Int J Clin Exp Med 2015;8(8):13154-13162
www.ijcem.com /ISSN:1940-5901/IJCEM0011965

Original Article

Osteogenic differentiation of CD271$^+$ cells from rabbit bone marrow cultured on three phase PCL/TZ-HA bioactive scaffolds: comparative study with mesenchymal stem cells (MSCs)

Alessia Colosimo[1], Cristina Rofani[2], Elisa Ciraci[2], Aurelio Salerno[3], Maria Oliviero[4], Ernesto Di Maio[4], Salvatore Iannace[5], Paolo A Netti[3], Francesco Velardi[6], Anna C Berardi[2]

[1]Faculty of Veterinary Medicine, University of Teramo, Italy; [2]Research Laboratory, "Stem Cells", U.O.C. Immunohematology and Transfusion Medicine, Hospital Santo Spirito, Pescara, Italy; [3]Interdisciplinary Research Centre on Biomaterials (CRIB) and Italian Institute of Technology (IIT), Naples, Italy; [4]Department of Materials and Production Engineering, University of Naples Federico II, Naples, Italy; [5]Institute of Composite and Biomedical Materials, National Research Council (IMCB-CNR), Naples, Italy; [6]Neurosurgery, Bambino Gesù Children's Hospital, Scientific Institute (IRCSS), Rome, Italy

Received June 26, 2015; Accepted August 11, 2015; Epub August 15, 2015; Published August 30, 2015

Abstract: Tissue engineering is one of the major challenges of orthopedics and trauma surgery for bone regeneration. Biomaterials filled with mesenchymal stem cells (MSCs) are considered the most promising approach in bone tissue engineering. Furthermore, our previous study showed that the multi-phase poly [ε-caprolactone]/thermoplastic zein-hydroxyapatite (PCL/TZ-HA) biomaterials improved rabbit (r) MSCs adhesion and osteoblast differentiation, thus demonstrating high potential of this bioengineered scaffold for bone regeneration. In the recent past, CD271 has been applied as a specific selective marker for the enrichment of MSCs from bone marrow (BM-MSCs). In the present study, we aimed at establishing whether CD271-based enrichment could be an efficient method for the selection of rBM-MSCs, displaying higher ability in osteogenic differentiation than non-selected rBM-MSCs in an *in vitro* system. CD271$^+$ cells were isolated from rabbit bone marrow and were compared with rMSCs in their proliferation rate and osteogenic differentiation capability. Furthermore, rCD271$^+$ cells were tested in their ability to adhere, proliferate and differentiate into osteogenic lineage, while growing on PCL/TZ-HA scaffolds, in comparison to rMSCs. Our result demonstrate that rCD271$^+$ cells were able to adhere, proliferate and differentiate into osteoblasts when cultured on PCL/TZ-HA scaffolds in significantly higher levels as compared to rMSCs. Based on these findings, CD271 marker might serve as an optimal alternative MSCs selection method for the potential preclinical and clinical application of these cells in bone tissue regeneration.

Keywords: Rabbit bone marrow, mesenchymal stem cells, CD271, osteoblast, biomaterials, bone tissue engineering

Introduction

Each year bone fractures and trauma-related injuries account for a large number of surgical procedures, while reconstructive surgery requiring bone grafts is carried out on a daily base in worldwide hospitals. Bone tissue can self-regenerate and self-repair. However, wide pathological fractures and anatomical predisposition of specific sites to fracture, as well as insufficient blood supply and infections may cause a delayed or difficult healing, accounting for the failure of bone repair. Autologous or allogenic bone graft is commonly used as a medical strategy for bone repair. Nevertheless, there are many limitations in the use of the graft, as recently reviewed by Ahmad et al. [1]. Bone tissue engineering promises such an alternative, because its ultimate objective is to develop a microenvironment that holds osteogenic, osteoinductive and osteoconductive properties. More recently, it has been demonstrated that the combined use of appropriate scaffolds and stem cells may improve *in vivo* bone regeneration. Until now, many researchers have developed strategies based on the seeding of

mesenchymal stem cells (MSCs) into three-dimensional biodegradable polymeric scaffolds. Previously, we have demonstrated that the biomaterial made by poly(ε-caprolactone), thermoplastic zein and hydroxyapatite (PCL/TZ-HA) improved rabbit MSCs adhesion, proliferation and osteogenic differentiation capability in an *in vitro* study [2]. In addition, Udehiya et al [3] showed that the seeding of hydroxyapatite scaffolds with MSCs, isolated from bone marrow (BM), induced faster and better healing of bone segmental defects in a rabbit model, as compared to hydroxyapatite alone.

MSCs are usually isolated from different sources: bone marrow [4], adipose tissue [5], skin [6], umbilical cord blood [7] and foetal membranes [8, 9], are considered a valuable cell type for bone regeneration, due to their differentiation ability into osteogenic, as well as adipogenic, myogenic and chondrogenic lineages [10, 11]. They express the STRO-1, CD73, CD90 and CD105 cell-surface markers, while are negative for the CD45 and CD34 ones. Despite this knowledge, MSCs isolation remains a difficult task due to the lack of own specific identifying markers and, as such, there is no universal agreement on the optimal harvesting strategy. The most common method for MSCs isolation is based on their plastic adherence ability, which in turn leads to a heterogeneous population. The identification of a selective marker would ensure the selection of pure MSCs that could be employed in several tissue engineering-based therapies. Recent studies have demonstrated that the low-affinity nerve growth factor receptor (LNGFR or CD271) antibody identified human BM cells with the characteristics of MSCs: they rapidly adhered to plastic and differentiated into osteoblasts, adipocytes and chondroblasts [10, 11]. So far, CD271 represents one of the most selective markers for the enrichment of BM-MSCs [12]. In fact, BM-MSCs isolated by CD271 positive selection demonstrated a higher cell proliferation capacity compared to the same cells isolated by plastic-adherence ability [12].

In this study we investigated whether CD271+ cells isolated from rabbit BM might be used for bone marrow tissue engineering. To this aim, rabbit (r) CD271+ cells were compared to rMSCs. Pours sponge scaffold made of poly-caprolactone (PCL), a biocompatible and completely biodegradable polymer, were used. In more detail, to enhance osteoconductivity, as well as, to improve mechanical properties, cell survival and proliferation, a particular type of PCL scaffold, named PCL/TZ-HA, that contains hydroxyapatite and the thermoplastic vegetal protein zein, was used.

Materials and methods

Scaffolds preparation

PCL (MW = 65 kDa, Tm = 59-64°C and Tg = -60°C) and maize zein powder (cod.: Z3625, batch: 065K0110, Sigma-Aldrich, St. Louis, MO). Poly(ethylene glycol) (PEG) 400 (Fluka, Sigma-Aldrich) and used as plasticizer for the preparation of the TZ. HA granules (ENGIpore, batch 071105, 250-355 μm size, Finceramica, Faenza, Italy) and used as a bio ceramic filler for the preparation of the composite biomaterial scaffolds. The TZ was prepared as previously described in details [2]. The biomaterials were prepared by using a twin counter rotating internal mixer connected to a control unit (Rheomix 600 and Rheocord 9000, respectively, Haake, Germany), at 70°C, 80 rpm for 6 min. PCL pellets were first melted at 70°C, 20 rpm for 2 min and, subsequently, TZ and HA were added into the mixing chamber and mixed at 80 rpm for 6 min. Finally, the biomaterials were compression molded at 80°C and 30 bar into 1 or 2 mm-thick plates by a hot press (P300P, Collin, Germany).

Rabbit mesenchymal stem cells (rMSCs) isolation and expansion

The adult MSCs were isolated from bone marrow of 8 New Zealand White adult rabbits weighing approximately 3-4.5 kg. The animal experiments were performed in accordance with the institutional ethical protocol (No. 116/92) for the protection of animal experimentations. The bone marrow (BM) was harvested according to procedures previously described [2]. BM was isolated from the distal and proximal left femur under sterile conditions and the aspirated material was settled in a tube containing sodium heparin (20 U mL^{-1} of aspirated material). Then, mononuclear cells (MNCs) were separated on Ficoll-Hystopaque 1077 gradients (Sigma-Aldrich). The cells were washed and resuspended at a density of 10^5 cells/mL in Mesencult basal medium contain-

ing MSC stimulatory supplements (Stemcell Technologies, Vancouver, Canada). Cells were then seeded in T12.5 flasks and incubated at 37°C, in a 5% CO_2 atmosphere and 95% of relative humidity. Adherent cells were allowed to reach approximately 80% confluence (12-17 days for the first passage). Cells were detached following treatment with trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco BRL, Grand Island, NY) and replaced every 6-8 days at approximately 80% confluence. The second passage (P2) of the cells was used if not otherwise stated. When culture flasks were nearly confluent, the cells in the primary culture were sub-cultured and collected for scaffold preparation.

Flow cytometric analysis

rBM-MNCs cells were analysed for the presence of CD271 cell-surface marker according to standard protocols. The monoclonal antibodies (mAbs) allophycocyanin (APC)-CD271 (Miltenyi Biotec, Bergisch Gladbach, Germany) was used. Background levels were measured with isotype-matched APC-conjugated antibody. Cells were analysed on a FACS-Scan flow cytometer using CellQuest software (Becton-Dickinson, San Jose, CA). Cells were electronically gated according to light scatter proprieties to exclude cell debris and 10,000 events were collected per analysis.

Selection of rCD271+ cells from rBM-MNCs

rCD271+ cells were isolated from rabbit BM-MNCs by magnetic microbeads selection, using a high-gradient magnetic field and mini-MACS columns (Miltenyi Biotec). A second column run was performed to obtained CD271+ cells of higher purity. At the end of the process the CD271+ cell purity was >95%.

rCD271+ cells expansion

The rCD271+ cells were washed and resuspended at a density of 10^4 cells/mL in Mesencult basal medium containing MSC stimulatory supplements (Stemcell Technologies). Cells were then seeded in T12.5 flasks and incubated at 37°C, in a 5% CO_2 atmosphere and 95% of relative humidity. The cells were cultivated until the cells at the base of the flasks reached confluence. After reaching confluence, the adherent cells were detached using trypsin-EDTA (Gibco BRL) and replaced. At the end of second passage (P2), when culture flasks were nearly confluent, the cells were detached following treatment with trypsin-EDTA and subcultured/or collected for scaffold preparation.

Osteogenic differentiation of rCD271+ cells and rMSC

rCD271+ cells were differentiated toward the osteoblastic lineage as described [13]. Cells were cultured in osteogenic induction medium using OsteoDiff Medium (StemCell Technologies INC) for 21 days. Differentiation into osteoblasts was verified through the extracellular calcium deposits by staining the cells with Alizarin Red S (ARS) (Sigma-Aldrich St. Louis, MO), following the manufacturing instructions. In each experiment, rMSCs were used as control.

Scaffold seeding and osteogenic differentiation of rCD271+ cells and rMSCs

2D non-porous scaffolds for cells culture experiments (10 mm in diameter and 2 mm thick) were γ-sterilized before seeding. For seeding, 2 × 10^4 cells/scaffold, resuspended in 40 μL of medium, were statically seeded onto the PCL-TZ-HA scaffolds. The scaffold/cell constructs were then placed in 24-well culture plates and incubated for 45 min in a humidified atmosphere (37°C, 5% CO_2) to allow cell attachment. Cell-free medium (Mesencult medium) was then added to bring the total well volume to 1.5 mL. After incubation at 37°C for 24 h, the medium was replaced with osteogenic differentiation medium. rCD271+ cells and rMSCs (control cultures) were maintained in Mesencult and osteogenic differentiation medium.

Scanning electron microscopy

To assess adhesion activity of the cells, total rMSCs and rCD271+ cells were plated onto scaffolds in the presence of differentiation medium. After 24 h and 21 days of culture, the medium was removed, the scaffolds washed with PBS, and the cells fixed using 2.5% glutaraldeyde in 0.2 M cacodylate buffer (pH 7.3), post-fixed in 1% OsO4 dehydrated in ascending grades of ethanol and with hexamethyldisilazane (HM-DS), and then gold coated by sputtering (BALZERS SCD 050). Samples were examined with a Cambridge 360 scanning electron microscope.

DNA quantification

Cell proliferation was determined by DNA analysis. At predetermined time point, three replicate scaffolds were washed with phosphatise-buffered saline (PBS) (Euroclone, Wetherby, UK) and transferred to centrifuge tubes containing 1 mL deionised molecular-grade pure water. The tube were then transferred and stored at -20°C. After thawing, the samples were treated ultrasonically in an ice-water bath for 10 min and the released amount of DNA was measured from supernatant. Total amount of DNA was detected using Hoechst 33258 dye (DNA quantification kit, Sigma-Aldrich). Sample fluorescence was measured with a luminescent spectrometer (LS50B, Perkin-Elmer, Waltham, USA) at an excitation and emission wavelengths of 360 and 460 nm, respectively. Samples were compared to the standard curve to determine DNA content per composite.

Analysis for alkaline phosphatase activity

For measuring the alkaline phosphatase activity (ALP), cells cultured in monolayer and scaffold-cell constructs after 21 days of cultures were homogenized in 800 µL 0.1% Triton-X100 + 0.01% NaCl followed by sonication for 6 min. ALP activity was measured using a biochemical assay (Sigma-Aldrich) based on conversion of p-nitrophenyl phosphatase to p-nitrophenol which was measured spectro-photometrically at 405 nm. ALP activity is expressed as micromoles of p-nitrophenol released. Scaffolds without cells served as control groups.

Detection and quantification of mineralization

Mineralized matrix synthesis was analyzed by Alizarin Red S (ARS) analysis. Cells cultured in monolayers and scaffold/cell constructs were washed with PBS (Euroclone) and fixed in 10% (v/v) formaldehyde (Sigma-Aldrich) at room temperature for 10 min. The monolayer and scaffold/cell constructs were then washed twice with excess dH_2O prior the addition of 1 mL of 40 mM ARS solution at pH 4.1 (Sigma-Aldrich). Samples were incubated at room temperature for 8 min in gentle shaking. After aspiration of the unincorporated dye, the wells were washed four times with 2 mL dH_2O while shaking for 5 min. The plates were stored at -20°C prior to dye extraction. Stained cells monolayers were visualized by inverted microscope. For quantification of staining, 800 µL 10% (v/v) acetic acid was added to the samples and plates were incubated in gentle shaking at room temperature for 30 min. Cells cultured in monolayer were removed by scraper and transferred into microcentrifuge tube. Scaffold/cell constructs were sonicated in an ice-water bath sonicator for three times 2 min and the supernatant was removed to a microcentrifuge tube. After vortexing, the slurry was overlaid with 500 µL mineral oil (Sigma-Aldrich), heated to 85°C for 10 min, and transferred to ice for 5 min. The slurry was centrifugated at 14,500 × g for 15 min, the supernatants were collected in a new microcentrifuge tube. Then 200 µL of 10% ammonium hydroxide was added to each sample. Aliquots (150 µL) of the supernatant were read in triplicate at 405 nm. Statistical analyses on three readings were carried out using Student's t test, and *P* values of less than 0.05 were considered significant. Scaffolds without cells served as control groups.

Semi quantitative RT-PCR measurements

The expression of the specific osteogenic marker, osteopontin (OPN), was evaluated by RT-PCR measurements. To isolate RNA, cells were lysed directly in the culture scaffold by adding 0.5 mL of TriReagent (Sigma-Aldrich). Total RNA was extracted according to the manufacturer's instructions. 2 µL of total RNA were used for quantification by Standard Agilent 2100 bioanalyzer Agilent. 200 ng of total RNA for each sample was reverse transcribed using SuperScript RT (Gibco) and oligo dT priming. 5 µl aliquots of cDNA (50 ng equivalent of total RNA) were then amplified with specific primers in a Gene Amp PCR System 9600 thermocycler (Perkin-Elmer). PCR conditions were as follows: 11' of initial denaturation at 94°C, and 30 cycles of 30" at 94°C, 30" at 57°C, 45" at 72°C plus 7' of final extension at 72°C. The following primers were used: Osteopontin (*OPN*) sense 5'-GCTCAGCACCTGAATGTACC-3', Osteopontin (*OPN*) antisense 5'-CTTCGGCTCGATGGCTAGC-3' (292-bp product); *GAPDH* sense 5'-TCATTTGAAGGGCGGAGCCAA-3', *GAPDH* antisense 5'-ATGCCTGCTTCACCACCTTCT-3' (465-bp product). All primer sequences were exon/intron overspanning to prevent possible signals from genomic DNA. PCR conditions and the number of PCR cycles for each primer pair (30 cycles for *GAPDH* and *OPN*) were chosen according to preliminary experiments, in which the PCR product was detectable in an amount directly

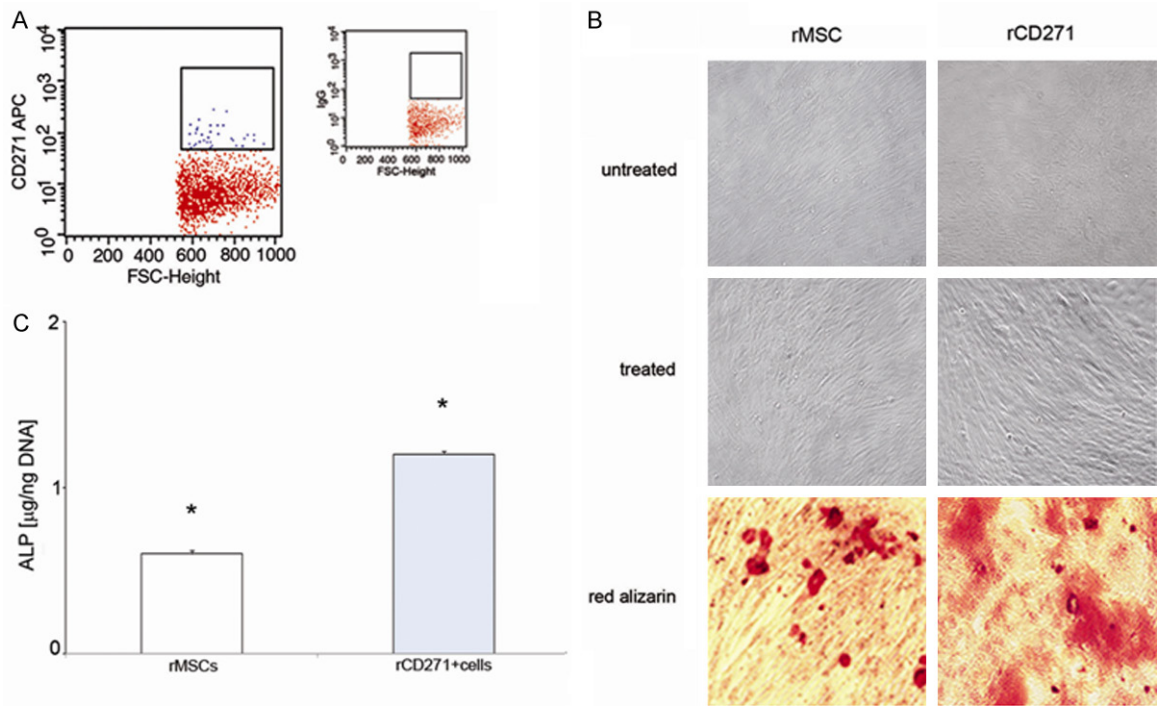

Figure 1. A. Representative flow cytometry plots showing the presence of rCD271+ cells from rabbit bone marrow mononuclear cells; B. Left panels shows cultures of rMSCs that have been isolated by plastic adherence ability and right panels shows cultures of rCD271+ cells isolated by antibody selection (14 days cultures, respectively). Untreated cells are expanded in vitro. Treated cells show nodular aggregates that are typical of osteogenesis. Alizarin Red S staining (ARS) show extracellular calcium deposits; C. Alkaline phosphatase activity measured after 21 days post induction. Original magnification × 100. Data are presented as means ± SD.

proportional to the quantity of starting cDNA. RT-PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide and run with a 100 bp ladder to confirm the predicted size. Relative levels of each PCR product were quantified by densitometric analysis of gel photographs using the Quantity One software and normalized to the signal from the housekeeping gene *GAPDH*. The ratio between the *OPN* and *GAPDH* RNAs was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Scaffold without cells served as control groups.

Statistical analysis

Paired Student t-test was used for data analysis using SPSS software version17. *P*-values less than 0.05 were considered statistically significant. Data were presented as mean ± standard error of mean (SEM).

Results and discussion

Bone is the second most commonly transplanted tissue after blood [14]. Since bone autografts and xenografts cause different and several problems, tissue engineering has been introduced in the last decades as a valuable therapeutic option. Tissue engineering involves the use of scaffolds, specific growth factors, cells and, more recently, stem cells [1]. Several authors and our previous studies demonstrated that the combined scaffold-BM-MSCs might have a higher potential in osteogenic differentiation and mineralized formation, compared to cells cultured in absence of scaffolds, both in *in vitro* and *in vivo* settings [15-17]. As such, BM-MSCs with multi-lineage differentiation capacities have been demonstrated to be a potential candidate for bone regeneration.

In this study, primary cultures of rabbit MSCs were derived from bone marrow (BM). Adherent cells, that were expanded *in vitro* until passage 2, displayed morphologic features that are typical of MSCs. The morphology of the rMSCs was determined by phase contrast microscopy (**Figure 1B**, untreated). At day 10, cells reached 80% confluence. At day 13, cells displayed a uniform spindle shape and reached 100% confluence. Among the several cell surface mark-

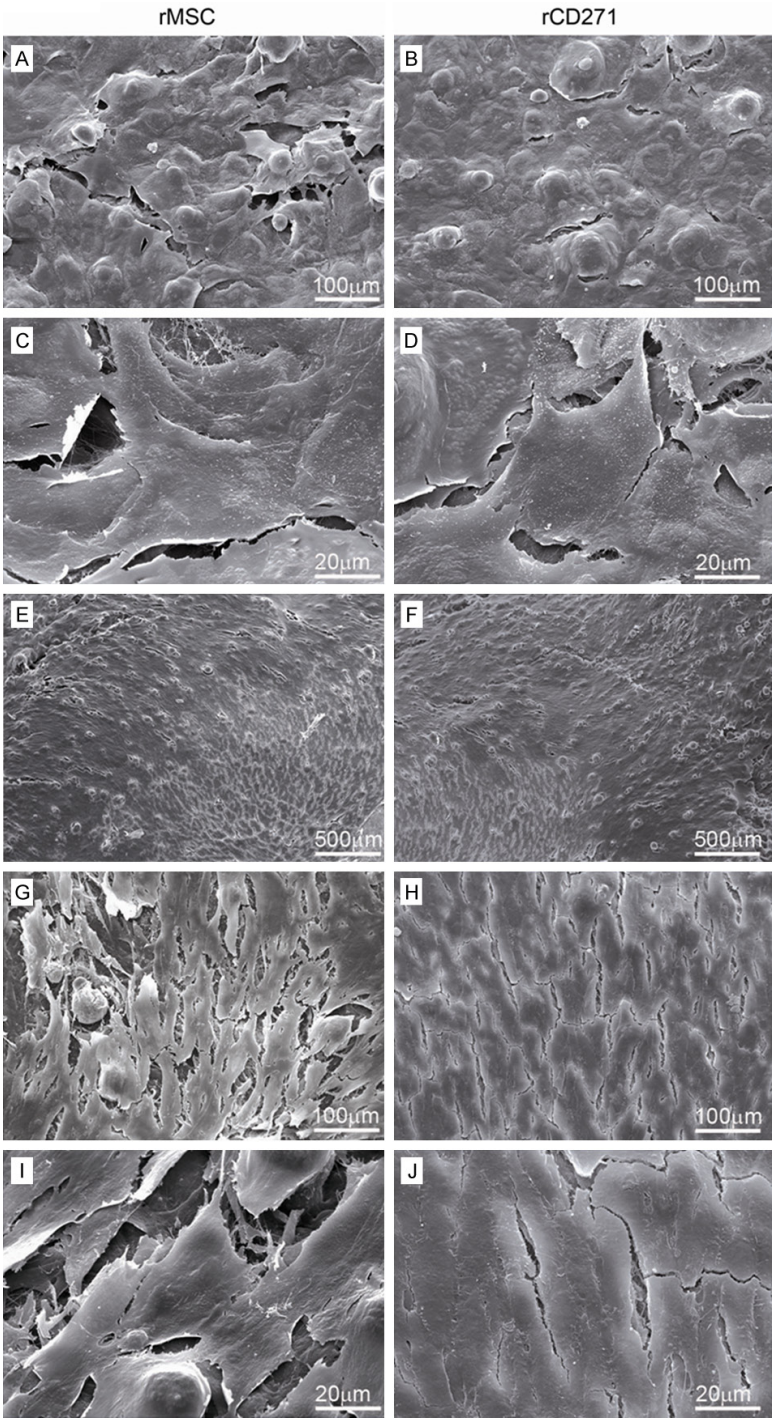

Figure 2. SEM micrographs of rMSCs and rCD271+ cells seeded on three-phases PCL/TZ-HA: (A) rMSCs and (B) rCD271+ cells on PCL/TZ-HA surfaces, (C) rMSCs and (D) rCD271+ cells on PCL/TZ-HA after 24 h of culture; SEM micrographs after 21 days of culture of the (E, G, I) rMSCs and (F, H, J) rCD271+ cells on PCL/TZ-HA. The SEM images illustrate rCD271+ cells and rMSCs populations after 24 h that were attached on the scaffold surface; after 21 days the cells proliferated on the entire surface of the scaffold. Bars = 100 μm, 20 μm, 500 μm.

ers that are expressed in MSCs, we choose to use the CD271 marker [18, 19]. To identify the rCD271+ cells in rabbit BM-MNCs population, cells were stained with monoclonal antibody against CD271. Gating on all CD271+, the frequency of CD271+ cells in our analyzed sample of BM-MNC fractions was 0.95±0.2% (range, 0.22% to 2%; median 0.65%) (**Figure 1A**). CD271+ cells were enriched from rabbit BM-MNCs by positive magnetic selection with an average purity of 95%±5.5%. rCD271+ cells and rMSCs were cultured as described in the previous section. After 15 days of culture, in both rCD271+ cells and rMSCs samples an almost homogeneous population of fibroblastic like cells was observed throughout the flask, with little evidence of round or floating cells (**Figure 1B**, untreated). To investigate the nature of rCD271+ cells, we examined their chondrocitic and adipogenic differentiation ability *in vitro*. In five independent experiment, we observed that both rCD271+ cells and rMSCs differentiated into both lineages (data not shown). These results are in agreement with other studies [12].

Next, we examined whether rCD271+ cells had osteogenic differentiation property. To this end, rCD271+ cells and rMSCs were plated and treated for osteogenic differentiation for 3 weeks. Osteogenic differentiation was evaluated by morphological and biochemical methods both in cells grown in osteoblastic (Ob) medium and in cells cultured in culture medium alone, which represented control cells. Proliferation and differentiation of control cells and Ob cultures were compared by phase contrast micros-

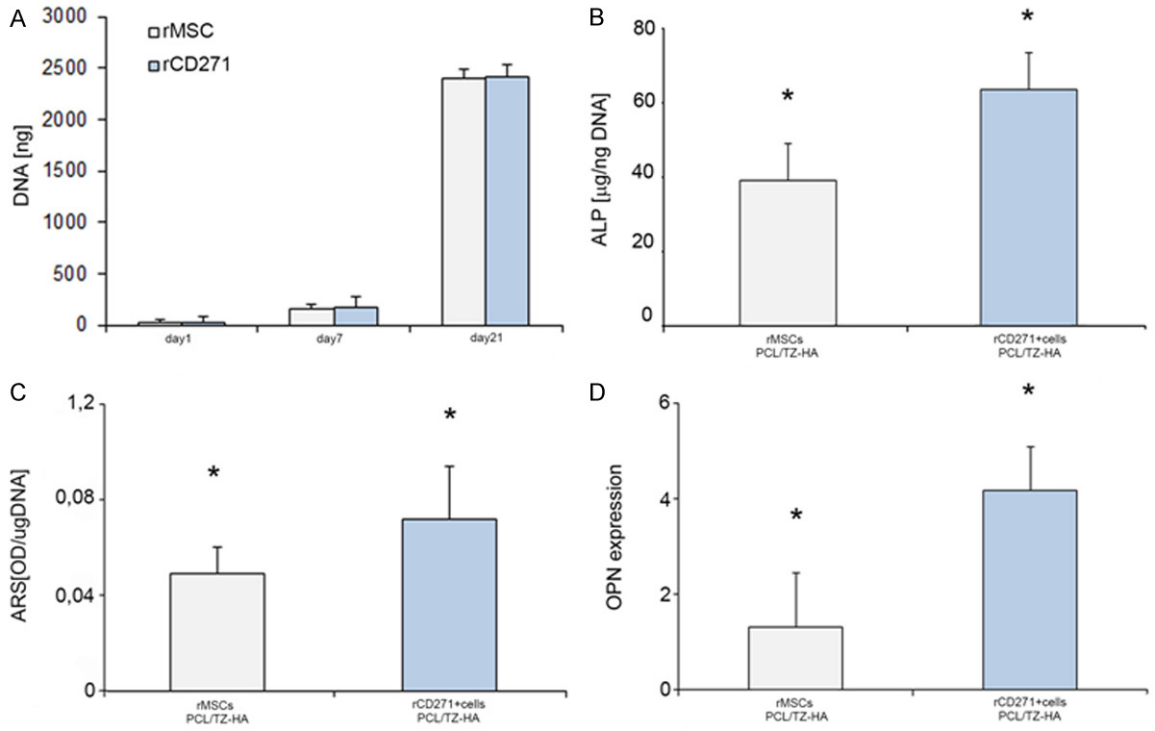

Figure 3. Quantitative assays. rMSCs and rCD271+ cells on three-phase PCL/TZ-HA scaffolds A. DNA content; B. ALP activity; C. Total calcium content, ARS; D. RT-PCR analysis of osteogenic marker osteopontin (OPN). Data are presented as means ± SD.

copy. After 7 days of cultures, nodular aggregates became evident in Ob cultures and increased after 3 weeks of culture (**Figure 1B**, treated). These aggregates were characterized by deposit of amorphous material. In rMSCs culture the aggregates were smaller as compared to rCD271+ cells (**Figure 1B**, treated). The nodular aggregates in Ob cultures stained with Alizarin Red S (ARS), demonstrated that the observed amorphous deposits were calcium deposits. ARS positive nodular aggregates were present after 2 weeks of culture. After 3 weeks, positive ARS aggregates were larger and stained more intensively in rCD271+ cells compared to rMSCs (**Figure 1B**, ARS), indicating that a more extensive calcium deposition had occurred. Total calcium content was measured after 3 weeks. Enhanced ALP activity was present in rCD271+ cells Ob cultures with respect to rMSCs cells after 3 weeks of cultures (**Figure 1C**). All together, these results indicate that both cell populations represent rabbit bone marrow-derived cells endowed with the ability to give rise to osteogenic cells.

An effective cell therapy for bone defects requires support from biomaterials or scaffolds. In the restoration of tissue defects, scaffolds can deliver cells or growth factors, provide a structure to which cells can attach and form tissue, and promote cell growth into the implant, both *in vitro* and *in vivo* [20].

To investigate whether rCD271+ cells could be used for bone regeneration, utilizing scaffolds as support, we tested the three-phase PCL/TZ-HA biomaterials to study the phenomenon in an *in vitro* system. Moreover, because in our previous study we have shown the rMSCs had already this capacity [2], we used the rMSCs population isolated from the same rabbits, as control. rCD271+ and rMSCs cells were cultured onto PCL/TZ/-HA scaffolds and SEM analysis was carried out to verify their capacity to adhere and proliferate on the surface of the scaffolds. SEM images showed that after 24 h most of both rCD271+ cells and rMSCs populations were attached to the scaffold surface, and that after 21 days the cells were still adherent, growing on the entire surface of the scaffold. There was no evident difference between the rCD271+ cells and the rMSCs (**Figure 2**). The obtained results thus confirmed the ability of both populations to adhere and proliferate onto the PCL/TZ-HA scaffolds.

Data of cell proliferation indicated that the number of cells continued to increase from day 1 to day 21 of culture in all different samples. The results demonstrated that the rCD271+ cells proliferated in a significantly higher number as compared to rMSC ($n = 6$, $P<0.05$) (**Figure 3A**) on PCL/TZ-HA scaffolds. Alkaline phosphatase (ALP) activity is one of the most commonly used marker of osteogenesis and is assumed to reflect the degree of osteogenic differentiation. As show in **Figure 3B**, all scaffolds expressed relatively high ALP activity. The one expressed by the rCD271+ cells was significantly higher than the activity expressed by the rMSCs cells cultured on the PCL/TZ-HA scaffold ($n = 8$, $P<0.05$). Total calcium content of each scaffold was measured at 21 days by staining with Alizarin Red S (**Figure 3C**). Significant higher calcium deposition was found after 21 days of cultures in osteogenic media for the rCD271+ cells compared to the rMSCs population on PCL/TZ-HA scaffold. RNA expression of one specific osteogenic differentiation marker, osteopontin (OPN), was evaluated by semiquantitative RT-PCR. mRNA levels of *OPN* were normalized to *GAPDH* mRNA levels within the linear range of amplification. *OPN* transcript levels increased in rCD271+ cells on the PCL/TZ-HA after 21 days of culture, and were significantly higher if compared to the ones of rMSCs, thus showing a higher ability of osteogenic differentiation of the CD271-selected cells (**Figure 3D**).

All these data indicate that rCD271+ cells were more efficient in the process of osteogenic differentiation than rMSCs. In agreement with other observations [21], we hypothesized that the higher capacity of proliferation and differentiation is the consequence of a relatively homogeneous population of stem cells, in comparison with the MSCs that represent a non-homogeneous population.

In summary, the results demonstrated that rCD271+ cells were able to proliferate in a higher significant rate compared to rMSCs. Alizarin Red S staining, ALP activity measurement and *OPN* RT-PCR analysis indicated that the rCD271+ cells seeded on the PCL/TZ-HA scaffolds produced higher levels of osteogenic biomarkers than rMSCs. However, the difference between the two cell populations could be ascribed to stem cells subpopulation homogeneity. We would also underline that the rCD271-based selection method could avoid cell manipulations such as plastic adherence (which potentially gives rise to a non-homogeneous population of stem cells). This study confirms that the use of CD271 as molecular marker represents a rapid and reliable method to obtain MSCs from BM with possible direct applications, avoiding cultivation costs, time and risk of contamination. Although these cells are very promising, in order to apply them for bone marrow regeneration, further investigations are needed to select the most efficient cell-scaffold combination approach.

Acknowledgements

This work was partially supported by "Fondazione PCFF" Pescara and "Associazione Donatori Sangue, F.I.D.A.S. Pescara".

Disclosure of conflict of interest

None.

Address correspondence to: Anna C Berardi, Research Laboratory, "Stem Cells", U.O.C. Immunohematology and Transfusion Medicine, Hospital Santo Spirito, Via Fonte Romana 8, Pescara 65125, Italy. Tel: +39 333 4338 414; E-mail: annac.berardi@ausl.pe.it; annacberardi@yahoo.it

References

[1] Oryan A, Alidadi S, Moshiri A, Maffulli N. Bone regenerative medicine: classic options, novel strategies, and future directions. J Orthop Surg Res 2014; 9: 18.

[2] Salerno A, Oliviero M, Di Maio E, Netti PA, Rofani C, Colosimo A, Guida V, Dallapiccola B, Palma P, Procaccini E, Berardi AC, Velardi F, Teti A, Iannace S. Design of novel three-phase PCL/TZ-HA biomaterials for use in bone regeneration applications. J Mater Sci Mater Med 2010; 21: 2569-81.

[3] Udehiya RK, Amarpal, Aithal HP, Kinjavdekar P, Pawde AM, Singh R, Taru Sharma G. Comparison of autogenic and allogenic bone marrow derived mesenchymal stem cells for repair of segmental bone defects in rabbits. Res Vet Sci 2013; 94: 743-52.

[4] Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 1970; 3: 393-403.

[5] Eagan MJ, Zuk PA, Zhao KW, Bluth BE, Brinkmann EJ, Wu BM, McAllister DR. The suitability of human adipose-derived stem cells for the

engineering of ligament tissue. J Tissue Eng Regen Med 2012; 6: 702-9.
[6] Vaculik C, Schuster C, Bauer W, Iram N, Pfisterer K, Kramer G, Reinisch A, Strunk D, Elbe-Bürger A. Human dermis harbors distinct mesenchymal stromal cell subsets. J Invest Dermatol 2012; 132: 563-74.
[7] Vasaghi A, Dehghani A, Khademalhosseini Z, Khosravi Maharlooei M, Monabati A, Attar A. Parameters that influence the isolation of multipotent mesenchymal stromal cells from human umbilical cord blood. Hematol Oncol Stem Cell Ther 2013; 6: 1-8.
[8] Campagnoli C, Roberts IA, Kumar S, Bennett PR, Bellantuono I, Fisk NM. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood 2001; 98: 2396-402.
[9] Colosimo A, Russo V, Mauro A, Curini V, Marchisio M, Bernabò N, Alfonsi M, Mattioli M, Barboni B. Prolonged in vitro expansion partially affects phenotypic features and osteogenic potential of ovine amniotic fluid-derived mesenchymal stromal cells. Cytotherapy 2013; 15: 930-50.
[10] Chan TM, Harn HJ, Lin HP, Chou PW, Chen JY, Ho TJ, Chiou TW, Chuang HM, Chiu SC, Chen YC, Yen SY, Huang MH, Liang BC, Lin SZ. Improved human mesenchymal stem cell isolation. Cell Transplant 2014; 23: 399-406.
[11] Raggi C, Berardi AC. Mesenchymal stem cells, aging and regenerative medicine. Muscles Ligaments Tendons J 2012; 2: 239-42.
[12] Álvarez-Viejo M, Menéndez-Menéndez Y, Otero-Hernández J. CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture. World J Stem Cells 2015; 7: 470-6.
[13] Quirici N, Soligo D, Bossolasco P, Servida F, Lumini C, Deliliers GL. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. Exp Hematol 2002; 30: 783-91.
[14] Brune JC, Tormin A, Johansson MC, Rissler P, Brosjö O, Löfvenberg R, von Steyern FV, Mertens F, Rydholm A, Scheding S. Mesenchymal stromal cells from primary osteosarcoma are non-malignant and strikingly similar to their bone marrow counterparts. Int J Cancer 2011; 129: 319-30.
[15] Hassani A, Motamedi MH, Saadat S, Moshiri R, Shahmirzadi S. Novel technique to repair maxillary sinus membrane perforations during sinus lifting. J Oral Maxillofac Surg 2012; 70: e592-7.
[16] Fang GW, Liu XS, Lv TZ, Shu HS, Shao H. A new choice for the treatment of segmental tibial bone defects: intramedullary nail internal fixation combined adipose-derived stem cells technique. Med Hypotheses 2013; 80: 422-4.
[17] Velardi F, Amante PR, Caniglia M, De Rossi G, Gaglini P, Isacchi G, Palma P, Procaccini E, Zinno F. Osteogenesis induced by autologous bone marrow cells transplant in the pediatric skull. Childs Nerv Syst 2006; 22: 1158-66.
[18] Yang Y, Wu J, Jin G, Li L, Li Z, Li C. Research of osteoblastic induced rat bone marrow mesenchymal stem cells cultured on β-TCP/PLLA porous scaffold. Int J Clin Exp Med 2015; 8: 3202-9.
[19] Caneva L, Soligo D, Cattoretti G, De Harven E, Deliliers GL Immuno-electron microscopy characterization of human bone marrow stromal cells with anti-NGFR antibodies. Blood Cells Mol Dis 1995; 21: 73-85.
[20] Spector M. Biomaterials-based tissue engineering and regenerative medicine solutions to musculoskeletal problems. Swiss Med Wkly 2007; 137 Suppl 155: 157S-165S.
[21] Cuthbert RJ, Giannoudis PV, Wang XN, Nicholson L, Pawson D, Lubenko A, Tan HB, Dickinson A, McGonagle D, Jones E. Examining the feasibility of clinical grade CD271+ enrichment of mesenchymal stromal cells for bone regeneration. PLoS One 2015; 10: e0117855.