MESENCHYMAL PROGENITOR CELLS IN HUMAN DENTAL PULP: PURIFICATION AND CHARACTERISATION

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Doctor of Dental Surgery

The University of Edinburgh

2021

DECLARATION

This dissertation is my own work 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.

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ABSTRACT

Introduction

Characterising the dental pulp perivascular cells will allow understanding whether these blood vessel associated cells have tissue regenerative potential that can be used for craniofacial regeneration, including bone, nerve, and tooth repair. Perivascular cells (pericytes and adventitial cells) can be purified from multiple human organs including placenta, bone marrow, pancreas, fat and muscle, and exhibit in culture and *in vivo* multilineage mesodermal developmental potential, implying the perivascular origin of the elusive, culture-derived mesenchymal stem cells (MSCs). Perivascular cells are ubiquitous in the organism and their developmental potential is partially imprinted by their tissue of origin. Pericytes in the dental pulp, which are deriving from primary or permanent teeth, originate from migrating cranial neural crest cells, which, theoretically, could exhibit superior potential to regenerate neural tissue. To test this, my aim was to characterise dental pulp cells and to evaluate their regenerative capacity.

Material and methods

To achieve this goal, we identified and isolated perivascular cells present in the human dental pulp. Dental pulp tissue was isolated from healthy third molar teeth from adults younger than 30 years old. Firstly, immunohistochemistry was performed to determine the presence of cell surface markers on dental pulp pericytes and adventitial cells

using markers such as αSMA, PDGFRβ, LepR, CD107a, CD146, NG2 and CD34. Blood vessels were next characterised using MSC markers such as CD44, CD90, and CD29 and endothelial cell markers CD54, CD34, UEA-1, and vWF on pulp sections by immunofluorescence microscopy. Secondly, purification of dental pulp perivascular cells was performed using flow cytometry, followed by culturing and differentiation assays towards osteogenic, adipogenic and neurogenic cell lineages.

Results

We found that the phenotype of pericytes in the dental pulp *in vivo* is similar to that of pericytes in other human organs previously documented. They express CD146, ALP and PDGFRβ and lack endothelial cell markers such as CD34, vWF, CD54 or UEA-1. Importantly, we also found that pericytes are heterogeneous and can be further discriminated based on NG2, αSMA and LepR that coincide with a particular blood vessel type or blood vessel diameter. For example, αSMA is mainly expressed in microvascular pericytes and occasionally in capillaries, similar to LepR. NG2 is not expressed in venules but it is expressed in capillaries and arterioles. In contrast, MSC markers CD90, CD44, and CD29 were expressed on all pericytes suggesting that MSCs reside in heterogeneous perivascular niches.

Dental pulp mesenchymal stromal cells were next derived from CD146+CD34-CD56-CD45- pericytes or NG2+ and NG2- pericyte subsets and from CD146-CD34+CD56-CD45- adventitial cells, another perivascular cell source of MSCs located around large vessels. Our data show that, similar to MSCs, all perivascular cell subsets we purified were able to adhere to plastic dish, to expand in culture, and to efficiently accumulate

calcium deposits upon osteogenic induction, reminiscent of bone development. However, most of cell lines tested were not adipogenic with the exception of rare cells in the NG2+ pericytes subset. Indeed, lipid droplets were consistently found accumulating only in these purified pericytes from 14 days onwards, albeit at a low frequency.

Finally, we discovered that, *in situ,* neural cells, positive for CD56 and vimentin, share markers with pericytes such as NG2 and CD146. We also found that *in vitro*, our perivascular cell-derived mesenchymal stromal cell lines express tubulin β III upon neuronal induction although these results require further confirmation.

Conclusions

We documented the molecular signature of pericytes in the human dental pulp. We demonstrated that perivascular cell subsets can be prospectively purified to homogeneity and that perivascular cell-derived stromal cell cultures can be established *in vitro*. Intriguingly, our *in vitro* MSC functional assays showed that these cells are not multipotent, and thus are not genuine MSCs. They are, in contrast, very potent bone progenitors with a low frequency of arterial pericytes being adipogenic. Importantly, we here showed a marker overlap between neural cells and pericytes. Whether these cell types are developmentally linked remains to be established.

GRAPHICAL ABSTRACT

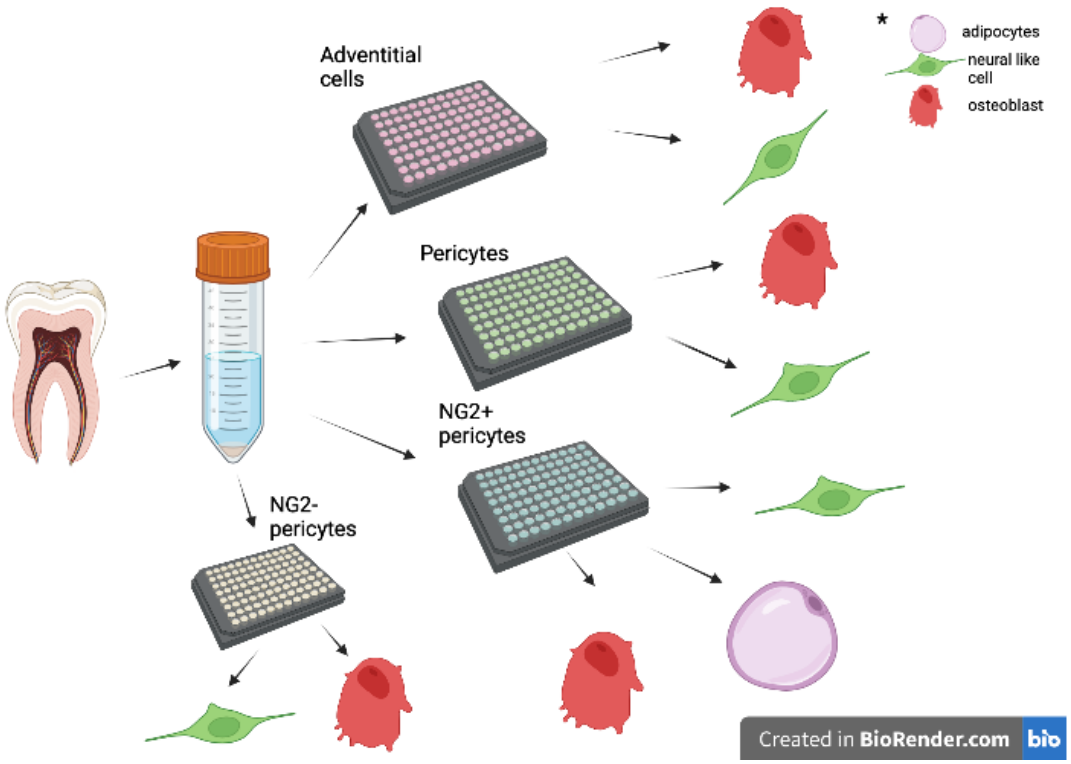

Figure 1. Graphical abstract.

Human dental pulp perivascular mesenchymal cell (DPpvMSCs) types following sorting demonstrated proliferation capacity as well as ability to differentiate into different cell lineages when exposed to specific media.

LAY SUMMARY

Replacement of lost bone or teeth continues to be a challenge for dental surgeons. Treatment modalities currently available include removable prostheses, fixed crown and bridgework, and inserted titanium implants. An alternative possibility to regenerate lost dental tissue could result in a significant improvement in quality of life for many patients.

Some stem cells called “mesenchymal stem cells” are found along blood vessels and can be cultured and multiplied for the treatment of numerous diseases. When these cells are surrounding large vessels, they are called adventitial cells, and when they are around small blood vessels, they are called pericytes.

The perivascular stem cells (PSCs) can be isolated from all vascularized tissues, including the dental pulp, and exhibit dramatic regenerative potential in bone, skeletal muscle, and other tissues. Although PSCs are ubiquitous in the organism, their developmental potential is partially imprinted by their tissue of origin.

My project was to characterise and purify human dental pulp perivascular cells and assess their ability to form nerve, bone or fat tissue *in vitro.* To this aim, we have first identified the different cell types on frozen thin sections of the dental pulp, by microscopic detection of molecules – or “markers” – that are unique to each of these cell types. Next, we have purified these cells by flow cytometry, a technology whereby cells are sorted according to the marker(s) they express, which have been tagged with a fluorescent compound detected by a laser beam. Finally, purified candidate stem cells have been exposed in culture to molecular factors that can drive their

development and maturation into bone cells, fat cells, or neural cells, which are recognizable in the culture dish by their morphology and expression of new molecular markers. Our results show that the human dental pulp contains perivascular cells – pericytes and adventicytes – that can give rise to bone and cells that resemble those found in the neural system although the latter needs further confirmation. Furthermore, we observed that only a subset of pericytes, called NG2+ pericytes, had the ability to yield fat cells in culture although only a few were observed. All together, these early results pave the way to the identification of easily accessible stem cells that might be used therapeutically for dental and maxillofacial regeneration and engineering.

ACKNOWLEDGEMENTS

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I would like to thank my supervisors Prof. Bruno Peault, Dr. Mihaela Crisan and Prof. Angus Walls for their support, guidance and inspiration.

I offer my sincere appreciation to Prof. Bruno Peault for the opportunity provided to work as a member of his team.

I would really like to thank Dr. Mihaela Crisan; this work would not have been possible without her mentoring, patience and encouragement from the very beginning of this project until the final draft of this dissertation.

I am extremely grateful to Dr. Bianca Vezzani, Dr. Leticia Fracaro and Dr. Mario Armando Gomez-Salazar for their patience, advice and understanding, without whom I would not have made it this far.

I would also like to thank all the members of both laboratories for their help and support: Dr. Joan Casamitjana, Dr. Isaac Shaw, Dr. Diana Sa Da Bandeira, David Craig and Dr. Zaniah Nashira.

I am really grateful for the help of the people from the core facilities at the institute: Fiona Rossi, Claire Cryer, Bertrand Vernay and Matthieu Vermeren as well as all the members of the Oral Surgery team at the Edinburgh Dental Institute and Mrs. Norma O'Connor. Without their help, I would not have been able to finish my project.

Finally, I wish to acknowledge the support of my family, my sister, Christina; my brother, George; my mother, Emily and my friends Aman, Marialena, Keith, Joanna, Spiridoula. They kept me going on and this work would not have been possible without their input.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AD-MSCs - adventitial mesenchymal stem cells

CNS - Central Nervous System

DAPI - 4’,6-diamidino-2-phenylindole (DAPI,

DPSCs - dental pulp stem/ stromal cells

DPpvSCs - dental pulp perivascular stem cells

DMEM – Dulbecco's modified Eagle medium

FACS - fluorescence activated cell sorting

hDPSCs - human dental pulp stem cells

MSCs - mesenchymal stem/stromal cells

NG2 - Neuron-glial antigen 2

PC- MSCs - pericyte mesenchymal stem cells

PDGFRβ - platelet derived growth factor receptor beta

SHEDs - stem cells from human exfoliated deciduous teeth

UEA-1 - Ulex europaeus receptor

vWF - von Willebrand factor

CHAPTER 1

INTRODUCTION

Dental tissue

Anatomy of human teeth

Humans are diphyodonts, with an initial primary or deciduous dentition, followed by a secondary or permanent dentition. With very few exceptions, the deciduous teeth erupt into the mouth after birth. These exceptions include natal and neonatal teeth with incidence of 1 in 2000-3000 births and frequently occur in children born with a cleft lip and palate. (Haith and Benson, 2008). After the age of approximately three years, all the primary teeth are usually present. The permanent teeth start to appear at the age of around six years. The first permanent tooth that erupts is the lower first molar which has no deciduous predecessor. At around the same period of time, the anterior deciduous teeth begin to exfoliate, and these are replaced by their permanent successors. This process is normally complete by the age of 18 years. Considering that the average life is around 75 years, the functional lifespan of the primary teeth contributes only to 5% of it (Figure 2).

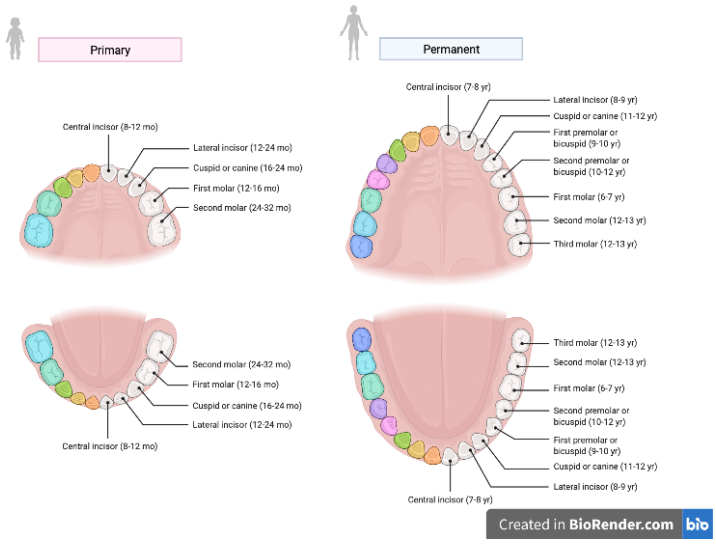

Figure 2. Eruption Chart for Primary and Permanent Teeth. **Image created using BioRender.**

Both primary and permanent teeth have three forms: incisiform, canineform and molariform. Incisiform teeth consist of the central and lateral incisors; they have thin crowns like blades for cutting. Canineform teeth are made up of the canines; they are used for tearing or piercing and have a pointed crown like a cone. Finally, the molarfiiform teeth include the molars and premolars; these are used for grinding food and have a number of cusps. Premolars or bicuspid teeth are only present in the permanent dentition.

A human tooth consists of two main parts, namely the crown and the root. The crown is the portion of the tooth visible in the oral cavity and has an outer layer of enamel. The root part is covered by cementum and is usually attached to the alveolar bone of the jaws by a network of periodontal ligaments (Figure 3).

The enamel is the hardest and most mineralized substance in the human body It covers the outer layer of the teeth and it is partially responsible for tooth colour. The enamel consists mostly of minerals; primarily hydroxyapatite and its colour may vary from light yellow to a grey looking white, as it is semi translucent. Enamel forms a very strong barrier that protects the inner, more sensitive layers of the tooth from any pathology such as decay. Enamel has a very important role in protecting teeth from decay. Enamel unlike other tissues of the human body, such as bone, does not contain any living cells, so it cannot regenerate. On the other hand, the recent research from Elsharkawy and colleagues was very promising for enamel regeneration with the use of elastin-like recombinants (ELRs) (Elsharkawy et al., 2018). Dentine has less minerals and it is a living tissue since it contains the od processes. The odontoblasts derive from ectomesenchyme cells originated by migration of neural crest cells at the

early phase of the craniofacial development. The dental pulp is a soft, fibrous connective tissue and lies within the central cavity of the tooth enclosed by the dentine (Figure 3). Blood vessels are responsible for the he dental pulp vitality and neuros for the transmission of sensory signals. Odontoblasts are located in the outermost layer of the dental pulp. Through this, it creates the pulp-dentine border as a layer of columnar shaped polarised cells able to deposit dentine matrix under pathological and physiological conditions (Goldberg and Smith, 2004). At the root surface, the cementum covers the dentine and the periodontal ligament (PDL) is responsible for connecting and supporting the tooth with the alveolar bone (Figure 3). The dental and surrounding tissues which house the tooth within the alveolar bone are termed collectively as the periodontium (Nanci and Bosshardt, 2006).

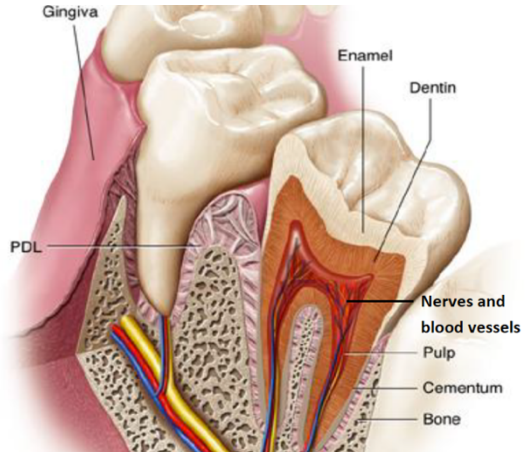

Figure 3. Tooth anatomy; The dental pulp tissue is located in the centre of the tooth.

The odontoblasts are cells based in the pulp and produce dentine, which surrounds and protect the dental pulp. Enamel covers the dentine at the crown level. At the root part, the cementum covers the dentine of the tooth and also allows the periodontal ligament (PDL) to attach to the alveolar bone in order to stabilize the tooth. Image adopted from Cate 1998. (Cate, 1998).

Embryology and morphology of dental pulp

Tooth development is a biological process that occurs in the developing embryo and it involves complex signaling between ectodermal and neural crest derived mesenchymal tissue (Tucker and Sharpe, 2004). At the sixth week of embryogenesis, after migration of the neural crest cells into the head and neck mesenchyme, ectoderm covering the stomodeum begins to proliferate giving rise to the dental lamina. From the dental lamina, following ecto-mesenchymal interactions, ovoidal structures start to separate and develop into tooth germ (Figure 4). The neural crest cells differentiate into the dental organ, called dental papilla, and dental follicle, forming the main part of the dental and periodontal structures (d'Aquino et al., 2008). Therefore, dental pulp is made of mesodermal components, containing neural crest cells that display plasticity and multipotential capability (Kerkis et al., 2006). Once the mineralisation is complete, the pulp resides in an environment that is protected from potential differentiation stimuli. The dental pulp ,in human ,is thus formed by four layers which are, from the outer to the inner part a) the odontoblasts, which are producing dentine, b) the cell-free zone, rich in extracellular matrix components, c) the cell-rich zone containing progenitor cells, and, d) the inner layer, which comprises the vascular area and nervous plexus (Graziano et al., 2008). Yamazaki and team reported the presence of neural crest cell-derived mesenchymal cells in developing teeth (Yamazaki et al., 2005)

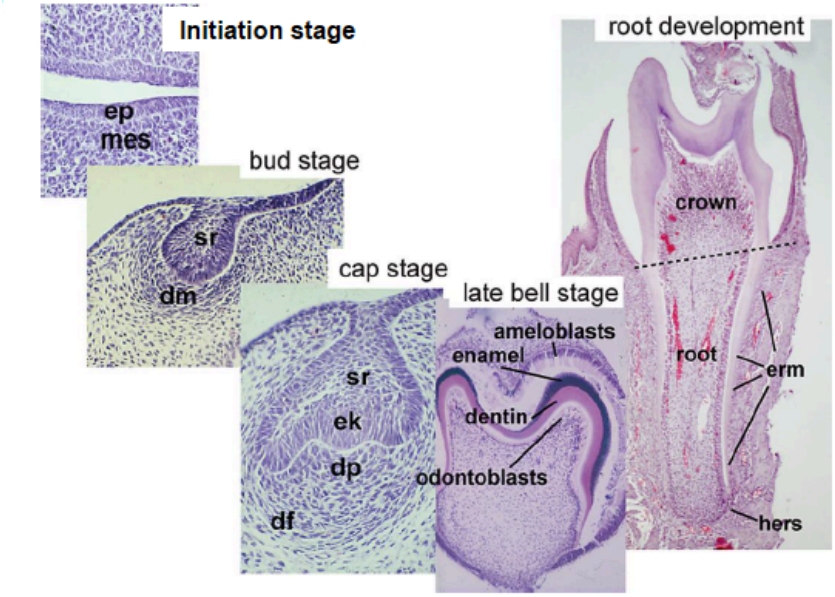

Figure 4. Tooth development stages: histological.

Crown formation is the initial stage followed by root development after the completion of the first one. At the bell stage, the epithelium derived ameloblasts and odontoblasts originating from the mesenchyme deposit of the enamel and dentine respectively. The root is covered initially by dentine and cementum which are quite soft as ameloblasts and enamel are missing. Ep: epithelium, mes: mesenchyme, sr: stellate reticuculum, dm: dental mesenchyme, dp: dental papilla, df: dental follicle, ek: enamel knot, erm: epithelial cell rests of Malassez, hers: Hertwig's epithelial root sheath (Thesleff, 2008).

Mesenchymal stem cells

Definition and discovery of mesenchymal stem cells

The presence of cells with osteogenic potential in the bone marrow was described by Tavassoli using entire fragments of bone-free bone marrow (Tavassoli and Crosby, 1968). However, Alexander Friedenstein was the first one to describe these cells in a more detailed manner when he found that a population of adherent cells from human bone marrow gives rise to clones, and could give rise to fibroblasts in culture (Friedenstein, 1976; Friedenstein et al., 1974, 1966). The observation he made was that these cells are not affiliated to the hematopoietic cell lineage and have the ability to give rise to bone and cartilage-forming cells.

Further characterisation of these cells performed by Owen and Friedenstein (Owen and Friedenstein, 1988) and Piersma and team (Piersma et al., 1985) and in addition to further investigations (Friedenstein et al., 1987; Wakitani et al., 2002) demonstrated that these cells, isolated by plastic adherence, can form osteoblasts, chondrocytes, adipocytes and myoblasts. Accordingly, 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). Caplan drew a parallel with the stem cells at the origin of mesodermal tissues in the embryo, and he was a pioneer to grow these cells from human tissues (Caplan, 1991). These studies led to a first definition of MSCs

as non-hematopoietic multipotent progenitors that have the potential to differentiate into cell types of the mesodermal lineages like adipocytes, chondrocytes and osteoblasts (Chamberlain et al., 2007) (Figure 5).

The first organ to be studied as a source of MSCs was the bone marrow. Bone marrow has been considered the usual source of adult stem cells and is generally used in the treatment of haematological diseases, despite its collection being a painful procedure and with a low yield of MSC (Haynesworth et al., 1992; Pittenger et al., 1999). Haynesworth and colleagues cultured and expanded bone marrow MSCs from the iliac crest of human donors and they demonstrated that human bone marrow has also cells with osteogenic potential (Haynesworth et al., 1992b). Furthermore, antibodies identifying SH-2 and SH-3 as unique cell surface antigens on MSCs were created by the same group (Haynesworth et al., 1992a). The ligands of the SH-2 and SH-3 antibodies were described later by Barry and colleagues as CD105 and CD73, respectively (Barry et al., 2001, 1999). Identifying MSCs could be based on ability to adhere and proliferate in culture, expression of cell surface markers: CD73, CD90, CD105, CD44, CD124 and capacity to produce mesodermal cell lineages *in vitro* (Haynesworth *et al.,* 1992a; Barry *et al.*, 1999, 2001). Pittenger and team isolated human bone marrow MSCs from more than 50 donors that were expanded, and were differentiating to osteogenic, adipogenic and chondrogenic lineages upon induction. The cells showed a normal karyotype and telomerase activity (Pittenger et al., 1999).

. Due to the difficulty of the bone marrow collection and the low number of cells obtained, other sources of MSCs have been investigated such as dental pulp (Gronthos et al., 2000a), skin (Shih et al., 2005), adipose tissue (Rodeheffer et al., 2008; Rodriguez et al., 2005; Xu et al., 2005; Zuk et al., 2002), placenta (In ’t Anker et

al., 2004), amniotic fluid (Nadri and Soleimani, 2007), umbilical cord blood (Crisan et al., 2008b; Erices et al., 2000), skeletal muscle (Crisan et al., 2008b).

As stated earlier though human MSCs have been firstly isolated from the bone marrow (Caplan, 1991), since then, they have been isolated from the stroma of practically all post-natal tissues (Meirelles et al., 2006; Sagar et al., 2018; Zuk et al., 2002). MSCs do not have a specific marker that they express and they are a mixed unique population of cells (Keating, 2012). The International Society for Cellular Therapy (ISCT) set the following characteristics in order to identify an MSC (Dominici et al., 2006):

- Be adherent to plastic
- Be able to self-renew
- Express the cell surface markers CD105, CD90, CD44 and CD73
- Do not express the surface markers CD45, CD19, CD14, CD11b, CD34, CD79α, and HLA-DR
- Be able to differentiate into osteocytes, chondrocytes and adipocytes.

Mesenchymal stem cells, apart from their differentiation properties *in vitro*, also provide *in vitro* support to hematopoietic stem cells and they are considered of high interest for cell therapy due to their regenerative and immunomodulatory properties (Majumdar et al., 1998). MSCs have been already used in almost 1000 clinical trials, though not all were successful (ClinicalTrials.gov).

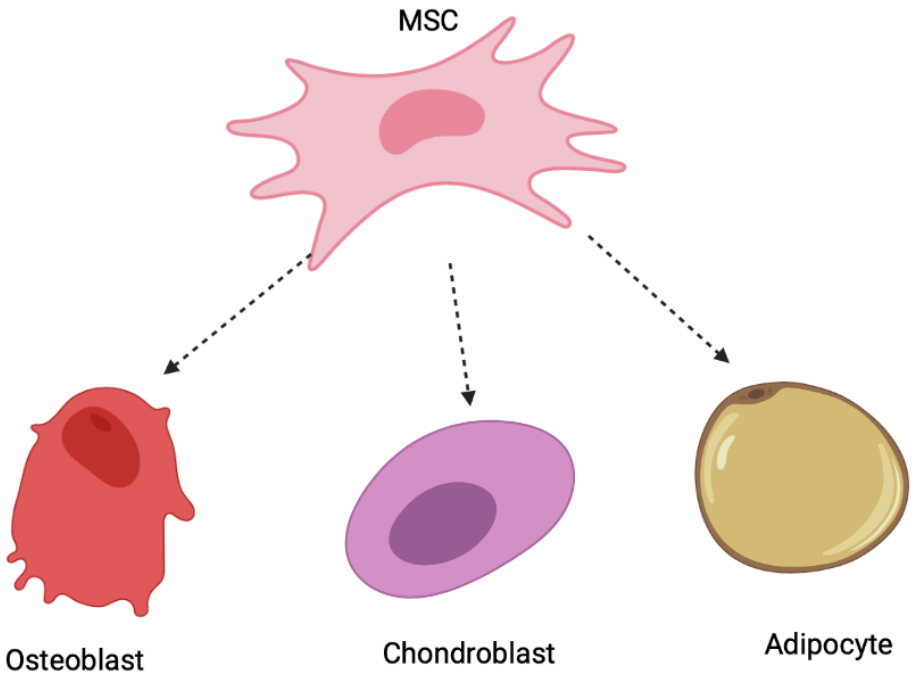

Figure 5. Mesenchymal stem cell tri-differentiation potential.

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

Immunomodulatory properties of MSCs

Mesenchymal stem/stromal cells are extremely valuable for cell therapy due to their regenerative and immunomodulatory properties (Kode et al., 2009). The isolation and culture expansion of human bone marrow MSCs were reported in 1992 (Haynesworth et al., 1992b), which were subsequently expanded and reinfused to patients, 1993-

1995 (autologous transplantation) (Lazarus et al., 1995). The MSCs have also been used to treat patients with breast cancer (Koç et al., 2000). Clinical trials have been performed following infusion procedures. It is now well documented that MSC are highly beneficial due to the release of growth factors and cytokines along with extracellular vesicles to trigger cell proliferation, prevent apoptosis, and finally improve regenerative responses (Shigemoto-Kuroda et al., 2017; Y. Yang et al., 2017). Moreover, MSCs can also modulate responses by interacting with different immune cells, either by direct contact or through soluble factors released (Figure 6). Nevertheless, the process which MSCs follow to prevent inflammation and to boost healing is not entirely understood.

The interaction of MSCs with immune cells including natural killer cells and T 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 following signalling by cytokines (Augello et al., 2005). Similarly, the production of antibodies by B cells is also inhibited by MSC administration. On the other hand, interactions between MSCs and B cells are poorly understood, and probably needs other intermediates (Fan et al., 2016). In fact, CD3+ T cells are required for B-cell inhibition (Rosado et al., 2014).

The fact that MSCs do not activate the immune system is multifactorial. That could be due to the lack of expression of the co-stimulatory molecules CD80 and CD86, needed for immune activation, the lack 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 essential

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 takes part in the production of anti-inflammatory IL-10 by macrophages (Németh et al., 2009) and prevents differentiation of monocytes into dendritic cells (DCs) (Spaggiari et al., 2009).

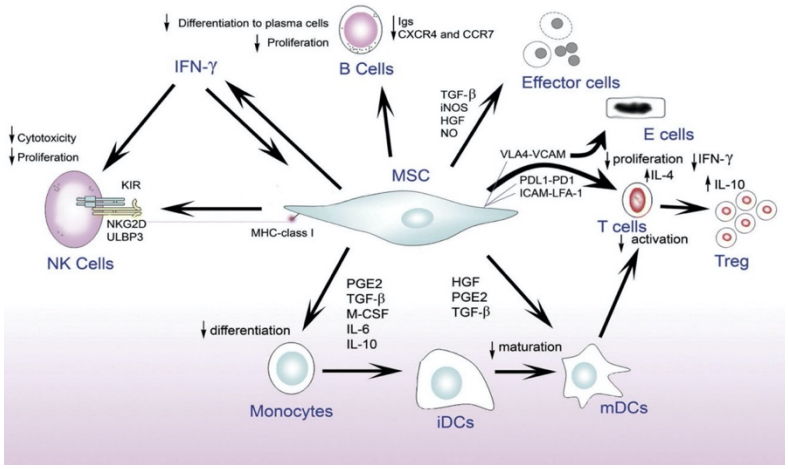

Figure 6. Immunomodulatory properties of MSCs.

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. (Kode et al., 2009)

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 through soluble factors release. One of the major mechanisms of MSC-driven regeneration described is the promotion of angiogenesis (Amado et al., 2005). Moreover, the direct injection of perivascular MSC-progenitors into ischemic hearts resulted in improvement of vascularization in the cardiac muscle (Chen et al., 2015). This improvement is believed to be due to the release of different factors such as vascular endothelial growth factor (VEGF) which activates angiogenesis as well as the nitric oxide synthase (NOS) that blocks fibrosis (Sorrell et al., 2009, Ferrini et al., 2002). Finally, and importantly, 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 scaring (Lai et al., 2010; Liu et al., 2018). It has been shown in a model of carbon tetrachloride (CCL4)-induced liver fibrosis, MSC-derived exosomes alleviated tissue damage (Jiang et al., 2018). However, whilst the use of MSC-derived EVs for cell therapy seems promising, more research is needed to completely appreciate the mechanisms of regeneration. For example, EVs are themselves highly heterogeneous, and developing specifications for their isolation and characterization constitute important guidelines for standardization in the field (Théry et al., 2018).

MSCs in clinical trials and alternative approaches to improve clinical outcome

Clinical trials and problems

Over the last twenty years we have seen clinical use of MSCs, and more than 980 registered MSC trials are listed by the FDA (www.clinicaltrials.gov). The clinical trial results have sometimes been inconclusive. For example, in a phase III trial for the treatment of steroid-refractory graft-versus-host disease (GVHD) where MSCs (Prochymal) were used, there was no significant difference after 28 days when MSC treatment was compared to the placebo control (Martin et al., 2010). Nevertheless, the stratification of the data showed a better response to MSC treatment by children, which then lead to approval of Prochymal in Canada (Reicin et al., 2012). A similar example was the use of cardiopoietic primed bone marrow derived MSCs for the treatment of ischemic heart failure by Celyad, a Belgium-based company, with promising preliminary results. However, further trials revealed no substantial differences between placebo and the MSC treatment (Bartunek et al., 2016, 2013). Finally, MSC treatment has been approved in other countries as well and it is becoming reality despite the controversial results from clinic trials. 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 in Europe, the European Medicine Agency (EMA) recommended the approval of Alofisel as a treatment option for Crohn's disease (Sheridan, 2018).

One of the main problems regarding MSC therapy is the inconsistent nature of the results. The clinical outcomes can be influenced by many variables like the tissue of origin, the donor's gender and the medical history. Other factors that could alter the results are the processing of the tissue, the culture conditions, freezing and thawing of cells along with the administration routes (reviewed by Galipeau and Sensébé, 2018). In addition, MSCs are expanded for long periods in order to gain enough cells for therapeutics, which might induce changes in gene expression and allows clonal selection, therefore affecting biological properties and the heterogeneity of these cells (Gomez-Salazar et al., 2020).

The heterogeneity of MSCs along with the diversity of the tissues they are isolated from adds to this complexity in the nature of MSCs. Bone marrow is the most commonly used tissue to isolate MSCs followed by adipose tissue and cord blood (Gao et al., 2016). MSCs can be obtained from all vascularized organs including skeletal muscle (Corselli et al., 2012a; Crisan et al., 2008b), brain (Lojewski et al., 2015) as well as dental pulp (Shi and Gronthos, 2003a). The MSC's secretome can be influenced by the tissue of origin, as well as from the health status of the donor (Kalinina et al., 2015). The importance of the immune system has been highlighted in the literature; MSCs obtained from diseased donors have shown not promising results , as their angiogenic activity might be affected or decreased, due to the pre-existing pathology (Dzhoyashvili et al., 2014). Importantly, recent studies revealed that age-related dysfunctions also occurred in dental pulp stem cells (DPSCs) (Yi et al., 2017a). Examples of how the age can affect DPSCs include their cell shape that appears enlarged, decreased cell proliferation and altered differentiation potential (Yi et al., 2017b). Also, increases of SA-β-gal activity and $p16^{INK4A}$ expression were

observed in adult DPSCs in comparison to cells from younger populations (Feng et al., 2011).

Many clinical trials have also been contacted aiming to assess the potential of dental pulp stem cells in dentistry. For example, D'aquino and team were assessing bone formation following dental extractions using DPSCs and collagen sponge in comparison of collagen sponge alone. They follow it up for three years and they identify using radiographic and clinical examination, that vertical repair and complete restoration of periodontal tissue were higher at the test site, DPSCS and collagen sponge, than the control site, collagen sponge only (d'Aquino et al., 2009b). On the other hand, in a more recent clinical trial by Barbier and team , who also investigated socket preservation with the use of DPSC, they did not reveal any significant difference between the testing and control group in a six month follow up period (Barbier et al., 2018). Furthermore, clinical trials investigating the success rate of periodontal treatment with the use of DPSCs have taken placed too. For example, Ferrarotti and team contacted a RCT, randomised control trial, using pulp micro-grafts and collagen sponge for the test group and collagen sponge only for the control group. They did follow it up for six and twelve months and clinical and radiographic examination revealed a significant periodontal reduction and a better done defect fill in the test group (Ferrarotti et al., 2018).

As mentioned earlier and as the above examples illustrated, cell therapy can change based on processing technical conditions, donor demographics and organ source. Therefore, cell therapy using MSCs must be customized to a disease or a specific type of injury which might include the pre-conditioning of cells to enhance clinical outcome and screening of the host's immune response (Pittenger et al., 2019).

Despite the high use of these cells for cell therapy, the destiny of transplanted MSCs is not well documented (autologous, allogeneic, or xenogeneic transplantation). The direct contribution of injected MSCs to new tissue formation is little, with only a minor proportion of xenogeneic (human) cells engrafting mouse tissues (Chen et al., 2015b), and those cells not engrafted being cleared from the tissue 72 h post administration (Gholamrezanezhad et al., 2011; Lee et al., 2009; von Bahr et al., 2012). However, this clearing of MSCs may be of clinical interest. It has been revealed that dying transplanted MSCs inundated by recipient macrophages release immunosuppressive soluble factors (Galleu et al., 2017), suggesting that death within host tissues plays an important role in the valuable effects of MSCs.

Alternative approaches to improve the clinical use of MSCs

- Serum free

The ideal conditions for MSCs to be used for cell therapy include the use of medium with no animal products. Fetal calf serum is one of the most common additives for cell culture which properties differ between batches. Passaging of cells, in which proteolytic enzymes are used, is essential to obtain this optimal cell expansion; hence inducing cell damage (Penna et al., 2015).

- Oxygen level

An element that might compromise the therapeutic benefits of MSCs is that MSCs are cultured at high oxygen levels. 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 (Gomez-Salazar et al., 2020). Indeed, hypoxia has been shown to increase proliferation of MSCs (Zhu et al., 2016) although the undifferentiated status and multipotency of the cells has not been affected (Basciano et al., 2011)

- Scaffolds and their characteristics

Different technologies have been proposed to improve the clinical benefit of MSC therapy. One of these technologies is, the use of scaffolds that were first seeded by MSCs prior to engraftment, and are able to provide a higher regeneration. The method of MSC-based scaffolds has been successfully utilized for bone and cartilage regeneration (Kim et al., 2019), as well as for the reproduction of blood vessels (Pinnock et al., 2016), cardiac tissue (Ichihara et al., 2018; Rashedi et al., 2017), and skeletal muscle (Witt et al., 2017). An important factor that can influence tissue replacement efficiency is the physical features of the scaffolds (Alakpa et al., 2017; Jeon et al., 2017; Mouser et al., 2018), as each mechanical property can alter the fate of the transplanted cells. For example, concave textured surfaces can be determinant to the pace of MSC differentiation into osteogenic and vascular cell lineages in comparison with convex surfaces (Graziano et., al 2007). Also, as Alakpa and colleagues showed, stiff matrices can lead to MSC differentiation into osteogenic or chondrogenic cell lineages (Alakpa et al., 2016a), while on the contrary softer substrates can advocate myogenic development (Gilbert et al., 2010). Cell differentiation can be influenced by dimensionality, stiffness, and degradability of the matrix (Caliari et al., 2016).

-3-Dimensional printing, tissue engineering

Another approach to improve clinical outcomes of MSC treatment is the use of 3-dimensional printing (Poldervaart et al., 2017) to copy organ microenvironment (Fatehullah et al., 2016). Tissue engineering is also useful in order to boost MSC residence after administration. With this method MSC-based scaffolds have been used along with either biodegradable or non-degradable polymers to form hydrogel matrices (Park et al., 2018), which can be boosted with growth factors (Gomez-Salazar et al., 2020).

- Cell passages

When human stem cells cultured in the laboratory for several passages are illustrating a typical three-step cell viability profile (Alaminos et al., 2007; Martin-Piedra et al., 2013); an initial adaptation to *ex vivo* cell culture conditions associated with a slight decrease of cell viability, following by an increasing period when cell viability rises and cells gain the top cell viability levels (the most appropriate time period for the use of the cells in regenerative medicine) and finally a decreasing phase. At this stage the cells tend to lose viability and death cell occurs.

The expansion of MSCs chooses the fastest growing clones (Selich et al., 2016). Additionally, MSC clones have different mesodermal differentiation potentials (Muraglia et al., 2000). Regarding the hDPSCs research have shown that eleven to fourteen passages of these cells have adequate cell function, proliferation and viability (Martin-Piedra et al., 2014).

- Uncultured cells

One way for the use of uncultured cells depends 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 conclusion, transplantation of uncultured cells may be the answer to enhance clinical outcome, despite the fact that fewer numbers of cells were obtained than in culture conditions and sometimes may be insufficient for conventional treatment. This is possible only if we were able to purify the presumptive MSC populations that reside *in vivo* in various organs (James and Péault, 2019; Murray and Péault, 2015). To identify the MSC origin *in vivo* in various organs, extensive work has been performed in the recent years by various laboratories including ours.

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 passagings. However, the 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., 2008b). 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 were shown to be MSC progenitors (**Error! Reference source not found.**) (Corselli et al., 2012a; C risan et al., 2008b).

Pericytes as MSC progenitors

Pericytes (PCs), which are also called mural cells or Rouget cells, are cells embedded in the basement membrane of microvessels. Eberth and Rouget were the first to describe them (Rouget, 1873). Zimmermann called them pericytes due to their proximity to endothelial cells (Zimmermann, 1923). Pericytes interact with endothelial cells through different signalling 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 control 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 leads to confusion between the term of a pericyte and a smooth muscle cell. The contractile function of pericytes is strictly regulated. Pericytes respond to β-adrenergic stimuli leading to relaxation, whereas α-

adrenergic signals produce contraction (Rucker et al., 2000). Pericyte contraction is also regulated by oxygen levels. For example, hypoxia increases the contraction of pericytes *in vitro* and CO2 induces relaxation.

Pericytes *in vivo* react to vasoactive substances in the skeletal muscle (Hirschi and D'Amore, 1996). Pericytes have also been described to act as progenitors post-injury (Dellavalle et al., 2011).

MSC progenitors were outlined to be in the perivascular niche. However, identifying pericytes is not easy due to the lack of 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 various organs (Crisan et al., 2008b).

Whether endogenous pericytes are mobilised upon injury *in situ* was further studied. Lineage tracing experiments showed that skeletal muscle pericytes can differentiate into muscle fibres upon injury (Dellavalle et al., 2011) and pericytes that reside in the adipose tissue were shown to be adipogenic (Tang et al., 2008). However, more recently, the involvement of endogenous pericytes *in vivo* has been questioned. Guimarães-Camboa and team showed that in mice, 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). This conclusion

was not supported by the experimental design. Indeed, Tbx18, used to trace pericytes, was questionable since not all pericytes express it. This study suggests however, that not all pericytes contribute to regeneration *in vivo* by directly differentiating into a particular cell type. They may have, however, been implicated indirectly in tissue repair by releasing pro-regenerative molecules. This is in line with one of our studies which 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.

Adventitial cells: another source of MSCs

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 and 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. The tunica adventitia is the outermost layer and consists of connective tissue, fibroblasts, inflammatory cells, *vasa vasorum* and other cells (Moreno et al., 2006) (Figure 7).

For a long time, the adventitia layer was regarded as a supportive 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., 2013, 2006).

Upon injury, cells in the adventitial 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 adventitial layer harbours a population of cells ($CD34^+$ $CD31^-$) able to differentiate into endothelial cells and participate in vessel formation (Hu and Xu, 2011; Zengin et al., 2006). Interestingly, a similar population of cells in the adventitia layer with the following immunophenotype: $CD34^+$ $CD31^-$ $CD146^-$ shows characteristics of stem cells, and give rise to MSCs *in vitro* when purified by FACS (Corselli et al., 2010a, 2012a). 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 participate in calcification (Kramann et al., 2016).

In conclusion, these studies suggest that the adventitia layer acts as a reservoir of progenitors revealing 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.

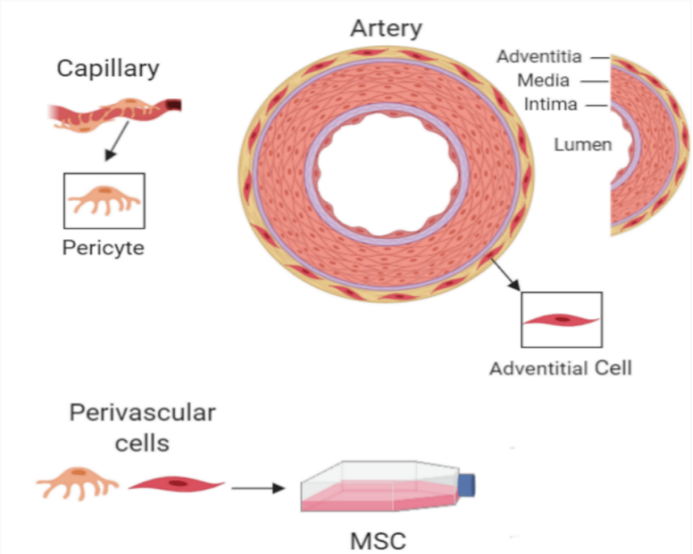

Figure 7. MSC progenitors are located in capillaries and large vessels.

Schematic of MSC progenitors in blood vessels. Figure was made using BioRender.

Heterogeneity of perivascular progenitors and derived MSCs

MSCs are heterogenous, due to the fact that they derive from cultures of total cell suspensions, even though this high heterogeneity is decreased over time *in vitro*, allowing a better protocol standardization. In fact, clonal analysis of MSC cultures for long periods of time has shown that diversity is dramatically decreased after multiple passages (Selich et al., 2016). Additionally, MSC clones exhibit diverse differentiation potentials (Muraglia et al., 2000).

The heterogeneity of conventional MSCs also reflects the diversity of their native progenitors (pericytes and adventitial cells). Research of perivascular MSCs has shown that the phenotype and function of these cells are variable, which also appears to be true to different organs and tissues. A developmental grading of pericytes and adventitial perivascular cells has been set in human adipose tissue (Hardy et al., 2017).

Dental pulp stem cell research and potential applications

Stem cells have been successfully harvested form different dental tissues such as dental pulp (Gronthos et al., 2000a), periodontal ligament (Seo et al., 2004), apical papilla, dental follicle (Zhou et al., 2019), periosteum from maxillary tuberosity (Giordano et al., 2011) as well as from naturally exfoliated deciduous teeth (Miura et al 2003). As stem cells are easily accessible from extracted teeth and with a quite minimal intervention, this source of stem cells has been quite popular in the field of regenerative medicine (Kim et al., 2012).

In 2000, Gronthos first identified and isolated odontogenic progenitor cells from the dental pulp (Gronthos et al., 2000b). These post-natal dental pulp cells showed characteristics of stem cells such as high proliferation capacity and the ability to self-renew following *in vivo* transplantation, for which reason they were called dental pulp

stem cells (DPSCs) (Gronthos et al., 2000b). The self-renewal-activity of stem cells is well known and documented, however only 1% to 10% of the whole dental pulp cell population was identified as stem cells (Gronthos et al., 2002). DPSCs can produce more than eighty passages while retaining their stem cell characteristics (d'Aquino et al., 2009a).

Gronthos and team showed that DPSCs can produce dentine-pulp like complex after transplantation into immunodeficient mice (Gronthos et al., 2000a). The dentin / pulp like complex consisted of mineral matrix with tubules lined with odontoblasts as well as fibrous tissues with blood vessels in a similar arrangement to human tooth dentin - pulp complex (Gronthos et al., 2000a). The ability of DPSCs to differentiate into cells with osteogenic, adipogenic, chondrogenic, neurogenic and angiogenic characteristics was shown by Gronthos and other teams (Gronthos et al., 2000b; Lan et al., 2019; Saito, 2015; Suchánek et al., 2010; Yu et al., 2007).

DPSCs can easily be obtained and due to the fact that their biological function is to form mineralised hard tissue during tooth development, there is a large amount of research investigating their ability for osteogenic differentiation (Kim et al., 2012; Awais et al., 2020). Indeed, DPSCs have been shown to express in culture bone markers including: bone sialoprotein, type I collagen, osteonectin, alkaline phosphatase and osteocalcin (Buchaille et al., 2000; Kuo et al., 1992; Monterubbianesi et al., 2019). Paino and team illustrated the successful fabrication of woven bone-like structure derived from DPSCs after four days in culture. Following that, the woven bone-like structure was transplanted into immune-compromised rats and this

transplantation led to the production of highly vascularised bone tissue (Paino et al., 2017). Similarly, Ikeda and team observed bone tissue formation from cultured hDPSCs with recombinant human BMP-2 (rhBMP-2) (Ikeda et al., 2011). Ikeda and team suggested the treatment with rhBMP2 in osteogenic medium could potentially lead in using dental mesenchymal tissues as an effective source of cells for bone tissue engineering (Ikeda et al., 2011).

On the other hand, the chondrogenic differentiation of DPSCs was not equally promising and quite low compared to mesenchymal stem cells deriving from bone marrow (Balic et al., 2010). More recently, Longoni and team showed that the dental pulp is a valuable stem cell source for the regeneration of fibrocartilaginous tissues, including condylar cartilage, for TMJ regeneration (Longoni et al 2020).

Regarding adipogenesis, Gronthos and colleagues stated that they were unable to develop adipocytes using DPSCs (Gronthos et al., 2000b). However, two years later, this group reported that it was possible to induce DPSCs to form characteristic oil red O-positive lipid-containing adipocytes using a more potent adipogenesis-inductive culture medium (Gronthos et al., 2002), these data were not reproduced by others (Monterubbianesi et al., 2019).

Taking into consideration the neural crest origin of the dental pulp, it comes as no surprise that DPSCs exhibit intrinsic neurological characteristics and can differentiate into neural and vascular endothelial cells (Karaöz et al., 2011). In tone with the above,

Kaukua and team showed that a sub-population of DPSCs have glial cell origin (Kaukua et al., 2014). Young and team have suggested that dental pulp may give a likely alternative source of stem cells for replacement therapy following central nervous system damage (Young et al., 2013). This was shown initially in an animal study by Yu and team (Yu et al., 2002). The potential of DPSCs to differentiate into neuron-like cells was also supported by Rafiee and team (Rafiee et al., 2020).

By the viral induction of Oct3/4, Sox2, Klf4 and c-Myc transcription factors the pluripotency of dental pulp cells was proven (Tamaoki et al., 2010). However, the transcription factors work by integrating to the host DNA and that could trigger tumour genesis (Tamaoki et al., 2010).

As DPSCs have the ability to differentiate after cryopreservation (Zhang et al., 2006), private companies have pioneered dental stem cell banks in order to use these cells in the future (Kim et al., 2012).

DPSC potential clinical applications

When transplanted with hydroxyapatite/tricalcium phosphate (HA/TCP) or nanofibrous poly-L-lactic acid (PLLA), DPSCs differentiated into odontoblasts and formed dentine in immunodeficient mice (Lee et al., 2011; Sun et al., 2014; Wang et al., 2010). The use of the DPSCs was investigated by Gandia and team for the treatment of myocardial infarction in rats (Gandia et al., 2008a). DPSCs were injected intramyocardially in rats in which myocardial infarction was induced. That lead to an improvement in cardiac function and reduction of the infarct size four hours later along with angiogenesis (Gandia et al., 2008b).

The ability to treat muscular dystrophy using DPSCs in golden retrievers was investigated by Kerkis and team (Kerkis et al., 2008). The results of the above research were promising as clinical improvement was noted in one dog which had monthly arterial injections (Kerkis et al., 2008).

Clinical studies have shown the successful use of DPSCs cells in oro-maxillo-facial (OMF) bone repair (d'Aquino et al., 2009). They have used DPSCs on collagen sponge scaffolds and they revealed that DPSCs have good adherence and good vascular bone tissue formation on microconcavity surface textures. These outcomes enforce the concept that DSPCs cells can be used for OMF bone repair and tissue engineering (d'Aquino et al., 2009).

Other studies suggested that DPSCs also have the potential to help with corneal reconstruction. Indeed, Gomes and team demonstrated the successful ocular surface recontraction in an animal model of complete limbal stem cell deficiency with the transplantation of human DPSC (Gomes et al., 2010; Monteiro et al., 2009).

Neurogenesis was also investigated by Nakashima and colleagues. Acceleration of neovascularisation at the ischemic zone as well as neuronal regeneration and decrease of the ischemia and improvement in the functional outcome was seen following the transplantation of human DPSCs into the striatum of the ischemic rat (Nakashima et al., 2009).

There is an increased interest and many attempts have been carried out to generate neural cells using adult stem cells from various resources (Kim et al., 2012). All trials are aiming to regenerate nerves, which could be used for the treatment of nerve injuries and neurodegenerative disorders. Yang and team investigated the potential use of DPSCs on repairing the complete transection of rat spinal cord (Yang et al., 2017). Song and team investigating potential therapies for degenerative diseases, showed both *in vivo* using a rat stroke model and *in vitro* in an ischemia model that DPSCs might be a more promising source in cell therapy for ischemic stroke than human bone marrow mesenchymal cells (Song et al., 2017). In another study, where a rodent ischemia model was used, Nito and team demonstrated that DPSC transplant helped to improve functional recovery of brain damage after acute cerebral ischemia (Nito et al., 2018).

Although some successful methods have been reported and set criteria have been suggested, up to this moment there is no definitive path for the trans-differentiation of MSCs to neural cells (Hernández et al., 2020; Krabbe et al., 2005).

The brain neural stem cells have been shown to express nestin (Takeyasu et al., 2006). Nestin is a neural stem cell marker which is expressed at the first developmental stages and then downregulated in mature tissues (About et al., 2000). The ability of the DPSCs to express nestin as well as GFAP (glial fibrillary acid protein) was shown by Gronthos and team (Gronthos et al., 2002). Also, the differentiation of the DPSCs into neurons, neurospheres , odontoblasts and glial cells has been

reported (Sasaki et al., 2008; Kim et al., 2012). Under neural differentiation conditions, *in vitro,* DPSCs have been tested positive for the following neuronal markers : calcium/calmodulin- dependent protein kinases II, GFAP, TUJ1 (β tubulin III) and tyrosine hydroxylase (Takeyasu et al., 2006). Further research performed by Arthur and team showed that adult human DPSCs developed a neuronal morphology and expressed neuronal specific markers at gene and protein levels (Arthur et al., 2008). They also found that these cells had the ability to produce a sodium current consistent with functional neuronal cells when exposed to neuronal inductive media (Arthur et al., 2008). The same team reported secreted factors which coordinate axon guidance while using human DPSCs in an avian embryonic model (Arthur et al., 2009).

The DPSCs as a new source of mesenchymal stem cells could be a tool for regenerative dentistry and medicine. DPSCs have demonstrated ability to influence angiogenesis (Mattei et al., 2021). Angiogenesis is the formation of new vessels from pre-existing vessels. It is controlled by mutual interactions between perivascular and endothelial cells and has a pivotal role in tissue repair and regeneration (Pittenger et al., 2019). Perivascular and endothelial cells can form a vascular network *in vitro* and *in vivo*. Characterising these dental pulp perivascular cells and exploring their ability towards differentiation *in vitro* will help us to understand their mechanism and the extent of their efficacy and role in regenerative medicine and dentistry.

Project objectives

Characterising and isolating the dental pulp stem cells (DPSCs) will provide further understanding of the potential of these cells in craniofacial regeneration in the event of an injury. Previous published work, including ours, has shown that perivascular cells can be prospectively purified from multiple human organs including placenta, pancreas, fat and muscle and demonstrated their multilineage developmental potential, characteristic of mesenchymal stem cells (MSCs) (Corselli et al., 2010b; Crisan et al., 2008c). Pericytes are therefore of potential interest for regenerative medicine.

Teeth, which are lost in the course of normal development (primary teeth), and commonly extracted in young adults (third molar or wisdom teeth), can be a source of such autologous stem cells, especially since dental pulp is rich in blood vessels. Pericytes in the dental pulp might originate from the migrating cranial neural crest cells (Nayak et al., 1988; Zhao and Chai, 2015) in contrast to pericytes isolated from other tissues, which originate from the mesoderm (Téclès et al., 2005). This led to the hypothesis that the dental pulp pericytes exhibit different characteristics and differentiation potential. Regenerative DPSCs and their mode of action are not fully identified and characterised *in situ.* Evidence from literature suggests that these cells reside within the blood vessels. To test this, my overall objective was to identify and characterise human dental pulp perivascular cells *in situ* and their mesenchymal differentiation potential *in vitro* upon isolation and culture.

Strategy to address the question:

Identify, localise and characterise mesenchymal perivascular stem cells in human dental pulp (molar and premolar). To do this,

a) I performed immunohistochemistry on frozen sections using combinations of markers that distinguish pericytes, endothelial cells and neural cells.
b) I performed flow cytometry to characterise, quantify and purify perivascular cells from the dental pulp.
c) I established perivascular cell lines in culture and I investigated their differentiation potential *in vitro*.

My approach relied first on immunohistochemistry experiments to determine the presence of cell surface markers that could be utilized to sort dental pulp pericytes. Following the above, I then confirmed that sorted dental perivascular cells could be expanded in culture and are multilineage mesodermal progenitors.

Hypothesis

Dental pulp pericytes are heterogeneous and they are endowed with the ability to differentiate into different lineages.

Aim 1: Characterise dental pulp perivascular cells by immunohistochemistry

Markers of pericytes (αSMA, PDGFRβ, LepR, CD107a, CD146, NG2), adventitial cells (CD34), MSCs (CD29, CD44, CD90), endothelial cells (CD54, CD34, UEA-1, vWF) and neural cells (CD56, vimentin) were localised on pulp sections. The tissue sections

were further analysed on a Zeiss Observer inverted microscope and imagine was taken.

Aim 2: Purify dental pulp perivascular cells by flow cytometry

Multi-colour fluorescence-activated cell sorting, FACS, was used to purify to homogeneity pericytes and adventitial cells from the dental pulp. Yield and purity post-sort were assessed by FACS re-analysis prior to culture.

Aim 3: Determine the developmental potential of dental pulp perivascular cells, *in vitro*

The ability of cultured PCs, ADCs, NG2+ PCs and NG2- PCs (6 to 8 passages) to differentiate into adipogenic, osteogenic and neurogenic cell lineages were evaluated in culture using the appropriate inducing medium.

CHAPTER 2
MATERIAL AND METHODS

Dental pulp extraction

Dental pulp will be harvested from impacted third molar teeth in patients younger than 30 years, at the Oral Surgery Department under local anaesthetic at the Edinburgh Dental Hospital. Following extraction, all periodontal tissue will be removed gently from the root surface with Gracey curettes and the teeth will be split by placing a groove longitudinally along the mesial and distal surfaces of the root and crown. Within two hours the tooth will then be split mechanically with a mallet and chisel and the dental pulp retrieved with a straight probe.

MSC isolation and cell culture

Following the above procedure, the dental pulp was digested with collagenases I, II, and IV (final concentration in DMEM +20% fetal bovine serum (FBS)) at 37 ˚C for 45 minutes under gentle agitation in a water bath at 37^0C.

After the tooth pieces were removed and washed with phosphate buffered saline (PBS) + 2% FBS. All material obtained was centrifuged at 1500 rpm for 10 minutes. The supernatant was removed, PBS + 2% FBS was added and homogenised. The samples were then filtered using strainers (100 μm, 70 μm and 40 μm pore size) and again centrifuged at 1000 rpm for 10 minutes. Part of the samples were cultured (MSCs) and another part was submitted to FACS sorting. For MSCs, cells were plated into flasks containing DMEM high glucose supplemented with 1% penicillin-streptomycin and 20% FBS. Cultures were stored in an incubator at 37 °C with 5% CO_2. The culture medium was replaced twice a week. When the cultures reached approximately 80–90% confluence, cells were dissociated using 0.25% trypsin/EDTA (Invitrogen) and re-plated (passage 1).

Immunohistochemistry

After removing the dental pulps, the samples were frozen in Tissue Freezing Medium (Triangle Biomedical Sciences) and cryosectioned at 10-μm thickness. Tissue sections were fixed in ice cold methanol for 10 minutes, air dried for 15 minutes and

then washed with PBS three times for 5 minutes. Sections were next incubated with 5% goat serum (Gibco) in PBS (blocking solution) for 1 hour at room temperature (RT) in a wet chamber to prevent antibody non-specific binding. Excess blocking solution was removed from the slides and the tissue sections were incubated with primary antibody (Table 1) overnight (ON) at 4˚C.

Marker	Supplier	Catalog	Dilution
CD34	BD Pharmingen	550390	1:100
CD146	BD Pharmingen	550314	1:100
PDGFRβ	Abcam	ab32570	1:100
LEPR	Abcam	ab5593	1:100
NG2	Abcam	ab104535	1:100
CD44	BD Pharmingen	550392	1:100
CD90	BD Pharmingen	555593	1:100
CD54	Abcam	ab2213	1:100
ALP	R&D Systems	MAB1448	1:100
CD29	BD Pharmingen	09351D	1:100
αSMA	Sigma Aldrich	F3777	1:300
vWF	Abcam	ab8822	1:200
CD56 (Biotinylated)	Ancell	208-030	1:250
UEA-1 (Biotinylated)	Vector	B1065 20806	1:200
Vimentin			1:200

Table 1. Primary antibodies and isolectin used for detection of perivascular cells in human

The following day, sections were washed twice with PBS for 5 minutes. Appropriate conjugated secondary antibodies were next used (Table 2) for 1 hour at RT.

Marker	Supplier	Catalog	Dilution
Alexa Fluor 488	Invitrogen	A21428	1:250
Alexa Fluor 555	Invitrogen	A21422	1:250
Alexa Fluor 488 conjugated Streptavidin	Life Technologies	532354	1:250

Table 2. Secondary antibodies used to detect primary antibodies

When two antibodies were used simultaneously, the sections were incubated for 1 hour at RT with a second primary antibody that was already conjugated. After two additional washes with PBS, the sections were then stained with 4',6-diamidino-2-phenylindole (DAPI, 1:2000, Molecular Probes) for 5 minutes at RT to visualize nuclei. Coverslips were mounted with Gel/Mount mounting medium containing anti-fading agents (Biomeda Corp.).

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.

Fluorescence-activated cell sorting and cell culture

Freshly digested dental pulp cells were washed with PBS. The tissue was next treated according to our established protocol (Corselli et al., 2012a; Crisan et al., 2008b). Cells were washed and stained with fluorescent antibodies for 30 minutes at 4 ˚C.

Marker	Fluorochrome	Supplier	Catalog	Dilution
CD146	BV711	BD Horizon	563186	1:100
CD31	V450	BD Horizon	561653	1:100
CD34	PE	BD Pharmingen	555822	1:100
CD45	V450	BD Horizon	560368	1:100
CD56	V450	BD Horizon	560360	1:100
NG2	FITC	Millipore	Ab532044	1:100

Table 3. Antibodies used for cell sorting

Cells were then washed with PBS+2% FBS and resuspended in 200 µl PBS+2% FBS. The cell suspension was filtered with 40 µm cell strainer. A FACS Aria dual-laser fluorescence activated cell sorter (Becton-Dickinson) was used to isolate adventitial

cells (CD146-CD31-CD34+CD45-CD56-); pericytes (CD146+CD31-CD34-CD45-CD56-); NG2 positive pericytes (CD146+CD31-CD34-CD45-CD56-NG2+) and NG2 negative pericytes (CD146+CD31-CD34-CD45-CD56-NG2-). DAPI was used to exclude dead cells.

Sorted cells (adventitial and NG2 positive pericytes and NG2 negative pericytes) were cultured at 37˚C, 5% CO_2 in Endothelial Cell Growth Medium 2 (EGM-2, Cambrex Bioscience Inc.) on 96-well tissue culture plates coated with 2% gelatin (Calbiochem) at 10,000 cells per well. The culture medium was replaced twice a week. When the cultures reached approximately 80–90% confluence, cells were dissociated using 0.25% trypsin/EDTA and re-plated (passage 1). After passage 1, medium was changed to DMEM high glucose (GIBCO) with 20% FBS and 1% penicillin-streptomycin.

Osteogenic differentiation

Cells were plated at a concentration of 25,000 cells/cm^2 in triplicate in 24-well plates. The cells were kept in an incubator at 37 °C with 5% CO_2 until reaching 80% confluence. The osteogenic differentiation medium contains DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 50ug/ml L-ascorbic acid (Fisher Biotech), 100mM ß-glycerophosphate (Sigma), and 100nM dexamethasone (Sigma). For control, cells were cultured with DMEM supplemented with 10% FBS without differentiation factors or inducers. The cultures were maintained for 28 days, and the

culture medium was replaced every two to three days. Cultures were checked at day 7, 14, 21 and 28.

To evaluate the presence of calcium crystals, cells were washed with PBS and fixed with 4% PFA for 15 minutes at RT at different time points (7, 14, 21 and 28 days). After fixation the samples were rinsed with distilled water and incubated for 15 minutes in Alizarin Red S (Sigma-Aldrich). Excess Alizarin Red was removed with three washes of distilled water.

Quantification of Alizarin Red S in osteogenic differentiation

To quantify alizarin Red S in osteogenic differentiation, a solution of 10% acetic acid was added to alizarin red-stained cells and incubated with shaking for 30 minutes. Following that, the acetic acid was removed along with the cells by scraping the bottom of the well and transferred into a 1.5 ml Eppendorf tube. On to the next step, the tubes were heated for 10 minutes at 85°C, and were transferred to ice to cool down for 5 minutes. The tubes were then centrifuged for 10 minutes at 21952 RCF, next 500ul were taken into a new tube and then 200ul of ammonium hydroxide was added. After mixing the tubes, 50ul were transferred into a well of a 96 well plate, then absorbance at 450nm was measured in a FLUOstar Omega Microplate Reader (BMG Labtech).

Adipogenic differentiation

Cells were plated at a concentration of 25,000 cells/cm^2 in triplicate in 24-well plates. The cells were kept in an incubator at 37 °C with 5% CO_2 until reaching 80% confluence. The adipogenic differentiation medium contains DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 0.5 mM of 1-methyl-3-isobutylxanthine (Sigma), 1uM dexamethasone (Sigma), 0.01 mg/ml insulin (Cell Sciences), and 0.2 mM indomethacin (Sigma). For control, cells were cultured with DMEM supplemented with 10% FBS without differentiation factors or inducers. The cultures were maintained for 28 days, and the culture medium was replaced every two to three days. Cultures were checked at day 7, 14, 21 and 28.

To evaluate the presence of lipid vacuoles, cells were washed with PBS three times and fixed with 4% PFA for 15 minutes at RT, following by washing with 70% ethanol for a few seconds. The samples were incubated with Oil Red O (Sigma-Aldrich) for 20 minutes at RT at different points (7, 14, 21 and 28 days). Afterwards, the samples were washed with 70% ethanol and distilled water.

Neurogenic differentiation

Cells were seeded at 25,000 cells/cm^2 in 24-well tissue culture plates and coated with laminin (LN511, Biolamina) 10µg/ ml final concentration ON at 4°C. Wells were washed gently with PBS (with Ca++ and Mg++) prior adding the neurogenic medium. The neurogenic medium consists of: Neurobasal A Medium, 100 u/ml penicillin, 100

ug/ml streptomycin, 1 x B27 supplement, 20mg/ml epidermal growth factor (EGF) and 40mg/ml basic fibroblast growth factor (bFGF). Neurogenic medium was prepared the same day and it was replaced twice per week for 28 days. For control, cells were cultured with DMEM supplemented with 10% FBS without differentiation factors or inducers.

At the final day, day 28, the medium was removed from the wells. Wells were washed once with PBS and 4% PFA for 10 minutes was added. Wells were then washed twice with PBS. Following that 0.5% triton in PBS for 3 minutes was added and was removed by washing twice with 0.2% BSA in PBS. Wells were blocked with 10% goat serum for 1 hour at RT, the excess of serum was removed and β tubulin III was added ON at 4˚C. (TUJ1/ β tubulin III MAB1195, 1:1000). The following day β tubulin III was removed, wells were washed 3 times for 10 minutes with 0.2% BSA and the appropriate conjugated secondary antibodies were used (Table 2). After two additional washes with PBS, the wells were stained with 4',6-diamidino-2-phenylindole (DAPI, 1:2000, Molecular Probes) for 5 minutes at RT to visualize nuclei. Wells were analysed on a Zeiss Observer inverted microscope.

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 4X 10X, 20X and 40X were used to take images.

Statistical analysis

Data were analysed using GraphPad Prism software. Number of biological replicates is indicated as (n). Every biological replicate has 3 technical replicates. 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
RESULTS

Participants / Demographics

Participants were identified at the new patient consultation clinic at the Oral Surgery department at the Edinburgh Dental Institute by a single clinician (EB). Information leaflets were given to the patients and they were asked if they wish to participate in the study (appendix section).

Inclusion criteria were:

- Participants should be younger than 30 years of age.
- Attending for removal of a non-carious tooth under local anaesthetic.
- Non-surgical extraction

The day of the appointment, patients were re-consented for the procedure of non-surgical removal of treated tooth, and they confirmed their decision to participate in the study for “Tissue collection of tissue, biospecimens and data for research”

following by the signing of the participant consent form (appendix section). The consent form was next signed by the clinician treating the patient at the day.

The collection of teeth started in the June 2016 and completed in April 2019.

Fifty-four participants, twenty-one males and thirty-three females, were recruited with an age range of 20 to 29 years. The mean age for the total sample was 24.5 years (male 23.4 years, female 25.2 years). From the sample of 54 subjects 83 teeth were collected. From these, 31 teeth were from the male subjects and the remaining 52 were from the female subjects (Table 5). Only four out of the 83 teeth were premolars (3 upper premolars and 1 lower premolar which were extracted for orthodontic preposes). Seventy-nine teeth were third molar teeth which were extracted due to pericoronitis. From the majority of the participants one or two teeth were extracted at the same appointment, 29 and 22 teeth respectively. Three subjects were the exception as four teeth were extracted from one participant and three teeth from the other two participants. 49% of the extracted teeth were maxillary teeth (41 maxillary teeth and 42 mandibular teeth) (Figure 8). Seven of the above teeth were not used due to failure to remove the dental pulp atraumatic (four teeth), incomplete extraction / fracture of root (two teeth) and due to early detection of caries (one tooth).

	Participant number	***Mean Age (years)***	***Teeth number***
Males	21	23.4	31

Females	33	25.2	52
Total	54	24.5	83

Table 4. Demographics of the study, mean of age for males and females and number of teeth

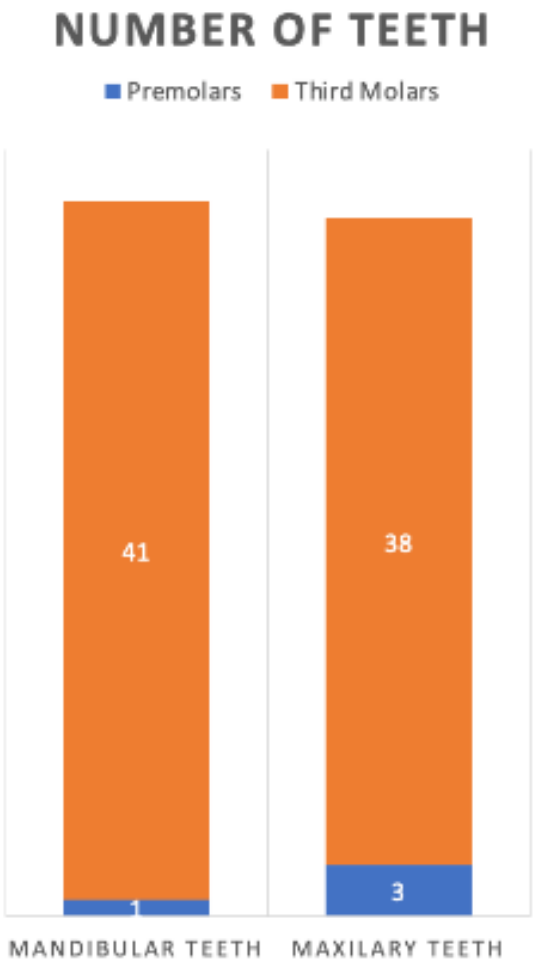

Figure 8. Type of teeth and number of teeth included in the study.

Characterise dental pulp perivascular stem cells by immunohistochemistry

Identification and characterisation of pericytes in the dental pulp

We used immunohistochemistry and various combinations of markers such as αSMA, CD146, PDGFβ, LepR, CD107a to identify and characterise pericytes in the human dental pulp. These markers were previously used by our laboratory to identify pericytes in other human organs (Corselli et al., 2010b; Crisan et al., 2008c, 2012a; Xu et al., 2019, 2020). Since pericytes are localised around the microvasculature we used CD34, UEA-1, vWF and CD54 to mark endothelial cells. The detection of the endothelial cell markers showed the presence of a developed vascular network in the human dental pulp independent of the tooth types tested.

We examined five human dental pulp samples in total, three molars and two premolars, from both female and male subjects aged 20 to 29 years. In the human dental pulp, we noticed the characteristic expression of αSMA by perivascular cells

surrounding capillaries (diameter < 10μ) and arterioles and venules (diameter 10 to 100μm). For example, I found that αSMA expression pattern in the dental pulp is similar to that in other organs. Indeed, αSMA is expressed in larger vessels (Figure 9A-F) and only in a few capillaries (Figure 9C). I have also investigated the expression of leptin receptor (LepR). Also known as CD295 and OB-R, LepR is a type I transmembrane glycoprotein, class I cytokine receptor (Zhang et al., 1997). LepR is expressed in microvessels, in perivascular cells, in human adipose tissue (Corselli et al., 2013) and in healthy and inflamed human dental pulp (Martín-González et al., 2013). Although Martín-González and team showed that LepR is expressed in human dental pulp, they did not identify in which cell type LepR is expressed. (Martín-González et al., 2013). In line with this, we confirmed that LepR is indeed expressed in healthy human dental pulp and we have identified LepR expression in bigger vessels (arterioles and venules) (Figure 10 B, D, E) but not always in capillaries (Figure 10 A, B, C, D). CD107a, a lysosomal membrane protein -1 (LAMP), with functions in cancer metastasis and in immune response (Eskelinen et al., 2003), has also been shown by our laboratory to be expressed in perivascular cells in human adipose tissue, with only 82% of pericytes and 32% of adventitial cells being positive (Xu et al., 2020), highlighting the heterogeneity within these cell compartments. Research from our team had further demonstrated that CD107a- pericytes have higher osteogenic ability (Xu et al., 2020). We have tested CD107a in the human dental pulp, and we found that its expression is associated with pericytes in microvessels (Figure 10 E).

The CD146 marker was also used in our experiments. CD146 or M-CAM or MUC18 is a membrane glycoprotein which is essential for the interaction between endothelial cells and pericytes by binding to laminin a4 (Ishikawa et al., 2014; Shih, 1999). CD146

is expressed by human pericytes and some endothelial cells (Crisan et al., 2008c), smooth muscle cells, B and T lymphocytes (Covas et al., 2008; Tormin et al., 2011) as well as in glioma stem cells (Yawata et al., 2019). We have shown that CD146 is expressed in pericytes in the dental pulp in both capillaries and microvessels (Figure 11 A-C) as well as neural cells (Figure 11 A). Other markers we used that are expressed by pericytes are platelet derived growth factor receptor β (PDGFRβ) and neural glial antigen 2 (NG2). Research has revealed the PDGFRβ fundamental role during capillary sprouting recruiting pericytes due to PDGFβ/PDGF-b signalling as well as the PDGFRβ expression on smooth muscle cells, glial cells and interstitial fibroblasts (Bonner, 2004; Crisan et al., 2008c; Hellstrom et al., 1999; Robbins et al., 1994; Stapor et al., 2014). In the dental pulp, we found that all perivascular cells express PDGFRβ irrespectively of the blood vessel type (Figure 11 D, E, F).

NG2 also known as chondroitin sulphate proteoglycan 4, regulates cell proliferation and migration at vasculogenic stage and binds to collagen α2 and β1 integrin (Stallcup and Huang, 2008). NG2 is expressed on pericytes in the arterial system as well as on macrophages, glial cells and different tumour cells (Crisan et al., 2009; Murfee et al., 2006, 2005). As illustrated in figure 12, we found that not all pericytes express NG2. Indeed, NG2 is expressed in human dental pulp pericytes in capillaries and microvessels (Figure 12 A-D) but is absent in venules (Figure 12 A, B).

In conclusion, human dental pulp pericytes express multiple cell markers that include NG2, PDGFRB, αSMA, LepR and CD107 of which combination changes based on the blood vessel type where they reside. We here confirm that human dental pulp perivascular cells do not express the endothelial cell markers von Willebrand factor (vWF), CD34, Ulex europaeus lectin ligand and CD54 (Figure 9-12). Therefore, we

identify the molecular signature of PCs in dental pulp as CD146+ αSMA+/- NG2+/- PDGFRβ+ LepR+/- CD107a+ ALP+ CD34- CD54- vWF- UEA-1R-.

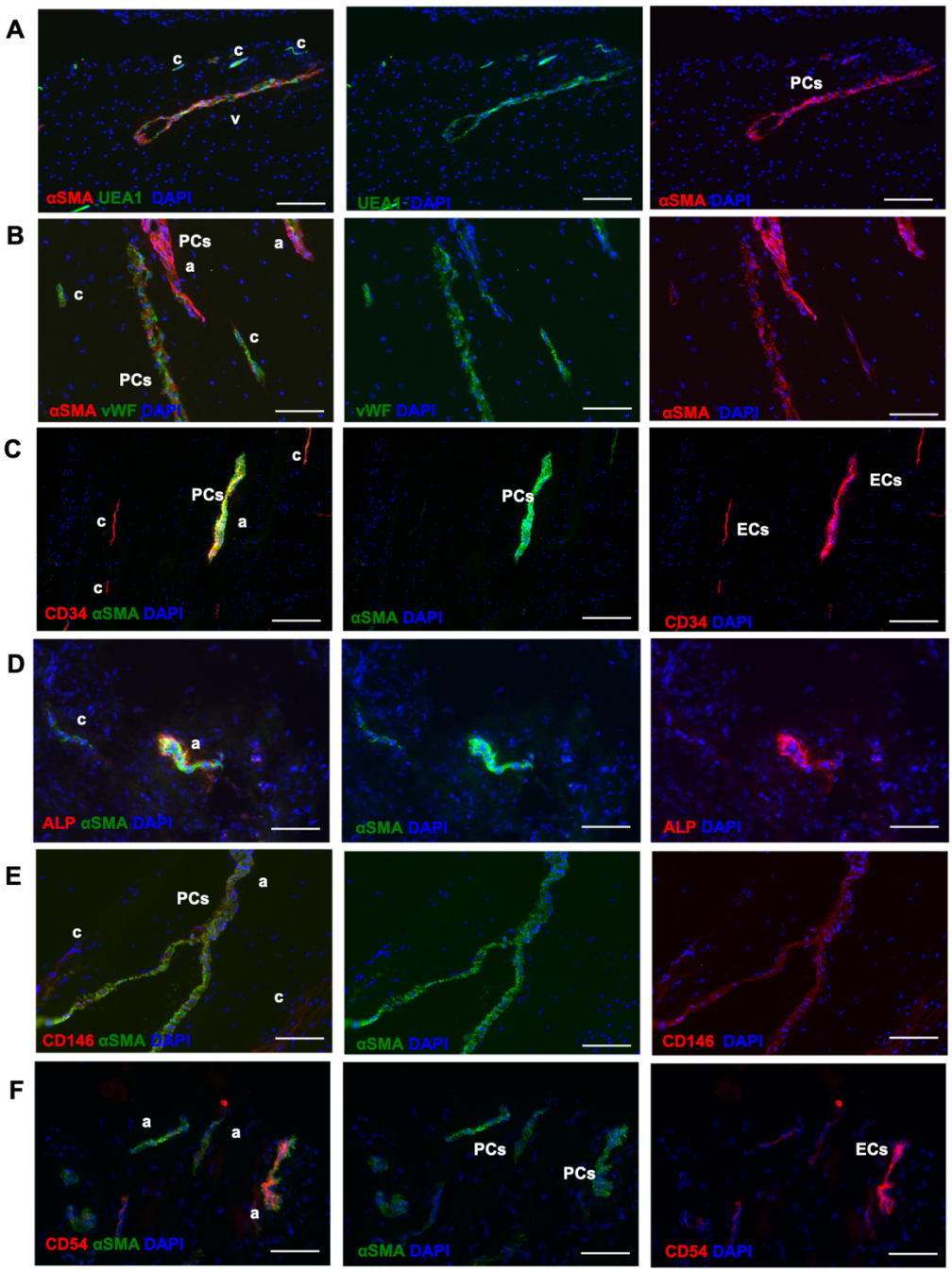

Figure 9. Expression of αSMA on human dental pulp frozen sections in combination with endothelial cell markers UEA-1 (A), vWF (B), CD34 (C), CD54 (F) and pericyte markers ALP (D) and CD146 (E).

n=5 teeth tested (molars/premolars= 3/2, males/female=2/3). All nuclei are stained by Dapi (blue). ECs: endothelial cells PCs: pericytes, a: arterioles, c: capillaries, v: venules. Scale bar= 10μm. (A ,C X10 B ,D ,E ,FX20).

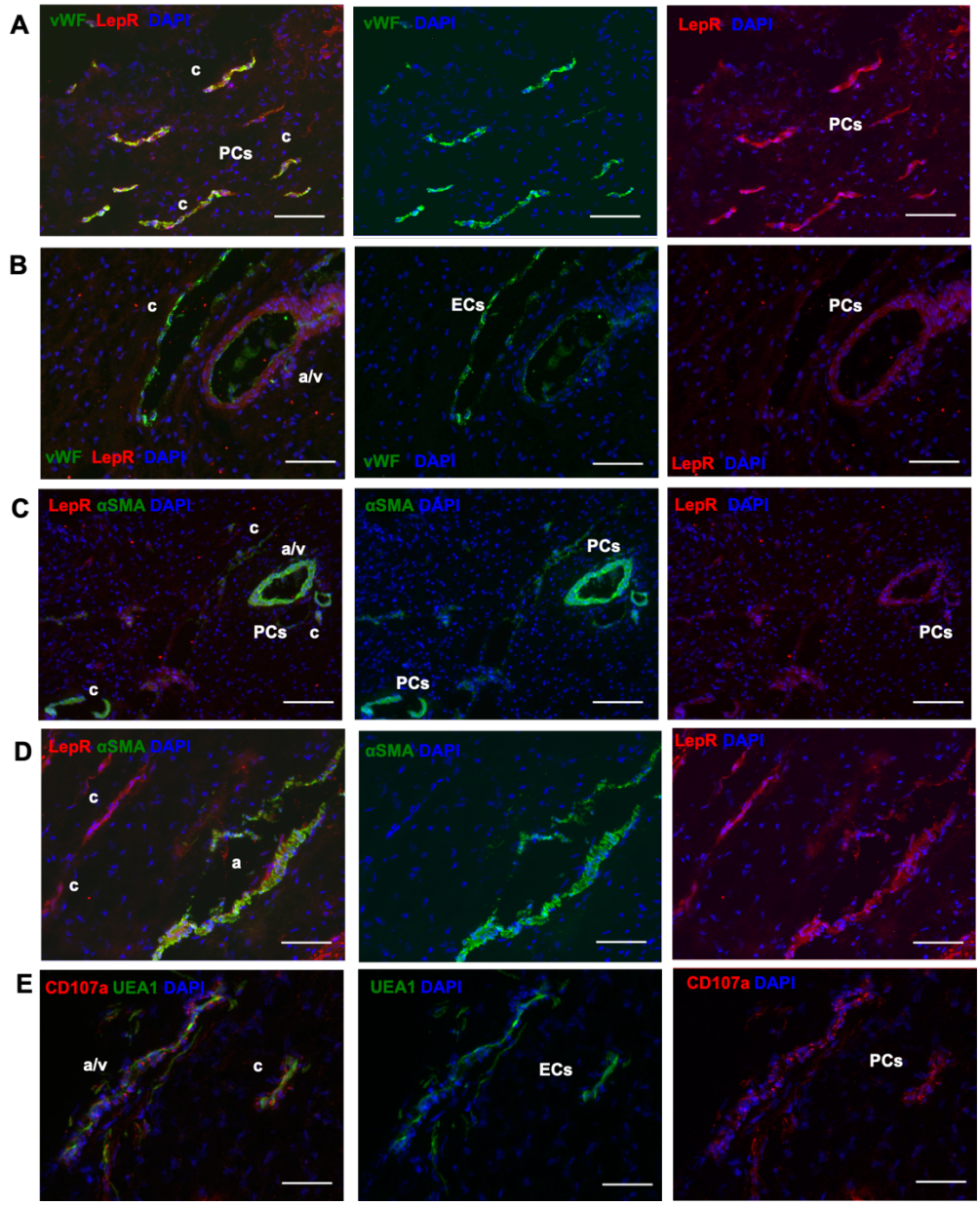

Figure 10. Expression of LepR and CD107a on human dental pulp frozen sections in combination with endothelial cell markers vWF (A, B), UEA-1 (E) and pericyte marker αSMA (C, D)

n=2 teeth tested, molars (A, D, E) /premolars (B, C) = 1/1, both females. All nuclei are stained by Dapi (blue). ECs: endothelial cells PCs: pericytes, a: arterioles, c: capillaries, v: venules, a/v: arterial or venule. Scale bar= 10µm**. (**A,C X10 B,D,E X20).

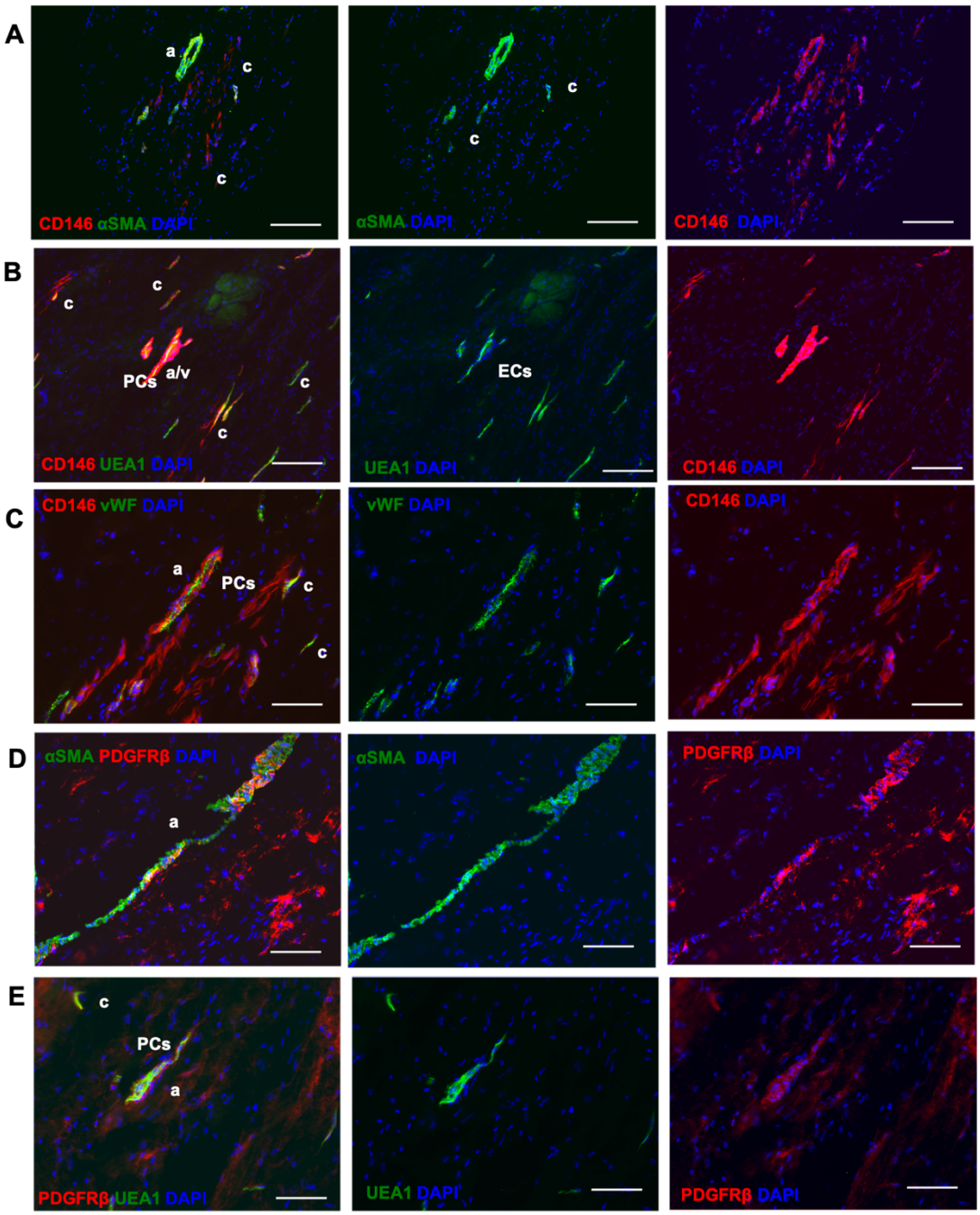

Figure 11. Expression of CD146 and PDGFβ on human dental pulp *in situ* in combination with αSMA pericyte marker (A, D) and endothelial cell markers vWF (C, F) and UEA-1 (B, E).

n=2 teeth tested, both molars and from male subjects. All nuclei are stained by Dapi (blue). ECs: endothelial cells PCs: pericytes, a: arterioles, c: capillaries, v: venules a/v: arterial or venule. Scale bar= 10μm. (A, B, C, E X10 D, F X20).

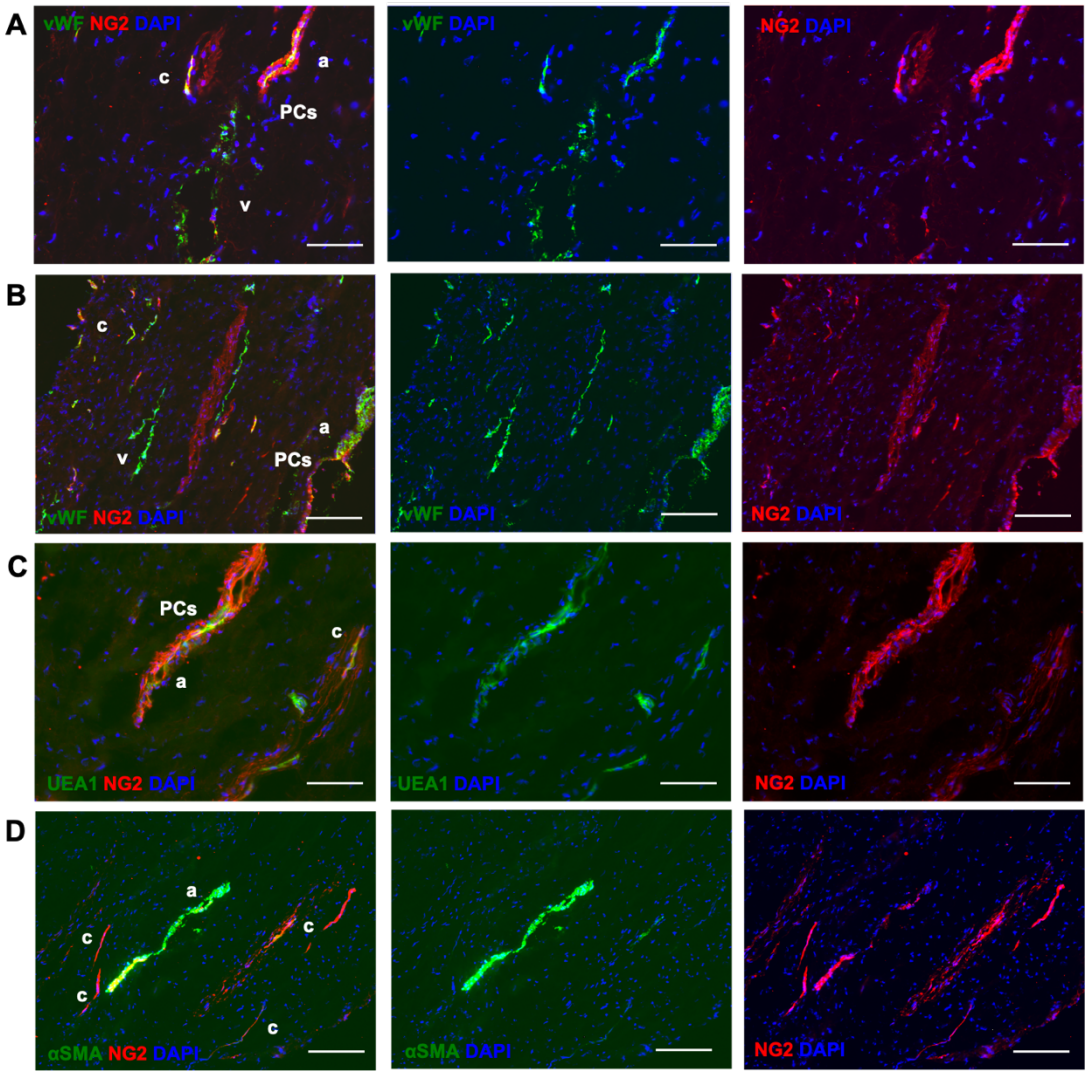

Figure 12. Expression of NG2 on human dental pulp frozen sections in combination with endothelial cell markers vWF (A, B), UEA-1 (C) and pericyte marker αSMA (D). Immunohistochemistry showing pericyte heterogeneity, NG2+ and NG2- pericytes.

n=4 teeth tested (molars/premolars= 3/1, males/female=2/2). All nuclei are stained by Dapi (blue). ECs: endothelial cells PCs: pericytes, NC: neural cells a: arterioles, c: capillaries, v: venules. Scale bar= 10μm. (A, D X10 B, C X20).

Pericytes in the dental pulp co express MSC markers

Since pericytes in other human organs express classical MSC markers both *in situ* and in culture (Crisan et al., 2012a), we next examined some of these MSC markers including CD29, CD90 and CD44 by immunohistochemistry in the human dental pulp in combination with αSMA and vWF. We found that all three markers tested are expressed in human dental pulp and that their expression coincides with that of αSMA (Figure 13 A, B, D). CD44 was not expressed from all endothelial cells as there is no overlapping expression with vWF (Figure 13 C).

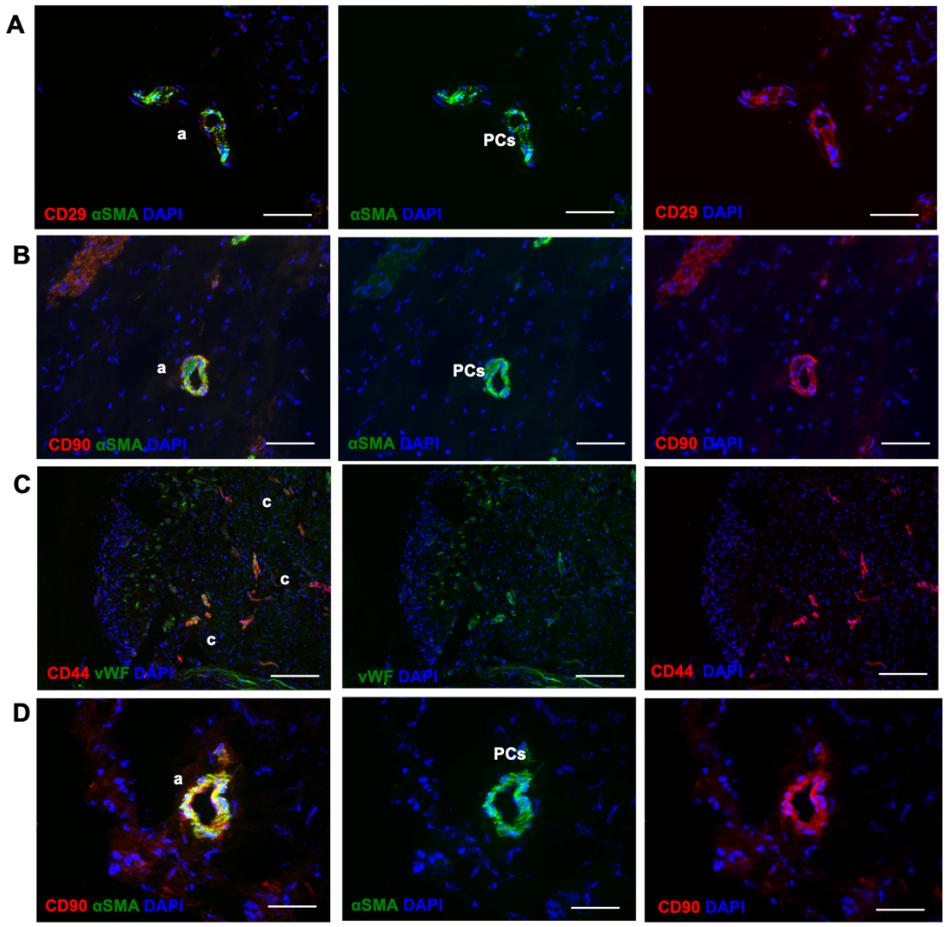

Figure 13. Immunohistochemistry on human dental pulp using antibodies against MSCs, pericytes, and endothelial cells. MSC biomarkers: CD29 (A, D), CD90 (B), CD44 (C), pericyte biomarker αSMA (A, B, D) and endothelial cell marker vWF (C) were used.

Blue marks DAPI staining of cell nuclei. ECs: endothelial cells PCs: pericytes, a: arterioles, c: capillaries. Scale bar= 10μm. (A, B X20 C X10 DX40).

Identification of neural cells in the dental pulp

To identify nerve cells, we used neuronal markers CD56 and vimentin, and combined with the pericyte markers NG2, CD146, LepR and αSMA in both males and females molar and premolar teeth. CD56 or neural cell adhesion molecule (NCAM) is a marker for natural killer, neural and skeletal muscle associated satellite cells (Plappert et al., 2005). CD56 is a member of the immunoglobulin family. CD56 is expressed on neurons and glia in both the developmental and mature nervous system and it has a key factor in synaptic plasticity and memory formation of the mature nervous system (Fields and Itoh, 1996; Jessell, 1988). Vimentin is an intermediate filament protein which can be found in different types of immature cells in the central nervous system (CNS) and body such as neuroepithelial cells. It can also be found in mature cells in the CNS such as: endothelial cells, smooth vascular musculature, fibroblasts (Harvey B. and Flores-Sarnat, 2013).

Interestingly we found that the expression of neural markers coincides with the pericyte expression of LepR, NG2 and CD146 pericyte markers as shown in Figure 14 A, B, C and E. It is important to highlight that not all pericyte markers overlap with neural cell markers. Indeed, we found that neither CD56+ nor vimentin+ neural cells express αSMA (Figure 14 B, F).

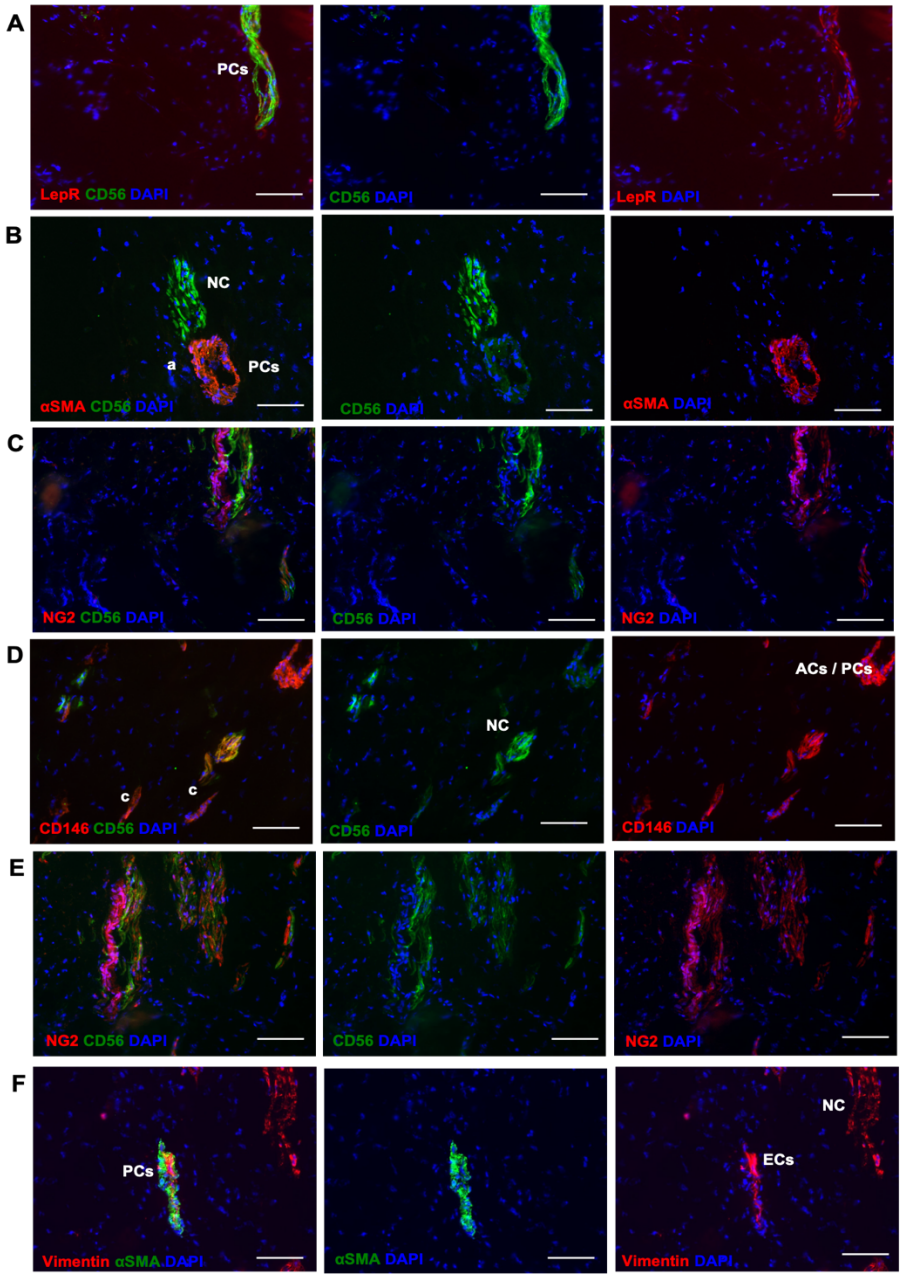

Figure 14. Immunohistochemistry of human dental pulp frozen sections with neural cell, pericytes and endothelial cells markers. Neuronal cell biomarkers: CD56 (A-E), vimentin (F), pericyte biomarkers: LepR (A), αSMA (B, F), NG2 (C, E), and pericytes/endothelial cell marker CD146 (D).

molar/premolar=2/1, male/female=2/1. Blue marks DAPI staining of cell nuclei. ECs: Endothelial cells, NCs: Neural cells, PCs: pericytes, a: arterioles, c: capillaries. Scale bar= 10μm. (A, B, C, D, FX20 EX40).

Purification of dental pulp perivascular cell subsets by flow cytometry

To study the biological function of various perivascular cell populations, we next dissociated the dental pulp in single cell suspension and purified these cells by flow cytometry. Viable cells were selected while excluding debris, dead cells and doublets (Figure 15 A). CD56+ cells were then gated out of dental pulp cell suspensions to avoid contamination due to the overlap with pericyte markers. In order to remove endothelial cells and hematopoietic cells we used negative selection for CD31 and CD45 (Figure 15 B, C). Pericytes were then identified by the expression of CD146 (Figure 15 D). We repeated this experiment three using 4 samples obtained from 1 male and 3 females.

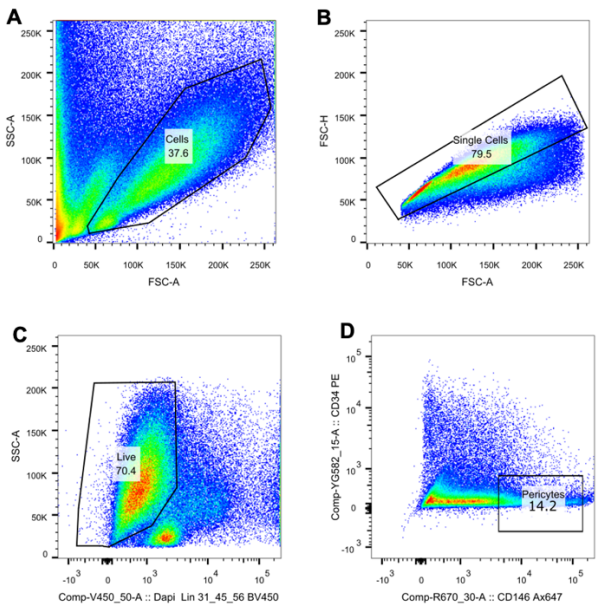

Figure 15. Pericyte isolation from human dental pulp.

(A, B &C) Perivascular cells are identified as live single cells, negative for CD31, CD45, CD56. **(C)** Pericytes were gated based on the expression of CD146. n=3, (4 samples pooled together)

Adipogenic, osteogenic and neural differentiation of MSCs and pericytes from human dental pulp

We found that perivascular cells *in situ* co express MSC markers. These observations lead us to address the question whether pericytes can be cultured *in vitro* and differentiate towards MSC lineages. To do this, we used adipogenic and osteogenic media to induce adipogenic and osteogenic differentiation in our CD146+ pericytes and MSCs and check their ability at various time points such as 7, 14, 21 and 28 days. Results at day 28 are shown in figure 16. We found that both MSCs and CD146+ pericyte cultures after the use of adipogenic medium were unable to accumulate lipid droplets at any time points studied (Figure 16 A). Both cell populations are sharing osteogenic differentiation abilities. Indeed, after the induction of the osteogenic medium, evidence of calcium deposits were visible in both cell populations (Figure 16B). In addition, we also found that pericytes and neural cells share markers *in situ* suggesting a link between pericytes and neural system. Interestingly when neurogenic differentiation was induced in MSCs and CD146+ pericytes, tubulin β III expression was detected by immunofluorescence. However, cell morphology was not indicative to neural cells. Cells appeared with a flat, large elongated body and with long processes extending out of the body. No changes were seen when the control medium was used (Figure 16 C, D)

Nevertheless, whether if this staining is specific remains to be investigated, as no negative control for tubulin β III has been used.

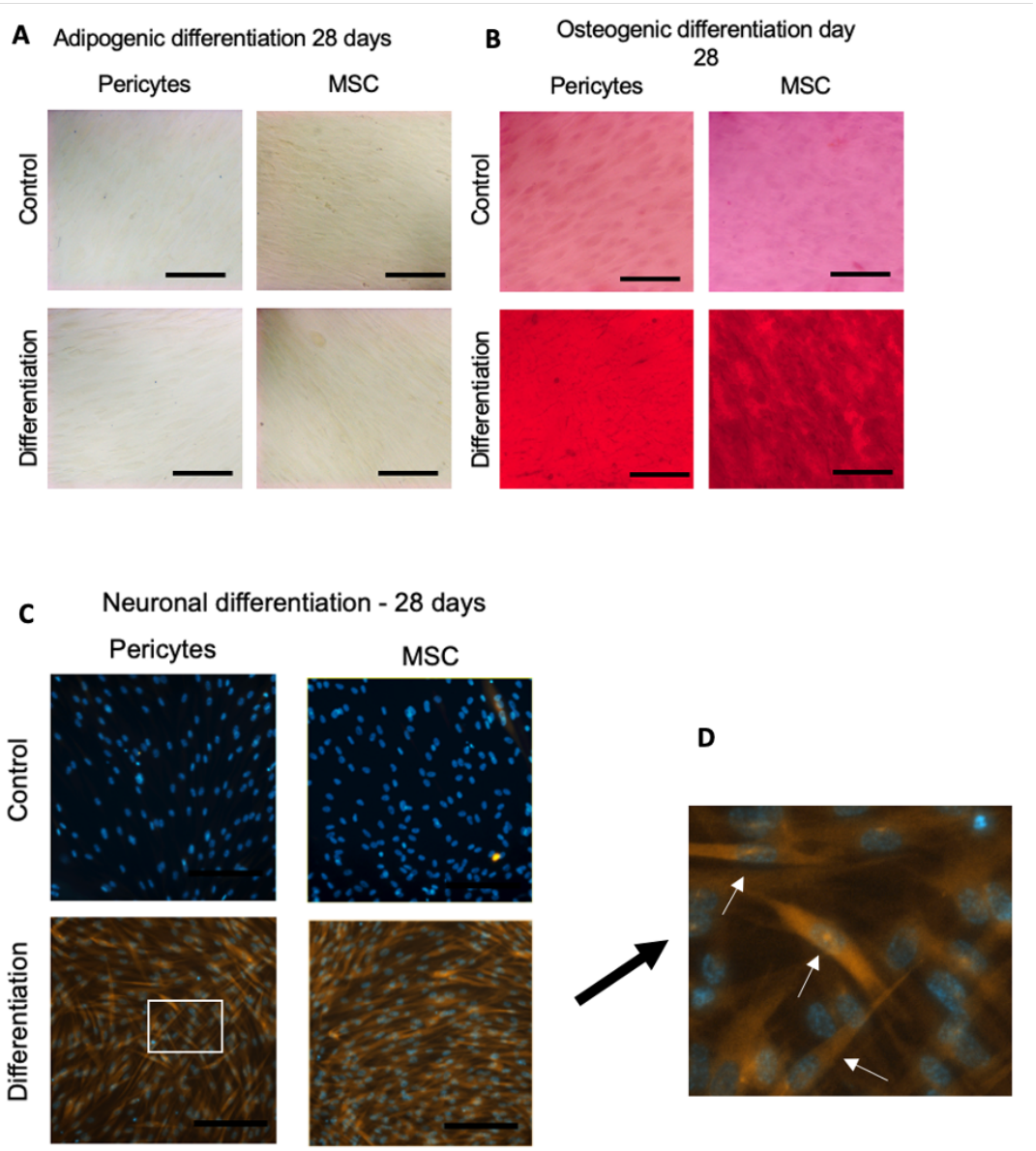

Figure 16. Images of Oil Red-O staining for adipogenic differentiation, Alizarin Red staining for osteogenic differentiation and β tubulin III staining for neural differentiation on day 28 cultured human dental pulp pericytes and MSCs.

(A) no evidence of adipogenic differentiation in pericytes or MSCs, **(B)** evidence of calcium deposits in red colour in both cell populations **(C)** evidence of tubulin III staining, in both cell populations. Cells appeared long, flat elongated with processes extending out from the end the cell body. Nuclei illustrated in the centre of the cell with a round/oval shape **(D)** Boxed area in c is showed enlarged. Arrows indicated pericytes, Scale bar = 50 μm.

Dental pulp perivascular cells isolation using flow cytometry

Perivascular cells are present in human tissues in different frequencies depending on the types of vessels present (Murray and Péault, 2015). Dental pulp is highly vascularised and is a great and easy source of mesenchymal stem cells. However, the frequency of each type of perivascular cells in the dental pulp is not known. To investigate this aspect, we performed eighteen individual experiments with teeth from both male (7) and female (11) subjects.

We enzymatically digested the dental pulp to obtain a single cell suspension and used flow cytometry to quantify perivascular cells. Viable cells were selected excluding debris, dead cells and doublets (Figure 17 A). CD56+ cells were then gated out of dental pulp cell suspensions to avoid contamination due to the overlap with pericyte markers. In order to remove endothelial cells and hematopoietic cells we used negative selection for CD31 and CD45 (Figure 17 C). In parallel, we derived MSCs in culture from total unfractionated samples.

Moreover, pericytes and adventitial cells were then identified by the expression of CD146 and CD34, as CD146+CD34- and CD34+CD146- respectively (Figure 17 A, iv). On average, we found that the dental pulp consisted of 10% pericytes 1-2% adventitial cells (Figure 17 B) with a significant difference between them ($p<0.05$).

Dental pulp pericyte subsets can be further sorted according to NG2 expression.

As we illustrated using immunohistochemistry, not all perivascular cells express NG2. To test this, we next analysed 18 dental pulp samples by flow cytometry and we confirmed that a subset of CD146+CD34-CD31-CD56- pericytes indeed express NG2. However, we found that the majority of pericytes in the dental pulp are negative for NG2. Indeed, NG2+ pericytes represent around 5.9% of the total dental pulp pericyte population and this is significantly lower ($p<0.001$) than NG2- pericytes (Figure 17 C).

To further characterise these pericyte subsets and to test their differential potentials *in vitro,* we next sorted these cells based on NG2 expression and established culture cell lines (Figure 17 C).

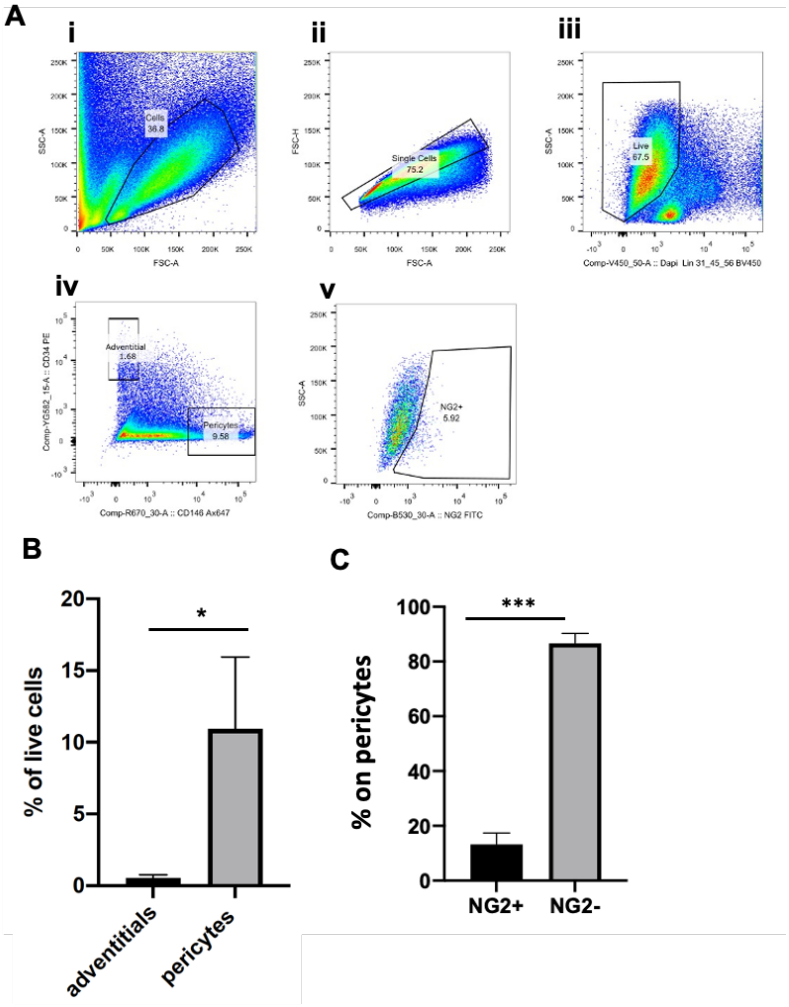

Figure 17. Quantification of the perivascular cell compartment in the dental pulp

(A) Perivascular cells are sorted as live single cells, negative for CD31, CD45 and CD56. Pericytes are CD146+CD34-. Adventitial cells are CD34+CD146-. Pericytes are further divided into 2 subgroups, NG2+ and NG2- then sorted. **(B)** The comparison between pericytes and adventitial cells in the dental pulp shows that pericytes are significantly more abundant. Unpaired t-test with Welch's correction was used. Data are shown as mean with SEM, N=18, *p=0.0445. **(C)** Percentages of NG2+ and NG2- pericytes in the dental pulp. Unpaired t-test with Welch's correction was used. Data are shown as mean with SEM. N=18, ***, P<0.001.

Determine the developmental potential of dental pulp-derived perivascular cell subsets, *in vitro*

Sorted dental pulp perivascular cells were seeded according to our established protocol (Crisan et al, 2008). In short, freshly sorted cells were cultured in endothelial cell medium, on gelatin-coated plates. To derive these cell populations a particular cell density was needed in order for the cells to be cultured successfully. Cell viability was low when less than 1,000 cells were seeded after sorting. So, all our samples that have grown in culture had more than 1,000 cells. One week after culture initiation, EGM-2 medium was replaced by DMEM supplemented with 20% FBS and 1% penicillin-streptomycin (PS). Recently attached cells exhibited mixed elongated, spindle and polygonal shapes. After cells were passaged once, they exhibited star-like shapes with prominent nuclei and multiple cytoplasmic extensions.
Ten samples were cultured up to passage 8 for about 2 months.

Adipogenic, osteogenic and neurogenic potentials of the same sample populations of dental pulp adventitial cells, NG2+ pericytes and NG2- pericytes sub-populations, at passage six, were evaluated at 7, 14, 21 and 28 days.

Adipogenic differentiation

Any cells undergoing adipose differentiation change their morphology and form intra-cytoplasmic lipid droplets. These lipid deposits are stained with Oil red O. The limited capacity of dental pulp- derived stromal cells to differentiate into adipogenic cells has

been reported by Fracaro and team (Fracaro et al., 2020). NG2+ pericytes differentiated into adipocytes, as illustrated by Oil Red O staining of lipid droplets into the cytoplasm compared to the control where these changes were not observed (Figure 18). Indeed, the differentiation started at day 14 and was maintained in both day 21 and 28. Interestingly, NG2- pericytes or adventitial cells were not able to accumulate lipid droplets at any time points studied (Figure 18).

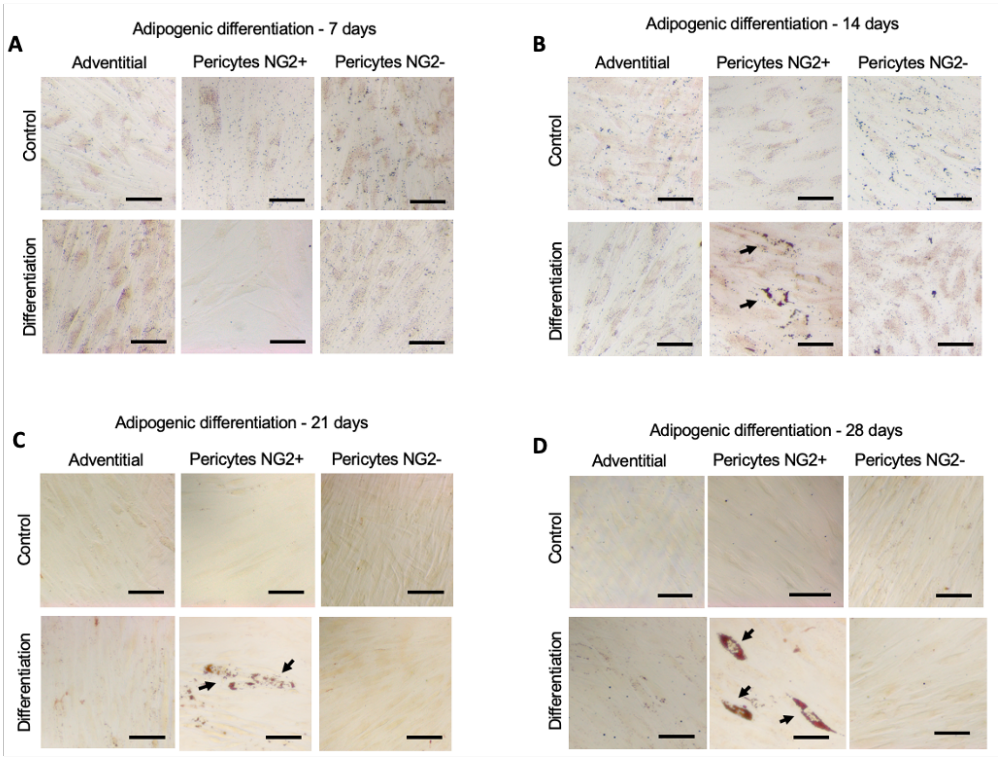

Figure 18. Adipogenic differentiation of cultured sorted perivascular cells *in vitro* on day 7, 14, 21, 28 in adventitial cells, NG2+ and NG2- pericytes from cultured human dental pulp, in control medium (first row), and adipogenic medium (second row).

(A) Day 7: No differentiation detected in any of the three cell groups **(B)** Day 14: NG2+ pericytes are differentiating, black narrows indicate lipid droplets. **(C)** Day 21: NG2+ pericytes show are differentiating (red-brown lipid droplets) whereas NG2- pericytes and adventitial cells do not. (**D)** Day 28: adipogenic differentiation revealed from NG2+ pericyte population with the appearance of red-brown lipid droplets. Staining Oil Red-O, black arrows indicate lipid droplets in brownish colour. Scale bar = 50µm

Osteogenic differentiation

Cells undergoing osteogenic differentiation accumulate calcium deposits that are revealed with alizarin red staining. Adventitial cells, NG2- pericytes and NG2+ pericytes exposed to osteogenic medium differentiated into calcified mineral matrix-secreting cells as early as 14 days following the induction of osteogenic differentiation (Figure 19). Osteogenic capacity was quantified by extraction of alizarin from the calcium deposits with acetic acid and subsequent analysis using spectrophotometry (Figure 20).

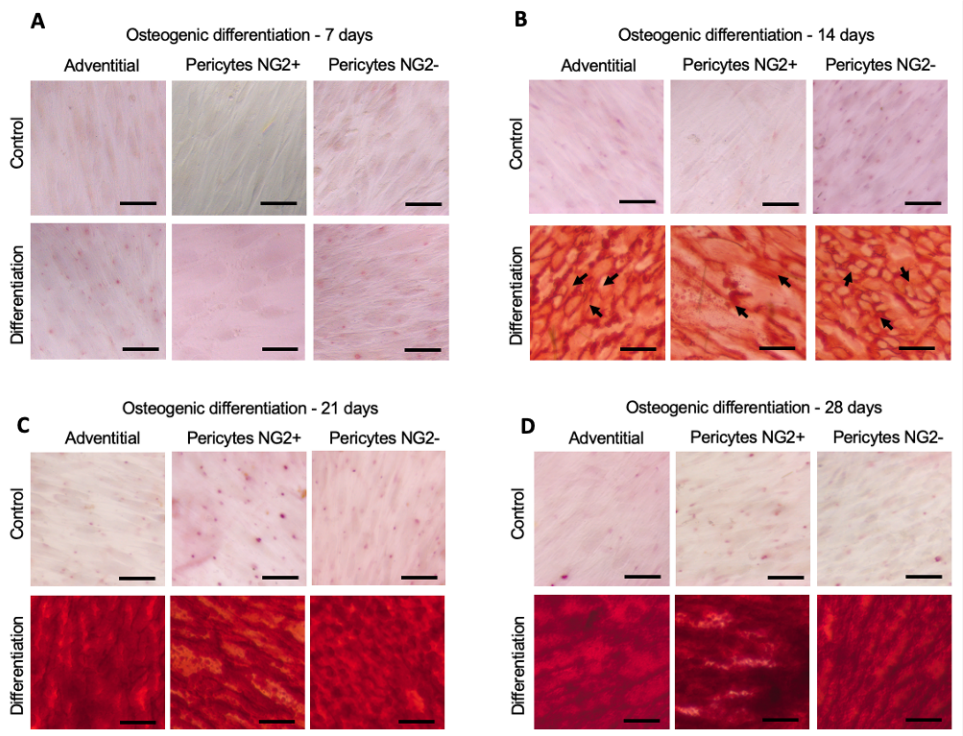

Figure 19. Osteogenic potential of adventitial cells and NG2+ and NG2- pericytes revealed by Alizarin Red staining on day 7, 14, 21 and 28 of cultured human dental pulp cell populations in control (first row) and differentiation conditions (second row).

(A) Day 7: no osteogenic differentiation **(B)** Day 14: calcium deposits (red colour) are evident in all three groups (black arrow) **(C)** Day 21 and **(D)** Day 28: further osteogenic differentiation. Staining Alizarin red, black narrow indicates calcium deposits in red colour. Scale bar = 50μm.

Subsets of NG2 pericytes and adventitial cells in the dental pulp illustrated similar osteogenic potential using quantification for alizarin red staining.

Osteogenic differentiation of dental pulp perivascular cells has been seen in eight independent experiments and it was identified by alizarin red staining and spectrophotometry. Further spectrophotometry technology was used to quantify osteogenic output of subsets NG2+ and NG2- pericytes, and adventitial cells to identify any differences in these three cell groups at 7-, 14-, 21- and 28-days post-induction. Firstly, we observed that after 7 days of differentiation there was no osteogenesis in either of these three cell populations analysed (Figure 20 A). The osteogenic differentiation started at day 14 as shown in figure 20 B. For that time point on, we observed statistically significant differences between the undifferentiated control and the differentiated cells in all the cell populations (Figure 20 B, C, D). The osteogenic differentiation ability was similar for all the three cell groups as no statistical difference at day 14, 21 or 28 was found (Figure 20 B-D).

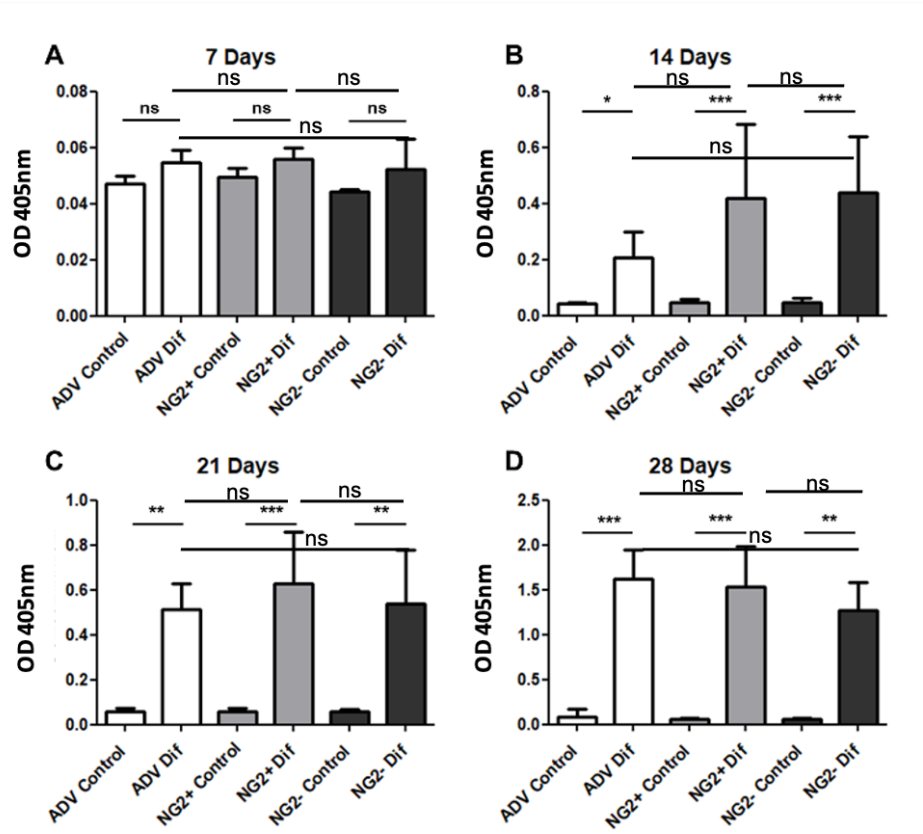

Figure 20. Quantification of osteogenic differentiation of cultured adventitial cells, NG2+ and NG2- pericytes.

(A) 7 days **(B)** 14 days **(C)** 21 days **(D)** 28 days. Unpaired t-test with Welch's correction was used. Data are shown as mean with SEM. N=8, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns: not significant. OD: Optical density.

Neurogenic differentiation

Adventitial cells, NG2- and NG2+ pericytes revealed positive response for tubulin β III after 28 days when compared to control media when treated with basic fibroblast growth factor and epidermal growth factor. Tubulin β III is a neural marker; hence this result may indicate that these human dental pulp-derived perivascular cells were

developed to phenotypic neural-like cells following treatment with appropriate growth factor *in vitro.* The staining of tubulin β III might be indicative of neural cell differentiation. However, the cell morphology it was not. Neural cells have the following characteristics: pyramidal shaped soma and long axonal like extensions. In our experiment our cells did not present this morphology. Adventitial cells, NG2- and NG2+ pericytes, following the 28-day neural differentiation, appeared large, flat, elongated with processes that extending out from the ends of their body. (Figure 21).

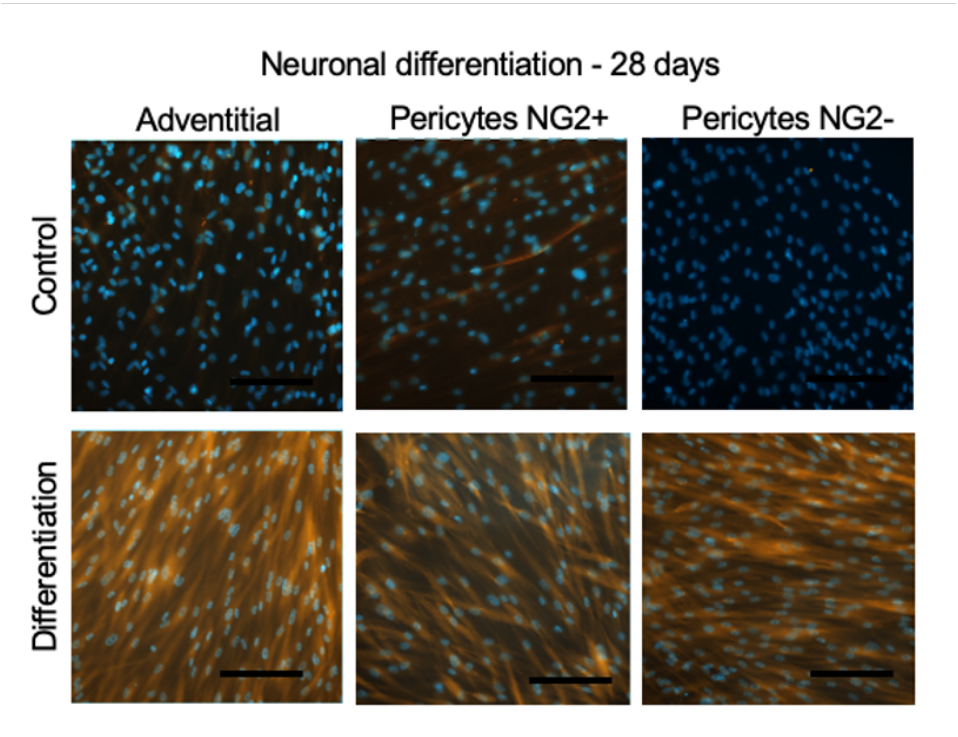

Figure 21. Immunofluorescence showing β tubulin III staining for neural differentiation on day 28 of cultured human dental pulp adventitial cells, NG2+ and NG2-pericytes in control medium (first row) and in neural medium (second row).

Evidence of tubulin β III staining in all cell populations with cells appearing long, flat elongated with an oval centred nuclei and processes extending out from the end of their body. Scale bar = 50 μm.

CHAPTER 4

CONCLUSIONS AND FUTURE RESEARCH

Conclusion

As has been shown, perivascular cells are a highly heterogeneous population of cells. Both pericytes and adventitial cells are located in different types of vessels. Nevertheless, both cell types are considered an *in situ* counterpart of mesenchymal stem cells (MSCs) (Corselli et al., 2010c, 2012a; Crisan et al., 2008b). In this thesis, we investigated the presence of perivascular cells in human dental pulp, their different aspects including the location of these cells, and whether dental pulp perivascular cells and MSCs are linked.

Firstly, we have focused on identifying and characterising the dental pulp perivascular cells *in vivo.* Our data show that dental pulp perivascular cells express the classical pericyte markers identified in other human organs such as αSMA, CD146, PDGFRβ, LepR, CD107a and NG2, but not the endothelial cell markers CD34, Ulex europaeus receptor, CD54 and vWF. The illustration of the above endothelial cell markers on dental pulp sections proved the presence of a developed vascular network in the adult human tooth. Having a functional vascular network includes the participation and interaction of different cell types including endothelial cells, pericytes, macrophages, essential for angiogenesis (Hur et al., 2004; Rafii and Lyden, 2003). The data of our study are in agreement with previous studies by our group and others pertaining to the isolation of pericytes from other tissues (Corselli et al., 2013; Crisan et al., 2012b, 2008b, 2008a; James et al., 2017; Péault et al., 2007; Shi and Gronthos, 2003b). Moreover, we have illustrated that not all pericytes express the same markers and localisation of pericytes is one of the reasons for this heterogeneity. Indeed, we have shown that αSMA is not always expressed in capillaries but is consistently present in

arterioles and venules. CD146 and PDGFRβ are expressed in capillaries, arterioles and venules, NG2 and LepR are expressed in both capillaries and arterioles but not venules. Alliot-Licht and team have utilized total pulp cells and have shown that a population of αSMA positive cells can form mineralised nodules *in vitro*, suggesting that the bone/dentin progenitors are perivascular cells (Alliot-Licht et al., 2001; Shi and Gronthos, 2003b) . The expression of leptin in the dental pulp has been shown before (J. Martín-González et al., 2013) but it was not illustrated which cells expressed LepR. It was important to identify if all pericytes express LepR especially as shown to be essential for alveolar bone regeneration (Zhang et al., 2020). Our work found that LepR was not always expressed in pericytes in capillaries (Figure 10). Leptin is the ligand of LepR, a hormone which derives from adipocytes, and was initially associated with lipid metabolism and obesity (Coleman, 1978). LepR/Leptin signaling has been identified to be part of the immune homeostasis, angiogenesis, haematopoiesis, reproduction, bone formation, haematological malignancies and as a possible regulator for inflammation associated with dental infections (Chen and Yang, 2015; Konopleva et al., 1999; Jénifer Martín-González et al., 2013). Also, cells expressing LepR have been shown to be essential for alveolar bone regeneration (Zhang et al., 2020).

Giulio Cossu (Dellavalle et al., 2007) used ALP to isolate pericytes from human skeletal muscle for example, and we confirmed that ALP is also a pericyte marker of cultured pericytes in other organs (Crisan et al., 2008b). Interestingly, we found that dental pulp perivascular cells also express ALP. To summarize, we have identified the molecular signature of pericytes in dental pulp as described: CD146+ a-SMA+/- NG2+/- PDGFRβ+ LepR+/- CD107a+ ALP+ CD34- CD54- VWF- UEA-1-.

Secondly, using immunohistochemistry, we also found that dental pulp perivascular cells express CD29, CD90 and CD44, MSC markers. Numerous studies have been carried out from our group, and others too, demonstrating that in other organs perivascular cells are the *in vitro* counterpart of the MSCs. Initially it was assumed that all pericytes are MSCs (Caplan, 2008).However, later it was shown that MSCs arise from perivascular cells as well as the fact that MSCs come from various locations, such as pericyte and adventitial layers. Perivascular cells have been characterised as an ubiquitous “niche” regenerative cell population with exceptional developmental plasticity (Alakpa et al., 2016b; Corselli et al., 2012b, 2010b; Crisan et al., 2008b).
Furthermore, we have investigated the co-expression of neuronal markers (CD56 and vimentin) with pericyte markers (LepR, NG2, CD146) and interestingly we also showed here that some neural cells are expressing pericyte markers such as αSMA, NG2, CD146, LepR, but not always (Figure 14). The neurogenic ability of pericytes was stated and investigated by Geranmayeh and team who reviewed the different subsets of pericytes and their neurovascular ability (Geranmayeh et al., 2019). The higher the pericyte density is in the CNS, the more likely is for the brain potency to develop a blood brain barrier integrity. Two different subtypes of pericytes in muscular tissues have been shown by Birbrair and team: the pericyte group which had the ability to transdifferentiate towards adipogenic and fibrogenic lineages (Birbrair et al., 2013) and the second pericyte type which possessed the ability towards neurogenic, angiogenic and myogenic differentiation (Birbrair et al., 2014)

Following immunohistochemistry results, we identified dental pulp perivascular cells, their frequency as well as their ability to differentiate into various cell lineages in

culture. We demonstrated successful isolation of dental pulp-derived perivascular cells, namely pericytes and adventitial cells which were consistent between experiments. More precisely, we have illustrated the existence of adventitial cells (CD34+ CD146- CD31-CD56-) which consist of around 1-2% and pericytes (CD146+CD34-CD31-CD56-) of approximately 10-15% of the sorted dental pulp perivascular cells. Carlile and team have shown a higher number of perivascular cells in the dental pulp (Carlile et al., 2000). In their study, they obtained dental pulp from eight wisdom teeth from patients age 17 to 25 years and they found that the dental pulp which was rich in capillaries composed of both endothelial and peri-endothelial (pericytes, transitional cells and fibroblasts) cells in a 1 to 4 ratio. (Carlile et al., 2000). It has been demonstrated that cultured DPSCs contain a population expressing the pericyte marker NG2 (Delle Monache et al., 2019). In line with this, we have illustrated that a subset of pericytes indeed express NG2 both on section and by flow cytometry. However, we found that NG2 is also expressed in neural cells raising the question about the origin of NG2+ cells in DPSCs. The importance of NG2 in proteoglycan molecular interactions and biological functions has been illustrated before (Girolamo et al., 2019). We have seen that all dental pulp perivascular cells are highly proliferative and multipotent, same as it was also demonstrated for perivascular cells derived from other human tissues (Alakpa et al., 2016; Corselli et al., 2012b, 2010b; Crisan et al., 2008b). Furthermore, Q-PRC, quantitative polymerase chain reaction, studies for the validation of the immunostainings that performed in this study could be done in the future.

Our next step was to investigate the *in vitro* adipogenic, osteogenic and neurogenic ability of all different perivascular cell populations. There are contradicting results in

literature and the limited ability of DPSCs towards adipogenesis has been reported before (Fracaro et al., 2020; Gronthos et al., 2000a; Isobe et al., 2016; Monterubbianesi et al., 2019; Tamaki et al., 2013). In our study we found that the NG2+ pericyte population was able to differentiate into adipocytes although the frequency seemed low. On the other hand, NG2- pericytes, pericytes, adventitial cells and MSCs were not adipogenic. These data suggest that the NG2+ pericytes or at least a few of them are endowed with a higher adipogenic ability compared to the NG2- counterpart that may be "blocked" by the latter when all pericytes are cultured together.

The quantitative osteogenic ability of all five different cell groups we tested here had been shown to be similar. In addition, no delay was observed. The calcium deposition was found at day 14 post-induction, in all cell lines tested.

Moreover, the *in vitro* neurogenic ability of all five different cell groups 28 days post-induction was also revealed in our study with the use of tubulin β III staining. However, cell morphology was not indicative of neural cell differentiation. Tubulin β III has been used before in order to identify neuron-like cells (Isobe et al., 2016; Tamaki et al., 2013). Several other studies have shown that hDPSCs have the ability to differentiate into neuronal-like cells or dopaminergic neuron-like cells (Ullah et al., 2016; Young et al., 2016). Pisciotta and team compared two subpopulations of hDPSCs based on CD34 expression and demonstrated different properties on the expression of neural markers (Pisciotta et al., 2015). They isolated two populations of hDPSCs based on STRO-1 and c-Kit expression associated or not to CD34. Pisciotta and team found that these two hDPSCs populations (STRO-1+/ c-Kit+/CD34- and STRO-1+/c-Kit+/CD34+) are illustrating marked behaviors regarding cell proliferation rate,

stemness maintenance and cell senescence/ apoptosis upon late passages. Moreover the CD34+ population demonstrated *in vitro* a stronger tendency after 3 weeks towards the neurogenic differentiation; whereas the differentiation ability of both hDPSCs populations towards mesoderm linages was similar.(Pisciotta et al., 2015)

In our study cell morphology was not indicative to neural cells though the staining of tubulin β III might suggest that longer exposure in culture was required. Nevertheless, in our experiments we did not have a negative control for tubulin β III. Thus, if this staining is specific remains to be investigated. This can be clarified in the future, in several ways such as by adding a negative control of the staining, or by doing the same staining on neural cells using them as positive control (for example brain cells), or by using the tubulin β III staining on epithelial cells and other unrelated cells that would have another negative control.

Furthermore, tubulin β III was the only neural markers we used, in contrast with Pisciotta and team who used tubulin β III as well as MAP 2, NEU-N synapsin, a glial specific marker (GFAP) and anti-Notch 3 ,Neurogenic locus notch homolog protein 3), antibody (Pisciotta et al., 2015).

There is an extensive need to develop safe and effective methods to regenerate soft and hard tissues for craniofacial diseases such as head and neck cancers, trauma, birth defects/ orofacial cleft, periodontal disease and pulp injuries. Cellular approaches provide an attractive option to develop therapies targeted for the craniofacial regeneration. Engineering cellular therapies for craniofacial regeneration requires the understanding of the cells and their regenerative potential.

With the above approach we set the step for a well-characterised dental pulp perivascular stem cell population of high purity that can be expanded and differentiate. Dental pulp-derived perivascular cells should therefore be amenable to study and develop targeted dental therapies as well as nerve and bone regeneration strategies. Interestingly, this project shows that pericytes and adventitial cells in culture are not functional MSCs *in vitro*, but rather bone progenitors (at least in our conditions). They express MSC markers *in situ*, they are adherent to plastic dishes, they have MSC morphology, but they are not multipotent *in vitro*. However, they are highly osteogenic and on sections they share markers with pericytes.

Specifically, dental cell therapies have recently garnered a lot of attention. Dentin, dental pulp, and cementum-periodontal complex regeneration has been shown with DPSCs, stem cells from human exfoliated deciduous teeth (SHEDs) and periodontal ligament stem cells (Shi et al., 2020). Root-periodontal complex regeneration has been shown with cells from apical papilla and periodontal ligament (Y.-J. Chen et al., 2015). Hard tissue formation with rat dental pulp cells has also been shown *in vivo* (Yang et al., 2009). FACS isolated dental pulp perivascular cells, namely pericytes and adventitial cells are promising a unique cell source to assess the *in vivo* differentiation potential of these cells into dental tissues. Due to the availability of deciduous teeth and third molar teeth, dental pulp perivascular cells can provide a convenient source of therapeutic cells to regenerate dental tissues as well as tissues of other mesodermal lineages such as bone, nerve and fat. Nevertheless, current data does not support yet a neuronal differentiation as more markers should be tested such as MAP2 and a glial specific marker and more experiments should also take place.

Our experiments for neurogenic differentiation followed the protocol by Arthur and team (Arthur et al., 2008). Although we both used adult human teeth, and same media and conditions, Arthur and team used other supplementary staining: nestin, polysialic acid-neural cell adhesion molecule (PSA-NCAM), neurofilament-medium chain (NF-M), mature neuronal marker neurofilament-heavy chain (NF-H) and differentiation took place for 3 weeks instead of 28 days. They also found an increase in β tubulin III cell expression following the induction of the neuronal medium (Arthur et al., 2008). The reason we choose to use the β tubulin III is that this molecule is the only phosphorylated tubulin. It is considered to be a neuronal-specific marker that is expressed at early brain development and is down-regulated as the brain develops into adulthood (Jiang and Oblinger, 1992)

Future research

This research project provides an important base on which further studies could build on. It was mentioned before that using the FACS analysis at an early passage is vital for future research on dental pulp derived cells as guarantee for a pure stem cell.

Further questions to be addressed:

1. What is the difference between dental pulp adventitial cells and pericytes, and which one is better in bone regeneration? Wang and team illustrated that adipose-derived CD146+ pericytes and CD34+ adventitial cells display functionally distinct yet overlapping and complementary roles in bone defect repair (Wang et al., 2019). Does the same occur with dental pulp pericytes and adventitial cells? Could we

inject them in mouse models where bone/tooth was injured? Could dental pulp be used as a source for regeneration of other types of bone such as long bone?

2. Are dental pulp pericytes and adventitial cells developmentally linked? Is one giving rise to another and vice versa?

3. Will it be possible to sort and culture CD56+ cells *in vitro* and differentiate into neurons or into pericytes? Are they MSCs as well?

4. Will be possible to Replace the missing dental pulp tissue, endodontics?

Stem cell treatment is a fascinating dynamic aspect of medical research and using perivascular cells in an accurate way might lead to an important foundation for further developments and treatments.

CHAPTER 5
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Appendix

Publication in Preparation

Ethical Approval

Patient Information Leaflet

Consent Form

BioResource Report Form

Publication in preparation

Besi, E., Vezzani, B., Fracaro L., Gomez-Salazar, M., Crisan, M., Péault, B. **Mesenchymal progenitor cells in human dental pulp: purification and characterisation**.

Ethical Approval

LOTHIAN NRS BIORESOURCE
SAMPLE REQUEST ANSWER FORM

Sample Request number:	SR823
Name of Researcher:	Eleni Besi Norma O'Connor
Address of Researcher:	Oral Surgery Department, Edinburgh Dental Institute, Lauriston Place, Edinburgh EH3 9HA
Study Title:	Continuation of study :An investigation of the ability to derive pluripotent stem cells from human dental pulp of extracted teeth
Ethical status:	15/ES/0094
Material Requested	Collection and use of the following, from consented patients, is approved for the purposes of the above project: Dental pulp from extracted teeth, that would otherwise be discarded into the clinical waste Data to be handled as described on the application form.

REQUEST AUTHORISED

Date:	24-Mar-2017
Authorised by:	

REQUEST REJECTED

Date:	
Authorised by:	
Reason	

Patient Information Leaflet

Participant Information Sheet (Stem cell version 1.2)
Lothian NRS BioResource
Information about the Use of Surplus Tissue for Medical Research and Education.

You are being invited to donate tissue samples to the Lothian NRS BioResource. Before you decide whether or not to do this, it is important for you to understand what the BioResource is and what will be involved. Please take time to read the following information carefully. Talk to others about the study if you wish. Contact us if there is anything that is not clear or if you would like more information. Take plenty of time to decide whether or not you wish to take part.

What is the purpose of the study?
The Lothian NRS BioResource is funded by the CSO (Chief Scientist Office) which is part of the Scottish Government Health Directorates. This is a collaboration which has been set up between Scottish Universities and Health Boards. Its aim is to help translate discoveries made in research laboratories into improved care for patients.
The Lothian NRS BioResource will collect and store **biospecimens** (small pieces of tissue, cells and samples of body fluids) and build up a collection of these for use in medical research and education. This BioResource is being run by NHS Lothian, and has been approved by a Research Ethics Committee. The samples will only ever be used in research applications which have been approved by a scientific review committee.

What is tissue and why is it required for research and education?
The human body is made up of cells which are the basic building blocks for tissues. Organs such as lungs, liver, kidney and appendix are made up of tissue. There are many different types of cells and tissues in the human body. Body fluids such as blood, urine and saliva contain cells. Material taken during a cervical smear test also contains cells. Doctors and scientists need human tissue and other biospecimens for medical research. From these they can see and understand how diseases start and develop. They can also try out different drugs and tests on the tissue. This may help them find new medicines and treatments, and possibly even ways of diagnosing diseases earlier. They may grow adult stem cells from your tissue to help develop ways of repairing injured or diseased cells.
As well as providing care and treatment for patients, the NHS is also responsible for educating and training doctors, nurses and other healthcare workers. Human tissue is needed for this as well.

Why have I been asked to take part?
You are currently attending a clinic and are due to have an operation or medical procedure in a hospital within NHS Lothian. During the procedure, you may have some cells or tissue removed by the surgeon. If some cells or tissue are left over, and you agree, we would like to use this material for research.

What will this involve?
During your investigation(s) or operation(s), which are part of your present treatment regime the doctor may take some tissue from your body. This piece of tissue will be sent to a pathologist for diagnosis.
A pathologist is a doctor who specialises in the examination of tissue, to diagnose or confirm what is wrong with you. Some of the tissue is used for your tests, and then becomes part of your medical record. This means that it can be looked at again if you are ill in the future.
However, some tissue is usually left over and would normally be disposed of. We are asking you to donate some of this surplus tissue for medical research and education.
During your treatment, the doctor may take body fluids e.g. blood and urine for testing. Some of these may also be "left over" and if you agree, could also be used for medical research and teaching. . If you agree, we may ask you to provide us with an extra blood sample, and this would possibly mean an extra venepuncture.
We may also ask you to provide an extra urine sample.
We would also like to use some information from your medical notes. This will be information about your physical condition, but not about your mental health.
Your name and address and anything else that could identify you will be removed before allowing researchers to use any of your samples or data.

Do I have to do this?
No, it is entirely up to you if you want to donate biospecimens to the BioResource. Whatever you decide, it will not affect your treatment, care, or diagnosis. You can also change your mind at any time, without giving a reason. If you change your mind later on however, some of your samples may already have been used in research. It would be too late for us to stop your sample being used, but we would dispose of any tissue that hadn't been used yet.

What are the possible benefits of taking part?
It is unlikely that you will personally benefit from the research. This research often involves testing large numbers of samples from many different people to try to identify factors that influence medical conditions and disease, and it can take many years to produce advances in treatmen. However, if at any point during the programme this information becomes of use in guiding your treatment, the clinical care team looking after you in the hospital will be made aware of this and they will explain the information to you

You can benefit from the knowledge that you are personally helping research to prevent or treat illness. The tests and treatments being used for you were developed with the help of patients who took part in research years ago. Research might make faster progress as more human samples are studied, helping the health of future generations

What are the possible disadvantages or risks of taking part?
The only risk would be the possibility of some pain or bruising from giving an extra blood sample. Otherwise, there will be nothing extra happening to you as any of your surplus tissue stored by the BioResource would normally have been collected and disposed of anyway.

Where will my tissue sample be used?

- The NHS
- Universities
- Research Institutions
- Commercial companies

Tissue samples may also be sent abroad. You can be sure, however, that all researchers whether in this country or abroad must have proof that they are following legal and ethical guidelines for their research. Researchers working abroad will be required to sign a form agreeing to follow the same rules and regulations which apply in the UK.
Your donated tissue will not be used in animal research, research about termination of pregnancy, or reproductive cloning.

How long will tissue be stored?
The tissue you donate will stay in the NHS, or with approved researchers until it is all used, or disposed of should you decide to withdraw your consent. All tissues will be disposed of lawfully and respectfully, and a record will be kept of this. If you decide to withdraw consent, you can tell a member of your healthcare team, or contact us at the BioResource on the telephone number or email address in the "Further Information" section.

Will my taking part be confidential?
Yes, only the BioResource staff will be able to identify you. They will abide by the Data Protection Act 1998 at all times and make sure your name, address, and any other information that would identify you are removed from your medical information before it is given to any researchers. The information held on computer will be kept secure, and all written information will be held in locked filing cabinets.

Will my medical notes be used?
Medical research is of more value if the researcher has information about the medical history of the person who donated the tissue. We would like your permission to use and store information from your medical notes now, and possibly in the future as a follow up. All information collected and stored will be kept strictly confidential. Your personal information like your name and address will be removed from your medical notes before being given to anyone for their research. Only the BioResource staff and your Healthcare team will be able to link your information to your medical notes.

Can researchers find new information about my health?
It is possible, but the research on your tissue will normally have nothing to do with your own care or treatment. Future research may give us information about what type of treatment would be most suitable for particular medical conditions but this data is primarily intended for research and it is very unlikely that your sample alone will give us this type of information. However, if any information might be of use in your clinical care, the doctors looking after you may discuss how the information could be used to guide your treatment.

Will researchers carry out genetic tests on my tissue?
Genetic testing on your DNA may be carried out on your tissue. These tests may include whole genome sequencing. Whole genome sequencing means studying the complete pattern of the DNA to help understand the biology of genes. If whole genome sequencing was carried out on

your sample, this would be a research test and not a clinical test and no results would be fed back to you unless your doctor felt it would improve your treatment in some way. A lot of research today focuses on the study of genetic material from healthy individuals compared to people with known diseases. This comparison helps researchers to discover genetic differences which in turn helps in the development of new drugs and treatments.

Will anyone make money from my tissues?
It is illegal to sell tissue for profit. The NHS may charge researchers a fee for your tissue, but this is to cover the costs of running the BioResource.
If researchers develop a new drug, treatment or test, a pharmaceutical company or other researcher may then make a profit. It will not be possible for you to claim any money because you donated tissue.
However, any new drug, treatment, or test would potentially help all of us in the future.

I want to donate…what should I do next?
When you come into hospital or attend a clinic, you will be asked if you agree to let your surplus tissue from any investigation(s) or operation(s) be used for medical research. You will then be asked to sign a consent form. You should keep a copy of this Patient Information Leaflet and Consent Form for your records.

I do not want my tissue to be used in research.
When you come into hospital or attend a clinic and we ask if you agree to let us use your surplus tissue for education or research, simply say no. We will not put you under any pressure, and you do not have to give a reason. **Your decision will not affect your healthcare.**

What if there is a problem?
If you believe that you have been harmed in any way by taking part in this study, speak to the research team in the first instance. If you are still unhappy, you have the right to pursue a complaint and seek any resulting compensation through NHS Lothian who are acting as the research sponsor. Details about this are available from the research team. Also as a patient of the NHS, you have the right to pursue a complaint through the usual NHS process. To do so, you can submit a written complaint to the Patient Liaison Manager, NHS Lothian Complaints Office, 2nd Floor, Waverley Gate, Edinburgh telephone 0131 465 5708. Note that the NHS has no legal liability for non-negligent harm. However, if you are harmed and this is due to someone's negligence, you may have grounds for a legal action against NHS Lothian, but you may have to pay your legal costs.

Who has reviewed the study?
The East of Scotland Research Ethics Committee 1, has examined the proposal and has raised no objections from the point of view of medical ethics. It is a requirement that your records in this research together with any relevant medical records, be made available for scrutiny by monitors from NHS Lothian whose role is to check that research is properly conducted and the interests of those taking part are adequately protected.

Further Information
If you have any further questions, please ask the person who is witnessing your consent. If you think of anything else later, you can contact us at 0131 465 5456, Tissue Governance, Public Health Office, Waverley Gate, Edinburgh.
email rie.tissuegovernance@luht.scot.nhs.uk

We will try to supply this information in different languages and formats if requested.

Thank you for taking the time to read this Information Sheet and for considering taking part in this study.

Author	: Frances Rae	Date : 01-Jun-2015
Authority for Issue	: Craig Marshall	Date : 01-Jun-2015
Quality checked	: Craig Marshall	Date : 01-Jun-2015

Consent Form

Document Number	QF-TGU-A-CONSSCR VERSION 1.2	page	1 of 2	Review date	01-Jun-2019

Lothian NRS BioResource

The collection of Tissue, Biospecimens and Data for Research

PARTICIPANT CONSENT FORM (Stem cell version 1.2)

Explanation of consent procedure

After you have had time to read the Participant Information Sheet (Stem cell version 1.2) discuss it with a member of your healthcare team or GP and consider whether you wish to donate your surplus tissue to the Lothian NRS BioResource. Take time to discuss it with your family and friends.
If you decide to participate, we would ask that you **initial the boxes for numbers 1 to 8, circle YES or NO for numbers 9, 10, and 11. and sign at the bottom (overleaf)**. All of your information will be treated strictly confidentially, and all BioResource staff will follow the principles of the Data Protection Act 1998.

Please initial

1. I confirm that I have read this consent form and Participant Information Sheet (Version 1.2) and have had the opportunity to ask questions about them. ☐
2. I understand that some surplus tissue or body fluids may be left over during the course of my operation(s) / investigation(s) within NHS Lothian and I agree that this surplus material may be stored in the Lothian NRS BioResource for research use. ☐
3. I declare that I have given my consent voluntarily to the storage of this surplus tissue. ☐
4. I agree that my donated sample(s) may be used by clinical, academic or commercial researchers, and may be used abroad. ☐
5. I understand that I am free to withdraw at any time, without giving any reason, and that my medical care will not be affected. ☐
6. I give the Lothian NRS BioResource permission to access and store information about my general physical health and current and past episodes of illness from my medical records. I understand that all information collected about me will be kept strictly confidential by the BioResource research team. ☐
7. I understand that the information obtained through any research carried out is not likely to have any direct medical benefit to me but may help society as a whole in the overall understanding of the causes, detection and diagnosis of diseases, medical conditions, and responses to treatment. ☐
8. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test. ☐

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Quality Checked	: Craig Marshall	Date : 01-Jun-2015

9. I agree that my surplus tissue or other biospecimen may be stored and used for future genetic testing including DNA testing and possibly whole genomic sequencing.. **Please clearly circle YES or NO** — YES / NO

10. I agree to provide an extra blood sample, by venepuncture, and/or urine sample if neccessary. **Please clearly circle YES or NO.** — YES / NO

11. I agree that my surplus tissue may be stored and used for adult stem cell research **Please clearly circle YES or NO** — YES / NO

Name of patient (please print)	**Signature**	**Date**
……	……	……

Name of person witnessing consent	**Signature**	**Date**
……	……	……

Thank you for agreeing to take part in this research

3 Copies:
1 to Participant, original to Lothian NRS BioResource, 1l to be stored in Patient File

Contact details for BioResource team:

The Tissue Governance Manager
NHS Lothian
Public Health Office
Waverley Gate
Edinburgh

Tel 0131 465 5456
Email rie.tissuegovernance@luht.scot.nhs.uk

Study ID label:

BioResource Report Form

Document Number	QF-TGU-A-QUREPFOR VERSION 1.2	Review date	22-DEC-2018

BioResource Reporting Form

Please complete and return within a week of receipt. Ongoing approval for the research being conducted is dependent upon the provision of this information.

Tissue Request No.	SR823
Project Title	An investigation of the ability to derive pluripotent stem cells form human dental pulp of extracted teeth (continuation study)
Lead Researcher	Eleni Besi (eleni.besi@nhslothian.scot.nhs.uk) Norma O'Connor (norma.o'connor@nhslothian.scot.nhs.uk)
Start Date	March 2017

Is this study still active?	yes
Is this study still collecting samples?	yes
Estimated completion date?	07/2019

Number of Participants consented from 1st April 2017 – 31st December 2017	13
Number of samples collected from 1st April 2017 - 31st December 2017	18