Non-Adherent Mesenchymal Progenitors from Adipose Tissue Stromal Vascular Fraction

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In primary human bone marrow cultures, the initial adherent cell fraction has been shown to provide a microenvironment for self-renewal of primitive non-adherent mesenchymal progenitors (non-adherent progenitors of bone marrow stroma [BM-NAMP]), with increased differentiation potential compared to adherent colony-forming units-fibroblast (CFU-f). The present study investigates whether NAMP exist also in cultures of stromal vascular fraction (SVF) cells derived from human adipose tissue. Adipose-tissue NAMP (AT-NAMP) were shown to be stably non-adherent and their number correlated with the number of the initial adhering CFU-f. Unlike BM-NAMP, AT-NAMP did not propagate in suspension in serial replating experiments and the number of colonies steadily decreased with each replating step. However, when AT-NAMP were kept on the initially adhering SVF cells, they could significantly expand without loss of clonogenic, proliferation, and differentiation potential. Although AT-NAMP progeny differentiated into mesodermal lineages similar to that of adherent CFU-f, it was enriched in early mesenchymal progenitor populations, characterized by increased expression of SSEA-4 and CD146. Furthermore, FGF-2 supported AT-NAMP survival and could not be replaced by another mitogenic factor, such as platelet derived growth factor BB. In conclusion, these data suggest that the SVF adherent fraction provides niche signals that regulate the expansion of adipose non-adherent mesenchymal progenitors with the maintenance of their potency. The biological differences described between BM- and AT-NAMP further qualify the properties of the stroma from different tissues and will be relevant for the selection of a cell source for specific regeneration strategies.

Introduction

IN THE LAST DECADES, mesenchymal stromal/stem cells (MSC) from bone marrow (BM) have been widely investigated, due to their capacity to extensively proliferate *in vitro* while maintaining their differentiation potential into several mesodermal lineages.¹ More recently, adipose tissue has been identified as a promising source of multipotent progenitors because of its abundance, easy accessibility, and minimal donor site morbidity.² The freshly isolated stromal vascular fraction (SVF) of human adipose tissue is a heterogeneous population of mononuclear cells, which include mesenchymal progenitors referred to as adipose-derived stromal/stem cells (ASC).³ SVF presents the advantage of containing a higher number of clonogenic cells per volume of tissue sample compared to human BM,⁴ as well as a population of vasculogenic cells.⁵ Several reports have shown differences between BM-MSC and ASC after isolation and expansion on plastic, regarding their phenotype and differentiation capacity.⁶ For example, BM-MSC maintain a good

osteogenic potential upon limited *in vitro* expansion. On the other hand, although SVF cells have been shown to generate osteogenic grafts with intrinsic vasculogenic properties,⁵ expanded ASC display a poor osteogenic capacity both *in vitro* and *in vivo* in the absence of osteoinductive signals.^{7,8} This suggests heterogeneity in the stage of commitment of the isolated cell population and results in a limited potential of expanded ASC for bone regeneration. Therefore, it is key to understand the biological signals regulating the maintenance of SVF properties in ASC.

In vivo, ASC have been described to express CD34 and to occupy a perivascular position.^{6,9,10} However, it has not been clarified yet whether ASC, in analogy to stem cells of other tissues, require their own regulatory environment to preserve their progenitor characteristics. We have previously shown that non-adherent progenitors of bone marrow stroma (BM-NAMP) represent a primitive compartment of MSC, which display increased proliferation and differentiation capacity *in vitro* and a greater bone formation *in vivo* than the corresponding colony-forming unit-fibroblast (CFU-f).¹¹ The

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BM adherent fraction provides a niche function for BM-NAMP at least in part through humoral signals, promoting their expansion without loss of proliferation and differentiation capacity. Based on these results, we investigated whether NAMP represent a progenitor population also present in the SVF of adipose tissue with niche-dependent self-renewal potential. We found that adipose tissue-NAMP (AT-NAMP), although not able to propagate as non-adherent progenitors upon serial replating, are enriched for markers of multipotency and are able to expand on an *in vitro* niche, maintaining their clonogenic, proliferative, and differentiation capacity.

Materials and Methods

Cell isolation

Subcutaneous adipose tissue in the form of lipoaspirates was obtained from 12 healthy donors (21–69 years old) during routine lipoaspirations, after informed consent from the patient and following protocol approval by the local ethical committee. The tissue was digested in 0.075% collagenase type 2 (355 U/mg; Worthington) for 45 min at 37°C on an orbital shaker. The suspension was thereafter centrifuged at 300 g for 10 min, and the resulting SVF pellet was washed once with phosphate-buffered saline (PBS), resuspended in alpha-minimal essential medium (MEM) (Gibco), and finally filtered through a 100- μ m strainer (BDFalcon; BD Biosciences). Nucleated cells were counted after staining with Crystal Violet 0.01% (Sigma) in PBS (pH 7.2).

Cell culture

To determine colony-forming efficiency (CFE) and for serial replating experiments, cells were plated at clonal density (nine cells/cm²) and cultured in alpha-MEM (Gibco) with 10% fetal bovine serum (FBS). When indicated, the medium was further supplemented with FGF-2 (5 ng/mL; R&D System) or platelet derived growth factor BB (PDGF-BB; 10 ng/mL; R&D Systems). After 2 weeks, cells were then washed with PBS, fixed with 3.7% formaldehyde in PBS, stained with Crystal Violet (Sigma) for 10 min, washed with tap water, and the colonies were counted. Only colonies that were macroscopically visible and composed of fibroblastic cells were considered to exclude possible sporadic macrophage aggregates. All determinations were performed in triplicate, and CFU-f frequency in the fresh SVF sample was used to calculate the population doublings of first-confluence cultures.

For progeny expansion, freshly isolated SVF cells were plated at a density of 1×10^5 cells/cm² (Flask0, CFU-f progeny cells). For AT-NAMP progeny, SVF cells were plated at a density of 1×10^5 cells/cm², and at each medium change, the non-adherent fraction was resuspended in fresh medium and replated into Flask0. After 14 days, the non-adherent fraction was plated into a new flask and expanded in the presence of FGF-2 (AT-NAMP progeny cells). Aliquots of the pooled cells were used in *in vitro* differentiation assays. Flow cytometry analysis was performed on cells harvested 4 days after the replating to minimize phenotype changes due to expansion on plastic.

In vitro adipogenic differentiation

Adipogenic differentiation was induced in 2D cultures as previously described.¹² Briefly, CFU-f and NAMP progeny cells were seeded in six-well plates at a density of 5×10^3

cells/cm² and cultured in alpha-MEM with 10% FBS until they reached confluency. The medium was then supplemented with 10 μ g/mL insulin, 10^{-5} M dexamethasone, 100 μ M indomethacin, and 500 μ M 3-isobutyl-1-methyl xanthine (adipogenic induction medium) for 72 h and subsequently with 10 μ g/mL insulin (adipogenic maintenance medium) for 24 h. This 96-h cycle was repeated four times, and cells were then cultured for an additional week in the adipogenic maintenance medium.

In vitro osteogenic differentiation

Osteogenic differentiation was induced as previously described.¹³ Briefly, CFU-f and AT-NAMP progeny cells were seeded in six-well plates at a density of 5×10^3 cells/cm² in alpha-MEM supplemented with 10% FBS, 10 mM β -glycerophosphate (Sigma), 10 nM dexamethasone (Sigma), and 0.1 mM L-ascorbic acid-2-phosphate (Sigma) and cultured for 3 weeks, with medium changed twice per week. Cells layers cultured in the osteogenic medium were washed twice with PBS, fixed for 10 min in 4% formalin, and washed twice with water. For qualitative assessment, fixed cells were then incubated for 10 min with 2% alizarin red in distilled water and thereafter extensively washed with water. Osteoimage assay kit (Lonza) was used for hydroxyapatite deposition quantification.

Flow cytometry analysis

Freshly isolated, CFU-f and AT-NAMP progeny cells ($3-5 \times 10^5$ cells) were resuspended into 200 μ L of 0.5% BSA in PBS (fluorescence-activated cell sorting [FACS] buffer) with fluorochrome-conjugated anti-bodies against the indicated protein or an isotype control and were incubated for 30 min at 4°C. The antibodies used were CD146-PE, CD90-FITC, CD73-PE, CD14-PE, CD45-PE, CD31-FITC, CD34-APC, CD29, SSEA4-FITC, IgG1-FITC, IgG1-PE, IgG-APC (all from Becton, Dickinson and Company, www.bd.com) and CD105-FITC (Serotec Ltd., www.serotec.com). All the antibodies were used at a dilution of 1:50, except CD105-FITC, which was used at 1:20. Cells were washed twice with FACS buffer, resuspended in PBS, and analyzed with a FACS-Calibur flow cytometer (Becton, Dickinson and Company).

Statistical analysis

Data are presented as mean $\pm$ standard deviation. The difference between experimental groups was evaluated using analysis of variance (ANOVA) followed by the Bonferroni test for multiple comparisons. $p < 0.05$ was considered statistically significant.

Results

A population of stably non-adherent mesenchymal progenitors is present in adipose SVF cultures

To assess whether the non-adherent fraction of adipose SVF cultures contains a population of mesenchymal progenitors and to determine their number, nucleated cells were isolated from fresh human adipose tissue, plated at clonal density on cell culture dishes (Plate0) in the presence of FGF-2, and after 3 days, the non-adherent fraction was collected, resuspended in fresh medium, and replated in a new dish (Plate1, Fig. 1A). After 14 days from the plating, the dishes

FIG. 1. A population of stably non-adherent mesenchymal progenitors is present in human adipose tissue SVF cultures. **(A)** Schematic representation of the replating of the non-adherent fraction after 3 days of culture; **(B)** colony frequency for initial adhering CFU-f (Plate0) and AT-NAMP (Plate1) expressed as number of colonies per 100 SVF cells plated; **(C)** NAMP were discarded 3 days after plating (Plate0) or replated in the same dish at every medium change (Plate0*); **(D)** colony frequency for Plate0 and Plate0* conditions expressed as number of colonies per 100 SVF cells plated. CFU-f, colony-forming units-fibroblast; SVF, stromal vascular fraction; AT-NAMP, adipose tissue-non-adherent progenitors.

were stained to assess the number of colonies. Plate1 contained on average 10.5 ± 6.8 colonies/plate ($n=13$), corresponding to $2.1\% \pm 1.4\%$ of the SVF cells plated (Fig. 1B) and to $18.4\% \pm 11.9\%$ of the initial CFU-f (Plate0 = 56.9 ± 19.9 colonies/plate, $n=13$), indicating that a population of non-adherent mesenchymal progenitors (AT-NAMP) was present in adipose tissue SVF cultures.

To investigate whether these clonogenic cells were stably non-adherent and not simply displayed delayed adherence, nucleated cells of freshly isolated adipose tissue were plated at clonal density in the presence of FGF-2, and at each medium change, the supernatant containing the non-adherent fraction was either discarded (Plate0) or collected, resuspended in fresh medium, and replated into the same dish (Plate0*) (Fig. 1C). After 14 days, the colonies were counted. CFU-f frequency in Plate0 and in Plate0* did not differ significantly (Plate0 = 63.1 ± 16.7 colonies/plate and Plate0* = 57.6 ± 14.2 colonies/plate, corresponding to $12.6\% \pm 3.3\%$ and $11.5\% \pm 2.8\%$ of the SVF cells plated, respectively, $p=n.s.$, $n=4$, Fig. 1D). Colony diameter was also measured to compare the proliferation capacity of single CFU-f, and no significant difference was found (Plate0 = 4.3 ± 0.8 mm and Plate0* = 4.6 ± 0.2 mm, $p=n.s.$, $n=4$).

AT-NAMP do not self-maintain upon serial replating

We next investigated whether AT-NAMP could maintain themselves as non-adherent progenitors or if they would exhaust themselves by generating adherent colonies. To address this question, we performed serial replating experiments, in which at every medium change the non-adherent fraction was collected, resuspended in fresh medium, and replated in a new dish (Fig. 2A). Non-adherent aggregates or spheres were never observed, and AT-NAMP could grow either as free-floating cells or in physical contact with the adherent cells. Since the non-adherent fraction was composed of different cell types, including hematopoietic cells at

all replating steps, it was intrinsically not feasible to visualize AT-NAMP during their growth, and their identification was possible only retrospectively from colony formation. At each replating step, the number of colonies decreased compared to the initial CFU-f (Plate1 = $16.0\% \pm 9.0\%$, Plate2 = $8.4\% \pm 9.7\%$, Plate3 = $1.6\% \pm 2.9\%$, Plate4 = $0.1\% \pm 0.3\%$, $n=11$, Fig. 2B). Three of the analyzed donors showed colonies only in the first replating step, and only two of them, instead, showed few colonies up to the last replating. Although statistical analysis was not possible due to the low frequency of colonies in the last replating steps, the size of the colonies display a tendency to increase from Plate0 to Plate1 (4.3 ± 0.5 mm and 6.1 ± 0.7 mm, respectively, Fig. 2C). In the following two replating steps (Plate2 and Plate3), there was no increase in the colony size (5.9 ± 1.4 mm and 5.9 ± 0.8 mm). Interestingly, the few colonies present in Plate4 displayed a further increased diameter (9.5 ± 0.7 mm, Fig. 2C), more than twice as large as that of the initially adherent CFU-f in Plate0.

AT-NAMP require FGF-2 for initial survival

We next assessed whether FGF-2 could have a role, as for BM-NAMP, for AT-NAMP survival. Serial replating experiments were performed in the presence or the absence of FGF-2. Confirming the results in Figure 2B, in the presence of FGF-2 (Fig. 3, red line), although the number of colonies decreased, some colonies were found in all replating steps (Plate1 = $21.3\% \pm 7.5\%$, Plate2 = $17.1\% \pm 21.8\%$, Plate3 = $4.6\% \pm 8.4\%$, Plate4 = $4.3\% \pm 11.6\%$, $n=8$). Removal of FGF-2 (Fig. 3, black line) resulted in the absence of colonies already by the second replating (Plate1 = $4.8\% \pm 3.6\%$, Plate2 = $0.3\% \pm 0.6\%$, no colonies were present in Plate3 and Plate4, $n=8$). We then investigated whether FGF-2 was specific for AT-NAMP survival or if they could be maintained by other mitogenic growth factors. PDGF-BB similarly stimulated initial CFU-f proliferation (size of the colonies 4.7 ± 0.4 and 4.1 ± 0.9 mm,

FIG. 2. AT-NAMP are not able to expand through serial replating. (A) Schematic representation of serial replating experiments. (B) Relative number of colonies present in each replating step. (C) Average diameter of the colonies formed at each replating step.

for FGF-2 and PDGF-BB, respectively). As shown in Figure 3 (blue line), the number of colonies present in all replating steps with PDGF-BB was similar to the condition with serum alone (Plate1 = $11.5\% \pm 8.2\%$, Plate2 = $0.9\% \pm 1.5\%$, no colonies were present in Plate3 and Plate4, $n=3$), almost disappearing by the second replating, suggesting that PDGF-BB cannot substitute for FGF-2 in maintaining AT-NAMP.

FIG. 3. AT-NAMP require FGF-2 for initial survival. Effect of FGF-2 (red line) and platelet derived growth factor BB (PDGF-BB) (blue line) on NAMP regeneration as non-adherent progenitors upon serial replating (black line indicates the condition with serum alone). Color images available online at www.liebertpub.com/tea

Initially adhering cells promote AT-NAMP maintenance and proliferation as non-adherent progenitors

We previously found that when replated on the initially adherent BM fraction, BM-NAMP were able to proliferate in suspension without loss of their progenitor properties, indicating a niche function for the initially adhering BM fraction.¹¹ The data described above showed that AT-NAMP were not able to maintain themselves upon serial replating, so we then asked whether keeping them in contact with the initially adhering SVF fraction, they could proliferate as non-adherent progenitors and preserve their colony-forming potential.

SVF cells were plated at clonal density in the presence of FGF-2, and at each medium change, the non-adherent fraction was resuspended in the fresh medium and replated into Plate0. After 14 days (Plate4*), the non-adherent fraction was plated into a new dish. As shown in Figure 4A, AT-NAMP generated a significantly higher number of colonies when transferred in a new dish after culture on Plate0 compared to when they underwent a serial replating for the same time (Plate4 = $0.1\% \pm 0.3\%$, Plate4* = $129.1\% \pm 66.0\%$, $p < 0.01$, $n=4$). Furthermore, the initially present AT-NAMP (Plate1 = $18.4\% \pm 11.9\%$) were able to significantly expand in

FIG. 4. The initially adhering SVF cells promote AT-NAMP maintenance and proliferation as non-adherent progenitors. Relative number (A) and size (B) of colonies formed when the non-adherent fraction was serially replated for 3 (Plate1) or 14 days (Plate4) or maintained in the original dish for 14 days (Plate4*); ** $p < 0.05$. The number of colonies is shown as the percentage of those generated in the original dish (Plate0).

FIG. 5. AT-NAMP are enriched with early mesenchymal progenitor populations. **(A)** Expression of the mesenchymal stem/stromal cell markers (CD90, CD73, CD29, CD49a, CD105), hematopoietic markers (CD14, CD45), and endothelial markers (CD31, CD34) on the progeny of CFU-f (blue plots) and AT-NAMP (green plots). Isotype controls for the CFU-f and NAMP progeny are shown by red and orange plots, respectively. **(B)** Expression of CD146 and SSEA-4 on CFU-f and AT-NAMP progeny. Color images available online at www.liebertpub.com/tea

suspension by about sixfold when kept in contact with the initially adherent SVF cells for 14 days (Plate4* = 129.1% ± 66.0%, $p < 0.01$). The colony diameter assessment showed that by maintaining AT-NAMP in contact with the initially adherent CFU-f, it was possible to expand them while preserving their proliferation capacity. In fact, there was no significant decrease in the size of the colonies, despite the fact the AT-NAMP underwent significant expansion in suspension (Plate0 = 4.3 ± 0.8 mm, Plate1 = 6.1 ± 0.7 mm, Plate4* = 6.1 ± 1.8 mm, $n = 4$, $p = \text{n.s.}$, Fig. 4B).

AT-NAMP are enriched with early mesenchymal progenitor populations with adipogenic and osteogenic capacity *in vitro*

The progeny of AT-NAMP and CFU-f showed a similar expression of CD90 and CD73, CD29, CD49a and CD105 (Fig. 5A), which were previously described to identify specific clonogenic subpopulations.^{14,15} Both progenies also comprised a small population of hematopoietic (CD45⁺ and CD14⁺ cells) and endothelial (CD31⁺/CD34⁺) cells (Fig.

5A). However, AT-NAMP progeny was enriched with cells expressing SSEA-4 (28% vs. 2%) and CD146 (45% vs. 3% Fig. 5B), which were associated with early multipotent mesenchymal progenitors.^{16,17}

Following adipogenic stimulation *in vitro*, AT-NAMP were able to form mature adipocytes to the same extent of initially adhering CFU-f (Fig. 6A–D), as confirmed by quantification of oil red-O absorbance (Fig. 6H, $p < 0.01$ for both vs. control and $p = \text{n.s.}$ for AT-NAMP vs. CFU-f). In osteogenic conditions, the AT-NAMP progeny differentiated similarly the initial adhering CFU-f, as it was shown by alizarin red staining (Fig. 6E, F) and by quantification of hydroxyapatite deposits (Fig. 6G), indicating the preservation of their osteogenic potential.

AT-NAMP frequency does not correlate with the presence of specific subpopulations but is positively related to the initial clonogenicity

A direct analysis of AT-NAMP phenotype is not feasible due to their extremely low frequency in the freshly isolated

FIG. 6. *In vitro* differentiation of CFU-f and AT-NAMP progeny. (A, B) Representative fields of oil red-O staining of CFU-f (A) and AT-NAMP progeny (B, size bar=200 μ m) after culture with adipogenic growth factors. (C, D) The lower panels represent a higher magnification view of the area indicated by the black marks in the upper panels. Scale bar=50 μ m. (E, F) Alizarin red staining of CFU-f (E) and AT-NAMP (F) progeny (scale bar=200 μ m). (G) Hydroxyapatite deposition quantification. (H) Oil red-O quantification by absorbance expressed as OD. OD, optical density. Color images available online at www.liebertpub.com/tea

population. Alternatively, we investigated whether the presence of NAMP and their number correlate with the fraction of specific SVF subpopulations, which are known to be variable in preparations from different donors.^{3,10} Therefore, we analyzed freshly isolated SVF cells for the expression of markers specific of mesenchymal (CD105, CD90, CD73, CD146, SSEA-4), endothelial (CD31, CD34), and hematopoietic (CD14, CD45) lineages and investigated a possible correlation between the frequency of positive cells and the CFE of the first replated AT-NAMP. A statistically significant correlation would suggest an enrichment of AT-NAMP in the subpopulation positive for that marker. AT-NAMP frequency, indicated by the CFE of 3 days replated cells, did not correlate with any of the markers, even with those previously described to characterize early mesenchymal progenitors.

AT-NAMP frequency did, instead, significantly correlate with the initial CFE ($R^2=0.705$, $p=0.046$, Fig. 7A) but not with the diameter of the initially adherent colonies ($R^2=0.009$, $p=0.816$, Fig. 7B).

Discussion

The data presented in this study show that a specific population of non-adherent mesenchymal progenitors (AT-NAMP) is present in human adipose tissue SVF cultures. In addition, AT-NAMP: (1) are stably non-adherent; (2) require FGF-2 for their maintenance *in vitro*; (3) are enriched for markers of multipotency; (4) can be expanded on an *in vitro* niche preserving their clonogenic, proliferative, and differentiation capacity.

SVF cells are a heterogeneous population, which includes hematopoietic and endothelial cells, as well as different subpopulations of multipotent progenitors characterized by the expression of mesenchymal markers, such as CD105, CD90, CD73, or CD146.^{10,18} AT-NAMP frequency did not correlate with the frequency of any specific mesenchymal subpopulation in the freshly isolated SVF cells, but it was positively related to the initial CFE, indicating that they represent a fixed fraction of the initial CFU-f.

AT-NAMP could not expand upon serial replating. In fact, unlike in the experiments performed with BM-NAMP,¹¹ in which clonogenic progenitors could expand in suspension, the number of colonies steadily decreased in each replating step, until only very few colonies were found in the last dish. These colonies had a significantly increased diameter, suggesting that, in these conditions, AT-NAMP may undergo a selection for the rare progenitors with the highest proliferation ability, but without an actual amplification. These data suggest that, although mesenchymal progenitors have been identified in several tissues, the stromal compartment is

FIG. 7. AT-NAMP frequency correlates to initial clonogenicity. Correlation between AT-NAMP CFE after 3 days of culture and the number (A) and the diameter (B) of initial CFU-f. CFE, colony-forming efficiency.

characterized by specific features, which are different depending on the nature of the tissue source, in line with what has been recently shown.^{1,19–22}

Surface marker analysis showed that AT-NAMP progeny was enriched with cells expressing CD146 and SSEA-4. Tri-potent BM-MSC clones express CD146, which was indicated as a potential marker of multipotency.¹⁷ SSEA-4, instead, has been used to monitor the maintenance of an undifferentiated state in embryonic stem cells^{23,24} and described to be upregulated by the most primitive mesenchymal progenitors in the BM.¹⁶ Recently, SSEA-4 has been used to prospectively isolate a subpopulation of ASC with increased endothelial and osteogenic differentiation potential from freshly isolated SVF cells.²⁵ Taken together, these data indicate that AT-NAMP display distinct phenotypic features from ASC, which also suggest a potential difference in functional properties. The identification of a unique set of markers to quantify AT-NAMP prevalence in the SVF population still remains a fundamental requirement for the exploitation of their potential. Moreover, *in vivo* differentiation assays, possibly in an orthotopic site, might be necessary to rigorously assess AT-NAMP differentiation and tissue regeneration capacity.

BM-NAMP required FGF-2 for their initial survival and their self-renewal as non-adherent progenitors. The experiments performed with the adipose tissue SVF cells indicate that FGF-2 plays a crucial role also for AT-NAMP, without, however, supporting their expansion as non-adherent progenitors when serially replated. FGF-2 is a pleiotropic factor, which has been described to have a complex and context-dependent role in the biology of several cell types. In particular, it has been implicated in the regulation of self-renewal of embryonic stem cells^{26,27} and has been shown to be required for the maintenance of a neural stem cell population in the mouse brain.²⁸ Medium supplementation with FGF-2 has been shown to promote the proliferation of mesenchymal progenitors from several tissues allowing the *in vitro* expansion necessary for clinical applications.²⁹ In BM-MSC, FGF-2 increases the frequency of tripotent progenitors, capable of osteogenic, chondrogenic, and adipogenic differentiation at a clonal level,³⁰ prolongs their lifespan, and selects a subset of pluripotent mesenchymal precursors with increased telomere length.²⁹ It has been also described as the major candidate for the maintenance of self-renewal ability of ASC *in vitro*, supporting their long-term propagation and maintenance of their multilineage differentiation potential.^{31,32} Our data indicate that FGF-2 has a function in the survival of AT-NAMP. In fact, PDGF-BB, although inducing a similar rate of proliferation of the initially adhering CFU-f, was not able to sustain AT-NAMP survival at a level comparable to FGF-2. This also indicates that AT-NAMP frequency does not depend on the proliferative status of the initially adhering CFU-f, excluding as well that they are fraction of the actively proliferating adherent cells.

Similar to BM-NAMP, AT-NAMP could undergo expansion as non-adherent progenitors when kept in contact with the initially adhering SVF cells. These data suggest that adherent SVF cells could provide niche signals for AT-NAMP and additional experiments should aim at investigating which cell population is responsible for their maintenance and whether humoral and/or cell-to-cell contact signals are involved.

In conclusion, the presence of NAMP appears to be a common property of the mesenchymal progenitor compart-

ment of different tissues and not specific to BM stroma alone. AT-NAMP are a population of early mesenchymal progenitors, with a multipotent phenotype, that require an *in vitro* niche for expansion without loss of potential. However, our data also show important tissue-specific biological differences, in particular in their ability to regenerate their own niche and therefore to self-renew upon serial replating. Further investigations are needed to elucidate signals that are responsible to maintain and expand most primitive ASC and to investigate whether the same cues are also established *in vivo*, aiming at improving their performance in regenerative medicine applications.

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Disclosure Statement

No competing financial interests exist.

References

1. Bianco, P., Cao, X., Frenette, P.S., Mao, J.J., Robey, P.G., Simmons, P.J., *et al.* The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat Med* **19**, 35, 2013.
2. Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., *et al.* Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279, 2002.
3. Bourin, P., Bunnell, B.A., Casteilla, L., Dominici, M., Katz, A.J., March, K.L., *et al.* Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy* **15**, 641, 2013.
4. Fraser, J.K., Wulur, I., Alfonso, Z., and Hedrick, M.H. Fat tissue: an underappreciated source of stem cells for biotechnology. *Trends Biotechnol* **24**, 150, 2006.
5. Scherberich, A., Galli, R., Jaquiere, C., Farhadi, J., and Martin, I. Three-dimensional perfusion culture of human adipose tissue-derived endothelial and osteoblastic progenitors generates osteogenic constructs with intrinsic vascularization capacity. *Stem Cells* **25**, 1823, 2007.
6. Pachon-Pena, G., Yu, G., Tucker, A., Wu, X., Vendrell, J., Bunnell, B.A., *et al.* Stromal stem cells from adipose tissue and bone marrow of age-matched female donors display distinct immunophenotypic profiles. *J Cell Physiol* **226**, 843, 2011.
7. Mehrkens, A., Saxer, F., Guven, S., Hoffmann, W., Muller, A.M., Jakob, M., *et al.* Intraoperative engineering of osteogenic grafts combining freshly harvested, human adipose-derived cells and physiological doses of bone morphogenetic protein-2. *Eur Cell Mater* **24**, 308, 2012.
8. Muller, A.M., Mehrkens, A., Schafer, D.J., Jaquiere, C., Guven, S., Lehmcke, M., *et al.* Towards an intraoperative engineering of osteogenic and vasculogenic grafts from the stromal vascular fraction of human adipose tissue. *Eur Cell Mater* **19**, 127, 2010.
9. Crisan, M., Yap, S., Casteilla, L., Chen, C.W., Corselli, M., Park, T.S., *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* **3**, 301, 2008.

10. Maumus, M., Peyrafitte, J.A., D'Angelo, R., Fournier-Wirth, C., Bouloumie, A., Casteilla, L., *et al.* Native human adipose stromal cells: localization, morphology and phenotype. *Int J Obes (Lond)* **35**, 1141, 2011.
11. Di Maggio, N., Mehrkens, A., Papadimitropoulos, A., Schaeren, S., Heberer, M., Banfi, A., *et al.* Fibroblast growth factor-2 maintains a niche-dependent population of self-renewing highly potent non-adherent mesenchymal progenitors through FGFR2c. *Stem Cells* **30**, 1455, 2012.
12. Barbero, A., Ploegert, S., Heberer, M., and Martin, I. Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. *Arthritis Rheum* **48**, 1315, 2003.
13. Jaiswal, N., Haynesworth, S.E., Caplan, A.L., and Bruder, S.P. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*. *J Cell Biochem* **64**, 295, 1997.
14. Boiret, N., Rapatel, C., Veyrat-Masson, R., Guillouard, L., Guerin, J.J., Pigeon, P., *et al.* Characterization of non-expanded mesenchymal progenitor cells from normal adult human bone marrow. *Exp Hematol* **33**, 219, 2005.
15. Quirici, N., Soligo, D., Bossolasco, P., Servida, F., Lumini, C., and Delilieri, G.L. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. *Exp Hematol* **30**, 783, 2002.
16. Gang, E.J., Bosnakovski, D., Figueiredo, C.A., Visser, J.W., and Perlingeiro, R.C. SSEA-4 identifies mesenchymal stem cells from bone marrow. *Blood* **109**, 1743, 2007.
17. Russell, K.C., Lacey, M.R., Gilliam, J.K., Tucker, H.A., Phinney, D.G., and O'Connor, K.C. Clonal analysis of the proliferation potential of human bone marrow mesenchymal stem cells as a function of potency. *Biotechnol Bioeng* **108**, 2716, 2011.
18. Sengenès, C., Lolmede, K., Zakaroff-Girard, A., Busse, R., and Bouloumie, A. Preadipocytes in the human subcutaneous adipose tissue display distinct features from the adult mesenchymal and hematopoietic stem cells. *J Cell Physiol* **205**, 114, 2005.
19. Belicchi, M., Pisati, F., Lopa, R., Porretti, L., Fortunato, F., Sironi, M., *et al.* Human skin-derived stem cells migrate throughout forebrain and differentiate into astrocytes after injection into adult mouse brain. *J Neurosci Res* **77**, 475, 2004.
20. Choi, Y.S., Noh, S.E., Lim, S.M., Lee, C.W., Kim, C.S., Im, M.W., *et al.* Multipotency and growth characteristic of periosteum-derived progenitor cells for chondrogenic, osteogenic, and adipogenic differentiation. *Biotechnol Lett* **30**, 593, 2008.
21. De Bari, C., Dell'Accio, F., Tylzanowski, P., and Luyten, F.P. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum* **44**, 1928, 2001.
22. Dodson, M.V., Hausman, G.J., Guan, L., Du, M., Rasmussen, T.P., Poulos, S.P., *et al.* Skeletal muscle stem cells from animals I. Basic cell biology. *Int J Biol Sci* **6**, 465, 2010.
23. Henderson, J.K., Draper, J.S., Baillie, H.S., Fishel, S., Thomson, J.A., Moore, H., *et al.* Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. *Stem Cells* **20**, 329, 2002.
24. Riekstina, U., Muceniece, R., Cakstina, I., Muiznieks, I., and Ancans, J. Characterization of human skin-derived mesenchymal stem cell proliferation rate in different growth conditions. *Cytotechnology* **58**, 153, 2008.
25. Mihaila, S.M., Frias, A.M., Pirraco, R.P., Rada, T., Reis, R.L., Gomes, M.E., *et al.* Human adipose tissue-derived SSEA-4 subpopulation multi-differentiation potential towards the endothelial and osteogenic lineages. *Tissue Eng Part A* **19**, 235, 2013.
26. Dvorak, P., Dvorakova, D., Koskova, S., Vodinska, M., Najvirtova, M., Krekac, D., *et al.* Expression and potential role of fibroblast growth factor 2 and its receptors in human embryonic stem cells. *Stem Cells* **23**, 1200, 2005.
27. Xu, C., Rosler, E., Jiang, J., Lebkowski, J.S., Gold, J.D., O'Sullivan, C., *et al.* Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. *Stem Cells* **23**, 315, 2005.
28. Zheng, W., Nowakowski, R.S., and Vaccarino, F.M. Fibroblast growth factor 2 is required for maintaining the neural stem cell pool in the mouse brain subventricular zone. *Dev Neurosci* **26**, 181, 2004.
29. Bianchi, G., Banfi, A., Mastrogiacomo, M., Notaro, R., Luzatto, L., Cancedda, R., *et al.* *Ex vivo* enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. *Exp Cell Res* **287**, 98, 2003.
30. Muraglia, A., Cancedda, R., and Quarto, R. Clonal mesenchymal progenitors from human bone marrow differentiate *in vitro* according to a hierarchical model. *J Cell Sci* **113 (Pt 7)**, 1161, 2000.
31. Rider, D.A., Dombrowski, C., Sawyer, A.A., Ng, G.H., Leong, D., Huttmacher, D.W., *et al.* Autocrine fibroblast growth factor 2 increases the multipotentiality of human adipose-derived mesenchymal stem cells. *Stem Cells* **26**, 1598, 2008.
32. Zaragosi, L.E., Ailhaud, G., and Dani, C. Autocrine fibroblast growth factor 2 signaling is critical for self-renewal of human multipotent adipose-derived stem cells. *Stem Cells* **24**, 2412, 2006.

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