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Fibroblast Growth Factor-2 Maintains a Niche-Dependent Population of Self-Renewing Highly Potent Non-adherent Mesenchymal Progenitors Through FGFR2c

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ABSTRACT

Bone marrow (BM) mesenchymal stem/stromal cells (MSC) are a heterogeneous population of multipotent progenitors currently under investigation for a variety of applications in regenerative medicine. While self-renewal of stem cells in different tissues has been demonstrated to be regulated by specialized microenvironments called niches, it is still unclear whether a self-renewing niche also exists for MSC. Here, we show that primary human BM cultures contain a population of intrinsically non-adherent mesenchymal progenitors (NAMP) with features of more primitive progenitors than the initially adhering colony-forming units-fibroblast (CFU-f). In fact, NAMP could generate an adherent progeny: (a) enriched with early mesenchymal populations (CD146+, SSEA-1+, and SSEA-4+); (b) with significantly greater proliferation and multilineage differentiation potential in vitro; and (c) capable of threefold greater bone formation in vivo than the corresponding CFU-f. Upon serial replating, NAMP were able to regenerate and expand in suspension as non-adherent clonogenic progenitors, while also giving rise to an adherent progeny. This took place at the cost of a gradual loss of proliferative potential, shown by a reduction in colony size, which could be completely prevented when NAMP were expanded on the initially adhering BM fraction. Mechanistically, we found that NAMP crucially depend on fibroblast growth factor (FGF)-2 signaling through FGFR2c for their survival and expansion. Furthermore, NAMP maintenance depends at least in part on humoral signals distinct from FGF-2. In conclusion, our data show a niche/progenitor organization in vitro, in which the BM adherent fraction provides a self-renewing microenvironment for primitive NAMP. STEM CELLS *2012;30:1455–1464*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Bone marrow (BM) stroma comprises the connective tissue elements providing structural and functional support for hematopoiesis, among which resides a population of multipotent mesenchymal progenitors, commonly called mesenchymal stromal/stem cells (MSC). Human BM MSC are a heterogeneous population of progenitors at different stages of commitment and with different expansion potential [1], as shown in clonal experiments [2]. Although this heterogeneity was already described by Friedenstein in the 1980s [3] and despite recent interest on the hierarchical relationship among different MSC populations [4, 5], the mechanisms regulating MSC self-renewal are still largely unknown.

Recent studies have revealed that specific microenvironments, often referred to as "stem cell niches" regulate stem cell function by providing architectural support, together with humoral and contact-dependent signals [6]. In the BM, different niches are specialized to support different functions of hematopoietic stem cells (HSC) [7, 8]. At the endosteal surface, a subpopulation of osteoblasts regulates HSC self-renewal and inhibits their differentiation [9–11]. HSC have been also localized close to BM sinuses in vascular niches composed of endothelial cells [12, 13]. Furthermore, osteoprogenitors, that reside perivascularly in the BM, are able to regenerate the hematopoietic microenvironment in vivo after transplantation [14], suggesting a role for mesenchymal progenitors in the formation of HSC niches, in addition to differentiated osteoblasts. On the other hand, it still remains unclear if also MSC

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require their own niche to maintain their progenitor characteristics.

Recently, an additional source of mesenchymal progenitors has been reported in the non-adherent fraction of human BM cultures [15]. Furthermore, it has been shown that mesenchymal progenitors can be expanded in suspension [16, 17], maintaining a less committed phenotype [16, 18], compared to when cultured on plastic. In analogy to the regulation of HSC function by their stromal niches, here we investigated whether non-adherent mesenchymal progenitors (NAMP) could represent a specific population of early progenitors and whether the BM adherent fraction may provide a niche function for their maintenance in vitro. We found that: (a) NAMP can generate a highly potent adherent progeny while regenerating themselves as non-adherent progenitors, but with a gradual loss of their proliferative potential; (b) however, the immediately adhering BM fraction can provide a niche function for NAMP self-renewal, allowing their expansion in vitro without loss of proliferative ability; (c) mechanistically, NAMP function and their maintenance by the niche depend on fibroblast growth factor (FGF)-2 signaling through FGFR2c.

Materials and Methods

Cell Culture

BM aspirates were obtained from a total of 32 healthy donors (27–64 years old) during routine orthopaedic surgical procedures, in accordance with the local ethical committee (University Hospital, Basel) and after informed consent. Bone marrow nucleated cells (BMNCs) were counted after staining with Crystal Violet 0.01% (Sigma-Aldrich, Saint Louis, MO, http://www.sigmaaldrich.com) in phosphate-buffered saline (PBS), pH 7.2. To determine colony-forming efficiency and for serial replating experiments, cells were plated at clonal density (4.5×10^3 cells per square centimeter) and cultured in α-minimum essential medium (Gibco, Grand Island, NY, http://www.invitrogen.com) with 10% fetal bovine serum. Medium was further supplemented with FGF-2 (5 ng/ml; R&D Systems, Minneapolis, MN, www.rndsystems.com) at every medium change or, when indicated, with platelet-derived growth factor-BB (PDGF-BB) (10 ng/ml; R&D Systems), epidermal growth factor (EGF) (10 ng/ml; R&D Systems), and insulin-like growth factor-1 (IGF-1) (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 minutes, washed with tap water, and the colonies were counted. Only colonies that were macroscopically visible and composed of fibroblastic cells were considered in order to exclude possible sporadic macrophage aggregates. All determinations were performed in triplicate and colony-forming units-fibroblast (CFU-f) frequency in the fresh marrow sample was used to calculate the population doublings of first-confluence cultures.

For progeny expansion, freshly isolated BMNCs were plated at a density of 1×10^5 cells per square centimeter (Flask0, CFU-f progeny cells) and after 3 days, the non-adherent fraction was transferred into a new flask (Flask1, NAMP progeny cells). After 2 weeks of expansion in presence of FGF-2, the cells were detached with 0.05% trypsin/0.01% EDTA (Gibco) and counted for the population doubling determination. Aliquots of the pooled cells were used in vitro and in vivo differentiation assays or replated at a density of 2×10^3 cells per square centimeter for growth curve determination. Flow cytometry analysis was performed on cells harvested only 4 days after the replating to minimize phenotype changes due to expansion on plastic. To assess the effects of conditioned medium, only half of the medium volume was replaced with fresh medium, and compared to a condition in which the whole volume was renewed.

For sorting experiments, freshly isolated BMNCs were stained for 30 minutes at 4°C with an antibody specific for CD45 or an isotype control, both directly conjugated with phycoerythrin (PE) (BD Biosciences, Basel, Switzerland, http://www.bdbiosciences.com), in 0.5% bovine serum albumine in PBS. The CD45-negative population was then sorted with a FACS-Vantage SE cell sorter (BD Biosciences).

In Vitro Differentiation

In vitro differentiation was induced as previously described [19–21]. See Materials and Methods in Supporting Information.

Real-Time PCR

Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA, www.invitrogen.com), treated with DNase and retrotranscribed into cDNA, as previously described [22]. Real-time polymerase chain reaction was performed with the ABIPrism 77000 Sequence Detection System (Perkin Elmer/Applied Biosystem, Rotkreuz, Switzerland, www.appliedbiosystems.com) and expression levels of the genes of interest bone sialoprotein (BSP) and PPAR-gamma were normalized to the 18S rRNA. Primers and probe sets and PCR conditions for BSP and 18S were used as previously described [22]. PPAR-gamma primers and probe (Hs00234592_m1) were provided by Applied Biosystem.

Assessment of Bone Formation In Vivo

In vivo ectopic bone formation was assayed as previously described [23]. Briefly, on reaching their first confluence, 1×10^6 of CFU-f and NAMP progeny cells were resuspended in 30 μl of fibrinogen (20 mg/ml; Baxter, Austria, www.baxter.at), quickly mixed with 30 μl of thrombin (6 IU/ml; Baxter), and loaded onto 35 μg of bovine bone-derived granules (Bio-Oss, Geistlich, Switzerland, www.geistlich.com). The constructs were transferred for 15 minutes in a humidified incubator at 37°C with 5% CO_2 to allow fibrin polymerization and implanted subcutaneously in CD1 nu/nu nude mice (Charles River, Germany, www.criver.com). After 8 weeks, the constructs were harvested, fixed overnight in 4% formalin, completely decalcified with Osteodec (Bio-Optica, Milan, Italy, www.bio-optica.it) for 2 hours at 37°C, paraffin embedded, and 7-μm-thick sections were obtained from different levels. Sections were stained with hematoxylin and eosin (H&E) and observed microscopically to detect the formation of bone tissue for qualitative analysis and assessed by computerized bone histomorphometry as previously described [22, 24] for bone tissue quantification. Briefly, fluorescent images of sections at different depths of each construct were acquired (Scion Image; Scion Corp., Frederick, MD) and used to measure the area covered with bone tissue.

Flow Cytometry Analysis

CFU-f and NAMP progeny cells ($3–5 \times 10^5$ cells) were resuspended into 200 μl of 0.5% BSA in PBS (fluorescence-activated cell sorting [FACS] buffer) with fluorochrome-conjugated antibodies against the indicated protein or an isotype control and were incubated for 30 minutes at 4°C. The antibodies used were CD146-PE, CD49a-PE, CD90-FITC, CD73-PE, SSEA-1-FITC, CD31-FITC, CD34-APC, Nestin-PE, IgG1-FITC, IgG1-PE, IgG-APC (all from Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com), CD271-APC (Miltenyi Biotech, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com), CD105-FITC (Serotec Ltd., Oxford, U.K., http://www.serotec.com), and SSEA-4-FITC (R&D Systems, Minneapolis, MN, http://www.rndsystems.com). Staining for the intracellular protein Nestin was performed after cell permeabilization with the Fix/

Perm Buffer (Biolegend, San Diego, CA, www.biolegend.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).

FGF Receptor Signaling

To block specific FGF receptors, freshly isolated BM-MNC were incubated on ice for 30 minutes in the presence of anti-hFGF R1 IIIb isoform (1 μg/ml; R&D Systems) or anti-hFGF R3 IIIb, IIIc isoforms (1 μg/ml; R&D Systems) or anti-hFGF R2 IIIb, IIIc isoforms (1 μg/ml; R&D Systems). The cells were then seeded at a density of 4.5×10^3 cells per square centimeter and cultured in α-MEM supplemented with rhFGF-2. To stimulate specific FGF receptors, cells were cultured with rhFGF-5 (10 ng/ml; R&D Systems) or rhFGF-19 (10 ng/ml; R&D Systems) instead of FGF-2. After 3 days, the non-adherent fraction was resuspended in fresh medium, supplemented with the same blocking antibody and/or growth factors, and replated in a new dish. The medium was changed twice per week, and after 14 days, the colonies were stained and counted.

Statistical Analysis

Data are presented as means ± SE. The significance of differences was evaluated using analysis of variance followed by the Bonferroni test for every set of data on which multiple comparisons were performed. For single comparison, Mann-Whitney *U* test was used. $p < .05$ was considered statistically significant.

Results

BM Cultures Contain a Population of Intrinsically Non-adherent MSCs

Fresh human BMNCs were plated at clonal density, and after 3 days, the non-adherent fraction was replated in a new dish (d3). The d3 plate contained 24.40 ± 4.47 colonies per 10^6 BMNC plated ($n = 4$), corresponding to 20.4% ± 3.6% of the initial CFU-f (119.6 ± 22.1 colonies per 10^6 BMNC plated). Since in other progenitor systems the cells with earlier features display delayed adherence [25, 26], we assessed whether these clonogenic cells simply required more time to adhere. At each medium change (days 3, 7, 11, and 14), the non-adherent fraction was either discarded (CFU-f plate) or replated in the same dish (CFU-f* plate, Fig. 1A). The number of colonies was not increased by replating the non-adherent cells in the same dish ($n = 3$, 99.1 ± 6.9 vs. 97.8 ± 7.5 colonies per 10^6 BMNC plated, $p =$ N.S.), suggesting the existence of an intrinsically non-adherent population of mesenchymal progenitors (NAMP), which is capable of generating an adherent progeny only when replated in the absence of the initially adherent BM fraction. The diameter of the colonies, which indicates the proliferative potential of the initiating clonogenic progenitor, was measured and no significant difference was found between the average size in the CFU-f and CFU-f* conditions ($n = 5$, 5.5 ± 0.5 mm vs. 5.8 ± 0.6 mm, $p =$ N.S., Supporting Information Fig. S1A, S1B). Furthermore, also the distribution of colony sizes was similar between the two conditions (Fig. 1B), indicating that the constant presence of NAMP in the culture did not affect either the quantity or quality of initially adhering CFU-f.

NAMP Regenerate Themselves as Non-adherent Clonogenic Cells

We next asked whether NAMP would exhaust themselves in generating adherent colonies or could regenerate a non-adherent population after serial replating ($n = 5$, Fig. 1C). Non-adherent aggregates or spheres were never observed and NAMP could grow either as free-floating cells or in physical contact with the adherent cells. Since the non-adherent fraction was predominantly composed of hematopoietic cells at all replating steps, it was intrinsically not feasible to visualize NAMP during their growth, and their identification was possible only retrospectively from colony formation. The non-adherent fraction always contained clonogenic progenitors, whose number steadily increased during the three serial replating steps (d3 = 23.2% ± 3.9%, d7 = 49.1% ± 15.1%, d11 = 72.2% ± 12.5%, and d14 = 86.6% ± 26.5% of the initial CFU-f, $p < .05$, Fig. 1D). Furthermore, NAMP (Fig. 1F) gave rise to colonies that were twice as large as the initially adhering CFU-f (Fig. 1E), with an average diameter of 10.7 ± 1.2 mm and 5.5 ± 0.5 mm, respectively ($p < .01$, Fig. 1H). This increase in average size was due to a global shift in the diameter distribution (Fig. 1I, 90th percentile = 15 mm vs. 8 mm for d3 and CFU-f, respectively) and not simply by the presence of a few very large colonies, suggesting that NAMP are a distinct subset of progenitors with a significantly higher proliferative potential than the initially adhering CFU-f. The regenerated NAMP always gave rise to larger colonies than initial CFU-f at all times during serial replating, but these were gradually smaller (Fig. 1H) until the d14 replating, when diameters were again similar to initial CFU-f (6.4 ± 0.3 mm vs. 5.5 ± 0.4, $p =$ N.S., Fig. 1G, 1H).

NAMP Progeny has a Greater In Vitro Proliferation and Differentiation Capacity than CFU-F

On plastic, NAMP progeny expanded 16-fold more (four population doublings; Supporting Information Fig. S2) than CFU-f-derived cells (15.6 ± 1.2 vs. 11.9 ±.0.9 doublings, $n = 3$, $p < .05$). Furthermore, NAMP progeny consistently differentiated better than CFU-f-derived cells toward mesenchymal lineages in vitro ($n = 2$, Fig. 2). NAMP gave rise to 6.9 ± 2.5 times more adipocytes in culture and expressed 4.1 ± 0.7 greater levels of the adipogenic transcription factor PPAR-gamma (Fig. 2A--2C, $p < .05$). Under chondrogenic conditions, NAMP progeny produced pellets with an increased matrix deposition and clearer chondrocyte morphology (Fig. 2D, 2E), which was paralleled by a 1.8-fold higher glycosaminoglycan accumulation (Fig. 2F, $p < .05$). Following osteogenic stimulation, NAMP progeny produced significantly more calcium deposits (Fig. 2G, 2H) and expressed 7.8-fold more BSP mRNA (Fig. 2I, $p < .05$).

NAMP Progeny Forms Bone Tissue In Vivo More Efficiently than CFU-F

In order to rigorously assess the differentiation potential of NAMP progeny as compared to that of CFU-f, we determined their in vivo bone-forming efficiency. The constructs loaded with NAMP progeny contained much greater amounts of bone and more uniformly distributed throughout the grafts compared to those formed by CFU-f progeny (Fig. 3C, 3D and 3A, 3B, respectively). Thick matrix and the formation of clear osteocyte lacunae were evident in both conditions and autofluorescence imaging confirmed the deposition of compact collagen fibers in an orderly parallel orientation (Fig. 3B, 3D). Quantitative analysis showed that NAMP progeny generated a 3.1-fold higher amount of bone tissue ($n = 3$, Fig. 3E). The clonogenic fraction, assessed before implantation, was similar in both populations, and thus the number of clonogenic cells loaded on each construct was equivalent for CFU-f and NAMP progeny ($2.2 \pm 0.04 \times 10^5$ and $2.1 \pm 0.1 \times$

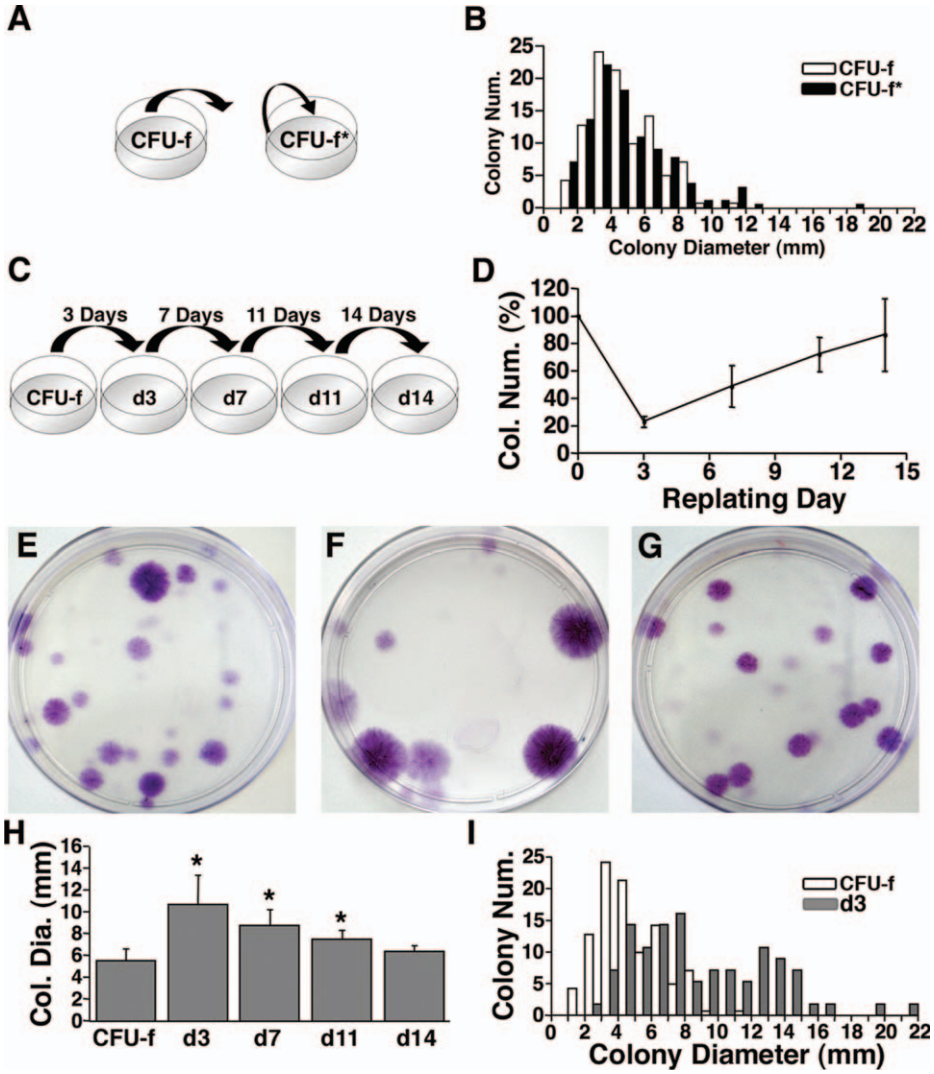

Figure 1. Non-adherent mesenchymal progenitors (NAMP) are intrinsically non-adherent clonogenic cells and can expand through serial replating. **(A):** NAMP were discarded 3 days after plating (CFU-f) or replated in the same dish at every medium change (CFU-f*). **(B):** Analysis of colony diameter distribution (CFU-f = white bar; CFU-f* = black bar). **(C):** Schematic representation of serial replating experiments. **(D):** Number of colonies generated at each replating step. **(E--G):** Representative colonies growing from the CFU-f (E), d3 (F), and d14 (G) populations. **(H):** Average diameter of the colonies generated at each replating step. **(I):** Distribution of diameters of the colonies generated by the CFU-f (white bars) and d3 (gray bars) populations. *, $p < .01$. Abbreviation: CFU-f, colony-forming units-fibroblast.

10^5, respectively). These results indicate an actual increase in bone-forming efficiency and an intrinsically greater osteogenic potency of the progenitors within NAMP progeny compared to CFU-f. Interestingly, hematopoietic marrow was never observed within these ossicles.

NAMP Progeny is Enriched with Early Mesenchymal Populations

We then asked whether the superior differentiation ability of NAMP was related to a specific phenotype. Because of the low frequency of NAMP in the non-adherent fraction, the analysis could be performed only on progeny cells after a minimal expansion on plastic (4 days, $n = 3$). NAMP progeny showed a similar or slightly lower expression of CD73, CD105, CD49a, CD271, CD90, and Nestin (Fig. 4A--4F), which were previously described to identify specific clonogenic subpopulations [14, 27-29]. Neither NAMP nor CFU-f progeny expressed CD31 or CD34 (Fig. 4G, 4H). However, NAMP progeny was enriched with cells expressing CD146 (60% vs. 40%) as well as SSEA-4 (70% vs. 50%) and SSEA-1 (1.2% vs. 0.2%; $n = 2$, Fig. 4I--4N), which were associated with early multipotent mesenchymal progenitors [4, 30, 31].

NAMP Depend on FGF-2 Signaling Through FGFR2c

Since FGF-2 was previously shown to select a subset of MSC with early progenitor characteristics [32], we investigated whether FGF-2 was necessary for NAMP maintenance. Removal of FGF-2 completely abolished the presence of NAMP (Fig. 5A, dashed line), as only 2.9% ± 0.4% of the initial number of colonies was present after 3 days and none could be observed at any following replating step.

We next determined whether NAMP presence may simply require active proliferation of adherent CFU-f. FGF-2 was replaced with PDGF-BB, EGF, or IGF-1, which also efficiently stimulate mesenchymal cell proliferation in culture

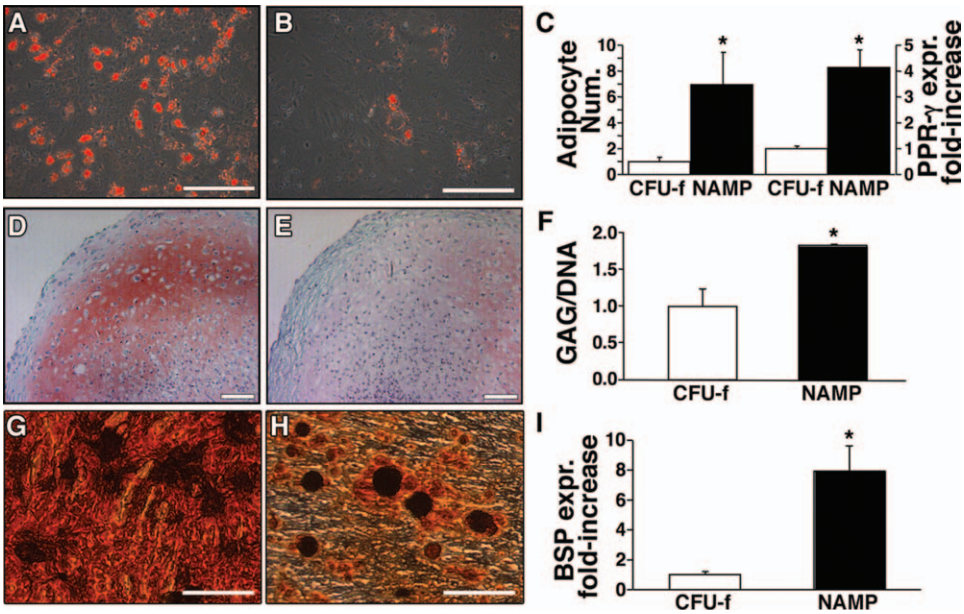

Figure 2. In vitro differentiation of NAMP and CFU-f progeny. **(A, B):** Representative fields of oil red-O staining of NAMP (A) and CFU-f progeny (B, size bar = 200 μm); **(C)** number of adipocytes per field (left side) and PPAR-gamma mRNA expression (right side); **(D, E)** histological sections of cell pellets obtained from NAMP (D) and CFU-f (E) progeny and stained with Safranin-O (size bar = 100 μm); **(F)** deposition quantification of GAG production normalized by DNA content; **(G, H)** alizarin red staining of NAMP (G) and CFU-f (H) progeny; **(I)** quantification of BSP mRNA expression. *, $p < .05$. Abbreviations: BSP, bone sialoprotein; CFU-f, colony-forming units-fibroblast; GAG, glycosaminoglycan; NAMP, non-adherent mesenchymal progenitor; PPAR, peroxisome proliferator-activated receptor gamma.

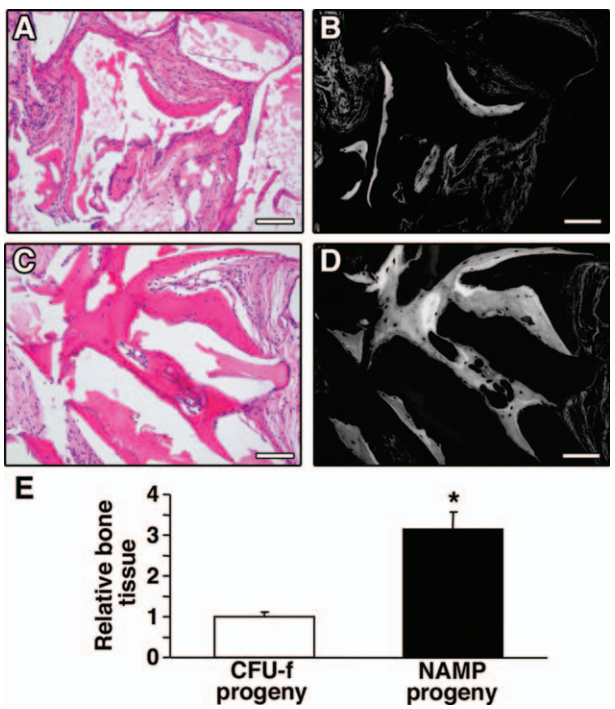

Figure 3. In vivo bone-forming efficiency of NAMP and CFU-f progeny. Representative fields of histological sections of osteogenic constructs loaded with CFU-f **(A, B)** and NAMP **(C, D)** progeny stained with hematoxylin and eosin (A, C) and imaged under fluorescent light (B, D). Size bar = 100 μm. **(E):** Bone tissue formation quantification. *, $p > .05$. Abbreviations: CFU-f, colony-forming units-fibroblast; NAMP, non-adherent mesenchymal progenitor.

[33–35]. However, under those conditions, NAMP disappeared similarly to the condition with serum alone (Fig. 5A dotted line and Supporting Information Fig. S3).

To determine whether FGF-2 was also necessary for the maintenance of NAMP function throughout culture after initial plating, and conversely whether NAMP could be induced by FGF-2 from the initially adherent fraction at a later stage, FGF-2 was removed or added only after the first or second replating steps (3 or 7 days). As shown in Figure 5C ($n = 3$), when FGF-2 was removed after the first 3 days of culture (dash-dotted line), the number of colonies in the following replating steps dropped to zero like in the serum-alone condition (dashed line). When FGF-2 was removed after 7 days (dotted line) instead, the number of NAMP-derived colonies decreased in the following replating steps, but did not disappear completely. No colonies were ever observed when FGF-2 was added after the first 3 or 7 days of culture, suggesting that NAMP depend on FGF-2 for their initial survival and cannot simply be induced later.

FGF-2 is a pleiotropic factor that can bind five different receptors (FGFR1b, FGFR1c, FGFR2c, FGFR3c, and FGFR4) [36]. We investigated which receptor mediates NAMP maintenance in vitro taking advantage of a combined approach with specific blocking monoclonal antibodies against FGFR1b, FGFR2, and FGFR3, and other members of the FGF family of growth factors that activate specifically only FGFR1c/2c (FGF-5) [37] or FGFR4 (FGF-19) [36]. As shown in Figure 5D, in the presence of FGF-19, the number of NAMP-derived colonies was drastically reduced compared to the FGF-2 condition and similar to the no-FGF condition, suggesting that FGFR4 is not involved in NAMP maintenance. Blocking antibodies to FGFR1b and FGFR3 did not significantly reduce NAMP-derived colonies in the presence of FGF-2. However, FGFR2 blocking antibody significantly inhibited the number of NAMP-derived colonies induced by FGF-2 ($p < .05$) to levels similar to the no-FGF condition (p = N.S.), although it did not affect the number of initial CFU-f ($n = 3$, 155.00 ± 13.26 vs. 158.00 ± 10.78 colonies per 10^6 BMNC plated, respectively). Furthermore, by stimulating selectively FGFR1c and FGFR2c with FGF-5, it was possible to induce a number of NAMP-derived colonies similar to FGF-2 (p

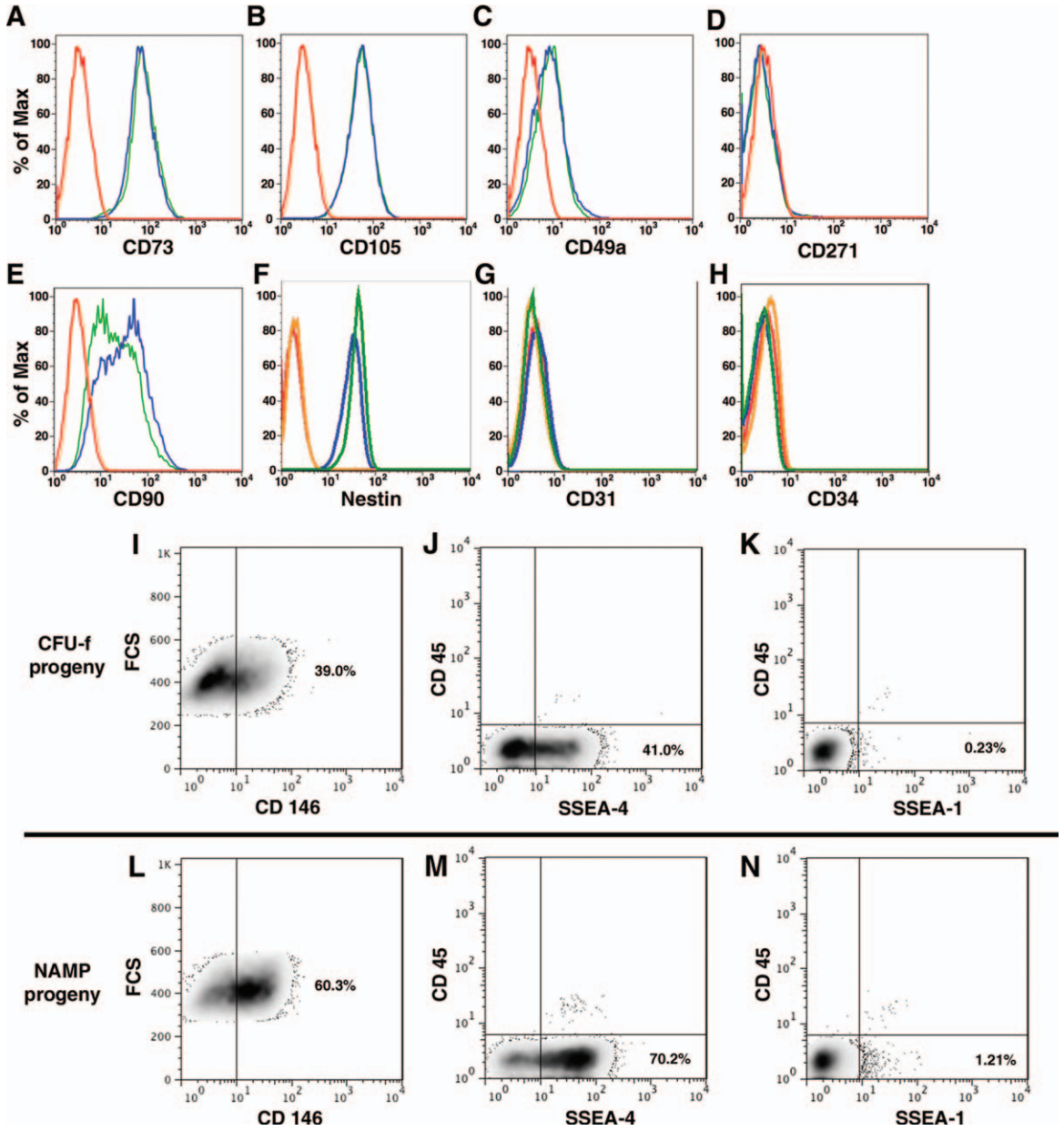

Figure 4. Surface phenotype of NAMP and CFU-f progeny. **(A--E):** Expression of the mesenchymal stem/stromal cell markers CD73 (A), CD105 (B), CD49a (C), CD271 (D), CD90 (E), Nestin **(F)**, CD31 **(G)**, and CD34 **(H)** on the progeny of NAMP (green plots) and CFU-f (blue plots) was evaluated by flow cytometry. Isotype controls for the NAMP and CFU-f progeny are shown by the almost perfectly superimposed orange and red plots, respectively; **(I--N)** expression of CD146, SSEA-4, and SSEA-1 for CFU-f (I--K) and NAMP progeny (L--N). For SSEA-4 and SSEA-1, CD45 expression was also evaluated to exclude contaminating mature hematopoietic cells. Abbreviations: CFU-f, colony-forming units-fibroblast; FCS, forward scatter; NAMP, non-adherent mesenchymal progenitor.

= N.S.). Although these results cannot exclude a role of FGFR1c, they together indicate the involvement of FGFR2c in FGF-2-mediated NAMP survival.

The Adherent BM Fraction Provides a Niche Function for NAMP

We next investigated whether the adherent BM fraction provides a specific niche function to preserve NAMP potential during in vitro expansion. In a loss-of-function approach, 3-day NAMP were transferred to agarose-coated dishes to prevent the formation of adherent colonies for 4 or 11 days ($n = 3$) and no colonies appeared in any condition, indicating that NAMP could not survive in suspension in the absence of an adherent fraction.

In a gain-of-function approach, we hypothesized that, if the BM adherent fraction provided a niche function for NAMP, culturing them together should prevent the gradual loss of their proliferative potential observed during serial replating. Therefore, the non-adherent fraction was maintained in the initial dish for 14 days before replating to a new culture dish ($n = 3$). As shown in Figure 6A, when NAMP were kept on the initially adherent fraction for 14 days (d14*), they generated greater than fourfold more colonies compared to when they underwent serial replating for the same time (d14 = 87.20 ± 9.96 vs. d14* = 373.00 ± 22.59 colonies per 10^6 BMNC plated, equal to 61.0% ± 8.0% vs. 260.8% ± 15.8% of the initial CFU-f, $p < .001$). Furthermore, as shown in Figure 6B, d14* NAMP

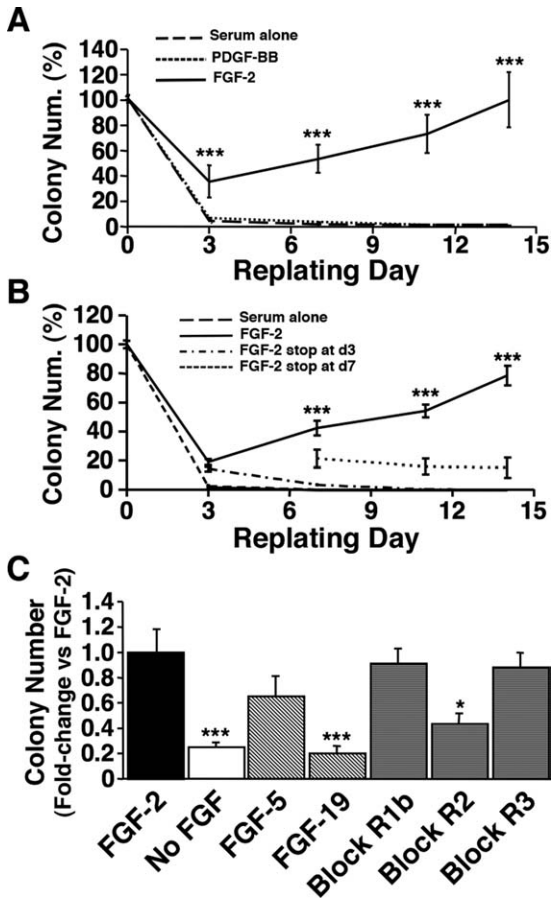

Figure 5. Non-adherent mesenchymal progenitor (NAMP) maintenance in vitro requires FGF-2 signaling through FGFR2c. **(A):** Effect of FGF-2 (black line) and PDGF-BB (dotted line) on NAMP regeneration as non-adherent progenitors upon serial replating (dashed line indicates the condition with serum alone); **(B):** Serial replating experiments performed removing FGF-2 after 3 (dash-dotted line) or 7 days (dotted line); (black line and dashed line represent the condition with FGF-2 and with serum alone, respectively); **(C):** Number of colonies generated by NAMP stimulating or blocking specific FGF receptors; striped and gray bars indicate stimulating and blocking strategies, respectively. *, $p < .05$; ***, $p < .001$. Abbreviations: FGF, fibroblast growth factor; PDGF-BB, platelet-derived growth factor-BB.

generated colonies with an average size much larger than those formed by NAMP serially replated (d14* = 9.8 ± 0.4 mm vs. d14 = 6.8 ± 0.3 mm, $p < .001$) and identical to that of d3 NAMP (9.3 ± 0.5 mm, p = N.S.). Interestingly, Nestin could not be detected in the cells of the non-adherent fraction (data not shown). We further asked whether the adherent BM fraction was specifically required or whether NAMP could be supported by another adherent cell type. After 4 days of culture on human skin fibroblasts, only a minimal number of colonies were generated compared to those formed when NAMP were kept in contact with the initial adherent BM fraction for the same time (2.67 ± 2.31 vs. 101.80 ± 20.28 colonies per 10^6 BMNC plated, $n = 2$) and none at all after 11 days ($n = 2$). These data together indicate that NAMP require specific signals for their maintenance and that the BM adherent fraction provides a niche function.

To investigate whether these signals were provided by mature hematopoietic cells, freshly isolated human BMNCs were sorted according to the expression of CD45. Both the CD45-negative fraction and the unsorted cells were plated at clonal density and the non-adherent fraction was maintained in the initial dish for 14 days before replating to a new culture dish ($n = 2$). The non-adherent fraction of CD45-negative cells generated the same number of colonies as compared to the non-adherent fraction of unsorted cells (Fig. 6C, p = N.S.). However, the colonies generated by the non-adherent fraction of CD45-negative cells were 25% smaller (Fig. 6C, $p < .001$), suggesting that cells of the mature hematopoietic lineage contribute a critical role to preserve NAMP proliferation capacity.

Since FGFR2c was found to be fundamental for NAMP survival in the absence of an adherent BM fraction during serial replating, we investigated whether this receptor was also involved in their maintenance by the niche. As shown in Figure 6D, FGFR2 blockage caused a significant reduction of approximately 35% in the number of colonies compared to the conditions with FGF-2 alone or FGF-2 + heparin ($n = 3$, $p < .001$). Interestingly, the colony size was similar among the three conditions (d14* = 8.22 ± 0.43 mm, d14* + heparin = 8.13 ± 0.3 mm, d14* + FGFR2 blocking = 8.07 ± 0.35 mm, p = N.S.), indicating that FGFR2 signaling regulates NAMP maintenance by the niche but not their proliferation potential.

Finally, we asked whether the niche function is mediated by humoral signals. NAMP were cultured in contact with the niche for 11 days and then kept for 3 days in the absence of an adherent fraction on agarose-coated dishes either with fresh or conditioned medium. Conditioned medium could preserve a significantly higher number of colonies compared to fresh medium (47.21% ± 4.21% and 30.93% ± 1.76% of the number of colonies generated by NAMP kept on the niche, respectively, $p < .05$, Fig. 5E). The fresh medium colonies also showed 15% decrease in diameter, which was prevented by conditioned medium ($p < .001$, Fig. 6E; Supporting Information Fig. S4). These data cannot exclude cell-to-cell interactions but suggest that the BM adherent fraction positively regulates NAMP survival without loss proliferative potential at least in part through secreted signals.

Discussion

The data presented in this study indicate that a specific population of non-adherent mesenchymal progenitors (NAMP) is present in human BM cultures. In particular, NAMP: (a) are a population of mesenchymal progenitors distinct from classic CFU-f with increased proliferation and differentiation potential; (b) establish a niche-progenitor system in vitro with the BM initially adherent fraction, which is required for their self-renewal as non-adherent progenitors; and (c) are dependent on FGF-2 signaling through FGFR2c.

Several lines of evidence indicate that NAMP are a population of mesenchymal progenitors distinct from classic CFU-f. In fact, if NAMP were just later adhering or a subpopulation of the initially adherent CFU-f progeny that became temporarily non-adherent during culture, for example during mitosis, they should: (a) generate colonies with equal efficiency both when replated in the original dish and in a new one and (b) be present in all conditions of active proliferation. Instead, we found that NAMP remain non-adherent and do not form colonies as long as they are maintained in the presence of the initially adherent fraction (Fig. 1), despite the fact that their population continues expanding between 3 and 14 days in these conditions (Fig. 6A) and they are able to efficiently generate colonies twice as large as the initial CFU-f at both time points (Figs. 1F, 6B). Furthermore, NAMP were dependent on FGF-2 signaling and disappeared completely in the presence of serum alone or of other MSC mitogens like PDGF-BB,

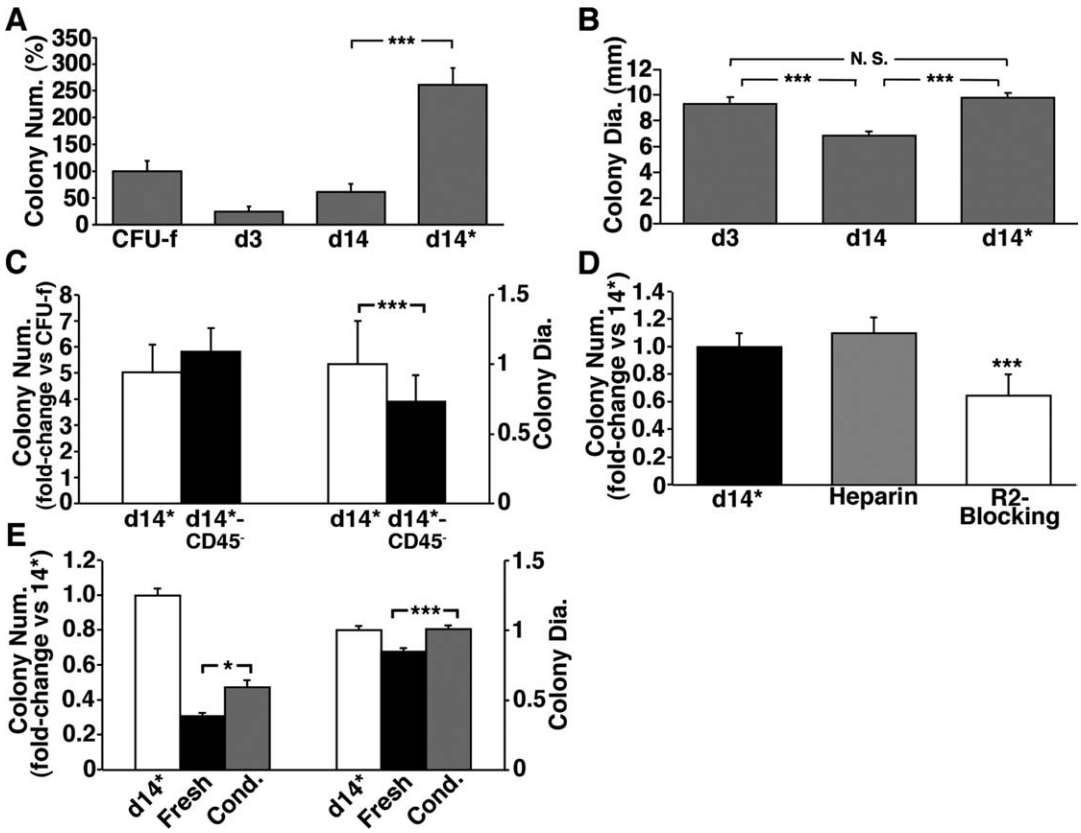

Figure 6. The initially adhering bone marrow fraction provides a niche function for non-adherent mesenchymal progenitor (NAMP). **(A, B):** Number (A) and size (B) of colonies formed when the non-adherent fraction was replated after 3 days (d3), serially replated for 14 days (d14), or maintained in the original dish for 14 days (d14*). The number of colonies is shown as the percentage of those generated in the original dish (CFU-f). **(C):** Number (left side) and diameter (right side, expressed as fold change vs. CFU-f) of colonies generated by the non-adherent fraction of unsorted (white bars) and CD45-negative cells (black bars). **(D):** Number of colonies formed by NAMP kept on the initially adherent fraction with fibroblast growth factor (FGF)-2, either alone (d14*), or with heparin, or with heparin and an FGFR2c blocking antibody (R2 block). **(E):** Number (left side) and diameter (right side, expressed as fold change vs. d14*) of colonies formed by NAMP after culture for 3 days in the absence of an adherent fraction with fresh or conditioned medium, compared with the condition kept on the adherent fraction the whole time. The number of colonies in (C–E) is shown as fold change compared with the control condition (d14*); *, $p < .05$; ***, $p < .001$. Abbreviations: CFU-f, colony-forming units-fibroblast; N.S., not significant.

EGF, and IGF-1 (Fig. 5A, Supporting Information Fig. S3), despite the active growth of colonies generated by the initially adherent CFU-f in both conditions. Even more importantly, readdition of FGF-2 at any time after initial plating could not induce any clonogenic cell in the non-adherent fraction, showing that NAMP are a specific population of mesenchymal progenitors, initially present in BM cultures, and not a proliferating fraction of CFU-f progeny.

BM-derived mesenchymal progenitors are a widely heterogeneous population in terms of both proliferation and differentiation potential [38]. In vitro studies of clonal-derived MSC strains have shown the presence of different classes of progenitors within the CFU-f population in a specific hierarchical relationship, with sequential loss of adipogenic and chondrogenic potential and progressive commitment to the osteogenic lineage [2], and a model has been proposed for their lineage hierarchy in vivo [39]. Our data did not define the possible hierarchical relationship between NAMP and CFU-f, which would require lineage tracing experiments with genetic models, but indicate that NAMP represent a more primitive entity since their progeny possesses a significantly higher proliferative and multilineage differentiation potential [40]. NAMP produced colonies twice as large as CFU-f, indicating a much higher proliferation activity at clonal density, which returned similar to CFU-f only after three serial replating steps. MSC differentiation potential is progressively lost during in vitro expansion, pointing to a gradual commitment of the population [41], limiting their potential for clinical application. CFU-f progeny differentiated into the three typical mesenchymal lineages and formed frank bone tissue upon ectopic implantation in nude mice as described in the literature, but, even though at the first confluence NAMP progeny had expanded 16-fold more (four population doublings), it still differentiated more robustly in all three mesenchymal lineages in vitro and generated bone tissue in vivo three times more efficiently. Moreover, surface marker analysis showed that NAMP progeny was enriched with cells expressing of CD146, SSEA-4, and SSEA-1. CD146 was recently found to be upregulated in tripotent compared to unipotent MSC clonal strains and proposed as a marker of multipotency [4], while SSEA-1 and SSEA-4 have been described to label the most primitive MSC in the BM [30, 31]. Taken together, these data indicate that NAMP possess distinct functional and phenotypic features from CFU-f, which identify them as an earlier class of osteoprogenitors. The identification of a unique set of markers, which could help isolate NAMP and quantify their prevalence in the initial BM cells, still remains a critical need for the exploitation of NAMP potential.

Self-renewal of a progenitor is defined as the ability to divide while generating at least one daughter equivalent to the mother

cell [42]. Our data show that NAMP are able to expand as a non-adherent population in vitro, but that they require the adherent BM fraction for the maintenance of their progenitor properties. Upon serial replating, NAMP were able to regenerate themselves as non-adherent clonogenic progenitors at every step, while at the same time giving rise to an adherent progeny. However, this expansion took place at the cost of a gradual loss of proliferative potential, shown by the stepwise reduction in colony size, until reaching a stage similar to that of initial CFU-f. A similar process takes place during in vitro expansion of skin stem cells, whereby the most primitive progenitors, which give rise to the largest colonies (holoclones) transition to intermediate meroclones and then terminal paraclones, which produce smaller and smaller colonies, as they exhaust their proliferative potential [43]. In the BM, the contact with the specialized microenvironment of the niche regulates HSCs survival and self-renewal [8]. Similarly, when maintained on the initial adherent fraction, NAMP underwent greater expansion compared to when they were serially replated (12-fold vs. 4-fold in 14 days, Fig. 6A) without any loss of clonogenic proliferative potential (Fig. 6B). Clonal studies have recently shown that MSC proliferation potential, and thus colony size, correlates with cell potency, both in healthy and pathologic conditions [4, 44-46]. Colonies with the largest diameter were multipotent, while slow-growing clones had limited differentiation potential [4, 45, 46], suggesting that extensive proliferative ability is a characteristic of early mesenchymal progenitors, whereas it is reduced with lineage commitment. Our data, therefore, suggest that the BM adherent fraction provides a niche function for NAMP, promoting their expansion and self-renewal as early progenitors in vitro. The BM adherent fraction comprises different cell populations of hematopoietic, endothelial, and mesenchymal lineage, one or more of which may establish the niche being responsible to release signals for the maintenance of NAMP in culture. Our data show that cells of the mature hematopoietic lineage are a component of this niche and that its function is at least in part mediated by released signals. Further experiments should aim at investigating whether cell-to-cell contact-dependent pathways are also involved in NAMP maintenance.

We found that, unlike initially adherent CFU-f, NAMP are dependent on FGF-2 both for their initial survival and maintenance in culture. In fact, removal of FGF-2 at different time points during serial replating always caused the disappearance of NAMP and its addition could not reinduce them at a later stage. This is in contrast to the effects on CFU-f, where FGF-2 induces, instead, an immediate and permanent selection of a subset of pluripotent mesenchymal precursors, even after a short exposure or when supplemented only after several days of culture [32]. The different biological response to FGF-2 reinforces the concept that NAMP are a distinct class of progenitors and not a proliferating fraction of the adherent CFU-f. Furthermore, we also showed that FGF-2 controls NAMP function in culture through FGFR2c. Interestingly, gain-of-function mutations of FGFR2c, which is expressed during osteogenesis [47], caused impaired differentiation of BM osteoprogenitors and osteoblasts [48], leading to bone malformations characteristic of the Crouzon and Pfeiffer syndromes in patients [49]. Conversely, in FGFR2c$^{-/-}$ mice, the balance between proliferation and differentiation of skeletal progenitors was shown to be shifted toward differentiation, leading to premature loss of osteoprogenitor self-renewal and early termination of bone formation [50]. These data collectively suggest the intriguing possibility that NAMP may represent the in vitro counterpart of in vivo early mesenchymal progenitors, whose expansion and self-renewal during osteogenesis are also regulated by FGFR2c.

Even though mesenchymal progenitor cells are the subject of extensive interest in regenerative medicine, their regulation in vivo and the need of a specific microenvironment for their maintenance still remains unclear. Sacchetti et al. [14] identified for the first time the presence of self-renewing MSC, which can be selected ex vivo only in the absence of FGF-2. This population is characterized by CD146 expression and is able to form bone, adventitial reticular cells, and the hemopoiesis-supporting niche in vivo, while self-renewing as clonogenic stromal progenitors in a model of intramembranous ossification. Our data suggest an expansion of this model, showing that FGF-2, instead, maintains a different population of progenitors from the common BM pool, the NAMP, which can generate a highly potent progeny and require the adherent BM fraction to provide a niche function supporting their own self-renewal in vitro, but lose the ability to support hemopoiesis in vivo in the same ossification model. In fact, despite an enrichment in CD146+ cells in NAMP progeny, they could not support BM formation in vivo. Moreover, recent data [51] suggest that endochondral ossification is required for the generation of the HSC niche by a specific population of CD105+/Thy1.1− murine osteoprogenitors directly implanted under the kidney capsule, while the CD105+/Thy1.1+ population formed only bone through intramembranous ossification. Our data are in agreement with this report, since NAMP progeny was positive for both CD105 and the human ortholog of murine Thy1.1, CD90 (Fig. 4).

Conclusions

We propose a niche/progenitor organization in vitro, in which the BM adherent fraction can provide a self-renewing microenvironment for primitive NAMP. The identification of NAMP is an important step toward clarifying the hierarchical organization of mesenchymal progenitors and further studies should aim at defining the phenotype of these progenitors and the specific signals provided by their niche. Our study opens the possibility to achieve self-renewing expansion of mesenchymal progenitors in vitro and prompts for the development of strategies to maintain/expand NAMP in culture, for example, based on (a) the maximization of interactions with the adherent BM cell fraction by using three-dimensional culture systems or (b) the presentation of niche-mimicking signals by smart materials. Efforts in this direction may ultimately help to improve the performance of MSC-based tissue-engineered grafts, which is so far rather unsatisfactory in clinical studies [52].

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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