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Review

Defining adipose tissue-derived stem cells in tissue and in culture

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Summary. Adipose tissue-derived stem cells (ADSC) are routinely isolated from the stromal vascular fraction (SVF) of homogenized adipose tissue. Similar to other types of mesenchymal stem cells (MSC), ADSC remain difficult to define due to the lack of definitive cellular markers. Still, many types of MSC, including ADSC, have been shown to reside in a perivascular location, and increasing evidence shows that both MSC and ADSC may in fact be vascular stem cells (VSC). Locally, these cells differentiate into smooth muscle and endothelial cells that are assembled into newly formed blood vessels during angiogenesis and neovasculogenesis. Additionally, MSC or ADSC can also differentiate into tissue cells such as adipocytes in the adipose tissue. Systematically, MSC or ADSC are recruited to injury sites where they participate in the repair/regeneration of the injured tissue. Due to the vasculature's dynamic capacity for growth and multipotential nature for diversification, VSC in tissue are individually at various stages and on different paths of differentiation. Therefore, when isolated and put in culture, these cells are expected to be heterogeneous in marker expression, renewal capacity, and differentiation potential. Although this heterogeneity of VSC does impose difficulties and cause confusions in basic science studies, its impact on the development of VSC as a therapeutic cell source has not been as apparent, as many preclinical and clinical trials have reported favorable outcomes. With this understanding, ADSC are generally defined as CD34+CD31- although loss of CD34 expression in culture is well documented. In adipose tissue, CD34 is localized to the intima and adventitia of blood vessels but not the media where cells expressing alpha-smooth muscle actin (SMA) exist. By excluding the intima, which contains the CD34+CD31+ endothelial cells, and the media, which contains the CD34-CD31- smooth muscle cells, it leaves the adventitia as the only possible location for the CD34+ ADSC. In the capillary, CD34 and CD140b (a pericyte marker) are mutually exclusively expressed, thus suggesting that pericytes are not the CD34+ ADSC. Many other cellular markers for vascular cells, stem cells, and stem cell niche have also been investigated as possible ADSC markers. Particularly the best-known MSC marker STRO-1 has been found either expressed or not expressed in cultured ADSC. In the adipose tissue, STRO-1 appears to be expressed exclusively in the endothelium of certain but not all blood vessels, and thus not associated with the CD34+ ADSC. In conclusion, we believe that ADSC exist as CD34+CD31-CD104b-SMA- cells in the capillary and in the adventitia of larger vessels. In the capillary these cells coexist with pericytes and endothelial cells, both of which are possibly progenies of ADSC (or more precisely VSC). In the larger vessels, these ADSC or VSC exist as specialized fibroblasts (having stem cell properties) in the adventitia.

Key words: Adipose tissue-derived stem cells, Mesenchymal stem cells, Vascular stem cells, Pericytes, CD34, STRO-1

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Introduction

The adipose tissue is an ideal source for cells with therapeutic potential

Stem cells are conventionally divided into embryonic stem cells (ESC) and adult stem cells (ASC). ESC are obtained from embryonic blastocysts and have the ability to differentiate into all tissue types. ASC are derived from various tissues of developed (adult) or

developing (fetus, infant, or child) individuals and can differentiate into certain but not all tissue types. The best-studied ASC is bone marrow stem/stromal cells (BMSC), and its research has progressed to clinical trials with favorable outcomes. Another type of ASC is adipose tissue-derived stem/stromal cells (ADSC), which holds several advantages over BMSC as a therapeutic cell source.

In mammals, the predominant type of adipose tissue is white, as opposed to brown adipose tissue, which is present in the newborns but practically absent in the adults. The white adipose tissue is mostly composed of mature adipocytes that are each filled with a single lipid droplet of various sizes. Interspersed between these mature adipocytes is the stroma, which is itself composed of blood vessels, fibroblasts, leukocytes, macrophages, and pre-adipocytes (not yet filled with lipid). Blood vessels in the stroma (the stromal vasculature) support the growth of the adipose tissue parenchyme, including both the hyperplasic and hypertrophic growth of adipocytes as well as the differentiation of pre-adipocytes into mature adipocytes.

Unlike most adult tissues, which are normally stable in size, the adipose tissue can grow and regress throughout adulthood. Parallel to this distinction, while the vasculatures of most adult tissues are quiescent (Hobson and Denekamp, 1984), the adipose tissue vasculature is active during adipose tissue expansion (Crandall et al., 1997; Hausman and Richardson, 2004; Christiaens and Lijnen, 2009). Specifically, it has been shown that adipose tissue angiogenesis often precedes adipogenesis (Crandall et al., 1997) and inhibition of angiogenesis results in adipose tissue regression (Rupnick, 2002; Christiaens and Lijnen, 2009). As such, the adipose tissue vasculature contains cell populations with large proliferative capacities and high differentiation potentials.

Both BMSC and ADSC are mesenchymal stem cells (MSC), which are increasingly considered to reside in perivascular locations (Shi and Gronthos, 2003; Brachvogel et al., 2005; Amos et al., 2008; Covas et al., 2008; Crisan et al., 2008; Zannettino et al., 2008). While the term "perivascular" implies "surrounding the blood vessels", MSC may actually reside within the blood vessel wall, that is, they are likely de facto cellular components of the blood vessel wall (Kovacic and Boehm, 2009). However, due to the lack of a definitive MSC marker, the exact location and the cellular identity of MSC remain elusive. Nevertheless, the generally accepted perivascular localization implies a close relationship between MSC and blood vessels, and thus, in terms of availability as a therapeutic cell source, the more abundant the blood vessels are within a donor tissue site, the higher yield of MSC can be expected of that tissue site. When MSC yield is sufficiently high, it provides the option of transplanting the freshly isolated cells into patients without the need of cell culturing – a process that can be problematic due to the introduction of undesirable animal products and the possibility of microbial contamination, cell type alteration, and human errors.

The adipose tissue is endowed with an abundance of blood vessels, with each adipocyte surrounded by an extensive capillary network (Crandall et al., 1997). Furthermore, a recent study shows that the MSC frequency correlates with blood vessel density in the adipose tissue (da Silva Meirelles et al., 2009). While reinforcing the concept of a vascular connection for MSC, this study also suggests that, being highly vascular, the adipose tissue could be an ideal source for harvesting large numbers of MSC. Indeed, it has been shown that, while the number of BMSC in bone marrow is approximately 1 in 25,000 to 1 in 100,000 nucleated cells, the average frequency of ADSC in processed lipoaspirate is approximately 2% of nucleated cells (Strem et al., 2005). Thus, the yield of ADSC from one gram of fat is approximately 5,000 cells, whereas the yield of BMSC is 100 to 1,000 cells per milliliter of morrow. Furthermore, while bone marrow can only be obtained in limited quantity, the adipose tissue is usually obtainable in abundance, especially in our increasingly obese society. The safety of the tissue isolation procedure is another advantage of ADSC over BMSC, as it has been shown that between 1999 and 2000 there were zero deaths on 66,570 liposuction procedures and a serious adverse event rate of only 0.068% (Housman et al., 2002). Thus, while ADSC and BMSC are virtually identical in their therapeutic potential, the difference in their applicability is obvious.

Histology of ADSC

ADSC originate from the stromal vascular fraction (SVF) of adipose tissues. Freshly isolated SVF cells are a heterogeneous mixture of endothelial cells, smooth muscle cells, pericytes, fibroblasts, mast cells, and pre-adipocytes (Pettersson et al., 1984). Culturing of these cells under standard conditions eventually (within the first few passages) results in the appearance of a relatively homogenous population of mesodermal or mesenchymal cells (Zuk et al., 2001) that were first named PLA (processed lipoaspirate) cells but are now more commonly called ADSC (or ASC, which can be confused with ASC for adult stem cells). However, many factors can influence the cellular composition of an ADSC culture, for example, species, age, tissue depot, isolation procedure, culture condition, passage number, and cell storage. Additionally, the choice of experimental methods and reagents may also affect the outcome of any given study concerning ADSC's expression profile, differentiation potential, and therapeutic capacity. Thus, in order to provide consistency, the following review of ADSC's histology will emphasize on human subcutaneous adipose tissue, human SVF cells, and human ADSC cultured in Dulbeco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For clarity, the discussion is organized according to the

antigens (cellular markers) that have been analyzed by immunohistochemistry, immunocytochemistry, immunofluorescence, and/or flow cytometry. These antigens generally belong to three categories of cellular markers, namely, stem cell, stem cell niche, and blood vessel.

Smooth muscle actin

Human ADSC cultured for 72 h contain a fraction (~29.2%) of cells that express SMA according to flow cytometric analysis (Zuk et al., 2001). These SMA+ cells are considered to be contaminating smooth muscle cells and/or pericytes (Zuk et al., 2001). In human adipose tissue SMA+ cells are identified in the tunica media (smooth muscle) of arteries, arterioles, veins, and venules (Lin et al., 2008). SMA+ cells are also found in adipose tissue capillaries, and because capillaries lack smooth muscle, these cells are possibly pericytes. Further discussion on the relationship between ADSC and pericytes will be given in the "Pericyte markers" section.

CD31

It has been shown that, at passage 0 (cells cultured for 72 h), less than 10% of human or murine ADSC express CD31 (Planat-Benard et al., 2004). Another study however showed that the percentage of CD31+ cells in passage 0 human ADSC is much higher at 24±17% (Mitchell et al., 2006). The latter study also showed that CD31 expression remained essentially unchanged at passages 3 and 4, and all other endothelial cell-associated markers (CD144, vascular endothelial growth factor receptor 2, von Willebrand factor) were also stably maintained. Despite such discrepancies, several recent review articles place ADSC in the CD31- and thus non-endothelial category (Gimble et al., 2007; Helder et al., 2007; Schaffler and Buchler, 2007; Locke et al., 2009; Mizuno, 2009). In human adipose tissue CD31 was identified in the tunica intima (endothelium) of arteries, arterioles, veins, and venules (Lin et al., 2008). It was also identified in capillaries, indicating endothelial cells. On the other hand, there is no evidence of CD31 expression in any non-endothelial cells, thus supporting ADSC being CD31-.

Wnt5

Wnt signaling has been shown to regulate self-renewal and differentiation of both hematopoietic and bone marrow stem cells, and importantly Wnt5a expression has been localized to the bone marrow niche environment (Baksh and Tuan, 2007; Nemeth et al., 2007). As such, we tested whether Wnt5a could be a stem cell niche marker in the adipose tissue. The results show that Wnt5a expression is localized to the tunica media of arteries, arterioles, veins, and venules (Lin et al., 2008). In capillaries, cells that were positively stained for SMA (possibly pericytes) were also stained positive for Wnt5a (Lin et al., 2008). This expression pattern implicates vascular smooth muscle cells and pericytes as possible niche cells for ADSC. In regard to the possibility that pericytes might be niche cells, a recent article has discussed the subject extensively (Diaz-Flores et al., 2009). In regard to Wnt signaling, a 2007 study showed that recombinant Wnt5a protein could induce the differentiation of ADSC into beating cardiomyocyte colonies in a dose-dependent manner (Palpant et al., 2007). Several other studies also showed that Wnt signaling is involved in the decision between adipogenic and osteogenic differentiation of ADSC (Cho et al., 2006; Kim et al., 2008b; Li et al., 2008; Park et al., 2008; Rajashekhar et al., 2008).

OCT4

OCT4 is a widely accepted marker for embryonic stem cells but its expression in adult stem cells is less certain (Ratajczak et al., 2007). To our knowledge, only one paper has provided experimental data concerning OCT4 RNA and protein expression in ADSC and it showed (by RT-PCR, immunofluorescence, and western blot) that OCT4 was abundantly expressed in cultured human and monkey ADSC (Izadpanah et al., 2006). By using the same research techniques, we found that OCT4 expression was indeed detectable but only at low levels in all tested tissue and cell samples (unpublished). In a recently published study, we show that only 1.9% of human ADSC were OCT4+ as determined by flow cytometry (Lin et al., 2008). In a more recent study Ramos et al (Ramos et al., 2009) show that murine SVF and the adipose "side population" cells lacked OCT4 expression as determined by RT-PCR. Furthermore, another recent study also reported very low levels of OCT4 expression in human ADSC, as determined by quantitative PCR (Sun et al., 2009). In human adipose tissue, OCT4+ cells were rarely detectable; some located in arterioles and capillaries (Lin et al., 2008). This in vivo result is consistent with the well-known fact that OCT4 is an embryonic transcription factor whose expression persists in only a limited number of cells in adult tissues (Ratajczak et al., 2007).

Telomerase

Similar to OCT4, telomerase is better accepted as a stem cell marker for ESC than for ASC (Hiyama and Hiyama, 2007). Using RT-PCR analysis, Kang et al. (2004) and Katz et al. (2005) respectively show positive and negative telomerase expression in monkey and human ADSC. Several other researchers detected variable levels of telomerase activities (by TRAP assay) in human ADSC (Rodriguez et al., 2005; Estes et al., 2006; Izadpanah et al., 2006; Kim et al., 2008a; De Francesco et al., 2009; Wolbank et al., 2009). Furthermore, in a comprehensive study using RT-PCR, western blot, immunofluorescence, and TRAP assay, Madonna et al. (2008) identified abundant telomerase

expression in murine, porcine, and canine ADSC. However, despite these mostly positive in vitro results, our cytometric analysis showed only 1% of human ADSC are telomerase-positive and our histological analysis of human adipose tissue identified very few telomerase-positive cells (Lin et al., 2008). In the artery, the rare telomerase-positive cells are located in the tunica adventitia. In the capillary, the telomerase-positive cells exhibit unusual morphologies, for example, being larger than what would be expected of endothelial cells. Thus, the identity of these telomerase-positive cells awaits further clarification.

SSEA-1

SSEA-1 is a marker for ESC and BMSC (Jiang et al., 2002; Anjos-Afonso and Bonnet, 2007). Interestingly, it has been shown that SSEA-1+ cells are the most primitive progenitors (having the highest differentiation potential) in the adult murine bone marrow mesenchymal compartment (Anjos-Afonso and Bonnet, 2007). In regard to ADSC, its expression was reported to be negative in human ADSC as determined by flow cytometric analysis (Rodriguez et al., 2005). However, our own cytometric analysis showed that 25.7% of human ADSC were SSEA-1+ and our histological analysis showed that SSEA-1+ cells were frequently visible in human adipose tissue (Lin et al., 2008). The positive cells are located in the tunica media and tunica adventitia but not the tunica intima of arteries. Their location in the arterioles is also in the tunica adventitia and perhaps in the tunica media as well. In the capillary SSEA-1+ cells are also identifiable. Thus, our data show that SSEA-1 is expressed both in the adipose tissue and in cultured ADSC.

STRO-1

In a 2007 review article Kolf et al. (2007) factually point out that STRO-1 is by far the best-known MSC marker. Indeed, since its publication in 1991, the STRO-1 antibody has played a critical role in hundreds of studies that relied on it for the identification and/or the isolation of MSC from a wide range of tissues. In the case of ADSC, which can be easily separated from the rest of the adipose tissue, there has been no need to rely on the STRO-1 antibody for its isolation. However, similar to the situation with other types of MSC, whether STRO-1 is expressed in ADSC is not without controversy. While there are several possible reasons why these controversies arose, our experience with the STRO-1 antibody made us suspect that its unusual characteristics may have played a role. Indeed, despite the fact that this antibody debuted nearly 2 decades ago and has been utilized extensively, its cognate antigen molecule remains unidentified. This is in sharp contrast to the case in which the STRO-3 antibody (a related but very rarely used antibody) was reported along with the identification of its cognate antigen molecule (Gronthos et al., 2007). More recently, the same group of researchers reported the production of the STRO-4 antibody, also along with the identification of its cognate antigen molecule (Gronthos et al., 2009). Thus, it is logic to assume that the STRO-1 antibody must possess certain unusual properties that make it difficult to identify its cognate antigen, and if so, it is also logic to question the accuracy of data produced with this antibody. Considering the important role it has played in stem cell research, the STRO-1 antibody thus warrants a detailed discussion.

The STRO-1 antibody was produced from a hybridoma resulting from the fusion of a mouse myeloma with B cells obtained from a mouse immunized with human CD34+ bone marrow cells (Simmons and Torok-Storb, 1991). The monoclonal antibody is of the IgM isotype, not the more common IgG, and can be purchased from one academic and four commercial sources, which all cite the same hybridoma clone as the origin of their products. In addition, all 4 commercial sources provide the same product information: (1) STRO-1 is a cell surface protein expressed by bone marrow stromal cells and erythroid precursors, (2) the frequency of colony forming units-fibroblasts (CFU-F) was enriched 100-fold in the STRO-1+/Glycophorin A- population from bone marrow cells, and (3) the subset of marrow cells that expresses the STRO-1 antigen is capable of differentiating into multiple mesenchymal lineages including hemato-poiesis-supportive stromal cells with a vascular smooth muscle-like phenotype, adipocytes, osteoblasts, and chondrocytes.

Despite having been used in numerous cytometric analyses and FACS for many different types of MSC, the STRO-1 antibody has never been used in a western blot in published studies. Thus, the molecular weight of its cognate antigen molecule remains officially unknown. Although we have tentatively identified a seemingly specific protein band in a western blot of STRO-1-selected rat ADSC, the results were not reproducible and therefore not published. In regard to the antibody's application in immunocytochemical or immunohisto-chemical experiments, exhaustive search of the literature found very few examples. In one such rare occasion, STRO-1 was reported to localize "not only to endothelial cells but also the perivascular cells of the blood vessel wall" (Bianco et al., 2001). In others, most notably in the dental stem cell field, the histological images are of insufficient magnification or quality and therefore difficult to judge. In our published (Lin et al., 2008) and unpublished studies we found that STRO-1 reactivity occurred usually in and around the vascular endothelium. However, we have also found that such endothelial localization occurred in some but not all of the blood vessels (Lin et al., 2008), and such a "differential" endothelial staining occurred not only in the adipose tissue but also in the prostate, urinary bladder, urethra, and penis (unpublished). Occasionally, we have also seen staining on some unknown "stuffs" in

these tissues. Thus, despite our great interest in this antibody and numerous efforts in pursuit, we still don't know what it really is or does.

In the ADSC field, the first reported use of the STRO-1 antibody is from Gronthos et al. (2001), and which reported negative results of cytometric analysis for human ADSC. However, in another study that was published the next year, Zuk et al. (2002) showed that 31.26% of human ADSC expressed STRO-1. Interestingly, Gronthos' group, which reported the initial negative findings, tested STRO-1 as an ADSC marker in a more recent study and found perivascular STRO-1 expression in human adipose tissues. Furthermore, they also found that ADSC could be enriched by selection for STRO-1 immunoreactivity (Zannettino et al., 2008). However, in a very recent study Goudenege et al. (2009) still reported negative cytometric results for human ADSC. In studies using immuncytochemistry, we and another group reported that a large percentage of rat ADSC stained positive (Ning et al., 2006; Radtke et al., 2009); however, a third group reported only 11.38% positivity (Kingham et al., 2007). On top of these conflicting reports, perhaps the most perplexing of all is that a recent short communication reported positive real-time RT-PCR results for rat ADSC (Rada et al., 2009). This of course implies that the authors have knowledge of the nucleotide sequence of the STRO-1 "gene". However, searches in gene bank databases failed to find its existence and inquiries made to the authors were not replied.

As mentioned earlier, the identification or isolation of ADSC does not rely on the STRO-1 antibody. However, many other types of MSC do, particularly those from dental tissues and articular cartilage (Kolf et al., 2007; Hombach-Klonisch et al., 2008; Otsuki et al., 2009; Sloan and Waddington, 2009). Thus, from a general stem cell research point of view, it is imperative that the mystery surrounding the STRO-1 antibody be solved. Before this becomes a reality, questions regarding the reliability of STRO-1 as a MSC marker will persist. For example, in a recent paper Grogan et al. (2009) suggests that the STRO-1 antibody may not be useful to identify progenitors in cartilage because a high number (>45%) of cells were positive throughout normal cartilage.

CD34

CD34 has long been regarded as a reliable marker for hematopoiectic stem cells (HSC), but recent studies have demonstrated the existence of CD34- HSC and that the two populations of HSC (CD34+ and CD34-) can differentiate into one another (Gangenahalli et al., 2006). Several papers, including ours, have shown that CD34 is highly expressed in freshly isolated ADSC (SVF cells) but is quickly lost in cultured ADSC (within 3 passages) (Ning et al., 2006; Gimble et al., 2007; Helder et al., 2007). This loss of expression is probably due to downregulation of CD34 expression rather than death of CD34+ cells (unpublished observation). In any event, loss of CD34 expression appears to have only minor effects on the in vitro properties of human ADSC, including differentiation potentials (Suga et al., 2009).

In human adipose tissue CD34 is abundantly expressed in all blood vessels in all of the adipose tissue samples we have examined (Lin et al., 2008). In the arteries, arterioles, veins, and venules, CD34 is localized to the tunica intima and tunica adventitia; thus, the cross section of these CD34-stained vessels have the appearance of two concentric circles (endothelium and adventitia) sandwiching the unstained smooth muscle layer. A similar staining pattern in subcutaneous tissue has been reported previously (Pusztaszeri et al., 2006). In the tunica intima, CD34 staining is easily distinguishable from that of CD31 because CD34 staining was homogeneous and contiguous while that of CD31 was discrete and more intense at intercellular junctions (Lin et al., 2008). This differential staining of the endothelium by anti-CD31 and anti-CD34 antibodies has been previously observed in blood and lymphatic vessels in non-adipose tissues (Sauter et al., 1998; Muller et al., 2002; Fiedler et al., 2006).

In the tunica adventitia, which is devoid of CD31 expression, CD34 is expressed most likely in cells that have been proposed to be progenitor/stem cells (Xu, 2008). First, a 2004 paper reported that the aortic adventitia harbors an abundance of vascular progenitor cells that are CD34+ (Hu et al., 2004). Second, a 2007 paper also reported the identification of CD34+ progenitor cells in the human coronary arteries (Torsney et al., 2007). Third, a 2006 paper reported the existence of CD34+CD31- cells in a distinct zone located between the smooth muscle and adventitial layers of the human vascular wall (Zengin et al., 2006). Furthermore, the report showed that the CD34+CD31- cells were capable of differentiating into mature hematopoietic cells, endothelial cells, and macrophages. Based on these consistent data, we believe that the CD34+ cells in the adventitia (or the "vasculogenic zone") of blood vessels in the adipose tissue are the de facto ADSC.

Pericyte markers

There have been increasing interests in the possibility that MSC are pericytes, which are traditionally defined as extensively branched cells located in nonmuscular microvessels, capillaries, and postcapillary venules (Diaz-Flores et al., 2009). In the ADSC field, the pericytic identity of ADSC has been examined in five 2008 papers, 4 of which reported favorable assessments mainly based on cytometric analyses. First, Traktuev et al. (2008) reported that 90% of freshly isolated CD34+CD31-CD144- ADSC co-expressed mesenchymal (CD10, CD13, and CD90), pericytic (chondroitin sulfate proteoglycan, CD140a, and CD140b), and smooth muscle (SMA, caldesmon, and calponin) markers. These cells however do not express another pericyte marker 3G5 (see next section), and the

authors proposed that pericytic identity should be established by physiological properties and function rather than by surface markers. Second, Crisan et al. (2008) reported that human ADSC expressed pericyte markers CD146, NG2, and PDGFR-, (CD140b). Third, Amos et al. (2008) reported that human ADSC expressed SMA (10%) and NG2 (8%). Forth, Rajashekhar et al (Rajashekhar et al., 2008), who are co-workers or collaborators of Traktuev et al, showed that NG2 mRNA (by quantitative PCR) was expressed in CD140b-sorted ADSC but not in CD31-sorted ADSC.

The report by Traktuev et al. (2008) suggested that ADSC could be defined by co-expression of CD34 and CD140b. Thus, we performed immunofluorescence staining for the two markers in human adipose tissue, and surprisingly, we found that these two markers are mutually exclusive in all of the capillaries (in the hundreds) that we have examined (Lin et al., 2008). In addition, we found that CD34 and SMA are also mutually exclusively expressed in all of the capillaries that we have examined. Thus, in their native tissue, ADSC do not seem to co-express CD34 and CD140b or CD34 and SMA. This finding is now supported by a recent publication (Suga et al., 2009), which, citing their previous paper (Yoshimura et al., 2006) and data not shown, disclosed that human ADSC in tissue and in freshly isolated SVF express CD34 but not pericyte markers, such as CD146 and NG2. In addition, this paper also showed that expression of pericyte markers is higher in CD34- than in CD34+ ADSC. Thus, because ADSC are known to lose CD34 expression during culture (see previous section), it is possible that the four above-mentioned papers (in favor of equating ADSC with pericytes) were looking at cells whose CD34 expression was on the decline and whose pericyte marker expression on the rise.

Additional markers

Although several additional markers have been examined in cultured ADSC, only those that have been investigated in the adipose tissue will be discussed here. CD90 and CD105, which are upregulated with passage of ADSC culture (Locke et al., 2009), and Sca-1, a marker for adipocyte progenitor cells (Rodeheffer et al., 2008) but also expressed by endothelial cells (van de Rijn et al., 1989), have been examined in murine adipose tissue (Yamamoto et al., 2007). Although all three markers appear to localize in the adipose stroma, whether they are associated with blood vessels cannot be assessed because the photographs are of low magnification. CD146 and 3G5, both of which are considered as pericyte markers but are also expressed in several other cell types (Bardin et al., 2001; Stramer et al., 2004), were examined along with STRO-1 in human adipose tissue by Zannettino et al. (2008). Although these three markers were all identified as positively expressed in or around two large blood vessels, the histology images are difficult to interpret due to the lack of reference landmarks (such as adipocytes). Furthermore, while the images show that all of the tested markers were localized to the vessel wall, they do not display sufficient details as to tell whether the markers are located in the intima or the adventitia. In one of the images, STRO-1 expression appears to be sandwiched between two concentric layers of CD146 expression, thus resembling the images of ours in which SMA expression is sandwiched between two concentric layers of CD34 expression (Lin et al., 2008). However, the inferred notion that STRO-1 is expressed in the tunica media (smooth muscle) and CD146 in the intima and adventitia would further complicate the identification of ADSC in vivo.

Concluding remarks

We have previously proposed that ADSC and MSC in general are vascular stem cells (VSC) (Lin et al., 2008). We also proposed that, due to the vasculature's dynamic capacity for growth and multipotential nature for diversification, VSC in tissue are individually at various stages and on different paths of differentiation. Therefore, when isolated and put in culture, these cells are expected to be heterogeneous in marker expression, renewal capacity, and differentiation potential. Although this heterogeneity of VSC does impose difficulties and cause confusions in basic science studies, its impact on the development of VSC as a therapeutic cell source has not been as apparent, as many preclinical and clinical trials have reported favorable outcomes.

Despite the growing appreciation of MSC's vascular origin, it remains unsettled where exactly MSC are located in the blood vessels. In a recent review, Kovacic and Boehm (Kovacic and Boehm, 2009) comprehensively summarized all possible locations, including all tissue layers (intima, media, and adventitia), and all cell types that may possess stem cell properties (endothelial cells, pericytes, and adventitial progenitor cells). However, in regard to ADSC's location, there appear to be only two possible sites, namely, the intima where pericytes may reside (Diaz-Flores et al., 2009) and the adventitia where adventitial progenitor cells reside (Hu et al., 2004; Zengin et al., 2006). At present, four studies (Amos et al., 2008; Crisan et al., 2008; Rajashekhar et al., 2008; Traktuev et al., 2008) favor the pericyte theory while we (Lin et al., 2008) consider the adventitia. Our objection to the pericyte theory is based on our finding that CD34 (ADSC marker) and CD140b (pericyte marker) are mutually exclusively expressed in the adipose tissue. This finding is now confirmed by a recent independent study (Suga et al., 2009). Therefore, while we cannot rule out the possibility that the CD34-CD140b+ cells in the adipose tissue may possess stem cell properties, these cells cannot be ADSC, which are classified as CD34+. However, since both SVF cells and freshly cultured ADSC do contain a mixture of CD34+ and CD34- cells and both CD34+ and CD34- cells are

capable of differentiation (Suga et al., 2009), it is also possible that both pericytes and adventitial progenitor cells contribute to what constitutes an experimentally obtainable ADSC culture.

In conclusion, we believe that ADSC exist as CD34+CD31-CD104b-SMA- cells in the capillary and in the adventitia of larger vessels. In the capillary these cells coexist with pericytes and endothelial cells, both of which are possibly progenies of ADSC (or more precisely VSC). In the larger vessels, these ADSC or VSC exist as specialized fibroblasts (having stem cell properties) in the adventitia.

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