

Existence of multilineage mesenchymal progenitor cells in human atrophic nonunion tissue

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Research article

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Abstract

Background : Though the treatment of atrophic nonunion is often challenging, bone union may occur in some patients with atrophic nonunion who underwent indirect treatment at the docking site. We aimed at explaining the reasons why does this phenomenon appear?

Methods : Five patients diagnosed with atrophic nonunion were enrolled in our study. Nonunion tissues were cut into strips and cultured immediately after being obtained. We got the adherent cells from atrophic nonunion tissues, and applied the flow cytometry to assess the expression of different cell-surface protein. The research in differentiation of the adherent cells was carried out under the induction of various lineage-specific factors, and Western-blot analysis was used to measure the differentiation-related protein expression.

Results : We found that the adherent cells from atrophic nonunion tissues have the characteristics similar to bone marrow stromal cells. These traits demonstrated by the flow cytometry consist of positive expression of markers CD29 and CD44, but negative expression of CD34 and CD45. The adherent cells could differentiate into osteogenic, chondrogenic, and adipogenic cells under the different lineage-specific induction factors in vitro. Alkaline Phosphatase (ALP), Col α , osteocalcin (OC), SOX9, lipoprotein lipase (LPL) and PPAR- γ 2 were high expressed, as measured by Western-blotting.

Conclusions : Mesenchymal stem cells obtaining from atrophic nonunion tissues have the potential of transforming into cartilage and bone-forming cells. Furthermore, we get conclusion that the atrophic nonunion tissues play an important role in the healing process.

Background

Fracture nonunion is a major cause of chronic disability and could generate a heavy burden to families and society [1–3]. There are about 5.6 million bone fractures in the United States every year, and the rate of failed and required further treatment fracture is up to 10% [4]. Customarily, aseptic nonunion is routinely classified into atrophic nonunion and hypertrophic nonunion basing on radiographic [5, 6]. The main features of atrophic nonunion consist of lacking the callus and poor vascularity at the fracture site [7]. Traditionally, the surgery is the most common treatment for atrophic nonunion, including decortication and stabilization together with bone grafting to stimulate vasculogenesis and osteogenesis [8]. The nonviable bone and nonunion tissues are required to be removed [5, 6]. The atrophic nonunion with bone defects were treated by bone transport technique and achieved successful outcomes as literature reported [9, 10]. However, they did not expose the docking sites to remove the nonunion tissues and did not use bone graft in these operations [11, 12]. The radiographic results of hypertrophic nonunion revealed having hypertrophic callus formation, which were considered to be biologically active [4]. Furthermore, most patients with hypertrophic nonunion were successfully treated by simple stabilization of the docking site without cleaning the nonunion tissues [13, 14]. Why the bone union occurs in some

nonunion patients with indirect treatment at docking site? There is little known regarding the biological reasons and molecular pathogenesis of nonunion [2, 15].

The nonunion cells (NCs), which are derived from nonunion tissues, reserved their ability to respond to bone morphogenetic protein in vitro similar to mesenchymal cells [16]. As we all known, hematomas were formed at the fracture site when a fracture occurs, and then hematomas were organized into granulation tissue, and transformed to nonunion tissue if the bone nonunion occurs. Some literatures have demonstrated that the hematomas at the fracture site contain multilineage mesenchymal progenitor cells, and these cells are key factors in bone healing [17–19]. Therefore, some authors hypothesized that one reason why bone union occurs with indirect treatment at docking site is that mesenchymal progenitor cells maybe reserved in nonunion tissue [4, 20]. The NCs of hypertrophic nonunion have the multilineage differentiation potential in vitro, which were first demonstrated by Iwakura T and their colleagues [4]. Similar to bone Mesenchymal Stem Cells (BMSCs), the NCs from hypertrophic nonunion can differentiate into osteogenic, chondrogenic, and adipogenic cells under the different lineage-specific induction factors in vitro [4]. Though previous studies suggested that the tissues in atrophic nonunions were avascular [7], some studies shown that the tissues at the atrophic nonunion were well vascularized and the number of blood vessels in atrophic nonunion reaches the same level as that in healing bone [21, 22]. The NCs from atrophic nonunion tissue of rats were proved to have osteogenic potential in vitro [22]. Mesenchymal stem cells (MSCs) in the atrophic nonunion site were discovered by Ismail HD et al. [23], but they did not make a further study to assess the differentiation capability of these cells. Therefore, there is no reports of detailed cellular analysis of the NCs from human atrophic nonunion. To study the NCs from the atrophic nonunion, we conduct the experiment as Iwakura T et al. [4] reported.

In present study, we targeted the NCs from human atrophic nonunion and examined whether NCs from atrophic nonunion have the ability of multilineage mesenchymal differentiation in vitro.

Materials And Methods

Study participants

This study involved five consecutive patients with long bone atrophic nonunion (Table 1, Fig. 1). The participants' average age was 25.4 years (range, 18 to 34 years). There were four males and one female, and the sites of nonunion were tibias in three and the femurs in two. One patient underwent initial surgery using intramedullary locking nail, and the four other patients underwent initial treatment with plate-and screw fixation. There is no evidence that patients have become infected after surgeries. The average interval time between the first and the current surgery for nonunion treatment was 21.0 months (range, 16–27 months). We have excluded the patients with infections, tumors, or some systemic bone-related diseases, or patients who underwent treatment with hormones, steroids, and someone with alcohol abuse, but treatment with nonsteroidal anti-inflammatory drugs is allowed for patients [4]. We obtained some nonunion tissues from the enrolled patients during surgery. All atrophic nonunion were healed at last follow-up through our surgical treatment method [11, 24]. This study was ethically approved by our

hospital committee (No.2010-S02) and written informed consent was obtained from the patients or their legal guardians.

Table 1
Patients' data

Case	Gender	Age (years)	Nonunion site	Duration from fracture (months)	Results
1	male	18	femur	18	Union
2	female	25	tibia	16	Union
3	male	21	tibia	23	Union
4	male	29	femur	21	Union
5	male	34	tibia	27	Union

Isolate and culture human NCs

We got a small amount of nonunion tissues at the nonunion sites after exposing the nonunion sites at the current operation. The nonunion tissues were obtained with attention paid to avoiding contaminating the bone, periosteum, and muscle after exposing the nonunion sites adequately. The NCs were isolated by the method as the previous study carried out [4, 25]. Then we cut the nonunion tissues into small pieces after washing them with phosphate-buffer saline (PBS). The cells were cultured on 100 mm diameter culture dish with complex medium, including α -Modified Minimum Essential Medium (Sigma, St. Louis, MO, USA), 10% heat-inactivated fetal bovine serum (FBS; Sigma), 2 mM L-glutamine (Gibco, Carlsbad, CA, USA), and antibiotics. Finally, the cells were incubated at 37°C with 5% humidified CO₂. The nonviable cells and debris were removed at the seven days after initial incubation. Thereafter, we changed the culture medium twice a week. About two or three weeks later, we obtained the adherent cells with 0.05% trypsin-0.02% Ethylene Diamine Tetraacetic Acid (EDTA) (Sigma, St. Louis, MO, USA), and these adherent cells were preserved to passage for further expansion. The cells from passage three or four were widely applied in the following differentiation assay. All experiments were performed with all the five samples.

Flow cytometry analysis

After the first passage, we had harvested the adherent cells from nonunion tissues. The cell-surface protein expressions of NCs were evaluated using the method of flow cytometry. In order to make a comparison with BMSCs, BMSCs were kindly provided by our hospital laboratory and were cultured in the same medium like NCs' conditions. The cells were washed with PBS-3% FBS for several times, and then were incubated with phycoerythrin (PE)-conjugated anti-mouse antibodies for 30 minutes at 4°C in the dark, which includes CD29, CD34, CD44, and CD45 (BD Biosciences, San Jose, CA, USA). We applied the nonspecific mouse PE-conjugated IgG (BD Biosciences) as an isotype control. After the incubation, we have washed the cells with PBS-3% FBS twice, and applied the facsaria flow cytometry system (BD

Biosciences) and CellQuest Pro (BD Biosciences) for data analyses. The positive staining of cells was scored as literature reported [19]. At least 10000 list mode events were collected for each sample.

Differentiation studies

Osteogenic induction

Aiming to induce osteogenic differentiation, we cultured the NCs under an osteogenic medium for three weeks, which have added 10 nM dexamethasone (Sigma), 10 mM β -glycerophosphate (Sigma), and 50 mg/mL ascorbic acid (Sigma) into the original medium [4]. However, we cultured the control NCs under the original medium without the osteogenic medium. Three weeks later, we evaluated the calcium deposition which is one common characteristic of osteogenic differentiation, and made a photo record of the cells staining after staining with 1% Alizarin Red S (Sigma).

Chondrogenic induction

Aiming to induce chondrogenic differentiation, we applied the three-dimensional culture structure, and constructed a pellet after centrifuging cells in the polypropylene tube at 2000 rpm for 4 minutes [4]. We cultured the NCs under an chondrogenic medium, which have added 100 nM dexamethasone, 50 ug/mL L-ascorbic acid-2-phosphate (Sigma), 0.4 mM proline (Sigma), 1% ITS⁺1(Sigma), 10 ng/mL recombinant human transforming growth factor (TGF)- β 3 (R&D Systems, Minneapolis, MN), and 500 ng/mL recombinant human bone morphogenetic protein (BMP)-6 (Sigma) into the original medium [4]. However, we cultured the control NCs under the original medium without chondrogenic medium. Three weeks later, chondrogenic differentiation was evaluated by staining with toluidine blue (Sigma) and the cells staining were photographed.

Adipogenic induction

Aiming to induce adipogenic differentiation, we cultured the NCs under an adipogenic medium for three weeks, which have added 1 uM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 10 mg/mL insulin (Sigma), 0.2 mM indomethacin (Sigma) into the original medium [4]. However, we cultured the control NCs under the original medium without adipogenic medium. Three weeks later, we evaluated the cellular accumulation of neutral lipid vacuoles which is one common characteristic of adipogenic differentiation, and made a photo record of the cells staining after staining with Oil-red O (Sigma).

Western blots analysis

Aiming to evaluate protein expressions which were related to each differentiation events, we obtained the differentiated and undifferentiated NCs. The NCs were collected and washed with PBS three times. The Western blots were implemented as previously described [26]. The cells were lysed with RIPA containing 1 mM protease inhibitor cocktail and 1 mM phosphatase inhibitor cocktail (Boster). We transferred the 30 ug protein samples, which were separated by SDS-polyacrylamide gels, into PVDF membranes. Then we blocked the membranes with 5% bone serum albumin for 1 hour, and incubated with appropriate antibodies at 4°C overnight. Subsequently, we had incubated blots with horse-radish peroxidase (HRP)-

conjugated secondary antibodies for 1 hour at room temperature. Then we used the Western ECL Substrate Kit (Thermo Pierce, USA) to detect the bands. Protein expressions about Alkaline Phosphatase (ALP), osteocalcin (OC), Col α , SOX9, lipoprotein lipase (LPL) and PPAR- γ 2 were determined by normalizing to GAPDH, and representative bands were shown.

Statistical analysis

We used the SPSS 21.0 software (SPSS Inc., Chicago, IL, USA) to analyze the data. Measurement data was described as the mean \pm standard deviation, and difference was assessed by student's t test. We considered it statistically significant when the p value was less than 0.05 for all tests.

Results

Morphological features and immunophenotypes of adherent NCs

Fibroblast-like cells became visible in the primary culture after five days, and the colonies of adherent cells were formed. We found that the colonies of NCs were formed like colony-forming unit-fibroblasts (CFU-F) of BMSCs, and the sizes of colonies were increased rapidly. Then a subconfluent monolayer of fibroblastoid cells was merged and formed after three to four weeks. We analyzed the cell-surface antigen profiles of the adherent NCs and compared these with BMSCs (Table 2). We found the adherent NCs were positive for mesenchymal stem cell (MSC)-related markers CD29 and CD44, and negative for hematopoietic markers CD34 and CD45, which were similar to BMCs.

Table 2

Immunophenotypic comparison of nonunion cells (NCs) and bone marrow derived mesenchymal stem cells (BMSCs)

Cell-surface markers	NCs*	BMSCs*
CD29	++	++
CD44	++	++
CD34	-	-
CD45	-	-

*, less than 1% were negative for a specific antibody; +, more than 40% but less than 75% were positive; ++, more than 75% cell were positive.

Multiple differentiation potential of the adherent NCs in vitro

We cultured the adherent NCs under the osteogenic, chondrogenic or adipogenic differentiation medium, respectively. The differentiated NCs, which were cultured under osteogenic conditions for three weeks,

have produced a mineralized matrix as assessed by Alizarin Red S staining. However, no mineralization was observed in undifferentiated NCs. We found cell pellets have a spherical and glistening transparent appearance after incubating in chondrogenic differentiation medium for three weeks. The differentiated NCs cultured under chondrogenic conditions have generated cartilage matrix as evaluated by toluidine blue staining, but not of undifferentiated NCs. And after three weeks culturing under adipogenic conditions, the adherent NCs have generated the neutral lipid vacuoles as visualized by Oil-red O staining. However, we did not discovered Oil-red O-positive lipid vacuole in control group without differentiation conditions. The results of all five samples showed multiple differentiation potential of the adherent NCs in vitro, including osteogenic, chondrogenic and adipogenic differentiation abilities (Fig. 2).

Western blots (W-B) analysis

Furthermore, the differentiation capacities of the adherent NCs were detected by the Western blot analysis. We discovered that the adherent NCs have potentials to express ALP and OC under osteogenic conditions, to express Col I and SOX9 under chondrogenic conditions, and to express LPL and PPAR- γ 2 under adipogenic conditions after three weeks culturing as evaluated by W-B analysis. The results showed the differentiation capacity of NCs under osteogenic, chondrogenic or adipogenic conditions was higher than these under undifferentiated conditions in the control group (Fig. 3a, Fig. 3b).

Discussion

Atrophic nonunion, which was described as the most difficult disease to treat, is clinical challenges among fracture nonunion. To our knowledge, the combination of biological and biomechanical therapeutic approaches is the key substance in bone formation [27]. Iwakura T et al. [4] found that mesenchymal stem cells obtaining from hypertrophic nonunion tissues has the potential of transforming into cartilage and bone-forming cells, and made a conclusion that hypertrophic nonunion tissues play an important role in the healing process. Takahara S et al. [25] also discovered the osteogenic cells getting from pseudoarthrosis tissue have the same cell surface markers as MSC-related and has the potential to increase proliferation of cells. Whether atrophic nonunion tissues also have the capability to proliferate is unknown. Although atrophic nonunion tissues have been considered as avascular disease for a long time [28], the tissues at the atrophic nonunion are well vascularized and the blood vessels in atrophic nonunion also can get the same level of these in healing bone as reported in the literatures [21, 22]. Therefore, the present study was aimed to explore whether the cells obtained from atrophic nonunion have the same biological activity as BMSCs.

Though our current study is relative simple, it is very interesting. Resembling the colony formation of BMCs, the adherent NCs obtained from atrophic nonunion can generate the colonies of fibroblase-like spindle shape cells in the primary culture. We also found the adherent NCs express MSC-related markers CD29 and CD44, but do not express hematopoietic-lineage markers CD34 and CD45 when cell-surface markers were analyzed by facsaria flow cytometry system [29, 30]. In present study, we discovered that the adherent NCs of atrophic nonunion can differentiate into osteogenic, chondrogenic, and adipogenic cells under the different lineage-specific induction factors in vitro, and these results were also confirmed by W-B analysis. These above findings indicated that mesenchymal stem cells obtaining from atrophic

nonunion tissues have the multiple potential of transforming into cartilage and bone-forming cells which were similar to BMSCs. Therefore, our above results can explain the reason why bone union occurs in some atrophic nonunion with treating the docking site indirectly, and the adherent NCs owning enough differentiation capacity may be an important factor.

In order to promote woven bone formation for the constitution of the bony callus, BMSCs were recruited and proliferated, and differentiated into chondroblasts and osteoblasts during the period of fracture repairing [31, 32]. In nonunion patients, the potential of BMSCs have been demonstrated with a reducing pool and proliferation [33, 34]. Hernigou P et al. [33] made a conclusion that not only the bone marrow of nonunion site but also the bone marrow of the iliac crest of nonunion patients contained low levels of progenitor cells compared with control bone marrow donors. In order to further research, Mathieu M et al. [35] assessed the pools and biological functions of BMSCs between nonunion patients and healthy patients, and found that the pool of BMSCs was decreased and their proliferation delayed. The NCs have the multilineage differentiation potential in vitro and their proliferation capacity are lower than that of the control BMSCs reported by Iwakura T et al [4]. While the pool of BMSCs in nonunion patients was decreased compared to healthy subjects, we inferred that the NCs were the BMSCs in nonunion patients. In atrophic nonunion, it is also an important factor to improve the biological environment [36]. The purpose of using bone morphogenetic proteins is stimulating osteogenic activity at the nonunion site [37]. We discovered the adherent NCs obtained from atrophic nonunion have the capability of osteogenic differentiation at our present study, and own the capacity of responding to osteogenic stimulation. Therefore, we speculated that it could be a potential treatment option for atrophic nonunions in the future with the preservation of the atrophic nonunions and local application of osteogenic stimulators.

There were some limitations in this study. Firstly, our present study had a small sample size of only five patients. Second, the stains of differentiation were not quantified because the quality of images was poor in some extent. Thirdly, the characteristics of the NCs were based on their general morphology and their function in the in vitro study, and these might not reflect the in vivo characteristic of NCs in the model. Therefore, further studies in vivo and with larger samples are required.

Conclusion

The atrophic nonunion tissue is important element during the healing process of atrophic nonunion. The mesenchymal stem cells obtaining from atrophic nonunion tissues have the multiple potential of transforming into cartilage and bone-forming cells.

Abbreviations

BMSCs: Bone marrow stromal cells;

ALP: Alkaline Phosphatase;

OC: Osteocalcin;

LPL: Lipoprotein lipase;

NCs: Nonunion cells;

PBS: Phosphate-buffer saline;

EDTA: Ethylene Diamine Tetraacetic Acid.

Declarations

Ethics approval and consent to participate

This study was ethically approved by the Second Xiangya Hospital committee for clinical research (No.2010-S02). Patients' data obtained in the course of the study were reserved confidential and used only for this study.

Consent for publication

Written informed consent was obtained from all the patients for publication of this article and any accompanying images.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding authors on reasonable request.

Competing of interests

The authors declare that they have no competing interests. No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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Authors' contributions

XH Z, T L and ZW T were accountable for the execution of the research, the integrity and analysis of the data, and the writing of the manuscript. J W, GY J, DS Y and XS Z were accountable for the execution of the research and analysis of the data. All authors have read and approved the manuscript.

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Author details

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Figures

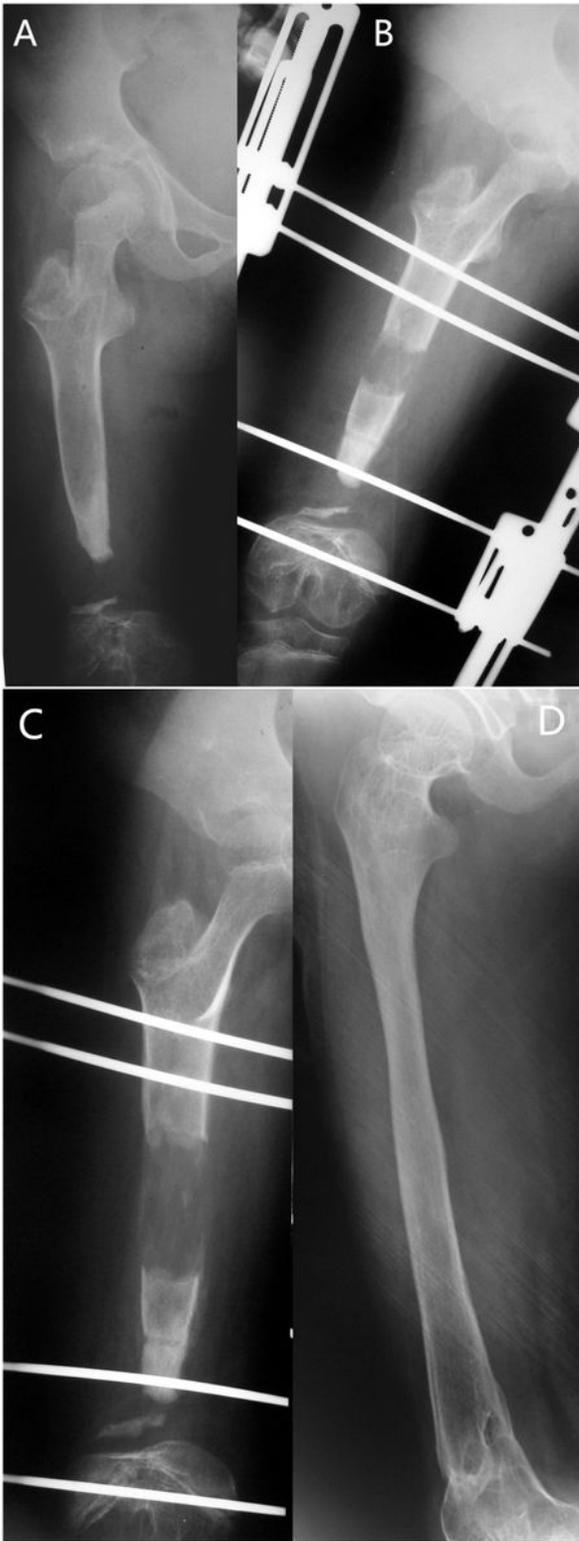
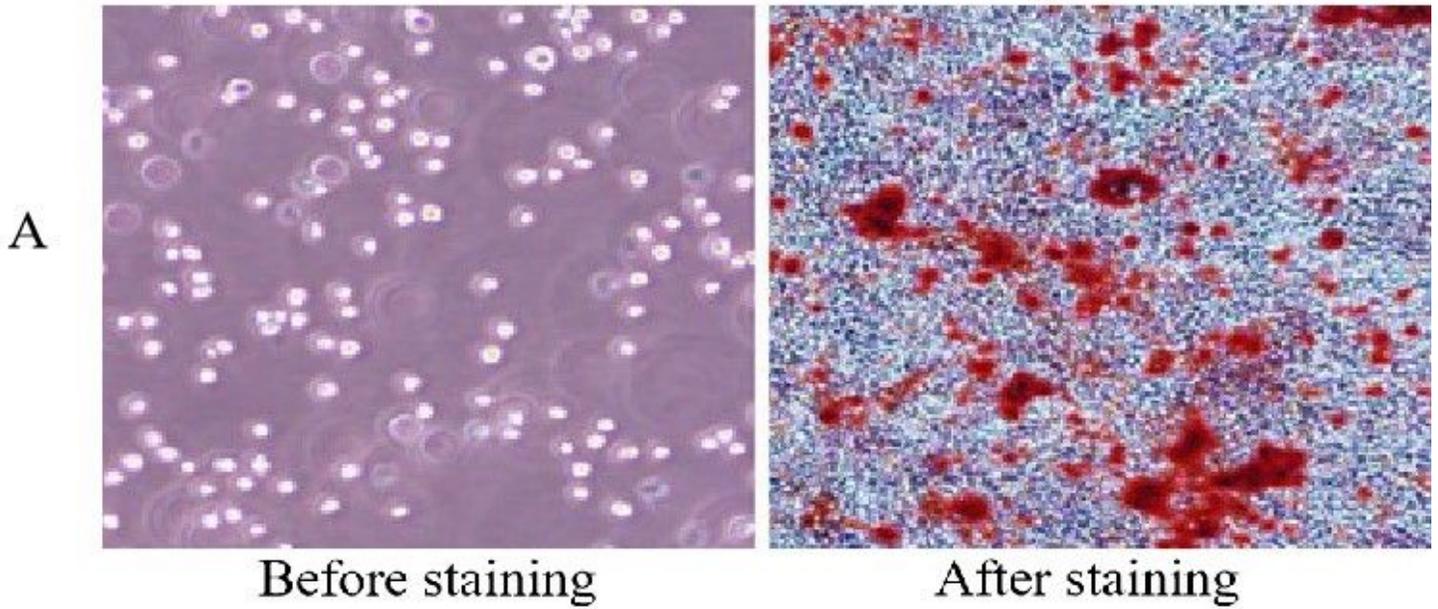


Figure 1

Radiographs of X-ray shown one patient with femoral atrophic nonunion and healed through our treatment at last follow-up.

Osteodifferentiation of Alizarin red staining



Adipodifferentiation of Oil red staining

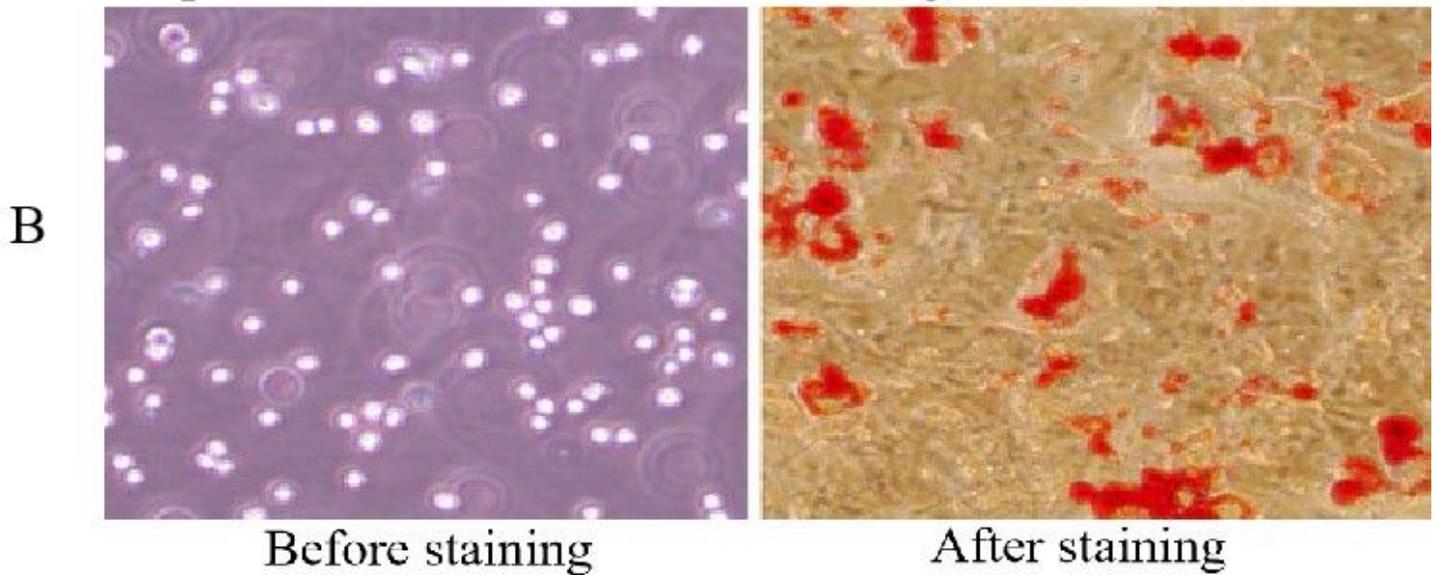


Figure 2

After three weeks incubation under osteogenic conditions and adipogenic conditions, induced NCs formed a mineralized matrix as evidenced by Alizarin Red S staining (A); adherent NCs showed the formation of neutral lipid vacuoles, visualized by staining with Oil-red O (B).

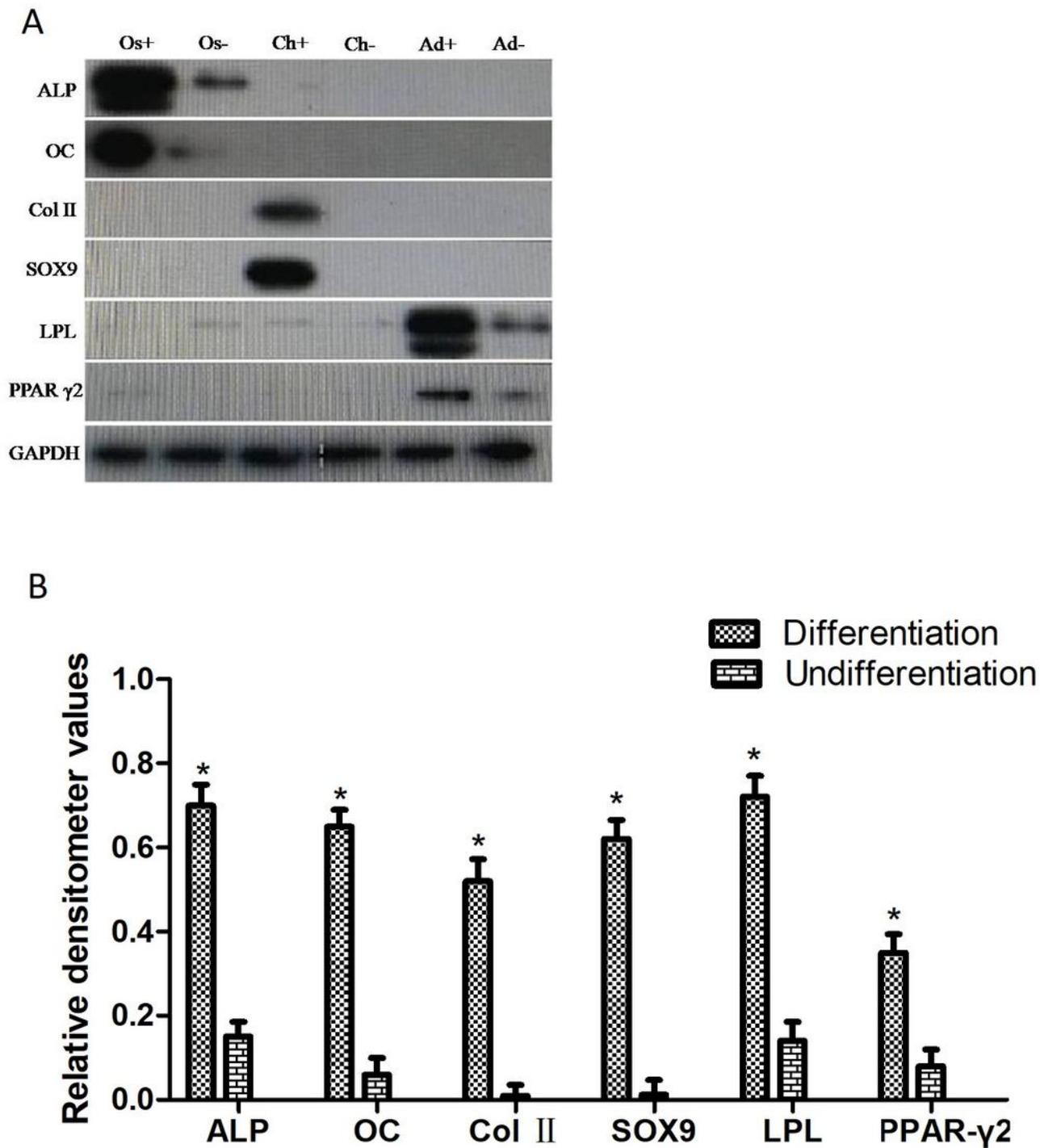


Figure 3

The expression of ALP, OC, Col II, SOX9, LPL and PPAR- γ 2 proteins of both group determined by western blot analysis (3a). Relative densitometer value of ALP, OC, Col II, SOX9, LPL and PPAR- γ 2 proteins of both groups, GAPDH used as internal control for quantification (3b). Data shown as mean \pm SD, * p <0.05.