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TOPIC HIGHLIGHT

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Adipose-derived mesenchymal stromal/stem cells: An update on their phenotype *in vivo* and *in vitro*

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Abstract

Adipose tissue is a rich, ubiquitous and easily accessible source for multipotent stromal/stem cells and has, therefore, several advantages compared to other sources of mesenchymal stromal/stem cells. Several studies have tried to identify the origin of the stromal/stem cell population within adipose tissue *in situ*. This is a complicated attempt because no marker has currently been described which unambiguously identifies native adipose-derived stromal/stem cells (ASCs). Isolated and cultured ASCs are a non-uniform preparation consisting of several subsets of stem and precursor cells. Cultured ASCs are characterized by their expression of a panel of markers (and the absence of others), whereas their *in vitro* phenotype is dynamic. Some markers were expressed de novo during culture, the expression of some markers is lost. For a long time, CD34 expression was solely used to characterize haematopoietic stem and progenitor cells, but now it has become evident that it is also a potential marker to identify an ASC subpopulation *in situ* and after a short culture time. Nevertheless, long-term cultured ASCs do not express CD34, perhaps due to the artificial environment. This review gives an update of the recently published data on the origin and phenotype of ASCs both *in vivo* and *in vitro*. In addition, the composition of ASCs (or their subpopulations) seems to vary between different laboratories and preparations. This heterogeneity of ASC preparations may result from different reasons. One of the main problems in comparing results from different laboratories is the lack of a standardized isolation and culture protocol for ASCs. Since many aspects of ASCs, such as the differential potential or the current use in clinical trials, are fully described in other recent reviews, this review further updates the more basic research issues concerning ASCs' subpopulations, heterogeneity and culture standardization.

Key words: Adipose-derived stromal/stem cells; Adipose tissue; Subpopulation; Heterogeneity; Phenotype; CD34; Mesenchymal stem cells

Core tip: Adipose tissue is a rich, ubiquitously available and easily accessible source for multipotent stromal/stem cells. This review gives an update of the recently published data on the origin and phenotype of adipose-derived stromal/stem cells (ASCs) both *in vivo* and *in vitro*. Furthermore, since many aspects of ASCs, such as the differential potential or the current use in clinical trials, are fully described in other recent reviews, this review also updates the more basic research issues concerning ASCs' subpopulations, heterogeneity and culture standardization.

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INTRODUCTION

The isolation and culture of mesenchymal multipotent

stromal/stem cells (MSCs), beginning with the pioneering work of Friedenstein *et al*[1,2] nearly 50 years ago, and the introduction of the nomenclature "marrow stromal stem cells[3]" and "mesenchymal stem cells[4]" (MSCs), opened up a new field of stem cell research. In order to address the discrepancy between the nomenclature and biologic characteristics of MSCs, the International Society for Cellular Therapy recommended that MSCs, regardless of their tissue origin, be termed multipotent mesenchymal stromal cells, while the term mesenchymal stem cells should only be used for the subset (or subsets) that meets specified stem cell criteria[5-7]. On the other hand, Arnold Caplan, who coined the term MSC, has recently further proposed naming these cells "Medicinal Signalling Cells" to preserve the MSC acronym and, in his opinion, correctly explain their function[8]. Obviously, this term only describes MSCs' function in pathological situations and, therefore, ignores their physiological functions as structural cells in the haematopoietic stem cell niche of the bone-marrow. Furthermore, whereas MSC differentiation may not be the main regenerative mechanisms in cell therapy, the multipotent character of these preparations has been shown *in vitro* and this is the main intention for their usage in tissue engineering approaches.

In general, MSCs are described as immature cells within the bone-marrow, peripheral blood, menstrual blood, and nearly all adult tissues (*e.g.,* adipose tissue, synovium, dermis, periosteum, deciduous teeth) and solid organs (*e.g.,* liver, spleen, lung)[9-11]. MSCs are a rare and quiescent population (or populations) within the perivascular niche (or are derived from perivascular cells or pericytes[12]) within fully specialized tissues. MSCs derived from different tissues not only share many similarities, but also seem to have many differences in terms of their marker expression and their biological properties (*e.g.,* differentiation potential). It has been shown, for example, that MSCs from different tissue origin of the same donor differ in some features[13]. Whereas MSCs isolated from the bone-marrow and cardiac tissue (cMSCs) shared a common stromal surface phenotype, their gene, microRNA and protein expression profiles were remarkably different. cMSCs were less competent in acquiring the adipogenic and osteogenic phenotype, but showed a higher cardiovascular differentiation potential.

There is a large number of studies showing that cultured MSC preparations are heterogeneous and consist of different populations of stem and progenitor cells with self-renewal properties and established multipotent differentiation profiles[14]. In general, MSCs are isolated by their capacity to adhere to cell culture plastic surfaces. The cells can be expanded in culture while maintaining their multipotency in standard culture conditions, and are phenotypically characterized *in vitro* by a specific panel of markers. In this context, it should be mentioned that the clear characterization of MSCs remains difficult due to the lack of a unique cellular marker[7]. In 2006, the International Society for Cellular Therapy proposed minimal phenotypic criteria for the definition of cultured MSCs: expression of CD73, CD90, and CD105, and lack of CD11b or CD14, CD19 or CD79, CD45, and HLA-DR expression[5,7]. [It should be noted that the main criteria for MSCs are (1) plastic adhesion; (2) the above described phenotype; and (3) their tri-lineage differentiation potential[5]]. In this position statement, the society also specified CD34 as a negative marker for MSCs[5], but recent reports have shown that this marker must be highlighted separately due to the tissue from which the MSCs were isolated (discussed later in this review).

Nevertheless, independent from the term used for MSCs and independent from its mechanism of action during repair or regeneration (*e.g.,* paracrine stimulation, immunomodulation, angiogenic effect, differentiation), it should be noted that MSCs have been proven to be beneficial in different medical treatments and exert positive therapeutic effects and proregenerative activities. It has been shown that MSCs secrete cytokines, growth factors and bioactive molecules with trophic, paracrine effects at variable concentrations in response to local microenvironmental cues, which seems to be the main (but maybe not the only) mechanism for their regenerative and repair potential[15]. MSCs have also been shown to possess immunogenic properties and a powerful immunosuppressive potential, which also make them attractive for allogenic cell therapy[16-19]. MSCs are attractive cells for clinical applications to repair or regenerate damaged tissues, especially because they hold no ethical concerns (in contrast to embryonic stem cells). Furthermore, MSCs from an autologous origin seem to be a safe source for cell-based regenerative approaches. However, ideal MSCs for use in therapeutic approaches need to be isolated with minimal harm to the patient, must be available in high cell numbers, proliferate in culture, and differentiate into a broad spectrum of lineages[7].

MESENCHYMAL STROMAL/STEM CELLS FROM FAT

It has been shown over the past few decades that adipose tissue is in addition to his main function as an energy reservoir also a abundant resource for multipotent stromal cells. The dissociation method and the biological characterization of these stromal cells from adipose tissue was first shown nearly 40 years ago[20,21], but their multipotent character was first confirmed only at the beginning of the recent millennium[22,23]. Adipose tissue seems to be the ideal source for multipotent stromal/stem cells as it has several advantages over other sources[7]. Subcutaneous fat is omnipresent in humans and is easily accessible in large quantities by liposuction aspiration. Liposuction is a well-tolerated and almost safe procedure yielding large quantities of tissue aspirate[7]. Furthermore, the lipoaspirate is finally discarded as medical waste, qualifying this as a good starting material for adipose-derived stromal/stem cell (ASC) isolation. In addition, the tissue contains a large number of multipotent cells which can easily be isolated and proliferated in culture. Stem and precursor cells in the freshly isolated stromal vascular fraction (SVF)

usually account for up to 3%, and this is approximately 2,500-fold more than the frequency of stem cells in bone-marrow (up to 0.002%)[24].

However, it is important to mention that many different names and abbreviations for these cultured adipose-derived cells can be found in the literature generating a confusing discrepancy. The terms "adipose-derived adult stem cells", "adipose-derived adult stromal cells", "adipose-derived stromal cells", "adipose stromal cells (ASC)", "adipose mesenchymal stem cells", "preadipocytes", "processed lipoaspirate cells", "vascular stromal/stem cells" and "adipose-derived stromal/stem cells (ASCs)" for cells isolated by an almost similar isolation procedure have been used in the last ten years. It should also be noted that others use the abbreviation ASC for adult stem cells in general. In order to eliminate this discrepancy, the International Fat Applied Technology Society (IFATS) reached a consensus to use the term "adipose-derived stromal/stem cells" (ASCs) to name the plastic-adherent, cultured and serially passaged, and multipotent cell population from adipose tissue[7,9,25]. In 2013, the IFATS published a revised statement to point out the minimal phenotypic criteria to characterize the uncultured SVF and the adherent stromal/stem cell population from adipose tissue[26]. In the SVF, native ASCs are now characterized as $CD45^-/CD235a^-/CD31^-/CD34^+$ cells, which represent approximately 20% of the whole SVF[26]. The authors proposed the inclusion of CD235a (glycophorin A) to monitor any contaminating erythroid lineage cells. The leukocyte common antigen CD45 should be used as a classic marker to identify cells of haematopoietic origin (except for red blood cells) and CD31 (PECAM-1) to detect endothelial cells and their progenitors (and also platelets and leukocytes)[26]. The authors further state that cultured ASCs are characterized as $CD73^+/CD90^+/CD105^+/CD44^+/CD45^-/CD31^-$ cells[26]. Furthermore, cultured ASCs can be distinguished from BM-MSCs by their expression of CD36 and their negativity for CD106[26]. Nevertheless, more characterization studies are needed to identify the *in vivo* counterpart(s) of the ASC population(s).

IS THE *IN SITU* LOCALIZATION AND PHENOTYPE OF ASCS SHOWN CONVINCINGLY?

Several studies have tried to identify the origin of the stromal/stem cell population within adipose tissue *in situ*. This is a complicated attempt because no marker has been described recently which unambiguously identifies native ASCs. Traktuev *et al*[27] demonstrated that ASCs are rarely distributed among adipocytes, but are predominantly associated with vascular structures in the walls of adipose microvasculature (with a $CD34^+/CD31^-$ phenotype). They detected a portion of $CD34^+$ cells co-expressing CD31 (capillary endothelial cells), and a separate and predominant population of $CD34^+/CD31^-$ cells (ASCs) in a perivascular location using immunofluorescence staining.

Corselli *et al*[28,29] proposed that blood vessels in virtually all tissues house MSCs in a perivascular niche. The group also described a perivascular cell subset (including pericytes in small vessels and adventitial cells around larger vessels), which natively expresses MSC markers and displays a multilineage differentiation potential *in vitro*[28,30-32]. The cells were extensively branched and are located in non-muscular vessels, capillaries and venules[30]. They demonstrated that these perivascular cells (or pericytes) express CD146, neuro-glial proteoglycan 2 (NG2), CD140β, and also co-express MSC-specific markers (CD44, CD73, CD90, CD105)[31]. However, this subset was shown to be negative for CD34 expression. Due to the phenotype with the expression of CD146 shown, the authors hypothesized that pericytes are an *in vivo* counterpart of cultured MSCs, but questioned whether all MSCs are derived from pericytes[29]. Zannettino *et al*[33] also described $CD146^+$ (co-localized with the mesenchymal marker Stro-1 and the pericyte marker 3G5) cells within adipose tissue, which reside perivascularly and show the biological characteristics of MSCs *in vitro*. Nonetheless, as shown by their CD146 expression, this cell population is clearly distinct from the population described by others[27,34,35] and it seems likely that these cells are a different subset of ASCs or pericytes.

In context with many other studies, it seems likely that the *in vivo* counterparts of ASCs express CD34. Maumus *et al*[36] have shown that ASCs are scattered *in situ* in the fat stroma, express $CD34^+$ and do not express pericyte markers such as NG2, CD140α, and α-smooth muscle actin (SMA) *in situ*. They identified ASCs *in situ* by their CD34 expression and discriminated them from endothelial, pericytes and other perivascular cells by immunofluorescence staining of human native adipose tissue[36]. Unfortunately, the authors did not further characterize these $CD34^+$ cells *in situ* by additional staining for other markers of ASCs. It has also been speculated that ASCs (and MSCs in general) are localized within blood vessels as a subset of pericytes or vascular precursor (stem) cells at various stages of differentiation located in the wall surrounding the vasculature[34,37]. The same group demonstrated in a newer publication that ASCs exist as $CD34^+/CD31^-/CD140\alpha^-/SMA^-$ cells in capillaries and in the adventitia of the vasculature[37]. They speculated that ASCs in capillaries coexist with pericytes and endothelial cells (and that both are progenies of ASCs), whereas ASCs exist in the adventitia of larger vessels as specialized fibroblasts with stem cell properties[37]. Zimmerlin *et al*[35] also encouraged the hypothesis of a perivascular localization of ASCs. This study has shown $CD90^+/CD34^+/CD31^-/CD146^-/smA^-$ cells in the outer adventitia of blood vessels, and postulated this population as supra adventitial ASCs. Furthermore, the authors detected cell populations which may represent transitional stages between undifferentiated stromal cells (ASCs) and perivascular cells (pericytes). These "transitional" cells were

characterized by their marker expression and their adipogenic differentiation potential, and clearly discriminated against endothelial cells. These perivascular cells are organized in two discrete layers ($CD146^+/CD34^-$ pericytes and $CD146^-/CD34^+$ supra adventitial ASC), whereas a $CD146^+/CD34^+$ subset suggests a population transitional between pericytes and ASCs.

In summary, the results from recent histological studies using immunological staining techniques suggest that ASCs reside in a (peri-)vascular location, where they coexist with pericytes and endothelial cells. Nevertheless, the exact location within the vascular niche (adventitia, inner intima, subendothelial) has not been precisely determined. It seems clear that there is a close relationship between tissue-resident stem/progenitor cells (MSCs/ASCs) and vascular pericytes. With regard to a subendothelial location, some authors concluded that pericytes are the *de facto* MSCs[9,12]. These authors suggest "MSCs (or even pericytes) stabilize blood vessels and contribute to tissue and immune system homeostasis under physiological conditions and assume an active role in the repair of focal tissue injury[9]". However, many studies demonstrated a phenotypic difference between ASCs and pericytes. Recent studies provided much evidence that native ASCs *in situ* express a $CD34^+/CD90^+/CD31^-/CD45^-/CD146^-$ phenotype. However, a definite phenotype of ASCs *in situ* has not been convincingly shown. Several studies contradict the expression of some markers (especially CD146 and CD34). Therefore, it is important to mention that the results of many studies suggest that ASCs (and MSCs in general) may be comprised of subsets or subpopulations at various stages, perhaps with varying differentiation potentials.

ASCs CHANGE THEIR PHENOTYPE EARLY IN CULTURE AND CONSIST OF SUBPOPULATIONS

In general, ASCs are isolated by plastic adherence from adipose tissue using the so-called SVF, regardless if isolated from a subcutaneous or a perirenal fat source or any other fat tissue. The percental cellular composition of the stroma vascular fraction has been described with large lab-to-lab variability, whereas it seems unquestionable that the SVF consists of adipose stromal (stem and progenitor) cells, and also endothelial cells, fibroblasts, lymphocytes, monocyte/macrophages, and pericytes, among others (*e.g.,* haematopoietic stem cells, erythrocytes)[19,26]. Several subpopulations in the SVF have been cytometrically identified, including potential ASCs ($CD31^-/CD34^+/CD45^-/CD90^+/CD105^-/CD146^-$), endothelial (progenitor) cells ($CD31^+/CD34^+/CD45^-/CD90^+/CD105^{low}/CD146^+$), pericytes ($CD31^-/CD34^-/CD45^-/CD90^+/CD105^-/CD146^+$), and blood-derived cells ($CD45^+$)[7,38]. However, native ASCs could not be clearly separated from the whole heterogeneous mixture as they share membrane antigens with other cells found in the SVF. This is indeed based on the fact that the term ASCs is related to the plastic adherent and cultured population, which dramatically changes the phenotype very early during cell culture.

After adherence to cell culture plastic, these ASC preparations are less heterogeneous than the SVF, but they are not a homogeneous culture. However, more than 85% of the initially adhering cells are shown to express CD34 and do not express CD31, CD45, and CD146[39]. The cells of this fraction are characterized early during primary culture by a slightly heterogeneous morphology indicating different stem and pregenitor cell subsets, and (perhaps) more differentiated cells (dedifferentiated endothelial cells, smooth muscle cells and pericytes)[7,39]. Many researchers have described and compared the expression profile of the cultured ASC. They have shown the alterations during culture passaging, and described a dynamic phenotype which changes during cell culture[17,22,39-42]. Immediately after the cell isolation procedure, ASCs do not consistently express all characteristic MSC markers which are supposed to be expressed no matter from where the ASCs are derived. It has been shown that some specific surface markers (*e.g.,* CD105, CD166) increase during culture, while the expression of others decreases (*e.g.,* CD34)[17,39]. Later on during culture, the heterogeneity of ASCs decreases, leading to the finding that the characteristic marker expression of ASCs (and MSCs in general) depends on the culture conditions (or environment) and time in culture. ASCs in passage 2 or 3 are morphologically a homogeneous population of fibroblastoid cells. These cells uniformly express the characteristic MSC markers: CD29, CD44, CD73, CD90, CD105, and CD166, and lack expression of CD11b, CD14, CD31, and HLA-DR. Nevertheless, different subpopulations can also be detected in these cultures[43]. In a recent study, we analysed subsets/subpopulations of cultured ASCs by multicolour flow cytometry. In this study, we also characterized the overall phenotype of cultured ASCs using a high throughput technology with a screening panel of 242 antibodies and assessed the donor-dependent variations of the ASC phenotype[43]. Unfortunately, due to the high number of cells which are needed to perform this assay, we were not able to analyse ASCs very early after isolation to further investigate phenotypic alterations during time in culture. However, we analysed specific subsets of ASCs in culture (Passage 2-4, median culture time 45 d), demonstrating $CD34^+$, $CD36^+$, $CD200^+$ and $CD201^+$ subsets. All of them co-expressed the MSC-characteristic antigens CD73, CD90 and CD105[43]. Several other studies analysed the subpopulations of ASC preparations or the properties of the subsets. These subsets were characterized either in the SVF or whole ASC cultures or isolated using flow cytometric or immunomagnetic sorting. Kawamoto *et al*[44] sorted murine ASCs due to a different expression of CD90, and demonstrated $CD90^{high}$ and $CD90^{low}$ subpopulations. $CD90^{high}$ ASCs had a greater reprogramming capacity, and also showed increased numbers of alkaline phosphatase-positive colonies compared

to the CD90^{low} subpopulation. It has also been shown that sorted human CD90^{high} ASCs are more potent for osteogenic differentiation compared to CD90^{low}, CD105^{high} and CD105^{low} subpopulations[45].

Others demonstrated a small subpopulation of pluripotent stem cell-like cells, termed adipose-multilineage differentiating stress enduring (adipose-Muse) cells, which can be identified as CD90^{+}/CD105^{+}/SSEA-3^{+} cells with ASC preparations[46]. Importantly, this subpopulation of ASCs was shown to be able to cross the boundaries from mesodermal to ectodermal or endodermal lineages even under cytokine induction[46]. The existence of an ASC subpopulation that expresses SSEA-4, a marker usually associated with pluripotency, has also been shown and isolated by immunomagnetic cell sorting[47,48]. The cells have been shown to exhibit a higher potential for endothelial, osteogenic and adipogenic differentiation compared to whole ASCs. On the other hand, it has been shown that MSC preparations from adipose tissue lack a CD106^{+} subpopulation, whereas this subset is present in MSC preparations from bone-marrow and umbilical cord blood, and most prominent from placental chorionic villi[49].

AND WHAT ABOUT CD34?

CD34 was specified as a negative marker for MSCs in the position statement of the International Society for Cellular Therapy from 2006[5]. For several years, many researchers have followed this statement. It was generally accepted that ASCs do not express CD34 either, even though first reports about CD34 expression early after primary isolation were published[17,38]. One explanation is that many studies which showed the absence of CD34 used plastic-adherent cultured ASCs in higher passages, and did not investigate the expression of CD34 earlier in their cultures. For a long time, CD34 was solely used as a marker for haematopoietic stem and progenitor cells, but endothelial progenitor cells, skeletal muscle satellite cells and other precursors were also shown to express CD34[50,51]. Moreover, there are studies that provided convincing evidence that BM-MSCs also express CD34[52-54]. Nevertheless, expression of CD34 decreases in the following passages and gets totally lost during cell culture[17,38]. This cell culture-related loss of CD34 expression has also been described in other cells *in vitro* (*e.g.,* endothelial cells, haematopoietic stem cells)[52].

The percentage of SVF cells expressing CD34 has been reported with great variability among authors[35,39,42,55]. It has been shown that up to 85% of the cells in the stroma vascular fraction express CD34[39,50,56]. Two days after plastic adherence, more than 95% express CD34, co-express mesenchymal (CD10/CD13/CD90) and pericytic markers (CD140a and -b), and are CD31^{-}/CD45^{-}[27]. Furthermore, different CD34^{+} subpopulations were described[39,42,57]. Astori and co-workers identified two CD34^{+} populations (CD34^{dim} and CD34^{bright}) in the SVF with a marked difference in the intensity of antigen expression, the majority of the cells expressing CD34 at low intensity[42]. Nevertheless, they found that only approximately 7% of the SVF cells expressed CD34. Others also described several CD34^{+} subpopulations in the SVF[57]. Beside the assumed ASCs (CD34^{+}/CD31^{-}/CD146^{-}), they identified endothelial cells (CD34^{+}/CD31^{+}/CD146^{-}), haematopoietic stem-like cells (CD34^{+}/CD45^{+}) and vascular smooth muscle cells/pericytes (CD34^{+}/CD31^{-}/CD146^{+})[57]. An adipose tissue resident macrophage population expressing CD34 (and co-expressing the macrophage marker CD206) within the SVF has also been described[58]. Furthermore, several haematopoietic CD34^{+} subpopulations (co-expressing CD45) were also described, but these were eliminated by the following plastic adhesion.

Others isolated the CD34^{+} subpopulations from the SVF by an immunomagnetic method and characterized the native and cultured cells[36]. In this study, about 40% of the whole SVF cells were described as CD34^{+}, with an increase of CD34-expressing cells in the adhered and cultured cell population (to 80%). They also described that the proliferation rate of the isolated CD34^{+} population(s) was negatively correlated with the decrease of the antigen in the following passages. A study by Suga *et al*[59] opposed the sorted CD34^{+} to the CD34^{-} subpopulation of shortly cultured ASCs. They described that CD34^{+} cells proliferate faster and formed more colonies, whereas the CD34^{-} cells differentiate better into adipo- or osteocytes. In addition, the CD34^{+} subpopulation expressed some endothelial markers and, therefore, correlates with endothelial characteristics (progenitors?). On the other hand, the CD34^{-} cells expressed pericyte markers CD146 and NG2[59]. The authors further speculated that CD34 expression in human ASCs correlates with replicative capacity, differentiation potential, expression profiles of angiogenesis-related genes, and immaturity or stemness of the cells. The loss of CD34 expression may be related to the physiological process of commitment or differentiation. Others concluded that the decrease of CD34 expression depends on the environment because of the cultured cells' lack of their specific *in vivo* microenvironment[52]. Furthermore, the kinetics of decrease seems to vary strongly between different studies, depending on the culture conditions (*e.g.,* plating density, culture medium)[38,50]. In a recent study, we showed a specific subset in cultured ASCs (passage 2-4) which is positive for CD34 and co-expressed the MSC characteristic antigens CD73, CD90, and CD105[43]. We also detected other subsets (*e.g.,* CD36^{+}, CD200^{+}, CD201^{+}), but detected no double positive subpopulation for these markers (*e.g.,* a CD34^{+}-CD36^{+} subpopulation). However, it should be mentioned that the percentages of these subsets varied between isolations from different donors. It has also been described that the culture medium influences the decrease of CD34[38]. CD34 expression was maintained for at least 10-20 wk by using a cell culture medium supplemented with acidic fibroblast growth factor[38]. Scherberich *et al*[50] described that the CD34 expression in ASCs is maintained when the cells were cultured in a model recapitu-

lating the complex microenvironment of their niche. In this three-dimensional physiological environment, ASCs persist in a $CD34^+/CD31^-/CD105^-$ phenotype for up to six weeks in culture.

Finally, there are some technical difficulties concerning the verification of CD34 expression which should also be pointed out. Firstly, we were only able to detect $CD34^+$ cells using a PE-labelled antibody. If we stained the same cell population with a FITC-labelled antibody, we were not able to detect these cells in our multicolour cytometric analyses. Furthermore, it has been described that there are multiple classes of CD34 antibodies recognizing unique immunogens and influencing the signal[60]. Bourin *et al*[26], therefore, recommended the use of class Ⅲ CD34 antibodies (*i.e.*, clone 581 or 4H11) for SVF cell characterization[60].

According to current published data, CD34 is a potential marker that can be used to identify an ASC subpopulation *in situ* and after a short culture time[26]. Nevertheless, it should be noted that there is the possibility that $CD34^-$ ASC subsets *in vivo* also exist. As the proof of CD34 expression in ASCs has only quite recently been accepted, it is not surprising that little is known about the functional role of CD34 in ASCs. Recently, it has become more and more accepted that MSCs (or subsets) *in vivo* also express CD34[52]. Two recent reviews excellently summarized the current knowledge of the expression of CD34 by MSCs in general[52] and ASCs[50].

DO WE COMPARE "THE SAME ASCs"? THE PROBLEM WITH DONOR-SPECIFIC DIFFERENCES AND CULTURE STANDARDIZATION

One of the main problems in comparing results from different laboratories is the lack of standardized methodologies to culture ASCs. The composition of ASC subpopulations varies between different isolations[61] and the phenotype of ASCs display a dynamic phenotype during cell culture also due to culture conditions. Heterogeneity of ASC and MSC preparations and cultures has been discussed in many publications[7,62-64]. It has been described, for example, that single-cell-derived clonal MSC populations are also highly heterogeneous and contain undifferentiated stem/progenitors and lineage-restricted precursors with varying capacities to proliferate and differentiate[65,66]. Therefore, the resulting variability limits the standardization of MSC-based repair strategies and impedes the comparison of clinical study outcomes[65].

The heterogeneity of ASC preparations may result from different reasons; some of them can be influenced by the researchers, some not. First of all, the donors from which ASCs are isolated. These donors differ in age, body mass index, gender, ethnicity, and existing diseases[7]. The negative correlation of the body mass index, for example, and the number of stromal cells per gram and their differentiation capacity has been shown[67]. Our recent data also revealed donor-specific differences in the composition of ASC subpopulations[43]. A total of forty-nine cellular surface markers in a comprehensive phenotyping study showed a high variability in their expression between the donors. Anyway, all cells expressed the main characteristic markers (CD73, CD90, CD105). Expression of CD36 and CD34 from different donors, for instance, varied highly from no expression, scattering of fluorescence intensity to highly expressed. In summary, albeit positive for the main characteristic markers, the cells also differ in their expression of some other markers[43]. In conclusion, it is extremely difficult, if not impossible, to standardize these donor-related variables[7].

On the other hand, further points to consider are the liposuction procedure, which may differ between different clinics, the time lapse until cell isolation procedure starts, or the temperature at which the lipoaspirate is stored until cell isolation. It has been reported that liposuction side and liposuction procedure influences the cell yield, proliferation capacity and frequency of isolated stromal cells[57,67-70], but it is unclear whether this promotes different subpopulations in the isolates.

Next, a standardization of the isolation and culture conditions may increase the comparability of the results from different laboratories[7]. The first critical step is the point in time after which the initial cultures are washed (*i.e.*, the initial time for adherence). It has been shown that ASCs' heterogeneity can be reduced by a washing procedure early after plating the SVF[71], indicating that several subsets require different time points to adhere to the cell culture plastic. Another effort to reduce the heterogeneity of ASCs was carried out by using flow cytometric sorting or immunomagnetic separation, either by positive or by negative selection for a specific marker[7,71-74]. Nevertheless, usage of such techniques only select specific subpopulations which must be evaluated separately, and studies using such isolation cannot be compared with results from ASCs.

In addition, no unique and standardized culture protocol for ASCs has been accepted overall. There are different variables that may impair ASCs (and their subsets) in their undifferentiated state: density of initially plated cells, surface-coating, culture medium composition, supplements (bovine serum, human serum, platelet lysate, or growth factors), oxygen partial pressure, antibiotics, method of subculturing, and method of cryopreservation. Only limited information is available about which medium optimally expands ASCs by maintaining the undifferentiated character *in vitro*[59,75,76]. In MSC cultures, it has been shown that basal medium, glucose concentration, quality of fetal calf serum, cell plating, and cell density highly affects the final outcome resulting in the expansion of populations with totally different potential[7,77]. Culture medium composition also affects the expression stem cell-related transcription factors NANOG, Oct-4, Sox-2, and Rex-1[7,76]. Many investigators use Dulbecco's modified Eagle medium (DMEM) as a standard medium for ASCs, but no further description of the DMEM

used in the study is given. We always utilize low-glucose DMEM (physiological glucose content, 100 mg/dL), whereas others use DMEM with a higher glucose content because ASCs cultured in this medium show a much better proliferation rate. Nevertheless, the glucose content is one variable which must be considered to be near to the *in vivo* situation. Using high glucose medium raises the question about the effects of such a "diabetic" environment on the cells. In most cases, ASCs were cultured with foetal calf or bovine serum as a proliferation supplement. Related to a possible use of ASCs in human therapeutic approaches, there are many concerns about the usability of foetal calf or bovine serum (infectious complications, host immune reactions)[78]. The usage of low-serum containing culture media supplemented with recombinant growth factors [*e.g.*, epidermal growth factor, platelet-derived growth factor and/or basic fibroblast growth factor] has been described[79-82]. Low doses of bone morphogenic protein 4 have also been shown to stimulate ASC proliferation[83]. Nevertheless, the gold standard for culturing ASCs will be a medium absolutely free from animal serum or factors, with well-known ingredients[7].

In summary, modifications in the isolation and/or culture conditions might select for the expansion of subpopulations and have a huge impact on the differentiation potential of the cells cultured, albeit the primary cells could be phenotypically identical if characterized with a standard marker panel[64]. Therefore, standardization of the isolation and culture procedure is highly necessary for a good reproducibility of results from different laboratories and studies[7].

WHAT NEEDS TO BE RESOLVED?

Although ASC preparations are already used in different clinical trials, many questions concerning their counterparts and biology *in situ*, differentiation potential *in vitro* and *in vivo*, and also the mechanism of repair or regeneration (paracrine effects, differentiation, immunomodulation) are not completely understood or still unsolved. Research goes on and therapy approaches are also possible without the exact knowledge of the mechanisms as long as they are safe and beneficial for the patient.

However, what are the main questions in basic ASC research which need to be resolved? Many recent histological studies have tried to identify native ASCs (or ASC subsets) *in situ* due to their expression of some markers, but the exact phenotypic definition of the cells/cell populations we call ASCs relies solely on the analysis of a culture-expanded preparation. Therefore, there is the possibility that the phenotype and potential of ASCs/MSCs varies between *in vivo* and *in vitro* settings provoked by the isolation technique and culture conditions. Despite intense investigation, the physiological role of the native ASCs in adipose tissue (and MSCs in general) *in vivo* is not fully understood. Therefore, it is extremely important to overcome the lack of standardization in order to abolish the variability in cell quality (if not solely based on donor-specific variabilities). In addition, alternative culture methods should be developed to avoid the loss of CD34 expression and to preserve a physiological phenotype[50]. Research progress has also been hampered by the limited knowledge of the subsets/subpopulations of ASCs, due to the lack of unique subset markers for their characterization[7]. A lot of work still remains.

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