Groningen, June 2013

The origin and applications of adipose-derived stem cells (ADSCs)

Eva Houtsma (s1983067), supervisor: M. Harmsen & G. Krenning, Department of Regenerative Medicine, RUG.

Abstract

Recently, it has been found that mesenchymal adipose-derived stem cells (ADSCs) have many functions. ADSCs have similar properties as bone marrow mesenchymal stem cells, thereby capable of repairing and regenerating damaged tissue. ADSCs can be isolated from the stromal vascular fraction (SVF), within adipose tissue. The exact location of in vivo ADSCs in relation to their in vitro and in vivo applications is unknown. There is leading evidence that ADSCs find their origin in the perivascular environment, interacting with the vascular network. This thesis aims to look at the ADSC origin, associated with in vitro and in vivo applications.

The experimental results show that mesenchymal stem cells (MSCs), including ADSCs, belong to a subset of perivascular cells, with ADSCs located in the tunica adventitia. Moreover, a pericyte subset that may be transitional between pericytes and ADSCs was found. The possible perivascular origin of ADSCs suggests that these cells interact with the vascular network, supported by various growth factors. Moreover, in vitro cultured ADSCs can be used for in vivo ADSC implantation by using scaffolds capable of vascularisation in vivo.

To conclude, adipose-derived stem cells are located in a perivascular environment, thereby contributing to their in vitro and in vivo applications with regard to vascularisation. ADSC-pericyte subsets may be transitional between pericytes and ADSCs, suggesting a progenitor/progeny relationship in interaction with the vascular network. ADSCs can provide an additional way of stem cell therapy for tissue regeneration, besides bone marrow mesenchymal stem cell therapy. In the future, ADSCs may be used for in vivo applications, providing therapy for patients with obesity, neurodegenerative disease, leukaemia, ischemic diseases and cardiovascular diseases.

Key words: adipose-derived stem cells (ADSCs), stromal vascular fraction (SVF), pericytes, vascularisation.

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Introduction

White adipose tissue is a multifunctional organ, regulating many physiological processes in the body such as inflammation and endocrine responses. Adipose tissue contains various cellular types, including mature adipocytes and the stromal vascular fraction (SVF). Mature adipocytes primarily compose adipose tissue, regulating energy storage as fat. The stromal vascular fraction is a source of adipose-derived stem cells (ADSCs) and can be isolated from the human adipose tissue. ADSCs are comparable to the bone marrow mesenchymal stem cells (BM-MSCs). Both BM-MSCs and ADSCs belong to the mesenchymal stem cells (MSCs) and are derived from the embryonic mesenchym. BM-MSCs are currently being used for stem cell therapy, which can help patients with damaged tissue, for example patients with burn injury. The isolation process of ADSCs is less invasive than the isolation of BM-MSCs. In this way, ADSCs can be a promising source of stem cells in the future [Gentile et al., 2012].

Stem cells are cells with at least two important characteristics: self-renewal capacity and the potential to differentiate. The cells can be divided into embryonic stem cells (ESCs) and adult stem cells (ASCs). The stem cell potential of ESCs is enormous, but because of ethical issues, the ESC use in the clinics is very limited. ASCs, including hematopoietic stem cells and mesenchymal stem cells, are an alternative for the ESCs [Zuk et al., 2002].

The stromal vascular fraction is a mixture of adipocyte progenitors, immune cells, fibroblasts, blood endothelial cells (BECs), pericytes, stromal cells and stem cells. The SVF can be obtained from the lipoaspirate, which is the product of liposuction. This concerns mostly liposuction of the subcutaneous adipose tissue (SAT) [Koh et al., 2011]. Since obesity is a common disease nowadays, SAT is readily accessible and can be used for the collection of ADSCs. However, during liposuction, the subsequent scar formation should be carefully monitored [Bunnell et al., 2008].

ADSC have stem cell properties and additional functions, such as being pro-angiogenic, anti-apoptotic, pro-mitogenic, anti-inflammatory and matrix remodelling [Harmsen, 2013]. ADSCs are regulated by genetic (e.g. developmental genes), hormonal (e.g. pancreatic hormones) and metabolic (e.g. fatty acids) factors [Cignarelli et al., 2012].

ADSCs obtained by isolation from the SVF can be used for in vitro and in vivo purposes. In vitro, ADSCs can differentiate along the mesenchymal lineage into adipocytes, osteoblasts and chondreoblasts. Other studies have found that ADSCs can differentiate into other mesenchymal cell types, such as cardiomyocytes [Cignarelli et al., 2012]. The in vitro differentiated ADSCs can be implanted for application in vivo. However, the outcome of implanted ADSCs has not been measurable in terms of cell survival and function. Moreover, the intrinsic stemness of freshly isolated SVF is rarely maintained during culture. The in vitro ADSC differentiation is not readily reproducible in vivo, which can cause unintended cell populations after ADSC implantation [Koh et al., 2011].

For the in vivo ADSC implantation, the vascular network is an essential component. Several investigations on the relation between vascularisation and adipose tissue have been performed, confirming the hypothesis that vascularisation plays a critical role in the development of adipose tissue. This is to say, adipose tissue is an important organ when it comes to metabolic and endocrine functions. In turn, adipose tissue excretes angiogenic factors, critical for the development of the vascular network. There might be an important role for the ADSCs when it comes to the collaboration between the vascular system and the adipose tissue [Lin et al., 2008].

The functions of ADSCs have been well described and the importance of having these cells is known for many years. However, the in vivo origin and identity of ADSCs remains elusive. To fully understand the possible in vitro and in vivo applications of ADSCs, it would be interesting to investigate the origin of these mesenchymal stem cells. Knowledge

about the ADSC origin can be used to develop better therapies for patients with damaged tissue, using specific targeting drugs [Chen et al., 2012].

Important cells belonging to the vascular network, are the vascular associated pericytes, which support the vascular system in its function. Various investigations have found leading evidence for pericytes being mesenchymal stem cells. In addition, specific pericyte populations are present in the adipose tissue, the so-called adipose resident pericytes. ADSCs often express pericyte markers on their cell surface, suggesting that ADSCs may find their origin in the perivascular environment [Zimmerlin et al., 2010].

This thesis aims to look at the relation between the ADSC origin and possible in vitro and in vivo applications. Since the vascular network, including pericytes, is crucial during adipose tissue development, I speculate that ADSCs may have a perivascular origin. Moreover, the probable causes for differences between in vitro and in vivo ADSCs are explained. The question to be answered is: *Where do ADSCs find their origin and can this origin be associated with in vitro and in vivo applications of ADSCs?*

Overview of research

Adipose tissue: fat depots & embryology

Adipose tissue is found throughout the body except for the brain, with distinct fat depots originating from different microenvironments. Fat depot origins in adipose tissue have been examined to see whether different depots cause different preadipocyte differentiation. As will be explained later on, adipose-derived stem cells (ADSCs) and preadipocytes share many features, including the ability to differentiate into adipocytes. Innate differences in adipose cell characteristics can contribute to the variation in preadipocyte differentiation, thereby explaining why it is so difficult to regulate ADSC differentiation in vivo. These innate characteristics can cause variation in lipid accumulation and lipogenic enzyme activities. Using several adipogenic transcription factors, it was found that fat depot origin affects the capacity of preadipocytes to differentiate. For example, visceral preadipocytes are less responsive to the triggered differentiation process compared to subcutaneous preadipocytes [Tchkonia et al., 2002]. This finding is of importance when observing obese individuals. Perhaps there are ADSC differences between obese and normal individuals. The knowledge on adipocyte differentiation in different fat depots, can create specific therapeutic targets in the treatment of obese patients. Obesity can occur in various forms, including subcutaneous obesity and the more dangerous visceral obesity [Tchkonia et al., 2002]. The distribution of body fat is more important than the total amount of body fat for the development of obesity-related comorbidities. Obesity-related comorbidities, linked to visceral adiposity, can be type-2 diabetes, hypertension, dyslipidemia and cancer [Cignarelli et al., 2012].

Fat depots in the body can have different embryonic origins and can therefore differ in phenotype. Knowledge about the embryology of adipose tissue can give possible origins of ADSCs. Most fat depots, for example visceral and subcutaneous depots, have a mesodermal origin, suggesting that ADSCs find their origin in the mesoderm as well. It is unknown which exact mesodermal compartments are the origin of fat depots. Adipocytes from the head are not derived from the mesoderm, but from the embryonic neural crest. Although the origin of head adipocytes suggests that they may share a few features with neural stem cells, evidence for this hypothesis was not found. For example, the differentiation of ADSCs into mature neurons is not evident. ADSCs don't have the capacity of acting as neural stem cells, such as the ability of self-renewal, thereby replacing lost neurons. However, ADSCs can support neural repair by other mechanisms, such as the secretion of several trophic and growth factors. Additional research is needed to consider these mechanisms of neural support, to get new insight into the ADSC origin and function. Perhaps in the future, ADSCs can provide a promising therapy for patients with neurodegenerative diseases [Wrage et al., 2008].

ADSC isolation and characterization/identification

ADSCs are isolated from the stromal vascular fraction (SVF), by first collecting adipose tissue through liposuction. By extensively washing, incubating and centrifuging the tissue fragments, you can easily gain the SVF. The SVF is found pelleted in the collection tube, with mature adipocytes floating on top. After collecting the SVF, the ADSCs can be isolated, cultured and expanded in this order [Bunnell et al., 2008]. Freshly isolated ADSCs differ in displaying markers compared to cultured ADSCs, but both ADSCs types are capable of multipotential differentiation [Lin et al., 2008].

The stromal vascular fraction is a heterogeneous population and contains many different cellular types. The heterogeneous SVF makes it difficult to isolate ADSCs. Although near pure ADSCs can be isolated from the SVF, ADSCs can be further purified, thereby minimizing disadvantages for tissue regeneration. Human perivascular stem cells (hPSCs), a purified population of adipose-derived stem cells, are readily accessible from the SVF. These purified ADSCs also provide more tissue regeneration in vivo compared to the unpurified

ADSCs. hPSCs may be the first cells to be used uncultured, by using fluorescence activated cell sorting (FACS)- purified hPSCs [James et al., 2012]. The advantages of hPSCs include specific use of the intended cell population and precise product characterization. In addition, hPSCs are found abundantly in all vascularised tissue, therefore hPSCs can be easily harvested [Askarinam et al., 2013]. The perivascular origin of the hPSCs indicates that all ADSCs may originate from the perivascular environment.

Several studies have characterized and thereby identified ADSCs using markers, such as CD34. Using flow cytometry, is was found that ADSCs are CD34+ expressing cells before culturing. Although this marker is not specific for ADSCs only, comparing markers on different cellular types can still give an indication of the cell characteristics [Traktuev et al., 2008]. Examining MSC marker expression in general may be an indication for ADSC marker expression as well. However, various studies have found that MSC and ADSC marker expression patterns are not comparable. For example, Zimmerlin & Rubin et al. showed that MSCs derived from the bone marrow are CD34-, while ADSCs are CD34+. Possibly, the fact that MSCs are cultured can contribute to this finding. This suggests that freshly isolated MSCs should be examined to determine their CD34 expression. By investigating both ADSC and MSCs when freshly isolated, results based on culturing differences can be excluded [Zimmerlin & Rubin et al., 2013].

ADSC differentiation

ADSCs have the ability to differentiate into cell types of multiple different lineages. *Figure 1* represents an overview of the differentiation potentials for ADSCs. In vitro, ADSCs can be induced to differentiate into specific cell types. The most common cell types resulting from ADSC differentiation, are adipocytes, osteocytes and chondrocytes. ADSCs have the potential to differentiate in additional cell types as well, such as myocytes [Bunnell et al., 2008]. It seems logical that ADSCs form adipocytes, since the ADSCs are adipose-derived. However, the reason why ADSCs seem to have a preferable differentiation potential, is not clear. It could be that the osteocyte and chondrocyte formation is most similar to adipocyte formation.

The finding of the multipotent ADSC is not in line with previous findings of Hauner et al. In this report, ADSCs have been termed preadipocytes, able to differentiate into adipose tissue cells only. This contradicts with the findings of Guilak et al., who claimed that ADSCs and preadipocytes are similar but different cell types, because of differences in protein secretion. Remarkable is that both the ADSCs and the preadipocytes are able to differentiate into adipocytes. Hauner et al. did not investigate the ability of preadipocytes to differentiate into cell types other than adipocytes [Hauner et al., 1989 & Guilak et al., 2006]. Skillington et al. have proved that preadipocytes can differentiate into osteocytes in response to extracellular signalling cues. It should be noted that the differences or similarities between preadipocytes and ADSCs are not discussed in this report [Skillington et al., 2002]. Also, the report of Hauner et al. dates in 1989, until Guilak et al. presented their report in 2006, more extensive research was done. Despite the fact that Hauner et al. and Guilak et al. disagree when it comes to the distinction between ADSCs and preadipocytes, they both state that the cell types are equally able to differentiate into adipocytes [Hauner et al., 1989 & Guilak et al., 2006]. This indicates that both cell types have similar properties and perhaps the same origin.

The in vitro ADSC differentiation is different from the in vivo ADSC differentiation, indicating that the development of ADSCs is regulated differently in vitro and in vivo. That is to say, in vitro and in vivo ADSCs have the same preference regarding cell differentiation, but the regulation of differentiation differs [Bunnell et al., 2008]. It is important to note that functional in vivo assays should be used to confirm these results [Guilak et al., 2006]. The regulation of differentiation includes the rate of differentiation and which specific cell types are formed. The variety in differentiation rate can be explained by slow-cycling ADSCs in vivo, so ADSC expansion in vitro may be contrary to their expected natural behaviour. Expanding ADSCs in vitro may be relatively fast, making it difficult to keep their undifferentiated state [Da Silva Meirelles et al., 2008]. Moreover, the origin of ADSCs can also contribute to the different differentiation potentials in vitro and in vivo, as will be explained later on. The

difference in, in vitro and in vivo ADSC differentiation is an indefinite happening which should be extensively investigated in the future.

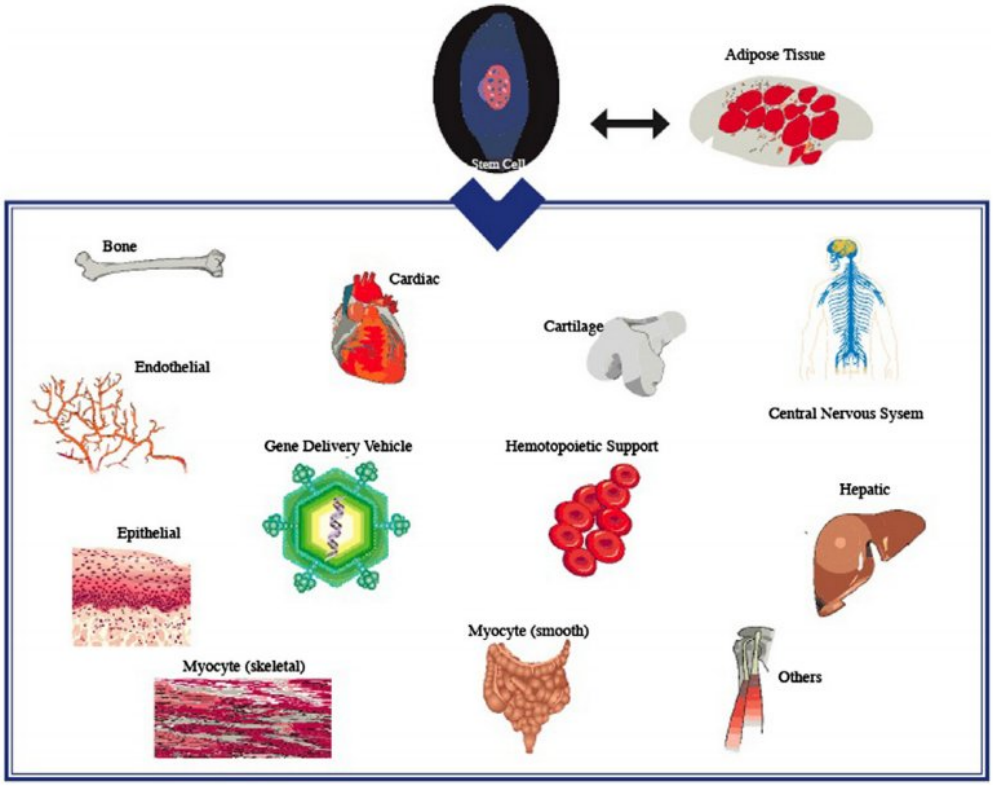

Figure 1: ADSC differentiation. Multi-lineage differentiation potential for adipose-derived stem cells [Bunnell et al., 2008].

Origin of ADSCs

Mesenchymal stem cells (MSCs), including ADSCs, are of unknown origin. To determine the origin of ADSCs, it is therefore important to determine the origin of MSCs in general. Crisan et al. found that MSCs are associated with the arterial and venous blood vessel wall by looking at several MSC markers. Immunohistochemistry was used to make a combination of markers, useful for MSC detection. Long-term cultured perivascular cells expressed MSC markers, indicating that MSCs belong to a subset of perivascular cells and may have a perivascular origin [Crisan et al., 2008]. This is an interesting feature, since MSCs are stem cells, capable of self-renewal and differentiation. However, it is not entirely clear whether ADSCs are real stem cells. To clarify, it is well known that ADSCs are able to differentiate, but their capacity of unlimited self-renewal remains elusive. This is the same for pericytes, capable of differentiation but not as capable to provide self-renewal [Schäffler & Büchler, 2007].

The statement of MSCs having a perivascular origin, can be supported by the embryonic origin of MSCs. MSCs and pericytes are most certainly descendants of cells from the embryonic dorsal aorta, called mesoangioblasts. Mesoangioblasts are vessel-associated stem cells, which can leave the vessels during tissue development and adopt the fate of the tissue. The notion of ‘vessel-associated stem cells’ refers to the fact that mesoangioblasts reside in the vascular network, rather than having a specific role in the vascular network. Mesoangioblasts differentiate into tissues of mesodermal origin, including white adipose tissue and possibly ADSCs. This differentiation process of mesoangioblasts can occur during embryonic development, postnatal development or during regenerative processes, supported by local signalling factors [Minasi et al., 2002].

To continue on the MSC origin, possible stem cell niches for MSCs and ADSCs have been investigated. Stem cell niches are found throughout the body, consisting of stem cells supported by surrounding cells. Zannettino et al found that ADSCs reside in perivascular stem cell niches in vivo, associated with perivascular cells (*Figure 2*). The perivascular stem cell niches include vascular wall (VW)-resident niches, located between pericytes. In small vessels, such as in capillaries, stem/progenitor cells are found in the sub-endothelial zone. In large and medium-sized blood vessels, stem/progenitor cells are localized in the 'vasculogenic zone' within the adventitia. The mesenchymal stem cells, including the ADSCs, are coloured in pink [Ergün et al., 2011 & Zannettino et al., 2008]. It could be that in vitro, the differentiation behaviour of ADSCs is changed because they are removed from their natural environment. Perhaps ADSCs miss support from the perivascular niche, causing ADSCs to have different differentiation potential in vitro and in vivo. The support can include cell-cell and cell-matrix adhesion and signalling factors, including endocrine, paracrine and autocrine factors [Kolf et al., 2007]. This can possibly explain the fact why in vitro and in vivo ADSCs are so different. Perhaps a perivascular stem cell niche can be created in vitro, to exclude niche-related ADSCs differences.

In the perspective of perivascular stem cell niches for ADSCs, it should be noted that ADSCs and preadipocytes may be the same type of cell, despite the fact this is not confirmed. If ADSCs and preadipocytes are the same, adipocytes may derive from specific adipocyte niches instead of perivascular niches [Harmsen, 2013]. However, mesenchymal stem cells, including ADSCs, are found located in the vascular wall. It could be that ADSCs have not been characterized properly as real stem cells, since their unlimited self-renewal capacity is questionable. In this view, ADSCs can be seen separately from MSCs and therefore be located somewhere different from adipocyte stem cell niches, such as in the vascular wall.

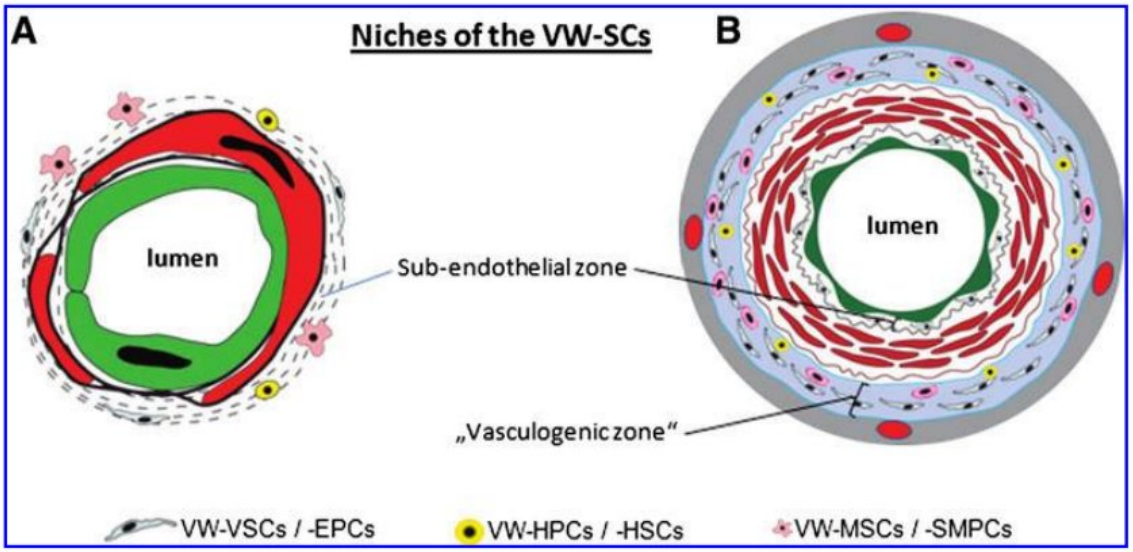

Figure 2: Vascular wall (VW)-resident niches for stem and progenitor cells. (A) In small vessels, such as in capillaries, VW resident endothelial progenitor cells (VW-EPCs), hematopoietic stem cells (VW-HSCs), and mesenchymal stem cells (VW-MSCs) are localized in the sub-endothelial space, located between or around pericytes (red) covering endothelium (green). (B) In large and medium-sized blood vessels, stem and progenitor cells are mainly localized in the 'vasculogenic zone' within the adventitia [Ergün et al., 2011].

Traktuev et al. took a more extensive look at the origin of MSCs, specifically in adipose tissue. Over de past few years, it was found that the non-endothelial population within the SVF, including ADSCs, is highly enriched in CD34 expressing cells. Clusters of differentiation molecules (CDs) are generally used to identify various cell types, mainly by cell-cell adhesion. CD34 is a stem cell marker found on the cell surface, mediating cell-cell and cell-matrix adhesion. Using immunofluorescent analysis and several assays, Traktuev et

al. wanted to localize in vivo CD34+ ADSCs in adipose tissue. On the basis of the CD34+ marker among others, they showed that ADSCs are perivascular located in close proximity to the pericytes, which are also CD34+. Besides, ADSCs also express pericytic markers after culturing. This suggests that in adipose tissue, pericytes are similar to ADSCs [Traktuev et al., 2008]. However, the findings of Lin et al. contradict with the finding of pericytes in adipose tissue being CD34+. Lin et al. found that pericytes in adipose tissue do not express CD34. This makes it difficult to confirm the similarity between pericytes and ADSCs on the basis of CD34 characteristics [Lin et al., 2008]. The contradicting findings of Traktuev et al. and Lin et al. may be due to the pericyte and ADSC culturing over several days. Passaged pericytes have been characterized CD34-, which indicates that uncultured pericytes should be used to characterize them properly [Lin et al., 2008 & Traktuev et al., 2008]. Lin et al. have repeated their experiments with uncultured ADSCs and pericytes. They showed that both ADSCs and pericytes were CD34+ [Lin et al., 2010]. Despite the fact that Lin et al. found CD34- pericytes in their previous research, their methods were more suitable for the experiments when compared to the methods of Traktuev et al. The study of Lin et al. shows sections of different types of blood vessels including specific sizes. This in contrast to the study of Traktuev et al., showing only the longitudinal view of two blood vessels of unknown sizes. Their histological analysis is therefore of limited usefulness, giving a distorted view of the adipose cellular composition [Lin et al., 2008].

Zimmerlin et al. have also used the current knowledge on MSC markers to investigate the possible perivascular origin of MSCs in adipose tissue (ADSCs). Flow cytometry and immunostaining were used to investigate different CD markers on vessel-associated stromal cells and stem/progenitor cells. In this way, SVF subpopulations could be characterized. Two mesenchymal and two endothelial populations from the SVF were isolated, cultured and tested for adipogenesis, which is the formation of adipocytes. It was found that the two mesenchymal populations, including pericytes and supra adventitial-adipose stromal cells (SA-ASCs, a subset of ADSCs), have adipogenic potential. The two endothelial populations, including endothelial progenitors and mature endothelial cells, did not show adipogenic potential in culture. Flow cytometry also revealed a pericyte subset that may be transitional between pericytes and SA-ASCs. This suggests that there is a progenitor/progeny relationship between pericytes and SA-ASCs. Since pericytes are located in contact with the intimal surface of arterioles and venules, ADSCs may be located inhere as well [Zimmerlin et al., 2010]. *Figure 3* reveals the locations of the mesenchymal and endothelial cells in arterioles and venules, by means of different markers. CD31 is an endothelial marker, α-SMA is a perivascular marker, CD34 is a stem cell/endothelial marker and CD146 is a perivascular marker. In vivo, pericytes (CD31-, α-SMA+, CD34- and CD146+) appear as cells in contact with the intimal surface of small vessels. SA-ASCs (CD31-, α-SMA-, CD34+ and CD146-) are found in the outer adventitial stromal ring. Endothelial progenitors (CD31+ and CD34+) were especially found in the capillary endothelial cells, whereas mature endothelial cells (CD31+ and CD34-) were found in small vessel endothelium [Zimmerlin et al., 2010].

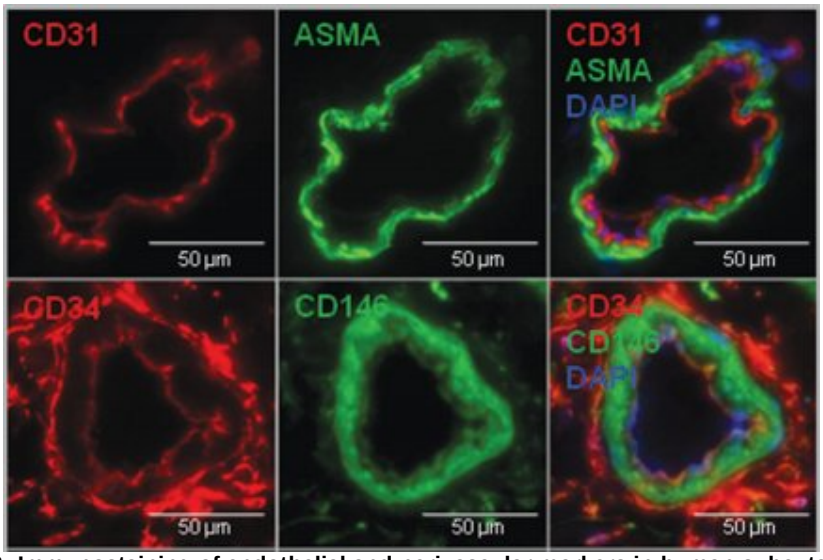

Figure 3: Immunostaining of endothelial and perivascular markers in human subcutaneous fat tissue. Top row: CD31 staining was visualized at the luminal surface of capillaries and small blood vessels. ASMA was detected on perivascular cells. Bottom row: CD34 staining was visualized on capillaries and small blood vessels, dim on the luminal endothelium and bright in the supra-adventitial layer. CD146 detection was mostly on perivascular cells, but endothelial staining was also observed. Nuclei were stained blue with DAPI [Zimmerlin et al., 2010].

After the study of Zimmerlin et al. investigating ADSC and pericyte coherency, MSC markers on ADSC were investigated. In the follow-up study performed by Zimmerlin & Donnenberg et al., MSC markers on freshly isolated ADSCs were examined. Besides the pericyte-SA ASC subset, in the current study, a mesenchymal/pericyte-endothelial subset was also found. The principle of this subset can be found in the 'mesenchymoangioblast', a common precursor for mesenchymal and endothelial cells. The mesenchymoangioblast exists together with the previously named vessel-associated stem cells, the mesoangioblasts, which are the probable ancestors of ADSCs and multipotent pericytes. This suggests that mesenchymoangioblasts and mesoangioblasts together may regulate the pericyte/SA-ASC/endothelial cell development [Zimmerlin & Donnenberg et al., 2013].

There are other lines of evidence suggesting that ADSCs are vascular stem cells (VSCs), according to different markers and differentiation patterns. Lin et al. designed their study to investigate whether ADSCs indeed are vascular stem cells (VSCs) at various stages of differentiation. Both the immunohistochemistry and immunofluorescence techniques were used for marker localization, to provide optimal histology and to enable co-localization, respectively. The localized markers may or may not be ADSC specific. For example, non specific ADSC markers were used to visualize the distribution of blood vessels in adipose tissue. Based on the experimental results and literature investigation, Lin et al. invented the 'vascular stem cell theory'. This theory suggests that ADSCs and MSCs in general are vascular stem cells, localized in proximity with the vascular network. VSCs can differentiate in vivo into tissue-specific cell types, for example into adipocytes in adipose tissue. In vitro, an individual VSC can be induced to differentiate into various cell types, depending on its degree of fresh isolation. By reasoning from the assumption of ADSCs being part of VSCs, this can explain why the yield of ADSCs is much greater than the yield of bone marrow mesenchymal stem cells (BM-MSCs). This because the adipose tissue is highly vascularised, with the property of angiogenesis, suggesting that ADSCs can be an optimal source of adult stem cells. Moreover, the vascular origin of ADSCs suggests that the vascularisation process plays a critical role in the development of adipose tissue. The exact ADSC location in or near the blood vessels was not examined [Lin et al., 2008]. In a follow-up study of Lin et al., they continue on the vascular stem cell theory, thereby examining several cellular markers to localize the VSCs or ADSCs. They use the CD34 marker as an indicator for the presence of ADSCs. The experimental results show that CD34 is localized in the intima and adventitia of

arteries and veins, but not in the media. The intima and media of larger vessels can be excluded as candidates for ADSC localization, since inhere endothelial cells and smooth muscle cells are located, respectively. The adventitia is the only possibly location for the CD34+ ADSCs or VSCs. In the capillaries, ADSCs exist together with pericytes and endothelial cells, which are both possible progenies of ADSCs [Lin et al., 2010].

Both Zimmerlin et al. and Lin et al. showed that ADSCs and pericytes are located in the adventitia of blood vessels. Zimmerlin et al. showed this in small blood vessels, whereas Lin et al. showed this in large blood vessels. However, Zimmerlin et al. also found that pericytes can be located in contact with the intimal surface of small vessels [Zimmerlin et al., 2010 & Lin et al., 2010]. An explanation for this can be that there might be migration of adventitial cells out of the tunica adventitia into the tunica media and intima, thereby altering the location of, in this case, pericytes [Chen et al., 2012].

There is leading evidence that MSCs, including ADSCs, may support the HSCs in the in vitro haematopoiesis. Corselli et al. investigated a possible perivascular niche for hematopoietic stem cells (HSCs) besides their niche in bone marrow. Since this report is investigating the origin of ADSCs, knowledge on HSC origin can be used herein, if MSCs do support the HSCs. The perivascular marker CD146 was used to define human CD146+ perivascular cells as a subset of MSCs, extracted from the non-haematopoietic adipose tissue. The ability of these MSCs to support in vitro HSC maintenance was examined. It was shown that CD146+ perivascular cells support HSCs in vitro, by enhancing repopulation ability and self-renewal potential. For the support of HSCs, cell-cell contact was needed, possibly by the CD146 marker itself. Notch activation was also found to be required in HSC support, although the specific role of this pathway is not known. The HSCs reside in a distinct, hematopoietic perivascular niche, suggesting that MSCs find their origin in proximity as well. However, Zimmerlin et al. found that the CD146 marker is not expressed ADSCs. The CD146- appearance on ADSCs can be due to the ADSC culturing, thereby altering the marker expression patterns. Further research has to prove whether the support of HSCs is also observed in vivo. By gaining this knowledge, HSCs can probably be supported by the CD146+ perivascular cells in pathological conditions such as leukaemia [Corselli et al., 2013 & Zimmerlin et al., 2010].

To summarize the marker characteristics of ADSCs and pericytes, *table 1* represents marker characteristic data for in situ stainings and for (un)cultured isolated fractions. All the adipose tissue was harvested subcutaneously, to minimize fat depot dependent differences in marker expression. When comparing the marker expressions patterns observed in situ and in vitro (uncultured and cultured), it is shown that ADSCs change their phenotype during culturing. The ADSC/pericyte resemblance may be shown by the common CD34 marker expression, although extensive research has to confirm this statement.

Table 1 **Marker characteristics of ADSCs and pericytes**

Marker	in situ		isolated (uncultured)		isolated (cultured)	
	+/-	location of harvest	+/-	location of harvest	+/-	location of harvest
CD34	ADSC: +, pericyte: +/-	Human SAT	ADSC: +	Human SAT	ADSC: -	Human SAT
CD31	ADSC: -, pericyte: -	Human SAT	ADSC: -	Human SAT	ADSC: -	Human SAT
α-SMA	ADSC: -, pericyte: +	Human SAT	ADSC: -	Human SAT	ADSC: -	Rat SAT
CD146	ADSC: -, pericyte: +	Human SAT	ADSC: -	Human SAT	ADSC: +	Human SAT

Table 1: Marker characteristics of ADSCs and pericytes. CD34: stem cell/endothelial marker, CD31: endothelial marker, α-SMA: perivascular marker, CD146: perivascular marker, human SAT: human subcutaneous adipose tissue, rat SAT: rat subcutaneous adipose tissue. Marker characteristics of pericytes were included when possible. References: in situ [Zimmerlin et al., 2010 & Traktuev et al., 2008], isolated (uncultured) [Traktuev et al., 2008 & Suga et al., 2009], isolated (cultured) [Lin et al., 2008 & Cao et al., 2008 & Miyahara et al., 2006].

ADSC and vascular network interaction

The possible perivascular origin of ADSCs suggests that these cells interact with the vascular network. Traktuev et al. did not only investigate the perivascular origin of ADSCs, but also looked at the interaction with the vascular network. They found that when adipose tissue vasculature was diminished, adipose tissue was reduced, suggesting that the amount of adipose tissue mass can be regulated. Besides, ADSCs support endothelial cells (ECs) in their survival and in the vascular network formation, whereas ECs help ADSCs during their mitotic process. Moreover, mutual chemo-attraction was demonstrated by paracrine interactions. The ADSC-EC interactions were supported by various growth factors, such as the vascular endothelial growth factor (VEGF). The role of ADSCs in the ADSC-EC interaction pattern is similar to the role of pericytes, when interacting with ECs [Traktuev et al., 2008]. This is again an indication for ADSCs having a perivascular origin. Worth notable is the ability of ADSCs to secrete certain growth factors it selves. Rehman et al. found that subcutaneous ADSCs are able to secrete angiogenic and anti-apoptotic factors, with potentially synergistic functions. Using the real time polymerase chain reaction technique, especially VEGF and HGF (hepatic growth factor) were observed [Rehman et al., 2004].

Since ADSCs are multipotent and interact with the vascular network, the ADSC differentiation into endothelial cells was investigated. Cao et al. showed that cultured ADSCs do not express the endothelial marker CD31. To induce endothelial differentiation, ADSCs were cultured with VEGF. After 48 hours, ADSCs showed morphology of mature endothelial cells, thereby expressing CD31 and other endothelial markers. This indicates that the ADSCs have developed endothelial function, contributing to vascular network function [Cao et al., 2005].

Traktuev et al. speculated that ADSCs could be useful for the enhancement of vascular supply, limiting ischemic tissue loss. In this way, patients with ischemic diseases can be aided using ADSC therapy, also by the ability of ADSCs to secrete various growth factors [Traktuev et al., 2008 & Rehman et al., 2004 & Cao et al., 2005]. An important point to consider when using ADSCs for such vascularisation processes, is to use freshly isolated ADSCs. Otherwise, the vascular network formation will be limited, due to excessive SVF culture passages [Koh et al., 2011].

In vitro and in vivo applications

Induced in vitro differentiation of ADSCs can provide various cell types, which can be used for in vivo implantation. ADSC implantation in vivo can be done using several techniques. Various clinical trials investigated the effect of ADSCs in the formation of tissue, for example the formation of bone tissue. The implantation of ADSCs is mostly performed by using scaffolds, since inhere tissue can grow supported by various growth factors which are embedded in the scaffolds. In this way, scaffolds provide an appropriate environment for the regeneration of tissues. Hicok et al. observed the effect of subcutaneous ADSC implantation in vivo, using Collagraft scaffolds. Collagraft scaffolds are composed of collagen, ceramic and marrow, the substances needed to have the most effective bone graft substitute. Six weeks after scaffold implantation, the implants were removed and analyzed for osteoid formation. Osteoid is the unmineralized portion of the bone and when mineralized, bone is formed. It was shown that the ADSCs are capable to form osteoid, when combined with appropriate biomaterials. Moreover, the matrices were also highly vascularised, an essential feature during bone formation. It should be noted that, despite the appropriate Collagraft scaffold, ADSCs can be lost because of mechanical stress. However, this was not found in the study of Hicok et al. They used a calcein marker to detect viable cells and found that >95% of the ADSCs on the matrix were positive for this marker, thereby showing minimal cell loss [Hicok et al., 2004].

Adipose-derived stem cells can also be placed in the body using ADSC monolayers. Miyahara et al. showed that monolayered ADSCs repair scarred myocardium after myocardial infarction. After induced myocardial infarction, ADSCs were acquired, cultured and plated on a temperature-responsive dish. The ADSCs on the dish were cultured and

centrifuged, before the temperature was reduced to below 37°C. The ADSCs detached from the temperature-responsive dish and floated up into the medium as monolayered cell grafts. The ADSC monolayers were transferred to the myocardial scar and attached on its surface. In situ the ADSC monolayers developed into a thick stratum, including newly formed vessels and cardiomyocytes, thereby increasing cardiac function. Monolayered ADSCs may be a better strategy than injected ADSC therapy, because of the cell-cell connections and paracrine regulation. Paracrine regulation can be growth factor-mediated, thereby inducing neovascularisation. In addition, harvesting ADSCs is easy since cardiovascular patients often have abundant adipose tissue. This suggests that ADSC monolayers can be a new strategy for cardiac tissue regeneration [Miyahara et al., 2006].

ADSCs can also be implanted by intravenous or intralesional administration. However, direct injection of cells into soft tissue results in cell loss by mechanical stress. Zimmerlin & Rubin et al. have developed a matrix of biocompatible material, more suited for in vivo application than matrices in general. The so-called 'fibrin tissue sealant Tisseel' can serve as a vehicle for liquid cell delivery, that rapidly polymerizes forming a web of cells embedded in a fibrin structure. Unpassaged SVF cells could be suspended in Tisseel fibrin sealant and delivered by an aerosol spray system, while still having the ability to differentiate into endothelial tubules and adipocytes. SVF cells include large amounts of ADSCs and pericytes, which are possibly the progenitors of ADSCs. It was found that the fibrin tissue sealant Tisseel suspended with SVF cells can grow tissue in vitro and in vivo. Moreover, endothelial progenitors in the SVF can provide vascularisation in vitro and in vivo. The fibrin tissue sealant Tisseel may also participate in anti-inflammatory responses, by the secretion of cytokines mediated by the ADSCs. Therefore, the regenerative process should be enhanced even further. One point worth notable, is that too high a fibrin density can result in less cell survival, therefore fibrin density should be carefully monitored [Zimmerlin & Rubin et al., 2013]. During in vivo ADSC implantation, growth factors play an important role, supporting ADSC in their regenerative capability. ADSCs can secrete several growth factors themselves and be influenced by growth factors secreted by other cells. Growth factors can support ADSCs in their proliferation. In addition, growth factors can regulate the process of angiogenesis, thereby maintaining the in vivo implanted ADSCs [Rehman et al., 2004].

A combined ADSC-growth factor therapy can be an interesting way of stem cell therapy. Since the substantial potential of human perivascular stem cells (hPSCs, a purified population of ADSCs) to regenerate tissue, these cells have been examined more extensively. Askarinam et al. have observed the effects of hPSCs in combination with the novel craniosynostosis-associated protein, the Nel-like molecule I (NELL-1). Craniosynostosis is a condition in which fibrous tissue is formed into bone tissue, causing premature fusion of sutures in the skull of infants. Craniosynostosis-associated proteins, including NELL-1, can accelerate the formation of bone tissue in the regenerative process. NELL-1 is a growth factor and may support hPSCs in the regenerative process of especially blood vessel growth. An intramuscular ectopic bone model was used to investigate whether hPSCs can cause bone formation, including bone vascularisation. Bone growth was evaluated using histology, tomography and immunohistochemistry. It was shown that hPSCs can mediate bone formation and in combination with NELL-1, vasculogenesis was observed. These findings implicate that the cell/protein combination therapy hPSC+NELL-1 is promising for vascularised bone regeneration [Askarinam et al., 2013].

The fact is that in vitro and in vivo ADSCs are very different [Harmsen, 2013]. A possible explanation could be that in vivo, donor ADSCs are rejected, thereby altering their in vivo appearance. In the case when ADSCs are not rejected, it is difficult to induce intended cell differentiation. This unintended in vivo differentiation may be repaired by implanting ADSCs in a specific lesion or environment, thereby mediating intended differentiation, for example with the support of specific growth factors. The exact reason why the ADSCs behave so differently with respect to their in vitro and in vivo differentiation, remains elusive. With the growing knowledge on ADSC implantation, therapies targeting specific lesions can possibly be developed in the future.

To summarize the overview of research above, *Figure 4* is presented as own interpretation.

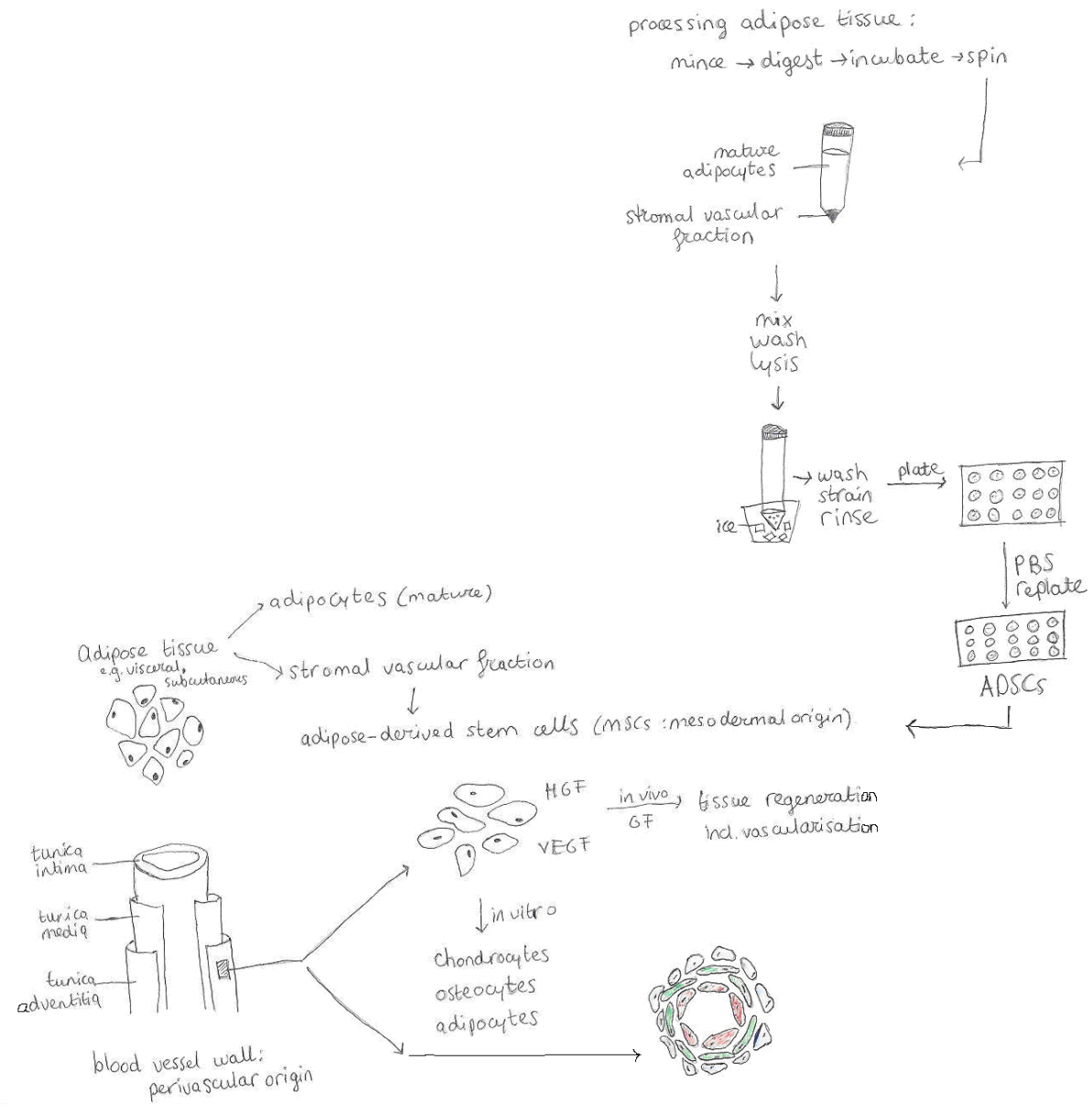

Figure 4: ADSC origin and applications. Adipose tissue is composed of adipocytes and the stromal vascular fraction (SVF). When processing adipose tissue, the SVF can be isolated. Additional steps are taken to isolate the ADSCs from the SVF [Bunnell et al., 2008]. The adipose tissue is highly vascularised and in the arteries and veins, ADSCs are located in the tunica adventitia. In vitro, ADSCs can differentiate mainly into chondrocytes, osteocytes and adipocytes. In vivo, ADSCs can be used for tissue regeneration including vascularisation, supported by growth factors. Intersection blood vessel: endothelial cells (red), smooth muscle cells (green) and adipose-derived stem cells (grey/blue) (see *Figure 2*). VEGF: vascular endothelial growth factor, HGF: hepatic growth factor, GF: growth factor.

Future perspectives

Elaborate and additional research is needed to use ADSCs in a responsible way. To begin with, the signalling pathways driving adipose progenitor cells to differentiate into mature adipocytes remain indistinct. Zimmerlin et al. speculate that the Wnt-signaling pathway may have a critical role in this process [Zimmerlin et al, 2010]. In addition, it would be useful to know more about the precise relationship between ADSCs and pericytes and their exact mesodermal origin, since their resemblance is meaningful for the functioning of both. The pericyte identity herein should be confirmed by its physiology, rather than by surface markers. It may be that the diverse marker expressions on pericytes differ because of local environments throughout the body [Traktuev et al., 2008]. The developmental relationship between pericytes and ADSCs is remarkable, since it is not evident which cells are the progenitors and which cells are the progenies. This suggests that ADSCs and pericytes are very similar cells with similar functional characteristics. This raises the question: why are ADSCs only being used for stem cell therapy? Can pericytes provide a stem cell source as well? How can the perivascular environment contribute to the pericyte stem cell properties? Is the similarity between ADSCs and pericytes the reason why ADSCs are perivascular located or the other way around? Further research may give an answer to these questions, before in vivo application can be seriously considered.

In this thesis, I aimed to investigate the relation between the ADSC origin and associated in vitro and in vivo applications. I speculated that ADSCs may have a perivascular origin. Besides, the differences between in vitro and in vivo ADSCs and their applications were discussed. The question to be answered is: *Where do ADSCs find their origin and can this origin be associated with in vitro and in vivo applications of ADSCs?*

In summary, adipose-derived stem cells are located in a perivascular environment, thereby contributing to their in vitro and in vivo applications with regard to vascularisation. ADSCs have many pericyte properties, while still able to differentiate along distinct lineages. The ADSC-pericyte subsets, originated from common intermediates, may be transitional between pericytes and ADSCs. This suggests that there is a progenitor/progeny relationship between pericytes and ADSCs, indicating that ADSCs have a perivascular origin. Moreover, ADSCs were found in the adventitia of both arteries and veins, interacting with the vascular network. When using freshly isolated ADSCs, ADSCs can be used for in vitro and in vivo applications. The distinction in ADSCs in vitro and in vivo can be due to the cycling rate of ADSCs and the specificity of implantation environment. In addition, innate differences in ADSCs can explain why it is so difficult to regulate ADSC differentiation in vivo. In vivo, ADSCs can be implanted using scaffolds in combination with several growth factors. To conclude, ADSCs can provide an additional way of stem cell therapy for tissue regeneration, besides bone marrow mesenchymal stem cell therapy. In the future, ADSCs may be used for in vivo applications, providing therapy for patients with obesity, neurodegenerative disease, leukaemia, ischemic diseases and cardiovascular diseases.

Acknowledgements

I would like to thank M. Harmsen and G. Krenning for their supervision.

References

Askarinam A, James AW, Zara JN, Goyal R, Corselli M, Pan A, Liang P, Chang Rackohn T, Stoker D, Zhang X, Ting K, Péault B, Soo C. Human perivascular stem cells show enhanced osteogenesis and vasculogenesis with Nel-like molecule I protein. Tissue Eng Part A. 2013 Jun;19(11-12):1386-97.

Bunnell BA, Flaat M, Gagliardi C, Patel B, Ripoll C. Adipose-derived stem cells: isolation, expansion and differentiation. Methods. 2008 Jun;45(2):115-20.

Cao Y, Sun Z, Liao L, Meng Y, Han Q, Zhao RC. Human adipose tissue-derived stem cells differentiate into endothelial cells in vitro and improve postnatal neovascularization in vivo. Biochem Biophys Res Commun. 2005 Jul 1;332(2):370-9.

Chen CW, Corselli M, Péault B, Huard J. Human blood-vessel-derived stem cells for tissue repair and regeneration. J Biomed Biotechnol. 2012. Epub 2012 Feb 2.

Cignarelli A, Perrini S, Ficarella R, Peschechera A, Nigro P, Giorgino F. Human adipose tissue stem cells: relevance in the pathophysiology of obesity and metabolic diseases and therapeutic applications. Expert Rev Mol Med. 2012 Dec 10;14:e19.

Corselli M, Chin CJ, Parekh C, Sahaghian A, Wang W, Ge S, Evseenko D, Wang X, Montelatici E, Lazzari L, Crooks GM, Péault B. Perivascular support of human hematopoietic stem/progenitor cells. Blood. 2013 Apr 11;121(15):2891-901.

Crisan M, Corselli M, Chen WC, Péault B. Perivascular cells for regenerative medicine. J Cell Mol Med. 2012 Dec;16(12):2851-60.

Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J, Péault B. A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell. 2008 Sep 11;3(3):301-13.

Da Silva Meirelles L, Caplan AI, Nardi NB. In search of the in vivo identity of mesenchymal stem cells. Stem Cells. 2008 Sep;26(9):2287-99.

Ergün S, Tilki D, Klein D. Vascular wall as a reservoir for different types of stem and progenitor cells. Antioxid Redox Signal. 2011 Aug 15;15(4):981-95.

Gentile P, Orlandi A, Scioli MG, Di Pasquali C, Bocchini I, Cervelli V. Concise review: adipose-derived stromal vascular fraction cells and platelet-rich plasma: basic and clinical implications for tissue engineering therapies in regenerative surgery. Stem Cells Transl Med. 2012 Mar;1(3):230-6.

Guilak F, Lott KE, Awad HA, Cao Q, Hicok KC, Fermor B, Gimble JM. Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. J Cell Physiol. 2006 Jan;206(1):229-37.

Harmsen M. 2013 Jun.

Hauner H, Entenmann G, Wabitsch M, Gaillard D, Ailhaud G, Negrel R, Pfeiffer EF. Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. J Clin Invest. 1989 Nov;84(5):1663-70.

Hicok KC, Du Laney TV, Zhou YS, Halvorsen YD, Hitt DC, Cooper LF, Gimble JM. Human adipose-derived adult stem cells produce osteoid in vivo. Tissue Eng. 2004 Mar-Apr;10(3-4):371-80.

James AW, Zara JN, Corselli M, Askarinam A, Zhou AM, Hourfar A, Nguyen A, Megerdichian S, Asatrian G, Pang S, Stoker D, Zhang X, Wu B, Ting K, Péault B, Soo C. An abundant perivascular source of stem cells for bone tissue engineering. Stem Cells Transl Med. 2012 Sep;1(9):673-84.

Koh YJ, Koh BI, Kim H, Joo HJ, Jin HK, Jeon J, Choi C, Lee DH, Chung JH, Cho CH, Park WS, Ryu JK, Suh JK, Koh GY. Stromal vascular fraction from adipose tissue forms profound vascular network through the dynamic reassembly of blood endothelial cells. Arterioscler Thromb Vasc Biol. 2011 May;31(5):1141-50.

Kolf CM, Cho E, Tuan RS. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthritis Res Ther. 2007;9(1):204.

Lin CS, Xin ZC, Deng CH, Ning H, Lin G, Lue TF. Defining adipose tissue-derived stem cells in tissue and in culture. Histol Histopathol. 2010 Jun;25(6):807-15.

Lin G, Garcia M, Ning H, Banie L, Guo YL, Lue TF, Lin CS. Defining stem and progenitor cells within adipose tissue. Stem Cells Dev. 2008 Dec;17(6):1053-63.

Minasi MG, Riminucci M, De Angelis L, Borello U, Berarducci B, Innocenzi A, Caprioli A, Sirabella D, Baiocchi M, De Maria R, Boratto R, Jaffredo T, Broccoli V, Bianco P, Cossu G. The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. Development. 2002 Jun;129(11):2773-83.

Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H, Ishino K, Ishida H, Shimizu T, Kangawa K, Sano S, Okano T, Kitamura S, Mori H. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat Med. 2006 Apr;12(4):459-65.

Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, Pell CL, Johnstone BH, Considine RV, March KL. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation. 2004 Mar 16;109(10):1292-8.

Schäffler A, Büchler C. Concise review: adipose tissue-derived stromal cells--basic and clinical implications for novel cell-based therapies. Stem Cells. 2007 Apr;25(4):818-27.

Skillington J, Choy L, Derynck R. Bone morphogenetic protein and retinoic acid signaling cooperate to induce osteoblast differentiation of preadipocytes. J Cell Biol. 2002 Oct 14;159(1):135-46.

Suga H, Matsumoto D, Eto H, Inoue K, Aoi N, Kato H, Araki J, Yoshimura K. Functional implications of CD34 expression in human adipose-derived stem/progenitor cells. Stem Cells Dev. 2009 Oct;18(8):1201-10.

Tchkonia T, Giorgadze N, Pirtskhalava T, Tchoukalova Y, Karagiannides I, Forse RA, DePonte M, Stevenson M, Guo W, Han J, Waloga G, Lash TL, Jensen MD, Kirkland JL. Fat depot origin affects adipogenesis in primary cultured and cloned human preadipocytes. Am J Physiol Regul Integr Comp Physiol. 2002 May;282(5):R1286-96.

Traktuev DO, Merfeld-Clauss S, Li J, Kolonin M, Arap W, Pasqualini R, Johnstone BH, March KL. A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. Circ Res. 2008 Jan 4;102(1):77-85.

Wrage PC, Tran T, To K, Keefer EW, Ruhn KA, Hong J, Hattangadi S, Treviño I, Tansey MG. The neuro-glial properties of adipose-derived adult stromal (ADAS) cells are not regulated by Notch 1 and are not derived from neural crest lineage. PLoS One. 2008 Jan 16;3(1):e1453.

Zannettino AC, S Paton, A Arthur, F Khor, S Itescu, JM Gimble and S Gronthos. (2008). Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. J Cell Physiol 214:413–421.

Zimmerlin L, Donnenberg VS, Pfeifer ME, Meyer EM, Péault B, Rubin JP, Donnenberg AD. Stromal vascular progenitors in adult human adipose tissue. Cytometry A. 2010 Jan;77(1):22-30.

Zimmerlin L, Donnenberg VS, Rubin JP, Donnenberg AD. Mesenchymal markers on human adipose stem/progenitor cells. Cytometry A. 2013 Jan;83(1):134-40.

Zimmerlin L, Rubin JP, Pfeifer ME, Moore LR, Donnenberg VS, Donnenberg AD. Human adipose stromal vascular cell delivery in a fibrin spray. Cytotherapy. 2013 Jan;15(1):102-8.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002 Dec;13(12):4279-95.