activity. Derived clones were expanded then induced to differentiate into osteoblasts. Quantification was performed by extracting Alizarin red from osteoblasts and determining concentration using spectrophotometry. OD405= optical density at 405 nm.

4.3.6 RNAseq reveals transcriptional differences between ALDH low and high adventitial cells before culture

In order to corroborate the findings obtained from single cell qPCR analysis (Hardy *et al.*, 2017), I performed RNAseq analysis on different populations of perivascular cells in both settings, freshly sorted and after they become MSC-like cells in culture. Principal component analysis (PCA) shows the clustering of the different perivascular cell populations before and after culture (Figure 23). Cultured ALDH low and high adventitial cells cluster together, due to similarities between these cell types. On the other hand, cultured pericytes appear to be different compared to the other cultured perivascular cells. In addition, the freshly sorted whole population of adventitial cells and ALDH subsets were similar. As expected, pericytes are different from adventitial cells at the transcriptomic level and this is maintained in culture.

We next looked at the genes that are upregulated or downregulated when comparing freshly sorted ALDH subsets of adventitial cells (Figure *24*, A). We did not find a clear difference between ALDH low and high in non-cultured cells. Importantly, upon culture, ALDH subsets are similar (Figure *24*,B). This corroborates what we have seen in the functional assays, that culture conditions make the cells similar.

We found that pericytes have a different transcriptomic profile when compared to freshly sorted ALDH low and high adventitial cells, and that these differences

are maintained in culture *(Figure 25*). This raises questions about the heterogeneity of common MSCs that are isolated by plastic adherence, where they may be clonal selection. Moreover, the stromal vascular fractions contain pericytes and adventitial cells and whether a specific type of perivascular cells proliferate faster and if there are functional differences.

Finally, by analysing transcripts in ALDH subpopulations I found genes that were uniquely expressed by ALDH high adventitial cells. Although there were many genes expressed exclusively by the ALDH high subset, only the ones with the most transcripts were selected (Table 8). I used RPMK (reads per kilobase of exon per million mapped reads) which takes into account both library size and gene length in within-sample comparisons. These genes may help us identify the function and find new markers to identify this ALDH high population *in situ*. Indeed, by querying the list of these genes uniquely expressed by ALDH high adventitial cells on The PANTHER (protein annotation through evolutionary relationship) classification system (http://www.pantherdb.org/), which analyses genes sets by gene function, ontology, and pathways, enrichment in different molecular pathways was found (*Figure 26).* Among the pathways enriched, we found angiogenesis, T cell activation, apoptosis signalling, PDGF, ATP synthesis and endothelin, among others. This suggests that ALDH high adventitial cells have specific functions after activation after injury or tissue turnover, being involved in activation of immune system, along with cytokine release and angiogenesis.

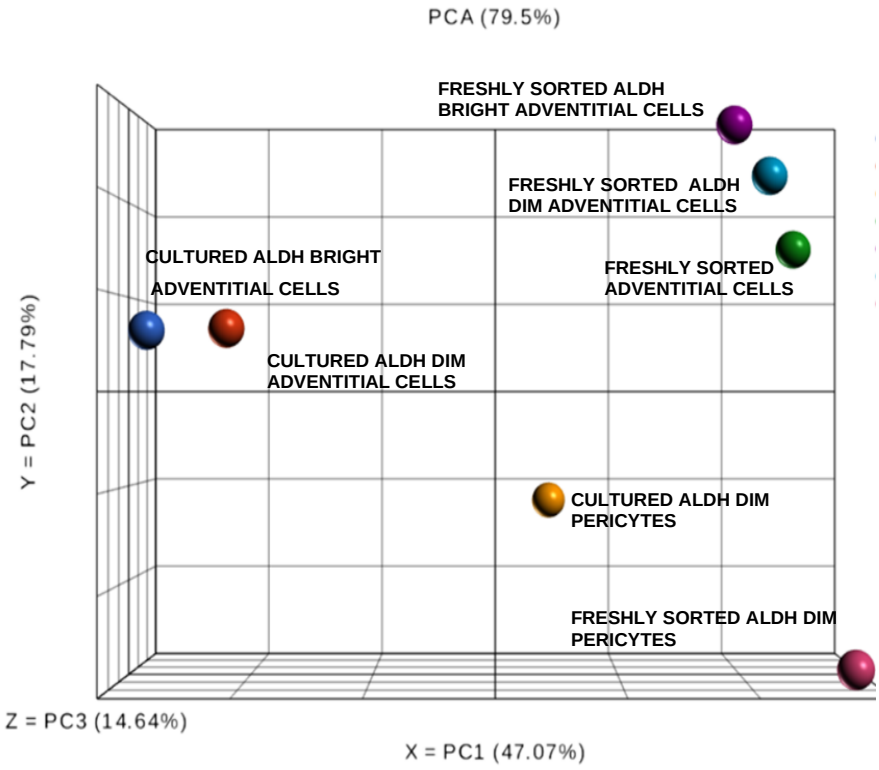


Figure 23 Principal component analysis (PCA) shows correlation between different subsets of perivascular cells.

Sub-populations of perivascular cells were sorted according to their ALDH activity. RNA seq was performed in freshly sorted cells and cultured cells. Principal component analysis shows groups of cells clustering in different planes PCA was done using the sofware PARTEK. n=1 (3 human donors pooled together)

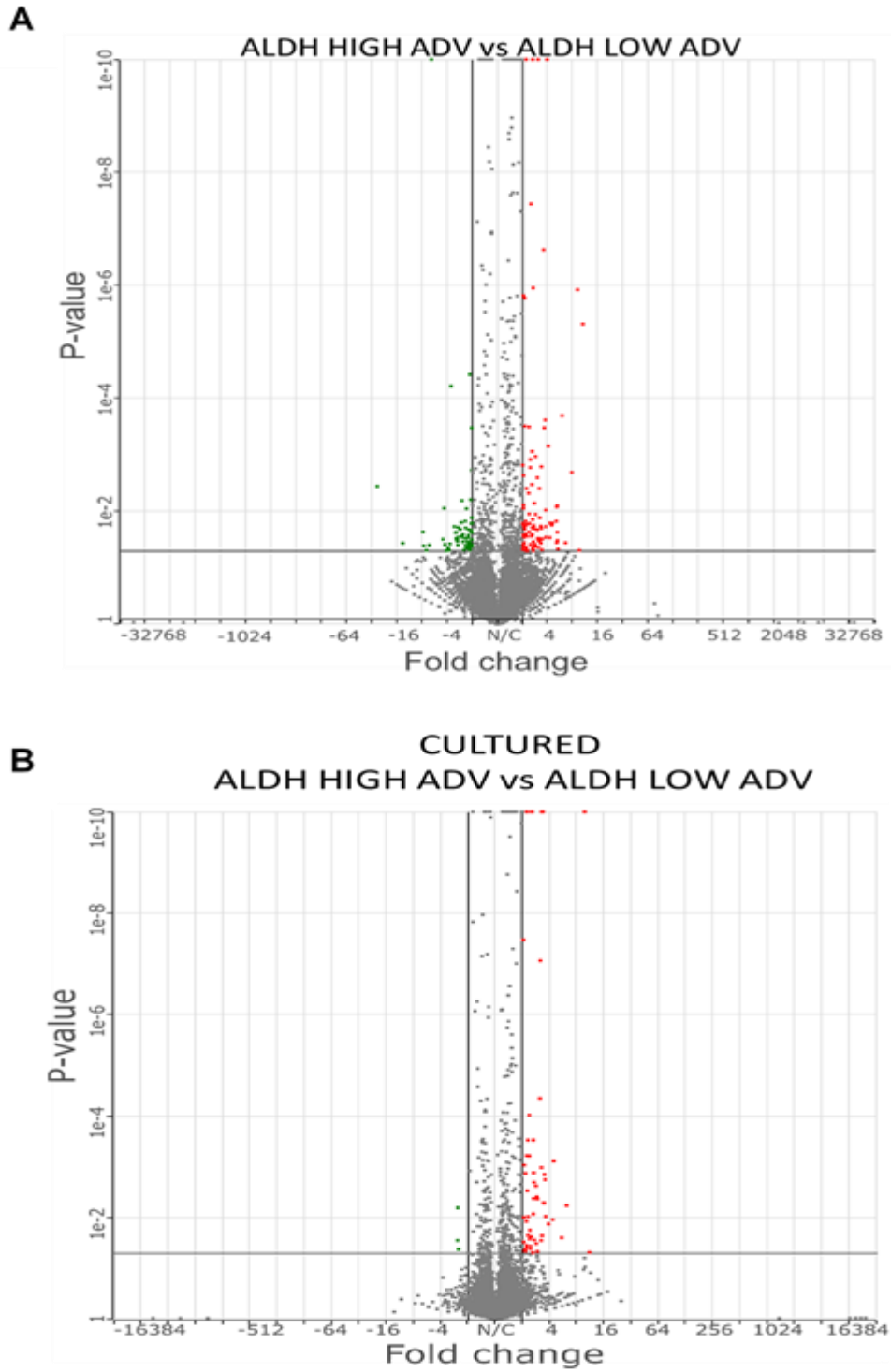


Figure 24 Volcano plots of ALDH subpopulations before and after culture.

Sub-populations of perivascular cells were sorted according to their ALDH activity. RNA was sequenced in freshly sorted cells and cultured cells. Volcano plots show the genes up and down regulated in ALDH subsets of adventitial cells freshly sorted and after culture**. A** comparison of genes up/down regulated in freshly sorted ALDH low vs freshly sorted ALDH high adventitial cells. **B** comparison of genes up/down regulated in cultured ALDH low vs Culture ALDH high adventitial cells

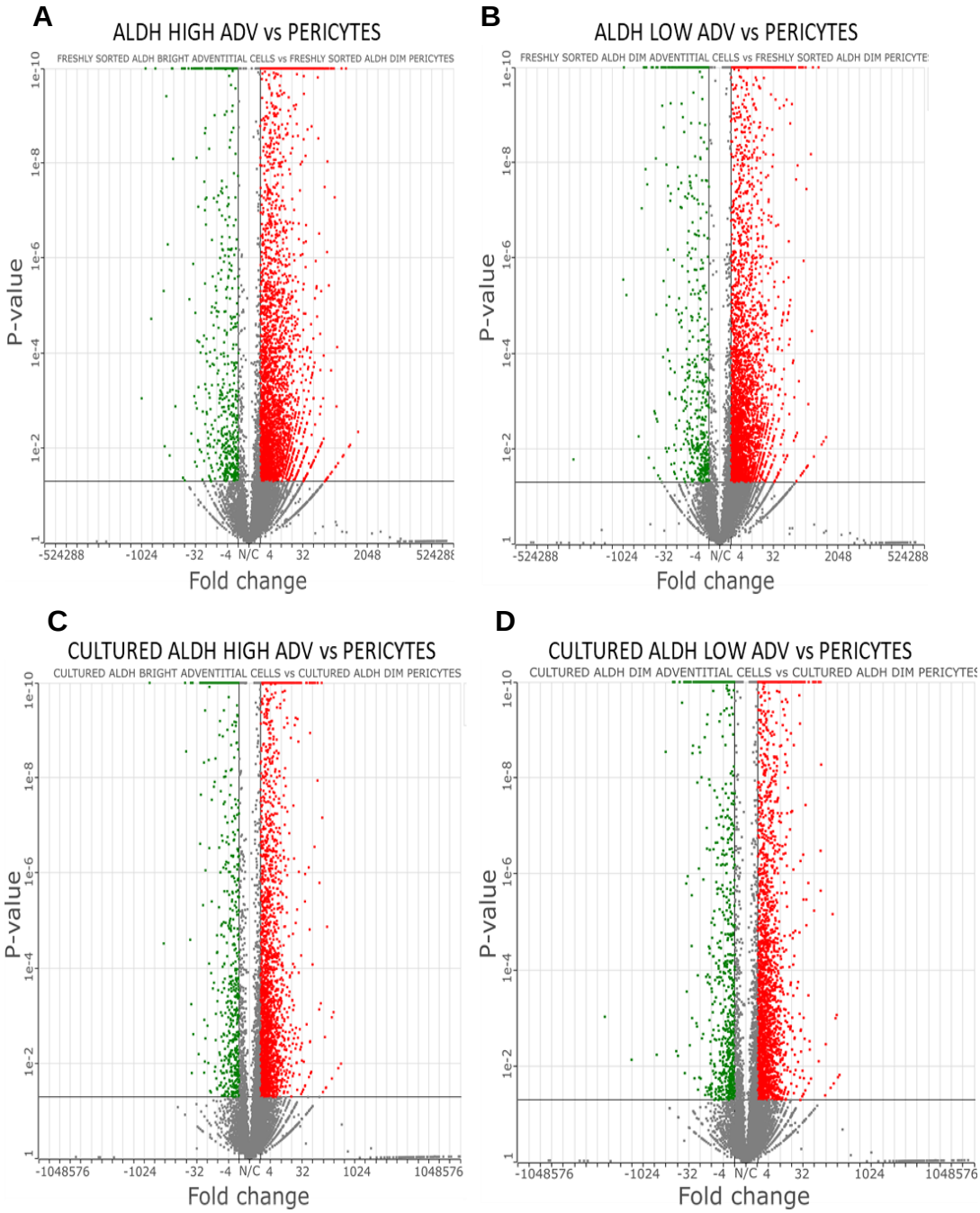


Figure 25 Volcano plots of ALDH subpopulations compared to pericytes before and after culture.

Sub-populations of perivascular cells were sorted according to their ALDH activity. RNA was sequenced in freshly sorted cells and cultured cells. Volcano plots show the genes up and down regulated of ALDH subsets in freshly sorted adventitial cells and pericytes, before and after culture. **A**. Freshly sorted ALDH high adventitial cells vs freshly sorted pericytes. **B**. Freshly sorted ALDH low adventitial cells vs freshly sorted pericytes. **C**. Cultured ALDH high adventitial cells vs cultured pericytes. **D**. Cultured ALDH low adventitial cells vs cultured pericytes

Table 8 Genes uniquely expressed by ALDH high adventitial cells with the higher number of transcripts

Gene	ALDH LOW ADV (RPMK)	ALDH HIGH ADV (RPMK)
GSN	1	38261
FOS	1	38219
COX1	1	27297
COX3	1	26612
ND4	1	21370
ND4L	1	26449
CYTB	1	24050
COX2	1	14607
ND2	1	12598
ATP6	1	7868
ND1	1	7731

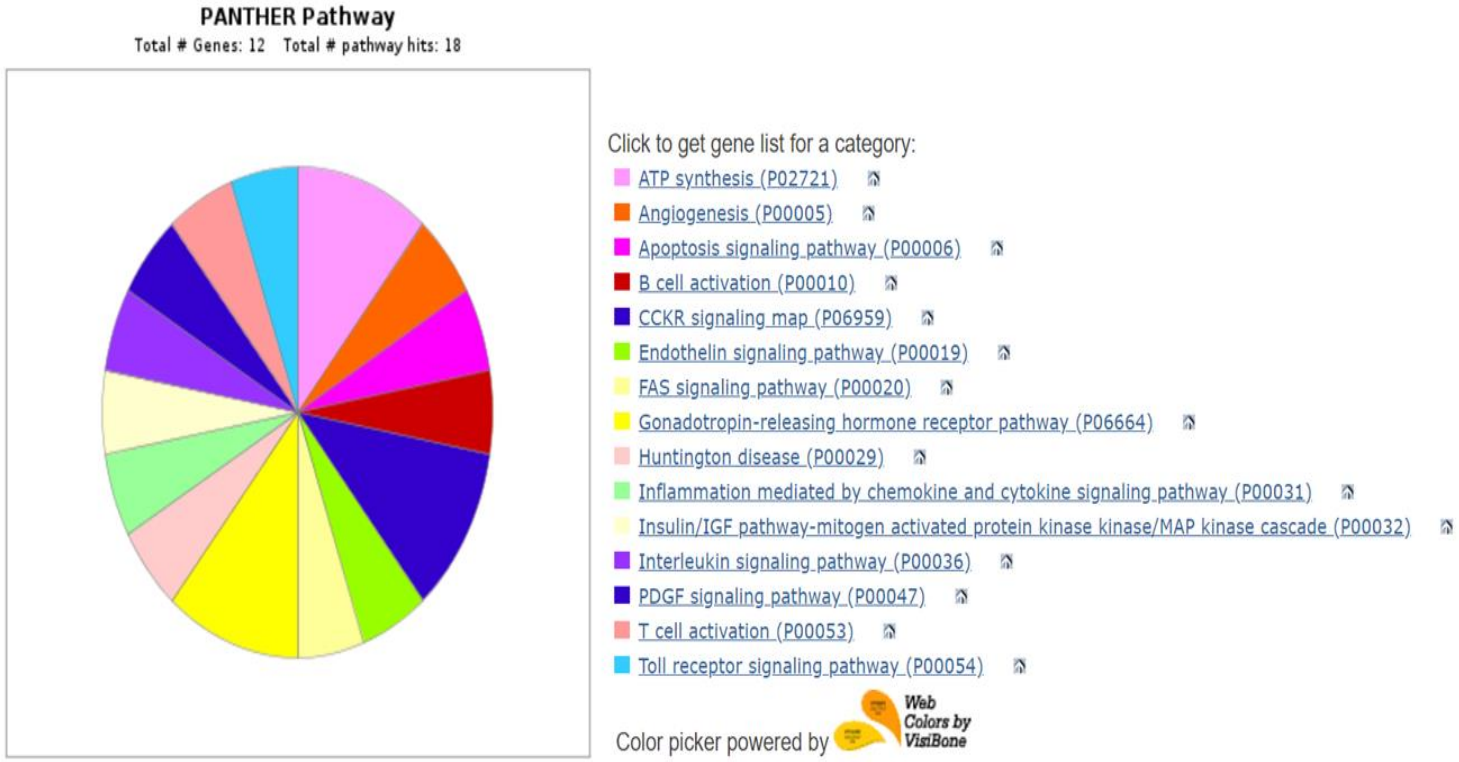


Figure 26 Protein classification in PANTHER

Genes uniquely expressed in ALDH high adventitial cells were queried in PANTHER to identify specific pathways related to these genes.

MSCs and their *in vivo* counterparts, perivascular cells are involved in many processes such as angiogenesis, adipogenesis, osteogenesis, tissue repair, among others. For that reason, we analysed the expression of genes involved in those different processes in freshly sorted cells and after they have been expanded *in vitro*. We created heatmaps that cluster genes according to their expression using the R package “Pheatmap”.

In the cohort of genes expressed by freshly sorted cells *(*Figure 27*),* we saw a similar expression pattern in angiogenesis related-genes, although some genes differentially expressed in adventitial cells with high ALDH activity when compared to both, ALDH low adventitial cells and pericytes. We saw a similar trend in the other data sets analysed. Expression of tissue repair related genes was similar between ALDH subsets of adventitial cells, whereas in pericytes a group of genes clustered differently. In the case of adipogenesis, pericytes express a group of genes clustering differently than in ALDH subsets including ADIPOQ and leptin. Lastly, osteogenesis related genes in ALDH subpopulations are differentially expressed. We found COL1A1 only expressed in adventitial cells, and RUNX2 and BMP5 only expressed in the ALDH low adventitial cell population.

We also analysed the same group of genes in culture expanded perivascular cells (*Figure 28*). We found similar results as in fresh cells. Most clusters of genes are similar between the different cell types. However, the clustering

pattern is different from that in the freshly sorted cells, meaning that cultured cells may have a different effect in cell therapies. For angiogenesis, tissue repair, and osteogenesis, the expression pattern is similar. In adipogenesis, we observed that genes expressed in adventitial cells cluster differently, such as ADIPOQ, APOE, among others, whereas they are not expressed in pericytes. This indicates a different capacity to differentiate into adipocytes *in vitro*.

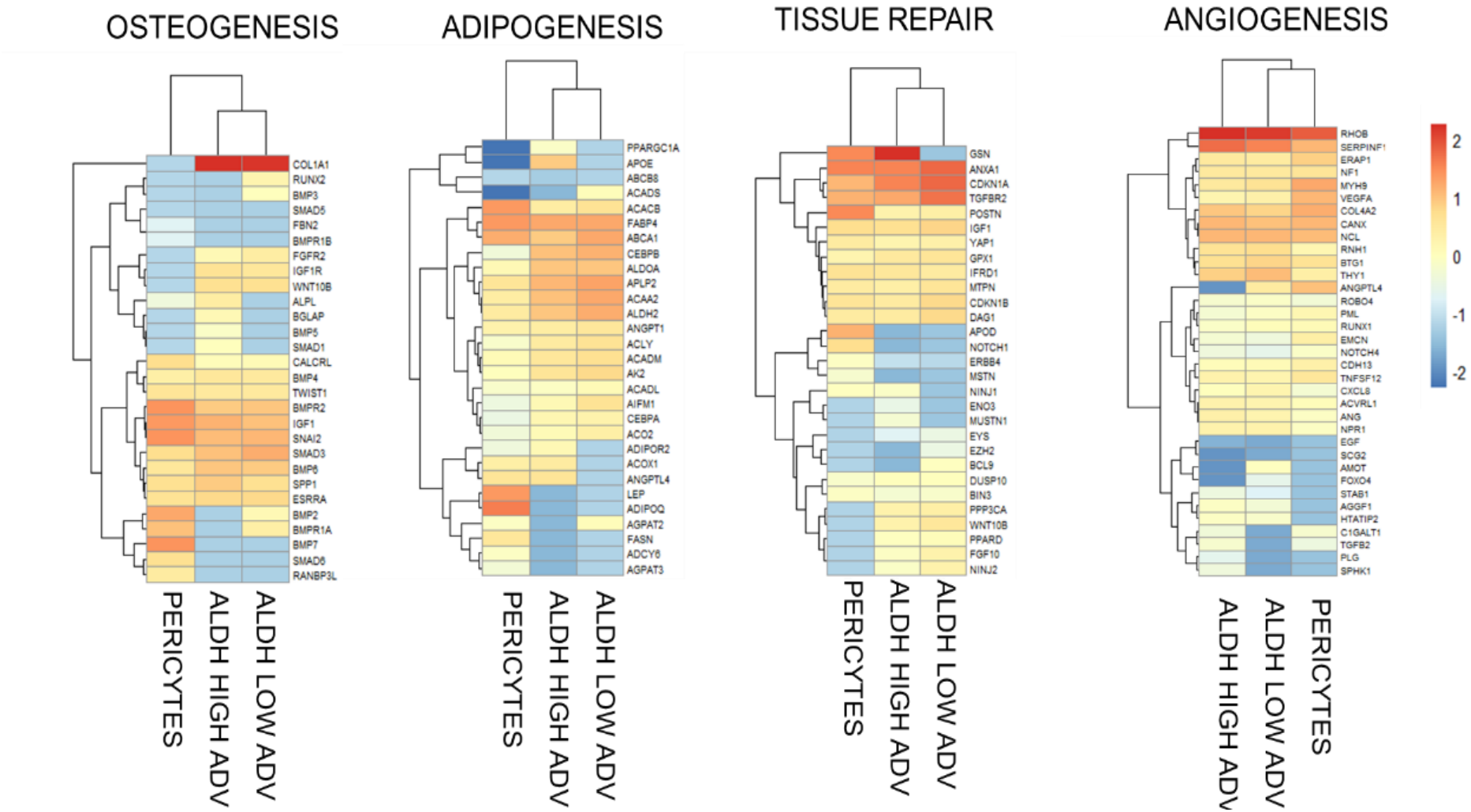


Figure 27 Heatmaps of different gene sets of freshly sorted perivascular cells

Heatmaps were created using Rstudio and the library: Pheatmap. Genes involved in osteogenesis, adipogenesis, tissue repair and angiogenesis were selected and run in the program. the genes are clustered by the number of transcripts and the relation between the 3 groups of perivascular cells.

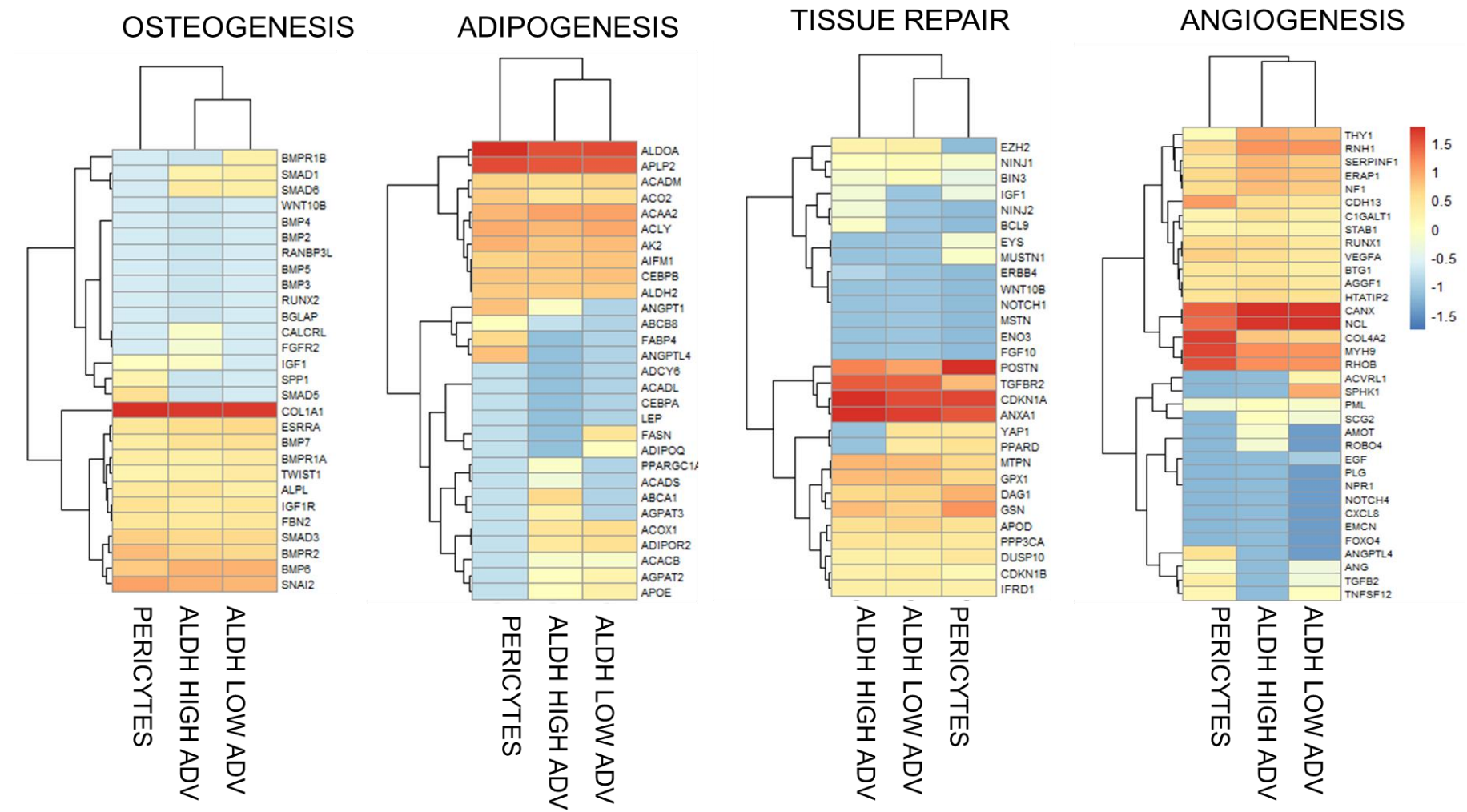


Figure 28 Heatmaps of different gene sets of cultured cells.

Heatmaps were created using Rstudio and the library: Pheatmap. Genes involved in osteogenesis, adipogenesis, tissue repair and angiogenesis were selected and run in the program. The genes are clustered by the number of transcripts and the relation between the 3 groups of perivascular cells.

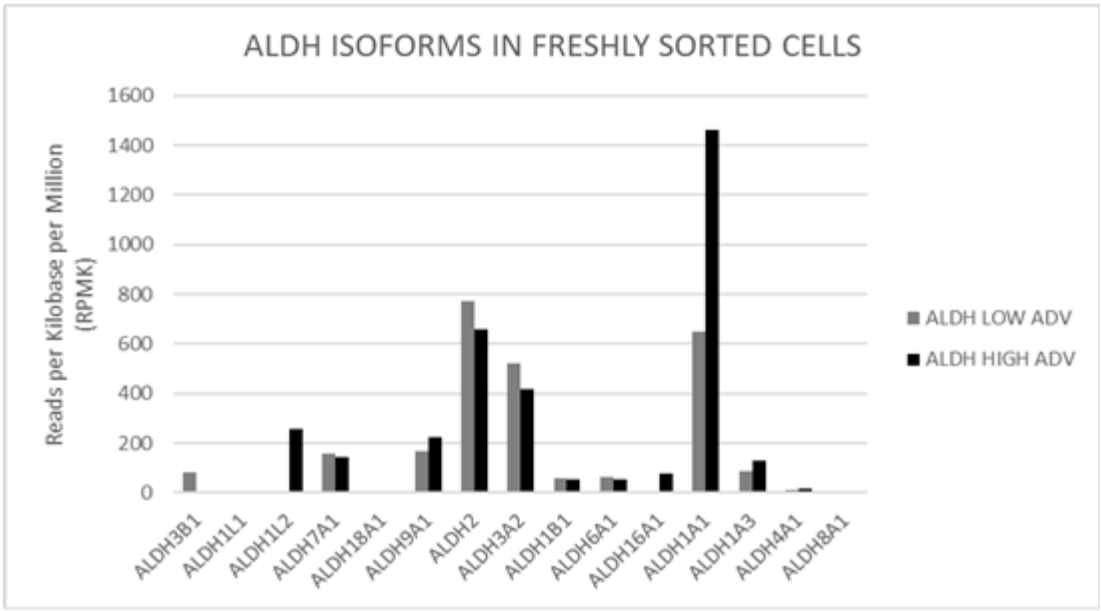
4.3.7 ALDH1A1 is the main ALDH isoform present in ALDH high adventitial cells, and it is lost upon culture

ALDEFLUOR assay was developed to detect ALDH1 family members, however it has been reported that in different tissues several isoforms can metabolize the substrate used in the assay.

In order to identify the main ALDH isoforms present in adventitial cells, we performed RNA sequencing on freshly sorted cells of the ALDH low and high compartments of adventitial cells. We found that ALDH1A1 is the isoform that appears to be more expressed in the ALDH high compartment (Figure *29*, A). Although mRNA levels may not be correlated with enzymatic activity this observation gives us an indication as to which enzyme is metabolizing the ALDEFLUOR reagent.

Since we observed that in culture the ALDEFLUOR activity was similar in ALDH low and high adventitial cells, we performed RNA sequencing on ALDH low and high adventitial cells after 3 passages of culture expansion. As expected, we found that mRNA levels of these two subtypes are similar in culture conditions (Figure *29*, B). Moreover, ALDH1A1 expression is completely absent after *in vitro* expansion.

A



B

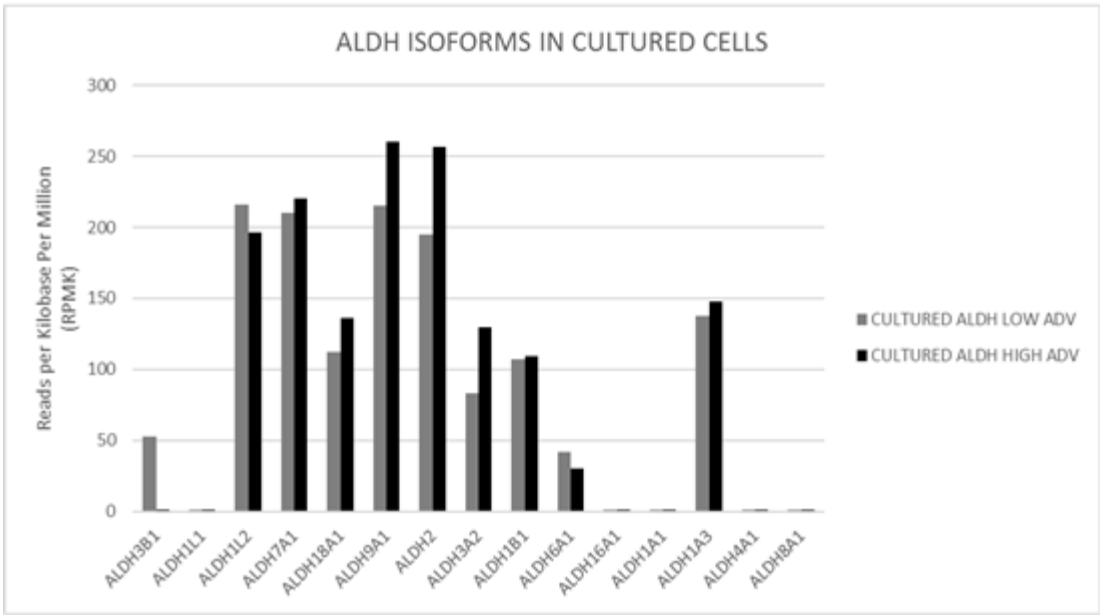


Figure 29 ALDH isoform expression in freshly sorted and cultured adventitial cells.

A. RNA sequencing performed in ALDH low and high compartments of adventitial cells reveals the expression of different ALDH isoforms showing ALDH1A1 highly expressed in the ALDH high compartment. **B.** Subsets of adventitial cells were cultured and RNA sequencing performed after passage three shows that cells express similar levels of the different ALDH isoforms. N=?

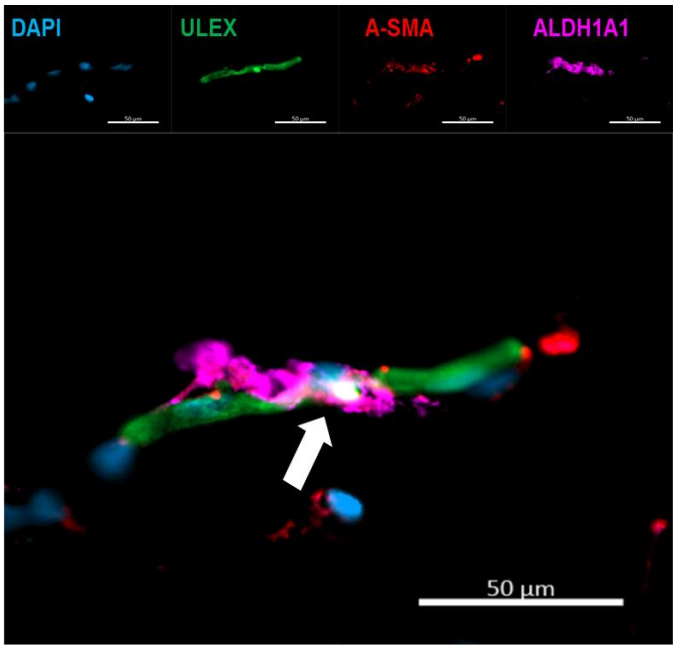
4.3.8 ALDH1A1 is present in perivascular cells of different human organs including adipose tissue, heart and uterus

Taking into account the finding that ALDH1A1 is the main isoform present in the ALDH high cell compartment, we decided to assess ALDH1A1 expression *in situ* in different foetal and adult human tissues including white adipose tissue, heart, and uterus.

Using different combinations of endothelial and perivascular cell markers, we were able to identify ALDH1A1 in the adventitial layer of adipose tissue in cells co-expressing CD34 (Figure 30). On the other hand, only few (almost none) pericytes expressed ALDH1A1 along with the pericyte marker α-SMA. These findings corroborate the data obtained by flow cytometry showing that adventitial cells have a higher percentage of ALDH high cells than pericytes. Next, human foetal heart was investigated to test whether ALDH1A1 expression is similar in different tissues. We found that only a few cells in the adventitia layer express ALDH1A1, suggesting that this subpopulation of adventitial cells expressing CD34 and ALDH1A1 is rare (*Figure 31*). We could not find pericytes expressing ALDH1A1 in the foetal heart.

Finally, adult human uterus in the proliferative stage shows homogenous ALDH1A1 co-expression with CD34 through the adventitial layer (Figure 32). These results suggest that ALDH1A1 expression in the adventitial layer of human uterus is dependent on tissue remodelling and proliferation. To address

this, analysis of tissues at different stages of remodelling or after injury is required. ALDH1A1 analysis was also performed in human foetal skeletal muscle, adult brain, and foetal limb. Data show that ALDH1A1 is primarily expressed in the adventitial layer, consistent with what was found in other tissues tested.



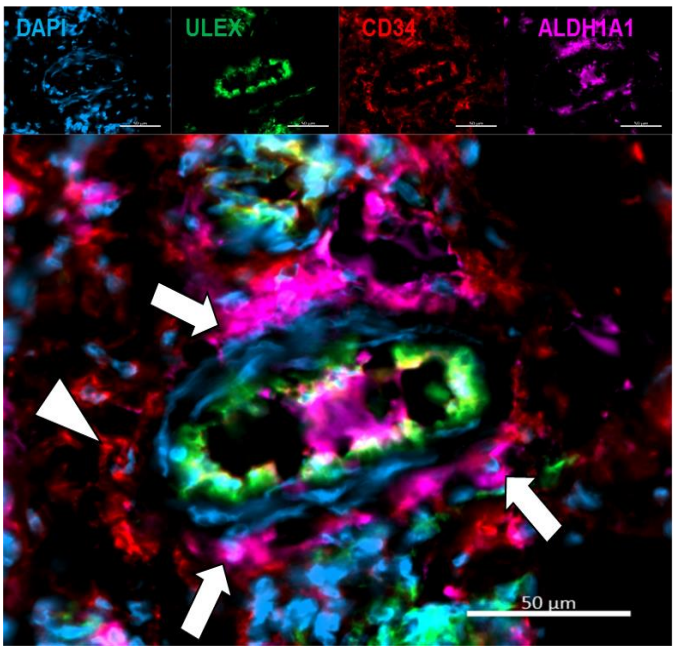


Figure 30 ALDH1A1 expression in the perivascular cells of human adipose tissue.
ALDH1A1 antibody was used along with CD34 and a-SMA marking adventitial cells and pericytes, respectively. Ulex europaeus lectin was also used to mark the endothelium along with CD34. It was found that adventitial cells expressing CD34 also express ALDH1A1 and that some pericytes co-express ALDH1A1 with a-SMA. Arrows mark CD34+ ALDH1A1+ cells , whereas arrowheads mark CD34+ ALDH1A1- cells. Scale bar represents 50 µm. n=3

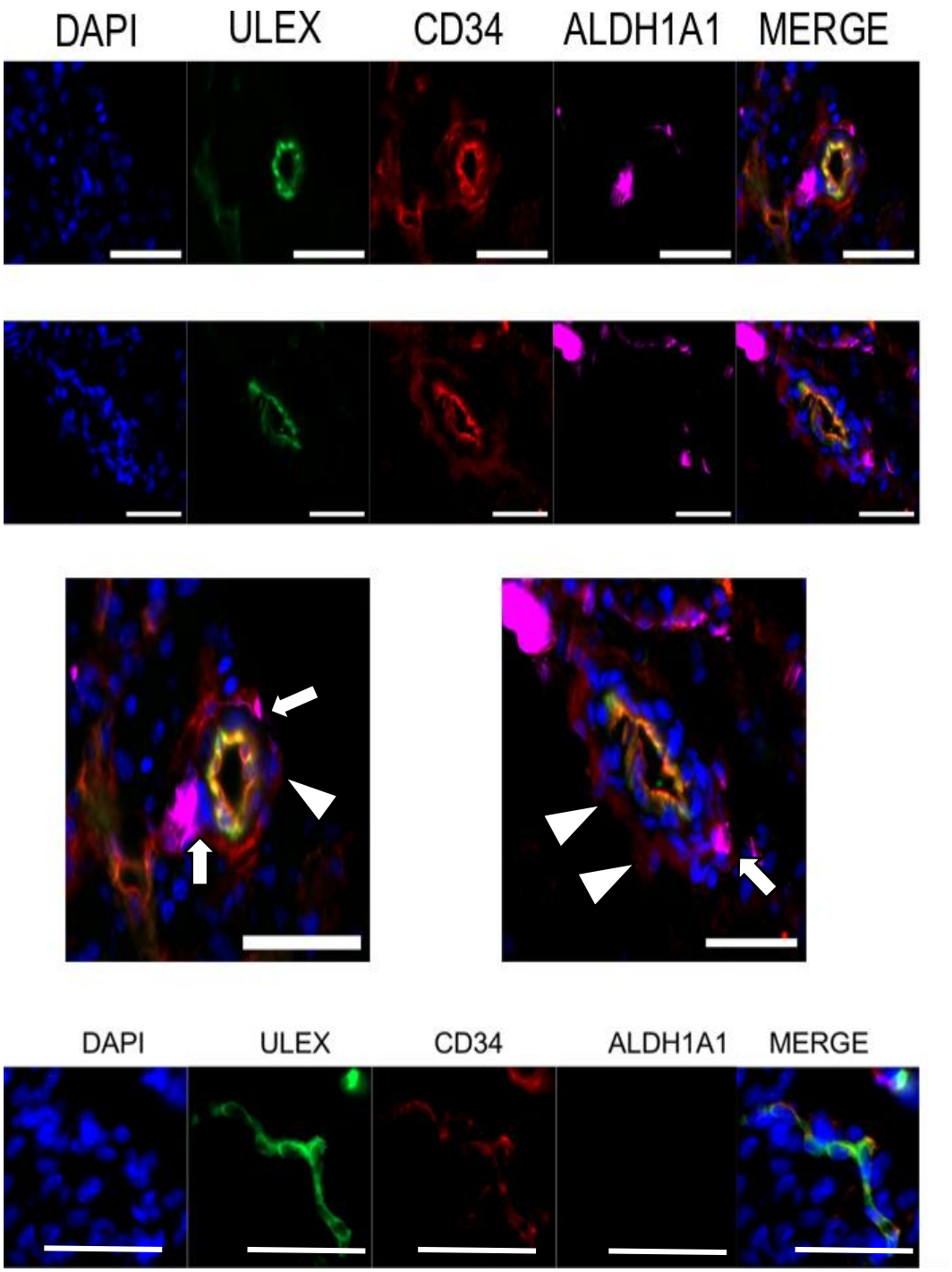


Figure 31 ALDH1A1 expression in the adventitial layer of blood vessels in the foetal heart.

A combination of perivascular and endothelial cell markers was used along ALDH1A1 to detect cells expressing ALDH1A1 in the adventitial layer. Cells expressing CD34 and ALDH1A1 (arrows) as well as ALDH1A1 negative (arrow heads) were found in various vessels throughout the tissue showing that the adventitial cells expressing CD34 include a sub-population expressing ALDH1A1, a marker of stem cells. Scale bar represents 50 µm. n=3

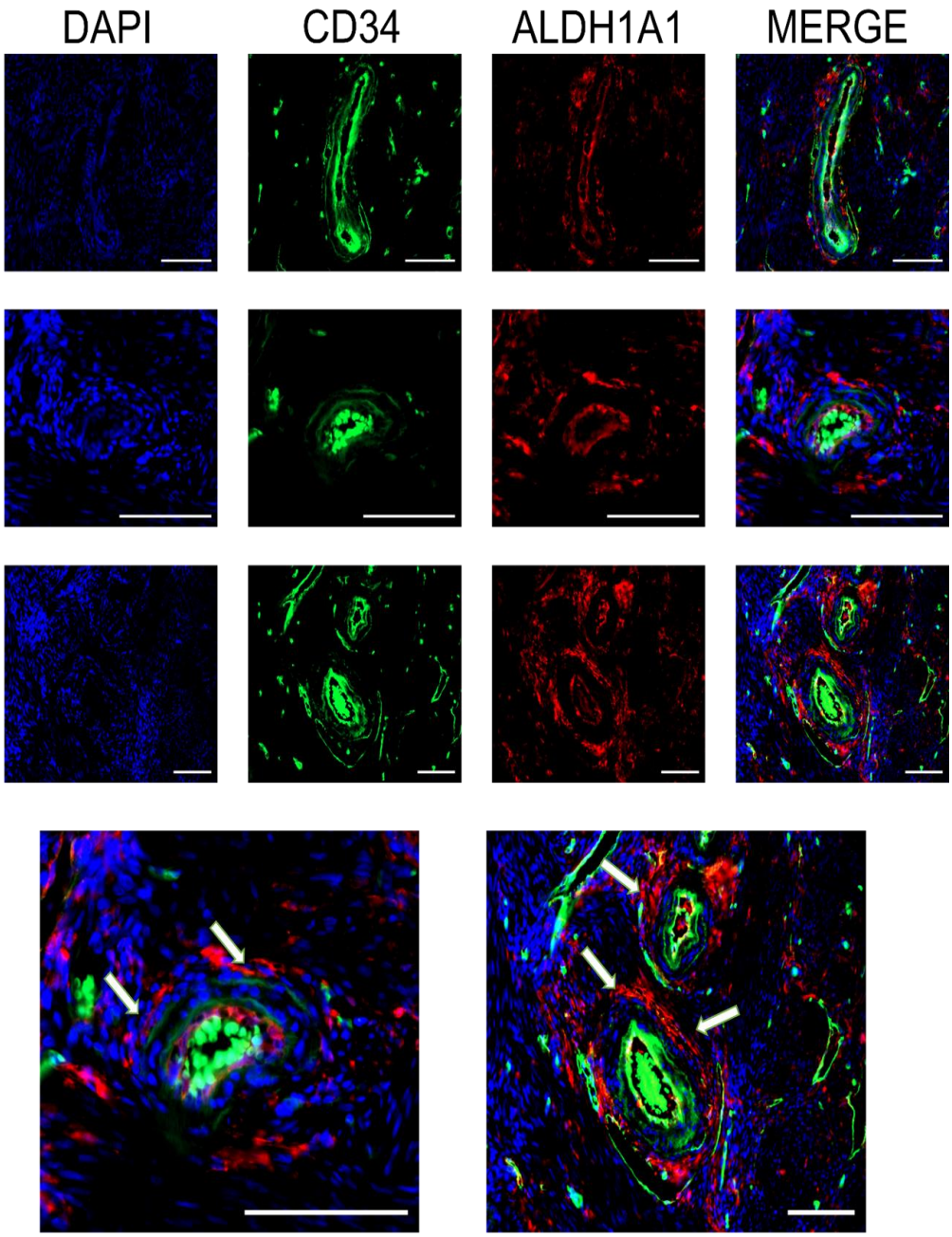


Figure 32 ALDH1A1 expression in the adventitial layer of adult uterus.

A combination of perivascular and endothelial cell markers was used along with ALDH1A1 antibody to detect perivascular cells expressing ALDH1A1. Cells expressing CD34 and ALDH1A1 were found in various vessels throughout the tissue showing that the adventitial cells expressing CD34 harbour a sub-population of cells expressing ALDH1A1 (arrows), a marker of stem cells, some endothelial cells also express ALDH1A1. Scale bar represents 100 µm. n=3

4.4 DISCUSSION

The work in this chapter sought to study a novel subset of perivascular cells with high ALDH activity, specifically, adventitial cells in large vessels, which showed higher ALDH activity than pericytes. In a series of different analyses including flow cytometry, *in vitro* assays, immunohistochemistry, and RNA sequencing, I characterized ALDH high cells in their *in vivo* niche and *in vitro*. I also identified ALDH1A1as a novel marker for adventitial cells that mark a subset of progenitor cells.

4.4.1 Characterisation of ALDH activity in pericytes and adventitial cells, and how culture modifies ALDH activity

First, it was corroborated that adventitial cells comprise the majority of the stromal vascular fraction, which may have implications when using adipose tissue-derived progenitors for cell therapy. Second, it was demonstrated that both types of perivascular cells can be isolated according to their ALDH activity, confirming previous research demonstrating ALDH activity being higher in adventitial cells (Hardy *et al.*, 2017). However, now we were able to quantify this by calculating the mean fluorescence intensity in the ALDEFLUOR assay. Moreover, I further analysed the ALDH high compartment of perivascular cells finding that pericytes and adventitial cells have the same percentage of ALDH high cells, but the activity is significantly higher in adventitial cells. We demonstrated that once perivascular cells are expanded *in vitro*, they lose this characteristic differential ALDH activity and become

similar in terms of percentage of ALDH high cells and ALDH activity, quantified by mean fluorescence intensity, and mRNA levels in cultured cells. To our knowledge, this has not been previously reported. ALDH has been used to characterize stem cells from different types of cancer (Tomita *et al.*, 2016; Mele *et al*., 2018; Zhou *et al.*, 2019). However, modifications caused by *in vitro* expansion need to be considered. Especially, cell density during expansion changes the percentage of cells in the ALDH high compartment.

I sought to analyse the relationship between the intensity of expression (flow cytometry) of the perivascular cell markers CD146 and CD34, and ALDH activity. Indeed, it was found that higher expression of the membrane receptor that distinguishes adventitial cells from pericytes, CD34, is correlated with higher ALDH activity. The stronger the signal of the cell marker in flow cytometry, the more ALDH activity. This finding led to the analysis of protein interaction between CD34 and ALDH1A1. Since CD146 expression levels did not correlate with ALDH activity and the project is focused on the most primitive cell type (adventitial cells), we did not analyse protein interaction of CD146 and ALDH. This ALDH1A1 isoform was chosen due to the fact that ALDEFLUOR assays were created to detect this specific isoform (Jones *et al.*, 1995; Storms *et al.*, 1999). Therefore, transcriptome analysis is needed to corroborate the ALDH1A1 specificity since different isoforms have been shown to metabolize the ALDEFLUOR reagent and it varies between cell types and tissues (Zhou *et al.*, 2019). Indeed, ALDH1A1 presence in the ALDH high compartment was later confirmed by RNA sequencing.

Interestingly, the interaction network of proteins showed molecules involved in vasculogenesis and extracellular matrix reorganization, which are known to play a role in vessel remodelling, suggesting that ALDH1A1 expression is needed when these processes are carried out by cells in the adventitia expressing CD34. Indeed, different subsets of adventitial cells have been involved in myofibroblast differentiation (Siow *et al*., 2003; Kramann *et al.*, 2015) as well as adipogenic and osteogenic differentiation (Ding *et al.*, 2019). It has been suggested that $VEGFR2^{+}$ adventitial cells give rise to cardiomyocytes (Mekala *et al.*, 2018). In conclusion, these data suggest along with different previous reports that ALDH1A1 marks a rare subset of primitive perivascular cells, especially adventitial cells. In order to characterise and prove this hypothesis, functional *in vitro* assays and RNA sequencing were performed.

The ALDEFLUOR assay has been used to isolate MSCs with high ALDH activity (Sherman *et al.*, 2017; Najar *et al.*, 2018). However, these cells have been expanded and the original identity of the cells that gave origin to these MSCs is not known. Here, a more prospective approach was used. We sorted MSC progenitors from the *in situ* niche according to ALDH activity, then cells were cultured. After expansion, the ALDEFLUOR assay was performed again and no difference between the subsets was found in terms of activity or percentage of ALDH high cells. Moreover, mRNA levels of ALDH isoform was similar in both ALDH subsets of adventitial cells. It was also shown that the

ALDH high compartment of cells is dependent on cell confluence. Previous studies showed that YAP/TAZ is involved in cell proliferation, and reacts by mechanical contact between the cells (Seo, 2018). This raises the question as to whether the ALDH high cells isolated from *in vitro* cultures may actually proliferate due to sensing mechanical cues (Kurpinski *et al.*, 2006) and not to ALDH activity. Cultured MSCs from the ALDH high compartment sorted and expanded again *in vitro* showed the same profile when the ALDEFLUOR assay was performed once more (not shown). This led me to speculate that ALDH activity *in vitro* marks proliferating cells but not a specific progenitor cell, and the use of the ALDEFLUOR assay *in vitro* may need to be better characterized. Other groups showed that freshly sorted ALDH high bone marrow cells are better at establishing blood flow after hind limb ischemia (Capoccia *et al.*, 2009), suggesting that freshly sorted cells may be more beneficial for cell therapy. However, high cell numbers are required for injection into patients. Hence, in vitro expansion may be unavoidable in most instances.

Next, ALDH low and high compartments of adventitial cells were analysed for expression of PDGF receptors. Cells expressing combinations of these receptors have been described to give rise to myofibroblasts but also muscle cells (Murray *et al.*, 2017; Jensen *et al.*, 2018). PDGFRβ+ perivascular cells expressing PDGFRα are highly fibroadipogenic, whereas single positive PDGFRβ+ cells are pro-myogenic (Jensen *et al.*, 2018). Indeed, PDGFRα has been shown to be highly involved in fibrosis (Hayes *et al.*, 2014; Uezumi *et al.*, 2014; Gallini *et al.*, 2016) in different tissues. Using flow cytometry, I analysed

the ALDH compartments of adventitial cells expressing either only PDGFRβ, or both receptors, PDGFRβ and α. The results showed no differences in the percentage of cells in either subset, indicating that ALDH activity may not be correlated with PDGF receptor expression. However, we cannot rule out that under pathological conditions the expression of these receptors changes. Indeed, published (Murray *et al*. 2017) and preliminary data from our laboratory showed that after heart injury, PDGFRα expression is upregulated in perivascular cells and contribute to organ fibrosis.

Finally, ALDH subsets of adventitial cells were characterised *in vitro* to analyse heterogeneity between clones. It has been reported that MSCs are clonogenic and suggested that only some clones in the whole population are real MSCs, able to differentiate into mesodermal cell lineages (Chamberlain *et al.*, 2007; Lv *et al.*, 2014). We found that ALDH low adventitial cell clones do not expand after sorting, whereas ALDH high adventitial cell clones reach confluency in 7 days. ALDH low cells were only able to adhere and proliferate when sorted cells were plated at high numbers. Although they proliferate slowly in the beginning, after 2-3 passages they proliferate almost at the same rate as ALDH high cells. This may be due to the harsh conditions of the cell sorting, that clones with low ALDH activity are less resistant to sorting. On the other hand, ALDH high adventitial cell clones were able to expand after sorting. Once under differentiation conditions, only a few clones were able to differentiate into the osteogenic lineage, providing more evidence that only a few clones are MSC progenitors. Moreover, freshly sorted ALDH high adventitial single

cells have been shown to be heterogeneous, as previously reported by our laboratory using single cell qPCR data (Hardy *et al.*, 2017) (Figure 33*).*

To summarise this section, ALDH activity marks a primitive population of perivascular cells. This ALDH high compartment does not differentially express PDGF receptors, involved in fibrosis, compared to the ALDH low compartment. During in vitro expansion this characteristic ALDH activity, that identified two subsets of adventitial cells, is lost. Moreover, in culture conditions ALDH activity is dependent on cell density. Lastly, only ALDH high adventitial cells are clonogenic and show heterogeneity in differentiation potential into osteoblasts.

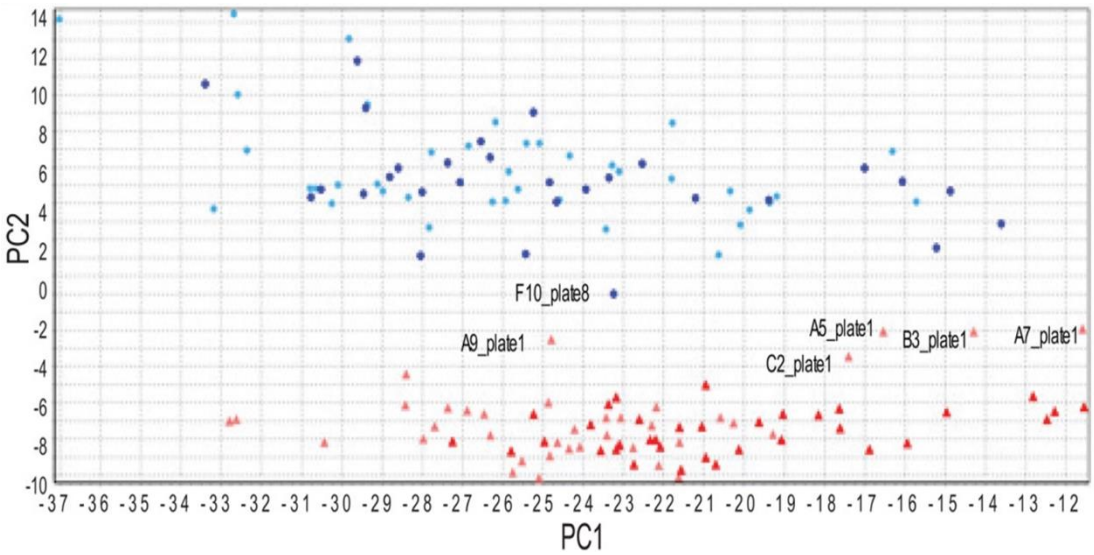


Figure 33 Principal component analysis of single perivascular cells from ALDH subsets.

Analysis of gene expression by single cells in populations of ALDH subsets of adventitial cells and pericytes showed heterogeneity of the different clones. Circles: ADV; triangles: pericytes; ALDH-high: light red or blue; ALDH-low: dark red or blue. Adapted from Hardy et al., 2017.

4.4.2 Transcriptomic analysis of ALDH subsets of adventitial cells and pericytes reveals differences between MSC progenitors.

Transcriptomic analysis allows for the comprehensive study of genes expressed in a specific cell population that may help reveal functional traits. Network analysis of 500 genes on single cells was performed by members of our laboratory showing that perivascular cells have a hierarchy according to ALDH activity, adventitial cells with high ALDH activity being the subset with the most primitive phenotype. However, a comprehensive analysis of the transcriptome of these ALDH subsets of perivascular cells has not been performed. Here, I showed that there are transcriptional major differences between pericytes and adventitial cells. Moreover, ALDH high adventitial cells exhibit a different transcriptome compared to the ALDH low counterpart before culture expansion.

Principal component analysis (PCA) is a method to simplify complex high dimensional data. PCA is similar to clustering in the sense that it is an unsupervised learning method that finds patterns with no background knowledge about the samples analysed (Lever *et al*. 2017). PCA analysis of our different populations of cells showed that adventitial cells (whole population) and derived ALDH subsets cluster together, although there are slight differences between them. As expected, pericytes, either freshly sorted or cultured, are transcriptionally different compared to adventitial cells. Next, volcano plot analysis showed the genes upregulated or downregulated when

ALDH subsets of adventitial cells were compared. Differences between ALDH subsets were minimal. Nevertheless, when pericytes and either ALDH subset of adventitial cells were compared, a clear difference in up/down regulated genes was observed. Pericytes and adventitial cells come from different locations in the human body and have different functions, so it was not unexpected that they will have different transcriptomes. Interestingly, this opens up the question of what approach is better to expand MSCs in culture. As it can be seen from my results, pericytes have an entirely different identity compared to adventitial cells after *in vitro* expansion.

In order to elucidate the functions of ALDH high cells *in vivo*, I found genes uniquely expressed by ALDH high adventitial cells and with the highest number of reads. As expected, many of these genes are involved in metabolism. The list of genes was queried in an ontology database (PANTHER) to find pathways related to the expression of this gene set. Angiogenesis, PDGF signalling, activation of immune cells, endothelin pathways, among others, are active in this ALDH high adventitial cell subset. Cells in the adventitia have been described to be involved in vascular remodelling, interact with immune cells, and differentiate into other cell types (Siow *et al*., 2003; Moos *et al.*, 2005; Campbell *et al.*, 2012; Kramann *et al.*, 2016; Ding *et al.*, 2019; Wang *et al.*, 2019). This analysis suggested that cells with high ALDH activity in the adventitia are prone to be involved in these processes.

Finally, four pathways involved in MSC differentiation potential (osteogenesis and adipogenesis) and therapeutic benefit related to MSCs (angiogenesis and tissue repair) were analysed in the ALDH subsets of adventitial cells and pericytes. Heatmaps were created by clustering the expression levels of the genes in every cell type in cultured or uncultured conditions. There were no clear differences in either of the pathways, although some clusters of genes were differentially expressed.

In other lineages there were some differences in gene expression. For example in osteogenesis, only subsets of adventitial cells expressed COL1A1 and, interestingly, only ALDH low adventitial cells expressed RUNX2 and BMP3. Lastly, in the adipogenesis related gene group, the expression profile was similar between all cell types. However, only pericytes expressed LEP and ADIPOQ, key genes involved in adipogenesis. A recent study has shown that human MSCs contain sub-populations of adipocyte progenitors (Min *et al.*, 2019). By isolating clones from adipose tissue, they found cell types with different adipogenic differentiation capacities. Interestingly, one of the genes correlated with adipogenesis was MCAM (CD146), a pericyte marker, which is consistent with our transcriptomic analysis suggesting that pericytes are more adipogenic that adventitial cells in non-cultured conditions.

On the other hand, once in culture, most genes drastically changed expression becoming more similar between the groups. LEP expression is downregulated in pericytes and ALDH high adventitial cells and only ALDH low adventitial cells express ADIPOQ. This highlights the complexity of studying perivascular cells and the dramatic changes in gene expression happening in culture. Once perivascular cells become MSCs it is difficult to tell whether they will behave as they would in their *in vivo* niche.

In summary, transcriptomic analysis shows that ALDH subsets of adventitial cells are different in freshly sorted conditions, but once they are expanded *in vitro* they become phenotypically similar. On the other hand, I also show that pericytes after in vitro expansion are transcriptionally different than adventitial cells. Lastly, it is worth noting that after in vitro expansion native pericytes and adventitial cells dramatically modified their transcriptome compared to their *in situ* counterparts.

4.4.3 ALDH1A1 as a novel marker for perivascular progenitor cells

The ALDEFLUOR assay was developed to analyse the activity of ALDH1A1. However, different isoforms of ALDH can metabolize the ALDEFLUOR substrate in different cell types (Zhou *et al.*, 2019). Indeed, my data showed that ALDH1A1 was the isoform with the highest number of transcripts in the ALDH high compartment, compared to the other isoforms. This led me to use an anti-ALDH1A1 antibody to detect this isoform in the native niche. ALDH1A1 was expressed primarily in the adventitial layer of large vessels in the human foetal heart, adipose tissue, skeletal muscle, and uterus. Interestingly, ALDH1A1 gene expression was absent once adventitial cells were placed in culture. Moreover, the other isoforms were equally expressed in both subsets of adventitial cells, corroborating what we saw in previous sections of this thesis, in which ALDH activity in cultured cell subsets of adventitial cells was not relevant due to their phenotypic similarities acquired upon culture expansion.

ALDH1A1 has been described as a stem cell marker in different tissues (Tomita *et al.*, 2016). It is mainly involved in retinoic acid mediated cell proliferation and differentiation (Vassalli, 2019). It has already been proposed that adventitial cells have a more primitive phenotype than pericytes (Hardy *et al.*, 2017), and that pericytes may not act as MSCs *in vivo* (Guimarães-Camboa *et al.*, 2017). These findings suggested that the adventitia is a source of MSCs in vivo after injury. Importantly, ALDH1A1 is expressed in a small

subset of perivascular cells. In the foetal heart, only a few cells express ALDH1A1 in the adventitia. On the other hand, pericytes expressing ALDH1A1 were absent in the foetal heart and very rare in other tissues. In adipose tissue, only one pericyte was expressing ALDH1A1 along with α-SMA *(Figure 30*).

Since ALDH1A1 is a stem cell marker, do ALDH1A1+ adventitial cells act as progenitor cells after injury or in disease?. As seen in the network analysis using ALDH1A1 and CD34, many genes involved in vascular remodelling and extracellular matrix organization interact with our two genes of interest. Furthermore, genes exclusively expressed in ALDH high adventitial cells showed that important factors such as PDGF, known to be involved in cell proliferation, angiogenesis, and the activation of immune cells were particularly associated with the ALDH high population *in situ*.

In conclusion, ALDH1A1 is a novel marker of adventitial progenitor cells and of some pericytes, and is consistently found in human organs. This new marker may identify a population of progenitors involved in tissue repair after injury or disease. However, the only way to document this is to use an animal model where we can track ALDH1A1+ cells in the adventitia after injury. Lastly, it would be interesting to investigate what subsets of perivascular cells are present in the ALDH1A1^{+} subset of adventitial cells.

Chapter 5 PRELIMINARY ANALYSIS OF ALDH1A1 EXPRESSING PERIVASCULAR CELLS IN PATHOLOGICAL CONDITIONS

5.1 Introduction

Perivascular cells are also involved in different pathological conditions. For example, cancer requires the formation of new vessels to grow and eventually spread to other parts of the body (Nishida *et al.*, 2006). Pericytes have been studied in this regard (Paiva *et al.*, 2018), but the complex interplay of different factors and the heterogeneity of perivascular cells make the research difficult.

ALDH1 expression in cancer has been described (Tomita *et al.*, 2016). It has been suggested that high ALDH activity in tumour endothelial cells marks a population of cells that drive angiogenesis during tumour growth (Ohmura-Kakutani *et al.*, 2014). Moreover, ALDH has also been used along with CD44 and other stem cell markers to isolate cancer stem cells (Li *et al.*, 2017). It is well documented that ALDH activity confers drug resistance as well as radio resistance (Januchowski *et al*. 2013). Recently, it has been shown that glioblastoma hijacks pericytes to promote tumour growth (Valdor *et al.*, 2019). However, whether a specific subset of pericytes or adventitial cells is involved in tumour growth has not been described.

On the other hand, perivascular cells, pericytes and adventitial cells, have been shown to be involved in different pathologies including atherosclerosis, arteriosclerosis and fibrosis (Birbrair *et al.*, 2014; Kramann *et al.*, 2015, 2016;

Shaw *et al.*, 2018). However, a study of specific subpopulations is needed to further categorise and classify which subtypes may be of therapeutic interest.

Expression of ALDH1A1 in perivascular cells, and specifically in pathologies such as cancer and fibrosis, to our knowledge, has not been thoroughly studied. In this chapter, I performed a preliminary characterisation of ALDH1A1^{+} perivascular cells in human cancer and fibrosis after rotator cuff chronic injury in the mouse.

5.2 Hypothesis and aims

5.2.1 Hypothesis

“ALDH1A1 marks a population of perivascular cells involved in pathological conditions such as cancer and fibrosis”

In the previous section a novel marker for perivascular cells and specifically of adventitial cells was found. ALDH1A1, a stem cell marker, is expressed in perivascular cells of different organs. This may indicate that ALDH1A1 marks a population of ubiquitous perivascular progenitors.

5.2.2 Aims

- To characterise ALDH1A1 expression in human brain cancer (glioblastoma multiforme)
- To test if ALDH1A1 expression is also found in mouse perivascular cells
- To analyse the expression of ALDH1A1 in skeletal muscle postinjury in the mouse

5.3 Results

5.4 ALDH in perivascular cells in glioblastoma multiforme (GBM)

In order to characterize ALDH1 in glioblastoma multiforme, the tissue was digested with collagenases. The ALDEFLUOR assay was performed on single cell suspensions, antibodies then added and finally samples were analysed by flow cytometry. We used CD56, an antigen that marks neural cells, to discard cells of neural lineage.

A small population of $CD146^+$ pericytes was found in the ALDH high compartment and comprises 13.35% ± 1.35% of all pericytes in the tissue (*Figure 34*). In this experiment we could not find a clear population of $CD34^+$ adventitial cells, and this was consistent between two donors.

Next, we investigated ALDH1A1 *in situ* as we did in other human tissues. We performed staining with UEA-1 to mark the endothelium and anti-ALDH1A1 antibody. Cells expressing ALDH1A1 in close contact with the endothelium were found in different areas of the tumour. The expression of ALDH1A1 in pericytes was present in only a small subset (*Figure 35*). Adventitial cells expressing CD34 and ALDH1A1 were found as well (*Figure 35*). However, this cell type was rare and only found in abnormal vessels with an aberrant adventitia layer, smaller in size compared to arteries and veins. This may be due to the modification caused in the tissue by abnormal growth of tissue characteristic of cancer.

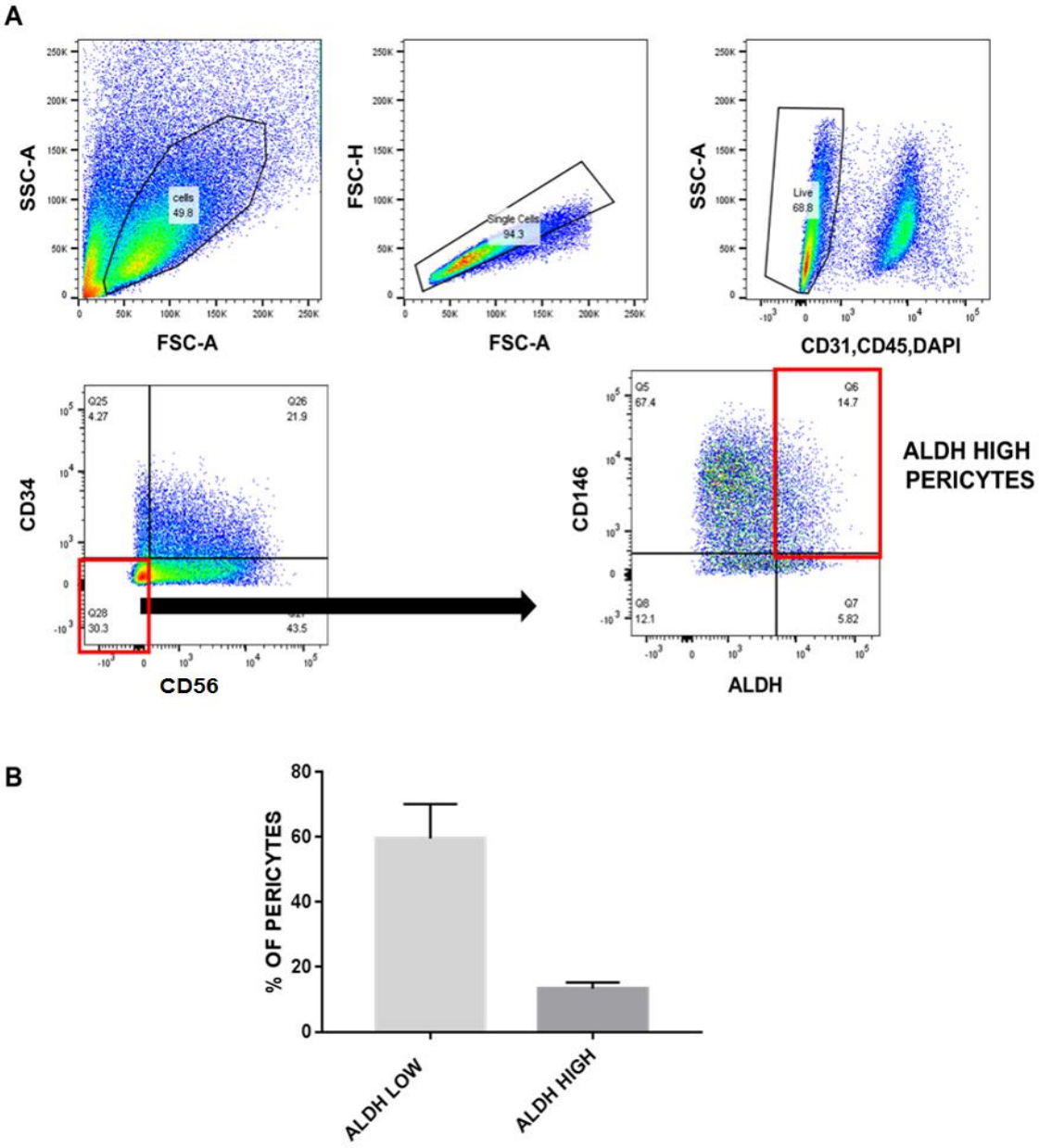


Figure 34 ALDH activity of pericytes from glioblastoma.

A. Tumour brain tissue with glioblastoma multiforme was digested and analysed with the ALDEFLUOR assay and perivascular cell markers. A population of pericytes expressing CD146 has high ALDH activity. **B.** Percentages of pericytes with low/high ALDH activity. n=2

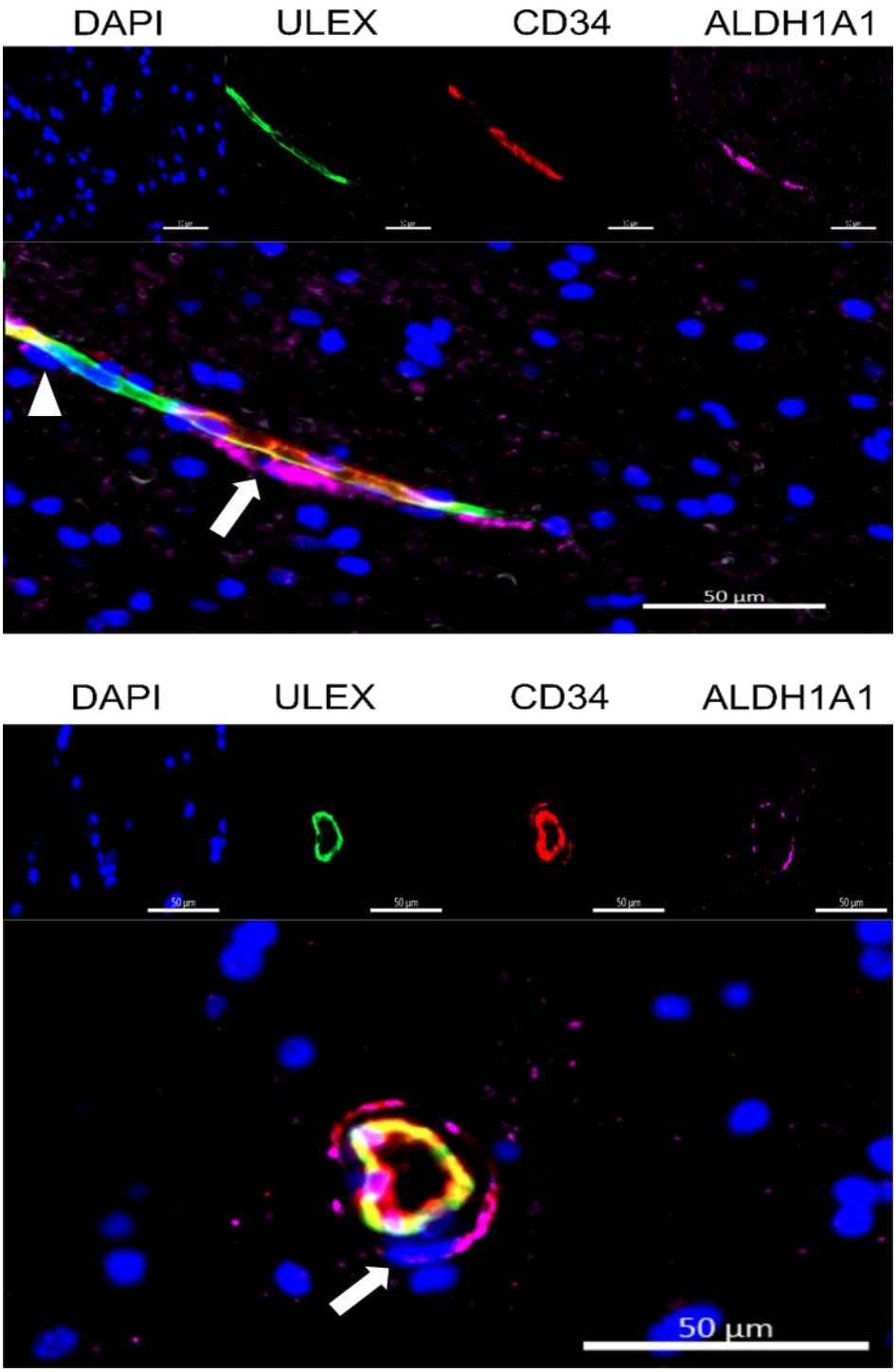


Figure 35 ALDH1A1 expression in perivascular cells in glioblastoma.

ALDH1A1 antibody was used along with perivascular and endothelial cell markers to assess the expression of this ALDH isoform in pericytes and adventitial cells in glioblastoma multiforme. Pericytes expressing ALDH1A1 were found in small vessels through the tissue. Adventitial cells expressing CD34 also express ALDH1A1, however this population was rare. Arrows mark ALDH1A1+ perivascular cells, whereas arrowheads mark ALDH1A1- perivascular cells. Scale bar represents 50 µm. ULEX: Ulex Europaeus Agglutinin I

5.5 ALDH1A1 characterization in mouse tissues after injury

5.5.1 ALDH1A1 validation in mouse tissues

Once we characterized the expression of ALDH1A1 in normal human tissues, we decided to investigate the role of this new marker after injury. First, we optimized the protocol for antibody staining on mouse tissues (*Figure 36*). A combination of α-SMA, to mark pericytes and smooth muscle cells, and ALDH1A1 was used in the mouse heart. ALDH1A1 expression was specific of the adventitial layer of the aorta (*Figure 36*, A), and found in some large vessels in the heart muscle and muscle cells (*Figure 36*, B&C).

This experiment confirmed the expression of ALDH1A1 in adventitial cells. The aorta shows high expression of ALDH1A1 whereas in other layers it is almost absent. Pericytes expressing ALDH1A1 were not found. This is consistent with what it was seen in the human foetal heart which showed that ALDH1A1 expression seems to be almost exclusively to the adventitial layer.

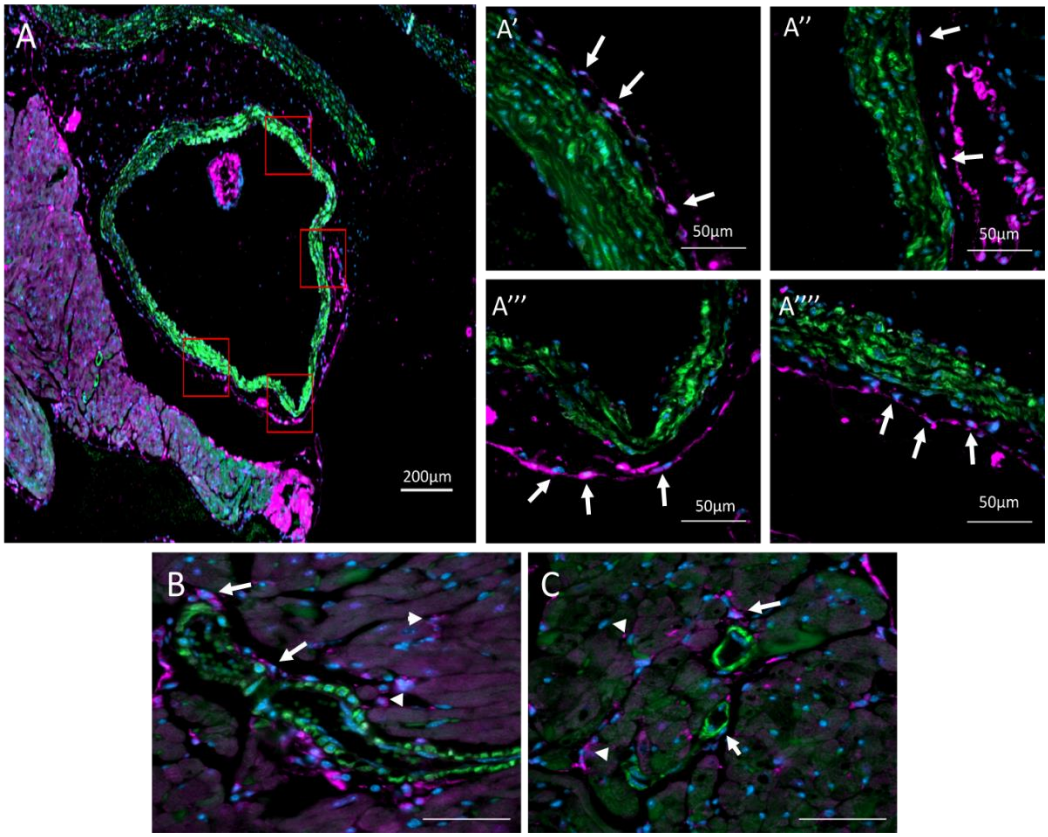


Figure 36 ALDH1A1 expression in the mouse heart.

The ALDH1A1 antibody was used in combination with anti α-SMA to identify pericytes and adventitial cells. α-SMA expression (green) marks the media layer of large vessels. Arrows show cells expressing ALDH1A1 (magenta) in the adventitia of large vessels and arrowheads point to cells expressing ALDH1A1 that are unrelated to blood vessels. Scale bar represents 50 µm. DAPI is shown in blue.n=2

5.5.2 ALDH1A1 expression increases in muscle cells in chronic muscle injury of the rotator cuff

It is known that perivascular cells contribute to fibrotic tissue in skeletal muscle (Kramann *et al.*, 2015; Murray *et al.*, 2017). Previously, members of our laboratory found that PDGFRβ+ cells contribute to fibrosis in mouse chronic rotator cuff injury (Jensen *et al.*, 2018). Therefore, using the same rotator cuff injury, we analysed how ALDH1A1 expression changes at different time points.

In uninjured tissue, ALDH1A1 was detected in the adventitial layer localised adjacent to α-SMA expression marking the media layer (*Figure 37*). Five days after injury, proliferation could be noticed in the muscle fibres in areas proximal to blood vessels with some cells expressing ALDH1A1 in blood vessels. Some muscle cells overexpress ALDH1A1 which was more obvious by 2 and 6 weeks post injury. ALDH1A1 expression in the perivascular area is absent after 2 weeks. By 6 weeks post injury, blood vessels appear to lack the adventitia layer.

In the site of proliferation of muscle cells, we found an upregulation of ALDH1A1 in myocytes and muscle fibres (*Figure 38*). Clearly, fibres with three to five nuclei inside are ALDH1A1 high. These fibres with nuclei in the centre are known to be regenerating fibres. Overall, after injury ALDH1A1 is upregulated in immature muscle fibres. There are also different levels of expression of ALDH1A1 among the tissue. Particularly, some fibres are

ALDH1A1 high compared to other ones, this could mean that new immature myofibers express high levels of ALDH1A1 and once they mature the expression goes back to low/basal levels. Even though, we could not find conclusive evidence that perivascular cells contribute to muscle cells, it does not mean that they do not act as progenitors.

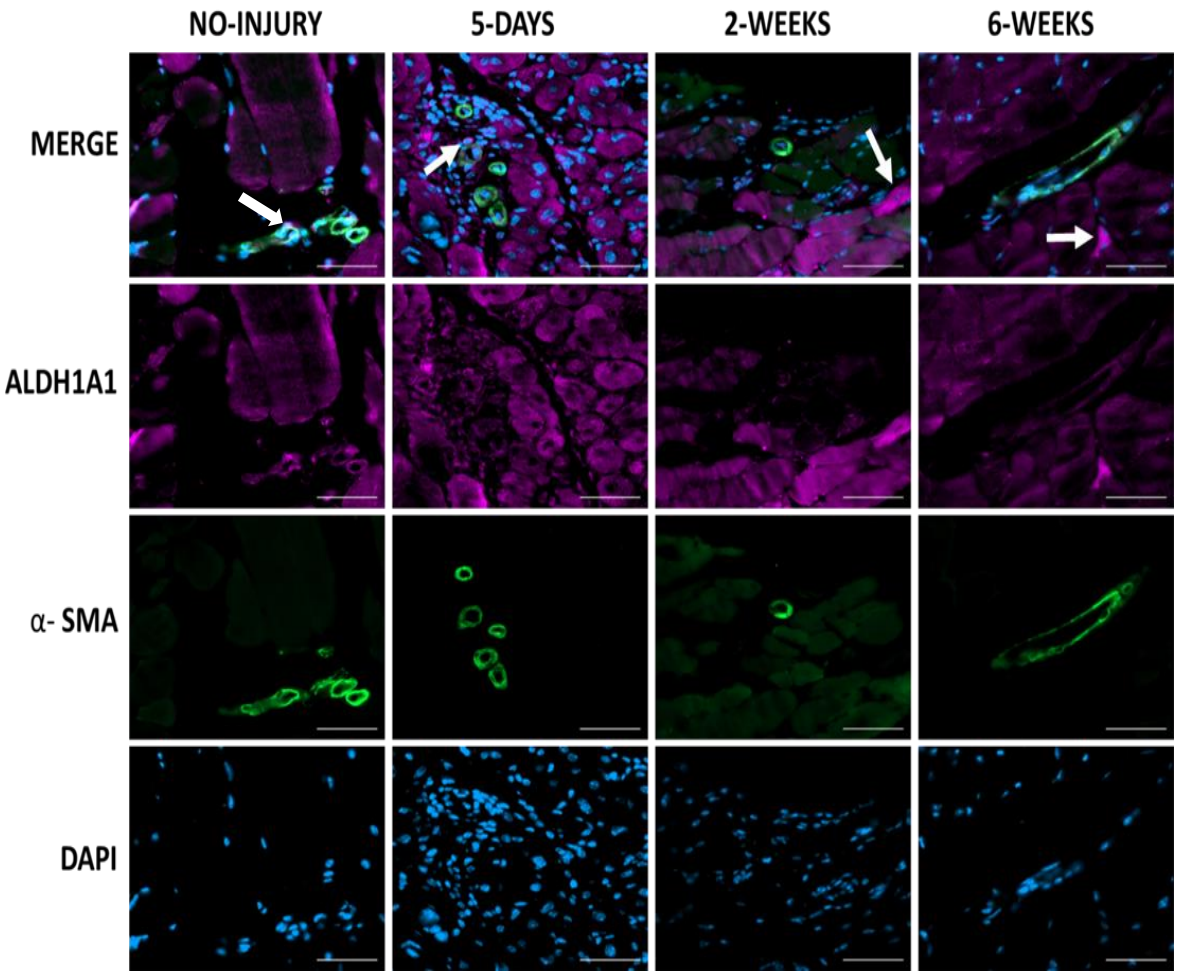


Figure 37 ALDH1A1 expression in chronic skeletal muscle injury.

Uninjured and injured rotator cuff muscles were stained with antibodies against ALDH1A1 (magenta) and α-SMA (green) at different time points (5 days, 2 weeks, and 6 weeks). ALDH1A1 expression increases after injury and is present in muscle fibres and some perivascular cells. After 2 weeks, ALDH1A1 expression in blood vessels is absent while some muscle fibres still express it. Arrows mark ALDH1A1+ cells. Scale bar 50μm. n=2

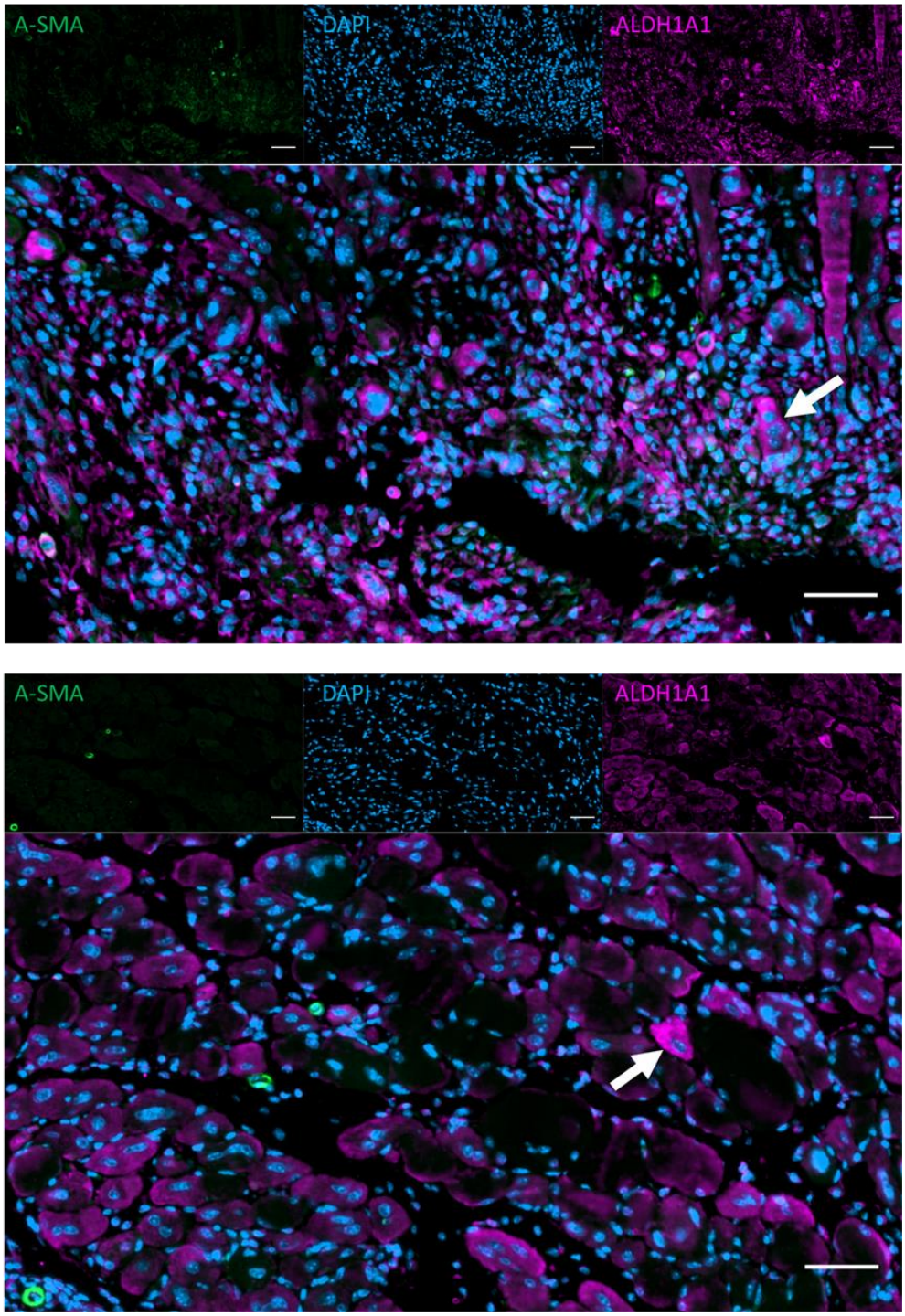


Figure 38 ALDH1A1 expression after 5 days of chronic rotator cuff injury.

Rotator cuff muscle tissue after 5 days of injury was stained with anti-ALDH1A1 antibody (purple) and α-SMA (green). Areas showing signs of cell proliferation upregulated ALDH1A1 in immature muscle fibres. Mature muscle fibres (nuclei on the side) have basal levels of ALDH1A1 whereas in cells that seem to be proliferating the expression in higher. Arrows mark fiber with high ALDH1A1 expression Scale bar 50µm. n=2

5.6 Discussion

In the previous chapter we showed that ALDH1A1 marks a novel population of perivascular cells. Now, I sought to obtain preliminary data on the expression of ALDH1A1 in pathological conditions (cancer and fibrosis). ALDH1A1 expression, as previously mentioned, has been described to mark cancer stem cells and endothelial cells with more angiogenic potential. However, the expression of ALDH1A1 in perivascular cells, pericytes and adventitial cells, has not been characterized in cancer. On the other hand, other subsets of perivascular cells contribute to fibrosis; however, whether these subsets also express ALDH1A1 is not known.

5.6.1 Glioblastoma multiforme contains a population with high ALDH activity, and the isoform ALDH1A1 can be found in pericytes

In 2017 cancer was the second cause of mortality worldwide (Hassanpour and Dehghani, 2017). One of the most lethal tumours is glioblastoma (GBM) which is the most common of central nervous system tumours, with a 5-year survival rate of 5% (Delgado-López and Corrales-García, 2016). One of the main components of the tumour environment is the vasculature which is involved in tumour growth and metastasis (Schaaf, Garg and Agostinis, 2018). While pericytes have been described to be important for tumour growth, little is known about the sub-populations involved.

In this study, I investigated ALDH activity in perivascular cells in fresh brain tissue with GBM and also the expression of ALDH1A1 *in situ* on tissue sections. It was found that there is a population of pericytes expressing CD146 with high ALDH activity which is consistent in at least 2 available donors. Endothelial cells with high ALDH activity exhibit a higher angiogenic potential *in vitro* and higher expression of angiogenesis related genes such as *VEGF-A* and *VEGFR2* (Ohmura-Kakutani *et al.*, 2014). Moreover, these endothelial cells show drug resistance (Hida *et al.*, 2017). Here, it was found that pericytes also include a population with high ALDH activity. Based on previous studies on endothelial cells, I speculate that these cells are also involved in angiogenesis and tumour progression. Furthermore, *in situ*, only a small subset of pericytes express the ALDH1A1 isoform. Could this mean that this sub-population of pericytes expressing ALDH1A1 are the main driver of angiogenesis and tumour growth?

Pericytes are particularly important for tumour growth. Indeed, glioblastoma stem cells can generate pericytes to support vessels which in turn promote tumour growth (Cheng *et al.*, 2013), and also immunosuppression (Sena *et al.*, 2018). Thus, targeting of pericytes from glioblastoma is a feasible strategy to improve chemotherapy (Zhou *et al.*, 2017; Guerra *et al.*, 2018). More recently it was found that glioblastoma modulates pericytes to assist its progression (Valdor *et al.*, 2019).

It is worth mentioning that large vessels were not found in the sections of glioblastoma multiforme, only venules and arterioles in rare occasions. However, these vessels were abnormal and smaller in size compared to the usual large vessels found in other tissues.

In conclusion, this novel population of pericytes expressing ALDH1A1 may be involved in tumour progression and targeting of this specific population may have therapeutic relevance. Indeed, ALDH1A1 has been described to promote angiogenesis in breast cancer (Ciccone *et al.*, 2018).

5.6.2 ALDH1A1 is expressed in adventitial cells of the mouse aorta and interstitial cardiac muscle cells

Next, the expression profile of ALDH1A1 was studied in the mouse. It was found that in normal uninjured heart and skeletal muscle, ALDH1A1 is expressed in perivascular regions of medium to large size vessels. In particular, the adventitial layer of the aorta was highly specific for ALDH1A1 expression. This is consistent with what was found in the transcriptomic and *in situ* analysis of ALDH1A1, which strongly suggests that ALDH1A1 is a marker of the adventitial layer.

The origin of myofibroblasts after injury is not yet clear. Several studies have implicated fibro-adipogenic progenitors and perivascular cells (Moyer and Wagner, 2011; Birbrair *et al.*, 2013, 2014; Kramann *et al.*, 2015; Murray *et al.*, 2017). Different subsets of perivascular cells have been shown to contribute to fibrosis. Nonetheless, other populations of perivascular cells that also give rise to muscle cells which regenerate the tissue has also been reported. PDGFRβ^{+} subsets of cells give rise to fibrotic tissue as well as to muscle fibres. Another type of perivascular subset that is involved in fibrosis is gli1^{+} cells. Kramann et al. have shown that gli1+ cells are involved in fibrosis in different organs and calcification during kidney injury. Furthermore, ablation of these cells prevents fibrosis and calcification (Kramann *et al.*, 2015, 2016; Schneider *et al*., 2017). In the same way, NG2^{+} perivascular cells have been involved in

the maintenance of hematopoietic stem cells (HSC) (Corselli *et al.*, 2013; Gao *et al.*, 2018).

Considering the results found in human in the previous chapter suggesting that native ALDH high cells are involved angiogenesis, proliferation and activation of immune system. Along with the fact that of stem cell marker ALDH1A1 is almost uniquely expressed in the tunica adventitia. I speculate that this subset of adventitial cells expressing ALDH1A1 act as progenitor and regulate immune responses.

5.6.3 ALDH1A1 is upregulated in skeletal muscle fibers

Lastly, chronic injury of the rotator cuff was analysed to assess the expression profile of ALDH1A1^{+} cells after injury. This same model was previously used by our collaborators at UCLA (Jensen *et al.*, 2018).

Here, it was found that ALDH1A1 is expressed by perivascular cells in different areas of the muscle. Interestingly, muscle fibres express ALDH1A1 at basal levels/low expression during homeostasis. Accumulation of cells, resembling proliferation, is seen at 5 days post injury along with upregulation of ALDH1A1 in muscle fibres suggesting a role of this enzyme in regeneration. By week 2 after injury, the tissue looks more normal with some muscle fibres negative for ALDH1A1 and others still expressing it. Finally, by week 6, muscle fibres

similar to the ones in the uninjured tissue are present. However, blood vessels lack an adventitia layer.

Next, I focused on an area with high proliferation at day 5 after injury. Muscle fibres and many cells upregulated ALDH1A1 expression. These cells may be immune cells, PDGFRβ^{+} progenitors, satellite cells, and other cells. The most interesting feature of ALDH1A1 expression in muscle fibres is that only cells part of immature/regenerating fibres upregulated ALDH1A1. Once muscle fibres are mature ALDH1A1 expression returns to basal level. Indeed, ALDH1A1 has been involved in the proliferation (Condello *et al.*, 2015). However, the majority of the research done on ALDH activity and the different isoforms was performed on cancer. Thus, it is difficult to link their findings to our data obtained from perivascular cells in normal tissues or in injury conditions.

Taking into account ALDH1A1 involvement in retinoic acid metabolism which affects proliferation and differentiation of progenitor cells (Tomita *et al*, 2016), it can be suggested that upregulation of ALDH1A1 in these cells is required for progenitors to proliferate. ALDH1A1 is regulated by YAP of the hippo pathway (Song *et al.*, 2018). The hippo pathway, particularly YAP, is involved in proliferation and differentiation of cells (Yu *et al.*, 2013; Varelas, 2014). On another hand, YAP expression in MSCs has been described in pro-osteogenic differentiation while suppressing adipogenesis of cultured bone marrow cells

(Pan *et al.*, 2018). In HSCs, inhibition of ALDH stops the differentiation and increases the numbers of cells, and confers radioprotection (Muramoto *et al.*, 2010). On the other hand, it has been reported that ALDH1A1 expression in HSCs does not affect stem cell function and that it is not a critical regulator of stem cells (Levi *et al.*, 2009). Therefore, ALDH1A1 likely has functional roles involved in differentiation and proliferation perivascular cells.

Overall, our preliminary data support the hypothesis that ALDH1A1 expression after injury marks proliferative cells that are differentiating. In order to further corroborate our data more analysis is needed. For example an animal model where ALDH1A1^{+} cells are labelled with green fluorescent protein (GFP). Moreover, ALDH1A1 function in stem cells is more complicated than previously thought. In some studies with ALDH1A1$^{-/-}$ mice HSCs were normal and stem cell function was maintained (Levi *et al.*, 2009), whereas in other studies ALDH1A1 is more important for stem cell functions (Tomita *et al*. 2016).

Chapter 6 CONCLUSIONS AND CONSIDERATIONS FOR THE FUTURE

6.1 Conclusions

Perivascular cells are a highly heterogeneous population of cells. Both pericytes and adventitial cells are located in different types of vessels. However, both cell types are considered an *in-situ* counterpart of mesenchymal stem cells (MSCs) (Crisan *et al.*, 2008; Corselli *et al.*, 2010, 2012). In this thesis, we investigated different aspects of perivascular cells, and tried to link the native cell type to the *in vitro* counterpart. Adventitial cells with high ALDH activity were the main subject of the study due to having the most primitive phenotype. However, pericytes were also included in the transcriptomic analysis, especially in the *in vitro* conditions in order to assess the identity of both types of perivascular cell once they become MSCs.

First, it was shown that ALDH activity marks a population of progenitors *in vivo* and importantly, culture conditions make ALDH activity dependent on cell density. Moreover, it was shown that expression levels of ALDH isoforms are similar in both ALDH subsets of adventitial cells once they are expanded in culture, confirming our results with the ALDEFLUOR assay. Overall, the use of ALDH activity as a marker of stem cells needs to be carefully assessed when using cells *in vitro*.

Second, *in vitro* assays of ALDH subsets showed that they become similar after series of passages during expansion showing no significant difference between the subsets in different experiments. However, donor variability

needs to be considered. In the last year, human adipose tissue became scarce due to hospital regulations. More donors will be needed to consider the real distribution of the population, and the donor variability. Another explanation is the clonal section *in vitro*. Indeed, Selich *et al*. showed that clonal selection takes place at early passages and that only a few clones gave rise to the whole population of cells (Selich *et al.*, 2016). Moreover, the phenotype change from perivascular cell to MSCs erases the perivascular identity of these cells at least in regards to adventitial cells.

Third, MSCs are the most used stem cells in clinical trials. However, there is a lack of understanding of the basic biology of these cells and where they come from. The majority of the research done on MSCs focuses on cells expanded *in vitro* without taking into account the origin of these cells and how this may affect therapeutic efficacy. Here, RNA sequencing analysis showed that freshly sorted pericytes and adventitial cells have different transcriptomes. Importantly, once they become MSCs after expansion *in vitro*, transcriptomic differences are maintained between pericytes and adventitial cells. This is especially important for therapeutics since MSCs from different types of perivascular cells have different gene expression profiles, they may have different functions and capacities to promote regeneration. Indeed, pericytes and adventitial cells have different abilities to promote angiogenesis after hind limb ischemia (Gonzalez *et al*., in preparation). Moreover, perivascular cells have different capabilities to regenerate bone and when used in combination,

adventitial cells and pericytes resulted in better bone regeneration (Wang *et al*. 2019).

In addition to transcriptomic differences between adventitial cells and pericytes in culture, the use of freshly sorted cells may also be better for cell therapy. Indeed, cells are similar *in vitro* and differ from their native identity. Others have used ALDH high cells with good results. However, freshly isolated cells were used for this experiment (Capoccia *et al.*, 2009). Therefore, more studies of how culture expansion may affect clinical efficacy are required. It is also worth mentioning that the ALDH high compartment contains other subsets of perivascular cells.

Finally, a novel marker for perivascular cells was identified. ALDH1A1, a stem cell marker, was found expressed in a subset of perivascular cells, especially in the adventitial layer of large vessels. This sub-population of adventitial cells may have specific functions in homeostasis and during injury, including acting as progenitors for myofibroblasts and muscle cells. However, an animal model where cells expressing ALDH1A1 are tagged is needed to analyse their contribution in injury and aging. Furthermore, ALDH1A1 was also expressed in a subset of pericytes in glioblastoma multiforme. These pericytes may be the drivers of angiogenesis for tumour growth as it has been demonstrated for tumour derived endothelial cells. Lastly, ALDH1A1 was expressed upon injury in muscle fibers in a model of chronic rotator cuff injury. Although this is not

conclusive it is indicative that ALDH1A1 expression may be needed for cells to proliferate and differentiate.

In conclusion, our results show that ALDH high adventitial cells in large vessels have a different transcriptome compared to ALDH low adventitial cells and pericytes and that the ALDH1A1 isoform is the enzyme active in the ALDH high compartment. Furthermore, we also documented how culture conditions change these perivascular cells once they become mesenchymal stem cells *in vitro*. We obtained preliminary data of perivascular cells expressing ALDH1A1 in brain cancer that may be involved in tumour growth. Finally, using two injury models, we found that ALDH1A1 is expressed by perivascular cells and muscle cells after injury, suggesting that ALDH1A1 is involved in the proliferation of progenitor cells.

6.2 Considerations for the future

MSCs come from perivascular cells and are used in many different disease settings. However, the following questions need to be answered in order to properly assess consistency of their use in cell therapy:

- Do pericytes and adventitial cells have different regenerative capacities after they become MSCs?

- Is there competitive advantage of one of the types of MSC progenitors and how consistent between donors will it be?

- Do MSCs derived from perivascular cells from different organs have differential tissue specific regenerative capacity?

Lastly, in order to complement the results found in this thesis, the following needs to be performed:

- Increase the donor pool for the *in vitro* analyses
- Do RNA sequencing in the ALDH subsets to have a sample size of at least 3 and perform statistics on them
- Perform injury in an animal model where ALDH1A1 cells are labelled to track them upon injury
- Inject freshly sorted and cultured ALDH high cells to further analyse what conditions may be better for therapeutics, culture expanded or freshly isolated cells?

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