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Novel nano-composite biomimetic biomaterial allows chondrogenic and osteogenic differentiation of bone marrow concentrate derived cells

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Abstract In clinical orthopedics suitable materials that induce and restore biological functions together with the right mechanical properties are particularly needed for the regeneration of osteochondral lesions. For this purpose, the ideal scaffold should possess the right properties with respect to degradation, cell binding, cellular uptake, non-immunogenicity, mechanical strength, and flexibility. In addition, it should be easy to handle and serve as a template for chondrocyte and bone cells guiding both cartilage and bone formation. The aim of the present study was to estimate the chondrogenic and osteogenic capability of bone marrow concentrated derived cells seeded onto a novel nano-composite biomimetic material. These properties have been evaluated by means of histological, immunohistochemical and electron microscopy analyses. The data obtained demonstrated that freshly harvested cells obtained from bone marrow were able, once seeded onto the biomaterial, to differentiate either down the chondrogenic and osteogenic pathways as evaluated by the expression and production of specific matrix molecules. These findings support the use, for the repair of osteochondral lesions, of this new nano-composite biomimetic material together with bone marrow derived cells in a "one step" transplantation procedure.

In memory of Professor Andrea Facchini who died during manuscript preparation.

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1 Introduction

Mesenchymal stem cells (MSC) with their unique peculiarity to differentiate toward different lineages are recently become one of the major players in the field of regenerative medicine [1]. In particular, they can differentiate into osteoblasts, adipocytes and chondrocytes [2] but their plasticity has been expanded to include contribution to cell lineages in brain [3], muscle [4], liver [5] and kidney tissue [6]. In the orthopedic field, MSC have been used in several preclinical and clinical studies with the aim to regenerate cartilage and bone [7–12]. However, despite their important characteristics, the use of MSC require an isolation and expansion in dedicated structures and no less important, their use implies risks of adverse immune reactions to some components such as foetal bovine serum (FBS) added to the culture and their possible malignant transformations [13]. Recently, the use of bone marrow directly concentrated in operating room has been advocated [14]. The rationale of its use lies in the concept of the "niche" that represents functional and structural unit able to regulate cell division and differentiation. Bone marrow contains different cell populations which act not only as precursors for some lineages but also as regulatory cells involved in tissues homeostasis. It has been proposed that cellular interactions could be mediated by paracrine signalling, cell

fusion or differentiation to specific cell types [15]. For these reasons, the transplantation of Bone Marrow Concentrate (BMC) seems to be an ideal strategy for the regeneration of muscoloscheletal lesions and in particular for those affecting cartilage and bone. Hernigou et al. demonstrated good results by using percutaneous autologous bone-marrow graft for the treatment of non unions fractures [16]; Gangji et al. [17] reported data on the treatment of osteonecrosis of femoral head with implantation of autologous bone marrow cells. BMC was also used in patients affected by talar osteochondral lesions and the results obtained at 4 years follow-up were good at both clinical and histological levels [18]. The regeneration of musculoskeletal tissues often requires the use of suitable biomaterials that support the delivery of the cells into the lesion sites, contributing in the meantime to their growth, proliferation and differentiation. Treatment of osteochondral lesions would require a scaffold that could reproduce both cartilage and bone structures able to support the growth and differentiation of transplanted cells but at the same time to possess the needed biomechanical characteristics. Recent data from our laboratory showed that MSC grown onto a novel nano-composite biomimetic material are able to differentiate either down the chondrogenic and osteogenic pathways in the suitable milieu [19].

The aim of the present study was to evaluate the behavior of BMC seeded onto the same scaffold in order to overcome all the aforementioned problems related to the use of expanded cells. BMC could represent a valid alternative as freshly harvested biological compound, suitable for the repair of osteochondral lesions in a "one step procedure".

2 Materials and methods

2.1 Bone marrow harvesting and concentration

Bone marrow was obtained from the iliac crest of five patients (mean age 28.0 ± 12.0; three females and two males) surgically treated for osteochondral defects. The study was approved by the Ethical Committee of the Institution and conducted following ethical principles of research. Informed consent was obtained by all the patients enrolled into the study.

As previously reported [18], bone marrow was harvested from the posterior iliac crest in a sterile regimen, with the patient in prone decubitus and in general or spinal anesthesia. The bone marrow was aspirated in small fractions from different points to maximize the harvesting of the stromal cells and reduce dilution by peripheral blood. The harvested bone marrow (60 ml) was reduced in volume directly in the operating room, by removing most of the red cells and plasma. Thus, it was possible to obtain 6 ml of bone marrow concentrate and in particular nucleated cells that are stem cells, monocytes, lymphocytes, and other bone marrow resident cells.

In this study, bone marrow was concentrated by Res-Q concentrator device (ThermoGenesis, Rancho Cordoba, CA, USA) and by IOR-G1 Kit (Novagenit, Mezzocorona, Italy) following the Manufacturer instructions.

2.2 Flow cytometric analysis

For identification of the BMC phenotype, a three-colour immunofluorescence was performed. Approximately 2.5×10^5 cells were incubated respectively, with anti-human CD45 conjugated with tetramethyl rhodamine isothiocyanate (TRITC), anti-human CD34 conjugated with phycoerythrin (PE) and anti human CD54, CD63, CD90, CD105, CD106, CD146, CD271 conjugated with fluorescein isothiocyanate (FITC) as previously reported [14]. Negative and isotype-matched controls were carried out. The percentages of specific markers were evaluated on CD34 and CD45 negative cells.

2.3 Colony-forming units-fibroblast (CFU-F) assay

The clonogenic ability of BMC was determined by a low-density CFU-F assay as previously reported [20]. 5×10^5 mononuclear cells were seeded in Petri dishes in α-MEM (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 20 % Fetal Bovine Serum (FBS) (Sigma Chemical Co, St. Louis, MO, USA). The medium was changed twice a week. On days 7 and 14 cells were washed with Phosphate-Buffer Saline (PBS), fixed with methanol and stained with Crystal Violet (Sigma). An aggregate of cells containing more than 50 cells was classified as a colony originating from one clonal cell.

2.4 Biomimetic scaffold

A nano-structured bio-mimetic three-layer gradient scaffold composed of type I collagen and magnesium enriched hydroxyapatite was evaluated (Fin-Ceramica Faenza, S.p.A.). This scaffold has a porous, composite three layered structure, able to reproduce the cartilaginous layer, the tidemark and the sub-chondral bone. The cartilaginous layer, consisting of equine type I collagen and has a smooth surface. The intermediate layer (tide mark-like structure) consists of a combination of type I collagen (60 wt%) and non-stoichiometric Mg-enriched hydroxyapatite (Mg-HA, 40 wt%), whereas the lower one is made of a mineralized blend of type I collagen (30 wt%) and Mg-HA (70 wt%) reproducing the sub-chondral bone tissue. In this study, we analyzed the whole scaffold and the cartilaginous and sub-

chondral bone layers separately for their ability to support the growth and differentiation of BMC. The chemical-physical characteristics, the mechanical performances and the bioactivity of the scaffold were previously reported [21–23].

2.5 BMC seeding and differentiation onto the different biomaterials

In the light of data obtained in our previous work that demonstrated the lack of chondrogenesis in the bone layer and the same for osteogenesis in the cartilagineous layer [19], we decided to induce chondrogenic differentiation only in the cartilaginous single layer and in the composite while osteogenic only in the bone single layer and in the composite. To this end, 0.3 and 0.15 ml of BMC (containing approximately 28 ± 4 x 10^6 and 13 ± 2 x 10^6 mononuclear cells respectively) were seeded onto composite osteochondral scaffold ($\sim$150 mm^3) and onto the single-layers ($\sim$75 mm^3 each).

BMC was allowed to adhere to biomaterials for 1 h at room temperature (RT) and then Dulbecco's modified Eagle medium (DMEM) (Sigma) with 10 % FBS was added. Chondrogenic and osteogenic differentiation was performed as previously reported [14]. Medium without the specific growth factors was used for chondrogenic and osteogenic controls respectively. Culture medium was changed twice a week. Each construct was evaluated on days 0 and 52 for cell viability, expression of specific extracellular proteins by histological/immunohistochemical analyses and ultrastructural observation to highlight the interaction between cells and the biomaterials. These experimental times were chosen on the basis of our previous experiences with the use of BMC grown onto a different scaffold [20].

2.6 Viability of BMC seeded onto the biomaterials

Analysis of cell viability was performed on days 0 and 52 using 3-4,5-dimethylthiazol-2yl-2,5-diphenyltetrazolium bromide (MTT) (Sigma) as previously reported [20]. Briefly, all constructs were washed with physiological solution and subsequently transferred into new plates containing 2 ml of MTT solution (1 mg/ml PBS) and incubated for 3 h at 37 °C. The color developed is due to the mithocondrial activity of the cells and thus proportional to their viability.

2.7 Light and transmission electron microscopy analyses

At each experimental time, constructs were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 for 3 h. After washings in cacodylate buffer the samples were post-fixed with 1 % osmium tetroxide, dehydrated and embedded in Epon. Sections were performed perpendicularly to the surface of seeding. Semithin sections were stained with Toluidine blue and used for light microscopy. The sections were observed with a Nikon Eclipse E 600 apparatus (Nikon, Tokio, Japan), and images captured using a Nikon Digital camera Dmx 1200 and ACT-1 software. Thin sections were stained with uranyl acetate and lead citrate; some sections were also stained with tannic acid and observed with Zeiss EM 109 electron microscope (Carl Zeiss, Oberkochen, Germany). Image were captured using a Nikon digital camera Dmx 1200F and ACT-1 software.

2.8 Immunohistochemical analysis

On days 0 and 52, single layers and composite osteochondral scaffolds were treated as previously reported (19). Immunohistochemical analyses were utilized to evaluate Collagen type II, proteoglycans, Sox-9, Collagen Type X and MMP-13 expression in the chondrogenic differentiated samples; Collagen Type I, alkaline phosphatase (ALP), Bone Sialon Protein (BSP), osteocalcin (OC) and osteopontin (OPN) in the osteogenic ones. Sections were incubated for 60 min at room temperature with monoclonal anti-human Collagen II diluted 1:100 (Chemicon International, Inc. Temecula, CA, USA), anti-human proteoglycans diluted 1:100 (Chemicon), anti-human Sox-9 diluted 1:150 (Chemicon), anti-human Collagen Type X diluted 1:1000 (Sigma), anti-human MMP-13 diluted 1:100 (R&D Systems, Minneapolis, MN, USA), anti-human Collagen Type I diluted 1:200 (Chemicon), anti- human alkaline phosphatase diluted 1:40 (Hybridoma Bank, Department of Biological Sciences, University of Iowa), anti-human BSP diluted 1:40 (Hybridoma Bank), anti-human OC diluted 1:50 (R&D) and anti-human OPN diluted 1:50 (Hybridoma Bank). Samples were rinsed and then incubated for 20 min at room temperature with multilinker biotinylated secondary antibodies and alkaline phosphatase-conjugated streptavidin (Biocare Medical, CA, USA). The reactions were developed using Fast Red substrate (Biocare). Negative controls were performed by omitting the primary antibodies. The sections were evaluated with bright field microscope (Nikon).

3 Results

3.1 Flow cytometric analysis

Flow cytometric analysis to evaluate the phenotype of BMC confirmed that these cells express, even if at low levels, CD54, CD63, CD90, CD105, CD106, CD146 and CD271 (Fig. 1).

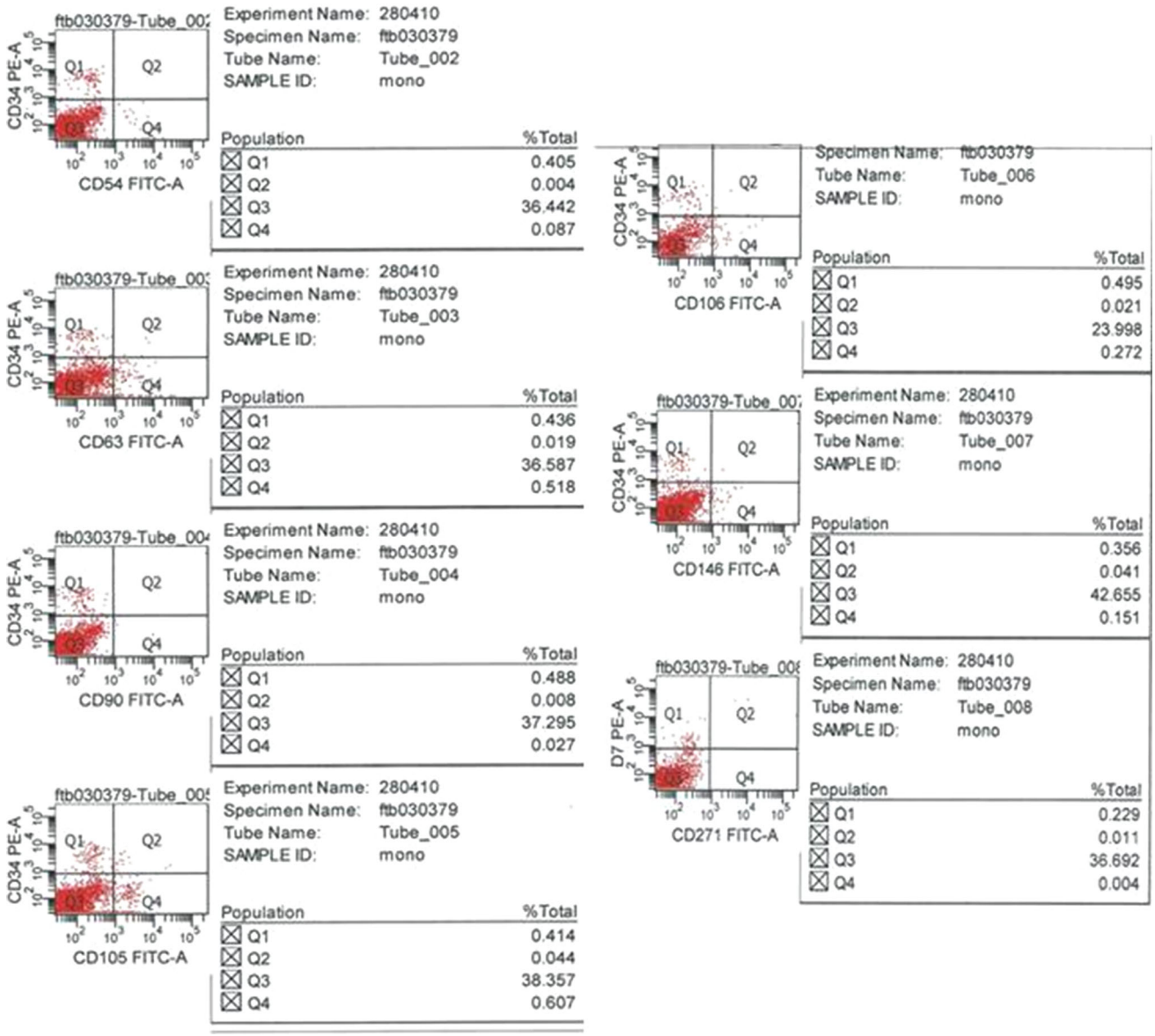


Fig. 1 Flow cytometry evaluation of BMC using the following antibodies: anti-CD54, -CD63, -CD90, -CD105, -CD106,-CD146, -CD271. Percentages of the different markers evaluated are also reported

3.2 Colony-forming units-fibroblast (CFU-F) assay

BMC was able to generate new cell colonies starting from single cells. At day 7 only small colonies were observed while an increase of the number of colonies was noticed at day 14 (Fig. 2).

3.3 Viability and proliferation of BMC seeded onto the biomaterials

BMC cultured onto the different single layers of the biomaterials and onto the composite were viable up to 52 days. Cells remained viable also when they were differentiated either down the chondrogenic and osteogenic pathways. This was confirmed by MTT testing, which is directly related to cell activity, and which showed increased values over time, demonstrating a cell proliferation inside the different scaffolds (Fig. 3).

3.4 Light and transmission electron microscopy analyses

To evaluate the cell distribution in the control and in the differentiated samples, the semithin sections of the single-layer scaffolds and of the composite were stained with Toluidine-blue and analyzed at 52 days (Fig. 4). As

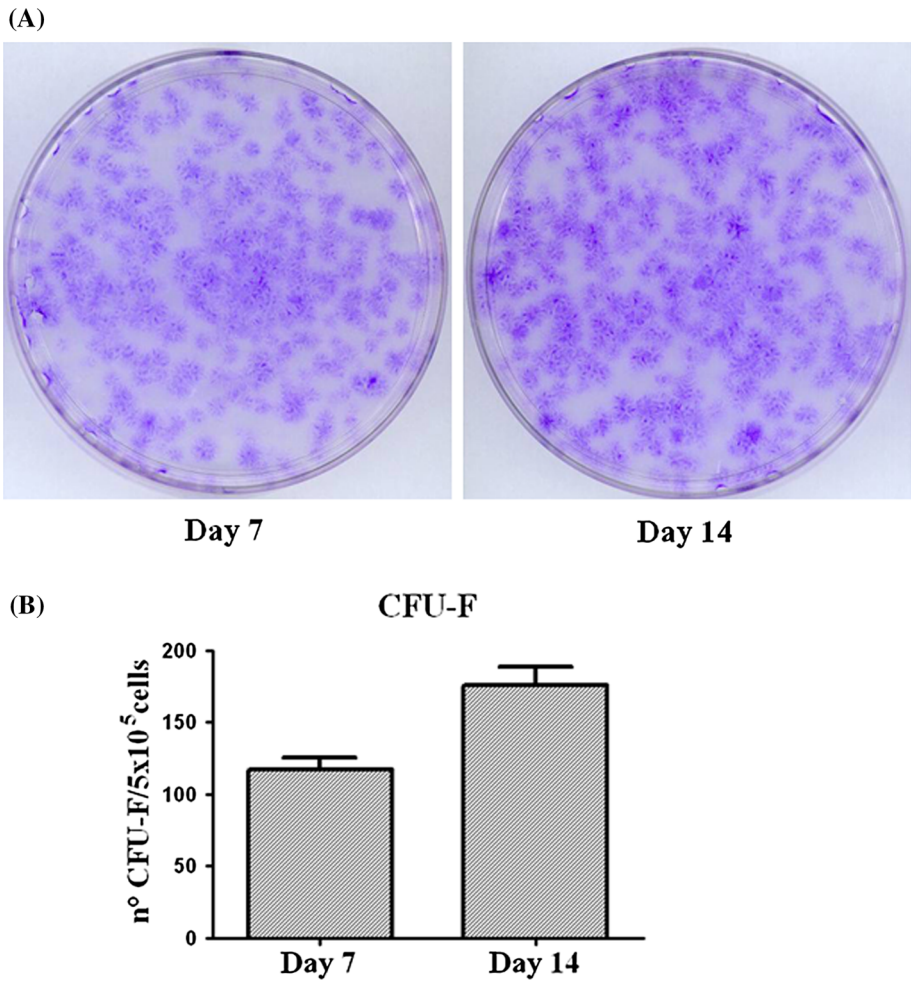


Fig. 2 **a** In the CFU-F assay (in the image a representative case; Crystal *violet staining*), an increased number of colonies are observed from Day 7 to Day 14 after the initial seeding of BMC. **b** Number of colonies at day 7 and day 14 expressed as n° CFU-F/5 $\times$ 10^5 cells (Color figure online)

concern chondrogenic differentiation, few cells are present inside the cartilaginous single-layer in the control sample, while a high amount of cells was present in the chondrogenic differentiated samples. This last sample showed organized areas of cartilage-like tissue mainly composed of groups of cells penetrating within the scaffold. These cells presented a ovoid/round morphology and appeared embedded in lacunae surrounded by extra cellular matrix (ECM) as shown at higher magnification in the inset (Fig. 4a). In the composite, chondrogenic differentiation occurs on the cartilaginous layer only after BMC induction where zones with cells that showed typical chondrogenic features were evident particularly at higher magnification in the inset (Fig. 4c).

For the bone single-layer scaffold, the cells remained located at the biomaterial surface in the control while after the osteogenic induction the colonizing cells showed a slight increased of ECM deposition as shown at higher magnification in the inset (Fig. 4b). Osteogenic differentiation of BMC seeded on composite showed that the cells formed a capsula around the cartilaginous layer similar to that observed in the control sample, while in all the other layers of the composite they penetrated more easily. A slight increased in ECM deposition was present at the surface level in intermediate and bone layers of differentiated samples as showed at higher magnification in the insets (Fig. 4d).

At the ultrastructural level (Fig. 5), the chondrogenic-differentiated BMC grown on the cartilaginous single-layer scaffold presented a typical round shape morphology of chondrocytes with respect to the control that presented a fibroblast like shape. The chondrocyte-like cells presented regular RER profiles, lipid droplets, Golgi apparatus, aggregate of glycogen particles and lysosomal bodies; abundant amounts of ECM could be observed (Fig. 5a). Chondrogenic-differentiated BMC grown on composite, showed a typical round shape morphology of chondrocyte-like cells surrounded by abundant ECM only in the cartilaginous layer. The cells showed regular RER profiles, lipid droplets, Golgi apparatus, aggregate of glycogen particles and lysosomal bodies. In control sample the cells appeared mainly elongated. ECM was present between

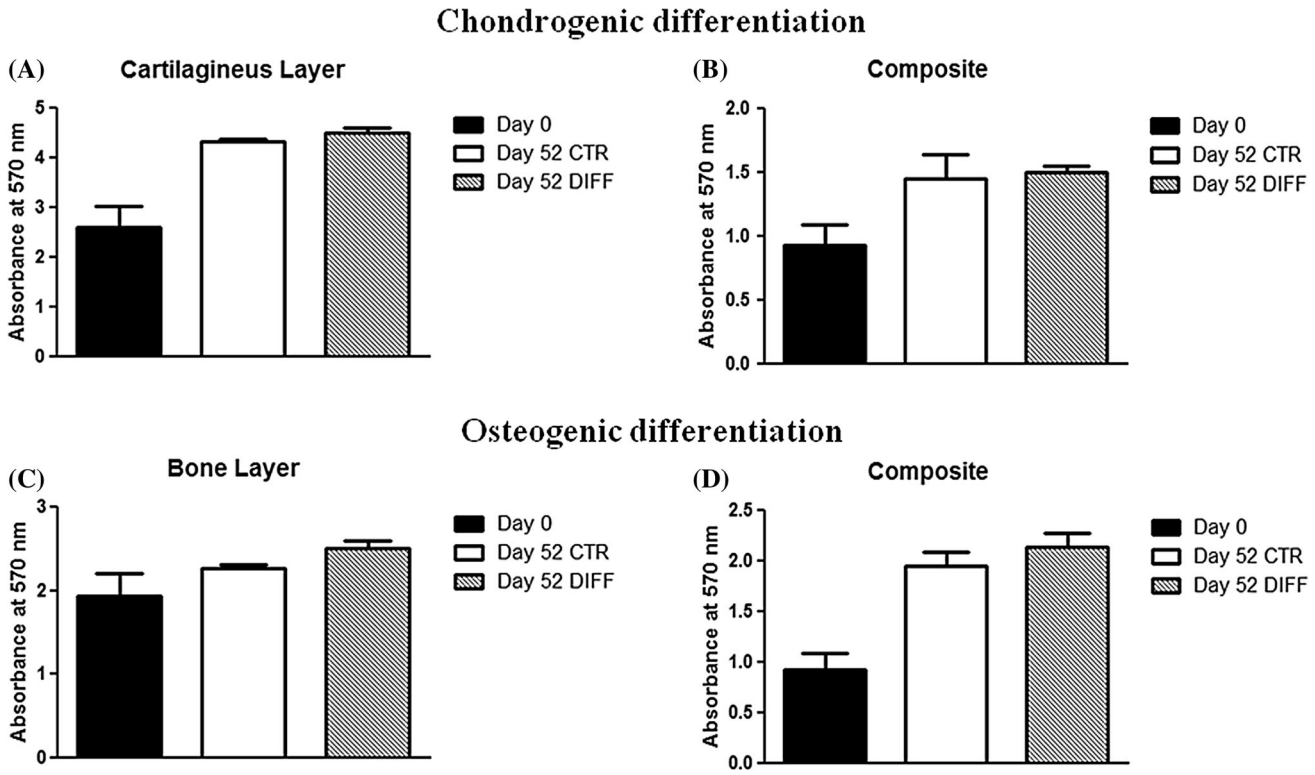


Fig. 3 MTT test performed on controls (CTR) and chondrogenic and osteogenic differentiated BMC (DIFF) seeded onto cartilaginous and bone layers (**a**, **c**) and onto the composite (**b**, **d**) at day 0 and 52. Data are expressed as mean optical density (OD) ± Standard Error (SE)

cells of intermediate and bone layers in small quantities in both control and differentiated samples (Fig. 5c). Electron microscopy analysis of BMC grown on the bone single-layer, indicated that the osteogenic-differentiated samples presented a slight increase of ECM with respect to control samples mainly in the superficial areas of the biomaterial. Mineralizing nodules were not detectable while residues of hydroxyapatite were evident also in control samples.

Both samples presented elongated cells with regular RER profiles, Golgi apparatus, mitochondria and lysosomal bodies (Fig. 5b). Elongated cells were observable in all three layers of composite in both control and osteogenic differentiated samples with an increase of ECM in the superficial area of the scaffold while calcium phosphate crystals, as sign of mineralized matrix, were not evident. Some degenerated areas of biomaterial were observed in all the samples examined (Fig. 5d).

3.5 Immunohistochemical evaluations

BMC differentiated down the condrogenic pathway in both cartilaginous single-layer and composite as shown by immunohistochemical analyses (Fig. 6). Collagen type II, proteoglycans, Sox-9, Collagen Type X, MMP-13 were negative on both cartilaginous layer and composite on day 0. On day 52, all the markers evaluated were negative in control samples with the exception of Collagen Type X that showed a slight positivity both in control and in the differentiated samples. A highly positivity was observed in differentiated samples for Collagen type II, proteoglycans, Sox-9 while MMP-13 was negative.

BMC differentiated down the osteogenic pathway in both bone layer and composite as shown by immunohistochemical analyses (Fig. 7). Collagen Type I, ALP, BSP, OC and OPN that were negative on both bone layer and composite on day 0 were highly positive in the differentiated samples on day 52. A slight positivity for Collagen type I was observed also in the control sample on day 52 and for osteocalcin in the composite. Osteogenesis did not occur in the other control samples.

4 Discussion

In tissue engineering field, the use of MSC isolated from bone marrow, adipose tissue, or other sources such as synovium and peripheral blood has shown promising results [24]. Bone marrow represents the ideal candidate for regenerative medicine due to its intrinsic characteristics; it contains not only hematopoietic and MSC but also elements that support angiogenesis and vasculogenesis processes [25]. The opportunity to use concentrated bone

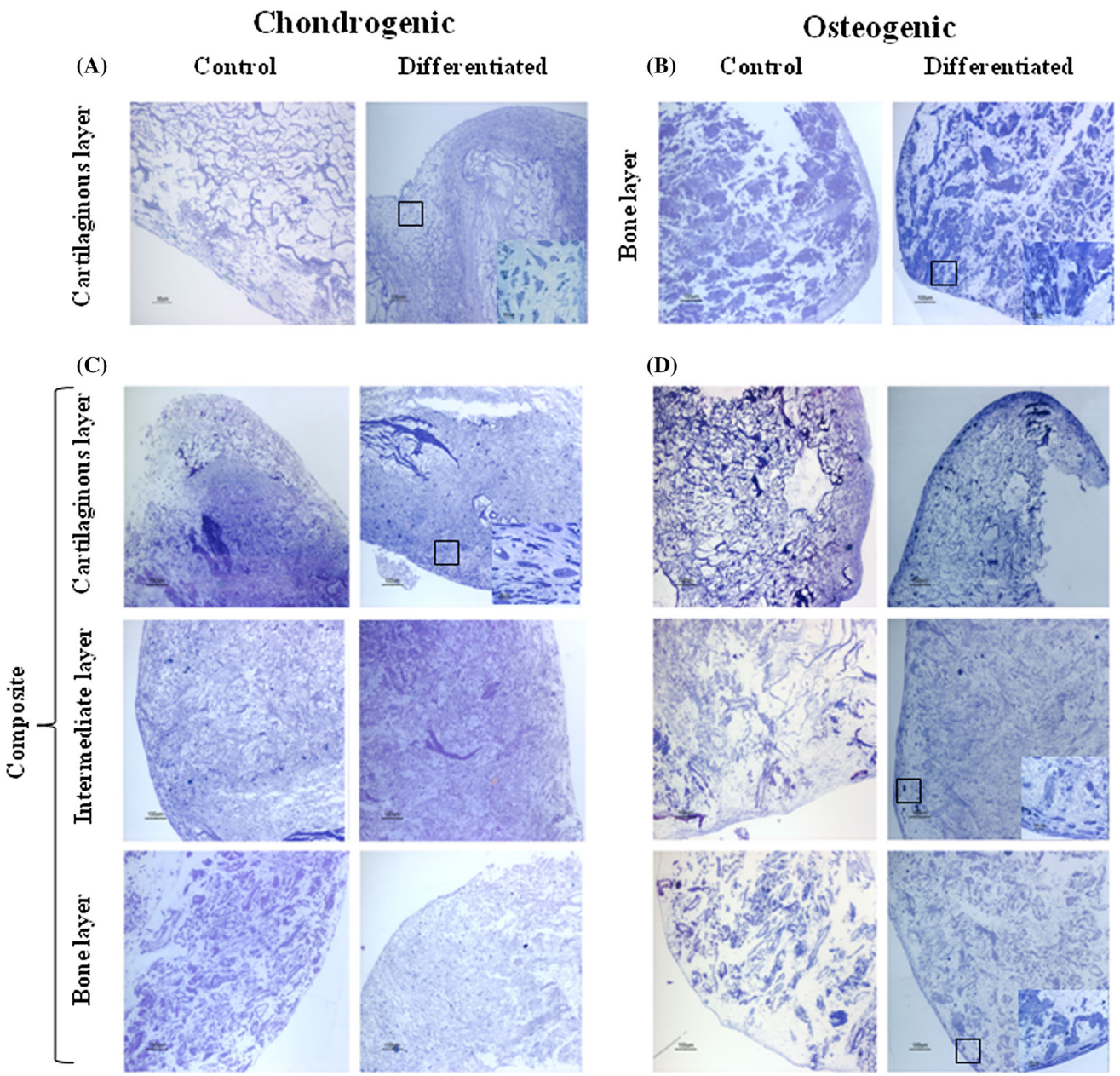


Fig. 4 Light microscopy analyses of representative controls and differentiated BMC on single layer and composite scaffold on 52 day of culture. Sections were cut perpendicularly to the surface of seeding. Toluidine *blue staining* was performed in control and in chondrogenic differentiated cells in the cartilaginous layer (**a**) and in the composite (**c**); in control and osteogenic differentiated cells in the bone layer (**b**) and in the composite (**d**). *Scale bars* 50 μm (**a**), 100 μm (**b**, **c**, **d**), 10 μm (*insets*) (Color figure online)

marrow enables a cell population surrounded by its "niche" to be implanted while avoiding all the complications related to the in vitro culture needed for cell expansion. The elements of the niche are able to regulate stem cell behavior through direct physical contact and the secretion of paracrine factors [26].

Despite the choice of the suitable population to be used in regenerative medicine, an important component of this therapy is represented by the scaffolds. For treatment of damages to musculoskeletal tissues, and in particular for the regeneration of osteochondral lesions, the ideal scaffold should present the optimal behavior for cell growth and differentiation and possess adequate mechanical properties. In a previous work, we evaluated the specific potentiality of a nano-structured bio-mimetic three-layer gradient scaffold composed of type I collagen and magnesium enriched hydroxyapatite in inducing chondrogenic or osteogenic of MSC [19]. We demonstrated that these

processes mainly depend by the local milieu which is fundamental for guiding cell differentiation. Taking into account these findings, the use of BMC allows to implant a heterogeneous population of fresh cells that may benefit the modulation and vascularization of the targeted tissue [27]. BMC, as reported above, are composed by different elements that constitute the stem cell niche, which is a microenvironment for stem cell self-renewal, proliferation, differentiation, mobilization and homing [26, 28]. In the present work, we showed that BMC seeded onto the different single layers and onto the composite of the same nano-structured bio-mimetic three-layer gradient scaffold, are able to colonize the biomaterial starting from the

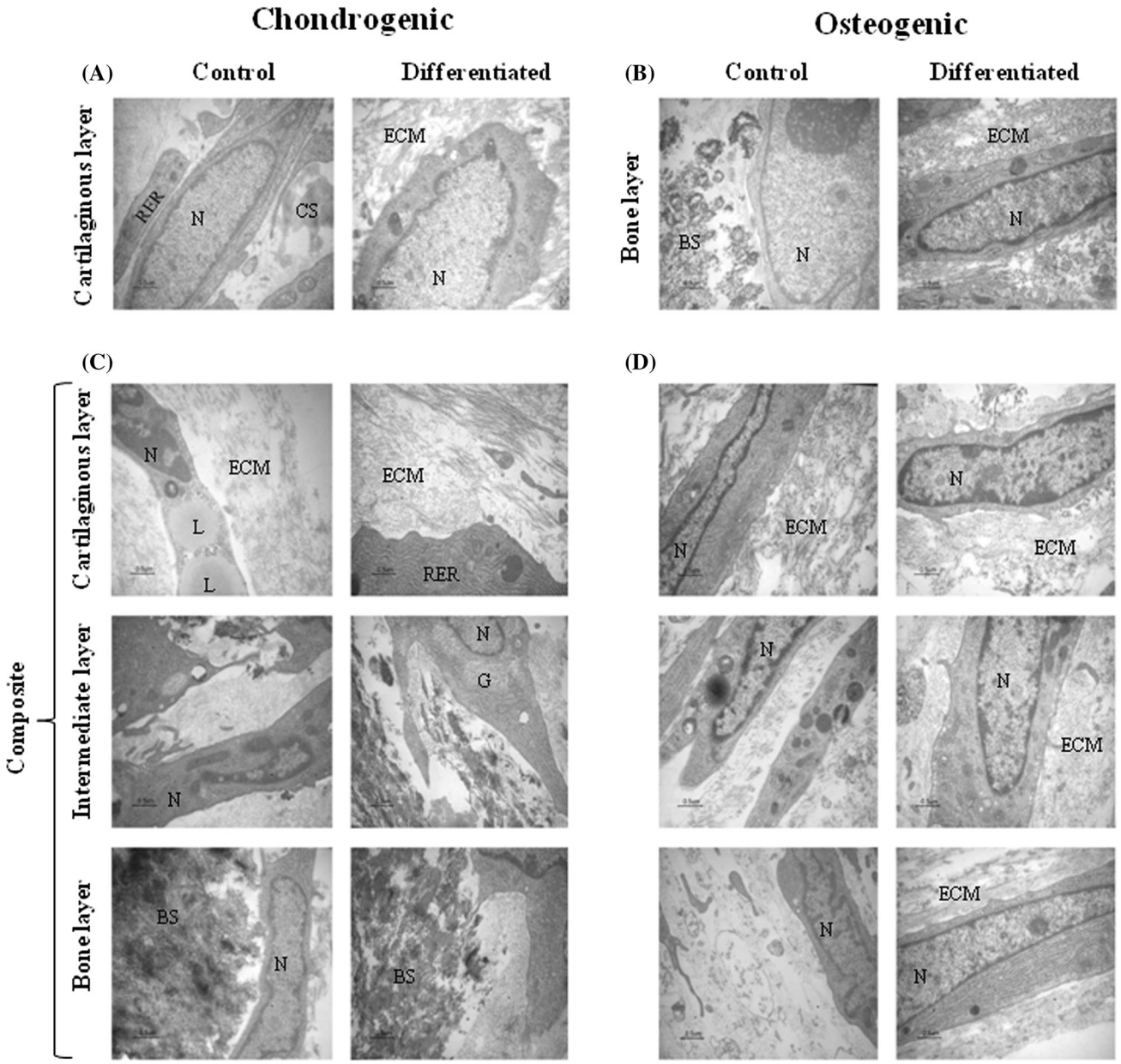
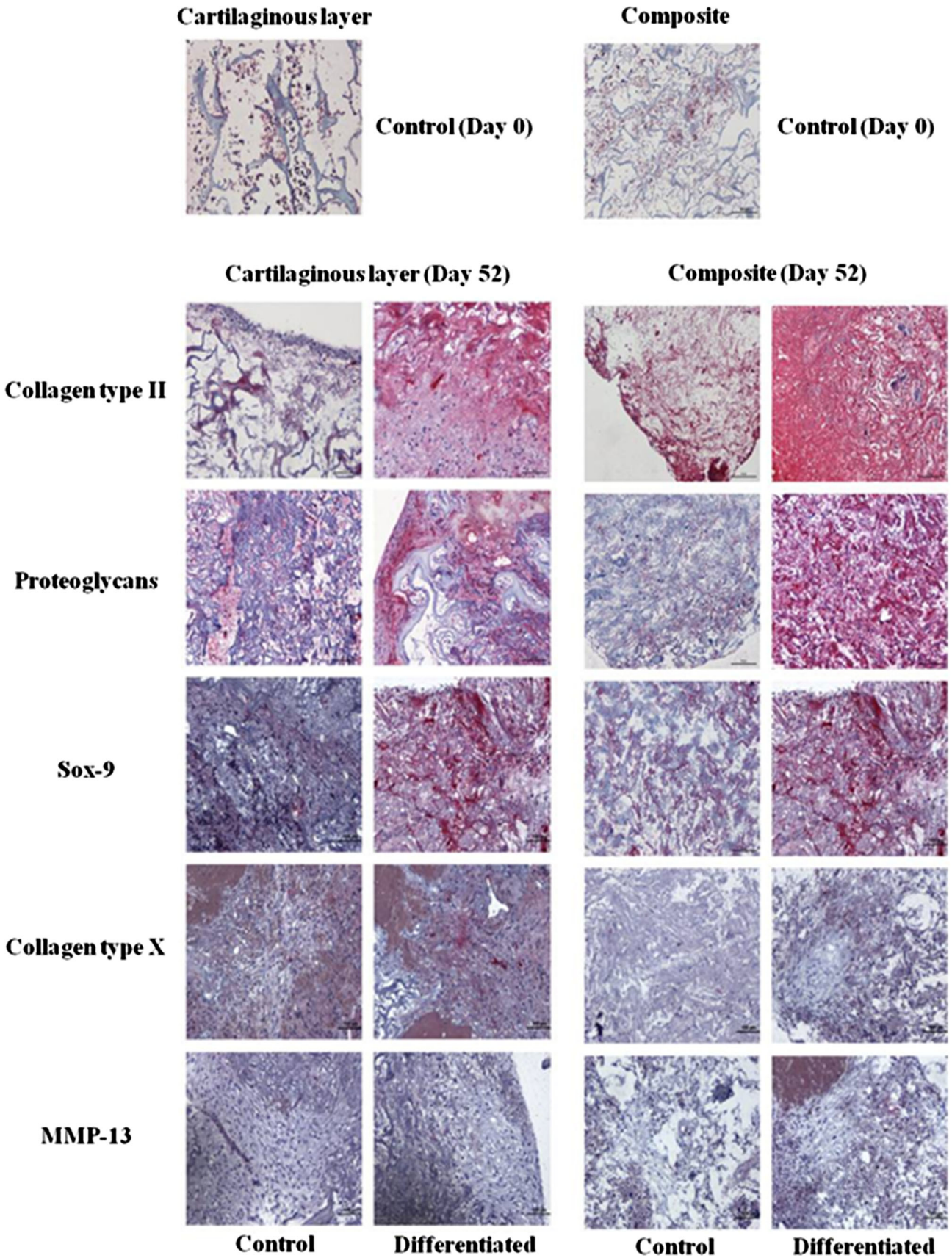


Fig. 5 Transmission electron microscopy analyses of representative controls and differentiated BMC on single layer and composite scaffold on 52 day of culture. Ultrastructure of control and chondrogenic differentiated cells in the cartilaginous layer (**a**), and in the composite (**c**); in control and osteogenic differentiated cells in the bone layer (**b**) and in the composite (**d**). *CS* cartilaginous scaffold, *BS* bone scaffold, *N* nucleus, *RER* rough endoplasmic reticulum profiles, *G* glycogen particles, *ECM* extracellular matrix, *L* lipid droplet. *Scale bars* 0.5 μm

Fig. 6 Immunohistochemical evaluation on both control and differentiated samples of several markers in the cartilaginous layer of the biomaterial and in the composite on day 0 (representative case) and day 52. *Scale bars* 200 μm


Cartilaginous layer
Composite
Control (Day 0)
Control (Day 0)
Cartilaginous layer (Day 52)
Composite (Day 52)
Collagen type II
Proteoglycans
Sox-9
Collagen type X
MMP-13
Control
Differentiated
Control
Differentiated

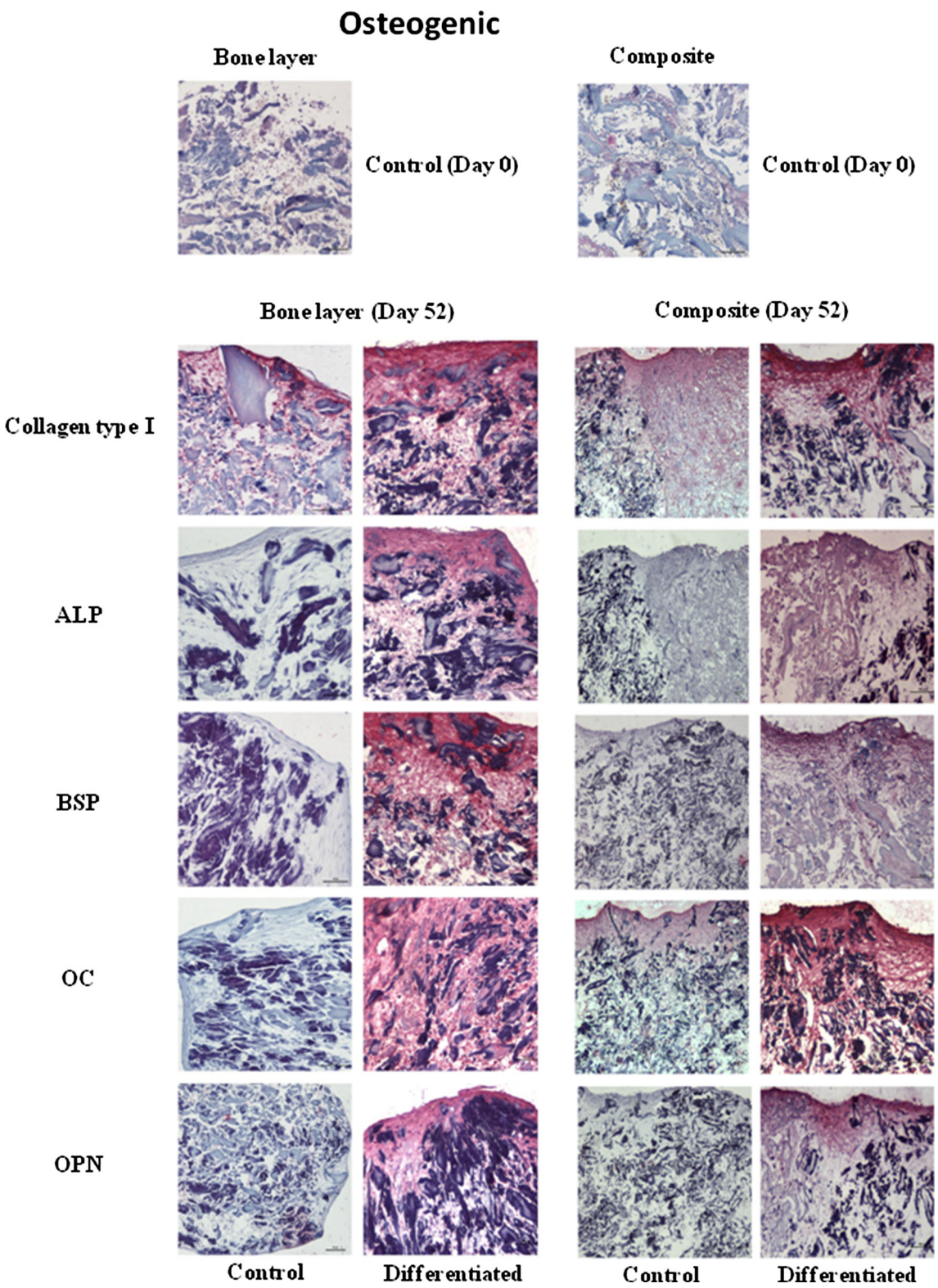


Fig. 7 Immunohistochemical evaluation on both control and differentiated samples of several markers in the bone layer of the biomaterial and in the composite on day 0 (representative case) and day 52. *Scale bars* 200 μm

superficial zone throughout all its thickness. Both chondrogenesis and osteogenesis induced by the use of specific growth factors and evaluated by means of immunohistochemical analyses on the main phenotypical markers expressed by the cells are coherently favored by the intrinsic composition of the scaffold that mimic the

respective native tissues i.e. cartilage and bone; in other words, the first was best induced in the cartilaginous layer, the last in the bone one.

In conclusion, even if this work present some limitations such as the lack of molecular biology data, we clearly showed the colonization and differentiation of BMC onto this nano-structured bio-mimetic scaffold that could represent one of the suitable biomaterial for the regeneration of osteochondral defects. Moreover, the use of BMC compared to that of isolated MSC, should represent an improvement of this therapeutic strategy for its important biological characteristics.

Its use is particularly indicated for the opportunity to inject it directly in operating room in a "one step" procedure". This easy strategy, as we stated in some previous pre-clinical and clinical works [14, 18], presents the advantages to overcome the major drawbacks of other cellular therapies that require the isolation and expansion of the cells in vitro and the need of two subsequent different interventions.

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Conflict of interest No potential competing interests have to be reported.

References

1. Das M, Sundell IB, Koka PS. Adult mesenchymal stem cells and their potency in the cell-based therapy. J Stem Cells. 2013;8(1):1–16.
2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143–7.
3. Brazelton TR, Rossi FM, Keshet GI, Blau HM. From marrow to brain: expression of neuronal phenotypes in adult mice. Science. 2000;290:1775–9.
4. Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F. Muscle regeneration by bone marrow-derived myogenic progenitors. Science. 1998;279:1528–30.
5. Alison MR, Poulsom R, Jeffery R, Dhillon AP, Quaglia A, Jacob J, Novelli M, Prentice G, Williamson J, Wright NA. Hepatocytes from non-hepatic adult stem cells. Nature. 2000;406:257.
6. Poulsom R, Forbes SJ, Hodivala-Dilke K, Ryan E, Wyles S, Navaratnarasah S, Jeffery R, Hunt T, Alison M, Cook T, Pusey C, Wright NA. Bone marrow contributes to renal parenchymal turnover and regeneration. J Pathol. 2001;195:229–35.
7. Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M, Fujinaga T. Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis. Biotechnol Bioeng. 2006;93:1152–63.
8. Grigolo B, Lisignoli G, Desando G, Cavallo C, Marconi E, Tschon M, Giavaresi G, Fini M, Giardino R, Facchini A. Osteoarthritis treated with mesenchymal stem cells on hyaluronan-based scaffold in rabbit. Tissue Eng Part C Methods. 2009;15:647–58.
9. Heng BC, Cao T, Lee EH. Directing stem cell differentiation into the chondrogenic lineage in vitro. Stem Cells. 2004;22:1152–67.
10. Majumdar MK, Banks V, Peluso DP, Morris EA. Isolation, characterization, and chondrogenic potential of human bone marrow-derived multipotential stromal cells. J Cell Physiol. 2000;185:98–106.
11. Marcacci M, Kon E, Moukhachev V, Lavroukov A, Kutepov S, Quarto R, Mastrogiacomo M, Cancedda R. Stem cells associated with macroporous bioceramics for long bone repair: 6- to 7-year outcome of a pilot clinical study. Tissue Eng. 2007;13:947–55.
12. Matsumoto T, Okabe T, Ikawa T, Iida T, Yasuda H, Nakamura H, Wakitani S. Articular cartilage repair with autologous bone marrow mesenchymal cells. J Cell Physiol. 2010;225:291–5.
13. Tonti GA, Mannello F. From bone marrow to therapeutic applications: different behaviour and genetic/epigenetic stability during mesenchymal stem cell expansion in autologous and foetal bovine sera. Int J Dev Biol. 2008;52:1023–32.
14. Cavallo C, Desando G, Cattini L, Cavallo M, Buda R, Giannini S. Bone marrow concentrated cell transplantation: rationale for its use in the treatment of human osteochondral lesions. J Biol Regul Homeost Agents. 2013;7:165–75.
15. Bluguermann C, Wu L, Petrigliano F, McAllister D, Miriuka S, Evseenko DA. Novel aspects of parenchymal-mesenchymal interactions: from cell types to molecules and beyond. Cell Biochem Funct. 2013;31:271–80.
16. Hernigou P, Poignard A, Beaujean F, Rouard H. Percutaneous autologous bone-marrow grafting for nonunions. Influence of the number and concentration of progenitor cells. J Bone Joint Surg Am. 2005;87:1430–7.
17. Gangji V, De Maertelaer V, Hauzeur JP. Autologous bone marrow cell implantation in the treatment of non-traumatic osteonecrosis of the femoral head: five year follow-up of a prospective controlled study. Bone. 2011;49:1005–9.
18. Giannini S, Buda R, Battaglia M, Cavallo M, Ruffilli A, Ramponi L, Pagliazzi G, Vannini F. One-step repair in talar osteochondral lesions: 4-year clinical results and t2-mapping capability in outcome prediction. Am J Sports Med. 2013;41:511–8.
19. Manferdini C, Cavallo C, Grigolo B, Fiorini M, Nicoletti A, Gabusi E, Zini N, Pressato D, Facchini A, Lisignoli G. Specific inductive potential of a novel nanocomposite biomimetic biomaterial for osteochondral tissue regeneration. J Tissue Eng Regen Med. 2013. doi:10.1002/term.1723.
20. Cavallo C, Desando G, Columbaro M, Ferrari A, Zini N, Facchini A, Grigolo B. Chondrogenic differentiation of bone marrow concentrate grown onto a hylauronan scaffold: rationale for its use in the treatment of cartilage lesions. J Biomed Mater Res A. 2013;101:1559–70.
21. Nicoletti A, Fiorini M, Paolillo J, Dolcini L, Sandri M, Pressato D. Effects conditions on the chemical-physical properties of a novel bio-inspired composite scaffold stabilized with 1,4-butanediol diglycidyl ether (BDDGE). J Mater Sci Mater Med. 2012;24:17–35.
22. Oliveira JM, Rodrigues MT, Silva SS, Malafaya PB, Gomes ME, Viegas CA, Dias IR, Azevedo JT, Mano JF, Reis RL. Novel hydroxyapatite/Chitosan bilayered scaffold for osteochondral tissue-engineering application: scaffold design and its

performance when seeded with goat bone marrow stromal cells. Biomaterials. 2006;27:6123–37.
23. Tampieri A, Sandri M, Landi E, Pressato D, Francioli S, Quarto R, Martin I. Design of graded biomimetic osteochondral composite scaffolds. Biomaterials. 2008;29:3539–46.
24. Murphy MB, Moncivais K, Caplan AI. Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. Exp Mol Med. 2013;15(45):e54.
25. Velazquez OC. Angiogenesis and vasculogenesis: inducing the growth of new blood vessels and wound healing by stimulation of bone marrow-derived progenitor cell mobilization and homing. J Vasc Surg. 2007;45:39–47.
26. Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. Nature. 2001;414:98–104.
27. Frenette PS, Pinho S, Lucas D, Scheiermann C. Mesenchymal stem cell: keystone of the hematopoietic stem cell niche and a stepping-stone for regenerative medicine. Annu Rev Immunol. 2013;31:285–316.
28. Li L, Xie T. Stem cell niche: structure and function. Annu Rev Cell Dev Biol. 2005;21:605–31.