Atrophic nonunion stromal cells form bone and recreate the bone marrow environment in vivo

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Abstract

Introduction: Nonunion is a challenging condition in orthopaedics as its etiology is not fully understood. Clinical interventions currently aim to stimulate both the biological and mechanical aspects of the bone healing process by using bone autografts and surgical fixation. However, recent observations showed that atrophic nonunion tissues contain putative osteoprogenitors, raising the hypothesis that its reactivation could be explored to achieve bone repair.

Methods: Here we characterized atrophic nonunion stromal cells (NUSC) in vitro, using bone marrow stromal cells (BMSC) and osteoblasts as controls of the osteoblastic lineage, and evaluated its ability to form bone in vivo.

Results: NUSC had proliferative and senescence rates comparable to BMSC and osteoblasts, and homogeneously expressed the osteolineage markers CD90 and CD73. Regarding CD105 and CD146 expression, NUSC were closely related to osteoblasts, both with an inferior percentage of CD105<sup>®</sup>/CD146<sup>®</sup> cells as compared to BMSC. Despite this, NUSC differentiated along the osteogenic and adipogenic lineages in vitro; and when transplanted subcutaneously into immunocompromised mice, new bone formation and hematopoietic marrow were established.

Conclusions: This study demonstrates that NUSC are osteogenically competent, supporting the hypothesis that their endogenous reactivation could be a strategy to stimulate the bone formation while reducing the amount of bone autograft requirements.

Keywords: atrophic nonunion, bone, fracture, nonunion stromal cell, osteogenic potential

1. Introduction

The process of fracture healing is influenced by several biological and mechanical factors, as described in the “diamond” concept,[1,2] and leads to the complete restoration of bone anatomy and mechanical function. Nevertheless, the occurrence of nonunion is not uncommon, and represents one of the most challenging conditions in fracture management, as it is related to multiple causing factors.[3–6]

Nonunions are classically classified as atrophic or hypertrophic, according to the biological viability of the bone segment and its mechanical stability.[7] While hypertrophic nonunion is attributed to an inadequate mechanical stability, atrophic nonunion is otherwise related to impairment in healing and vascular responses. Therefore, the current treatment of atrophic nonunion often involves the debridement of the fibrous tissue interposed between the bone ends, followed by eventual revision of fixation implants, and addition of autologous bone grafts as a strategy to augment the biological response.[8,9]

Studies in the field, however, provide evidence that atrophic nonunion is not avascular[10–12] and that the fibrous tissue contains a cell population with a phenotype similar to BMSC, which would be reminiscent of the cells initially recruited to drive the osteogenic response, whose differentiation process was subsequently interrupted.[13–17] In light of these findings, a notion was raised that bone healing could be achieved in atrophic nonunion patients by maintaining the fibrous tissue[18] and stimulating the reactivation of its endogenous cells, which would restart their differentiation program and contribute to new bone formation.[19] However, it remains unclear whether these NUSC[13] are indeed able to resume proliferation and form bone in vivo or if the altered signaling environment to which they were subjected during the course of the failed healing irreversibly affected their function. To address this hypothesis, we isolated NUSC from atrophic nonunion tissues and evaluated their proliferation and differentiation capacities within permissive in vitro and in vivo conditions. A thorough understanding of the properties of NUSC is a critical step to ascertain the validity of keeping nonunion tissue as an adjuvant to stimulate bone repair.

2. Materials and methods

2.1. Patient selection

Fifteen patients of both genders, aged 25 to 65 years, with atrophic nonunion in long bones (representative case in Fig. 1)
were selected and given written informed consent to participate in the study. All procedures were conducted according to the principles expressed in the Declaration of Helsinki. Nonunion was defined by the lack of bone healing after 9 months of the fracture. Radiographic evaluations were performed in 2 consecutive orthogonal x-rays taken within a 3-month interval. Patients with infected fractures, rheumatoid arthritis, renal or hepatic failures, and drug and/or alcohol abuse were excluded. Sample collection and use in this study was approved by the Institutional Ethics Committee (CAAE: 23348613.0.0000.5273). Patient demographics are shown in Table 1.

2.2. Biological samples

Sample collection was performed during treatment surgery. After exposure of the nonunion site, the fibrous tissue interposed between the bone ends was excised, along with adjacent osseous fragments of approximately 1 cm³ each (Fig. 2). In all patients, appropriate fracture fixation was performed, in association with autologous bone grafting obtained from the iliac crest. Nonunion tissue, fracture-adjacent cortical bone, and exceeding fragments of iliac crest trabecular bone containing marrow were collected for further analysis.

2.3. Cell isolation and expansion

NUSC[13] and osteoblasts were isolated from atrophic nonunion tissues and cortical bones, respectively. Collected tissues were washed with phosphate buffer saline (PBS) and dissected into small fragments. For bone samples, only the extremity facing the unaffected tissue was used. The fragments were subsequently digested with 1% collagenase (Sigma-Aldrich, St. Louis, MO) for 2 hours at 37°C. Harvested cells were centrifuged at 300 x g for 10 minutes and resuspended in Dulbecco’s modified Essential Medium (DMEM low-glucose, Gibco, Grand Island, NY) supplemented with 10% lot-selected fetal bovine serum (FBS, Gibco) and 10 μg/mL ciprofloxacin (Fresenius Kabi, Barueri, SP, Brazil). A total of 1.0 x 10⁶ cells were plated in 75 cm² flasks and let to grow until 70% confluence in a humidified atmosphere of 5% CO₂ and 37°C. Harvested cells were centrifuged at 300 x g for 10 minutes and resuspended.

BMSC were isolated as previously described, with minor modifications.[21,22] Briefly, iliac crest trabecular bone fragments were mechanically triturated; the bone marrow was resuspended and homogenized in PBS, and then allowed to stand for 30 seconds to enable sedimentation of bone spicules. The supernatant was collected and centrifuged at 300 x g for 10 minutes. Cells

Table 1

| Patient | Age | Gender | Affected bone | Comorbidities                     | Time of fracture, months |
|---------|-----|--------|---------------|----------------------------------|---------------------------|
| 1       | 31  | M      | Tibia         | No                               | 30                        |
| 2       | 57  | F      | Humerus       | No                               | 22                        |
| 3       | 42  | M      | Humerus       | No                               | 20                        |
| 4       | 58  | F      | Humerus       | Hypothyroidism                   | 9                         |
| 5       | 65  | F      | Femur         | No                               | 36                        |
| 6       | 52  | M      | Tibia         | Hypertension                     | 24                        |
| 7       | 25  | M      | Femur         | No                               | 24                        |
| 8       | 34  | F      | Humerus       | Hypertension, diabetes           | 32                        |
| 9       | 49  | F      | Ulna          | No                               | 16                        |
| 10      | 31  | M      | Humerus       | No                               | 120                       |
| 11      | 64  | F      | Femur         | No                               | 48                        |
| 12      | 49  | M      | Tibia         | No                               | 24                        |
| 13      | 54  | M      | Femur         | No                               | 13                        |
| 14      | 37  | M      | Humerus       | No                               | 32                        |
| 15      | 48  | M      | Humerus       | No                               | 63                        |
were resuspended in DMEM supplemented with 10% FBS and 10 μg/mL ciprofloxacin and plated at a density of 4.0 × 10⁴/mL in 75 cm² flasks. After 3 days at 37°C, nonadherent cells were discarded, and adherent cells were washed with PBS. The medium was changed, and cultures were allowed to grow for 11 additional days. After harvest by enzymatic digestion, cells were expanded until passage 3. Due to the low amount of tissue that could be collected from some patients, analysis could not be performed with all 3 cell types (BMSC, osteoblasts, and NUSC) isolated from the same donor in every experiment.

2.4. Histological evaluation of nonunion tissue
Nonunion tissue histology was evaluated through hematoxylin and eosin (H&E) staining. Briefly, tissue fragments were fixed with 10% buffered formalin, embedded in paraffin, sectioned, stained with H&E, and photographed using a Nikon E600 microscope (Nikon, Tokyo, Japan) equipped with a digital camera.

2.5. Population doubling
At each passage, the number of population doublings (PD) was calculated using the formula: PD = (Log Nf − Log Ni)/Log 2, where Nf is the harvested cell number and Ni is the initial cell seeding number. The cumulative PD was calculated at the end of passage 3 by summing each passage’s PD, that is, cPD = PD1 + PD2 + PD3. The doubling time (dT) was obtained by dividing the time, in days, required for cells to reach the end of passage 3 by the cumulative PD (dT = Δt/cPD).

2.6. Cell senescence
The percentage of senescent cells was assessed by β-galactosidase staining at pH 6.0. Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature and stained with a solution of 1 mg/mL X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galacto-pyranoside, Sigma), 5 mM potassium ferrocyanide, 2 mM magnesium chloride, and 150 mM sodium chloride in 0.2 M citric acid, 0.06 M sodium phosphate buffer for 24 hours at 37°C. Photomicrographs of 15 random fields were taken with an inverted microscope (Nikon Eclipse TS100, Nikon, Tokyo, Japan) equipped with a digital camera. The number of positive cells, identified by a blue staining in the cytoplasm, was counted in relation to the total number of cells per field of view.

2.7. Flow cytometry
A total of 1.0 × 10⁶ passage 3 cells were incubated per tube with PerCP Cy5.5-conjugated anti-CD90, APC-conjugated anti-CD73, FITC-conjugated anti-CD105, and PE-conjugated anti-CD146 antibodies (all from BD Biosciences, Franklin Lakes, NJ) for 30 minutes, at room temperature, and protected from light. Data were acquired using a BD Accuri flow cytometer (BD Biosciences) and analyzed with the CSampler Accuri Software (BD Biosciences).

2.8. Osteogenic differentiation and Von Kossa staining
At the end of passage 3, cells were plated at a density of 2.5 × 10⁴/cm² in 24-well plates in the expansion medium and allowed to grow until total confluence. Osteogenic differentiation was induced by incubation with DMEM 10% FBS and antibiotics, containing 10 mM β-glycerophosphate, 5 μg/mL ascorbic acid 2-phosphate, and 10⁻⁶ M dexamethasone (all from Sigma) for 21 days, with medium change at every 3 days. After this period, cell monolayers were fixed with 4% paraformaldehyde for 10 minutes at room temperature and incubated with a 2% silver nitrate aqueous solution for 1 hour, protected from light. Cells were washed 5 times with distilled water and exposed to UV light for 10 minutes. The quantification of mineralized areas was performed in 15 photomicrographs of random fields using the Image J software (https://imagej.nih.gov/ij/).

2.9. Adipogenic differentiation and Oil Red O staining
At the end of passage 3, cells were plated at a density of 2.5 × 10⁴/cm² in 24-well plates in expansion medium and allowed to grow until total confluence. Adipogenic differentiation was induced by incubation with DMEM 10% FBS and antibiotics, containing 0.5 mM isobutylmethylxanthine, 200 mM indomethacin, 10 mM insulin, and 10⁻⁶ M dexamethasone for 21 days (all from Sigma).
Lipid accumulation was assessed by Oil Red O staining. After fixation with 4% paraformaldehyde for 10 minutes at room temperature, monolayers were washed with propylene glycol P.A and incubated with 0.5% Oil Red O solution in propylene glycol for 20 minutes. After washing 2 times with 85% propylene glycol solution, monolayers were photographed using an inverted microscope to evaluate the presence of stained lipid-accumulating cells.

2.10. In vivo transplants

The analysis of in vivo heterotopic bone formation was performed as described in previous studies,[21,24,25] with minor modifications. Briefly, 1.0 × 10⁶ cells at passage 3 were suspended in 1 mL of expansion medium and incubated overnight with 40 mg hydroxyapatite/tricalcium phosphate powder (HA/TCP, Osteoset T, Wright Medical, Arlington, TN) in 1.5 mL tubes at 37°C. On the following day, the supernatant was carefully aspirated and 15 µL of 3.2 mg/mL human fibrinogen and 100 U/mL human thrombin were added (both from Sigma). After 30 minutes of incubation, the cell/HATCP mixture was collected and transplanted subcutaneously into the flank of immunocompromised mice (BALB/c n-u/n beige, IPEN, Sao Paulo, SP, Brazil), aged between 6 and 8 weeks. Animal use was approved by the institutional Animal Care and Use committee (protocol number 002/2014). Surgeries were performed under general anesthesia with intraperitoneal injections of 80 to 100 mg/g ketamine hydrochloride and 10 mg/kg xylazine. After 12 weeks, the mice were euthanized by deep anesthesia; the transplants were harvested and stained with hematoxylin-eosin technique for subsequent histological analysis.

2.11. Statistical analysis

Statistical analysis was performed with GraphPad Prism software (GraphPad software, La Jolla, CA, version 7.0). Data were compared using ANOVA One-Way and Tukey multiple comparison post-test. Values were expressed as mean ± SD. Values of \( P \leq 0.05 \) were considered significant.

3. Results

The histological appearance of the excised tissue was consistent with atrophic nonunion, consisting of connective tissue with a dense collagenous extracellular matrix, populated by fibroblast-like cells, and areas of vascularization (Fig. 3).

Following the isolation of the NUSC, we first performed a cellular characterization of this population, using BMSC and osteoblasts—two known distinct stages of osteogenic commitment—as positive controls. Similar to BMSC and osteoblasts, NUSC adhered to plastic dishes and had a fibroblastic morphology in vitro (Fig. 4A–C).

Analysis of NUSC proliferation showed that the time required to this cell population to double in number was, in average, 7.8 ± 3.8 days, which was not statistically different from BMSC (5.4 ± 1.8 days) and osteoblasts (9.0 ± 5.1 days) (Fig. 4D). To evaluate whether NUSC had a limited proliferation span as a result of the experienced failed healing conditions, the number of cells entering senescence during in vitro expansion was quantified. It was observed that the percentage of cells staining positive for β-galactosidase activity in NUSC cultures was comparable to those observed in BMSC and osteoblasts (Fig. 4E), indicating that NUSC could sustain proliferation to the same extent as the...
control lineages. Next, to certify that NUSC belonged to the osteoblastic lineage, we performed an immunophenotypic characterization, using the surface markers commonly expressed by osteogenic cells. Similarly to BMSC and osteoblasts, NUSC homogeneously expressed CD90 and CD73 (Fig. 5A–E).

However, the percentage of cells expressing CD105 in NUSC populations was significantly lower in comparison to BMSC (13.56% ± 8.86% vs 50.26% ± 21.64%, respectively), and similar to that of osteoblasts (28.98% ± 17.59%) (Fig. 5F). This indicates that NUSC mostly contained cells with a phenotype...
more closely related to the mature osteoblast stage. To further evaluate this finding, we then quantified the expression of CD146, a marker expressed by multipotent osteoprogenitors.

While BMSC cultures had an average percentage of 53.59% ± 39.81% of CD146+ cells, osteoblasts and NUSC had 4.0% ± 6.35% and 2.12% ± 2.94% of positive cells, respectively (Fig. 5G). When evaluating the percentage of cells simultaneously expressing both markers, NUSC had 3.78% ± 4.0% of CD105+/CD146+ cells, while osteoblasts and BMSC had 0.77% ± 0.9% and 39.6% ± 25.7%, respectively (Fig. 5H). Collectively, these results confirmed that NUSC indeed contained cells of the osteoblastic lineage, whose surface markers profile resembles that of cells in late-stage differentiation.

Next, we evaluated the ability of NUSC to respond to differentiation stimuli in vitro. Besides being able to differentiate into adipocytes (Fig. 6A–C), NUSC also deposited mineralized matrix positive for Von Kossa (Fig. 6D–F), similarly as BMSC and osteoblasts (Fig. 6G). To further confirm the ability of NUSC to differentiate and form bone in vivo, we next transplanted the cells into the subcutaneous of immunodeficient mice. After 12 weeks, histological examination of BMSC, osteoblasts, and NUSC implants confirmed the formation of ossicles from all cell types, with interconnecting bone matrix deposited over the surface of hydroxyapatite/tricalcium phosphate (HA/TCP) particles (Fig. 7B–D). Within this matrix, osteocytes were seen inside lacunae (Fig. 7B–D, arrowheads), indicating the viability of the new bone. In addition, the implants also contained cavities filled with hematopoietic cells (Fig. 7B–D, asterisks), demonstrating the ability of NUSC to also form a marrow-supportive stroma—therefore completely reconstituting the bone as an organ. Collectively, these results demonstrated that NUSC had a preserved differentiation potential.

4. Discussion

Nonunion is a challenging condition to treat and a major orthopaedic concern. Although it is known that the success of fracture healing depends on several factors, including appropriate mechanical stabilization, availability of blood supply, and the extent of injury to bone itself and to the surrounding soft tissues, the main biological determinants of nonunion are still unclear. Therefore, surgical interventions to manage this condition focus on stimulating both the biological and mechanical aspects of the healing process, which are currently pursued by using bone autografts and fixation revision.

A more recent line of thinking among orthopaedic surgeons, however, states that the majority of nonunions occur due to mechanical instability, and consequently, high strain at the fracture site. If this is so, it is argued that the nonunion will heal if the mechanical environment is corrected by surgery, with no need to excise the nonunion tissue or add autografts. This study is based on the concept that the tissue which is formed in the fracture site maintains its biological functions, despite the failed healing process.

Indeed, previous studies have shown that the nonunion tissue contained cells with similar characteristics as BMSC, from which emerged the idea that nonunion stromal cells could be reactivated in vivo to act as an adjuvant to stimulate bone formation. Nevertheless, none of these previous reports effectively evaluated whether nonunion cells were really capable of making bone in vivo. As nonunion cells experienced failed bone healing conditions, one could wonder that their proliferation and osteogenic capacities might have been irreversibly lost. To evaluate this hypothesis—the main objective of this study—we isolated the cells from atrophic nonunion tissues, to evaluate cell activity exempt of the confounding factors from the fibrous tissue environment.

Because bone marrow stroma is composed by several cell types, including osteoblasts, adipocytes, reticular cells, fibroblasts, and osteoprogenitors in several different commitment stages, and the identity of these distinct classes of osteoprogenitors are not yet fully known, in this study we used BMSC and osteoblasts—the best-known differentiation stages of the osteoblastic lineage—as parameters to characterize the isolated NUSC. The characterization step was of fundamental importance to certify that NUSC were indeed osteoprogenitors and not mere marrow fibroblasts.
Here we showed that, in vitro, NUSC had proliferative and senescence rates comparable to BMSC and osteoblasts, and homogeneously expressed the markers CD90 and CD73. However, the expression of CD105 and CD146 in NUSC was more closely related to that of osteoblasts, and significantly inferior in comparison to BMSC. In spite of this, NUSC differentiated along the osteogenic and adipogenic pathways in vitro, and when transplanted in vivo, formed ossicles displaying hematopoietic marrow, with the ability to host and support hematopoiesis.

Comparing the in vitro properties of NUSC versus BMSC isolated from healthy donors undergoing spinal fusion surgery, Bajada and co-workers reported that NUSC had a doubling time—a measure of how long a given cell culture takes to double in number in vitro—of 12 to 16 days and that the percentage of senescent cells in the cultures increased during cell culture expansion, in a way independent of patient’s age. In this study, however, NUSC doubling time was similar to that of BMSC and osteoblasts. Also, NUSC did not senesce over time. We attribute these differences to the fact that Bajada and collaborators compared NUSC to BMSC isolated from healthy donors, while we compared NUSC to BMSC and osteoblasts also isolated from nonunion patients. Evidence indicates that nonunion patients have polymorphisms in genes that regulate cell proliferation, such as FGFR1. Although a polymorphism does not directly dictate a loss of function, we cannot rule out the possibility that cells from nonunion patients could have a slower proliferation rate as compared to cells isolated from healthy subjects. In agreement with our findings, Takahara and colleagues showed that cells isolated from synovial pseudarthrosis, defined as an end-stage nonunion, could be expanded in vitro for 10 passages with minimal decline in their initial proliferative capacity. Therefore, we concluded that NUSC was not a senescence-prone population and was able to proliferate, under its own intrinsic rate, when appropriate signaling conditions were provided.

Regarding cell surface protein expression, 2 reports showed that NUSC expressed the BMSC-related markers CD29, CD44, CD166, and CD105. However, none of these markers are specific of osteoprogenitors. Here we chose to characterize the immunophenotypic profile of nonunion cells using the BMSC-related markers recommended by the International Society of Cellular Therapy. We also included CD146, which was shown to be expressed by the multipotent subpopulation that reside within the total BMSC fraction. CD146 expression had not been previously evaluated in NUSC populations. We observed that along with CD90 and CD73, only a small fraction (<10%) of NUSC was CD105+/CD146+. Considering that CD146 expression decreases as multipotent cells progress down the differentiation cascade toward osteoblasts, what was indeed confirmed in our BMSC and osteoblast cultures—we concluded that NUSC mostly contained cells that already progressed on the line of differentiation and were closer to the mature osteoblastic stage. This raised the hypothesis that the signaling disfunction that lead to the interruption of the healing process might have occurred after osteoprogenitors had been recruited to the fracture site and had initiated differentiation. Further transcriptomic profiling of NUSC would certainly contribute to confirm this question.

Figure 7. In vivo bone-forming potential. HA/TCP-empty control (A), BMSC (B), osteoblasts (C), and NUSC (D) were transplanted subcutaneously into immunocompromised mice. At 12 weeks after implantation, like BMSC and osteoblasts (C), NUSC (D) formed heterotopic ossicles containing hematopoietic marrow, a condition required for continued bone remodeling. (*) Marrow space; (arrowheads) osteoblasts contained within lacunae. H&E staining. Representative images of n = 3 experiments, performed with cells isolated from different donors, in quadruplicates. Scale bars: 50 μm.
After contributing to bone/marrow reconstitution, CD146+ multipotent cells are recruited back to perivascular niches, where they reside and are maintained at a quiescent state.\textsuperscript{[36,43]} Therefore, even in a small percentage, the remaining presence of CD146-expressing cells in NUSC indicated that nonunion tissues still contained multipotent cells. Indeed, when challenged in an in vivo environment, NUSC not only formed bone, but also established a normal hematopoietic marrow, thus confirming its potential to contribute to bone remodelling—a process strictly dependent on a functional bone marrow microenvironment.\textsuperscript{[44–46]}

In vivo transplantation assays have been strongly requested by experts of the bone biology field to reliably confirm the differentiation capacity of a given cell population.\textsuperscript{[25,30,32,37,47]} It has been shown that the in vitro differentiation assay is highly artifactual, and often confuses dystrophic calcification with the cells’ ability to form histology-proven bone.\textsuperscript{[23,32,37]} In this way, we considered it imperative to evaluate the capacity of NUSC to form bone through an in vivo assay, which had not been previously addressed by any study regarding NUSC properties.\textsuperscript{[13,14,16,28,29]} Given that NUSC from different donors formed bone in vivo in all experiments performed, we provided concrete evidence that NUSC were resting in the nonunion tissue due to an impaired environment, and not because they were inadequate cells. This means that once provided the right stimuli, NUSC can resume proliferation and differentiate to form bone.

This observation brings important new perspectives both in the clinical scenario and in nonunion etiology research. First, it supports the thesis of some clinicians that the mechanical environment is the strongest determinant of nonunion, and should, therefore, base the treatment.\textsuperscript{[18,19]} In other words, according to this notion, management of nonunion should rely on minimally invasive mechanical restitution of the fracture, in order to reduce the level of local strain, with no need to remove the nonunion tissue.\textsuperscript{[18]} Bone grafts or any biological adjuvants would only be applied to those cases where significant bone loss occurred.\textsuperscript{[18]} As in this context, the biological vector of the healing process is also severely compromised.

Then, considering the diamond concept proposed by Giannoudis and colleagues,\textsuperscript{[2,48]} the maintenance of nonunion tissues in critical-sized bone defects could be positive in 2 aspects: act as a scaffold to exogenously added cells and contribute with osteogenic cells that can help in bone formation. Following this strategy, the amount of bone graft required to fill the fracture gap and stimulate bone consolidation would be reduced. As the availability of bone autografts is often scarce and associated with a high donor site morbidity,\textsuperscript{[49,50]} the use of any adjuvant that could reduce the requirement of autografts is of great interest.

Finally, from the biological point of view, the knowledge that NUSC are able to form bone indicates that future studies should focus on the identification of specific signaling cues that could endogenously reanimate, that is, stimulate the proliferation and/or differentiation of the in situ NUSC, marrow, and periostial osteoprogenitors. Besides the mechanotransduction signaling provided by fixation revision, this reactivation could be achieved by providing specific growth factors and/or cytokines involved in bone formation. But unfortunately, a specific (or a combination of) signaling factor that can endogenously promote bone formation in a controllable and safe way is, at present, not known. To a certain extent, this has already been achieved with the percutaneous injection of bone marrow concentrates in the nonunion site.\textsuperscript{[51–55]} However, marrow preparations have the drawback of not having a homogeneous and reproducible composition of either signaling factors or cell types. Therefore, this strategy does not allow the predictability of results. Finding a strategy that combines the critical mechanical and biological healing-restoring components will be the next fundamental challenge for the development of less invasive and more effective treatments for nonunion.

### 5. Conclusions

The findings of this study support the view that the cells contained in atrophic nonunion tissues have preserved proliferation and osteogenic potentials; and reinforce the notion that the nonunion tissue could be maintained during fracture treatment to reduce the requirement of bone grafts and to explore the osteogenic potential of its endogenous cells, that once reactivated, would contribute to bone/marrow repair.

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