PROSPECTIVE IDENTIFICATION AND CHARACTERIZATION OF ADIPOGENIC AND MYOGENIC CELLS IN HUMAN ADIPOSE TISSUE

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Stem cells offer the hope of curing a variety of ailments such as diabetes, Parkinson's disease, myocardial infarct, muscular dystrophy and spinal cord injuries. In this regard, a detailed understanding of the origin and behavior of stem cells is invaluable to the advancement of public health. The adult human adipose tissue (hWAT) is an attractive and convenient source of therapeutic cells for use in the clinical setting. Previous studies have demonstrated that the stromal vascular compartment within hWAT contains multipotent cells, called adipose stem cells (ASC). However, the identity and anatomic distribution of ASC or progenitors within hWAT remain unclear. We addressed this issue through an a priori identification of different cell subsets within the hWAT stroma, by visualization of cells in their native state within the resident tissue, and analysis of their immunohistochemical profile. Endothelial cells, pericytes, as well as non-vascular cells from adult subcutaneous abdominal fat were separated and sorted to homogeneity based on CD34, CD146 and CD45 antigen expression. We first tested the adipogenic potential of the different purified stromal cell populations. A higher level of leptin mRNA, as much as a 20-fold difference, was observed in pericytes and the non-vascular cell fractions when compared to endothelial cells. High levels of leptin expression were maintained even after extensive expansion of the cells in culture. Additionally, we found

a reserve of brown adipocyte progenitors within the adult fat tissue vasculature, among pericytes, which challenges the notion that uncoupling protein-1 (UCP-1) expressing cells are confined to fetal and early human life. We also herein describe a precedently unsuspected role of adipose-derived pericytes as human muscle progenitors. When transplanted into cardiotoxin-injured NOD-SCID mouse muscles, pericytes generated a significantly higher number of myofibers than the other hWAT stromal cell populations. Quantitatively, the myogenic potential of adipose-derived pericytes was similar to that of a population of robustly myogenic cells within the skeletal muscle of adult humans, the "myogenic-endothelial cells". The long-term culture of hWAT pericytes did not diminish their capacity for myogenic differentiation. These results suggest that human adipose tissue is a viable alternative tissue source to skeletal muscle for muscle cell-mediated therapy.

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1.0 INTRODUCTION

1.1 SIGNIFICANCE

The observation made by several investigators in the last few years that human white adipose tissue contains progenitors with broad differentiation capabilities is of particular interest, inasmuch as fat tissue should represent an abundant, practical, and autologous source of therapeutic stem cells. In this study, we embark on the direct identification of these fat tissue-resident stem cells, which will be a step toward the identification of the elusive multipotent stem cells - mesenchymal stem cells (MSC), multipotent adult progenitor cells (MAPC) or muscle-derived stem cells (MDSC), shown previously to reside in adult organs. A characterization of the adipogenic and myogenic properties of individual cell subsets from fat tissue opens the possibility for therapeutic applications. From a biological standpoint, the differentiation of adipocytes from their precursor cells is an important aspect in the study of the pathogenesis of obesity. On the other hand, an effective utilization of progenitors to obtain mature adipocytes would be equally useful from a clinical perspective, especially with regard to tissue engineering and cosmetic or reconstructive applications. In the same context, the purification of an adipose tissue-derived cell population exhibiting a high capacity for muscle engraftment and repair may be an important tool for the treatment of muscular disorders through cell-mediated therapy.

1.2 HUMAN ADIPOSE TISSUE AS A SOURCE OF MULTIPOTENT CELLS

Multipotent stem cells, able to give rise to a diverse progeny of differentiated cells, were long believed to be restricted to embryonic tissues and to gradually disappear, along gestation, at the expense of tissue-restricted progenitors. It has however been discovered that some post-natal tissues, for example the bone marrow, brain and skeletal muscle can be a source of multipotent stem cells [1, 39, 40]. These cells could represent a panacea in regenerative medicine inasmuch as their potential is broad and they are easily accessible, for instance from a simple bone marrow aspiration. The human white adipose tissue (WAT), in particular, is believed to be a potentially excellent source of cells for cell-based therapy. This is in part because adipose tissue is abundant in the body, and a large amount of stromal cells (i.e. non-adipocytes) may be extracted from it. From a therapeutic standpoint, adipose tissue is also ideal as it may be obtained with minimal morbidity due to its superficial location in the body. Of note in this respect, an accessible and preferred source of WAT-derived cells is the subcutaneous abdominal fat, otherwise known as Camper's fascia.

The existence of a population of multipotent cells within cultured human adipose tissue has been previously documented [2, 3, 5]. Adipose-derived stromal cells have been shown to differentiate not only into adipocytes, but notably into other tissue lineages as well such as cartilage, bone and muscle [2, 3, 17]. These findings may be attributed to the fact that adipose tissue is derived from embryonic mesenchyme, and therefore, its stromal compartment may retain mesodermal lineage potential. Human and rodent white adipose tissues have recently been determined to contain progenitors which can

differentiate into cells of the mesodermal lineage. In 2001, Gronthos et al. have shown by FACS and immunohistochemistry analyses that the protein expression phenotype of human WAT stroma vascular cells was similar to that of human bone marrow stromal cells [16]. They showed that human WAT stroma vascular cells, when cultured under osteogenic conditions, can differentiate into osteoblasts. This observation was the first indication that human WAT stroma vascular cells are pluripotent.

In 2001, Zuk et al identified a putative stem cell population in human WAT lipoaspirates (LPA) [17]. In a subsequent study Zuk et al showed, by culturing human LPA under appropriate conditions, that it is possible to obtain differentiated adipocytes, osteoblasts, chondrocytes, myoblasts and putative neurogenic cells [2]. Culture at low confluence yielded clones with multilineage capacity. Furthermore, human LPA cells could be transduced efficiently with lentiviral vectors [18]. Human LPA cells transfected with the bone morphogenic protein (BMP)-2 gene produced more bone precursor products than did osteoblasts [19]. Human WAT stroma vascular cells might be an ideal source of mesoderm-lineage stem cells for transplantation and tissue engineering. Rat visceral WAT stroma vascular cells were also found to differentiate in vitro into osteoblasts, chondrocytes and neural cells, and rat or rabbit WAT stroma vascular cells were found to differentiate into cardiomyocytes [20, 21]. More recently, it was shown that mouse adipose-derived cells had the capacity to undergo adipogenic, osteogenic, chondrogenic, myogenic and hematopoietic differentiation in vitro, and readily differentiated to form bone and cartilage in vivo [56]. All of these findings support the existence of multipotent progenitors within the fat tissue. However, as stromal cells of adipose tissue are believed to represent a mixture of several committed progenitor cells

and their differentiated progenies, the exact identity and anatomical location of these stem cells remain elusive. Herein, we investigate the adipogenic and myogenic potentials of adipose-derived cells, after a prospective identification and, subsequently, separation of the stromal cell compartments into distinct cell populations.

1.3 ENDOTHELIAL CELLS AND PERICYTES AS STEM CELLS

Results obtained recently in distinct organ systems point to a role of endothelial and other vessel-related cells in the emergence and renewal of some mesodermal cell lineages [13, 14, 23, 24]. One of the earliest insights into this phenomenon were studies which suggested that during early development, blood cells and hematopoietic stem cells originate from a specified subset of endothelial progenitors in the floor of the dorsal aorta [10]. This led to the notion that other stem cells might be located in the same embryonic region. Indeed, it was later demonstrated that a contingent of cells associated with the embryonic dorsal aorta, named mesoangioblasts, have the capacity to contribute to muscle fiber formation during muscle development and regeneration [11, 12, 24]. These vessel-associated cells, which express both endothelial and myogenic cell markers, also had the ability to differentiate into multiple mesodermal cell lineages. This multipotent feature may in fact not only be limited to endothelial cells among cells lining the vasculature, but also observed for pericytes, which are suspected to be progenitors of different cell types. Pericytes, aka Rouget cells or mural cells, also named mesangial cells in the kidney and Ito cells in the liver, are intimately associated with endothelial

cells in blood vessels and microvessels [25, 26]. Pericytes contain alpha-smooth muscle actin (αSMA) and regulate microvessel contractility; pericytes can also inhibit, via TGFβ secretion, the division of endothelial cells [27]. Antibodies against desmin, αSMA and neuron-glial 2 (NG2) have commonly been used in the past to identify pericytes in tissue sections [25, 34]. Previous studies have suggested that pericytes are able to undergo adipogenic, chondrogenic and osteogenic differentiation [13, 14]. However, much of the evidence from these studies is indirect, as the differentiation experiments were performed on pericyte-containing cultures, and not on purified pericytes.

Notwithstanding, these findings, including the observation that the adipose tissue is rich in microvessels, from which compartment a major portion of the stromal cell fraction is contained, led us to hypothesize that progenitor cells from adipose tissue may also have a vascular origin.

1.4 PROJECT OBJECTIVES

1.4.1 Objective #1: Define and isolate by FACS sorting the various cell populations within human adipose tissue

Human white adipose tissue is primarily composed of two cellular compartments: mature adipocytes, which are filled with lipids, and a stromal vascular compartment (SVC) containing a highly heterogeneous cell population. Herein, we sought to identify and define the various cell populations that make up the hWAT stroma, with emphasis on blood vessel-associated cells. Because vascular cells are assumed to contribute a large proportion of the stromal cell fraction in fat tissue, and with a keen awareness of a

possible relationship between vessel-related cells and multipotent progenitors, we characterized the SVC using established endothelial cell and pericyte markers such as CD34, CD146 and UEA-1. In addition, as a prelude to differentiation studies, we sought to separate and purify the SVC into distinct cell populations through the use of FACS sorting.

1.4.2 Objective #2: Identify progenitors of mature adipocytes within the adipose tissue stromal cell fraction

The ability of humans to increase the number of adipocytes, depending on the localization of the adipose depot, the nature of the diet and environmental conditions, has long been known [43]. In well-nourished individuals where energy intake exceeds the needs of the body, excess calories are stored in the adipose tissue not only through an increase in the adipocyte cell volume, but also by expansion of the number of differentiated adipocytes. However, the number of adipocytes is generally believed to increase not because of proliferation of mature fat cells but because of the existence of a pool of adipocyte progenitors within adult fat tissue. In vivo labeling experiments have established that mature adipocytes do not incorporate H3-thymidine to a significant degree [22]. And though some investigators have observed otherwise- for example, Sugihara and coworkers have reported that approximately 2% of mature adipocytes from very young rats can undergo mitoses in culture, this phenomenon is believed to result from a rearrangement of the extracellular matrix in vitro following collagenase treatment of the abdominal subcutaneous adipose tissue [28].

To date, the identity of adipose precursor cells, or preadipocytes, remains to be determined. Preadipocytes correspond to interstitial cells which have not yet

accumulated triacylglycerol stores. In this objective, we sought to determine the adipogenic potential of different stromal cell populations, which in turn may provide some insights as to the most likely preadipocyte candidates within the hWAT.

1.4.3 Objective #3: Determine the differential ability of various adipose stromal cell populations for muscle regeneration

The existence of multipotent cells in hWAT, capable of differentiating into skeletal muscle and other lineages, has previously been shown retrospectively [2, 3, 8, 49]. However, the exact identity and anatomical location of these cells have not been determined. As such, investigators have used the general descriptive terms human multipotent adipose-derived stem (hMADS) cells or adipose-derived adult stem (ADAS) cells in reference to this population [2, 3]. In this objective, we sought to identify myogenic progenitors within the hWAT which we believe correspond to hMADS or ADAS cells. We accomplished this by transplanting purified cell populations into the gastrocnemius muscles of NOD-SCID mice injured by cardiotoxin, and comparing their respective abilities to generate human myofibers. After identifying a population of stromal cells with high myogenic capacity, we further investigated whether their expansion in culture is possible, and how this may affect the cell's myogenic potency in vivo.

2.0 CHARACTERIZATION OF THE ADIPOSE STROMAL COMPARTMENT

2.1 RESULTS

2.1.1 Immunohistochemistry of hWAT

Hematoxylin and eosin staining of the hWAT section shows adipocytes of varying sizes (Figure 2.1a). Located in between mature adipocytes are the non-lipid filled stromal cells (arrow, Figure 2.1b). Many of these cells are believed to be endothelial cells or pericytes within the capillaries. However, as the tiniest of blood vessels contain a single layer of only a few cells without any discernable lumen when seen under ordinary microscopy, such vessels cannot be positively identified based on structural appearance alone. In order to visualize the vascular cells contained therein, we labeled hWAT sections with endothelial cell markers such as UEA-1 (Figure 2.1c) and CD34 (Figure 2.1d). Stainings revealed the ubiquitous distribution of endothelial cells within the interstitium of the adipose tissue. We also analyzed the sections for CD146, an endothelial cell marker also reported to be expressed at the surface of pericytes. Larger-sized microvessels show CD146-positive cells which do not express CD34, in a typical pericytic location (Figure 2.1e), surrounding and closely adherent to the CD34-positive endothelial cells. The expression of CD146 by pericytes was also confirmed in arterioles and venules occasionally seen in adipose tissue sections, with pericytes assuming an abluminal location within the blood vessel wall (Figure 2.1f). Based on these

observations, we proceeded to isolate stromal cells from hWAT and purify endothelial cells and pericytes by FACS sorting, on expression of CD34 and CD146.

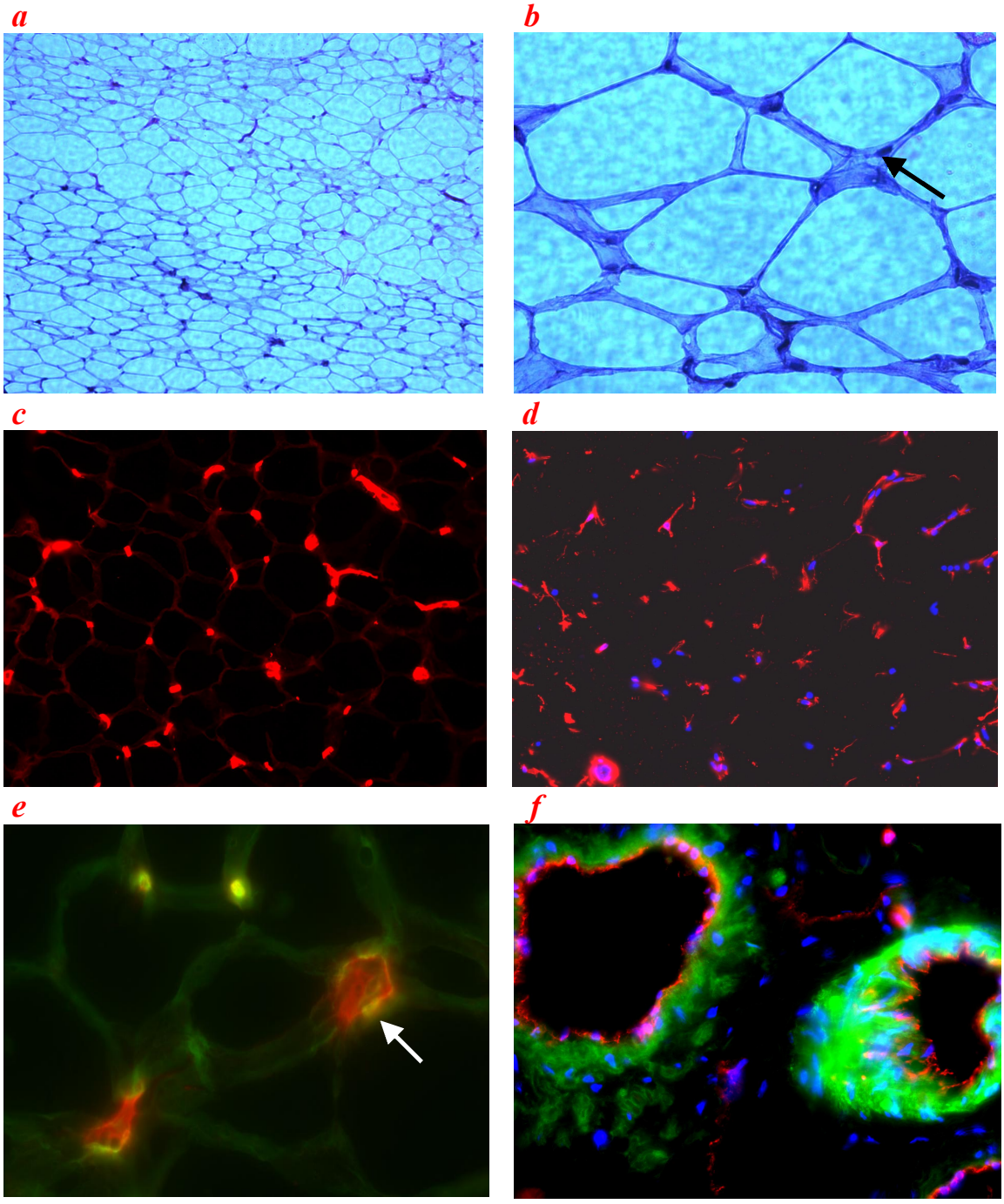

Figure 2.1 Immunohistochemistry on human adipose tissue.
(a) Hematoylin & eosin staining of a typical human subcutaneous abdominal fat tissue, the fat depot used throughout this study. (b) Staining of the same tissue as seen under high-power/oil magnification, showing interstitial/ stromal cells (arrow) and the peripheral nuclei of adipocytes, appearing as basophilic structures. Frozen sections were stained with UEA-1 (c) and anti-CD34 antibody (d), seen under low-power magnification; sections were also double-labeled for CD34 (red) and CD146 (green) (e), seen here under high magnification. (f) Occasionally, larger blood vessels such as this arteriole with its accompanying venule, may be seen. This section was double-labeled for CD144 (red) and CD146 (green).

2.1.2 Cell isolation and characterization

Whole adipose tissues used in this study were derived from the subcutaneous abdominal area (Figure 2.2a). Adipocytes were observed to account for more than 90%, while stromal cells less than 10%, of the volume of hWAT (Figure 2.2b). After separating by centrifugation the adipocytes from the stromal cells, we counted the latter and found that approximately 1 x 10^6 stromal cells are contained in one gram of adipose tissue. By counting adipocytes within a fixed volume of the lipid fraction, we further determined that adipocytes comprise approximately 20% of the total cells in hWAT. In terms of cell volume, adipocytes were on average 150 – 500 times larger than the stromal cells (Figures 2.2c and d). In order to separate endothelial cells from pericytes by FACS sorting, freshly isolated stromal cells were labeled with antibodies directed against CD34, CD146 and CD45. Hematopoietic lineage cells, which express CD45, and accounted for 5% of the starting population, were first gated out (Figure 2.2e), and the remaining cells were further analyzed for CD34 and CD146 expression (Figure 2.2f). From this analysis, we obtained and sorted four distinct adipose-derived, non-hematopoietic cell populations. (1) CD34+ CD146- CD45- cells and (2) CD34+ CD146+ CD45- cells represent the entire endothelial cell population within fat tissue. Two subsets of endothelial cells were thus observed, based on their expression of CD146. We named the endothelial cell population which do not express CD146, corresponding to population (1), as EC, while the endothelial cells which express CD146 (a.k.a. S-endo1), corresponding to population (2), were named S-EC. EC and S-EC accounted for the majority and comprised 67.5% and 10.1%, respectively, of the total non-hematopoietic stromal cells analyzed. (3) CD34-

CD146+ CD45- cells, corresponding to pericytes (PC), accounted for 14.6% of total cells. An approximate 1:6 ratio of pericytes to endothelial cells is thus observed in adipose tissue. The last, (4) CD34- CD146- CD45- cell population, comprises the non-vascular cells (NVC) and amounted to 7.8% of the stromal fraction. These consist of a heterogenous population of cells which would include fibroblasts from the connective tissue and smooth muscle cells (SMC) from the larger-sized arterioles and venules. A quantification of these stromal cell populations within hWAT is shown in Figure 2.2g, while Figure 2.2h shows the overall cell composition of hWAT.

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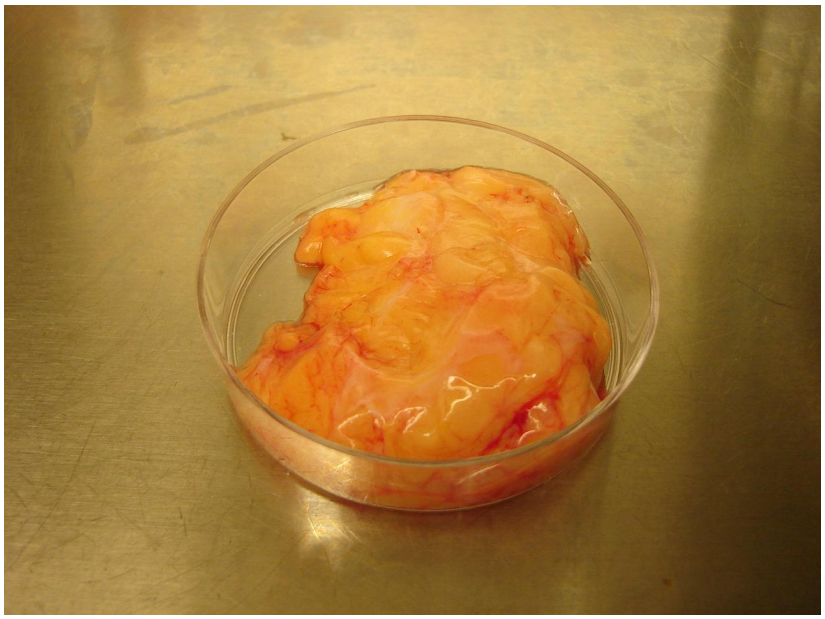

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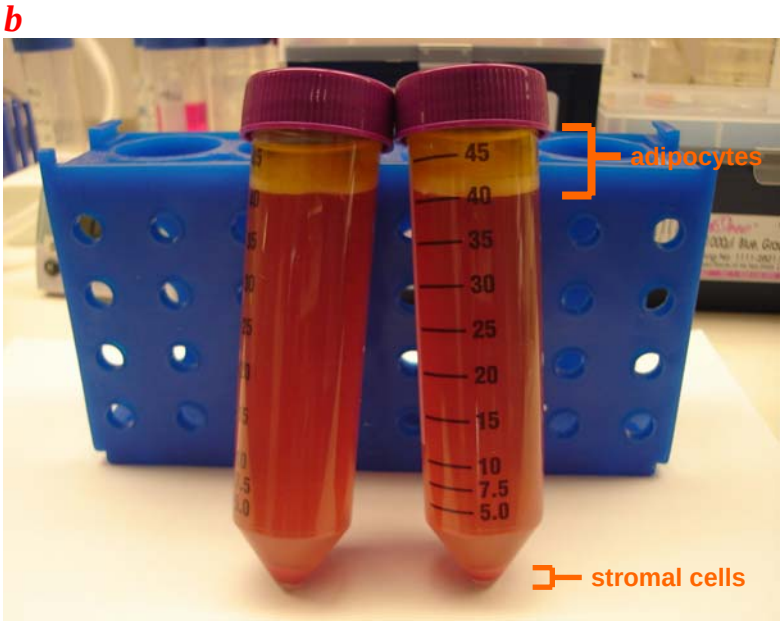

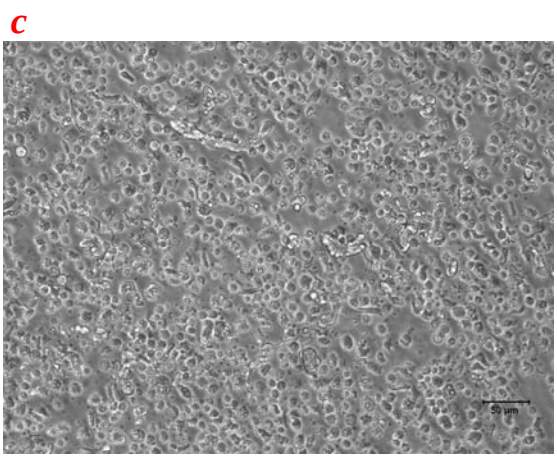

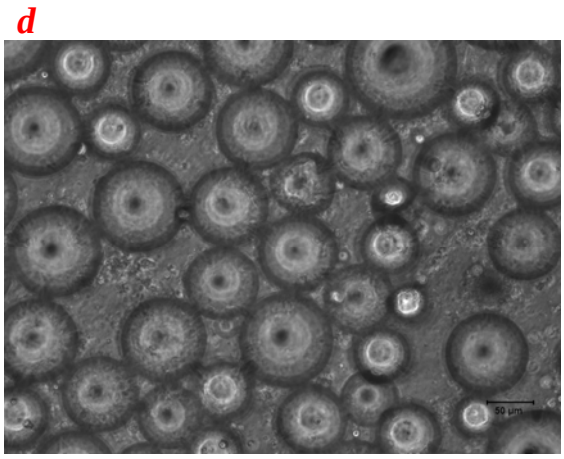

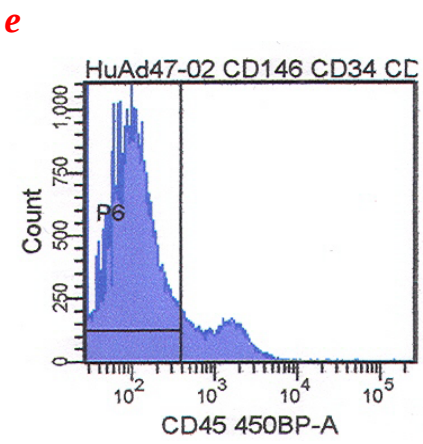

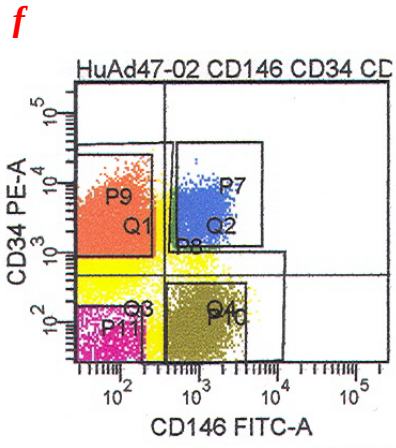

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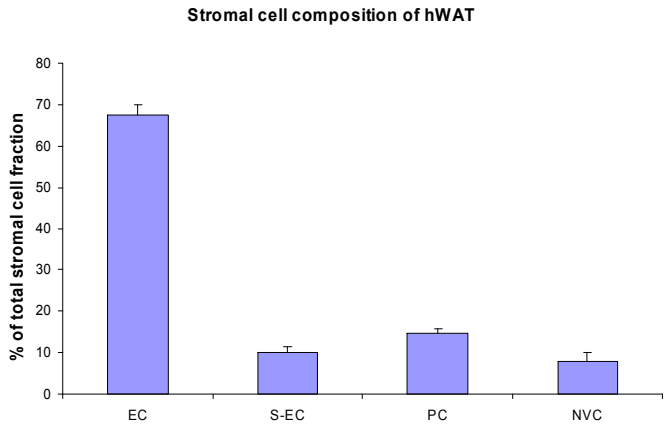

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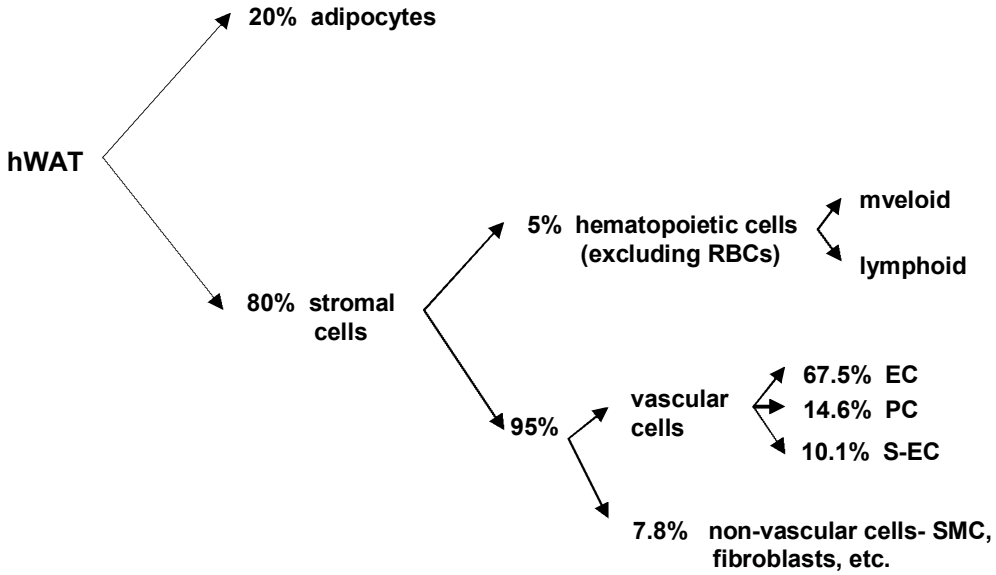


Figure 2.2 Isolation and purification of stromal cells from human adipose tissue
(a) Gross appearance of whole subcutaneous abdominal fat (to be distinguished from lipoaspirates). Digestion of the tissue yields mature adipocytes and the total stromal population; their relative volumes within the tissue are illustrated (b). Freshly isolated stromal cells (c) and mature adipocytes (d), as seen by light microscopy under similar magnifications. The total stromal cell population was analyzed by FACS, and CD45- cells were selected (e), and further separated on CD34 and CD146 expression (f). (g) The graph indicates percentages of the four identified cell populations within the hWAT stroma, expressed as fractions of the total non-hematopoietic cells. The results are the mean +/- s.e.m. of 4 different samples. (h) A more detailed diagram, showing the overall cell composition within the hWAT.

2.1.3 RT-PCR analysis of the sorted cells

To test the efficiency of our sort, we performed a RT-PCR analysis of the three sorted vascular cell populations (Figure 2.3). The results showed a good separation of the three populations based on their expression of CD34 and CD146. To further confirm the identity of the sorted cells, we analyzed them for desmin and vWF expression, which are well-established pericyte and endothelial cell markers, respectively [34]. Sorted pericytes were confirmed to express desmin, while the two endothelial cell populations did express vWF. These results also reveal that our sorting protocol using the CD34 and CD146 antigens enabled us to efficiently separate a pure population of endothelial cells uncontaminated by pericytes, as both EC and S-EC did not contain desmin. Likewise, PC did not express vWF, confirming that there was no contamination of sorted pericytes by endothelial cells. The RT-PCR analysis also showed that the two sorted endothelial cell populations, EC and S-EC, differed from each other with regard to NG2 expression, which is generally considered a pericyte marker but which has also been found to be expressed by proliferating endothelial cells during angiogenesis [4]. Lastly, we observed that both endothelial cell populations, but not PC, express CD56.

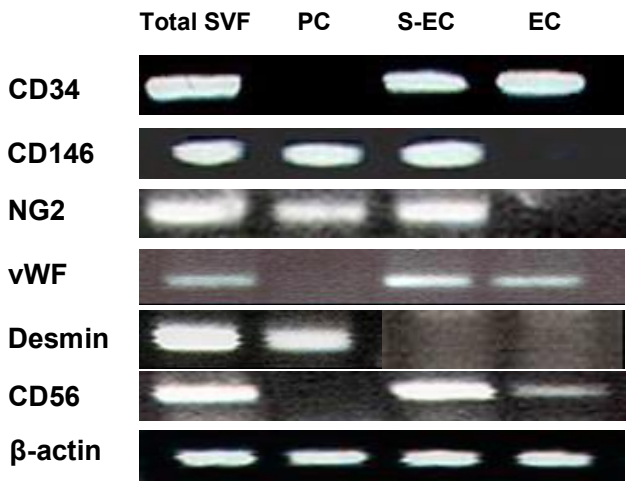


Figure 2.3 RT-PCR analysis
The three sorted vascular cell populations were analyzed by RT-PCR to confirm the efficiency of the sort and cell phenotypes. The vascular identity of the cells was also determined based on previously established antigens.

2.1.4 Further characterization of cells with UEA-1

We have thus far obtained a good separation of endothelial cells from pericytes as well as non-vascular cells, based on CD34 and CD146 expression, as confirmed by RT-PCR. However, we wished to further characterize CD34+ cells within the hWAT, as they account for close to 80% of the total non-hematopoietic stromal cell population. In particular, we wanted to determine the expression by the CD34+ cell population of additional endothelial cell markers, such as the UEA-1 ligand. To this end, we analyzed the four identified populations (Figure 2.4a) for expression of the receptor of the Ulex europaeus lectin, UEA-1, which is a marker of human endothelial cells (Figure 2.4b). As expected, the UEA-1 negative fraction contains the PC and NVC populations (Figure 2.4c). However, unexpectedly, we also find a population of CD34+ CD146- cells within this fraction (Figure 2.4c). On the other hand, the UEA-1 positive population consists

only of CD34+ CD146 low/high+ cells and does not contain any PC nor NVC. (Figure 2.4d). We also observed that CD34+ cells within the UEA-1 negative fraction account for the majority (70.8%) of the entire CD34+ cell population, while only 29.2% of all CD34+ cells express the UEA-1 ligand.

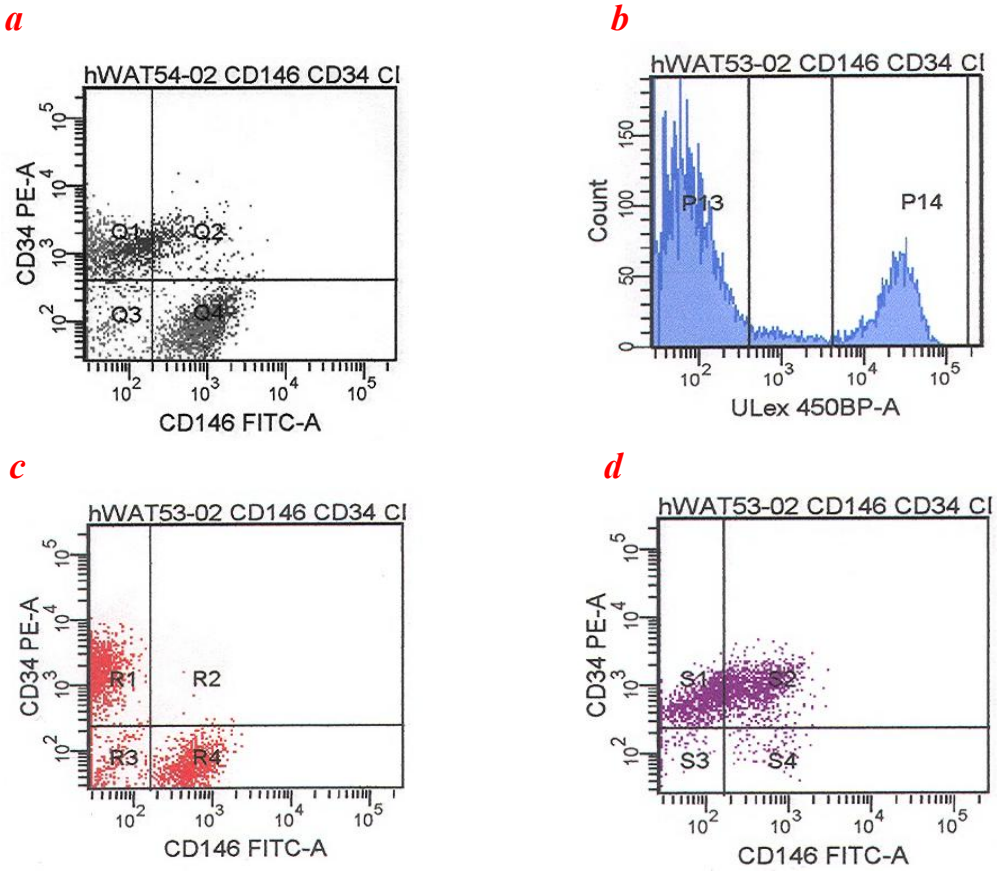


Figure 2.4 FACS analysis of adipose tissue vascular cells based on affinity for UEA-1
The total stromal cell population, after exclusion of hematopoietic cells, was analyzed by FACS using the CD34 and CD146 antigens, as described previously (a). Cells were further separated into UEA-1- and UEA-1+ cells (b). The UEA-1-negative cell subset contained CD34+ CD146- cells, CD34- CD146- cells and CD34- CD146+ cells (c), while the UEA-1-positive cell subset contained only CD34+ CD146 low/high+ cells (d).

2.1.5 Phenotype of the four stromal vascular cell populations identified in hWAT

In summary, we have separated and purified by FACS sorting four populations of stromal cells from hWAT. These cells exhibit the following phenotype:

1. **Endothelial cells** - CD34+ CD146- CD45- CD56- vWF+ NG2- Ulex- desmin-
2. **S-Endothelial cells** - CD34+ CD146+ CD45- CD56+ vWF+ NG2+ Ulex+ desmin-
3. **Pericytes** - CD34- CD146+ CD45- CD56- vWF- NG2+ Ulex- desmin+
4. **Non-vascular cells** - CD34- CD146- CD45- CD56- vWF- NG2- Ulex- desmin-

2.1.6 Culture of hWAT stromal cells

When freshly sorted cells were seeded in culture, it was observed that each of the different populations exhibited a distinctive morphology. Primary cultures of EC (Figure 2.5a) revealed cells with a spindle-shaped, narrow appearance, while S-EC were roundish, with transparent and smooth borders (Figure 2.5b). PC in culture had a very characteristic appearance that was typical of pericytes- rectangular and flat, with branching processes (Figure 2.5c). Lastly, NVC in culture were very long and narrow, with distinct edges (Figure 2.5d).

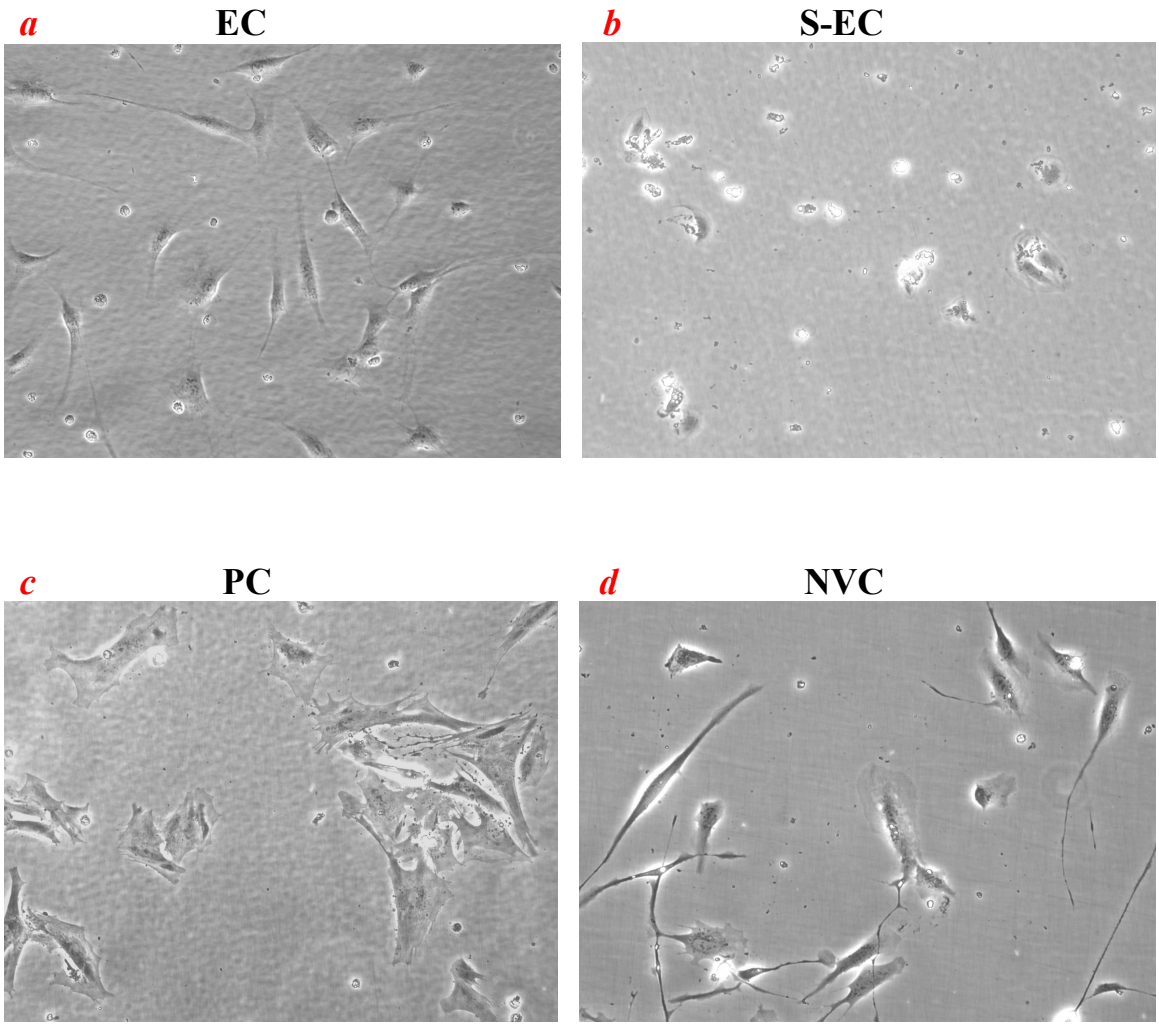
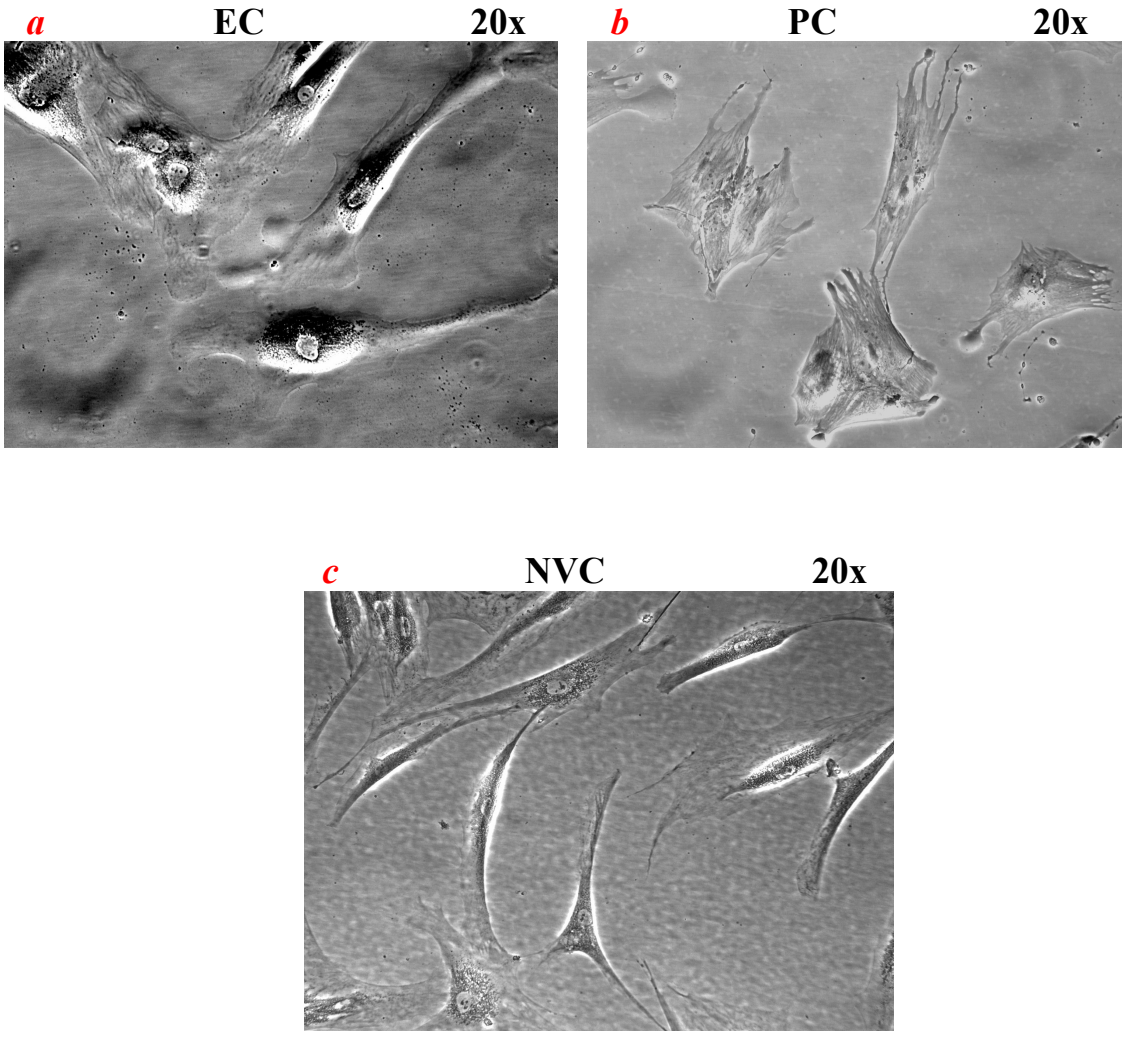


Figure 2.5 Primary culture of 4 different hWAT stromal cells
Phase contrast images of primary cultures of sorted EC, S-EC, PC, and NVC (a-d) during the first week in vitro, showing distinct differences in cell morphology.

2.1.7 Long-term culture of hWAT stromal cells

The four sorted cell populations were each cultured in DMEM + 20% FBS in subsequent replatings. It was again observed that the different cell populations had characteristic cell morphology and growth patterns, maintained after several passages. The EC and S-EC populations had almost similar morphologies, with very transparent and smooth cell borders (Figure 2.6a). PC, on the other hand, had distinct, sharp edges and branching

processes typical of pericytes (Figure 2.6b). NVC were either spindle-shaped or broad, but had smooth borders like EC (Figure 2.6c). It was further observed that PC, in particular, exhibit contact inhibition (Figure 2.6d), unless cells are seeded at close to 100% confluence during the initial plating or at the time of passage. This was in contrast to EC and NVC, which continue to grow until confluence (Figure 2.6e).



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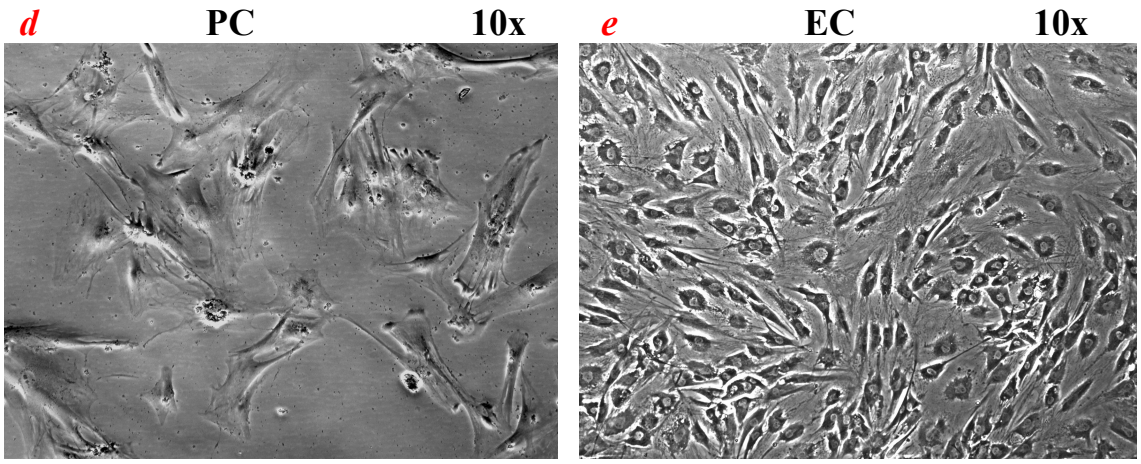


Figure 2.6 Long-term culture of 4 different hWAT stromal cells
Phase contrast pictures of sorted EC, PC, and NVC (a-c) after several passages in vitro, showing differences in cell morphology. Pericytes exhibited contact inhibition and never grew to confluence (d), unlike EC and NVC (e), as seen in this representative image of EC.

2.1.8 Fast and slow adherent hWAT stromal cells

When freshly sorted cells were plated in culture, it was observed that the different stromal cell populations exhibited characteristic growth patterns. EC, in particular, rapidly attached to the flask and proliferated at a high rate compared to the other cell fractions. We consistently observed that EC adhere within a few hours of plating, as compared to the other cell populations which normally require at least 4-5 days in order to adhere to the flask and begin proliferating. As depicted in the pictures below, within 24 hours of culture, more than 95% of all EC have attached and a few have spread out, forming fibroblast-like cells (Figure 2.6a). Conversely, the majority of S-EC and PC (at least 90%) have not attached at this time, as seen by the presence of mostly refringent cells in the culture (Figures 2.6b and c, respectively). The NVC population consisted of

roughly equal numbers of cells which have just recently attached and non-adherent/refringent cells after a period of 24 hours (Figure 2.6d).

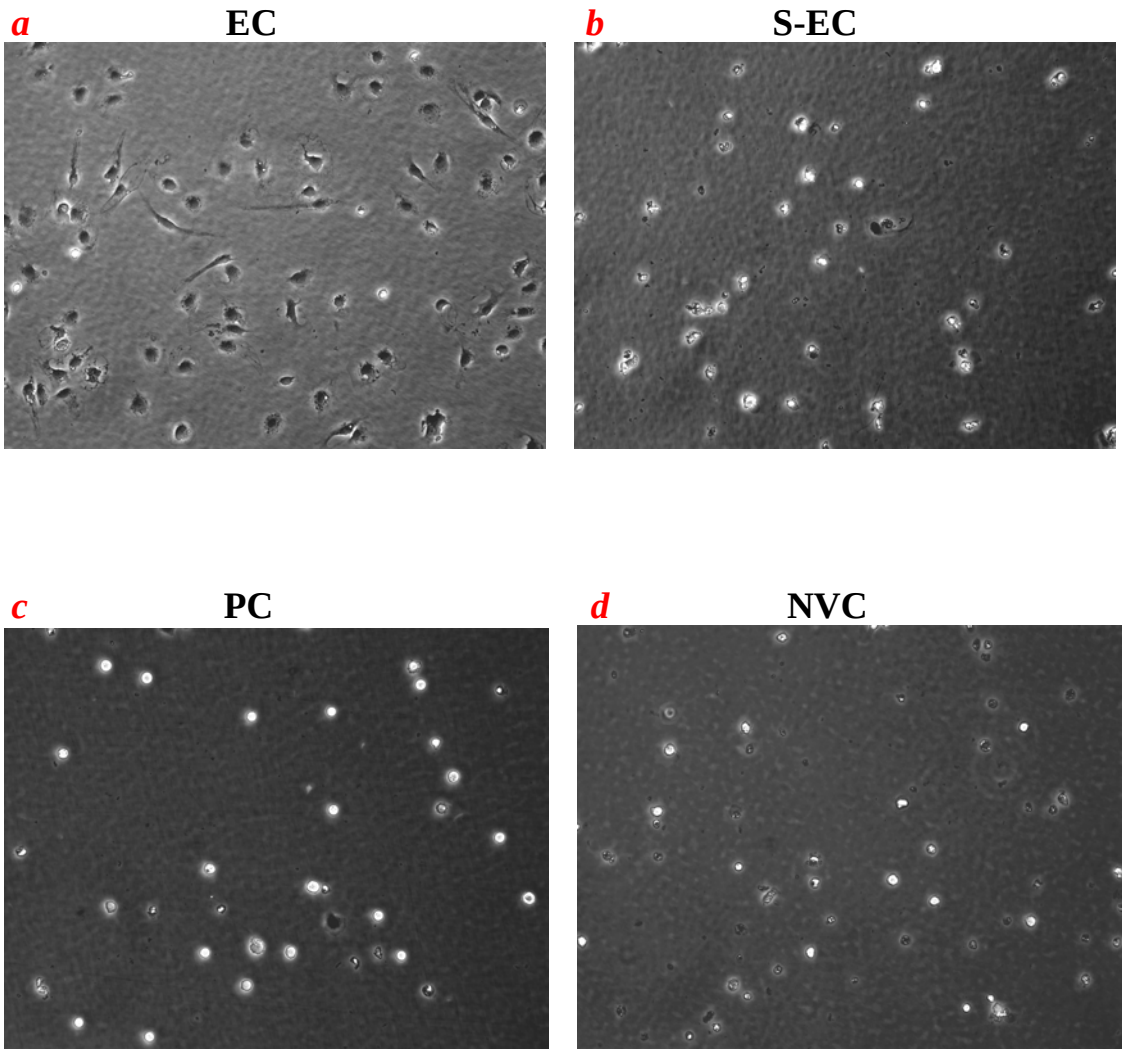


Figure 2.7 Adherence properties of the 4 stromal cell populations
Freshly sorted EC, S-EC, PC and NVC were seeded in culture (a-d). After a period of 24 hours, these phase contrast pictures were taken. Cells which have attached to the flask at this time exhibit a dark appearance, while non-adherent cells remain shiny and refringent.

Based on these initial observations of growth patterns, we proceeded to further analyze the adherence properties in culture of the cells, but this time in a retrospective manner (i.e. without the benefit of a prior knowledge of the identity of the cells). To this end, the

total unsorted stromal cell population was plated on a culture flask immediately after isolation from the adipose tissue. Approximately 1 hour after plating, cells which adhered to the bottom of the flask (herein named FA for fast-adherent cells) were collected and individual cells identified by FACS analysis. Cells which did not adhere to the flask after 1 hour (herein named SA for slow-adherent cells) were collected and analyzed in the same manner. FACS analysis of equal numbers of cells from the FA and SA fractions shows a visibly higher number of s-endothelial cells and pericytes within the SA fraction compared to the FA fraction (Figure 2.7a). Quantification of the different cell populations using the aforementioned analysis reveals the composition of cells within the FA and SA fractions, shown in Figure 2.7b. As seen in the figure, a large proportion of cells within the FA fraction consisted of endothelial cells, whereas the SA fraction consisted mostly of endothelial cells and pericytes in approximately equal proportions. When each cell population was analyzed individually for adherence properties, we observed that the majority of EC, approximately 66%, were fast-adhering cells. On the other hand, PC and S-EC were mostly slow-adhering cells, with about 80% and 74%, respectively, of the cells within these populations contained in the SA fraction. The NVC fraction consisted of about equal numbers of SA and FA cells (Figure 2.7c).

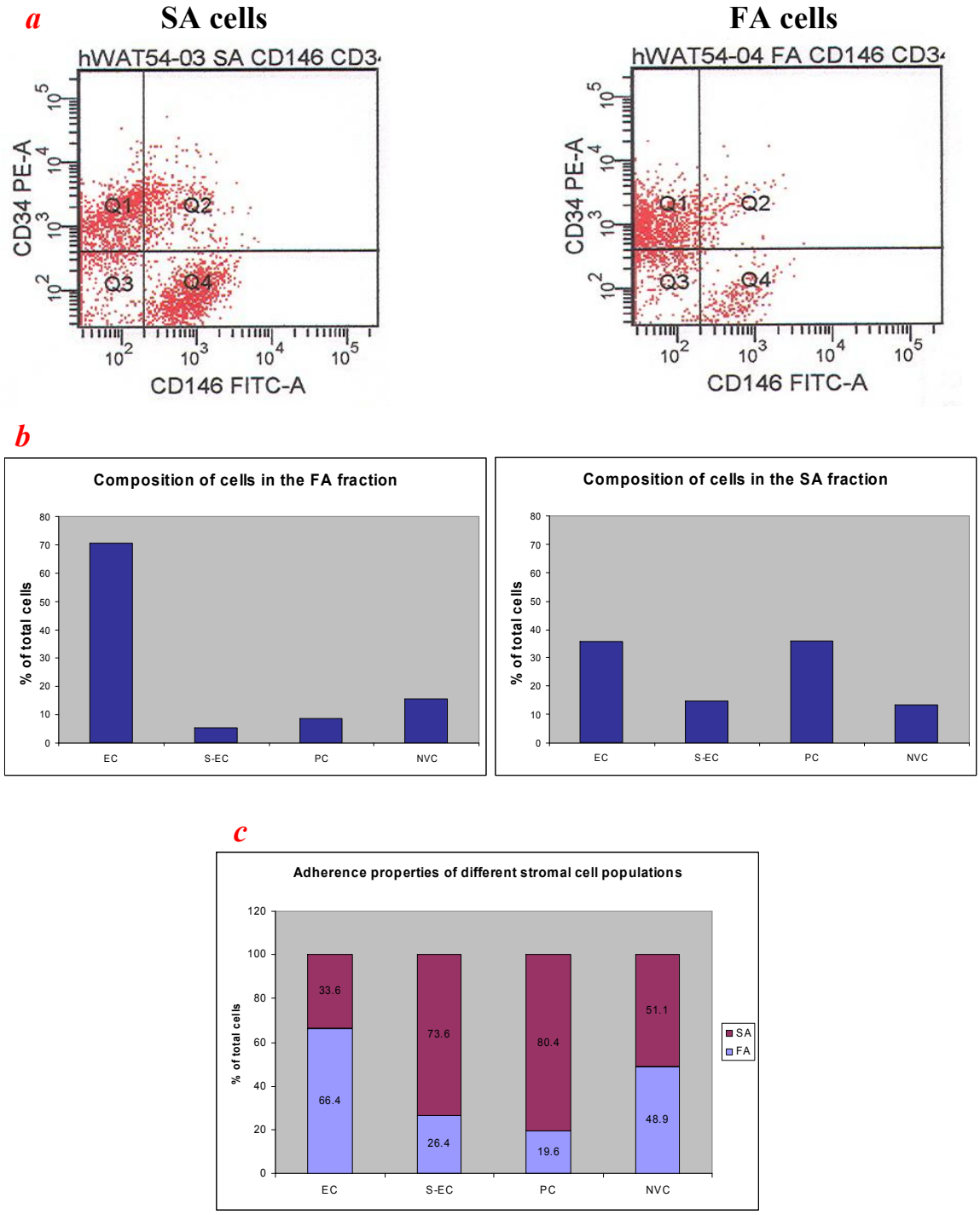


Figure 2.8 Preplating of hWAT stromal cells
The preplating method was performed on hWAT stromal cells. Freshly isolated, unfractionated cells were seeded in culture; after 1 hour and 15 min., cells which adhered to the flask/FA cells, and cells which did not adhere to the flask/SA cells, were each collected and analyzed by FACS separately (a). Percentages of the 4 stromal cell populations within the FA and SA fractions are shown (b). The composition of cells within each population, as defined on adherence properties, was also determined (c).

2.1.9 Population doubling time

When cultured on the long-term, it was observed that PC had a very slow proliferation rate compared to the other cell fractions. We measured the population doubling time (PDT) of PC and observed that it was more than twice that of the EC and NVC. The PDT of S-EC was not determined, as we were not able to propagate these cells effectively in culture.

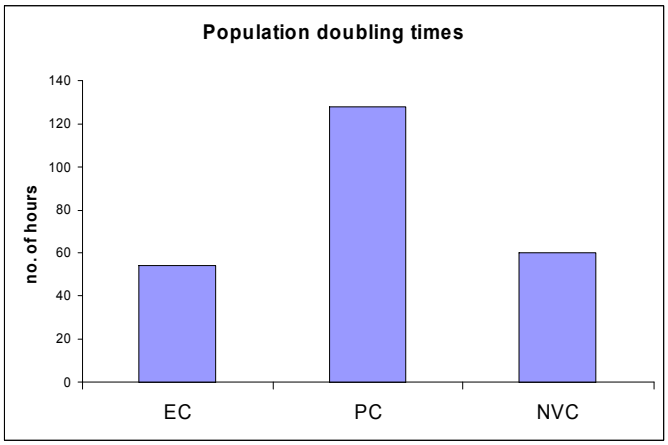


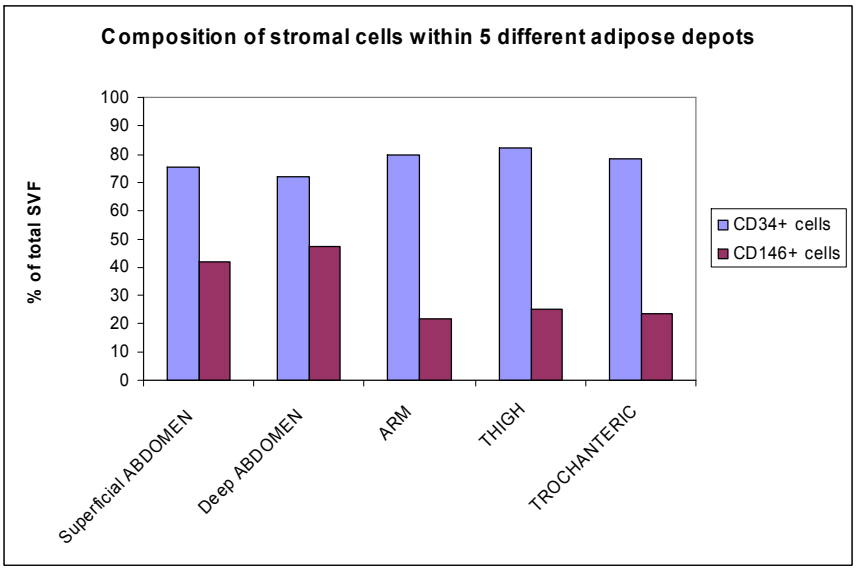
Figure 2.9 Pericytes exhibit a slow proliferation rate in vitro
The population doubling rate of EC, PC and NVC was determined. It was observed that the average PDT of PC was approximately twice that of EC and NVC when cultured under similar conditions.

2.1.10 Stromal cell composition of different fat depots

To determine the vascular cell composition within different fat depots of the human body, we analyzed lipoaspirates taken from 5 distinct anatomical locations: superficial and deep abdomen, arm, thigh, and trochanteric/hip area. We characterized the cells from each depot according to CD34 and CD146 expression. We found no noticeable differences in

the amount of total CD34+ cells among the different fat locations. However, we observed a higher percentage of total CD146+ cells within the total SVF of the abdominal fat depots compared to the arm, thigh, and trochanteric fat depots (Figure 2.10a). When the individual vascular cell populations were analyzed, a higher percentage of S-EC and PC, while a slightly lower percentage of EC, was seen within the stromal fraction of the abdominal fat depots compared to the other fat areas (Figure 2.10b).

a



b

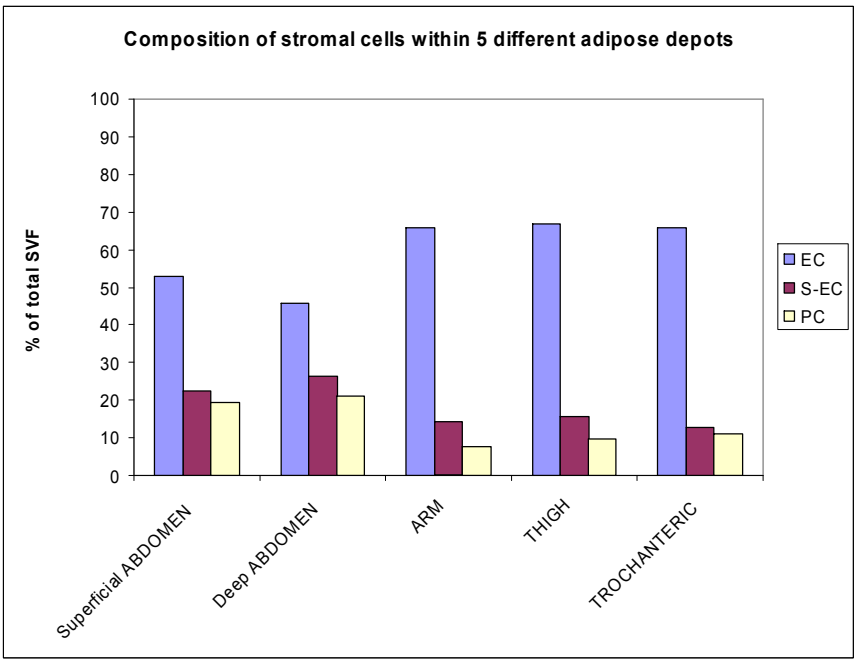


Figure 2.10 Vascular cell composition of different fat depots
(a) Percentages of total CD34+ cells within the SVF of the different fat depots are approximately the same. However, there are about twice as many CD146+ cells within the abdominal fat depots compared to the other 3 fat depots. (b) A slightly lower percentage of EC was observed within the abdominal fat SVF, compared to that in other fat depots. On the other hand, there are about twice as many S-EC and PC in the SVF of the abdomen compared to the 3 other fat depots. The results represent the mean of 2 distinct patient samples.

2.2 DISCUSSION

The stromal cell compartment within human adipose tissue was analyzed in this study. We aimed to identify the different cell subsets constituting the hWAT stroma. With a knowledge that the fat tissue is richly vascularized, our primary focus was on blood vessel-associated cells. We divided the vascular compartment into two distinct cell populations based on their antigenic profiles and location in blood vessel walls. Cells which line the lumen of blood vessels were considered as endothelial cells. Cells closely adherent to endothelial cells, and located on the abluminal surface, were identified as pericytes. After visualizing the anatomical location of these cells and determining their immunohistochemical profile on tissue sections, we next confirmed their existence by FACS analysis. This protocol eventually enabled us to effectively separate not only the two main types of cells embedded within the basement membrane of blood vessels- endothelial cells and pericytes- but also to obtain a population of non-vessel associated cells.

FACS analysis of the human adipose stromal cell fraction reveals that it is comprised predominantly of vascular cells (endothelial cells and pericytes), and only a small percentage of blood-derived cells and non-vascular cells. We have identified, by FACS analysis, a population of pericytes, defined as CD146+ CD34- CD45- cells, and two populations of CD34+ endothelial cells which differ with regard to CD146 expression. By RT-PCR, it was furthermore revealed that the S-EC population expresses NG2 while EC do not. Previous studies have shown that NG2 expression is a characteristic of activated endothelial cells. Therefore S-EC likely consist of

proliferating endothelial cells within the neovasculature, while EC represent the majority of endothelial cells which are quiescent.

In this study, we have arbitrarily categorized smooth muscle cells (SMC) as part of the NVC population. SMC have similar characteristics with pericytes, which are also contractile; however, SMC do not express CD146. Moreover, although SMC are found within the walls of the vasculature, they are located outside of the basement membrane of the arterioles and venules, in contrast to endothelial cells and pericytes which are embedded within the basal lamina of the blood vessel wall.

An interesting finding was the expression of CD56 by S-EC. CD56 is a marker of satellite cells in skeletal muscle, and of NK cells and some neural cells. Although the expression of CD56 by a subset of endothelial cells may seem unusual, we have already established, by confocal microscopy, the presence of CD56+ myogenic-endothelial cells within the blood vessel wall of the human skeletal muscle [38]. Vascular cells, termed mesoangioblasts, which express myogenic cell markers, have also been identified in the embryonic dorsal aorta [11, 12].

We also note that close to 80% of the non-hematopoietic stromal cell fraction in WAT consists of CD34+ cells. We believe that CD34+ CD45- cells represent the entire endothelial cell population in fat tissue. Indeed, the observed proportion of 1:6 pericytes to endothelial cells based on this categorization is consistent with established findings that endothelial cells outnumber pericytes in various tissues, with a normal ratio ranging from 1:5 to 1:10.

Cell culture was another method for us to confirm the identity of the sorted cells. Each purified cell subset- EC, S-EC, PC, and NVC- exhibited distinct cell morphology

and growth pattern. Pericytes, in particular, had the unique appearance of rectangular-shaped cells with irregular or ruffled edges, in contrast to the other cell populations which had smooth cell borders. In addition, pericytes were slowly-dividing and never grew to confluence in culture, in contrast to the other cell populations. We also demonstrate in this study the characteristic adherence properties of these different cells. Thus, it appears that the preplate technique may be utilized on the total SVC to select a desired population of cells, similar to the method used to obtain muscle-derived stem cells (MDSCs) [55]. Based on the data shown in this study, we could enrich certain cell populations based solely on preplating. For example, repeated preplatings of adherent cells would yield a population that is enriched for EC, as these would comprise the vast majority of the adherent fraction and would rapidly outgrow the slowly dividing PC. On the other hand, repeated preplatings of the non-adherent cells would yield a population enriched for PC, as we would expect the EC to diminish in successive preplatings of non-adherent cells. This suggests that freshly isolated cells from the hWAT, selected by a series of consecutive preplatings, would yield a culture that is enriched for pericytes.

2.3 METHODS

2.3.1 Human tissues

Whole adipose tissue from abdominal subcutaneous fat was obtained anonymously from patients who underwent abdominoplasty at the Department of Plastic and Reconstructive Surgery of UPMC. Donors were females ranging in age from 42 to 57 years old, with a mean age of 51 years. The described procedure was approved by the Institutional Review Board and the research protocol was reviewed and approved by the Animal Research and Care Committee at the Children's Hospital of Pittsburgh and University of Pittsburgh.

2.3.2 Immunohistochemistry

Adipose tissues were incubated overnight in phosphate buffer, then rinsed in PBS, impregnated in gelatin sucrose and finally frozen in gelatin sucrose in isopentane vapors prior to cryosectioning. 12um-thick cryosections were fixed in a 1:1 cold acetone/methanol mixture for 5 min and preincubated in 5% goat serum in PBS for 1 h at room temperature (RT). Sections were incubated with uncoupled primary antibodies overnight at 4^0C, or 2 hours at RT in the case of directly coupled antibodies. After rinsing, sections were incubated for 1 h at RT with a biotinylated secondary antibody, then reacted with fluorochrome-coupled streptavidin.

Anti-human primary antibodies used and their dilutions were: CD34 (Serotech, 1:50), biotinylated CD144 (BD, 1:100), and CD146-Alexa 488 (Chemicon, 1:200). Also used was biotinylated UEA-1 lectin (Vector, 1:100), which was then reacted with biotinylated

goat anti-mouse (DAKO, 1:1000 and Immunotech), followed by Cy3-conjugated streptavidin (Sigma, 1:1000).

2.3.3 Flow cytometry

Adipose tissues were finely minced, then digested in Dulbecco's modified Eagle medium (DMEM, Gibco) containing 3.5% bovine serum albumin (Sigma) and collagenase II (1mg/ml, Sigma) for 70 minutes on a shaker at 37^0C. Mature adipocytes were separated from pellets by centrifugation (2,000 rpm, 10 min). Pellets were resuspended in erythrocyte lysis buffer (155 mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA) and incubated for 10 min at RT. The pellets were resuspended and passed through a 70-um cell filter (BD Falcon). The approximate cell volume/size of stromal cells vs. adipocytes were calculated using the formula $4/3\ \pi r^3$. For FACS analysis, cells were incubated with one of the following directly coupled mouse anti-human antibodies: CD146-FITC (Serotec, 1:100), CD34-PE (DAKO, 1:100) and CD45-APC-Cy7 (Santa Cruz Biotechnologies). For the UEA-1 analysis, cells were first incubated with biotinylated UEA-1 (Vector, 1:100), followed by Pacific blue-conjugated streptavidin (Molecular Probes, 1:1000); cells were then labeled with directly coupled CD146, CD34, and CD45 mouse antibodies as described above. After washing and centrifugation, cells were incubated with 7-amino-actinomycin D (7-AAD, 1:100, BD) for dead cell exclusion, then run on a FACSAria flow cytometer (BD). As negative controls, cell aliquots were incubated with isotype-matched mouse IgGs conjugated to FITC (US Biological, 1:100), PE (Chemicon, 1:100) and APC-Cy7 (BD, 1:100) in the same conditions. Cell sorting was performed on

a FACSAria dual-laser fluorescence cell sorter (Becton-Dickinson). Sorted cells were re-analyzed in all experiments.

2.3.4 RT-PCR

Total RNA was extracted from 2 x 10^4 sorted cells using Trizol (InVitrogen). cDNA was synthesized with SuperScript ^{TM} II reverse transcriptase (InVitrogen), according to manufacturer's instructions. PCR was performed with Taq polymerase (Gibco) per manufacturer's instructions and PCR products were electrophoresed on agarose gels. The primers used for PCR are listed in Table 1.

2.3.5 Cell culturing

Cells were seeded at an initial density of 2 x 10^4 cells per cm^2 in EGM2 medium (Cambrex); all GIBCO-brand reagents were purchased from Invitrogen, Carlsbad, CA. At 70% confluence, cells were detached with trypsin/EDTA, replated after washing at densities between 2.0 and 3.0 x 10^3 cells/cm^2, and further cultured for up to 5 months in DMEM containing 20% FBS and 1% penicillin-streptomycin.

2.3.6 Preplating of adipose stromal cells

The total, freshly isolated stroma vascular fraction was plated in DMEM + 20% FBS. One hour and 15 minutes after seeding, the cells which did not adhere to the flask were collected and named as SA cells. Cells which attached to the bottom of the flask after this period were also collected and named FA cells. Cells were labeled for FACS analysis as described above.

2.3.7 Population doubling time

Long term cultures of PC, EC and NVC in DMEM + 20% FBS were used to calculate the population doubling time (PDT). For each population, cells were seeded into 3 wells of a 6-well plate at a density of 2.0×10^3 cells/cm^2. Cells were grown for 120 hours, and the PDT was calculated from the average of 3 wells using the formula: time / no. of doublings, where time = 120, and the no. of doublings = $\log_2 (N_{final} / N_{initial})$.

3.0 ADIPOGENESIS FROM ADIPOSE STROMAL CELLS

3.1 INTRODUCTION

The origin of fat cells remains unclear. However, there is evidence that during human fetal development, the appearance of adipocytes, which first occurs during the second trimester, is associated with the organization of a vascular structure [41]. At this stage, arterioles can be detected in the vicinity of small developing fat cell clusters that are surrounded by stroma. This observation may indicate, although indirectly, that adipocytes originate from cells contained within blood vessels. The relationship between tissue vascularization and adipocyte formation is further underlined by the fact that fat cell density appears to be positively associated with capillary density and that the largest fat cell clusters are located near the entry points of large blood vessels [42].

For these reasons, we hypothesized that during postnatal life, blood vessels within the fat continue to serve as a reservoir of preadipocytes, and that cells contained within the blood vessel wall contribute to the formation of new adipocytes according to the metabolic needs of the body.

3.2 RESULTS

3.2.1 Adipogenesis from total hWAT stromal cells

To test the efficiency of our adipogenesis culture medium, we grew freshly isolated total SV cells in EGM2 medium until confluence, followed by culture in adipogenic differentiation medium. Within four days of culture in the latter, we observed the emergence of clusters of lipid-filled cells (Figure 3.1). With this positive outcome, we then proceeded to test the adipogenic potential of our four sorted cell populations using the same culture conditions.

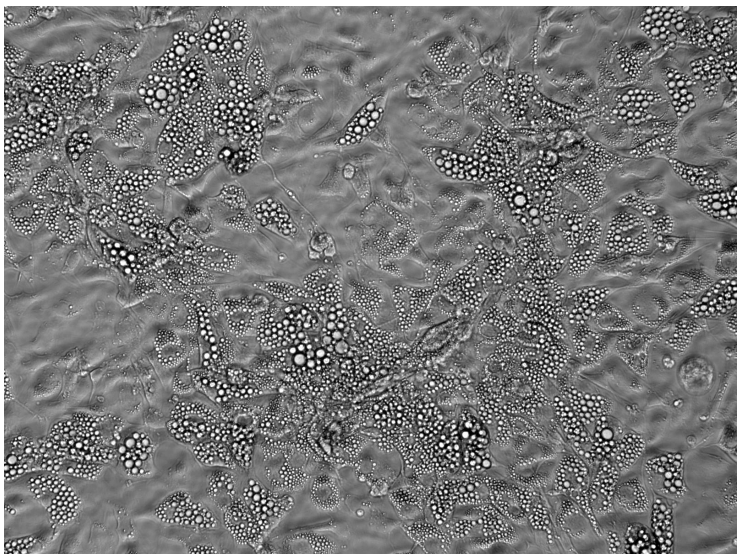
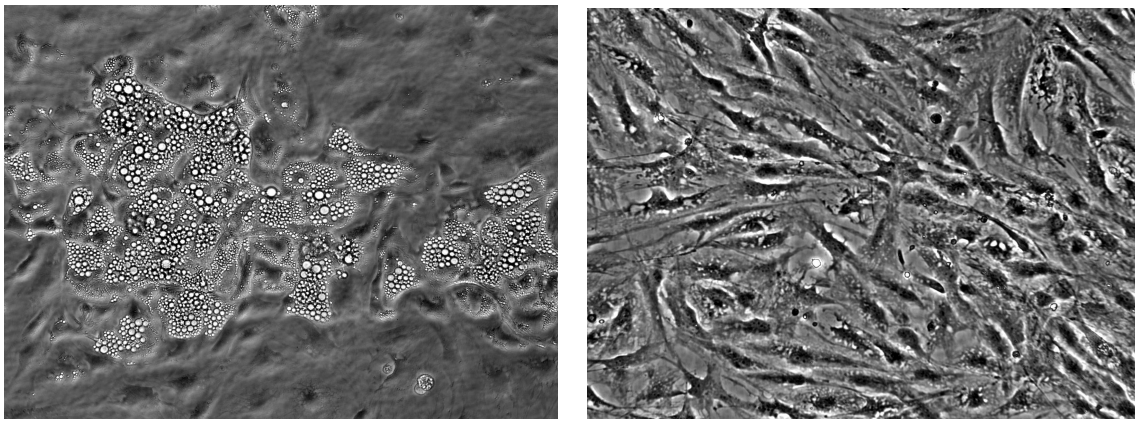


Figure 3.1 Adipocyte differentiation of total unfractionated adipose tissue stromal cells
Freshly isolated stromal cells were seeded in culture until confluence, then further cultured in adipogenic differentiation medium. Multilocular lipid-filled cells were observed after a period of four days in this medium, as seen in this phase contrast image.

3.2.2 Compared adipogenic potentials of four distinct hWAT-derived cell populations

Freshly sorted EC, S-EC, PC and NVC were cultured in EGM2 medium until confluence, then grown in adipogenic differentiation medium for an additional 8 days. At the end of this period, we observed that some of the cells within the EC (Figure 3.2a), PC (Figure 3.2c), and NVC (Figure 3.2d) populations differentiated into mature adipocyte-like multilocular cells. However, more densely aggregated and slightly larger clusters were observed in the EC population compared to the PC and the NVC populations (Figure 3.2a vs. 3.2c and 3.2d). We did not observe any adipocyte differentiation from the S-EC fraction (Figure 3.2b).

a **EC** ***b*** **S-EC**



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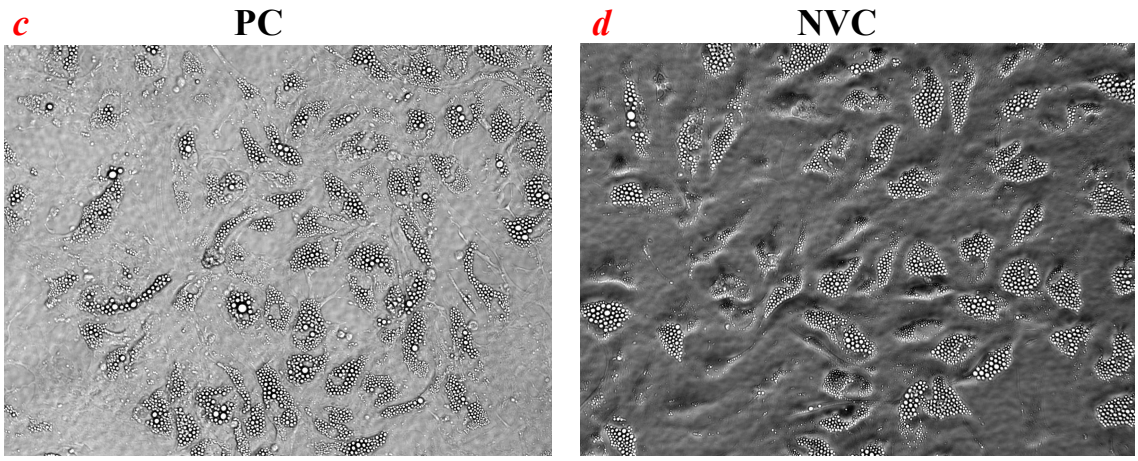


Figure 3.2 Adipogenic differentiation of 4 distinct stromal cell populations from hWAT
Phase contrast images of primary cultures of the four populations, taken 8 days after culture in adipogenic differentiation medium. No multilocular lipid-filled cells were derived from the S-EC population (b).

We further observed that the differentiation of EC into lipid-filled cells was more generalized, occurring in all areas of the well, as seen at lower magnification below. Approximately 60% of the surface of the well seeded with EC was covered with clusters of multilocular cells (Figure 3.3a). On the other hand, the differentiation of PC and NVC into lipid-filled cells occurred in a sporadic fashion, with approximately 70% of the surface of the well comprising non-multilocular cells (Figures 3.3b and 3.3c, respectively).

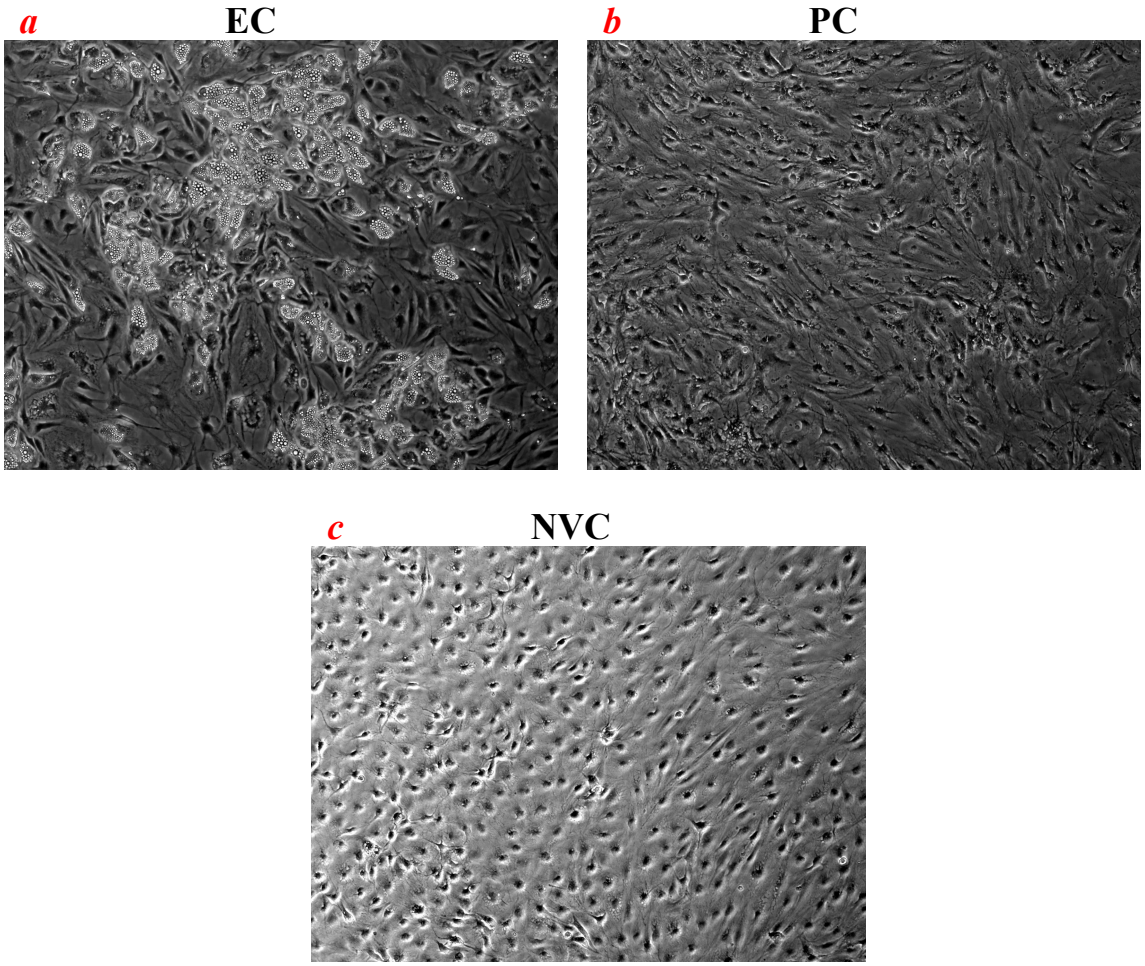


Figure 3.3 Efficiency of adipocyte differentiation from various WAT stromal cells
EC in adipogenic differentiation medium formed clusters of multilocular, lipid-filled cells which occurred in all areas throughout the well (a). On the other hand, most areas of the well seeded with PC (b) and NVC (c) contained cells which failed to differentiate into adipocytes, as seen in these phase contrast images.

3.2.3 Quantitative determination of leptin expression

We tested the presence of leptin mRNA in the four cell populations cultured in adipogenic differentiation medium, and found that there was a much higher level of leptin expression in the PC and NVC fractions, compared to the EC and S-EC fractions (Figure 3.4).

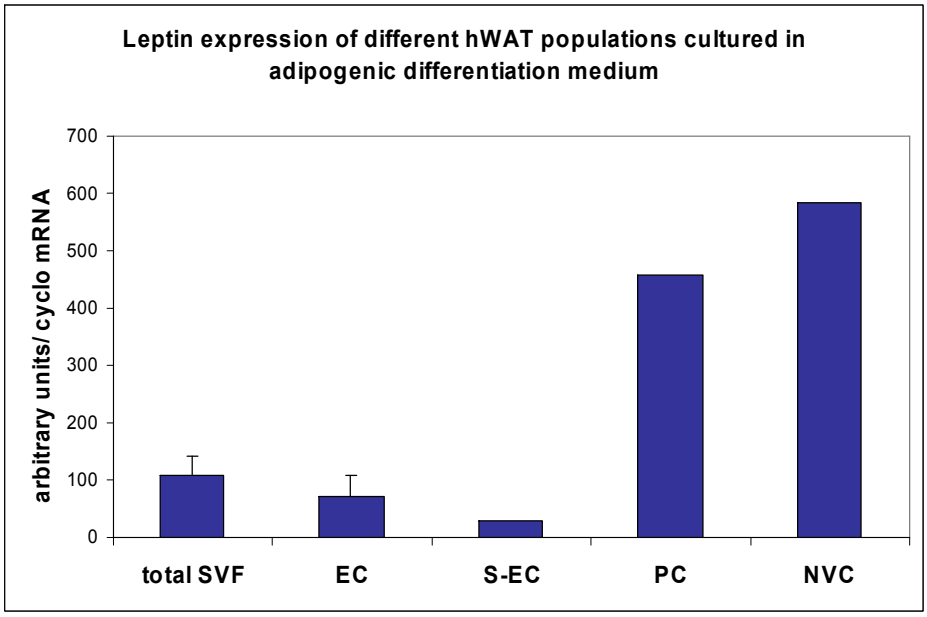


Figure 3.4 Determination of leptin expression
The graph represents a quantitative RT-PCR determination of leptin mRNA expression in EC, S-EC, PC, NVC, and total unsorted stromal cells after culture in adipogenic differentiation medium for 8-10 days. The results are mean +/- s.e.m. of arbitrary values normalized using the corresponding cyclophilin A values, n = 5, 2, 1, 1, 1 for total SVF, EC, S-EC, PC and NVC, respectively.

3.2.4 Quantitative determination of UCP-1 expression

We also tested the level of UCP-1 expression in each of the four populations, and found that only the PC fraction expressed a significant level of UCP-1, while the S-EC and NVC fractions had very low levels of the mRNA. No UCP-1 was detected from the EC fraction (Figure 3.5).

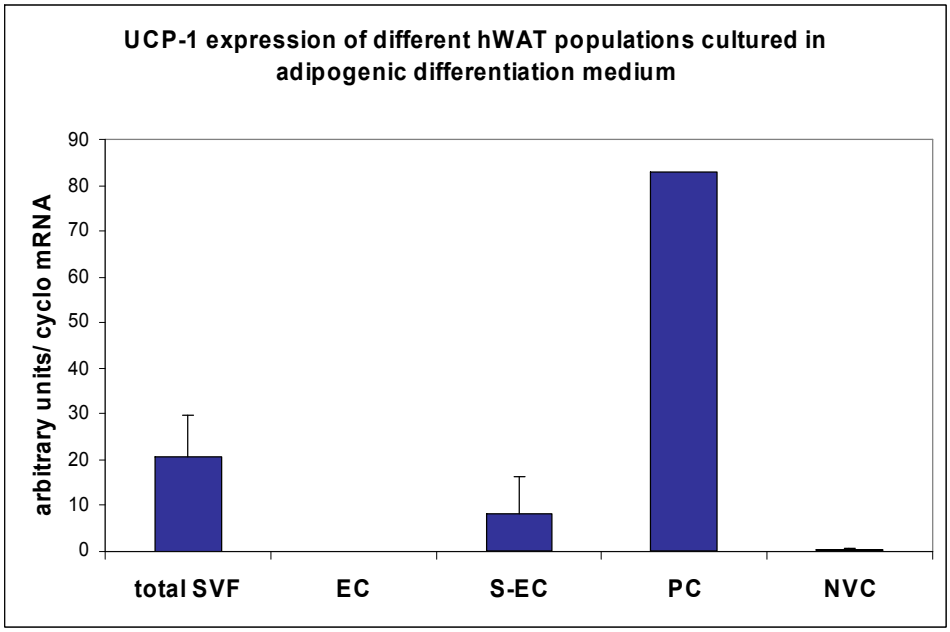


Figure 3.5 Determination of UCP-1 expression
The graph represents a quantitative RT-PCR determination of UCP-1 mRNA expression in EC, S-EC, PC, NVC, and total unsorted stromal cells after culture in adipogenic differentiation medium for 8-10 days. The results are mean +/- s.e.m., n = 5, 2, 2, 1, 4 for total SVF, EC, S-EC, PC and NVC, respectively.

3.2.5 Adipogenesis from long-term cultured hWAT-derived cells

As adipogenic differentiation obtained was thus far observed from primary cultures of WAT stromal cells, we wanted to determine whether the same cells could be expanded in culture while retaining their adipogenic potency. For this experiment, EC, PC and NVC cells were cultured for at least 14 weeks in DMEM + 20% FBS. To induce adipogenesis, cells were then plated in the same conditions as primary cultured cells. This time, only PC formed multilocular lipid-filled cells (Figure 3.6b). No lipid formation was observed from either the EC nor the NVC fractions (Figures 3.6a and 3.6c, respectively). We were not able to efficiently culture S-EC on the long term.

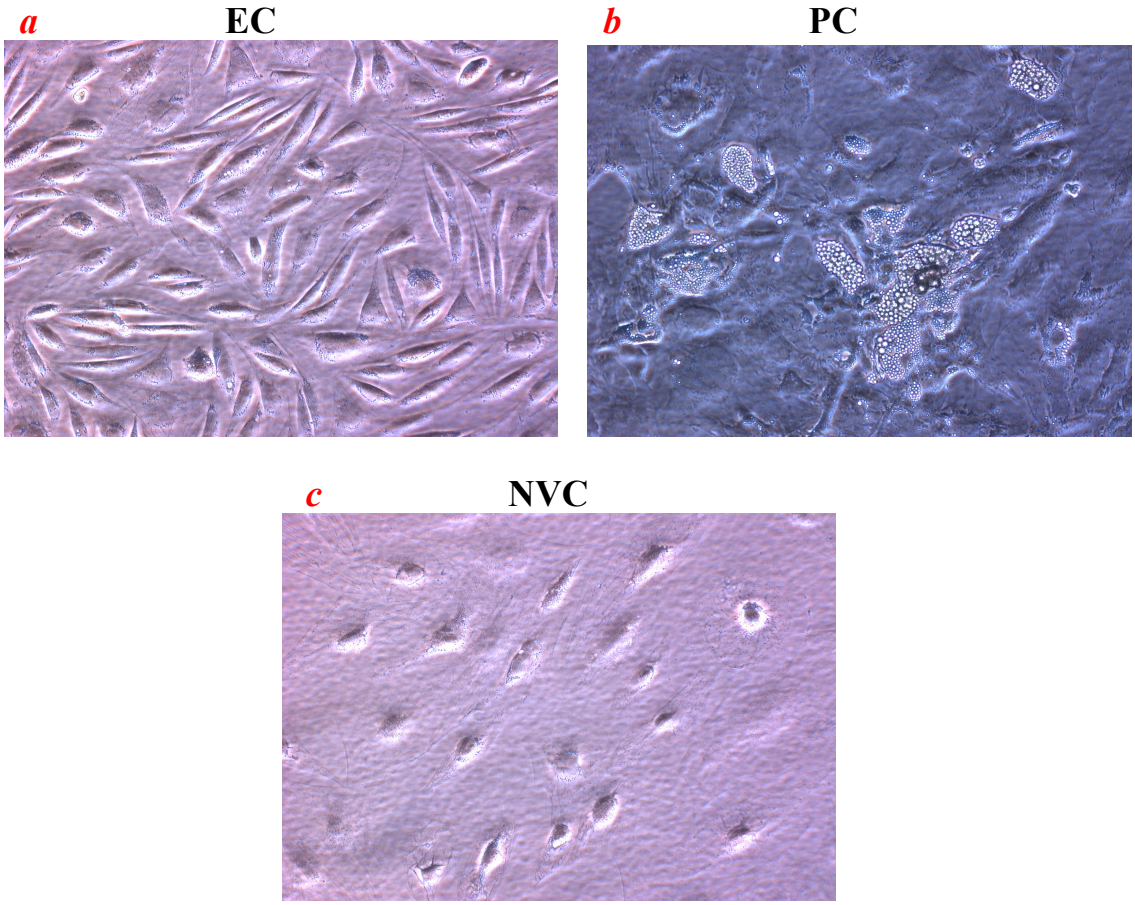


Figure 3.6 Adipogenic differentiation from long-term cultured cells
Phase contrast images of long-term cultured EC, PC and NVC, taken 15 days after culture in adipogenic differentiation medium. Only PC formed multilocular lipid-filled cells (a), while EC (a) and NVC (c) failed to differentiate into adipocytes.

3.2.6 Quantitative determination of leptin expression from LTC cells

We tested the presence of leptin mRNA in the EC, PC and NVC long-term cultured cells in adipogenic differentiation medium, and found a similar pattern of leptin expression with that observed from the primary cultures. There was again a much higher level of leptin expression in the PC and NVC fractions, compared to the EC fraction (Figure 3.4).

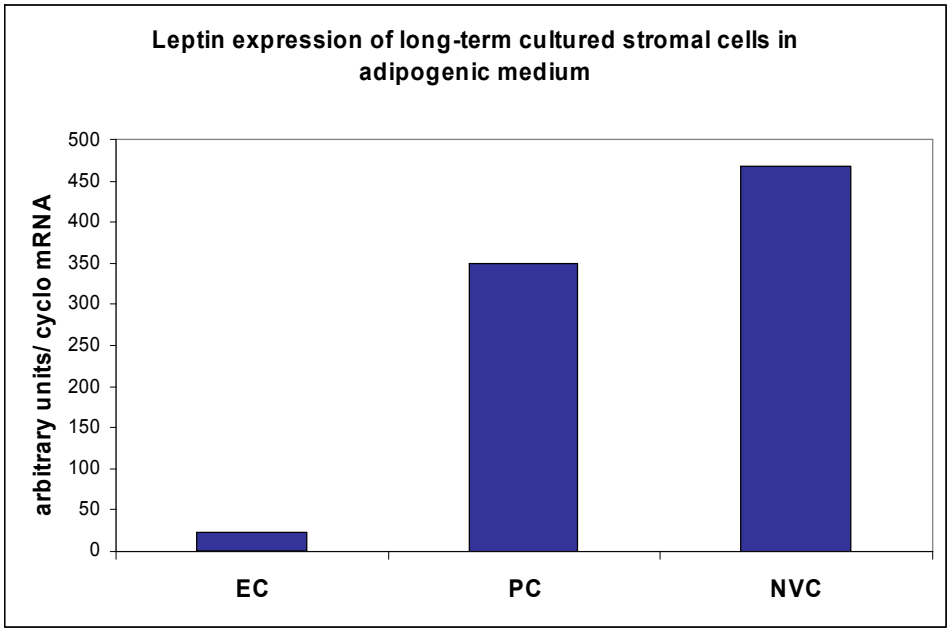


Figure 3.7 Determination of leptin expression from long-term cultured cells
The graph represents a quantitative RT-PCR determination of leptin mRNA expression in long-term cultures of EC, PC and NVC stromal cells, after exposure to adipogenic differentiation medium for 8-10 days. The results are mean +/- s.e.m., n = 1 each for EC, PC and NVC.

3.2.7 Quantitative determination of UCP-1 expression from LTC cells

No expression of UCP-1 was detected after long-term cultures of EC, PC and NVC were exposed to adipogenic differentiation medium.

3.3 DISCUSSION

We herein observed a differentiation of the EC, PC and NVC fractions of hWAT stromal cells into multilocular lipid-filled cells. We also tested the expression of leptin, a hormone secreted specifically by adipocytes, in the different stromal cells after their culture in adipogenic medium. We observed a much higher level of leptin, as much as a 20-fold difference, in both primary and long-term cultures of the PC and NVC fractions compared to the EC and S-EC fractions. The high levels of leptin expression by the PC and NVC populations were maintained even after their extensive expansion in culture. This result suggests that the pericyte and non-vascular cell fractions within fat most likely contain the precursors of mature adipocytes in humans.

When the different stromal cells were propagated in culture on the long term, only pericytes remained capable of differentiating into adipocytes, while the endothelial and non-vascular cells failed to differentiate into multilocular cells. This was an interesting finding, although it is consistent with earlier findings on the multipotency of pericytes in vitro; we remain however the first who have done it on purified pericytes. Indeed, we also observed that pericytes derived from non-adipose tissues have a greater propensity for adipocyte formation when compared to other cell populations.

Our findings also suggest that endothelial cells and non-vascular cells lose their potential to develop into adipocytes when cultured on the long term in vitro. Notwithstanding, long-term cultures of the non-vascular fraction maintained their high levels of leptin expression, suggesting that the cells might have been in the intermediary stages of their development into mature adipose cells. Indeed, the process of adipose cell

differentiation may occur in sequential stages, from stromal cells, into preadipocytes, then becoming immature adipose cells and finally mature adipocytes by which time they have already accumulated triglyceride stores.

The effective utilization of a stromal cell subset which may be expanded in culture and obtain adipocytes might be of importance in a clinical tissue engineering perspective. The use of autologous adipose tissue or cells derived from it, for cosmetic applications, is ideal and practical. This strategy may be used in such applications as lip and breast augmentations, buttock enhancement, and to restore youthful appearance to the face and hands, as when the technique is employed for the elimination of wrinkles or the reversal of atrophic tissue in the dorsal surface of the hands of the elderly. In the field of tissue engineering, the adipogenic potential of hWAT stromal cells may also be harnessed, as in the seeding of these cells into biodegradable scaffolds which may be engineered to contain adipogenic growth factors. Such scaffolds may be used to correct defects from patients who had undergone radical surgical procedures such as mastectomy for breast carcinoma.

We also determined the expression of UCP-1 by the different stromal cell populations after they were cultured in adipogenic medium. The identification of UCP-1 expressing cells in adipose stroma of adult humans is an important finding, and may bear a therapeutic relevance in the fight against obesity. UCP-1 is a main effector of thermogenesis, and the reactivation of this pathway in the adult may lead to an increase in the metabolic rate and thus, reduce excess energy that may be stored as fat in the body. We detected a higher level of UCP-1 in the PC fraction compared to the S-EC and NVC fractions, while no UCP-1 was detected in the EC fraction. Although the differentiation

of hWAT pericytes into genuine brown adipocytes is a significant finding, the UCP-1 mRNA expression by these cells is only about 22% of that found in CD34+ cells from the adult skeletal muscle, where the highest levels of UCP-1 expression among postnatal tissues are found [61].

3.4 METHODS

3.4.1 Cell culture

Freshly sorted cells were seeded at 2.10^4 cells per cm^2 in EGM2 (Cambrex Bio Science, Walkersville, MD) medium and cultured at 37ºC in plates coated with 0,2% gelatin until confluency. The EGM2 medium was then replaced by a modification of the adipogenic medium described by Rodriguez et al. consisting in DMEM-Ham's F-12 medium containing 0.86 μM insulin, 10 μg/ml transferrin, 0.2 nM triiodothyronine, 1 μM rosiglitazone (SmithKline Beecham, Research Triangle Park, NC), 100 μM 3-isobutyl-1-methylxanthine, 1 μM dexamethazone and 1% penicilline-streptomycine (PS), and the cells were grown 10 more days. Media were changed every 3 days. For adipogenic differentiation of long-term cultured cells, cells used had been in culture for at least 12 weeks, with passages between 6-9, before being exposed to adipogenic medium.

3.4.2 Quantitative RT- PCR

Total cell RNA was prepared using the kit NucleoSpin® RNAII (Clontech, Palo Alto, CA) according to the Manufacturer's Instructions and quantified by quantified by

Biophotometry (Biophotometer, Eppendorf). Oligo-dT primed first strand cDNA were synthesized using the Superscript™ II RNase H Reverse Transcription kit (Invitrogen, Carlsbad, CA). Analytical PCR was performed as described previously (Crisan et al., submitted) and quantitative real-time PCR was performed using ABI rapid thermal cycler system, and a SYBR Green PCR master mix according to the Manufacturer's Instructions (Applied Biosystems, Foster City, CA). Cyclophilin A was used as a control to account for any variations due to the efficiencies of the reverse transcription and PCR. The primers used are described in Table I. The conditions of PCR were a step at 50°C for 2 minutes followed by a denaturing step at 95°C for 10 minutes and by 50 cycles at 95°C for 15 seconds and 60°C for 1 minute. The upstream and downstream oligonucleotide primers were chosen on both sides of an intron to prevent amplification of possible contaminating genomic DNA.

3.4.3 Validation of the UCP1 amplicon

The PCR-amplified fragment was cloned into pCR2.1-TOPO vector through the TOPO-TA cloning system (Invitrogen, Carlsbad, CA) and purification of color-selected colonies was performed using the Qiaprep Spin Miniprep (Qiagen, Hilden, Germany). Sequences were determined with universal oligonucleotide M13 Reverse on the pCRII-TOPO vector using the Applied Biosystem Big Dye sequencing kit following the manufacturer's protocol on an ABI 3700 automated sequencer (Applied Biosystems, Foster City, CA).

4.0 MYOGENESIS FROM ADIPOSE STROMAL CELLS

4.1 INTRODUCTION

Duchenne muscular dystrophy (DMD) is a degenerative muscle disease characterized by a lack of dystrophin expression in the sarcolemma, a condition that leads to progressive muscle weakness [46, 47]. The lack of dystrophin in muscles of DMD patients disrupts the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix, resulting in muscle fiber necrosis and, eventually, weakness. The muscle weakness that occurs after the first few years of life is thought to be a result of replicative senescence and exhaustion of satellite cells [51]. Resarchers have extensively investigated various approaches to regenerate skeletal muscle or deliver dystrophin to the dystrophic muscle to alleviate the disease phenotype of DMD [48, 52, 54]. However, to date, there is still no effective treatment for this common inherited muscle disease. The use of cell therapy is one possible approach to restore damaged skeletal muscle and improve muscle function. In the past, efforts in this field have been hampered by the low migratory capacity of donor cells, immune rejection problems, and poor cell survival rates. The isolation and transplantation of a population of adipose-derived cells, exhibiting a high capacity for muscle engraftment and repair, would enhance the beneficial effects of cell therapy in skeletal muscle.

4.2 RESULTS

4.2.1 Comparison of myogenesis from 4 hWAT stromal cell populations

To determine the myogenic potential of various cell populations constituting adipose tissue, freshly sorted cells were immediately transplanted into NOD-SCID mouse skeletal muscles which had been injured 1 hour earlier by cardiotoxin injection. We observed that the injected cells from each population were able to regenerate human spectrin-expressing muscle fibers (Figure 4.1a). However, the number of myofibers formed by PC, expressed as a regenerative index per 1 x 10^4 cells injected, was significantly higher than that of the other cell populations (Figure 4.1b). A higher, although not significant, regeneration potential was also observed from the EC fraction compared to the S-EC and NVC fractions. Altogether, only a limited amount of human muscle regeneration was observed from these populations. To test whether engraftment with a mixed population of cells might offer an advantage in muscle regeneration compared to a homogeneous, pure cell population, we transplanted freshly isolated total unsorted stromal cells (total SVF) equal in number to that of the sorted cells, into NOD-SCID mouse muscle. The regenerative index from the total SVF was only comparable to that of the sorted EC, S-EC and NVC group.

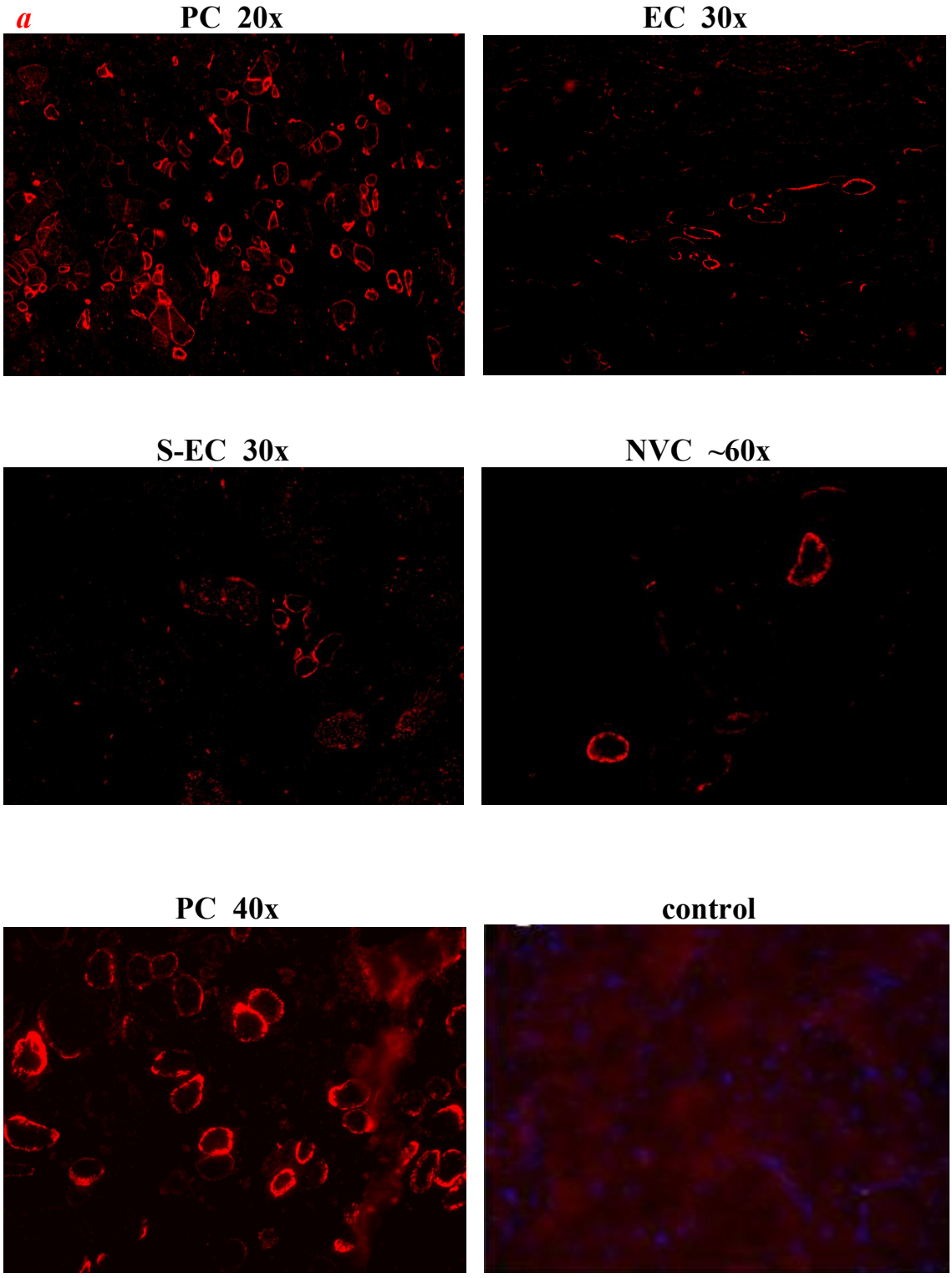


Figure 4.1a Transplanted hWAT-derived cells generated human myofibers
Expression of human-specific spectrin (red) in NOD-SCID mouse muscle implanted with freshly sorted PC, EC, S-EC, or NVC; included is a representative high-power/ oil magnification of myotubes developed from donor PC. For the control, a similar staining was done on injured NOD-SCID mouse muscle injected with PBS only.

b

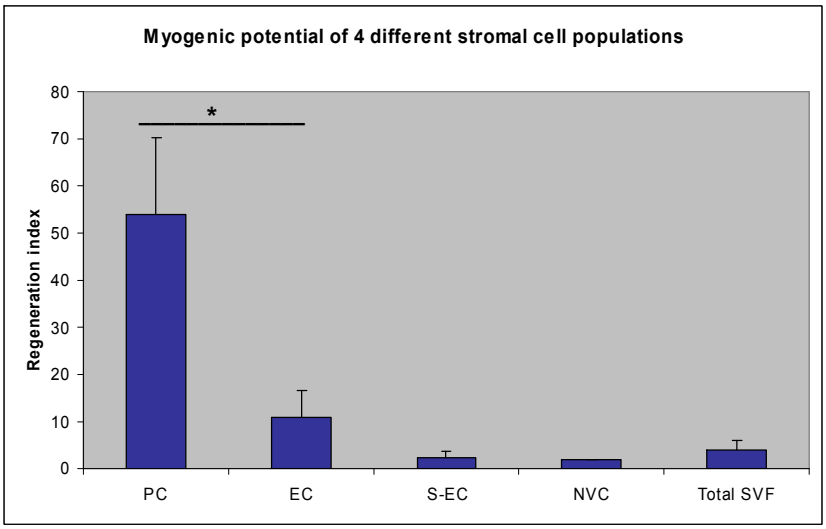


Figure 4.1b Pericytes display a superior muscle engraftment potential as compared to other adipose-derived cells
hWAT-derived pericytes displayed a significantly larger muscle regeneration index when compared to the other 3 sorted stromal cell populations, or the unsorted total stromal cell fraction.

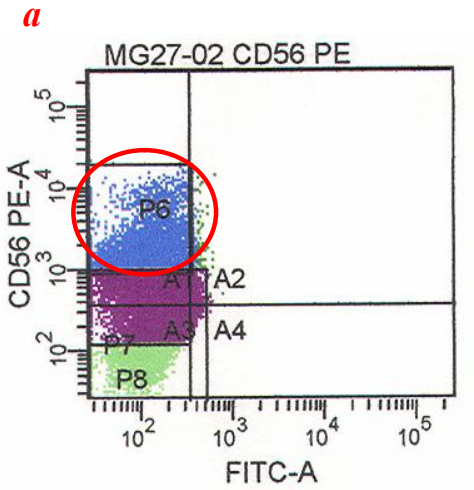
4.2.2 Pax7 expression of sorted hWAT pericytes

The identification of a highly myogenic population within adipose tissue raises the concern of contamination by muscle tissue, and by resident satellite cells contained therein. Therefore, we tested the expression of Pax7 in our sorted hWAT PC. The results showed that PC sorted from adipose tissue do not express Pax7 (Figure 4.2, with total cells from human muscle used as a positive control).

Figure 4.2 Sorted pericytes do not express Pax7
Total RNA was extracted from the unfractionated muscle cell population and from sorted hWAT pericytes; RT-PCR was used to assess Pax7 expression.

4.2.3 hWAT-derived pericytes vs. fetal muscle-derived satellite cells

Having established a superior muscle regeneration by PC from adipose tissue, we next asked how this myogenic potential of adipose-derived PC would compare to that of muscle satellite cells, which are the normal progenitors for muscle maintenance and repair in the human body. For this comparison, we used cells obtained from the skeletal muscle of 20-week fetuses, as this tissue, compared to adult skeletal muscle, is a very rich source of satellite cells. To obtain muscle satellite cells, we sorted the CD56+ cell population from fetal muscle (Figure 4.3a). Freshly sorted cells spontaneously formed muscle fibers in a robust manner when placed in culture (Figure 4.3b). Equal numbers of freshly isolated and sorted CD56+ cells were then injected into NOD-SCID mouse injured muscle. Analysis of the engrafted muscle (Figure 4.3c) shows a regenerative index by human muscle satellite cells that was on average comparable but not higher than that exhibited by adipose-derived PC (Figure 4.3d).



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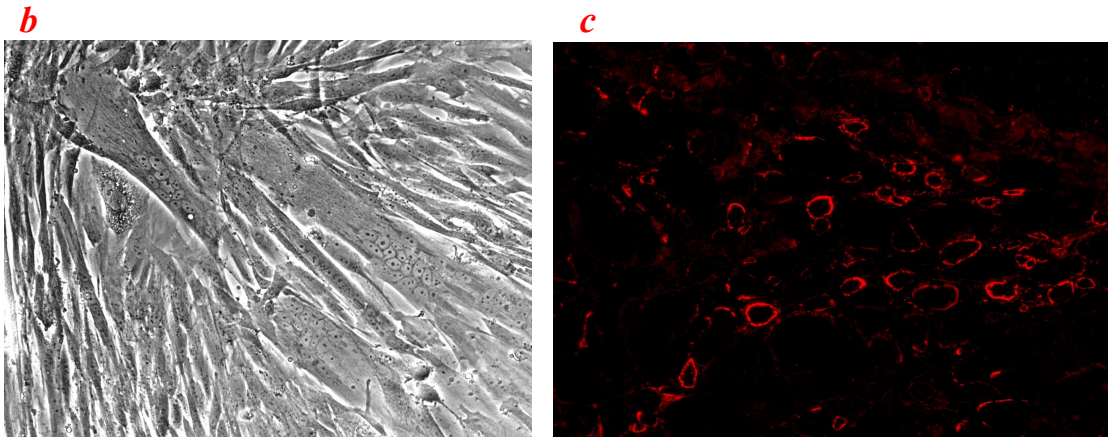


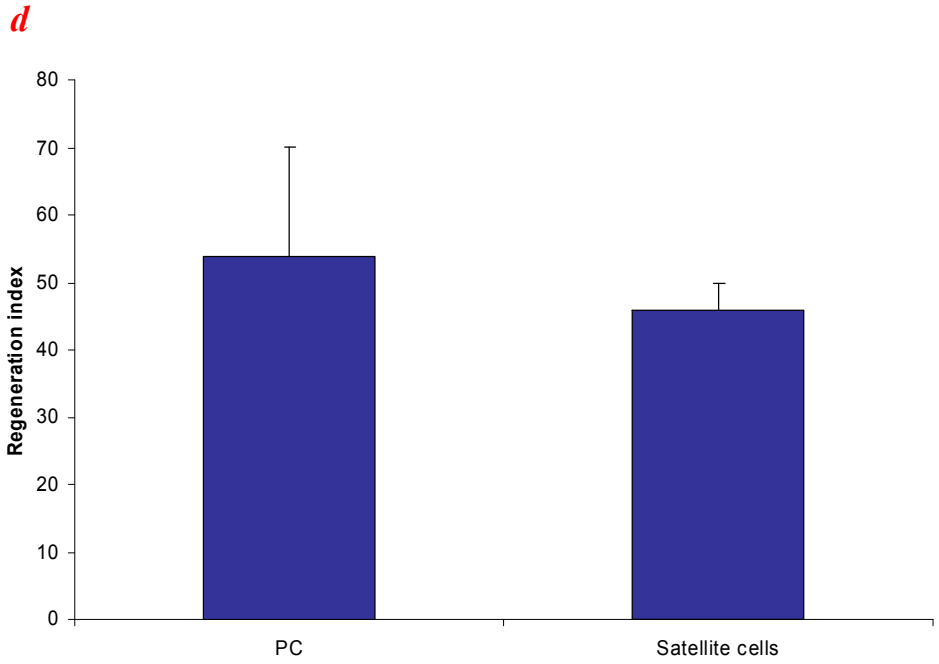


Figure 4.3 Muscle-derived satellite cells and hWAT PC display similar muscle regeneration efficiencies

(a) The total cell population from fetal skeletal muscle was analyzed for CD56 expression, and CD56+ satellite cells (encircled in red) were sorted by FACS. (b) When cultured in low serum-containing medium in vitro, satellite cells differentiated into multinucleated myotubes. (c) When the satellite cells were implanted into NOD-SCID mouse injured muscles, they generated myofibers which stained for human-specific spectrin (red). (d) A comparison of muscles engrafted with hWAT pericytes or muscle satellite cells shows no significant difference in muscle regeneration between the 2 donor cells. The results are the mean +/- s.e.m. of the no. of spectrin-positive myofibers. PC and satellite cells, n=5 and n=2, respectively; p = 0.78.

4.2.4 Myotube formation in vitro from hWAT-derived cells

To test the myogenic potential of hWAT-derived cells in vitro, we cultured the different cell populations in a fusion medium conditioned with human myofibers. Under these conditions, PC occasionally formed multinucleated myotubes (Figure 4.4a), but at a frequency that was significantly less than when human satellite cells were cultured in a non-conditioned fusion/low serum medium. No multinucleated myotubes were observed from the EC, S-EC nor the NVC fractions.

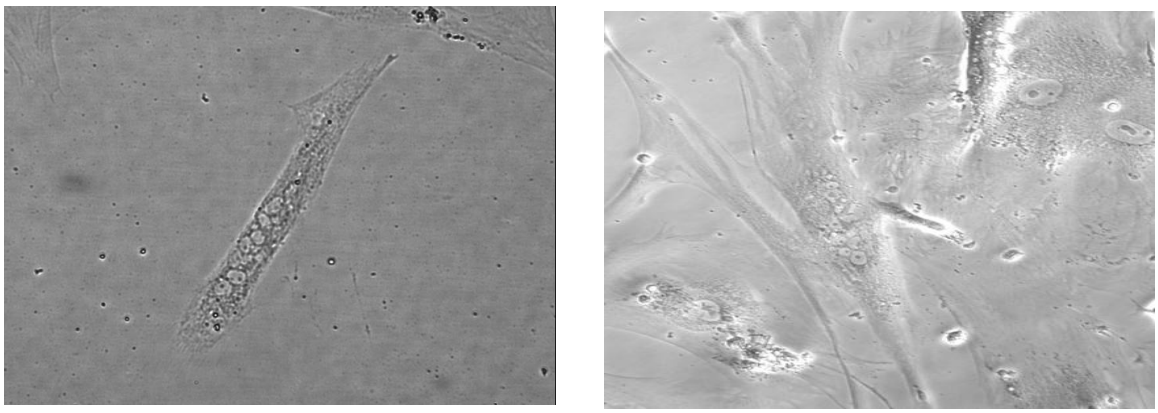
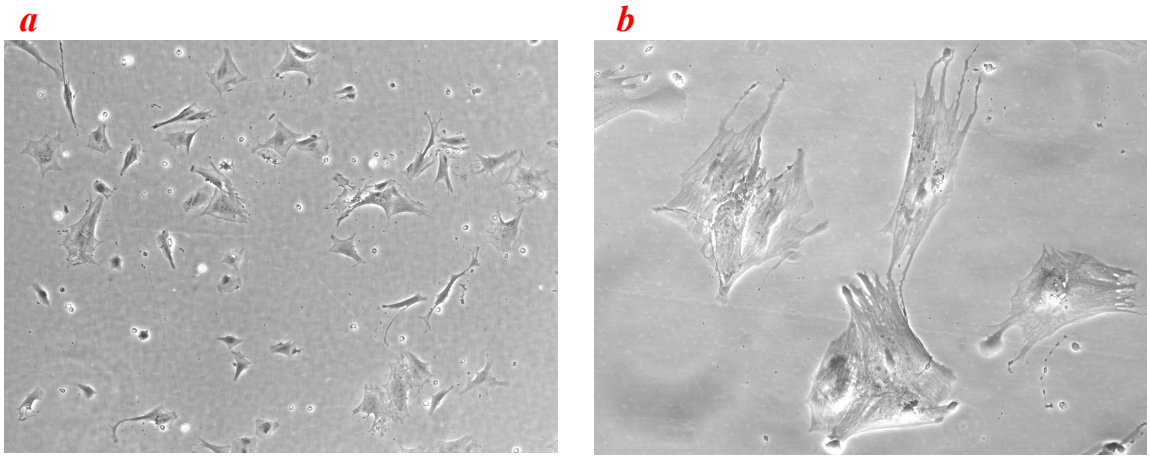


Figure 4.4 hWAT PC cultured in muscle differentiation medium
When hWAT pericytes were cultured in a low serum-containing medium from human myofibers, PC differentiated into multinucleated myotubes.

4.2.5 Long-term culture of hWAT PC

As the vigorous myogenic capacity of PC was thus far observed from cells freshly isolated from adipose tissue, we next asked whether purified PC can be cultured on the long term and still sustain myogenic potential. Sorted PC seeded in culture with DMEM

+ 20% FBS adhered and proliferated, and cultures could be maintained for at least 5 months by repeated plating at 70% confluence and replating at densities between 2.0 to 3.0 x $10^3/cm^2$. Figure 4.5a shows a phase contrast picture of a typical primary culture of PC during their first week in vitro, with most cells at their initial stage of attachment and growth. Figure 4.5b shows a similar magnification of this population after more than 10 weeks in culture, showing a significant increase in cell size but similar morphology. Immunostaining of long-term cultured PC in situ revealed that they express CD146, NG2 and αSMA (Figure 4.5c), confirming their pericyte origin. RT-PCR of cultured PC also revealed expression of CD146 and NG2 (Figure 4.5d).



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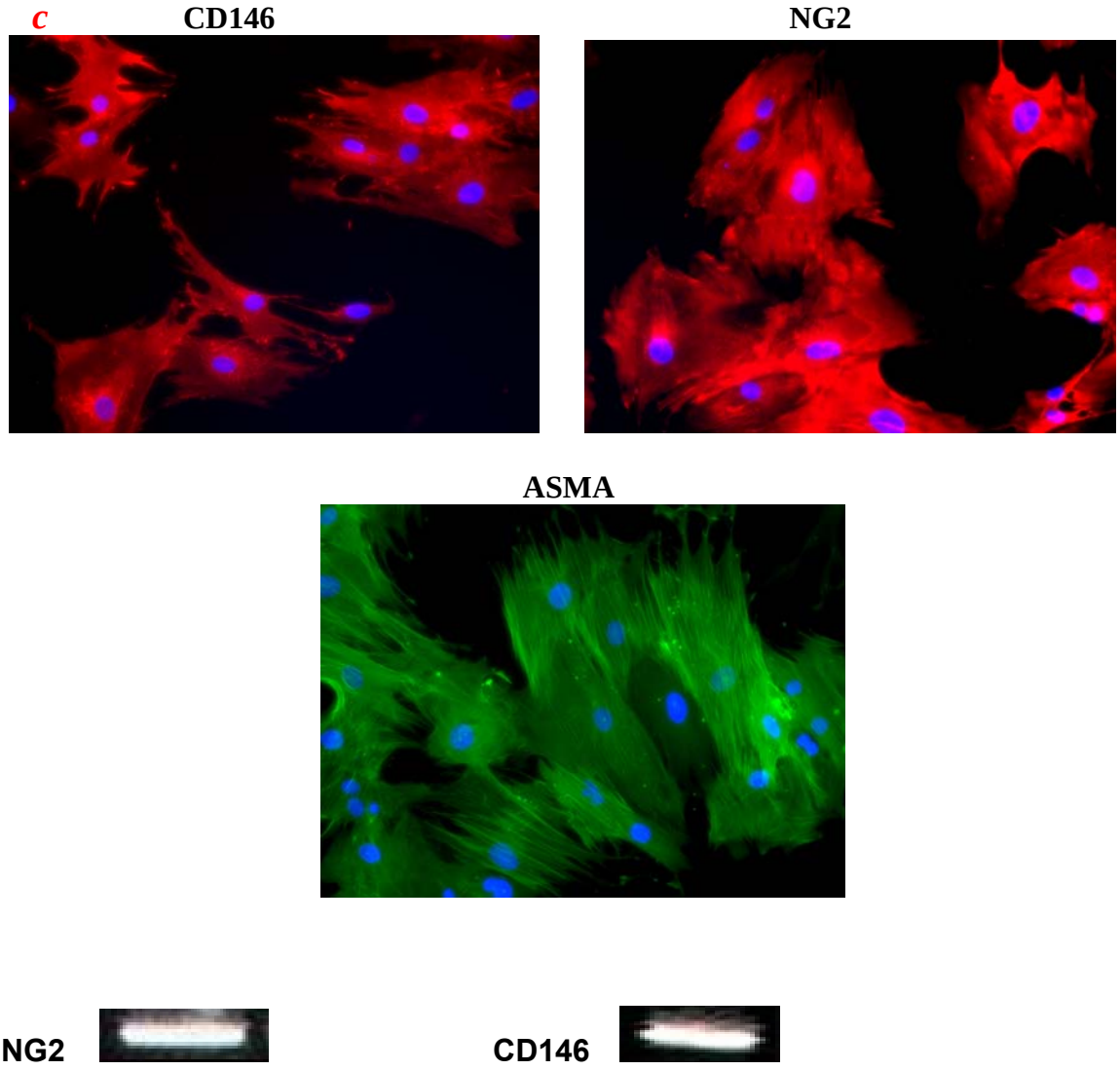
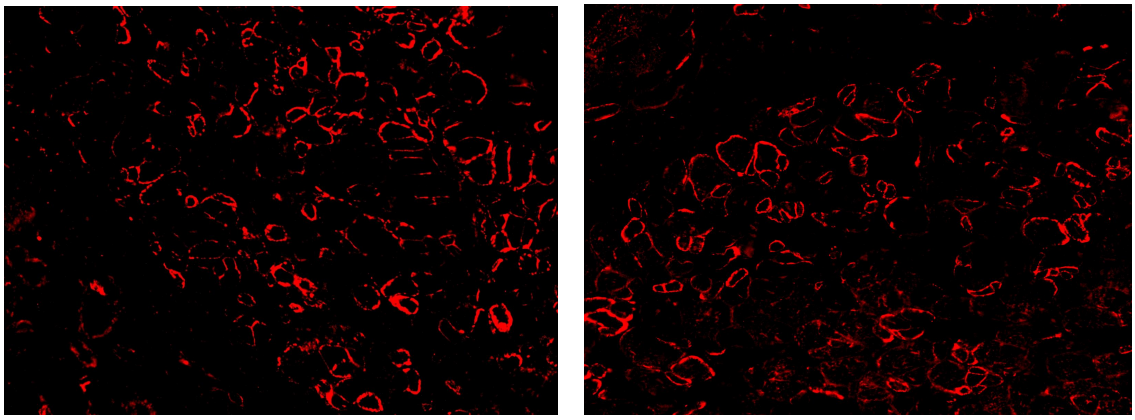


Figure 4.5 Long-term cultured PC retain their phenotype
(a) Pericytes during the first week of culture, showing cells which have attached as broad and rectangular, with sharp edges and branching processes. After culture on the long term, the cells increased in size but retained their distinct morphology (b). Long-term cultured PC also retain CD146 and NG2 expression, as tested by immunostaining and RT-PCR, and expression of ASMA as tested by immunostaining.

4.2.6 Myogenesis from long-term cultured hWAT PC

To determine the myogenic potential of long-term cultured PC, we transplanted them into NOD-SCID mouse muscle under the same conditions as freshly sorted cells. Long-term cultured PC remained capable of regenerating human muscle fibers in NOD-SCID mice (Figure 4.6a), and there was no decrease in their in vivo myogenic potential compared to freshly sorted cells (Figure 4.6b).

a



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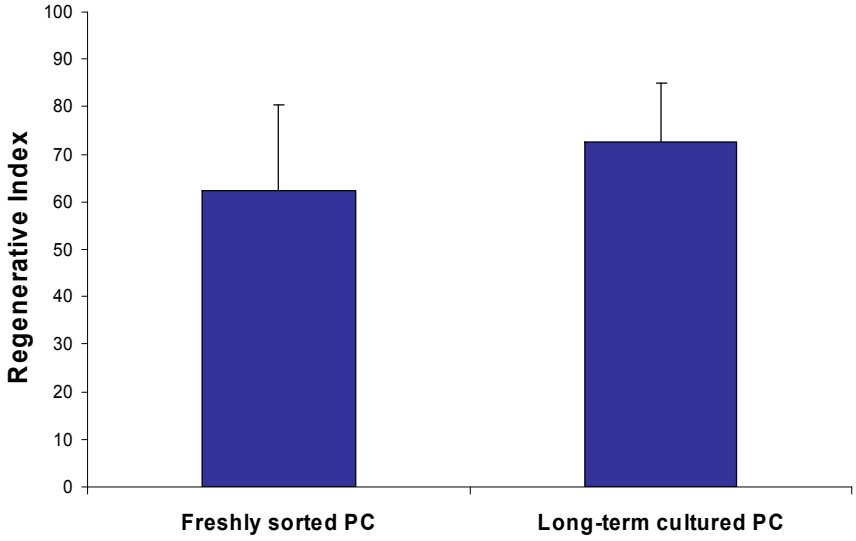


Figure 4.6 Long-term cultured pericytes retain myogenic potential
(a) hWAT pericytes, which were cultured for up to 16 weeks, generated human-spectrin positive myofibers (red) when injected into NOD-SCID mouse muscle. Shown are muscle sections engrafted with pericytes from 2 different patients. (b) There was no significant difference between the muscle regeneration capacity of freshly sorted PC and cultured PC. The results are mean +/- s.e.m. of the no. of spectrin-positive myofibers. Freshly sorted and long-term cultured PC, n=4 and n=2, respectively; p = 0.74.

4.3 DISCUSSION

We herein identified myogenic progenitors within the WAT stromal vascular fraction. Our study is unique in that it starts with the a priori identification of cells, followed by separation by FACS sorting into distinct groups, before myogenic potential is assayed.

We found that the myogenic potential of the human WAT stromal fraction is mainly associated with cells of the vasculature, notably pericytes and endothelial cells. In contrast, very little myofiber formation was obtained from the non-vascular cell

populations. Our observation that endothelial cells were capable of generating a few myofibers may not be surprising at all, since CD34 is one of the most well established stem cell markers. Pericytes exhibited a distinct advantage in the generation of muscle fibers compared to endothelial cells. Incidentally, this ability to form muscle was also observed from purified pericytes derived from non-adipose tissues, such as the pancreas and muscle [45]. We have also shown that transplantation of a pure population of pericytes instead of a heterogenous mixture of cells provided a higher muscle regeneration index. This indicates that cell-cell interaction between pericytes and the other SV cell populations was not a critical factor in enhancing its muscle differentiation.

Having established the vigorous muscle regeneration capacity of pericytes among adipose-derived cells, we aimed to determine how efficient this regenerative ability is when compared to a "gold standard", the muscle satellite cell. Surprisingly, the muscle regenerative capacity of satellite cells was comparable to that of WAT-derived PC when engrafted under similar conditions. The identification of a highly myogenic population within adipose tissue raises the concern of contamination by muscle tissue. However, the absence of CD56 and Pax7 expression by sorted PC, as confirmed by RT-PCR, excludes the presence of muscle satellite cells. We do not believe either in the infiltration of adipose tissue by other myogenic progenitors from peripheral blood having a phenotype similar to that of fat tissue pericytes. If present within peripheral blood, the number of these circulating myogenic progenitors would be extremely low, and unlikely to give the relatively high levels of muscle differentiation observed in this study.

In culture, we noted significantly less myotube formation from PC than from muscle satellite cells. This may be explained by the fact that satellite cells, being the

normal progenitors of muscle tissue, are already committed to the myogenic cell lineage. In vitro, simply decreasing the serum concentration of the medium was sufficient to allow proliferating satellite cells to exit the cell cycle and fuse to form multinucleated myofibers. In contrast, special culture conditions may be required for the efficient specification of adipose-derived PC into the myogenic cell lineage. The regenerating muscle of NOD-SCID mice after CTX injury, as shown in this study, provides an ideal environment whereby signals from the surrounding tissue promote the myogenic differentiation of donor pericytes to repair the host muscle. Under these conditions, pericytes are therefore able to generate a high number of muscle fibers, in a manner that was comparable to muscle satellite cells.

In summary, we have shown a superior muscle regenerative ability by pericytes among the different adipose-tissue derived stromal cells. Pericytes account for a relatively large proportion of the human adipose tissue stromal cell fraction. In addition, PC have the ability to extensively self-renew in vitro, while retaining their myogenic potency when stimulated under appropriate conditions. For these reasons, adipose-derived PC might be interesting candidates as autologous cells for the treatment of muscle disorders.

4.4 METHODS

4.4.1 Myofiber regeneration in vivo

The Institutional Animal Care and Use Committee, Children's Hospital of Pittsburgh, has approved the use of animals and surgical procedures performed in this study (Protocol no. 15-04).

A total of 1 x 10^4 freshly sorted PC, EC, S-EC, NVC and total adipose SVF from 5 independent adipose tissue samples, or satellite cells from a 22-week fetal skeletal muscle, were injected into the gastrocnemius muscle of 6 to 8-week old female NOD-SCID mice which had been injured 1 hr earlier by intramuscular injection of 15 µl of 50 µM cardiotoxin. For the myogenic differentiation of long-term cultured PC, cells were cultured as described previously (Section 2.3.5), for at least 10 weeks before being transplanted into NOD-SCID mouse muscles. Eighteen to 20 days after transplantation, the gastrocnemius muscles were harvested, snap frozen in liquid nitrogen-cooled 2-methylbutane, and serially sectioned (9 µm). Spectrin staining was performed on goat serum-blocked sections using a human specific anti-β-spectrin antibody (1:100; Novocastra, Newcastle upon Tyne, UK) to detect human cell derived myofibers. Sections were then washed in PBS and incubated with a biotinylated anti-mouse IgG antibody, followed by washing and incubation with Cy3-streptavidin (Sigma). Double-stained sections were labeled according to the aforementioned protocol, followed by mouse anti-Pax7 (Developmental Studies Hybridoma Bank, Iowa 1:100), then CD146-Alexa 488 (Chemicon, 1:200). The regeneration index represents the mean +/- s.e.m. of

the no. of human spectrin-positive myofibers after cell transplantation. n= 5, 5, 3, 3, and 2 for PC, EC, S-EC, NVC, and total unsorted cells, respectively.

4.4.2 Isolation and sorting of muscle satellite cells

Muscle biopsies from 20-22 week old fetuses were finely minced, then digested for 60 minutes at 37 ^{0}C with collagenase II (1 mg/ml, Sigma). The digested tissue was pelleted and resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) and poassed through a 70-um cell filter (BD Falcon). For FACS analysis, cells were incubated with an uncoupled anti-CD56 antibody (BD, 1:100), followed by goat anti-mouse-PE (DAKO, 1:1000). Cell sorting was performed on a FACSAria dual-laser fluorescence cell sorter (Becton-Dickinson). Sorted cells were re-analyzed in all experiments.

4.4.3 Culture of sorted cells and their myogenic differentiation in vitro

Sorted muscle satellite cells were seeded at an initial density of 2 x 10^4 cells per cm in proliferation medium (DMEM 10% FBS, 10% horse serum, 1% penicillin/streptomycin, 1% chick embryo extract; GIBCO-BRL). At 70% confluence, cells were detached with trypsin/EDTA, replated after washing at densities between 2.0 and 3.0 x 10^3 cells/cm^2. The ability of satellite cells to differentiate into myotubes was done by switching the medium to a fusion medium (DMEM 2% FBS, 1% penicillin/streptomycin).

4.4.4 Immunostaining of long-term cultured pericytes

Cultured cells were fixed with cold methanol/acetone for 10 min, washed in PBS, and incubated for 1 hour in PBS 5% goat serum. Cells were then stained with the following antibodies: uncoupled CD146 (BD Pharmingen, 1:100), uncoupled NG2 (BD Pharmingen, 1:300) and αSMA-FITC (Chemicon, 1:100). Uncoupled antibodies were reacted with biotinylated goat anti-mouse antibody (DAKO, 1:1000), followed by streptavidin Cy3 (Sigma, 1:1000).

5.0 CELL THERAPY FOR MUSCLE DISORDERS: ADIPOSE-DERIVED CELLS VERSUS MUSCLE-DERIVED CELLS

5.1 INTRODUCTION

Cell transplantation has been investigated as a way to regenerate skeletal muscle and improve the diseased phenotype of muscle disorders such as Duchenne muscular dystrophy. Researchers have investigated muscle repair from various cell types. The use of cells derived from post-natal human skeletal muscle has been the preferred method of autologous cell-mediated therapy for muscle-related diseases. This is due to the existence of native progenitors, known as satellite cells, located between the plasma membrane and basal lamina of muscle fibers, which are believed to be the normal cellular components for skeletal muscle maintenance and repair in the body. Muscle tissue is usually able to repair itself efficiently. However, the speed at which myogenic cells accumulate at sites of muscle damage seems too high for the small number of resident satellite cells to account for the muscle regeneration process after a massive injury. This suggests recruitment to myogenesis of resident or circulating non-satellite cells.

Indeed, our group has recently identified a population of human muscle cells which display a superior ability for engraftment when implanted in skeletal muscle. This novel population of cells, termed myogenic-endothelial cells, are located in the interstitial spaces between the myofibers and coexpress myogenic and endothelial cell markers [38]. When transplanted into damaged skeletal muscles of NOD-SCID mice, these cells generated a significantly larger number of human myofibers in comparison with other cell populations, most notably the resident satellite/ sublaminar cells of human muscle.

In the same context, undifferentiated progenitors from other tissue sources may have the capacity for myogenesis. Recent studies have indicated that progenitor cells isolated from bone marrow, the embryonic vasculature, the neuronal compartment and various mesodermal tissues including adipose tissue can also differentiate into the myogenic cell lineage and could contribute to muscle regeneration [1, 8, 24, 40, 49]. However, the efficiency for muscle engraftment and regeneration of these various non-muscle derived cells relative to native muscle cells is unknown.

Although our characterization of hWAT-derived pericytes indicates that they possess an enhanced capacity for skeletal muscle regeneration, their potential to generate muscle in comparison to human muscle-derived myo-endothelial cells has yet to be investigated. Our primary objective was to determine the respective abilities of these 2 cell populations for muscle engraftment and repair in the injured skeletal muscle of NOD-SCID mice.

5.2 RESULTS

5.2.1 Identification of myogenic-endothelial cells in human muscle

Much knowledge exists on muscle satellite cells and muscle-derived stem cells in the mouse. However, prior to this study, the identification and characterization of myogenic progenitors in human muscle were non-existing. In order to identify such human cells, and with an awareness of a possible relationship of vessel-associated cells to muscle regeneration, we stained adult human muscle sections with antibodies directed against myogenic and endothelial cell markers, and with an endothelium-specific lectin. Cells

which express Pax7, typical of satellite cells, were seen (Figure 5.1a, arrowhead). These cells were located underneath the muscle basement membrane and were, therefore, genuine satellite cells (Figure 5.1a, arrowhead, note relationship of Pax7+ cell to adjacent Dapi-marked nucleus). However, we unexpectedly also observed cells which coexpressed Pax7 and the endothelial cell antigen CD144 (ve-cadherin) (Figure 5.1a, arrow). This previously unidentified population was named myogenic-endothelial cells. Although the staining seems to indicate that myo-endothelial cells are within the interstitial spaces of the muscle (Figure 5.1a, arrow, note relationship of Pax7+ CD144+ cell to adjacent Dapi-marked nucleus), and therefore, non-satellite cells, we wanted a more definite proof that would indicate that the cells observed were not merely a subpopulation of sublaminar/satellite cells. In order to more accurately visualize the anatomical location of these cells within muscle, we next analyzed double-stained skeletal muscle sections by confocal microscopy. Confocal analysis of human muscle shows the presence of myogenic/CD56 positive cells (which correspond to the Pax7+ cells) that coexpress the receptor of UEA-1, an endothelium-specific lectin (Figure 5.1b). These cells were observed to be situated in interstitial spaces between muscle fibers. The relationship of these cells with the UEA-1 positive structures further suggests that these myo-endothelial cells are located within the blood vessel wall.

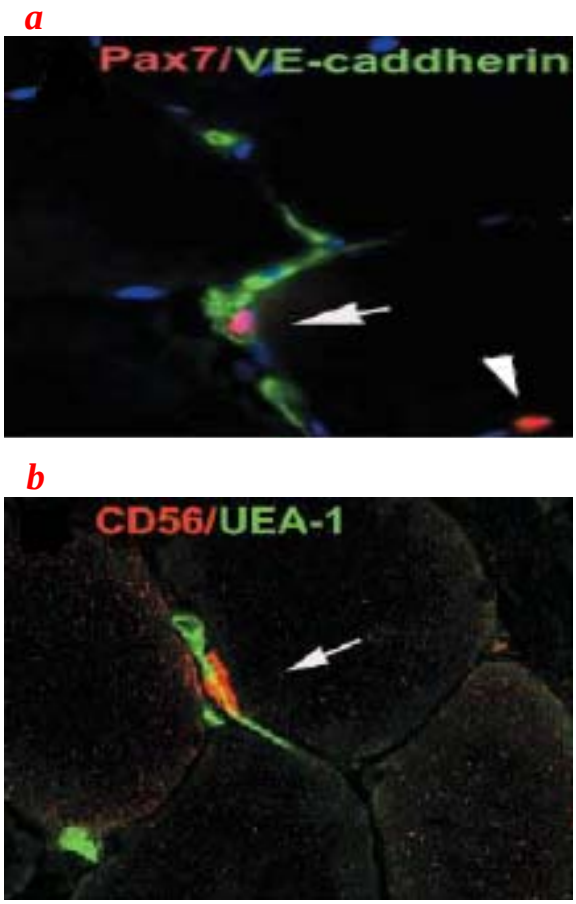


Figure 5.1 Co-localization of myogenic and endothelial cell antigens in adult human muscle
(a) Adult human muscle sections were double-labeled for Pax7 (red) and ve-cadherin (green); myo-endothelial cells (pink), as well as regular satellite cells (red), were detected. A confocal microscope was used to observe sections double-labeled for CD56 (red) and UEA-1 receptor (green), to determine the anatomical location of myo-endothelial cells in human muscle.

5.2.2 Isolation of myo-endothelial cells from human muscle by FACS sorting

Based on these findings, we further investigated and tried to confirm the presence of these myo-endothelial cells by FACS analysis of the total population derived from human skeletal muscle. Indeed, a subset of CD56+ cells from muscle coexpress the endothelial cell markers CD34 and CD144 (Figure 5.2). This prompted us to sort this subset of myo-endothelial cells and characterize their myogenic properties.

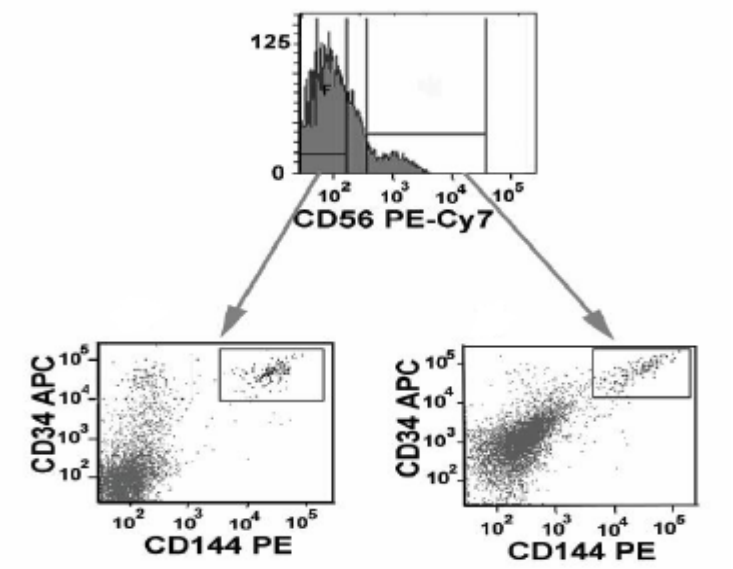


Figure 5.2 Flow cytometry analysis and sorting of myogenic endothelial cells
After excluding CD45+ cells from the whole muscle cell suspension (data not shown), cells were further separated into CD56- and CD56+ cells. The CD56+ cell subset contained CD34+CD144+ cells, representing myo-endothelial cells, which were then sorted.

5.2.3 Myo-endothelial cells regenerate muscle significantly better than other muscle-derived cells.

We explored the myogenic potential of sorted myo-endothelial cells in comparison with that of other muscle-derived cell populations. Thus, we also isolated and purified by FACS from human muscle a population of satellite cells (CD56+CD34-CD144+CD45-), believed to be the normal progenitors for muscle repair and regeneration, as well as endothelial cells (CD34+CD144+CD56-CD45-) within the capillaries of the muscle. The CD56- fraction, consisting mostly of vascular cells, comprise the greater part- more than 95%- of the cellular component of skeletal muscle. The sorted cell subsets were injected into SCID mouse skeletal muscles that had been injured by cardiotoxin. All three cell populations were able to regenerate human spectrin-expressing muscle fibers. However,

the myogenic potential of muscle-derived myo-endothelial cells is, quantitatively, much higher than that of endothelial cells, which are themselves more potent than muscle satellite cells (Figure 5.3).

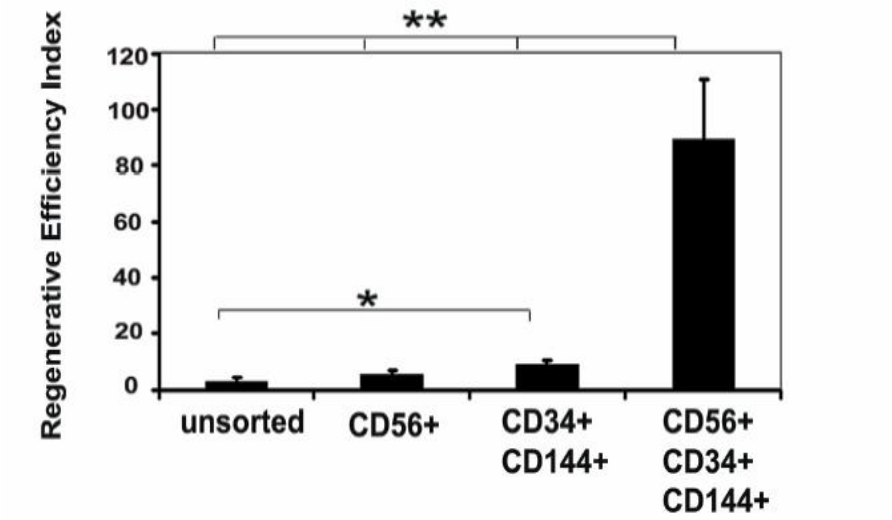


Figure 5.3 Muscle regeneration index of 3 muscle-derived cell populations
CD56+CD34+CD144+CD45- myo-endothelial cells generated the highest number of human spectrin-expressing cells among the three cell subsets tested.

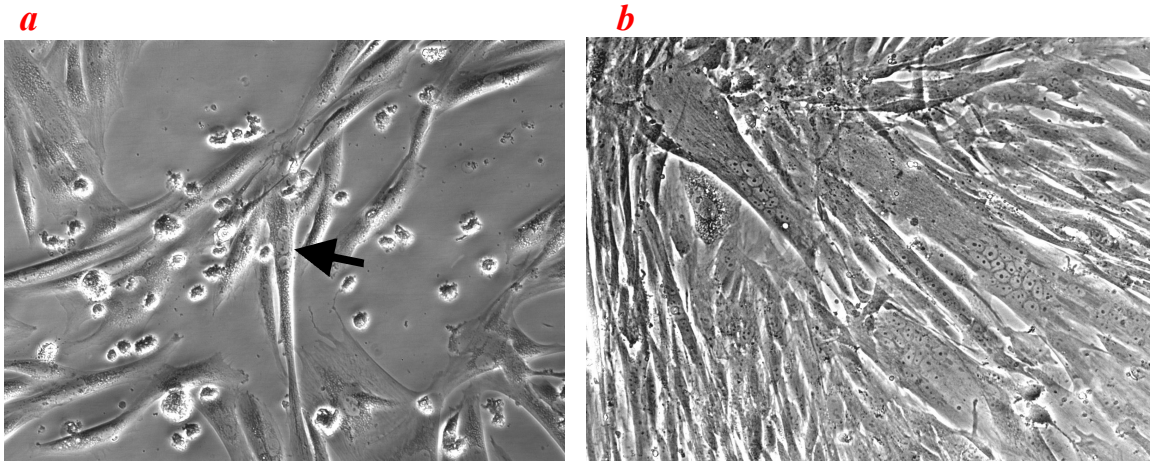
5.2.4 Frequency and yield of cells within the native tissue

We have thus far identified a population of cells with a high myogenic capacity from both muscle and fat, the myo-endothelial cells and hWAT PC, respectively. We next wanted to determine how much of these myogenic progenitors may be recovered from a given amount of tissue. Based on our isolation of cells from adult human muscles, it was observed that on average, about 6 x 10^4 cells are contained in one gram of tissue. FACS analysis further revealed that myo-endothelial cells account for about 0.37% of the total muscle cell population. On the other hand, an average of at least 1 x 10^6 stromal cells are contained in one gram of human adipose tissue, of which 14.6% are pericytes. Based on

this data, we gather that one gram of human muscle yields about 2.2 x 10^2 myo-endothelial cells while one gram of fat yields about 1.5 x 10^5 PC. Thus, there is on average 57-fold more of the potent myogenic cells within the adipose tissue compared to the skeletal muscle.

5.2.5 Myo-endothelial cells formed myosin-positive myotubes in culture

To test the myogenic potential of myo-endothelial cells in vitro, we cultured the cells in low serum medium. Under these conditions, the cells formed myotubes (Figure 5.4a), but they were smaller in caliber and contained fewer nuclei compared to the myotubes obtained from fetal muscle cells (Figure 5.4b). When stained for myosin fast and slow heavy chains, it was observed that unlike hWAT-derived pericytes, myo-endothelial cells are able to form myotubes which express myosin (Figure 5.4c).



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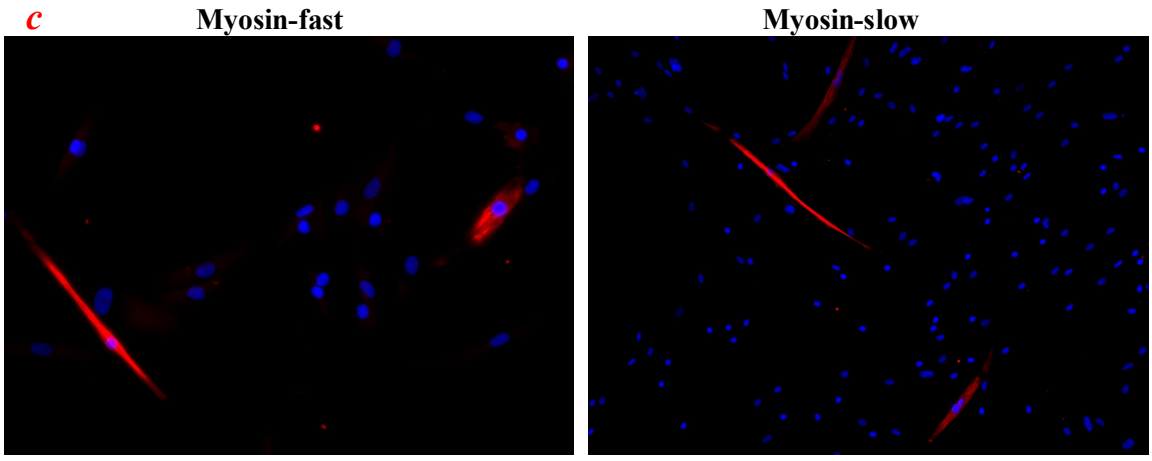
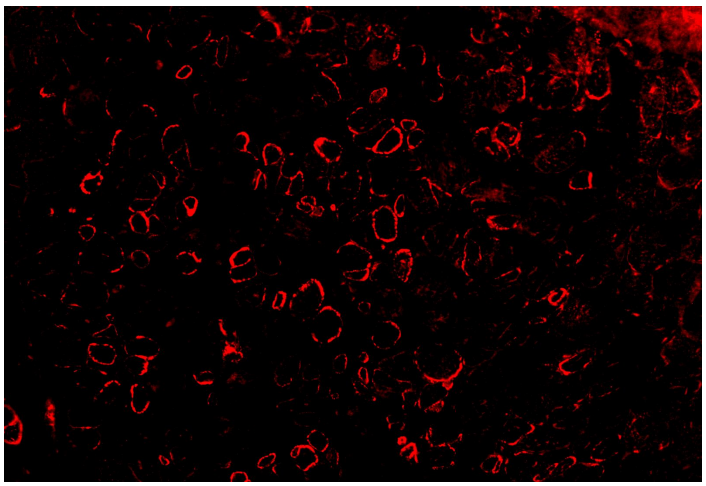


Figure 5.4 Myogenic differentiation of myo-endothelial cells in vitro
(a) When cultured in low-serum containing medium, myo-endothelial cells formed myotubes. (b) A representative image of myotubes formed under the same conditions, from fetal muscle-derived satellite cells. (c) Myo-endothelial cells differentiated into myotubes which were positive for both fast and slow myosin.

5.2.6 Myo-endothelial cells and hWAT-derived pericytes exhibit the same level of muscle engraftment and regeneration

To determine the differential abilities of myo-endothelial cells and hWAT PC for muscle repair in vivo, we injected long-term cultured myo-endothelial cells into injured NOD-SCID muscles under the same conditions as LTC hWAT PC. Myo-endothelial cells generated a large number of human myofibers (Figure 5.5a). However, when donor-derived myofibers were counted and compared to those developed in muscle which received hWAT PC, no significant difference in regeneration indexes was noted between the two cell populations (Figure 5.5b).

a



b

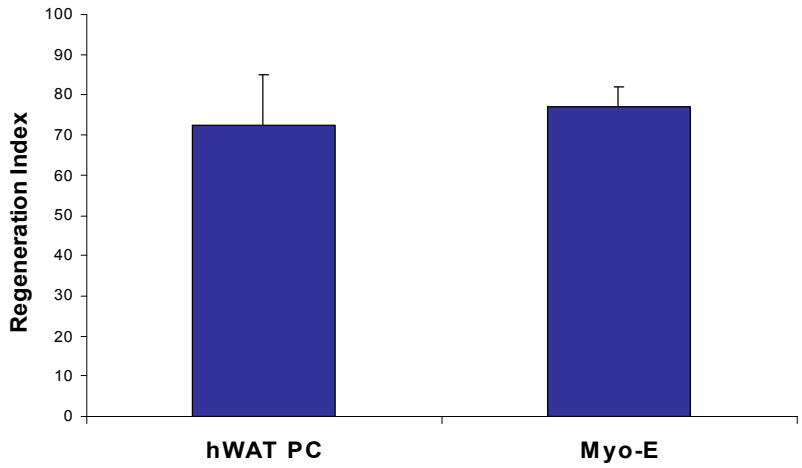


Figure 5.5 Myogenic potential of hWAT PC vs. myo-endothelial cells
(a) Myo-endothelial cells cultured on the long term generated myofibers, assessed by human-specific spectrin staining, after cells were injected into NOD-SCID mouse muscle. (b) There was no significant difference in the number of myofibers formed between muscles engrafted with hWAT PC and myo-endothelial cells. The results are the mean +/- s.e.m. of the no. of spectrin-positive myofibers per 1 x 10^4 cells injected. n=2 for both cell populations; p = 0.77.

5.2.7 Transplanted cells are able to replenish the satellite cell pool

We wanted to further determine whether some of the transplanted myo-endothelial cells did not develop into muscle fibers but were retained as myogenic/Pax7+ cells in the host muscle, which may contribute to muscle repair in future injuries. For this purpose, we double stained the engrafted muscle with antibodies to human-specfic spectrin and Pax7, and observed that a few human myofibers contained Pax7+ cells (Figure 5.6a, arrow). Likewise, similar staining was done on mouse muscles engrafted with hWAT PC. We also observed the presence of Pax7+ cells within a few of the human myofibers (Figure 5.6b, arrow), suggesting that some of the transplanted pericytes may have developed into satellite cells, which may contribute muscle repair in the event of a future injury.

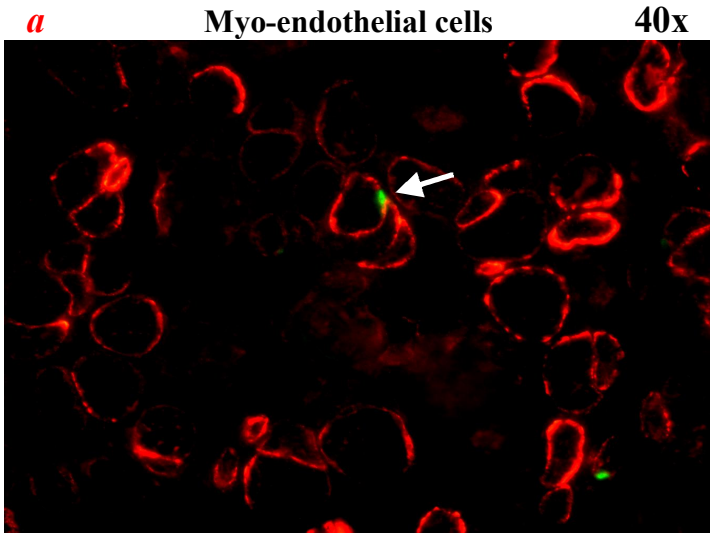
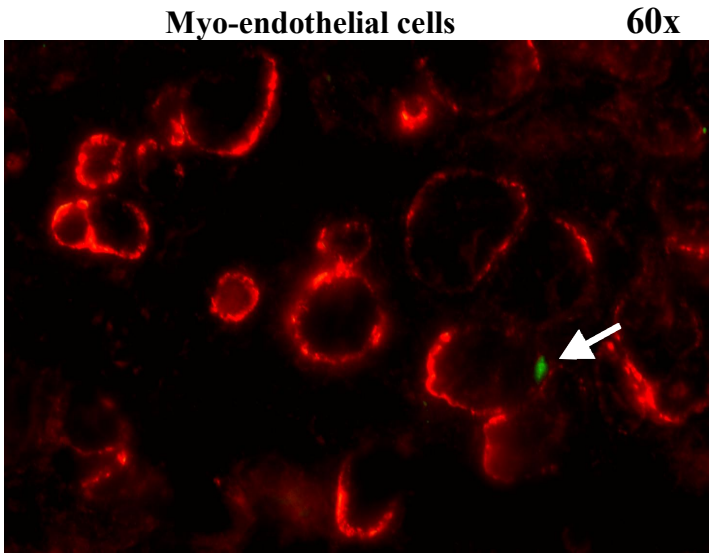


Figure 5.6 continued on the next page.



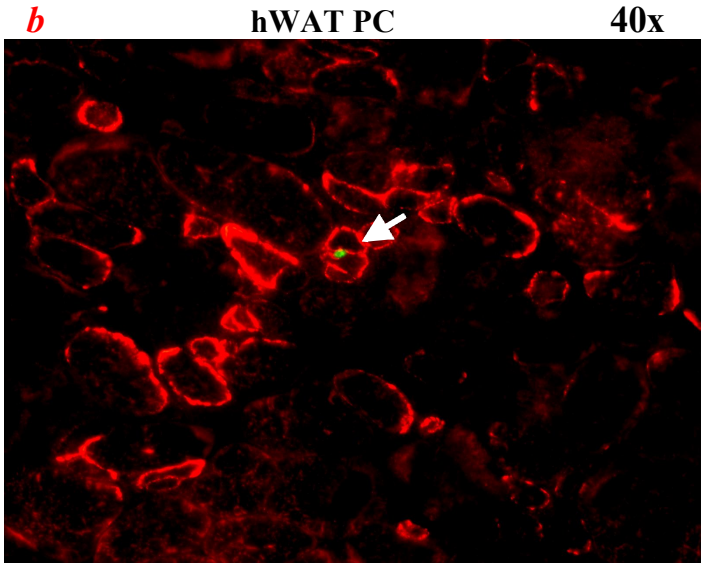


Figure 5.6 continued on the next page.

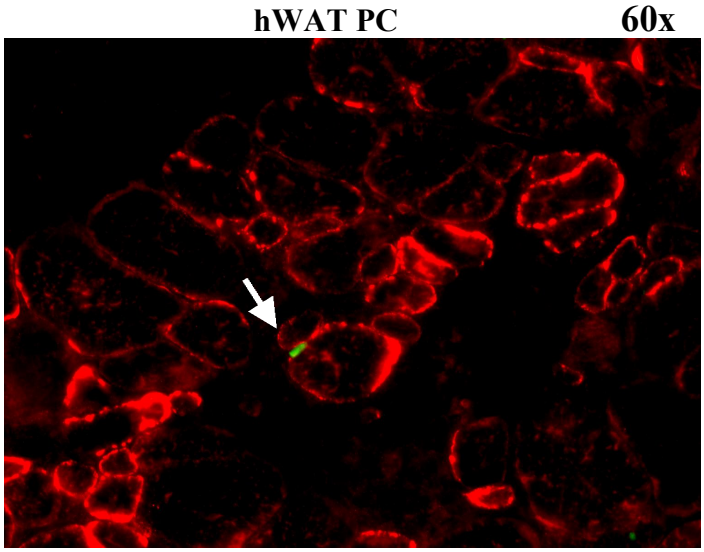


Figure 5.6 Co-staining of engrafted muscle for Pax7 and human spectrin
Human myofibers, assessed by human-specific spectrin staining (red), in NOD-SCID mouse muscles which received either myo-endothelial cells (a) or hWAT PC (b), contained sublaminar Pax7+ cells (green).

5.3 DISCUSSION

The superior ability of hWAT pericytes to generate muscle fibers in vivo was further confirmed in this experiment, where the cells were compared in the same setting with myo-endothelial cells, believed to be the human counterpart of mouse muscle-derived stem cells (MDSC) [39]. Previous studies have shown that cells from non-muscle sources such as the mesenchymal stem cells of the bone marrow are able to differentiate into muscle fibers when transplanted into degenerated muscles [1, 8, 24, 40]. In addition, it was demonstrated that some of the transplanted cells may develop into Pax7-positive cells in the host muscle [1]. These Pax7- positive cells were shown to contribute to subsequent regeneration of the muscle upon repetitive damage without additional cell transplantation [1].

Here, we also show that in addition to repair of damaged muscle, some hWAT pericytes may have developed into Pax7-positive cells in the engrafted muscle; likewise, some of the transplanted myo-endothelial cells may have been retained as Pax7- positive cells in the host muscle. We consider these satellite cells as donor-derived based on their location within human myofibers. However, co-labeling of the observed satellite cells within spectrin-positive myofibers with a human-specific marker or probe is needed to fully ascertain their origin. In this regard, further studies are also warranted to determine whether the spectrin-positive myofibers are a product of fusion of donor and host cells.

We also show that there exists a much larger reserve of highly competent myogenic progenitors within human adipose tissue, compared to skeletal muscle. For these reasons, the human adipose tissue appears to be a viable alternative tissue source to skeletal muscle to obtain autologous cells with a superior capacity for muscle repair in the treatment of muscle-related disorders.

5.4 METHODS

5.4.1 Human muscle samples

Muscle biopsies (mean age 27 yrs) were obtained during surgery or from the National Disease Research Interchange (NDRI). The described procedure was approved by the Institutional Review Board and the research protocol was reviewed and approved by the Animal Research and Care Committee at the Children's Hospital of Pittsburgh and

University of Pittsburgh. Samples were placed on ice in Hanks' balanced salt solution (HBSS) (Gibco) and transferred to the laboratory.

5.4.2 Immunohistochemistry on tissue sections

Samples were frozen in 2-methylbutane precooled in liquid nitrogen, then stored at -80^{0}C. 10-μm cryosections were fixed in a 1:1 cold (-20^{0}C) acetone/methanol mixture for 5 min and preincubated in 10% horse serum in PBS for 1 hr at room temperature. Primary antibodies and their dilutions in PBS were mouse anti-Pax7 (Developmental Studies Hybridoma Bank, Iowa, USA; 1:100), mouse anti-CD56 (BD Biosciences, 1:100) and rabbit anti-VE-cadherin (Sigma, 1:200). The primary antibody was incubated overnight at 4^{0}C, followed by PBS washes and incubation with its secondary antibody: biotinylated goat anti-mouse (Vector Laboratories, 1:250), followed by Cy3-conjugated streptavidin (Sigma, 1:500). Biotinylated UEA-1 (*Ulex europaeus* agglutinin-1) (Vector, 1:200), followed by Cy3-conjugated streptavidin (Sigma, 1:500), was also used.

5.4.3 Flow cytometry analysis

Muscle biopsies were finely minced, then digested for 60 mins at 37^{0}C with type-I and type-IV collagenases (100 μg/ml) and dispase (1.2 μg/ml; all from Gibco, Invitrogen Corporation). The digested tissue was pelleted and resuspended in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, triturated and then passed through a 40-μm filter to obtain a single cell suspension. Cells were then incubated with APC-Cy7-conjugated mouse anti-human CD45, APC-conjugated mouse anti-human CD34, PE-Cy7-conjugated mouse anti-human CD56 (all from BD Biosciences), and PE-conjugated

mouse anti-human CD144 (Beckman Coulter). Background staining was evaluated with isotype-matched control antibodies and a CompBeads Set (Bection-Dickinson) was used to optimize fluorescence compensation settings for multi-color analyses and sorts. Cell sorting was performed on a FACSAria dual-laser fluorescence cell sorter (Becton-Dickinson).

5.4.4 Long-term culture of sorted myo-endothelial cells

Sorted cells were plated in collagen-coated 96-well plates, at a density of 500 cells per well, in proliferation medium. At 70% confluency, cells were detached with trypsin/EDTA, replated after washing at densities between 1.0 and 2.5 x $10^3/cm^2$, and further cultured for 10 weeks before myogenic differentiation and cell transplantation experiments were performed.

5.4.5 Cell transplantation

Freshly sorted myogenic, endothelial and myo-endothelial cells were injected into the SCID mouse gastrocnemius muscle that had been injured 1 day earlier by intramuscular injection of 1 ug cardiotoxin (CTX, Molecular Probes, Inc.) in 20 ul HBSS. Animals were sacrificed 10 days post-injection and treated muscles were fixed and frozen as described above. Spectrin staining was performed on acetone-fixed sections using a procedure described previously (Section 4.4.1). For the comparison of the muscle regenerative ability of hWAT PC and myo-endothelial cells, cells were cultured for at least 10 weeks before their transplantation into NOD-SCID mouse muscles.

5.4.6 In vitro myogenic differentiation of myo-endothelial cells

Long-term cultured myo-endothelial cells (passage 8) were plated at 2.0 x 10^4 cells per well in 12 well-plates in proliferation medium, that was then replaced by fusion medium at 80% culture confluence. After 5 days in fusion medium, cultures were fixed and incubated with 2 monoclonal antibodies directed to slow myosin heavy chain (MyHC) (Sigma, 1:100) and fast MyHC (Sigma, 1:100), followed by biotinylated goat anti-mouse IgG (DAKO, 1:500) and Cy3-conjugated streptavidin (Sigma, 1:1000).

6.0 SUMMARY AND CONCLUSIONS

In this study, we have identified and purified different populations of stromal cells within human adipose tissue. Subsets of cells were first separated based on expression of the CD34 and CD146 antigens. Each purified cell population was reproducibly distinguished based on phenotype, morphology and growth pattern in culture. More importantly, our approach enabled us to prospectively assess the differentiation abilities of each cell compartment within adipose tissue. In this regard, we have established a previously unsuspected role of pericytes as human adipocyte progenitors, as confirmed by their high expression of leptin, a hormone specifically expressed by adipocytes. Moreover, pericytes from adult fat tissue were able to differentiate into genuine brown adipocytes under appropriate conditions. The developmental potential of human adipose-derived pericytes was not restricted to the adipogenic cell lineage. When injected into injured NOD-SCID mouse muscles, pericytes formed myofibers in a manner comparable to that observed from human muscle-derived myo-endothelial cells, a novel population of robust myogenic cells recently identified in our laboratory. These results suggest that microvessels within fat tissue serve as a repository of multipotent progenitors. The association of multilineage stem cells with the vasculature would enable their efficient distribution during tissue development and repair, and would explain that other groups have retrospectively documented the presence of multipotent cells in such diverse tissues as the bone marrow, brain, pancreas and skeletal muscle.

Table 1. Oligonucleotide sequence of PCR primers.

GENE	PRIMER	
hCD34 s:	CAT CAC TGG CTA TTT CCT GAT G	410bp
hCD34 as:	AGC CGA ATG TGT AAA GGA CAG	
hCD146 1s:	AAG GCA ACC TCA GCC ATG TCG	430bp
hCD146 1as:	CTC GAC TCC ACA GTC TGG GAC	
hNG2 s:	GTC TAC GCT GGG AAT ATT CTG	474bp
hNG2 as:	CTG GCC CAC GAA AGT GGA AG	
hVWF s:	GCA CCA TTC AGC TAA GAG GAG G	260bp
hVWF as:	GCT TCC CAC CTT GAC ATA CTG C	
hDESMIN s:	GAA GTG AAC CGG CTC AAG GG	260bp
hDESMIN as:	CGA GCT AGA TGA GCT GCA TC	
hCD56 s:	GTA TTT GCC TAT CCC AGT GCC	350bp
hCD56 as:	CAT ACT TCT TCA CCC ACT GCT C	
hPAX7 s:	ACC AGG AGA CCG GGT CCA TC	230bp
hPAX7 as:	CCC GAA CTT GAT TCT GAG C	
hβ-actin s:	CCT CGC CTT TGC CGA TCC	206bp
hβ-actin as:	GGA ATC CTT CTG ACC CAT GC	

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