Age-related changes in the features of porcine adult stem cells isolated from adipose tissue and skeletal muscle

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Perruchot MH, Lefaucheur L, Barreau C, Casteilla L, Louveau I. Age-related changes in the features of porcine adult stem cells isolated from adipose tissue and skeletal muscle. Am J Physiol Cell Physiol 305: C728–C738, 2013. First published July 17, 2013; doi:10.1152/ajpcell.00151.2013.—A better understanding of the control of body fat distribution and skeletal muscle development is of the upmost importance for both human and animal physiology. This requires a better knowledge of the features and physiology of adult stem cells in adipose tissue and skeletal muscle. Thus the objective of the current study was to determine the type and proportion of these cells in growing and adult pigs. The different cell subsets of stromal vascular cells isolated from these tissues were characterized by flow cytometry using cell surface markers (CD11b, CD14, CD31, CD34, CD45, CD56, and CD90). Adipose and muscle cells were predominantly positive for the CD34, CD56, and CD90 markers. The proportion of positive cells changed with age especially in intermuscular adipose tissue and skeletal muscle where the percentage of CD90+ cells markedly increased in adult animals. Further analysis using coimmunostaining indicates that eight populations with proportions ranging from 12 to 30% were identified in at least one tissue at 7 days of age, i.e., CD90+/CD34+, CD90+/CD34−, CD90+/CD31+, CD90+/CD56−, CD90+/CD56+, CD90−/CD34−, CD90−/CD56−, and CD90−/CD56+. Adipose tissues appeared to be a less heterogeneous tissue than skeletal muscle with two main populations (CD90+/ CD34− and CD90−/CD56−) compared with five or more in muscle during the studied period. In culture, cells from adipose tissue and muscle differentiated into mature adipocytes in adipogenic medium. In myogenic conditions, only cells from muscle could form mature myofibers. Further studies are now needed to better understand the plasticity of those cell populations throughout life.

A better understanding of the control of body fat distribution and skeletal muscle development is of the upmost importance for both human and animal physiology. In humans, the increase in the prevalence of obesity throughout the world highlights the need to prevent and/or treat this disturbance that is associated with increased risk of developing metabolic disorders (OECD obesity update 2012; http://www.oecd.org/dataoecd/1/61/49716427.pdf). These disorders have also been described to be associated with a decrease in muscle mass and a rise in intermuscular adipocytes (18). For farm animals, the lean-to-fat ratio is of special interest for production efficiency and meat quality traits (43). Several studies suggest a developmental and functional link between adipose tissue and skeletal muscle that needs to be further clarified (3, 20). Observations in primates and rodents indicate that manipulation of stem cells may be a way to control the number of differentiated cells (14). However, the mechanisms governing the number of adipose vs. muscle cells within the body or within skeletal muscle remain largely unknown whatever the species of interest. With respect to adipose cells within skeletal muscle, the few available data suggest that the origin of these cells may be different from that of nonmuscular adipocytes (13) and strengthen the need to identify the progenitors that could determine the number of adipose and muscle cells during development.

Both muscle and adipose cells are derived from mesenchymal stem cells (MSCs) also named mesenchymal stromal cells (30, 33). MSCs are considered as tissue resident stem cells that are preferentially localized in the stromal, interstitial, or perivascular areas. Since the quite recent description of adipose-derived stromal stem cells (53), there has been an explosion of research in this field. To date, one important point to consider is that available data are based mainly on the investigation of adult tissues and are dealing with human physiopathology and cell therapy (5, 15, 17, 33). With the importance of programming, it may be important to consider the period of growth that may influence later development (32) and to provide information on stem cell populations according to anatomical sites. Therefore, the present study was undertaken to enrich our knowledge on the phenotype and differentiation potential of skeletal muscle stromal vascular (SV) cells compared with adipose tissue SV cells in postnatal growing and adult animals. This study was based on the expression of a variety of well-characterized cell surface markers such as CD11b and CD14 for immune cells (36), CD31 for endothelial cells, CD34 for hematopoietic stem cells and other cells including cells from the endothelium (39), CD45 for hematopoietic cells, CD56 for muscle precursors (38, 39), and CD90 (Thy-1) for MSCs (31). In the current study, the pig was used not only for its important role in animal protein supply for human nutrition but also for its relevance as a suitable animal model for several human disorders (27, 44).

MATERIALS AND METHODS

Animals and sample collection. The care and use of pigs were performed in compliance with the European Union (Directive 86/609/CEE) and French (Décret No. 2001–464 29/05/01; http://ethique.ipbs.fr/sdv/charteexpeanimale.pdf; Agreement for Animal Housing...
No. C-35–275-32) legislations. Moreover, the scientific and technical staff involved in the experiment obtained an individual agreement from the French Veterinary Services to experiment on living animals. Female crossbred pigs (Piétrain x [Large White x Landrace]) free of the halothane-sensitivity allele (n) at the RYR1 locus were fed a standard diet and were slaughtered after an overnight fast by exsanguination after electrical stunning at 7 (D7; 3.5 ± 0.3 kg body wt, suckling piglets), 160 (D160; 110 ± 4.9 kg, commercial slaughter weight before puberty), or 400 (D400; 190 ± 17 kg, primiparous lactating sows) days of age (n = 5 per age group). Subcutaneous dorsal (SCAT) and intermuscular (interM; visible between semitendinosus and semimembranosus muscles) adipose tissues and skeletal muscles longissimus (LM; composed of ~11% type I oxidative, 7% type IIA oxydo-glycolytic, and 82% type IIB glycolytic myofibers), and rhomboideus (RM; composed of ~68% type I oxidative, 12% type IIA oxydo-glycolytic, and 20% type IIB glycolytic myofibers; Ref. 25) were rapidly excised for immediate cell isolation. Slices of adipose tissue (15 g) were placed in 37°C Krebs-Ringer bicarbonate buffer, whereas slices of muscle tissues (15 g) were placed in ice-cold Dulbecco’s phosphate-buffered saline (DPBS; Dutscher Brumath, France) containing 1% glucose (DPBS-glucose; Sigma, Saint-Quentin Fallavier, France). For histological analysis, samples of adipose tissue containing 1% glucose (DPBS-glucose; Sigma, Saint-Quentin Fallavier, France) were rapidly excised for immediate cell isolation. Slices of muscle tissues (15 g) were placed in ice-cold proliferation growth medium (PGM) containing DMEM supplemented with 10% FCS (Invitrogen, Cergy Pontoise, France), 3 μg/ml amphotericin D, and 20 μg/ml gentamycin and successively filtered through 200- and 50-μm Nylon membranes (Dutsch, Brumath, France).

Flow cytometry. Flow cytometry was performed on native cells isolated from adipose tissue and muscle. First, individual vials of cryopreserved cells were rapidly thawed in a 37°C water bath (1–2 min), suspended in 10 ml of FCS, and centrifuged at 90 g for 10 min at room temperature. Viable cells were then counted with the Vi-Cell XR analyzer using the trypan-blue exclusion test (Beckman Coulter, Paris, France). Cells with viability >90% were further analyzed for cell surface markers with human or porcine cross-reactive antibodies (Table 1). These cells (800,000 cells) were placed in PBS containing 0.5% FCS and incubated with appropriately labeled monoclonal antibodies (mAb) coupled to different fluorochromes: allophycocyanine (APC), FITC, phycoerythrin (PE), phycoerythrin-cyanine 7 (PE-Cy7), or R-phycocerythrin (RPE). All data were compared with appropriate isotype-matched negative controls. All tubes, protected from light, were incubated on ice for 30 min. After being washed in PBS, cells were fixed in 200 μl of PBS/1% paraformaldehyde. Labeled cells were then analyzed using a MACSQuant flow cytometer and software (Miltenyi Biotech, Paris, France). A minimum of 20,000 events was acquired for each sample.

Table 1. List of antibodies used for flow cytometry analysis or immunohistochemistry

<table>
<thead>
<tr>
<th>Marker</th>
<th>Isotype</th>
<th>Fluochrome (Flow Cytometry)</th>
<th>Clone</th>
<th>Supplier</th>
</tr>
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<tr>
<td>CD11b*</td>
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<td>PE-Cy7</td>
<td>ICRF44</td>
<td>Biolegend</td>
</tr>
<tr>
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<td>Mouse IgG2a</td>
<td>RPE-alexa647</td>
<td>TUK4</td>
<td>AbD Séroton</td>
</tr>
<tr>
<td>CD31†</td>
<td>Mouse IgG1</td>
<td>RPE</td>
<td>LCI-4</td>
<td>AbD Séroton</td>
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<tr>
<td>CD34</td>
<td>Mouse IgG1</td>
<td>PE</td>
<td>5E6</td>
<td>BD Biosciences</td>
</tr>
<tr>
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<td>FITC</td>
<td>K252-1E4</td>
<td>AbD Séroton</td>
</tr>
<tr>
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<td>PE-cy7</td>
<td>NCAM 16.2</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD90†</td>
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<td>APC</td>
<td>5E10</td>
<td>BD Biosciences</td>
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<tr>
<td>Laminin†</td>
<td>Rabbit</td>
<td></td>
<td></td>
<td>Sigma</td>
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<tr>
<td>MF20†</td>
<td>Mouse IgG2b</td>
<td></td>
<td></td>
<td>Sigma</td>
</tr>
</tbody>
</table>

APC, allophycocyanine; BD, Becton Dickinson; CD, cluster of differentiation; Cy7, cyanine 7; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; PE, phycoerythrin; RPE, R-phycoerythrin. *Antibodies directed against porcine markers. †Antibodies directed against human markers with porcine cross-reactivity.
mented with 10% FCS, 0.25 µg/ml amphotericin D and 20 µg/ml gentamicin) for cell culture.

Cell culture. Frozen cells isolated from adipose tissue and muscle and suspended in control medium were used for cell culture. Viable cells were first counted with the Vi-Cell XR analyzer using the trypan-blue exclusion test (Beckman Coulter). After centrifugation, some cells were maintained in the control medium (DMEM supplemented with 10% FCS, 0.25 µg/ml amphotericin D, and 20 µg/ml gentamicin) during 9 days to verify the absence of spontaneous differentiation. Other cells were placed in a medium allowing adipogenic or myogenic differentiation. For both conditions, cells were seeded at a density of 7,000 cells/cm² in six-well plates (9.6 cm²/well) coated with growth factor reduced Matrigel (1/50 vol/vol, BD Biosciences) and grown at 37°C under 95% air-5% CO₂.

Adipogenic differentiation. Adipose tissue- and muscle-derived cells including CD90⁺ and CD56⁺ selected cells were first placed in a medium (DMEM Glutamax High Glucose, 10% FCS, 20 µg/ml gentamicin, 0.25 µg/ml fungizone, 2.6 nM insulin, and 100 nM cortisol) allowing cell attachment. After 1 day of culture, the medium was changed and FCS was replaced by 10% swine serum. On day 3 of culture, the medium was switched to 2.5% swine serum. After 5 days of culture, cells achieved 80% confluence and were induced to differentiate in DMEM Glutamax High Glucose supplemented with 25 µM 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione, 20 µg/ml gentamicin, 0.25 µg/ml fungizone, 8 nM insulin, 1 µM dexamethasone, 10 µM trilatigon, 0.2 nM T3, 10 µg/ml transferrin, and 0.1 nM ascorbic acid. The medium was changed every day from days 5 to 9. After 9 days of culture, cells were fixed in PBS/4% formaldehyde for 5 min and stained with Oil Red O for 20 min to evaluate intracellular accumulation of lipid-rich vacuoles. Lipid accumulation in cells was estimated from three wells per animal and three random fields per well containing ~900 cells with ImageJ. Results were expressed as surface of Oil Red O staining divided by the total area of cells in the same field.

Myogenic differentiation. Adipose tissue- and muscle-derived cells were first placed in PGM during 5 days. The medium was changed every 2 days. After 5 days of culture, cells reached 80% confluence in PGM. Cells were then placed in a transition medium containing 10% FCS for 16 h. Cells were switched to a differentiation medium (DMEM containing 5% FCS, 1 µM insulin and 1 µM cytokine arabinoside to fully stop cell proliferation (Sigma)). From days 5 to 9 of culture, differentiation medium was changed every day. After 9 days of culture, cells were fixed as described previously and incubated in PBS/0.1% BSA with the following mouse primary monoclonal antibodies: anti-myosin heavy chain (MF20, 1:10; Developmental Studies Hybridoma Bank; University of Iowa, Iowa City, IA). After 2 h at room temperature, antibodies were visualized using a fluorescein-conjugated goat anti-mouse IgG (Jackson, Interchim). Cells were finally mounted in fluorescent mounting medium containing DAPI to stain nuclei in blue. The fusion index was estimated from three wells per animal and three random fields per well and expressed as the number of DAPI stained nuclei divided by the total number of nuclei in the same field. A Nikon DS-R1i epifluorescence microscope was used as previously described for immunohistochemistry.

Statistical analysis. Data were analyzed by ANOVA using the General Linear Model procedure of SAS (SAS Institute, Cary, NC). The first model used to study the expression of each surface marker in SV cells between tissues during development was a two-way ANOVA including the main effects of tissue type, age, and their interaction, as well as the animal within age effect. The effect of age was tested against the animal within age residual error. Least squares means values were compared using the PDIFF statement of the General Linear Model procedure. The second model used to analyze the occurrence of progenitor cell populations obtained from combined surface markers was a one-way ANOVA including the main effects of age within each tissue and cell population with Tukey’s post hoc test for multiple comparisons. All data are represented as means ± SE. Differences were considered significant at P < 0.05.

RESULTS

In situ localization of cell surface markers in adipose tissue and skeletal muscle. Immunohistochemical labeling for CD34⁺, CD56⁺, and CD90⁺ cells did not differ visually between stages, and the choice was made to show immunohistochemical pictures at only 7 days of age (Fig. 1). In SCAT, immunohistochemical staining showed the presence of CD34⁺, CD56⁺, and CD90⁺ cells with CD90⁺ labeling giving the most widespread signal (Fig. 1). CD90⁺ and CD34⁺ cells were mostly present in the stroma and also around capillaries and in the adventitia of larger vessels. CD56⁺ labeling was localized in the stroma mostly in the vicinity of vascular structures. In RM muscle of 7-day-old pigs, CD34⁺, CD56⁺, and CD90⁺ cells were also detected (Fig. 1). CD56⁺ cells were observed mostly within the perimysium and also between muscle fibers either in an interstitial position outside the basal lamina (28 ± 7%) or in a satellite cell position under the basal lamina in close contact with muscle fibers (12 ± 3%). CD56⁺ cells beneath the basal lamina surrounding each myofiber likely correspond to satellite cells. CD34⁺ cells were mainly

Fig. 1. Representative immunohistochemical pictures showing in situ localization of progenitor cells in subcutaneous adipose tissue (left) and rhomboideus skeletal muscle (right) of 7-day-old piglets. Cross-sections were stained in red for CD90, CD56, and CD34 cell surface markers with a blue DAPI counterstaining to localize nuclei. An antibody recognizing laminin (green) was also used to stain the basal membrane. In subcutaneous adipose tissue, white and grey arrows indicated stromal and perivascular structures, respectively. Scale bar = 20 µm.
located in the perimysium and interstitial space between muscle fibers with about 4 ± 2% of CD34+ cells exhibiting a satellite position. Finally, a strong CD90 staining was observed in the perimysium, whereas very few CD90+ cells were present within primary fascicles in the direct vicinity of muscle fibers.

Expression of individual cell surface markers in SV native cell. The number of SV cells isolated from all tissues was greater (P < 0.05) in 7-day-old than in older pigs (Table 2). Nevertheless, the number of isolated cells was lower (P < 0.01) in interM adipose tissue than in other tissues in the three examined stages. Flow cytometry analysis of cells isolated from adipose tissues and skeletal muscles revealed a significant expression of CD34, CD56, and CD90 markers (Fig. 2, A and B), whereas only 0 to 2% of the cells expressed hematopoietic markers including CD11b, CD14, and CD45 and the endothelial stem cell marker CD31. For this reason, the remaining portion of the article will focus on the expression of CD34, CD56, and CD90 markers.

The analysis of each marker separately showed a predominance of CD90+ cells in all tissues with proportions ranging from 10 to 48% (Fig. 2C). The proportion of CD90+ cells increased (P < 0.05) in adult animals in interM adipose tissue and in muscles whereas this proportion did not change with age in SCAT. The proportion of CD56+ cells was much greater (P < 0.05) in muscle than in adipose tissue up to D160. Thereafter, this proportion increased (P < 0.05) in adult adipose tissues and in adult LM muscle. The proportion of CD34+ cells did not exceed 10% in the examined tissues and did not change with age, except in SCAT where a fourfold increase (P < 0.05) was observed between D7 and D400.

Changes in progenitor cell populations based on surface marker coimmunostaining. Further analysis by flow cytometry using coimmunostaining indicates that the following cell populations with proportions ranging from 1 to ~36% were identified in both adipose tissue and skeletal muscle whatever the age considered: CD90+/CD34+, CD90+/CD34−, CD90+/CD56+, CD90+/CD56−, CD90−/CD56+, CD90−/CD56−, CD34+/CD56+, CD34−/CD56+, CD56+/CD34+, and CD56−/CD34+ cells (Figs. 3 and 4, A and B). In skeletal muscle, CD90+/CD34+ cells were also detected. In adipose tissues, CD90+/CD34− cells were predominant with proportions ranging from 18 to 38%. The percentage of these cells CD90+/CD34− cells decreased (P < 0.05) between D7 and D160 in both SCAT and interM adipose tissues. In interM, CD90+/CD56− cells were quite abundant (17–25%) but did not exhibit any age-related change. Other cell populations did not exceed 12% in adipose tissue. In addition, the percentage of CD34+ cells within the CD90+ cell population was <5% at D7 and D160 in SCAT but increased (P < 0.001) to ~10% at D400 in SCAT.

In RM muscle, six populations with proportions ranging from 12 to 30% were identified at D7 (CD90+/CD34+, CD90+/CD56+, CD90−/CD56+, CD56+/CD34+, and CD56−/CD34− cells; Fig. 3) and five populations with proportions ranging from 18 to 36% (CD90+/CD34+, CD90+/CD56+, CD90−/CD56+, CD56+/CD34−, and CD56−/CD34− cells) were identified at D400. In LM muscle, only three cell populations were over 10% (CD90+/CD56+, CD90−/CD56−, and CD56−/CD34− cells) at D7. At D400, four main cell populations were identified (CD90+/CD34−, CD90+/CD56+, CD90−/CD56−, and CD56−/CD34− cells). In RM and LM muscles, the percentage of CD90+/CD34− and CD56−/CD34− cells increased (P < 0.001) with age (Fig. 3).

Adipogenic and myogenic differentiation studies. Adipogenic and myogenic potentials of total SV cells and CD56+ and CD90+ cells isolated from SCAT and RM muscle were studied at 7 days of age. After 2 days of culture (D2), cells isolated from SCAT exhibited an elongated morphology, with grossly rectangular shape and cells isolated from RM muscle demonstrated also a typical morphology of muscle progenitor cells, with an elongated structure (data not shown). Cell differentiation was then evaluated on D9.

All SCAT cells placed in adipogenic conditions were filled with lipid droplets. Whereas 65 ± 7% of total SV cells differentiated into multilocular adipocytes, the percentage of differentiation reached 92 ± 17% for CD90− cells and 71 ± 6% for CD56− cells (Fig. 5). On D9, total stromal cells placed in myogenic conditions were aligned like myogenic cells before their fusion but they neither fused nor expressed the myosin heavy chain (MF20) marker (Fig. 5). This was also observed for CD90+ and CD56+ cells. In control medium, SCAT cells proliferated until confluence without accumulating lipid droplets and they did not fuse (data not shown).

All RM muscle cells placed in adipogenic conditions were filled with lipid droplets. For total stromal cells, 45 ± 9% of the cells differentiated into mature multilocular adipocytes (Fig. 5). The percentage of differentiated cells was 52 ± 7% for CD90− cells and 25 ± 7% for CD56− cells. In myogenic conditions, long multinucleated myotubes were present with a fusion index reaching 62 ± 9% for total stromal cells (Fig. 5). For CD90− cells, the fusion was 34 ± 14% and for CD56− cells, and the fusion was 78 ± 21%. In control medium, muscle cells proliferated until confluence. At day 9, cells did not fuse and did not differentiate into multilocular adipocytes (data not shown).

Table 2. Number of cells isolated from adipose tissue and skeletal muscle in 7-, 160-, and 400-day-old pigs

<table>
<thead>
<tr>
<th></th>
<th>D7</th>
<th>D160</th>
<th>D400</th>
<th>P Value</th>
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<td></td>
<td>20 × 10^6 ± 0.5 × 10^6*</td>
<td>6 × 10^6 ± 0.2 × 10^6;</td>
<td>2.6 × 10^6 ± 0.2 × 10^6;</td>
<td>&lt;0.05</td>
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<td>Adipose tissue</td>
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<td>SCAT</td>
<td>5.5 × 10^6 ± 0.4 × 10^6*</td>
<td>0.3 × 10^6 ± 0.03 × 10^6;</td>
<td>1.8 × 10^6 ± 0.3 × 10^6;</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>InterM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>22 × 10^6 ± 0.5 × 10^6*</td>
<td>8 × 10^6 ± 0.4 × 10^6;</td>
<td>7 × 10^6 ± 0.8 × 10^6;</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>RM</td>
<td>23 × 10^6 ± 0.2 × 10^6*</td>
<td>7 × 10^6 ± 0.8 × 10^6;</td>
<td>9 × 10^6 ± 0.4 × 10^6;</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LM</td>
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</table>

Results are number of cells isolated per gram of tissue and are presented as means ± SE (n = 5/age group), D, days old; SCAT, subcutaneous adipose tissue; InterM, intermuscular adipose tissue; LM, longissimus muscle; RM, rhomboideus muscle. Within the same row, means with different symbols differed, P < 0.05.
The current study provides phenotypic and functional information on adult stem cells present in the SV fraction of both adipose tissues and skeletal muscles in growing and adult animals. Despite the existence of functional links between muscle and adipose tissue, cells from these two tissues have been rarely investigated within the same study. Although numerous cell surface markers for adult stem cells have been described in many studies, there is no complete consensus on the antigen expression pattern of these cells. In agreement with this, recent studies have shown that both adipose and skeletal muscle cells express CD90, CD56, and CD34, but the proportion of cells positive for these markers varies significantly with age.

**DISCUSSION**

The current study provides phenotypic and functional information on adult stem cells present in the SV fraction of both adipose tissues and skeletal muscles in growing and adult animals. Despite the existence of functional links between muscle and adipose tissue, cells from these two tissues have been rarely investigated within the same study. Although numerous cell surface markers for adult stem cells have been described in many studies, there is no complete consensus on the antigen expression pattern of these cells. In agreement with this, recent studies have shown that both adipose and skeletal muscle cells express CD90, CD56, and CD34, but the proportion of cells positive for these markers varies significantly with age.

**Fig. 2.** Age-related expression of each surface marker in stromal vascular cells isolated from adipose tissue [subcutaneous (SCAT) and intermuscular (InterM)] and from skeletal muscle [rhomboideus (RM) and longissimus (LM)].

A: representative flow cytometry analyses of cells isolated from SCAT in 7-day-old piglets.
B: representative flow cytometry analyses of cells isolated from RM muscle in 7-day-old piglets. Analyses were performed using CD90-APC, CD56-PECy7 and CD34-PE and the corresponding isotypes. Signals for fluorescent isotypes are shown as black curves and antigen-specific signals as grey curves.

C: after cell isolation, percentages of positive cells for CD90 allophycocyanin (APC), CD56 phycoerythrin-cyanine 7 (PE-Cy7), and CD34 phycoerythrin (PE) were determined by flow cytometry at 7 (D7; white bars), 160 (D160; grey bars), and 400 (D400; black bars) days of age. Percentages of positive cells are expressed as means ± SE (n = 5 animals/age group). Within each marker, means with different letters differed, P < 0.05.
previous studies based on the investigation of freshly isolated SV cells from human adipose tissue (16, 28, 39) and from human (48) or porcine muscle (49), the current study indicates that SV cells did not express or only poorly expressed CD11b, CD14, CD31, and CD45 markers. Using both immunohistochemistry and flow cytometry, we provide evidence that the well-known mesenchymal CD90 marker, the hematopoietic stem cell CD34 marker and the myogenic CD56 marker were expressed at significant levels in all tissues whatever the age considered.

In adipose tissue, this study shows that cells expressing CD90, CD56, and CD34 markers were found in the stroma, in particular in the vicinity of vascular structures as reported previously for CD34<sup>+</sup>/H11001 cells in humans (26, 46). In this tissue, we show clearly that the proportion of CD90<sup>+</sup> cells was high compared with that of CD34<sup>+</sup> and CD56<sup>+</sup> cells whatever the age considered. Similar findings have been reported in humans (15, 28) and also in dogs and horses (40, 47). Further phenotyping of cells demonstrated that CD90<sup>+</sup>/CD34<sup>+</sup>/CD56<sup>+</sup> cells predominated in adipose tissue even though the proportion of these cells decreased during growth. The relative low proportion of CD34<sup>+</sup> cells agrees with data obtained in canine and equine adipose tissue (40, 47). In humans, whereas two studies support our findings (2, 12), other studies considered adipose tissue as a large reservoir of adult stem cells identified as CD34<sup>+</sup>/CD31<sup>+</sup> cells with a proportion ranging from 24 to 90% of SV cells (15, 16, 28, 35, 39, 41). These discrepancies may arise from the investigation of freshly isolated native SV cells in some studies and of cultured cells in others (15, 29, 39). It should be mentioned that the investigated fraction is not clearly...
specified in some studies. The conflicting results may also be linked to the lack of information of the studied subjects. Normal, overweight, and obese individuals are sometimes investigated within the same study with no distinction of their body weight group. Finally, another plausible hypothesis is that we have investigated cells harvested from growing animals, whereas most available data are based on the investigation of cells obtained from adults exhibiting most of the time different metabolic disorders. In the current study, the finding of a significantly higher number of CD34$^+$ cells in 400-day-old than in younger animals supports the idea that CD34$^+$ cells may be more abundant in adult than in young individuals. Compared with CD90 and CD34 markers, the expression of the CD56 marker that is usually considered as a myogenic marker has been poorly investigated in adipose tissue. Whereas the current study shows that SV cells isolated form adipose tissue expressed clearly CD56 (NCAM 16.2 clone), some studies in humans failed to detect this marker in cultured undifferentiated adipose-tissue-derived stromal cells (16, 22). In contrast, other studies demonstrated the presence of CD56 expressing cells in adipose tissue (4, 6). With such a paucity of data, it is difficult to determine whether these contrasted data may be related to differences between species or alternatively to the investigated period as discussed above.

In situ localization of cells positive for CD90, CD56, and CD34 markers in skeletal muscle are in accordance with

Fig. 4. A: representative flow cytometry analyses of cells isolated from SCAT. B: representative flow cytometry analyses of cells isolated from RM in 7-day-old piglets. Side scatter (SSC) and forward scatter (FSC) indicate the cell granularity and cell size, respectively. Histograms show the fluorescence intensity of double-stained cells. Each representation is illustrated by the isotype control (top) associated to its corresponding positive staining (bottom).
previous observations in humans (24, 37). As previously reported (7), CD90<sup>+</sup> cells were mainly found in the perimysial compartment. With respect to CD56, which is generally considered as a marker of myogenic cells, our results confirmed the presence of CD56<sup>+</sup> cells in a satellite cell position as reported previously (19). A small proportion of CD34<sup>+</sup> cells was also detected in a satellite cell position (4%).

As previously described in one study carried out in semitendinosus muscle of 8-wk-old pigs (49), we confirm that CD56<sup>+</sup> and CD90<sup>+</sup> cells were highly present in SV cells isolated from pig muscles. As observed in adipose tissue, we also demonstrated that the proportion of CD90<sup>+</sup> cells was high in skeletal muscle compared with that of CD34<sup>+</sup> cells. The proportion of these latter cells (11%) was similar to that reported in one human study (24) but lower than that reported in another recent study (53%; 37). In mice, the proportion of these cells has been shown to reach 42% in muscle (21). With respect to CD56<sup>+</sup> cells, their proportion was close to that of CD90<sup>+</sup> cells. These observations are not consistent

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**Fig. 5.** Features of total stromal vascular (SV), CD90<sup>+</sup>, and CD56<sup>+</sup> cells cultured for 9 days in adipogenic or myogenic conditions. Cells were isolated SCAT and RM muscle of 7-day-old piglets. Cell morphology was examined using phase contrast and arrows indicate lipid droplets. Adipogenic differentiation was evaluated with Oil Red O staining and myogenic differentiation with myosin heavy chain (MF20) immunostaining. The percentage of differentiated cells is mentioned on each figure. Scale bar = 10 µm. These data are representative of 3 consistent independent experiments for each cell type.
with data obtained in human adults showing that CD34+ and CD56+ cells were more abundant than CD90+ cells (24). Further phenotyping of cells based on the combination of several markers demonstrates the presence of several cell populations. Some of these identified populations have been reported to be exclusively myogenic progenitors (CD56+/CD34−), adipogenic (CD56−/CD34+), or myo-adipogenic (CD56+/CD34+) progenitors in human muscle (37, 38). Altogether, these data further support the idea that these cell populations may display different differentiation potentials.

The current findings confirm the mesenchymal nature of isolated cells and their multipotent potential but show marked differences between tissues. As previously demonstrated, cells including CD90+ and CD56+ cells isolated from adipose tissue can differentiate into an adipocyte phenotype (54) but did not have the ability to reach a complete myogenic phenotype in myogenic conditions even though these cells have been shown to participate to skeletal muscle regeneration in mice (9). In contrast, cells isolated from muscle can differentiate into a complete myogenic or adipogenic phenotype as already demonstrated in vitro with rat satellite cells (1, 51). At least, one of these cell populations residing in postnatal muscle may give rise to intramuscular adipocytes (45). For instance, CD90+ cells exhibited a higher adipogenic potential than CD56+ cells. Combined with phenotypic data showing the predominance of CD90−/CD34− cells in adipose tissue and the presence of several cell populations in skeletal muscle, these findings suggest that cells from adipose tissue are more homogeneous and globally less multipotent than cells isolated from skeletal muscle. These findings further illustrate the capacity of muscle cells to differentiate into different cell lineages. For instance, mice satellite cells can differentiate into brown adipocytes (52). With respect to the pig, such a differentiation is rather unlikely. Indeed, the pig is still considered as a species lacking brown adipose tissue. To clarify the differentiation potential of the different cell populations in muscle, further investigation based on cell sorting is needed. With the recent identification of beige adipocytes, cells intermediate between white and brown adipocytes (50), it may be relevant to determine whether those cells are present or not in the pig.

The current study documents for the first time the dynamic changes of progenitor cells in both adipose tissue and skeletal muscle of growing and adult animals. We clearly show that the number of SV cells per gram of tissue declined with age in both tissues as previously described in humans and bovine (11, 42). However, it is important to note that the largest difference was observed for SCAT in which this number was divided by eight compared with three in muscle. We demonstrate an increase in the proportion of CD90+/CD56− and CD34+ cells with age in both tissues. The dramatic decrease in the proportion of CD56+/CD34− cells in adult RM muscle, along with a decrease in the percentage of CD56−/CD34+ during growth likely denote a strong decrease in myogenic potential, whereas that of LM muscle seems to be preserved. Conversely, the age-related increase in the proportion of CD56−/CD34+ cells in both muscles suggests that the adipogenic potential of muscle progenitors may be higher in older animals. These observations are in agreement with data showing age-related changes differentiation potential with an increase in adipogenic potential of MSCs (23) and the increase in intramuscular fat content with age (13). To our knowledge, the dramatic increase in the percentage of CD90+/CD34− cells with age in RM and LM muscles and its decrease in SCAT and interM adipose tissue are original results. This population could correspond to multipotent mesenchymal stromal cells able to undergo osteogenic, adipogenic and chondrogenic differentiation ex vivo (10).

In conclusion, this study provides for the first time new information on adipose tissue and skeletal muscle in terms of adult stem cell populations during animal growth and tissue development. Our data indicate that muscle cells display a larger cell heterogeneity compared with adipose cells leading to greater plasticity since they can go through a myogenic and adipogenic complete phenotype, whereas adipose cells did not reach a complete myogenic differentiation. Further studies are needed to better understand the plasticity of those different cell populations throughout life.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES

8. De Clercq L, Mourot J, Genart C, Davids V, Boone C, Remacle C. An anti-adipocyte monoclonal antibody is cytotoxic to porcine preadipocytes.


