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Morpho-functional characterization of human mesenchymal stem cells from umbilical cord blood for potential uses in regenerative medicine

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ABSTRACT

Mesenchymal stem cells (MSCs) represent a promising source of progenitor cells having the potential to repair and to regenerate diseased or damaged skeletal tissues. Bone marrow (BM) has been the first source reported to contain MSCs. However, BM-derived cells are not always acceptable, due to the highly invasive drawing and the decline in MSC number and differentiative capability with increasing age. Human umbilical cord blood (UCB), obtainable by donation with a non-invasive method, has been introduced as an alternative source of MSCs. Here human UCB-derived MSCs isolation and morpho-

functional characterization are reported. Human UCB-derived mononuclear cells, obtained by negative immunoselection, exhibited either an osteoclast-like or a mesenchymal-like phenotype. However, we were able to obtain homogeneous populations of MSCs that displayed a fibroblast-like morphology, expressed mesenchym-related antigens and showed differentiative capacities along osteoblastic and early chondroblastic lineages. Furthermore, this study is one among a few papers investigating human UCB-derived MSC growth and differentiation on three-dimensional scaffolds focusing on their potential applications in regenerative medicine and tissue engineering. UCB-derived MSCs were proved to grow on biodegradable microfiber meshes; additionally they were able to differentiate towards mature osteoblasts when cultured inside human plasma clots, suggesting their potential application in orthopedic surgery.

INTRODUCTION

MSCs are known as non-hematopoietic, stromal cells that have the capability for self-renewal and multilineage differentiation into mesenchyme-lineage cell types, including osteoblasts, adipocytes, chondrocytes and myoblast. Moreover, given the appropriate microenvironment, MSCs in vitro could differentiate into cardomyocytes or even cells of non-mesodermal derivation including hepatocytes and neurons (1-5). MSCs can be isolated from various human sources, including BM, cartilage, periostium, synovium, tendons, adipose tissue and muscle. Fetal tissue, placenta, UCB and vasculature have been also reported to contains MSCs (6-8). The isolation and culture of MSCs, first described in the 1970s, from BM is achieved by the adherence to a plastic substratum followed by in vitro expansion (9). BM has been the most common source of MSCs, but aspirating BM from the patient is an invasive, painful

procedure and, in addition it has been demonstrated that the number and the differentiation potential of BM-derived MSCs decrease with age (10).

Therefore the identification of alternative sources of MSCs may provide significant clinical benefits with respect to ease of accessibility and reduced morbidity. The blood remaining in the umbilical vein following birth contains hematopoietic precursors and this has become an important alternative source for transplantation of hematopoietic stem cell (11-15). Most of the time UCB is still regarded as medical waste in the delivery rooms in contrast to BM aspiration; it is obtained by a simple, safe and painless procedure when the baby is delivered. UCB cells are good substitutes for BM-derived hematopoietic progenitors due to the immaturity of newborn cells (16). The immaturity of cells is associated with lower immunogenicity therefore UCB reduces graft-versus-host reactivity compared with adult-derived marrow grafts. Furthermore, UCB provides no ethical problems for basic studies and clinical applications.

Although the isolation of hematopoietic stem cell from UCB has been well established, the isolation and characterization of MSCs from UCB still need to be evaluated. Eriches et al. (17) reported that UCB-derived mononuclear cells gave rise to two adherent cell types, only one of them expressing MSC-related surface antigens. Mareschi et al. (18) reported that under given conditions, it was possible to isolate MSCs from BM, but not from UCB; Goodwin et al. (19) have reported the multi-lineage differentiation ability of UCB-isolated cells. Neither of these reports provided sufficient evidence to fulfill the qualifying criteria for MSCs because relatively heterogeneous cells were reported by both groups. Wexler et al. (20) have recently reported that UCB is not a rich source of human MSCs, while Romanov et al. (8) also suggested using umbilical cord endothelial cells as an alternative MSC source. Consistent findings within the literature include the extent of patient variability between each donor blood sample and the contamination of a large number of cells such as fibroblastic cells, dendritic cells, adherent monocytes, macrophages and osteoclastic cells arising within the cultures (18, 19, 21). The

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above observations indicate that this topic remains a complex and controversial area of research. This paper describes a methodological approach to the isolation and both phenotypical and functional characterization of MSCs from human UCB. Particularly several microscopic investigations were performed to characterize UCB-derived MSCs.

MSCs represent an attractive and promising field in tissue regeneration and engineering for treatment applications in a wide range of trauma and orthopedic conditions. Tissue engineering is an interdisciplinary field which applies the principles of engineering and of life sciences to the development of biological substitutes, to maintain or improve the function of damaged tissues and organs. The use of stem cells in tissue engineering has opened the possibility of regenerating every type of tissue in vitro. The composition of the scaffold influences the capability of adhesion, development and differentiation of the stem cells. In this study UCB-derived MSCs were cultured on different scaffolds (natural and synthetic) and both cell viability and differentiation potential towards osteoblastic lineage were evaluated by standard staining methods. From the three-dimensional culture on these scaffolds it was observed that the UCB-derived MSCs are able to adhere and colonize the supports. Therefore the clots of human plasma have proved to be suitable scaffolds not only for adhesion and growth of cord MSC, but also for the differentiation of these cells in osteogenic sense. Taken together, these studies support the hypothesis that multipotential stem cells derived from UCB exhibit morphological and phenotypical features similar to that observed in adult BM-derived stem cells; on the opposite, functional characteristics of UCB-derived MSCs differ from BM-derived MSCs. Their expansion and differentiation toward the osteoblast lineage on biodegradable scaffolds may provide a potential source of osteogenic material, which could be used in orthopedic surgery.

MATERIALS AND METHODS

Cell collection

Human UCB was collected from patients undergoing deliveries after receiving informed consent (Santa Chiara Hospital, Pisa, Italy). The UCB samples were harvested from normo-conducted pregnancies (n=100) between the 37th and the 42nd week of gestation after both vaginal and caesarean delivery. The blood was collected into heparinized tubes (5,000 IU/ml) or in bag system containing 17 ml of citrate phosphate dextrose anticoagulant (CB collect, Stemcare, Fresenius Kabi, Modena, Italy) and processed within 12 hours.

Isolation and culture of mononuclear umbilical cord blood cells

The buffy coat cells were obtained by centrifugation (400 g for 20 min) and the low-density mononuclear cells were obtained by negative immunodepletion of $CD3^+$, $CD14^+$, $CD19^+$, $CD38^+$, $CD66^+$ and glycophorin A^+ cells using a commercially available kit (RosetteSep Human Mesenchymal Stem Cell Enrichment Cocktail, StemCell Technologies, Vancouver, BC, Canada). In accordance with the manufacturer's instructions, 50 µl of RosetteSep was added per milliliter of undiluted blood, incubated for 20 min at room temperature, mixed 2:1 with Hank's balanced salt solution (Sigma-Aldrich, Saint Louis, MO). Gently overlaid onto Ficoll-Paque density solution (Histopaque®-1077, Sigma) and centrifuged at 300 g for 25 min. The enriched cell population was collected and plated in uncoated tissue culture flasks (CoStar Group, Bethesda, MD) in the expansion medium and was cultured at 37°C in 5% CO_2 concentration at 95% humidity. Expansion medium consisted of Minimum Essential Medium-alpha modification (α-MEM, Sigma) and 20% fetal bovine serum (FBS, Sigma)

supplemented with 100IU/ml penicillin (Pharmacia&Upjohn SpA, Milan, Italy), 100IU/ml streptomycin (Bristol-Myers Squibb SpA, Sermoneta, Italy) and 2mM L-glutamine (Cambrex Bioscience Inch., Baltimore). Cells were allowed to adhere overnight and non-adherent cells were washed out with medium changes. Medium changes were carried out twice weekly thereafter. On reaching confluence, cells were detached with 0.05% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) for further expansions. Cell growth was analyzed after the first passage (P1) by direct cell counts to determine the cumulative population doublings (PD). Number of PD were calculated using the formula $\log_{10}$ (N) / $\log_{10}$ (2) where N = cells harvested/ cells seeded (22) and results were expressed as cumulative PD per days of cultures (passages).

Cell samples cultured on cover glasses for 1 week were rinsed in phosphate buffer saline (PBS, Oxoid Limited, Basingstoke, Hampshire, UK), fixed using 10% formaline (Bio-Optica, Milan, Italy) for 10 min at 4°C, then washed and stained with hematoxylin and eosin (H&E, Sigma) for morphological analysis.

Immunophenotypic analysis

The phenotypical analysis was performed on cells cultured from passages 2 through 6, after incubation of the UCB-derived adherent cells with monoclonal antibodies (mAbs). MSCs were recognized by immunophenotype using mAbs specific for CD105, CD29, CD44 and CD90. The lack of hemopoietic (anti-CD45, CD14 and CD34 mAbs) and endothelial cell markers (anti-CD31 mAbs) was assessed. Anti-CD31, CD61, HLA-DR and HLA-ABC mAbs were also analyzed. Moreover the expression of the stage-specific embryonic antigen-4 (SSEA-4, Chemicon, Prodotti Gianni, Milan Italy) was evaluated on mononucleated cells isolated from UCB by density gradient centrifugation. A total of 100 µl of cell suspension (approximately 5×10^5 cells) was aliquoted per tube and appropriately labeled

mAbs were added for two-color analysis (FITC: fluorescein isothiocyanate and PE: phycoerythrin) (Immuno Tools, Friesoythe, Germany) and incubated for 30 min at 4°C; then washed twice and analyzed. Flow cytometer instrument was set using unstained cells. Cells were gated by forward versus side scatter to eliminate detris. A second gate was established on the CD45-negative population inside which the analysis of the various markers was then performed.

Appropriate mouse isotype antibodies served as respective controls. Ten thousand labeled cells were acquired and analyzed by using an Epics-XL flow cytometer (Becman Coulter, NC) running Software Expo32.

Characterization of MSCs

Atomic Force Microscopy (AFM) analysis

AFM represents the most wide employed probe microscopy in biomedical areas for surface characterization with unique resolution capability. The AFM techniques are based on an ultra-sharp probe (with tip radii less than 50nm) attached to a flexible cantilever and ceramic piezoelectric, which allow the sample to be scanned in the xy plane. The AFM large impact in biomedical areas is due principally to its ability to explore sample surfaces at the micro/nanoscale level under a variety of environmental conditions to simultaneously investigate different chemical or mechanical features of the same surface (23). MSCs were cultured on cover glasses for one week; afterwards they were fixed following the procedure recommended in literature to be observed with the AFM (24). Samples were gently rinsed with PBS, fixed using 4% phosphate-buffered para-formaldehyde (Bio-Optica) for 20 min at 4°C, rinsed with demineralized water (ddH_2O) to remove the fixative crystals, finally air died and stored at 4°C until observation. Topography was recorded via tapping-mode AFM, Veeco (Nanoscope III controller), with a TappingMode Etched Silicon probe (stiffness 0.24N/m, tip radius

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10nm). The measurements were performed in air at room temperature and relative humidity using a scanner of 100×100 micron^2.

Scanning Electron Microscopy (SEM) analysis

Cell samples cultured on cover glasses, prepared as described above, were observed by a Jeol scanning electron microscope model JSM-5600 LV (Jeol Ltd., Tokyo, Japan). Samples were mounted on aluminum stubs and covered with gold before SEM examination.

Osteogenic differentiation

To promote osteogenic differentiation, the cells were seeded at a density of 3-5×10^3 cells per cm^2 in 6-well plate (CoStar Group) and cultured in basal medium: α-MEM supplemented with 20% FBS, antibiotics and L-glutamine until they reached 60-70% confluence. As soon as subconfluence was reached, osteogenic differentiation of cells was induced by culturing them for 2.5 weeks, twice a week, with osteogenic induction medium consisting of 100 mM dexamethasone (Sigma), 10 mM β-glycerophosphate (Sigma), 100 µM ascorbic acid (Vitamin C, Roche, Indianapolis, IN) and 20% FBS in α-MEM. For the negative control, the cells were kept in basal medium. Osteogenic differentiation was investigated by the von Kossa staining, demonstrating the deposition of a hydroxyapatite matrix. The cells were fixed with 1% formalin (Bio-Optica) for 10 min at 4°C and stained for 15 min with 1% silver nitrate (Fluka, Millwaukee, WI, and Sigma). The stain was developed incubating the cells in 0.5% pyrogallol (Fluka) and then fixed with 5% sodium thiosulfate (Fluka) for 5 min. Finally the cells were counterstained with 0.1% nuclear fast red (Fluka). The samples were then dehydrated and mounted with DPX mountant (Fluka). Mineral deposition was evaluated as black granules using optical light microscopes.

Evaluation of osteogenic markers

Experiments were carried out to identify some osteogenic markers in differentiated cells. The samples, MSCs cultured in regular medium (controls, CTRL) and MSCs cultured in ostoegenic supplemented medium (osteoinduced cells, OST) for 10 and 20 days were harvested for quantitative assays: DNA content, alkaline phosphatase activity and osteocalcin production. Moreover a histochemical evaluation of alkaline phosphatase activity was performed to determine the percentage of positive cells.

DNA content

Cell number was quantified using the PicoGreen kit (Molecular Probes, Eugene, OR, USA) to measure double strand DNA content in lysates (25). Briefly, cell samples (1 sample for each cell type and time point) were scraped from the 6-well plates using 1.5 ml of cold ddH_2O, placed in eppendorf tubes and then stored at -20°C until analyses were performed. Samples were thus thawed at 37°C and vortexed for 10 s. Standards of DNA in ddH_2O in concentrations ranging from 0–6 µg/ml were prepared according to the manufacturer's instructions using reagents provided by the kit. Sample or standard was loaded with the fluorochrome solution and incubated in the dark; subsequently the fluorescence intensity was measured on a plate reader (Victor^3, Perkin Elmer, Waltham, MA, USA) using an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Cellularity was thus determined by considering 7.18 pg DNA/cell as the nuclear DNA content of a human diploid cell (26).

Alkaline Phosphatase Activity (ALP)

ALP activity is considered a marker of osteoblasts mostly expressed at early stages of their maturation although it is not specific only of these cells. ALP activity was quantified both using a cytochemical

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staining and a colorimetric end-point assay to determine the early differentiation of UCB-derived human MSCs towards the osteoblastic phenotype (27).

ALP activity was evaluated in cytocentrifuged samples (samples in duplicate) via cytochemistry to assess the number of ALP positive cells. ALP analysis was performed using an ALP staining (kit No 86, Sigma). Briefly, cells were fixed by acetone/citrate/formalin working solution, washed and stained with Naphtol AS-BI phosphate as substrate. The percentage of ALP positive cells was thus evaluated by direct counting with respect to the total cell number (approximately 200 cells were counted in each field).

Moreover, intracellular ALP activity per cell was quantified in aliquots of the same lysates used for DNA. As necessary, samples were diluted in ddH_2O to stay within the detection range of the assay. Standards of *p*-nitrophenol in concentrations ranging from 0–250 μM and substrate solution were prepared following the manufacture's instructions. Samples were incubated with the substrate for 1 h at 37°C and the reaction was stopped adding 0.3 M NaOH. The absorbance was then measured on a microplate reader (BioRad, Hercules, CA). All reagents used were purchased from Sigma-Aldrich, Milan, I. ALP activity was then expressed per cell as determined by the PicoGreen assay.

Osteocalcin (OC)

Osteocalcin (bone γ-carboxyglutamic acid) is a specific bone protein, synthesized by osteoblasts, which can be considered as a marker of metabolic activity proper of these cells. OC was evaluated in cascade on the same lysates used for ALP activity and DNA content, using an immunoenzymatic ELISA: N-MID Osteocalcin kit (Cobas, Roche, Indianapolis, IN), according to the manufacturer's instructions. OC production was then expressed per cell as determined by the PicoGreen assay.

Adipogenic Differentiation

Human MSCs committed towards adipocytic phenotype were cultured through 3-day induction cycles followed by 1-day rest periods, as described in the literature (28). The cells were seeded at a density of 3-5×10^3 cells per cm^2 in 6-well plate (CoStar Group) and cultured in basal medium: Dulbecco's Modified Eagle's Medium (DMEM, Sigma) low-glucose supplemented with 20% FBS, antibiotics and L-glutamine, until they reached 80% confluence. Induction was performed using adipogenic induction medium [complete DMEM supplemented with 10^{-6} M dexametasone (Sigma), 0.2 mM indomethacin (Fagron, Barsbüttel, Germany), 100 IU/ml insulin regular (Humulin-R, Lilly, Florence, Italy), 0.5 mM 3-isobutyl-1-methyl-xanthine (Sigma)] followed by rest using adipogenic maintenance medium (complete DMEM supplemented only with 100 IU/ml insulin regular). After three cycles, the induced cells were incubated for seven days more in adipogenic maintenance medium. Control cells were kept only in adipogenic maintenance medium. Adipogenic differentiation was analyzed by investigating the formation of neutral lipid-vacuoles that stained with Oil Red O (Sigma). The cells were fixed with 10% formalin for 10 min at 4°C, washed and incubated in 85% propylene glycol (Sigma) for 5 min. Then the cells were stained with a working solution of 2% Oil Red O in propylene glycol for 7 min and in 85% propylene glycol for 3 min. The nuclei were counterstained with H&E (Sigma).

Chondrogenic Differentiation

To promote chondrogenic differentiation 2.5x10^5 cells were gently centrifuged (150 g for 7 min) in a 15-ml polypropylene tube to form a pellet at the bottom of the tube (micromass culture). The cells were treated with serum-free chondrogenic medium for 3 weeks. Medium changes were carried out twice weekly without disturbing the pellet. Chondrogenic medium consisted in DMEM/F12 (1:1) (Sigma) supplemented with 1.25 µg/ml Bovine Serum Albumin (BSA, Sigma), 5.35 µg/ml linoleic acid (Sigma), 50 µg/ml ascorbic acid (Vitamin C, Roche, Indianapolis, IN), 100X ITS^+ premix (6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml selenius acid, 1.25 mg/ml BSA, 5.35 mg/ml linoleic

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acid; Invitrogen, Carlsbad, CA), 100 µg/ml sodium pyruvate (Sigma) , 10 ng/ml TGF-β1 (PeproTech, Rocky Hill, NJ). After the incubated period, cell morphology was investigated with H&E (Sigma), while the production of glycosaminoglycans (GAGs) was evaluated by Alcian Blue staining (Bio-Optica) at pH 2.5 and at pH 1, specific for non-sulphured mucins and for sulphured mucins, respectively. Briefly, samples were first incubated either in pH 1 or in pH 2.5 solutions, for 30 min. Then a detection solution was added for 10 min. After washing, nuclei were counterstained with 0.1% nuclear fast red (Fluka) for 5 min. Finally, the sections were dehydrated, clarified and mounted as described above.

Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cell cultures using High Pure RNA Isolation kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Extracted RNA was solved in diethylpyrocarbonate-treated water (DEPC-water), and RNA concentration was measured in a spectrophotometer at 260 nm. Identical amounts of RNA were reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis kit (Roche). cDNA was subsequently amplified by polymerase chain reaction (PCR) at 94°C for 30 seconds, 57°C for 30 seconds (58°C for 60 seconds for GAPDH), and 72°C for 30 seconds for 35 cycles (25 cycles for GAPDH), after initial denaturation at 94°C for 5 minutes. Primers used for amplification were: 5'-GCCGAGGTGATAGTGTGGTT-3' and 5'-TGAGGTGATGTCCTCGTCTG-3' amplifying a product of 101 bp corresponding to osteopontin (OPN); 5'-AAGGTCATGCTGGTCTTGCT-3' and 5'-GACCCTGTTCACCTTTTCCA-3' amplifying a product of 114 bp corresponding to collagen type Iα2 (Coll-I); 5'-AGCAGCAAGAGCAAGGAGAAG-3' and 5'-GCAGGCGTAGGAAGGTCATC-3' amplifying a product of 130 bp corresponding to collagen type II α1 (Coll-II); 5'-CCACCGGGACCATCAGCT-3' and 5'-CCAGGGATTCCAGGTGGTC-3' amplifying a product of 149 bp corresponding to collagen

type Xα1 (Coll-X); 5'-CCAACCCACGAATGCACTATC-3' and 5'-TAGTGAGTGGTGGCGGACATAC-3' amplifying a product of 92 bp corresponding to Runx2/cbfa-1 ; 5'-GCCAAAAGGGTCATCATCTCTG-3' and 5'-CATGCCAGTGAGCTTCCCGT-3' amplifying a product of 347 bp corresponding to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, the housekeeping gene) used as internal standard. The PCR products were loaded on a 2.5% agarose gel and stained with ethidium bromide.

Cell culture on three-dimensional scaffolds

Cell culture on fiber-mesh scaffolds

Biodegradable fiber meshes were investigated as synthetic scaffolds for cell growth. Meshes, shaped as rectangles (surface area 2×4 mm^2), were prepared using bioartificial hollow microfibers, based on Poly(L-lactic acid) (PLLA) and containing either dextran (PLLA/D meshes) or chitosan (PLLA/C meshes), which were produced by a method described elsewhere (29). The fibers were arranged in no-woven meshes that were stabilized by exposure to dichloromethane vapours. The produced meshes were then sterilized by ethanol washings, rinsed in PBS and then seeded with MSCs from human UCB with a density of 5×10^4 cells/sample in 96-well plates (CoStar group).

Cell culture was carried out in expansion medium for 20 days in a 5% CO_2 incubator. At the end-point, cell viability was investigated by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide test (MTT, Cell Proliferation Kit I, Roche). After 4 hours of cell incubation with the yellow-colored MTT solution (0.5 mg/mL), water insoluble formazan crystals, purple-colored, develop inside living and metabolically active cells that can be easily observed with an optical microscope.

Cell culture in human plasma clots

Plasmas for the clot preparation were obtained from peripheral blood of healthy adult donors. The blood was collected into tubes containing EDTA as anticoagulant and the plasma was obtained by centrifugation (1700 g for 20 min). The UCB-derived MSCs were seeded at density of 25×10^3, 5×10^4 and 1×10^5 /samples in 6-well plates (CoStar group) containing 500 µl of plasma. The clots containing the cells were produced with a published method (30). Briefly $CaCl_2$ (Dade Berhing, Marburg, Germany) was added to each well. Subsequently, the clots were incubated at 37°C in 5% humidified CO_2 atmosphere for seven days either with or without osteogenic medium. After seven days the clots were fixed in 4% neutral buffered formalin and embedded in paraffin. Osteoblastic markers were also identified. In particular, calcium deposition was tested on paraffin sections by von Kossa staining. The semiquantitative analysis used to assess the positivity of osteoinduced MSCs was determined by direct cell counts made by two independent observers.

Cell viability was tested after seven days of clot cultures. The presence of viable cells into the clots was qualitatively evaluated by a colorimetric assay, MTT test as describe above.

RESULTS

Isolation and culture of mononuclear cells isolated from UCB

A total of 100 UCB samples were used in this study. A representative experiment was carried out using two color-staining with SSEA-4 and CD105 mAbs to identify MSC in the whole pool of UCB-derived mononuclear cells. This experiment revealed that the SSEA-4 positive cells represents 0.52% of UCB-

derived mononuclear cells. The SSEA-4$^+$ population showed more than 7% of cells expressing the know MSC marker CD105.

Adherent cell populations were generated from the mononuclear fraction of human UCB samples. For the isolation of such cells a procedure of separation was used, by negative selection, with the RosetteSep Human Mesenchymal Stem Cell Enrichment Cocktail on a Ficoll-Paque density gradient. After about two weeks of culture in complete α-MEM, an adherent and stable cell layer was obtained. Under these conditions extensive interdonor variability was evident. In fact in 70% of the cord blood samples analyzed, two different cell populations were observed: an osteoclast-like cell (OLC) population, having oval or round morphology, and a mesenchymal-like cell population (MSC), showing elongated and spindle-like morphology (Fig. 1). H&E staining of OLCs showed multi-nucleated round shaped cells, with the nuclei as a circle around a central area, as shown in Fig. 1A. In this type of samples, the OLCs became more and more predominant on the MSCs along the culture time, as well as trypsin-EDTA resistant (Fig. 1B). On the contrary, in 30% of the analyzed samples, the primary cultures of mononuclear cells isolated from UCB gave rise to a layer of a single homogeneous adherent cell population, showing elongated morphology from initial stage of culture (Fig 1C and 1D). By 13-15 days, these cultures reached approximately 80% of confluence and were expanded and maintained in a continuous culture for 8 passages. Furthermore the cultures obtained from these blood samples did not present resistance to trypsin-EDTA treatment. Proliferation characteristics of mesenchymal-like cells through passage 2 to 6 were shown in Fig 4A.

Immunophenotypic analysis

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Adherent cells from 10 UCB samples at passages 1-6 were evaluated by flow cytometry for surface protein expression. The analysis of the two types of populations obtained, OLCs and mesenchymal-like cells, showed characteristic patterns of expression. The OLCs displayed expression of the hematopoietic antigens CD14 (> 50%) and CD45 (> 60%) (Fig. 2A) but no expression of the CD90 and CD105 antigens (data not shown) was revealed.

On the contrary, the mesenchymal-like cells were characterized for the absence of the CD45, CD14 and CD34 expression (more than 90% negative) (Fig.2B). These cells expressed on their surface the antigens typical of mesenchymal stem cells such as CD44 (95 ± 2.5%), CD90 (91.4 ± 3%), CD105 (56 ± 11%). They showed also a positive expression of CD13 (97 ± 2.6 %) and a more variable expression of CD29 (30.7 ± 9.8). Additionally these cells were negative for CD31 and CD61. Most importantly, immunophenotypic analyses showed that these cells expressed HLA-ABC (98 ± 1.3%) but they did not express HLA-DR molecules (Fig.2B).

Characterization of MSCs

The mesenchymal-like cells (named MSCs) at passages 4-6 were then investigated by an extensive morphological and functional characterization.

AFM and SEM analysis

A SEM image of isolated MSCs is shown in Fig. 3A, highlighting their extended morphology. The AFM micrographs showed the self-assembly capability of the MSCs on glass substrates, and their spatial orientation Fig 3B-C. The outgrowth of the MSCs generally followed one axial direction as shown by Fig. 3B (as confirmed by SEM, Fig. 3A). The MSC elongated filaments formed a network

communicating among different cells and investigating the surrounding environment (Fig. 3B). On contrary, a single MSC spatially well separated by the other ones seemed investigating the substrate through lateral pseudopodia (arrows in Fig. 3A and 3C) shorter than those of grouped cells (arrowheads in Fig. 3B).

Osteogenic differentiation

The UCB-derived MSCs were committed towards the osteoblast phenotype by supplemented culture medium for 21 days. Osteogenic markers such as ALP and OC were studied at two time-points (10 and 20 days). Intracellular ALP activity was investigated with two methods (cytochemistry, revealing the percentage of ALP positive cells per sample; and quantitative dosages, assessing the average ALP amount per cell) (Fig. 4B and 4C). The obtained outcomes highlighted that both CTRL and OST were able to express ALP. Moreover, after 20 days of culture, ALP was found quantitatively most abundant in CTRL than in OST cells. By comparing the two ALP analyses in CTRL, it also arises that the ALP quantity per cell increased much more than the percentage of ALP positive cells over 10-20 culture days. This suggests that intracellular ALP amount was increased contemporaneously to an enhanced ALP positive cell number. Differently, OST samples showed a high ALP positive cell number and a low intracellular ALP amount per cell after 10 days of culture, without any further increase up to 20 days. Summarizing, a sort of plateau seemed therefore to be reached both for CTRL and OST, being about 43% ALP positive cells, and the highest levels of intracellular ALP activity were detected in uncommitted UCB-derived MSCs at 20 days. Nevertheless the osteogenic differentiation of OST was confirmed by other analyses, such as OC dosages, cytochemistry for calcium matrix and RT-PCR for collagen I and OPN. OC, which is a specific osteoblast marker, resulted in fact more strongly produced by OST than by CTRL at all the time-points (Fig. 4C).

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Cytochemical staining using the Von Kossa method was performed after 21 days of culture. Osteoinduced cells showed morphological changes with respect to undifferentiated MSCs, and the deposition of a calcium matrix was observed (Fig. 5A and 5B). Finally, RT-PCR analysis showed that after 21 culture days, osteoinduced UCB-derived MSCs expressed OPN and slightly increased Coll-I mRNA levels compared to undifferentiated cells. Moreover, Runx2/cbfa-1 was positively expressed both in osteoinduced cells and controls (Fig. 6).

Adipogenic differentiation

The MSCs were committed towards the adipocyte lineage using induction stimula repeated for three consecutive cycles for a total of 4 weeks. The visualization of possible lipid vacuoles in induced cells was investigated by cytochemistry. However, cell samples resulted completely negative to Oil Red O staining (data not shown).

Chondrogenic differentiation

Chondrogenic differentiation of MSCs was performed for 4 weeks. Afterwards, cytochemical analysis with Alcian Blue was pursued to assess the deposition of GAGs. Chondroinduced cells stained positive at pH 2.5 (Fig. 5E), whereas they were completely negative at pH 1 (Fig. 5D). However the MSC morphology, as highlighted by H&E staining, showed a typical chondroblast shape (Fig. 5C).

The chondrogenic phenotype was shown with RT-PCR analysis by a strong increase in the expression of Coll-II, while Coll-X mRNA was not detected. Runx2/cbfa-1 was weakly expressed in chondroinduced cells compared to the controls (Fig 6).

Cell culture on three-dimensional scaffolds

Undifferentiated MSCs were cultured on PLLA-based fiber mesh scaffolds. After 20 days of culture, optical microscopy observation showed that all the scaffolds were completely covered by adherent cells, well spread out on the fiber surfaces. Furthermore, MTT test highlighted that MSCs were metabolically active both in PLLA/C (Fig. 7A) and PLLA/D (Fig. 7B) scaffolds, as proved by the deep purple color of the samples.

Conversely, MSCs cultured inside plasma clots were induced along the osteogenic lineage for 7 days. Clots having three different seeding densities were investigated, and resulted in different osteogenic performances, as shown by Von Kossa staining. In fact calcium deposition, clearly evidenced through black granules, was observed at the level of cells included on the clots at the highest cell density only. In these samples, that showed a percentage of calcium positive cells (i.e. mature osteoblasts) ranging from 82% to 95%, Von Kossa reactivity was also observed outside the cells (Fig. 7E).

Moreover the H&E staining analysis on plasma clots showed how the cells were able to adhere and proliferate not homogeneously inside this scaffold (Fig. 7C).

DISCUSSION

The presence of MSCs in cord blood is an interesting topic area of debate; many authors, in fact, contend this assumption as they have not been successful in isolating these cells from human cord blood (18). However, the results obtained by Campagnoli et al. (31) and Erices et al. (17), have suggested that MSCs are present in many fetal organs and circulate in fetal blood together with the hematopoietic precursors. Some authors have demonstrated that primary cultures of mononuclear cells from cord blood present morphological characteristics similar to the fibroblastic one and after a few weeks they form a homogenous layer of cells which adhere to the substrate (8, 32). Immunophenotypical analysis of these cells has confirmed the expression of surface antigens typical of BM-derived MSCs (32). Actually, in contrast to BM aspirates, UCB offers several advantages: ease harvest, absence of risks to donors, reduction of risks associated with transmitting infections, acceptable partial HLA mismatch and absence of ethical problems. In addition, due to their immaturity, UCB-derived cells virtually possess privileges immunoregulatory properties and could be considered good substitutes of BM-derived MSCs for many therapeutical uses. Nevertheless, the difficulty experienced by many investigators in UBC-derived MSC isolation, has reduced the accessibility of this cell source in tissue engineering applications. It is well known that the frequency of MSCs in UCB is lower than in BM and varies between 0 and 2.3 clones per 1×10^8 mononuclear cells (20**,** 21, 33). This low cell yield compared to BM is also due to the differences in the culture conditions needed for the two different samples: the composition of the culture medium, the serum, the pH and the trypsinization effects. A crucial point is also represented by the period of time between the UBC collection and processing, which should never exceed 10 hours. Finally, evident signs of either clotting or hemolysis

in the blood sample drastically affect the final results. Moreover, the net volume of an UBC sample should be at least 33 ml, and the number of mononuclear cells isolated from the cord blood should reach at least 1×10^8 cells (33). The high variability of UCB-derived MSCs seems to be additionally related to factors concerning the pregnancy (gestational age, presence of gestosis, caesarean or natural delivery, placental size and cord lenght), the donor (mother's age, ethnicity, alcohol and cigarette consumption, levels of hemoglobin) and the gestational time (34). Concerning to the late factor, evidence now suggest an inverse correlation between the gestational time and the frequency of MSC within UCB (35, 36). In conclusion all these factors, together with the different isolation and culture procedures, can influence the presence and the ability to obtain MSCs from UCB.

This study is one among a few papers investigating human UCB-derived MSC growth and differentiation on three-dimensional scaffolds focusing on their potential applications in regenerative medicine and tissue engineering. Herein, circulating stem cells in human UCB, not of hematopoietic origin, were isolated. Nevertheless a great variability, presumably due to all the above factors was observed even in the present study. From among 100 samples, about 70% led to the obtainment of a heterogeneous cell population containing two different cell types: MSCs and OLCs. OLCs were firstly characterized and named by Erices (17). According to the literature, we showed that OLCs were big multinucleated oval cells, while MSCs small stretched spindle-like cells. Cultures containing OLCs did not be passaged and tended to predominate over MSCs. The immunophenotypical analysis of such a population showed a monocyte-macrophage phenotype that was $CD45^+$ and $CD14^+$. Additionally the morphology of OLCs was confirmed by H&E staining.

In the 30% of samples a homogeneous population containing pure MSCs was obtained. In contrast to the OLCs-containing cultures, these homogeneous populations were characterized by the absence of hematopoietic markers and the expression of a specific pattern of adhesion molecules, such as CD90, CD105 and CD44. In addition these samples, consisting of mere MSCs, presented a durable

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proliferative capability in culture without evident morphological changes lasting for three months. Furthermore, the fact that UCB-derived mononuclear cells expressed high levels of SSEA-4 suggests the possibility that this antigen, whose expression is characteristic of human embryonic stem cells and MSCs (37), might also be expressed by UCB-derived adherent MSC.

Further investigation of MSCs by SEM confirmed a spindle-shape morphological pattern highlighting their elongated filaments. The advantages of light microscopy and electron microscopy is the ability to reconstruct an object from optical serial sections to get a three-dimensional impression of the distribution of labeled proteins in the specimen. However the resolution of images obtained with such techniques is limited to the optical diffraction limit of about half of the excitation wavelength. AFM, on the contrary, represents a fundamental option for imaging morphology and mechanical cell structures, making possible the identification of cellular components at the micro/nanoscale level. In this study the AFM was employed to investigate the cellular topography of UCB-derived MSCs cultured on glass substrates. Micrographs showed the self-assembly capability of these cells creating a network rich of thin intercommunicating cellular processes. In particular AFM images highlighted the ability of a single MSC to investigate the surrounding environment through fine lateral pseudopodia.

It has been reported that UCB-derived MSCs have the capability of differentiating into mature mesodermal phenotypes, typical of MSCs of various origins, and particularly into osteoblasts and chondrocytes (38, 39). In the present study, the UCB-derived MSCs showed differentiative capacities in vitro, into osteoblastic and early chondroblastic lineages. The data obtained by colorimetric and immunoenzymatic assays, cytochemistry and RT-PCR showed an expression profile proper of the osteoblast phenotype. In particular about 43% of osteoinduced cells resulted ALP positive, even if the intracellular ALP amount per cell resulted lower than the one of 20 day CTRL. Being ALP activity is an early marker of osteoblasts, it may be hypothesized that the peak in intracellular ALP concentration in OST was reached before 10 culture days, after which ALP could be secreted in the culture medium.

In fact, all the other investigated markers, either more specific or later-stage than ALP, confirmed the occurred cell maturation into the osteoblast phenotype. OC is associated with osteoblast-mediated matrix deposition and mineralization and is maximally expressed on day 21. OC amount per cell was found strongly superior in OST than in CTRL at all the time-points, reaching 0.05 pg/cell at day 20. Moreover, OPN was highly expressed after 21 days of differentiation, and under the same conditions, Coll-I, a marker of osteoprogenitor cells, increased its expression. Finally, mineralization of osteoinduced MSCs was also demonstrated with von Kossa staining after 21 days of culture.

Concerning the differentiation toward the osteoblastic phenotype, our results suggest that a slow spontaneous osteoblastic differentiation may occur in MSCs cultured in absence of osteogenic induction, as indicated by data of both ALP and RT-PCR. In fact, RT-PCR showed that the transcription factor Runx2/cbfa-1, an early osteogenic marker, was constitutively expressed in UCB-derived MSCs;

In this report UCB-derived MSCs did not show any adipogenic differentiation capability. Actually there are conflicting data concerning the adipogenic differentiation capability of UCB-derived MSCs (17, 19, 32, 40-42). Various authors pointed out how UCB-derived MSCs are less sensitive toward the adipogenic differentiation which might be related to the ontogenetic age of these cells (22, 33, 41). It is supported by the fact that the adipocytes reside in adult BM and adipose tissue but are absent in fetal BM, and by the observation of an increased adipogenesis correlated with age (43).

Chondroinduced cells showed the presence of both non-sulphurated GAGs and Coll-II. This may be retraced to an initial differentiation of the cells in chondroblasts; in fact the induced MSCs, as highlighted by H&E, showed a cellular morphology compatible with ongoing chondrogenesis. Furthermore mRNA of Coll-X, which is secreted primarily by hypertrophic chondrocytes, was not detected at the end of the differentiation time. Taken together, the overall data suggest that sulphurated GAG formation will probably appear in a long-term culture. Moreover, RT-PCR showed that the

transcription factor Runx2/cbfa-1 was down-regulated in the shifting from basal condition to chondrogenic environment.

Our experimental data indicate that by applying appropriate culture techniques, it is possible to isolate small populations of MSCs; interestingly we suggest that UCB-derived MSCs show different differentiative properties when compared to BM-derived MSCs.

At present UCB-derived MSCs have been proposed as a source of cell therapy for tissue engineering. To apply these cells in a surgical setting, the use of a scaffold is important to avoid cell dispersion and to allow the handling of samples. The ability of a cell to recognize and interact with the substrate represents the first and most important step, without which processes such as cell proliferation, migration and differentiation would not be possible. Therefore one of the fundamental requisites of biomaterial for tissue engineering is the ability of efficiently promoting the adhesion of the cells from which it will be colonized. Both the mechanical and biochemical properties of the material determine the efficiency and the avidity by which the cells recognize the material. In this report we showed that both PLLA/C and PLLA/D, as well as plasma clots, sustain both cell adhesion and proliferation. Viability of cells was maintained over the time of our experiments, as judged by the presence of purple-colored viable cells on MTT-treated scaffolds. Moreover plasma clots have proved to be suitable scaffolds not only for adhesion and growth of UCB-derived MSCs, but also for the differentiation of these cells in osteoblastic lineage as confirmed by von Kossa staining on treated clots.

Our previous work has recently demonstrated that the plasma clot as natural substrate could represent an ideal support for the delivery of human MSCs, and that its plasticity could allow the surgeon to adapt the material to the damaged surface (30). The present investigation indicates that the plasma clot is also an ideal scaffold for the proliferation and differentiation toward osteogenic lineage of UCB-derived MSCs. Therefore human UCB-derived MSCs could play a role in tissue engineering especially in the orthopedic setting.

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Figure legends

FIG.1 Morphology of UCB-derived cells. Light microscopy images show interdonor variability between mononuclear UCB-derived adherent cell populations after a 15-day culture period in expansion medium. (**A)** H&E staining of OLCs-containing populations showed large, round and multi-nucleated cells and (**B**) in these types of cultures OLCs were predominant respect of MSCs (original magnifications ×60 and ×40). (**C**) Morphology of MSCs displayed with H&E staining a spindle-shape phenotype (original magnifications ×20). (**D**) Inverted light microscopy image of MSCs isolated from UCB (original magnifications ×10).

FIG. 2 Flow cytometry histograms of UCB-derived cells. Adherent cells were obtained from the confluent monolayer at the end of the second passage. (**A**) OLC expressed hematopoietic markers, CD14 and CD45. (**B**) MSC expressed CD90, CD105, CD44, CD29, CD13, HLA-class I (HLA-ABC) and lacked the expression of CD45, CD34, CD14, CD31, CD61 and HLA-class II (HLA-DR). The respective isotype control was shown as open histogram.

FIG. 3 AFM and SEM analyses. (**A**) SEM image showing the extended morphology of UCB-derived MSCs. (**B-C**) AFM micrographs: (**B**) the self-assembly capability and the spatial orientation of the MSCs cultured on glass substrates is highlighted; (**C**) a zoomed cell with nucleus in the square panel. To note that a single MSC, spatially well separated by the other ones, keeps its axial orientation while it seems investigating the substrate through lateral pseudopodia (arrows in **A** and **C**) shorter than those of grouped cells (arrowheads in **B**).

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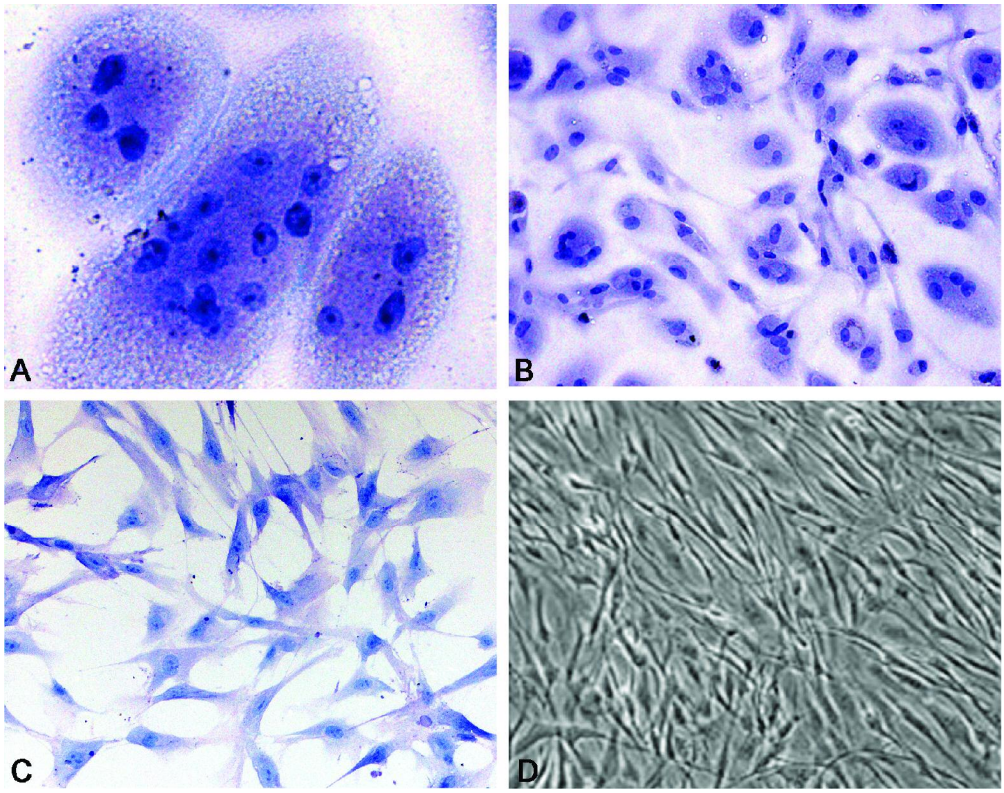
FIG. 4 Growth characteristics of undifferentiated UCB-derived MSC (**A**) and osteogenic marker [alkaline phosphatase (ALP) and osteocalcin (OC)] analyses of osteoinduced MSCs (B-C). Intracellular ALP activity evaluated via cytochemistry showed that a plateau was reached (about 43% positive cells) both for controls and for osteoinduced MSCs at 20 culture days (B). Dosages of intracellular ALP activity and OC showed that the highest ALP amount was in the controls at 20 culture days, whilst the highest OC amount was in osteoinduced MSC samples (C).

FIG. 5 Differentation of MSCs toward the osteoblast and chondroblast phenotype. (**A**) Osteogenesis is demonstrated by deposition of a mineralized matrix indicated by von Kossa staining on MSCs cultured for 21 days in osteogenic induction medium (original magnifications ×20). A mineralized nodule is shown (**B,** original magnifications ×40). Chondrogenesis is evaluated by Alcian blue staining on MSCs treated for 3 weeks with chondrogenic medium (**C-E**, original magnifications ×40). The induced-MSC morphology, as highlighted by H&E staining, shows a typical chondroblast shape (**C**). The cells are completely negative at pH 1 (**D**) whereas they result positive at pH 2.5 (**E**).

FIG. 6 Analysis of osteogenic and chondrogenic markers in both undifferentiated and differentiated UCB-derived MSCs obtained via RT-PCR. Osteoinduced UCB-derived MSCs expressed OPN and increased Coll-I mRNA levels compared to undifferentiated cells. Moreover, Runx2/cbfa-1 was positively expressed in both. A strong increase in the expression of Coll-II was detected in chondroinduced cells, while Coll-X mRNA was not revealed. Finally, Runx2/cbfa-1 resulted down-regulated in chondroinduced cells

FIG.7 Growth and differentation of UCB-derived MSCs on three-dimensional scaffolds via optical microscopy. MTT cell viability test on MSCs cultured for 2 weeks on PLLA-based fiber mesh scaffolds, shows purple colored viable cells both in (**A**) PLLA/C and (**B**) PLLA/D scaffolds (original magnifications ×35). (**C**) H&E staining of a clot section shows viable cells not homogeneously spread across the scaffold (original magnifications ×20). (**D**-**E** original magnification ×40) Osteogenic differentiation of 1×10^5 MSCs cultured for 7 days inside plasma clot is demonstrated by enhancement of calcium mineralization detected by von Kossa stain **(E)** with respect to undifferentiated controls **(D)**.



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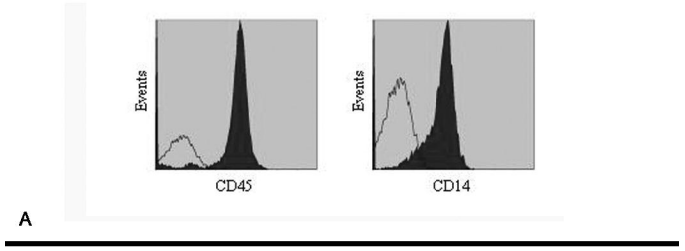
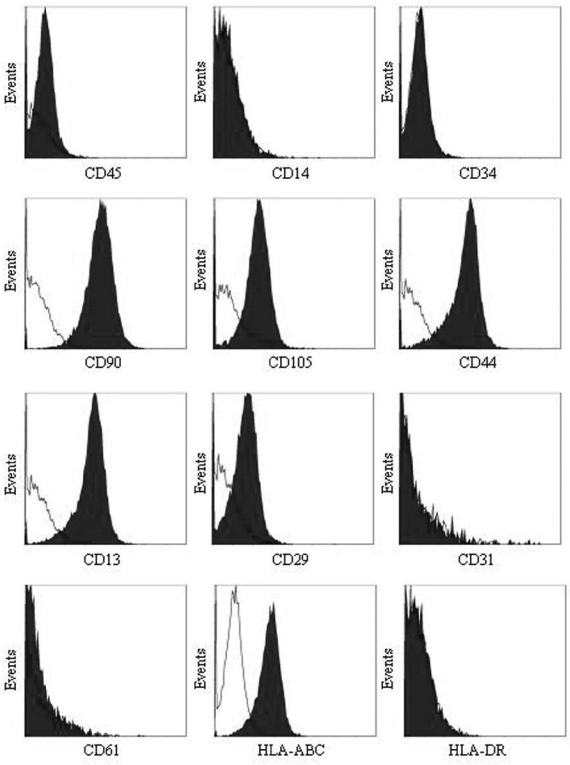


A



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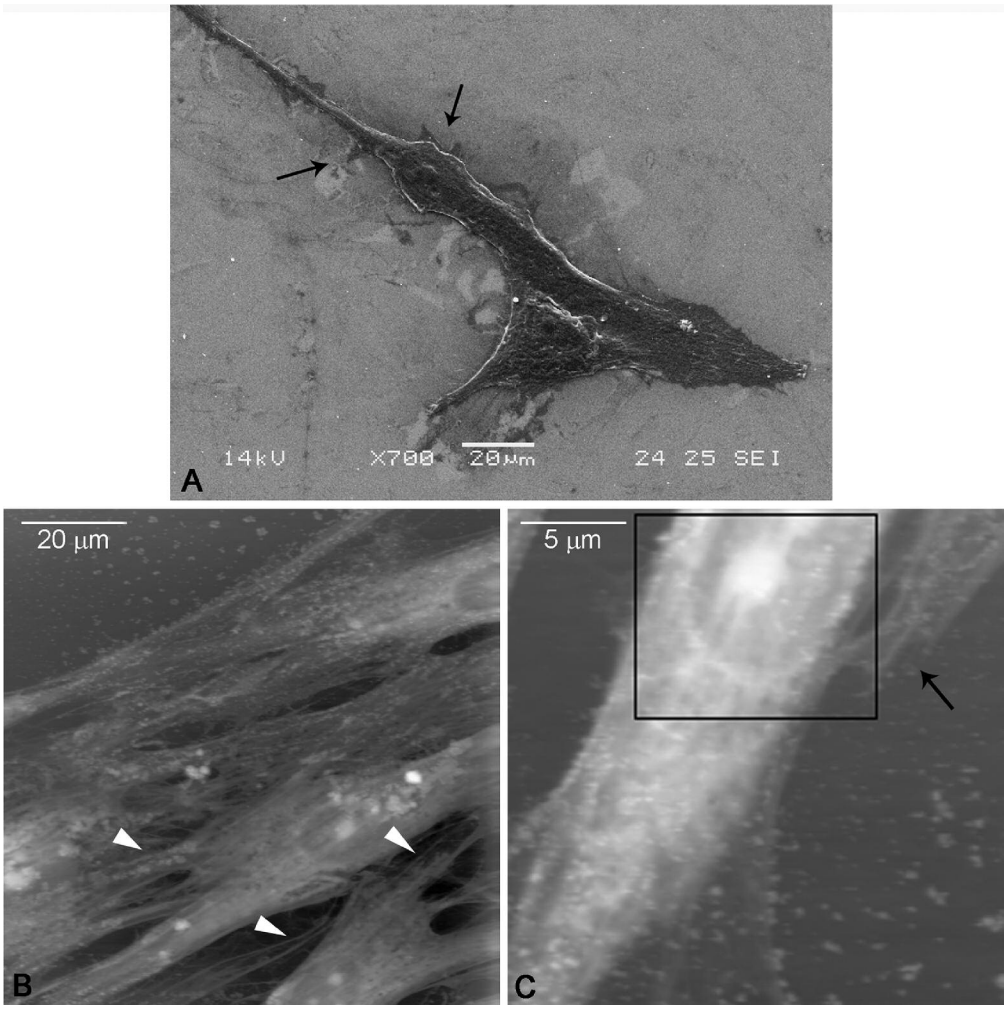

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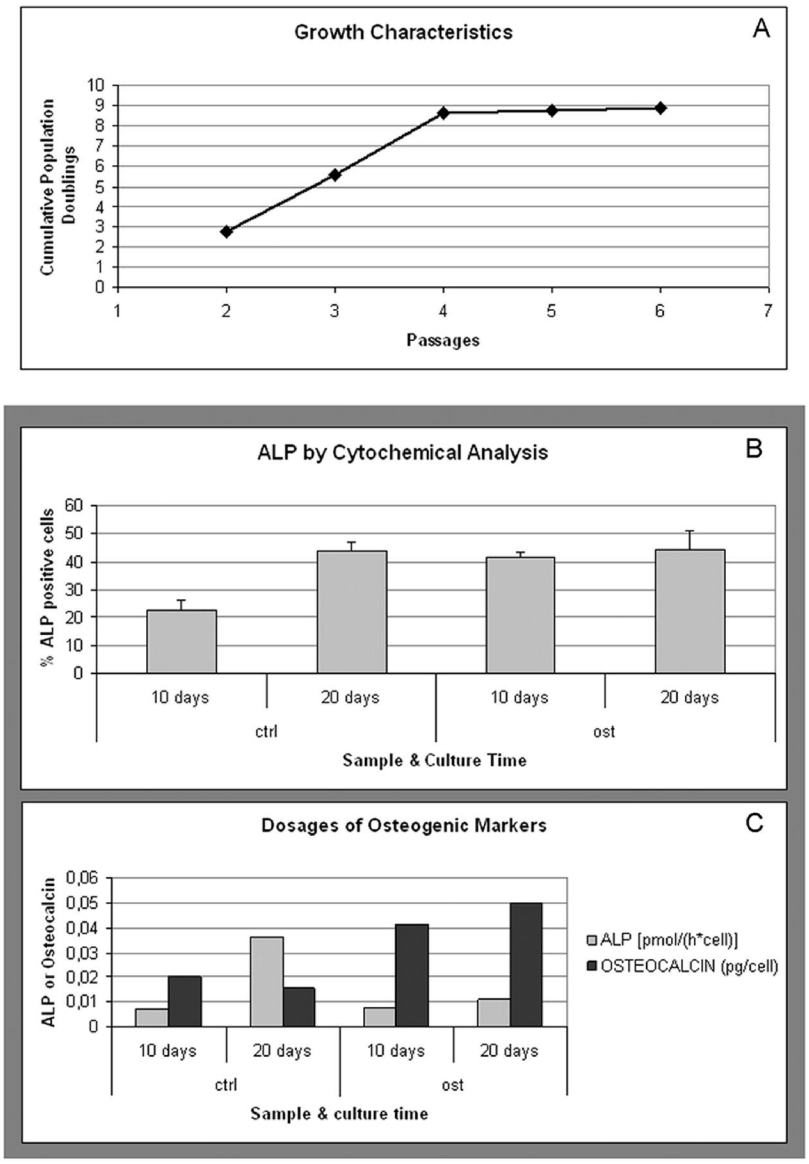

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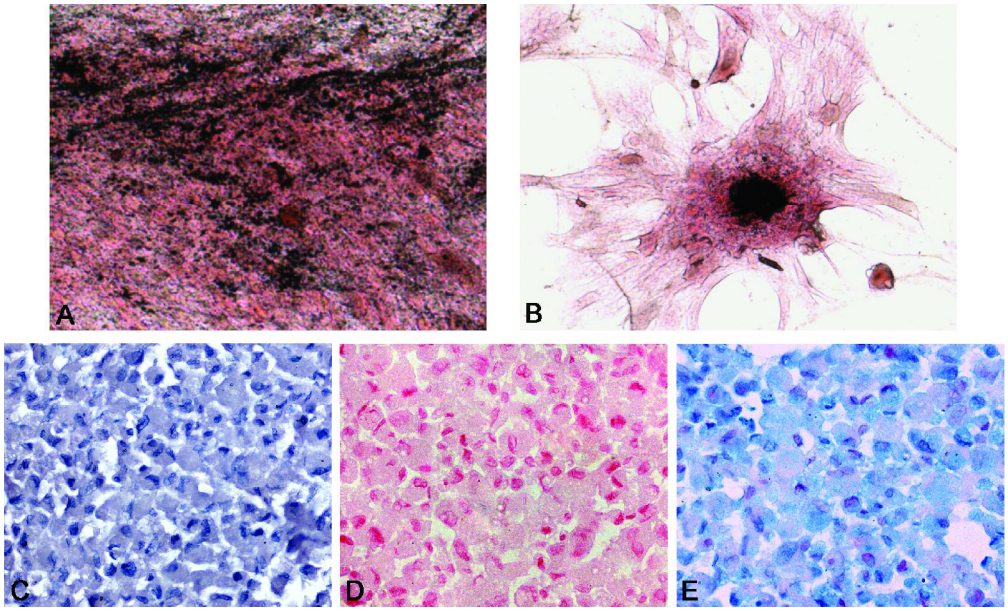

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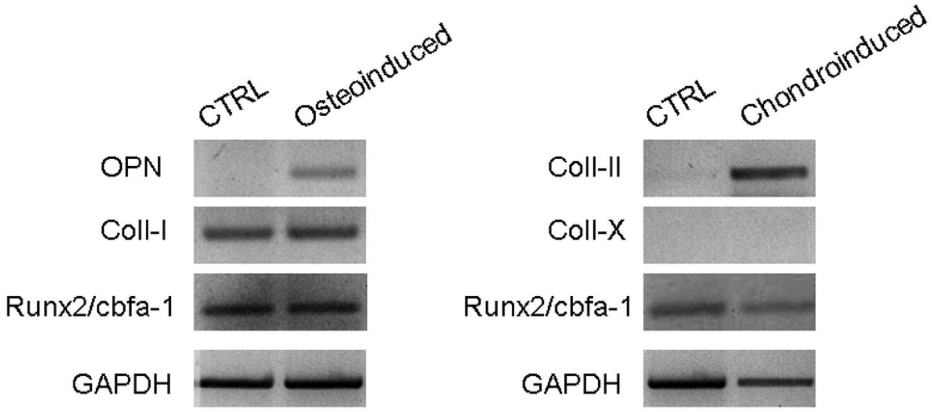

Fig. 6 Analysis of osteogenic and chondrogenic markers in both undifferentiated and differentiated UCB-derived MSCs obtained via RT-PCR. Osteoinduced UCB-derived MSCs expressed OPN and increased Coll-I mRNA levels compared to undifferentiated cells. Moreover, Runx2/cbfa-1 was positively expressed in both. A strong increase in the expression of Coll-II was detected in chondroinduced cells, while Coll-X mRNA was not revealed. Finally, Runx2/cbfa-1 resulted down-regulated in chondroinduced cells.
99x48mm (600 x 600 DPI)

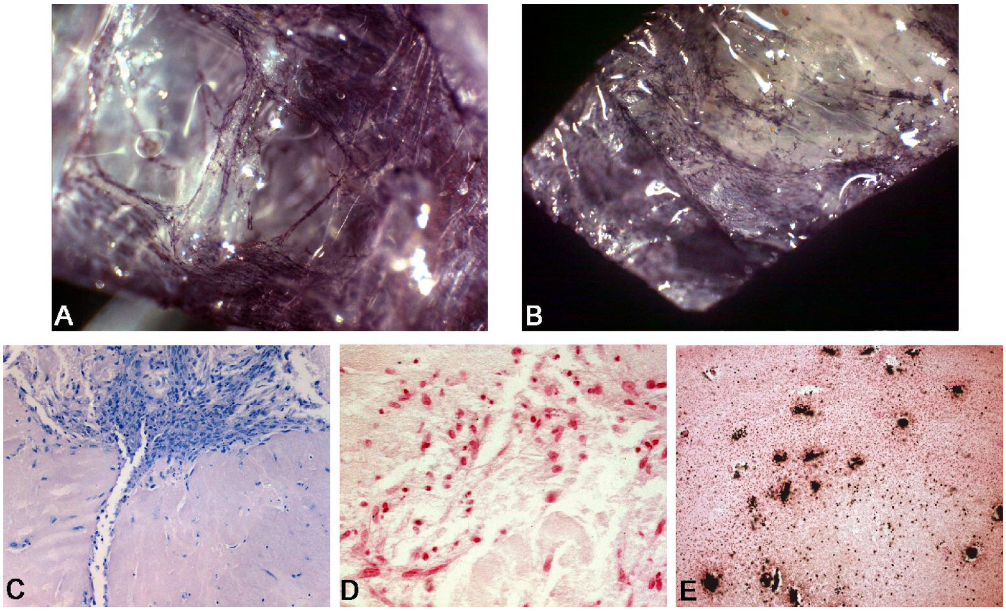

Fig. 7 Growth and differentation of UCB-derived MSCs on 3 dimensional scaffolds via optical microscopy. MTT cell viability test on MSCs cultured for 2 weeks on PLLA-based fiber mesh scaffolds shows purple colored viable cells both in (A) PLLA/C and (B) PLLA/D scaffolds (original magnifications ×35). (C) H&E staining of a clot section shows viable cells not homogeneously spread across the scaffold (original magnifications ×20). (D-E original magnification ×40) Osteogenic differentiation of 1×10^5 MSCs cultured for 7 days inside plasma clot is demonstrated by enhancement of calcium mineralization detected by von Kossa staining (E) compared to undifferentiated controls (D).
183x110mm (300 x 300 DPI)