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SELECTION AND INDUCTION OF RAT SKELETAL MUSCLE-DERIVED CELLS TO THE CHONDRO-OSTEOGENIC LINEAGE

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Abstract – Bone marrow (BM) has been long established as the main source of pluripotential mesenchymal stem cells (MSCs), and has been so far the main recognized source of osteoprogenitor cells that contribute to the turnover of the skeletal scaffold. The existence of an osteoprogenitor cell in other connective tissues such as skeletal muscle has been reported. In light of its availability and because of the relative ease of muscle cell isolation, skeletal muscle is an attractive source of cells for use in tissue engineering applications. The aim of this study was to explore the potential to differentiate into the chondro-osteoblastic lineage of a plastic adhering cell population, referred t as skeletal muscle-derived cells (SMDCs), obtained from biopsies of rat skeletal muscle. SMDCs displayed a fibroblast-like morphology. Our study revealed that the isolated cell population had a mesenchymal origin as indicated by abundant expression of STRO-1 and CD166. Osteogenic markers like osteocalcin (OC), bone sialoprotein (BSP) and osteopontin (OP) gene expressions were detected by RT-PCR. When these cells were cultured in the presence of an osteo-inductive culture medium, positive staining for alkaline phosphatase (ALP) and formation of mineralized matrix were increased. Furthermore SMDCs formed bone and cartilage tissues ***in vivo*** when placed inside of diffusion chambers and in demineralized bone matrix (DBM) cylinders, implanted subdermically into the backs of rat for 28 days. In conclusion, this experimental procedure is capable of selecting a cell population obtained from the skeletal muscle that is able to complete the differentiation pathway leading to the formation of cartilage and bone. In this respect SMDCs resemble BM stromal cells (BMSCs) and have demonstrated a potential application for cartilage and bone tissue engineering.

Key words: Skeletal muscle cells; Mesenchymal stem cells; Chondrogenesis; Osteogenesis; rhBMP-2; Demineralized bone matrix; Diffusion chamber; Rat.

INTRODUCTION

Bone, a dynamic tissue that provides structural support for soft tissues as well as stores for calcium and phosphate, depends for its normal function on a critical balance between formation and resorption. Since osteocytes and osteoclasts do not replicate, the maintenance of a healthy bone must rely on an exogenous source of cells. Although bone marrow stromal cells (BMSCs) have been long recognized as the main source of pluripotential mesenchymal stem cells (MSCs), several studies have suggested the existence of an osteoprogenitor cell in the skeletal muscle (9,35). Although not normally subject to rapid cell turnover, adult skeletal muscle also retains the ability to grow in response to increased work load and to repair and regenerate following damage. The mechanical functions of skeletal muscle are carried out by syncytial myofibers, each containing a highly

Abbreviations: **AB**, Alcian blue; **ALP**, alkaline phosphatase; **APC**, allophycocyanine; **BMP**, bone morphogenetic protein; **BMSC**, bone marrow stromal cell; **BSP**, bone sialoprotein; **Col I**, collagen type I; **Col II**, collagen type II; **DBM**, demineralized bone matrix; **FITC**, fluorescein isothiocyanate; **GT**, Goldner's trichromic stain; **IHC**, immunohistochemistry; **MSC**, mesenchymal stem cell; **OC**, osteocalcin; **ON**, osteonectin; **OP**, osteopontin; **Osx**, osterix; **PE**, phycoerythrin; **PSH**, picrosirius-hematoxylin; **rSMDC**, rat skeletal muscle-derived cell; **TB**, toluidine blue; **TGF**, transforming growth factor.

specialized contractile apparatus maintained by large numbers of postmitotic myonuclei. The capacity to generate new myonuclei resides in a population of mononucleated precursors, termed satellite cells, which lie sequestered between the basal lamina and the sarcolemma of each myofiber.

Satellite cells and vascular pericytes have been proposed as candidates. Satellite cells have demonstrated to possess multipotential MSC activity and are capable of differentiating into multiple lineages (3, 25). Muscle-derived stem cells exhibit the capacity to reconstitute the entire hematopoietic repertoire and to participate in the formation of new myofibers following tail vein injection into lethally irradiated mice (20). Likewise, it has been showed that pericytes have stem cell characteristics and can differentiate into osteoblasts, chondrocytes and adipocytes both ***in vitro*** and ***in vivo*** (14,15,25). Both populations express collagen type I, vimentin, fibronectin and desmin, but satellite cells are $CD34^+$ ALP^- whereas pericytes are $CD34^-$ ALP^+ (4,35).

Bone morphogenetic proteins (BMPs) were originally identified from demineralized bone matrix (DBM) as factors that induce ectopic bone formation when implanted into muscular tissue (33). In addition, several reports have shown that skeletal muscle-derived cells (SMDCs) can differentiate into osteogenic and chondrogenic lineages upon stimulation with BMPs (22, 23, 27-29, 31).

This report describes a reproducible method for the culture and differentiation into chondro-osteoblastic lineage of fibroblast-like cells from mature rat skeletal muscle connective tissue, which are capable of forming bone and cartilage *in vivo.*

Characterization of rat skeletal muscle-derived cells (rSMDCs) revealed that these cells have a mesenchymal origin ($STRO\text{-}1^+$ $CD166^+$), and have the potential to undergo both osteogenic and chondrogenic differentiation under appropriate culture conditions as indicated by expression of ***Runx2*** and ***Sox9***. Furthermore, rSMDCs express osteocalcin (OC), bone sialoprotein (BSP) and periostin mRNAs, considered to be important for osteoblastic differentiation.

We demonstrate that both the ALP staining and mineralization nodules formation gradually increase when rSMDCs are cultured in the presence of an osteoinductive medium. Moreover, we provide evidence of the ability of rSMDCs to form bone and cartilage when they are loaded in diffusion and DBM chambers and implanted ***in vivo***.

Thus, our results suggest that a population of muscle-derived cells have the capacity to differentiate into chondro-osteogenic lineage ***in vitro*** and ***in vivo***, as well as the own BMPs from DBM promote the formation of mature cartilage and bone tissue.

MATERIALS AND METHODS

Isolation of primary rSMDCs

Muscle biopsies were performed on the back legs of 4 weeks old rats (Charles River). The surrounded soft tissue was discarded and the samples were washed with DMEM 3% penicillin/streptomycin and 1.5% fungizone (Sigma) for 1hr, followed by an enzymatic digestion with 0.2% collagenase (Gibco), during 30 min at 37ºC with orbital shaking. The supernatant obtained was cultured with DMEM 10% FBS, 2.5 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1.25 µg/ml fungizone (Sigma). The culture medium was changed twice per week and the cells selected by their capacity to attach to the dish surface, discarding the floating cells in the first medium change at 72 hr.

RT-PCR Analysis

The expression of bone sialoprotein (BSP), alkaline phosphatase (ALP) and osteocalcin (OC) was semi-quantitatively evaluated for rSMDCs at passage 1 by reverse-transcription-polymerase chain reaction (RT-PCR). mRNA extracted from BMSCs was analyzed as a positive control. Total RNA was isolated using Real Total RNA Spin Plus (Real, Spain) and 1 µg of total RNA was used as a template for reverse transcription into cDNA by Transcriptor First Strand cDNA Synthesis Kit (Roche, USA). cDNA was amplified by PCR with the oligonucleotide primer sets outlined in Table 1. The expression of ß-actin was used to normalize gene expression levels. All primer sequences were determined through established GenBank sequences. DNA amplifications were performed at the occasion of an initial denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 1 min. The final cycle included 7 min for extension at 72˚C. PCR products from each sample were analyzed by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide. Table II shows each primer set.

Flow cytometry analysis of cells

In order to analyze the expression of surface markers characteristic for MSCs on rSMDCs, flow cytometry analysis using specific fluorochrome-conjugated monoclonal antibodies was used. Adherent cells at passage 1 were washed in PBS (without Ca^{2+}/Mg^{2+}), harvested in 0.25% trypsin/EDTA and washed twice in flow cytometry buffer (FACS buffer) consisting of 10 mM hepes (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mg/ml bovine serum albumin (BSA) (Sigma) in Leibovitz's L-15 medium (Gibco). After the washing step, cells aliquots ($1x10^6$ cells) were incubated in FACS buffer containing monoclonal antibodies to phycoerythrin (PE)-conjugated CD29 (integrin alpha-1 involves in cell adhesion mechanism), fluorescein isothiocyanate (FITC)-conjugated CD34, allophycocyanine (APC)-conjugated CD45 (both specific for hematopoietic cells) and ALP (all from R&D

Systems), FITC-conjugated CD166 (AbD Serotec. Specific antigen was used for MSCs) and STRO-1 (R&D Systems characteristic antigen for MSCs) with a PE-conjugated anti-mouse IgM (AbD Serotec), or an appropriate isotype control antibody (Sigma). After 30 min in the dark on ice, cells were washed again in FACS buffer before flow cytometry analysis. Four hundred-thousands events per sample were analyzed on a *MoFlo*® SP1338 (DakoCytomation, Denmark) using Summit software. Cells were gated on forwards and side scatter to exclude debris and cell aggregates, and dead cells were excluded by 7-Amino-Actinomycin D (7-AAD, BD Pharmigen) staining.

TABLE II. Primers sequences

Alkaline phosphatase (ALP)
5' –CGCCTATCAGCTAATGCACA– 3' (forward)
5' –AGGGAAGGGTCAGTCAGGTT– 3' (reverse)
Bone sialoprotein (BSP)
5' –AAAGAGCAGCACGGTTGAGT– 3' (forward)
5' –AGACCGTAGCACCATTCCAC– 3' (reverse)
Collagen type I (Col I)
5' –CTGCTGGAGAACCTGGAAAG– 3' (forward)
5' –GGAAACCTCTCTCGCCTCTT– 3' (reverse)
Msx1
5' –TCCTCAAGCTGCCAGAAGAT– 3' (forward)
5' –TTCACCTGGGTCTCGGTAAG– 3' (reverse)
Msx2
5' –TCCGCCAGAAACAGTACCTC– 3' (forward)
5' –CTTGCAAGGGGGAGTTGATA– 3' (reverse)
Osteocalcin (OC)
5' –GAGGGCAGTAAGGTGGTGAA– 3' (forward)
5' –AGGGTCGAGTCCTGGAGAGT– 3' (reverse)
Osteonectin (ON)
5' –AAACATGGCAAGGTGTGTGA– 3' (forward)
5' –GGTGACCAGGACGTTTTTG– 3' (reverse)
Osteopontin (OP)
5' –GAGGAGAAGGCGCATTACAG– 3' (forward)
5' –AAACGTCTGCTTGTGTGCTG– 3' (reverse)
Osterix (Osx)
5' –GCTGCCTACTTACCCGTCTG– 3' (forward)
5' –TGTGAATGGGCTTCTTCCTC– 3' (reverse)
Periostin/osteoblast-specific factor 2 (PN/OSF-2)
5' –TGCAAAAAGAGGTCTCCAAGGT– 3' (forward)
5' –AGGTGTGTCTCCCTGAAGCAGT– 3' (reverse)
Runx2
5' –GCCGGGAATGATGAGAACTA– 3' (forward)
5' –GAGGCGGTCAGAGAACAAAC– 3' (reverse)
Sox9
5' –CTGAAGAAGGAGAGCGAGGA– 3' (forward)
5' –TGTAATCGGGGTGGTCTTTC– 3' (reverse)
Transforming growth factor-β1 (TGF-β1)
5' –CTGCTGCTTTCTCCCTCAAC– 3' (forward)
5' –GACTGGCGAGCCTTAGTTTG– 3' (reverse)
β-Actin
5' –GTTGTCCCTGTATGCCTCTGGTCG– 3' (forward)
5' –TAGAAGCATTTGCGGTGCACGATG– 3' (reverse)

In vitro osteogenic differentiation

rSMDCs reached the confluence when they were subcultured and amplified in order to evaluate the osteogenic differentiation *in vitro* as well as the expression of the osteoblastic markers. Cells were seeded at a density of 2 x 10^4 cells/well in a 35 mm well-plate and osteoinduced at 70% of confluence during 14 days with 10^{-8} M dexamethasone, 10 mM ß-glycerophosphate and 50 µM ascorbate-2-phosphate.

DNA analyses

DNA content was quantified by a modification of a technique described previously (17). Triplicate cultures were rinsed twice with Tyrode's salt solution and stored at -70ºC until all cells had been collected. All samples from each preparation were analyzed for DNA content at once. At some time before the assay was conducted, the dishes were thawed, and then 1 ml of 0.1 N NaOH was added. The dishes were kept at room temperature for at least 1 hr, and then were returned to the freezer. On the day of the assay, after frozen cultures had thawed, the 0.1 N NaOH/cell lysis suspension was collected from each dish and transferred to a pre-labeled tube.

One ml of neutralizing buffer consisting of 4 M NaCl, 100 mM Na_2HPO_4 and 0.1 N HCl in water was added to each dish, collected while pipetting up and down to rinse off any remaining cells of the dish, and transferred in the appropriate tube. Appropriately 100 µl of diluted samples were transferred to a 96-well culture dish, and 100 µl of 2 µM Hoechst dye solution were added to each well. Samples were read at excitation of 360 nm and emission 460 nm with a fluorometer (BIO-TEK, FL600). A standard curve generated from a series of dilutions of calf thymus DNA was used to determine the DNA concentration of the samples. Differences in DNA content are assumed to reflect differences in cell number.

Quantitative analysis of ALP

After 14 days in culture in osteogenic medium, ALP enzyme activity in control and experimental groups was measured colorimetrically with a microplate reader (Elx800, Bio-Tek Instruments, Vennont, USA). Cultures were rinsed twice with Tyrode's balanced salt solution (Sigma), and 1 ml of a 1 mg/ml solution of ALP substrate (p-nitrophenyl phosphate, Sigma) in a buffer consisting of 50 mM glycine and 1 mM $MgCl_2.6H_20$ (pH 10.5) was added per 35-mm dish. After 10 min the solution was removed and transferred to a tube containing an equal volume of 1M NaOH. Appropriately diluted samples of the resulting solutions were transferred to a 96-well culture dish, and the absorbance was read at 405 nm. A standard curve generated from a series of dilutions of p-nitrophenol (Sigma) was used to determine the concentration of the enzyme reaction product. The ALP assay solution was removed, the cultures were rinsed twice with Tyrode's salt solution and stored at room temperature until used for cytochemistry. For each experiment, a minimum of 3 dishes were counted.

Cytochemical study

After 14 days in culture, confluent cells were stained for ALP using Kit. N85L-3R (Sigma) according to the manufacturer's instructions. The ALP positive cells stained red violet.

The deposition of a calcified matrix was evidenced by von Kossa staining in which calcium phosphate deposits are stained brown to black. Triplicate cultures were rinsed twice with Tyrode's salt solution, fixed for 15 min in 10% buffered neutral formalin at room temperature and rinsed 3 times with distilled water. One ml of 2% silver nitrate (Sigma) was added per dish, and the cultures were placed in a dark environment for 10 min. Cultures were then rinsed 3 times with distilled water and exposed to a 100-watt lamp light (while covered with water) for 15 min. Culture dishes were rinsed again with distilled water and then dehydrated with 100% ethanol.

In vivo implantation and histological study

One million of induced cells were inoculated into DBM (40 µl volume. Chambers made of cylinders from bone diaphysis) and diffusion chambers (130 µl volume. Commercial discs made of a plastic ring and two Millipore filters of 0.45 µm pore size) and then implanted subdermically into syngenic 10-week-old rats under anesthesia. Chambers, harvested 28 days after implantation, were processed for histology, fixed in Bouin or 4 % paraformaldehyde, dehydrated in alcohol, embedded in paraffin, sectioned 8 µm thick and the sections stained with picrosirius-hematoxylin (PSH), a technique that shows specificity for collagen I. Alcian blue (AB) reveals glycosaminaglycans from the cartilage matrix, toluidine blue (TB) colours in pink cartilage matrix, Goldner trichromic (GT) reveals mineralized collagen I fibers, and immunohistochemical antibodies to determine anti-collagen I and II.

Statistics

Four assays in triplicate, means and standard deviations were performed using Sigma Stat software (SPSS Inc., Chicago, IL). DNA content and ALP activity data for cultures were evaluated with one way analysis of variance (ANOVA) after the data passed normality and equal variance tests. Comparisons to determine significance between cells in control and in osteoinduced medium were done with the Student's t-test. Results were considered significantly different at $p>0.05\%$.

RESULTS

Cultured cell morphology

In primary cultures the cells showed a fibroblast-like polymorphic phenotype (Fig. 1A) at the time of the first change of media. Once the secondary cultures reached 70% of confluence and they were treated with either control or osteogenic medium during 14 days, the control situation showed cells with a spread-out morphology (Figs. 1B & 1C) while the osteoinduced cells were round in shape and aggregated in mineralized colonies (Figs. 1D-1F).

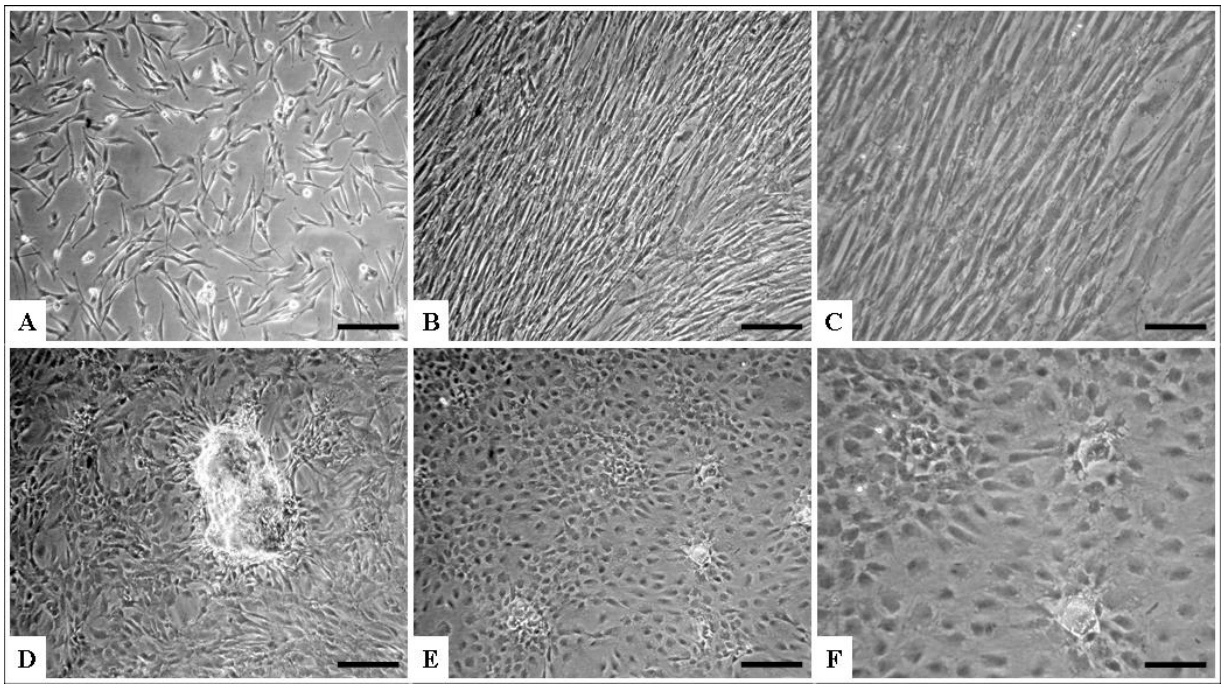

Figure 1. Rat SMDCs *in vitro* cultures. **(A)** Primary culture; **(B y C)** Secondary culture of control cells; **(D)** Detail of a mineralized colony of the osteoinduced secondary culture; **(E y F)** Secondary culture of the osteoinduced cells. Bar A-B and D-E, 150 µm; bar C and F, 75 µm.

RT-PCR analysis

We analyzed the expression of the mRNA for osteogenic and chondrogenic specific markers by RT-PCR and the signal was compared with cells derived from BM (Fig. 2). The osteogenic phenotype of the rSMDCs was suggested by demonstrating the expression of genes involved in the osteogenic lineage. ***Runx2***, independently or cooperatively with Osx, up-regulates osteoblast marker genes, Col I and ALP in early

differentiation stages, as well as BSP and OC in later stages. Osteonectic and OP, genes related with osteoblast maturation, and periostin, that is highly expressed in early osteoblastic cells ***in vitro***, were up-regulated in the rSMDCs. ***Msx1*** and ***Msx2***, genes involved in cell proliferation and in the prevention of osteogenic differentiation, were expressed through a very low signal.

The expression of ***Sox9***, a gene involved in the chondrocytes maturation, was also detected in the rSMDCs cultures, indicating the potential of these cells to differentiate to cartilage.

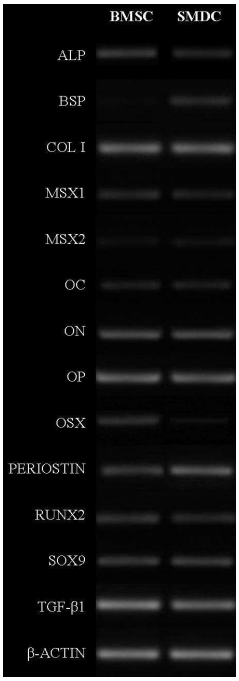

Figure 2. mRNA expression in rSMDC and BMSC by semi-quantitative RT-PCR. (ALP, alkaline phosphatase; BSP, bone sialoprotein; Col I, collagen type I; OC, osteocalcin; ON, osteonectin; OP, osteopontin; Osx, osterix).

Flow cytometry

The cell populations from rSMDCs were characterized by staining for surface marker proteins. From the set of accepted markers we choose six different cell surface markers and stained freshly isolated cells from rSMDCs (Table I).

TABLE I

Cell surface markers used for FACS analysis

CD locus	Detection of rSMDCs	Common name
ALP	Negative/Positive	Alkaline phosphatase
CD29	Negative	
CD34	Negative	
CD45	Negative	
CD166	Positive	ALCAM
STRO-1	Positive	

As shown in Fig. 3, we have found a relative heterogeneous cell population since the majority of the cells stained positive for STRO-1 and CD166, and negative for CD29, CD34 and CD45 while some cells were positive for ALP.

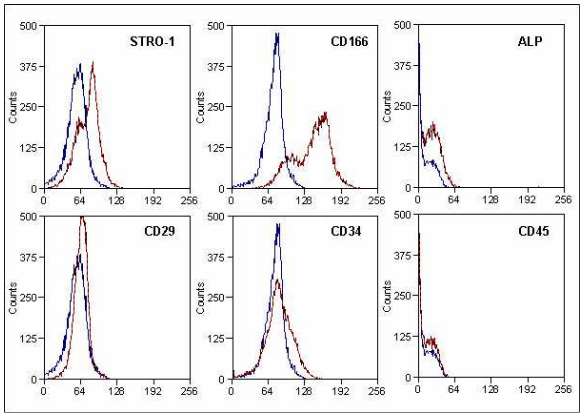

Figure 3. Phenotype of the cultured rSMDCs. Most of the cells were STRO-1^{+} CD166^{+} ALP^{+} CD29^{-} CD34^{-} and CD45^{-}. The blue line represents the corresponding control isotype.

Osteoblastic differentiation of culture expanded cells

The differentiation potential of the cultured rSMDCs into osteoblasts was also proved by analysis of quantitative/qualitative ALP as well as by the presence of dense mineralized nodules. The expression of ALP as a pre-osteoblast marker was tested on control cultures (day 0) and after osteoinduction. The quantification of ALP activity showed an increase of ALP per ng/ml of DNA with time, reaching a maximum level by day 14 (Fig. 4). The levels of ALP activity in the osteoinduced cells were significantly ($p<0.001$) higher in comparison with controls.

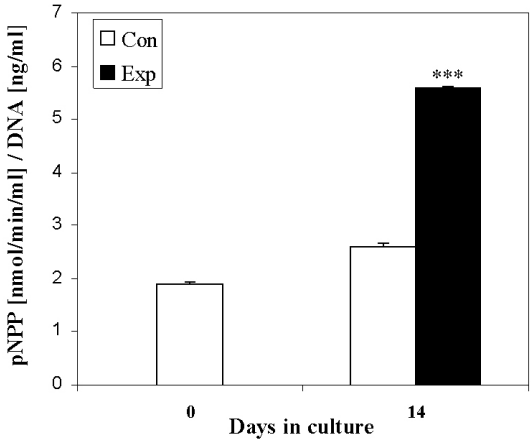

Figure 4. ALP expression in secondary cultures in presence and absence of osteoinducers. The asterisks indicate the significant difference between the control values and the experimental ones (*** p=<0,001).

rSMDCs after 14 days of culture formed an extensive network of a homogeneous distribution or grouped dense, multilayered nodules that stained positive for ALP (Figs. 5A-B) . Consistent with osteogenesis, several black regions with high mineral content, indicatives of a calcified extracellular matrix, were observed in cell colonies by von Kossa (Figs. 5C-D). In non-supplemented cell cultures no staining appeared.

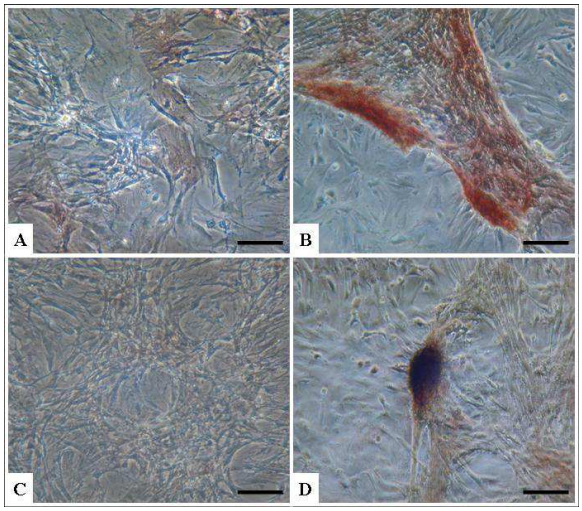

Figure 5. Rat SMDCs after 14 days of *in vitro* culture **(A-B)** ALP activity as an osteoblastic differentiation marker. **(A)** Cultured cells in control media; **(B)** Osteoinduced culture; **(C-D)** Von Kossa stain. **(C)** Negative control cells; **(D)** Detail of a mineralized colony in osteoinduced cultures. Bar, 150 µm.

In vivo tissue formation

Loading the cells into diffusion chambers and implanting them under the skin of rats provided a proof that rSMDCs have chondro-osteogenic potential. These chambers have previously been used to demonstrate the potential of BM MSCs to form bone and cartilage. Importantly, neither cartilage nor bone is formed when cells cultured with control medium are implanted in these chambers. The feature of the tissue formed by cells cultured in control conditions was of an homogeneous connective fibrous nature (data not shown). Figures 6A-C show examples of tissue formed within the diffusion chambers by cells cultured under chondro-osteogenic conditions: dense tissue condensations with osseous matrices localized adjacent to the Millipore filter positive for PSH and GT and immunoreactive for collagen I. Figures 6D-C show parallel sections with positive areas for AB and TB and negative stained for collagen II. Figures 7A and D-F correspond to a typical cartilage tissue positive for PSH, AB and TB, as well as immunoreactive antibodies for collagen II and negative for collagen I. This tissue contains a laguna of mature chondrocytes surrounded by a periostic layer positive for collagen I and negative for collagen II (Figs. 7A-C). Areas resembling fibrocartilage were localized towards the centers of the chambers (Figs. 6 and 7).

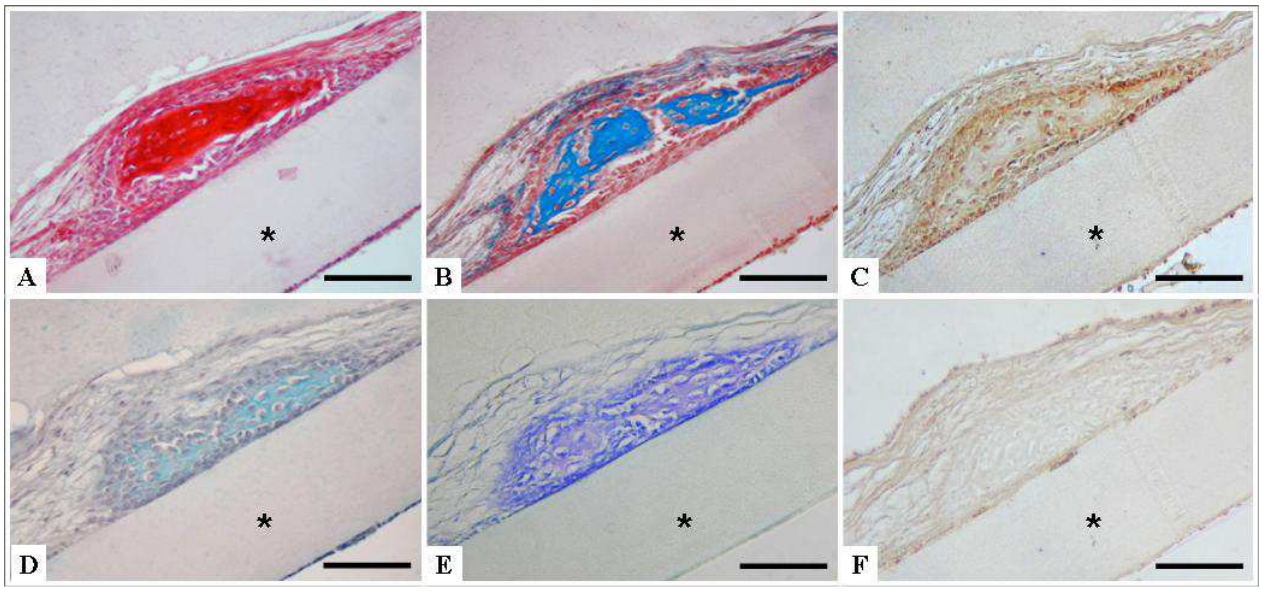

Figure 6. Histochemistry **(A-B y D-E)** and immunohistochemistry analyses **(C y F)** of the implants. **(A)** PSH stain; **(B)** GT stain; **(C)** Anti-Coll I stain; **(D)** AB stain; **(E)** TB stain; **(F)** Anti-Coll II stain. Asterisks represent the diffusion chamber filters. Bar, 100 µm.

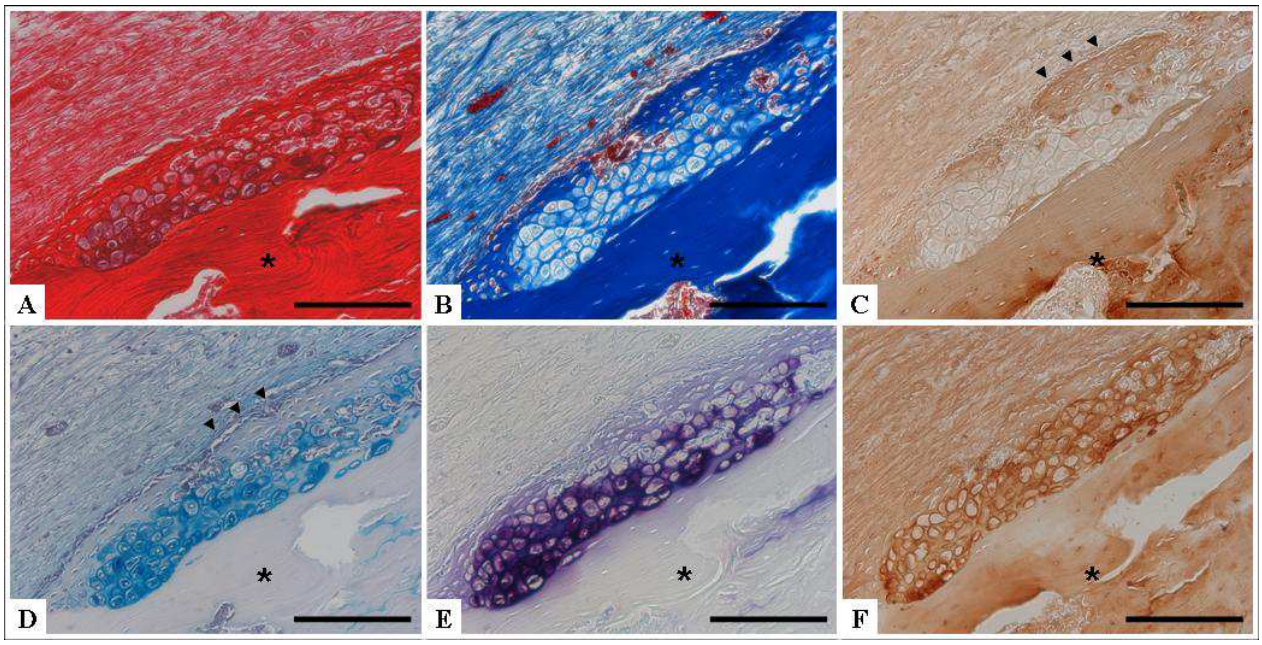

Figure 7. Histochemistry **(A-B y D-E)** and immunohistochemistry analyses **(C y F)** of DBM chambers. **(A)** PSH stain; **(B)** GT stain; **(C)** Anti-Coll I stain; **(D)** AB stain; **(E)** TB stain; **(F)** Anti-Coll II stain. Asterisks represent the DBM chamber filters.and the arrow heads represent the pericondrio. Bar, 200 µm.

DISCUSSION

The renewal of several terminally differentiated adult tissues is supported by populations of stem cells that both self-renew and generate a hierarchy of progressively lineage-restricted progenitors culminating in lineage-committed precursors fated to undergo terminal differentiation (36). In tissues with a high rate of turnover, such as blood, skin, and gut, the demands of replacement require a constant supply of precursors for terminal differentiation, such that progression from progenitor to functional, post-mitotic cell appears continuous. In adult skeletal muscle, however, new myonuclei are only required for growth and repair.

Several scientific groups have recently dedicated their studies to multipotent cell populations derived from the skeletal muscle, investigations realized through the necessity to find an osteogenic precursor source alternative to BM, together with the interest generated in the last years about the multipotent adult stem cells in regenerative medicine (9, 20, 22 ,23).

It is still not well known whether are capable of differentiation into osteogenic and adipogenic lineages. In this study, we present results about the isolation of a cell population obtained from rat skeletal muscle with similar characteristics to these ones of BMSCs. Rat SMDCs formed colonies in secondary cultures heterogeneous in shape and size that mineralize in the presence of the osteogenic conditions.

The characterization of rSMDCs indicated that these cells expressed high levels of ON and OP mRNAs, important specific markers of the skeletal lineage (8,11,30). Moreover, rSMDCs expressed both ***Runx2***, which has been widely accepted as the master osteogenic transcription factor because ***Runx2***-knockout mice display complete absence of bone due to arrested osteoblast maturation (20), and ***Sox9***, a master regulator of early phases of chondrocyte differentiation (7). Regarding other osteogenic markers, we found positive expression of the mRNA for OC, BSP and periostin.

Osteocalcin is considered to be important for osteoblastic differentiation although, as previously demonstrated (16), mRNA levels of BSP and OP appear more specifically associated to osteogenic cell differentiation than OC. We also report the low expression of transcription factor ***Msx1*** and ***Msx2***. Recent studies (13,18,24) demonstrate a negative role of ***Msx2*** during the early osteoblastic differentiation due to the blocking effect exerted over master transcription factors of the osteogenic commitment. ***Msx2*** seems to play its main effect in the expansion of the osteoprogenitor cell population in an initial phase, therefore promoting an indirect bone formation cascade.

Rat SMDCs were also characterized by mean of the expression of six cellular surface molecules (flow cytometry). The results show that this cell population is heterogeneous since most of the cells were STRO-1^+ $CD166^+$ $CD29^-$ $CD34^-$ and $CD45^-$ and only some of them express ALP. BMSCs are positive for CD29, CD44, CD73, CD90, CD105 (endoglin), CD166, and STRO-1 but negative for CD34, CD45 and HLA-DR (18). It is of particular interest to mention that some authors have described the isolation from mouse skeletal muscle of a cell population named as ***pp6*** which is $CD34^+$ and located in the basal laminae, the usual location for satellite cells (22).

In our cultures we also found a $CD34^+$ cell population. ***Pp6*** cell population, as well as a clonal subpopulation named ***mc13***, demonstrated their capacity to form bone ***in vivo***. Interestingly, **mc13** is $CD34^-$, therefore this cell population could be isolated from a small cell population $CD34^-$ located in the ***pp6*** cell population, or it was $CD34^+$ originally although it could lose such a marker due to a differentiation stateproduced during the selection period. Other cells derived from skeletal muscle that demonstrated their differentiation capacity to several mesenchymal lineages have been $CD34^-$ (23). However, for these authors the absence of this surface antigen, together with other cell characteristics such as the high expression of ALP, have led them to propose that the cell population isolated was in fact formed by vascular pericytes. Since the rSMDCs that we have selected are ALP^+ and $CD34^-$ we could propose that both are of the same cell type.

In addition to the own capacity of the rSMDCs to differentiate into the chondro-osteogenic lineage ***in vitro*** and when once they were implanted in rat, we have observed that the presence of ß-glycerophosphate, dexamethasone and ascorbate-2-phosphate into the culture media during 14 days increases the expression of an early osteoblastic marker as ALP and the formation of calcium phosphate deposit comparing to the untreated cells. As it have been published by others for BMSCs the addition of dexamethasone would accelerate the differentiation process originally induced by the growth factors and cytokines from FBS used in the culture media (6, 12, 30).

Levy *et al.* (24) have isolated a cell population from the human skeletal muscle that was ALP^+, adhering to the uncoated plastic dish and up regulating OC expression ***in vitro*** after a 24 hr induction with 1,25-dihydroxyvitamin D3. They failed nevertheless to demonstrate a real formation of cartilage and/or bone after implantation ***in vivo*** and they did not investigate the cell differentiation potential towards other mesenchymal lineages such as chondrogenic and osteogenic one's; Nevertheless it is likely that the cell population that we isolated is the same as that described by those authors. Therefore, the definitive proof of the rSMDC chondro-osteogenic potential was given through their ability to form cartilage and bone ***in vivo***.

Implants of induced rSMDCs loaded into DBM and diffusion chambers yielded cartilage and bone tissue formation, confirming that both tissues were originated from the implanted rat cells and not from the host. The formation of the cartilaginous tissue could be due to i) the presence of residual traces of BMPs coming from the osseous matrix of the DBM chamber (1,2,5), ii) a chemoattraction effect exerted by the DBM matrix towards the circulating BMPs, or iii) the vasculature invasion. It could also be the result of a combination of all of them. Some studies have published already the induction effect directed by the BMPs over this type of cells. A recent study revealed significantly better articular cartilage repair in rats that received BMP-4–transduced muscle-derived stem cells (21).

Moreover, it has been demonstrated that the differentiation of the muscle-derived ***pp6*** cells into the osteogenic lineage improves consequently bone healing enhanced by BMP-2 released from a gelatine scaffold (30). In this way, several authors repaired functionally and structurally 7-mm femoral segmental bone defects by using ***ex vivo*** gene therapy with rat primary muscle-derived cells expressing BMP-4 (22,29), and non-healing skull defect with ***mc13*** cells expressing BMP-2 (26).

In conclusion, our results consolidate that skeletal muscle is a promising source of progenitor, pluripotent mesenchymal stem cells that can undergo chondrogenic and osteogenic differentiation ***in vitro*** and ***in vivo*** under appropriate stimulation. Further experimentation will elucidate whether or not and satellite cells share a common phenotype and if both could represent the origin of chondrocytes and/or osteocytes *in vivo.*

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PS: Professor R.Wegmann, founder of the journal CMB® and former Editor-in-Chief has revised a certain number of words and phrases and corrected them in order to permit a better understanding of the text and to respect the true spelling and senses of the words, by example: writing sarcolemma instead of sarcolema, phenotype instead of fenotype, trichromic instead of tricromic, perichondrium instead of pericondrium, paraffin instead of parafine, glutamine instead of glutamina, & instead of y, Alcian blue instead of alcian blue , hematoxyline instead of hematoxiline, etc..etc..A perfect presentation of the text is representative of the high standard of the journal. Moreover it has been observed that the authors have perfectly well respected the remarks of the Peer Reviewers and corrected some small mistakes detected by the latter.

REFERENCES

1. Andrades, J.A., Han, B., Becerra, J., Sorgente, N., Hall, F.L. and Nimni, M.E., A recombinant human TGF-ß1 fusion protein with collagen-binding domain promotes migration, growth and differentiation of bone marrow mesenchymal cells. *Exp. Cell Res.* 1999, **250**: 485-498.

2. Andrades, J.A., Santamaría, J.A., Nimni M.E. and Becerra, J., Selection and amplification of a bone marrow cell population and its induction to the chondro-osteogenic lineage by rhOP-1: an *in vitro* and *in vivo* study. *Int. J. Dev. Biol.* 2001, **45**: 689-693.

3. Asakura, A., Komaki, M. and Rudnicki, M., Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. *Differentiation.* 2001, **68**: 245-253.

4. Beauchamp, J.R., Heslop, L., Yu, D.S., Tajbakhsh, S., Kelly, R.G., Wernig, A., Buckingham, M.E., Partridge, T.A. and Zammit, P.S., Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *J. Cell Biol.* 2000, **151**: 1221-1234.

5. Becerra, J., Andrades, J.A., Ertl, D.C., Sorgente, N. and Nimni, M.E., Demineralized bone matrix mediates differentiation of bone marrow stromal cells *in vitro:* effect of age of cell donor. *J. Bone Miner. Res.* 1996, **11** :1703-1714.

6. Beresford, J.N., Joyner, C.J., Devlin, C. and Triffitt, J.T., The effects of dexamethasone and 1,25-dihydroxyvitamin D3 on osteogenic differentiation of human marrow stromal cells *in vitro*. *Arch. Oral. Biol.* 1994, **39**: 941–947.

7. Bi, W., Deng, J.M., Zhang, Z., Behringer, R.R. and de Crombrugghe, B., Sox9 is required for cartilage formation. *Nat. Genet.* 1999, **22**: 85–89.

8. Bianco, P., Silvestrini, G., Termine, J.D. and Bonucci, E., Immunohistochemical localization of osteonectin in developing human and calf bone using monoclonal antibodies. *Calcif. Tissue Int.* 1988, **43**: 155–161.

9. Bosch, P., Musgrave, D.S., Lee, J.Y., Cummins, J., Shuler, T., Ghivizzani, T.C., Evans, T., Robbins, T.D. and Huard, J., Osteoprogenitor cells within skeletal muscle. *J. Orthop. Res.* 2000, **18**: 933-944.

10. Caplan, A.I., Mesenchymal stem cells. *J. Orthop. Res.* 1991, **9**: 641-650.

11. Chen, J., Singh, K., Mukherjee, B.B. and Sodek, J., Developmental expression of osteopontin (OPN) mRNA in rat tissues: Evidence for a role for OPN in bone formation and resorption. *Matriz.* 1993, **13**: 113–123.

12. Coelho, M.J. and Fernandes, M.H., Human bone cell cultures in biocompatibility testing Part II: Effect of ascorbic acid, bglycerophosphate and dexamethasone on osteoblastic differentiation. *Biomaterials.* 2000, **21**: 1095–1102.

13. Dodig, M., Tadic, T., Kronenberg, M.S., Dacic, S., Liu, Y.H., Maxson, R., Rowe, D.W. and Lichtler, A.C., Ectopic Msx2 overexpression inhibits and Msx2 antisense stimulates calvarial osteoblast differentiation. *Dev. Biol.* 1999, **209**: 298-307.

14. Doherty, M.J., Ashton, B.A., Walsh, S., Beresford, J.N., Grant, M.E. and Canfield, A.E., Vascular pericytes express osteogenic potential *in vitro* and *in vivo. J. Bone Miner. Res.*1998, **13**: 828-838.

15. Farrington-Rock, C., Crofts, N.J., Doherty, M.J., Ashton, B.A., Griffin-Jones, C. and Canfield, A.E., Chondrogenic and adipogenic potential of microvascular pericytes. *Circulation.* 2004, **110**: 2226-2232.

16. Frank, O., Heim, M., Jakob, M., Barbero, A., Schäfer, D., Bendik, I., Dick, W., Heberer, M. and Martin, I., Real-time quantitative RT-PCR analysis of human bone marrow stromal cells during osteogenic differentiation *in vitro. J. Cell. Biochem.* 2002, **85**: 737–746.

17. Gillery, P., Bonnet, A. and Borel J.P., A fluorometric deoxyribonucleic acid assay for tridimensional lattice cultures of fibroblasts. *Anal Biochem.* 1993, **210**: 374-377.

18. Gussoni, E., Soneoka, Y., Strickland, C.D., Buzney, E.A., Khon, M.K., Flint, A.F., Kunkel, L.M. and Mulligan, R.C., Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature.* 1999, **401**: 390-394.

19. Hu, G., Lee, H., Price, S.M., Shen, M.M. and Abate-Shen, C., Msx homeobox genes inhibit differentiation through upregulation of cyclin D1. *Development.* 2001, **128**: 2373-2384.

20. Jones, E.A., Kinsey, S.E., English, A., Jones, R.A., Straszynski, L., Meredith, D.M., Markham, A.F., Jack, A., Emery, P. and Mcgonagle, D., Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. *Arthritis Rheum.* 2002, **46**: 3349-3360.

21. Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R.T., Gao, Y.H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S. and Kishimoto, T., Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell.* 1997, **89**: 755–764.

22. Kuroda, R., Usas, A., Kubo, S., Corsi, K., Peng, H., Rose, T., Cummins, J., Fu, F.H. and Huard, J., Cartilage repair using bone morphogenetic protein 4 and muscle-derived stem cells. *Arthritis Rheum.*2006, **54**: 433-442.

23. Lee, J.Y., Qu-Petersen, Z., Cao, B., Kimura, S., Jankowski, R., Cummins, J., Usas, A., Gates, C., Robbins, P., Wernig, A. and Huard, J., Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. *J. Cell Biol.* 2000, **150**: 1085-1099.

24. Levy, M.M., Joyner, C.J., Virdi, A.S., Reed, A., Triffitt, J.T., Simpson, A.H., Kenwright, J., Stein, H. and Francis,

M.J., Osteoprogenitor cells of mature human skeletal muscle tissue: An *in vitro* study. *Bone.* 2001, **29**: 317–322.
25. Mastrogiacomo, M., Derubeis, A.R. and Cancedda, R., Bone and cartilage formation by skeletal muscle derived cells. *J. Cell. Physiol.* 2005, **204**: 594-603.
26. Ryoo, H.M., Lee, M.H. and Kim, Y.J., Critical molecular switches involved in BMP-2-induced osteogenic differentiation of mesenchymal cells. *Gene.* 2006, **366**: 51-57.
27. Sampath, T.K., Nathanson, M.A. and Reddi, A., *In vitro* transformation of mesenchymal cells derived from embryonic muscle into cartilage in response to extracellular matrix components of bone. *Proc. Nat. Acad. Sci. USA* 1984, **81**: 3419-3423.
28. Shen, H.C., Peng, H., Usas, A., Gearhart, B., Cummins, J., Fu, F.H. and Huard, J., Ex vivo gene therapy-induced endochondral bone formation: comparison of muscle-derived stem cells and different subpopulations of primary muscle-derived cells. *Bone.* 2004, **34**: 982–92.
29. Shen, H.C., Peng, H., Usas, A., Gearhart, B., Fu, F.H. and Huard, J., Structural and functional healing of critical-size segmental bone defects by transduced muscle-derived cells expressing BMP-4. *J. Gene Med.* 2004, **6**: 984-991.
30. Sugiura, F., Kitoh, H. and Ishiguro, N., Osteogenic potential of rat mesenchymal stem cells after several passages. *Biochem. Biophys. Res. Commun.* 2004, **316:** 233–239.
31. Sun, J.S., Wu, S.Y. and Lin, F.H., The role of muscle-derived stem cells in bone tissue engineering. *Biomaterials.* 2005, **26**: 3953-3960.
32. Termine, J.D., Kleinman, H.K., Whitson, S.W., Conn, K.M., McGarvey, M.L. and Martin, G.R., Osteonectin, a bone-specific protein linking mineral to collagen. *Cell.* 1981, **26**: 99–105.
33. Urist, M.R., Bone: formation by autoinduction. *Science.* 1965, **150**: 893-899.
34. Volek-Smith, H. and Urist, M.R., Recombinant human bone morphogenetic protein (rhBMP) induced heterotopic bone development *in vivo* and *in vitro. Proc. Soc. Exp. Biol. Med.*1996, **211**: 265-272.
35. Wada, M.R., Inagawa-Ogashiwa, M., Shimizu, S., Yasumoto, S. and Hashimoto, N., Generation of different fates from multipotent muscle stem cells. *Development.* 2002, **129**: 2987-2995.
36. Weissman, I.L., Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science.* 2000, **287**: 1442-1446.