Characterization of adult stem/progenitor cell populations from bone marrow in a three-dimensional collagen gel culture system

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Running title: Stem/progenitor cells in 3D culture.

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Abstract

Stem cell transplantation therapy using mesenchymal stem cells (MSCs) is considered a prominent strategy. Although MSCs are commonly isolated by exploiting their plastic adherence, several studies have suggested that there are other populations of stem and/or osteoprogenitor cells which are removed from primary culture during media replacement. Therefore, we developed a three-dimensional (3D) culture system in which adherent and non-adherent stem cells are selected and expanded. Here, we described the characterization of 3D culture-derived cell populations *in vitro* and the capacity of these cells to differentiate into bone and/or cartilage tissue when placed inside of demineralized bone matrix (DBM) cylinders, implanted subdermally into the backs of rat for 2, 4 and 8 weeks. Our results demonstrates that 3D culture cells were a heterogeneous population of uncommitted cells that express pluripotent, hematopoietic, mesenchymal and endothelial specific markers *in vitro* and can undergo osteogenic differentiation *in vivo*.

Key words: Stem/progenitor cells; Three-dimensional (3D) culture; TGF-β1; Osteogenesis; Demineralized bone matrix

INTRODUCTION

In recent years, stem cells have gained prominence as invaluable tools for research and as a promising resource for cell replacement therapies. The bone marrow is the remarkable reservoir of adult stem cells. Beside hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs), other populations of stem cells have been isolated from that tissue with different methods, such as the side population (SP) cells, multipotent progenitor cells (MAPCs), multipotent stem cells and pre-MSC, MIAMI

cells (Marrow-Isolated Adult Multilineage Inducible cells), VSEL cells (Very Small Embryonic Like stem cells) or endothelial progenitor cells (EPCs) (2,21,25,29,32).

MSCs are multipotent cells that persist in adult life in some tissue types, such as bone marrow stroma, fat, skeletal muscle, synovium, etc., without losing their capacity to proliferate and differentiate (23,31). Phenotypically, multiple studies have identified several markers that can be used to identify MSCs; however, even if isolated by density-gradient fractionation, they remain a heterogeneous mixture of cells with varying phenotype. Therefore, the International Society for Cellular Therapy (ISCT) established that MSCs must express CD73, CD90 and CD105, and lack expression of CD14 or CD11b, CD34, CD45, CD79α or CD19 and HLA-DR surface molecules (22).

MSCs are commonly isolated by exploiting their plastic adherence (38) and are expanded *in vitro* to obtain sufficient cell numbers for a range of tissue engineering applications, including bone and cartilage regeneration (6,15,43). Although there is no doubt that isolation of MSCs on the basis of plastic adherence clearly leads to the identification of a population of cells with multilineage potential *in vitro*, over the past several decades there has been a growing body of work suggesting that there are other populations of non-adherent cells in bone marrow, that also have characteristics of stem and/or osteoprogenitor cells (9,24). Włodarski *et al.* (2004) demonstrated that non-adherent cells, removed from primary bone marrow culture during media replacement, were able to produce bone *in vivo* (53).

In the last decade, our laboratory have successfully developed a three-dimensional (3D) collagen gel culture system supplemented with some growth factors, in which adherent and non-adherent bone marrow stem cells are selected and expanded in order to obtain cells capable to differentiate into bone and cartilage (3,4,5,10). Thus,

this report describes a further attempt to characterize this bone marrow-derived heterogeneous cell population by *in vitro* expression of undifferentiated, mesenchymal, hematopoietic and endothelial markers, and bone and cartilage formation when they are ectopically implanted *in vivo*. This heterogeneity can be advantageous for use in skeletal tissue engineering.

MATERIALS AND METHODS

Isolation of primary MSCs and 3D culture

Primary cultures of MSCs were established using bone marrow suspensions from human and rat origin. Human MSCs were used for flow cytometry and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis, and rat MSCs for *in vivo* experiments.

Human bone marrow cells were collected by aspiration from patients undergoing hip replacement surgery after informed consent and according to procedures approved by the local ethics committee. The MSC-enriched fraction was separated on Percoll (Sigma) gradient sedimentation at 20000 g for 15 minutes and suspended in Dulbecco's modified Eagle's medium (DMEM, Sigma).

Rat bone marrow cells were extracted from femurs and tibiae of syngeneic 8-week-old male Fisher 344 rats under Animal care procedures, conducted in accordance with the guidelines set by the European Community Council Directives (86/609/EEC) and approved by the local ethical committee. The epiphysis was removed and the medullar canal was washed with DMEM using a syringe. The medium containing the extracted cells was directed into a Falcon tube and the cells were suspended, homogenized, and centrifuged at 400 g for 10 minutes.

Human and rat cells were cultured in a 3D environment using a modification of a previously described method (3). Briefly, a collagen substrate for cell culture was prepared using a solution containing 0.85 mg/ml rat tail tendon type I collagen (BD), 1 M NaOH, 10x DMEM-F12, 100 U/ml penicillin, 100 µg/ml streptomycin and 1.25 µg/ml fungizone (Sigma) in MilliQ water at pH 7.4. Forty eight-well plates (Nunc) were coated with 150 µl of this solution and placed at 37°C for 30 minutes in order for it to solidify into a thin collagen gel matrix. After that, all of the cells were re-suspended in DMEM-F12 and mixed with the collagen solution plus 0.5% fetal bovine serum (FBS), in the absence (control cultures) or presence of 1 ng/ml recombinant human transforming growth factor-beta 1 (rhTGF-β1, R&D Systems) at a density of 2.5 x 10^5 cells/ 250 µl collagen/ well in 48-well plates. The culture plates were left 30 minutes at 37°C to allow the collagen to gel. Then, 250 µl/ well of culture medium that consisted of DMEM supplemented with 0.5% or 10% FBS, 2.5 mM L-Glutamine and the same amount of penicillin-streptomycin and fungizone as described above were added on top of the gel, in the presence or absence (control cultures) of rhTGF-β1 (1 ng/ml). Cells were incubated in culture medium containing 0.5% FBS for 10 days (selection period) and expanded in medium with 10% FBS for 4 days (amplification period). At the end of the culture, cells were incubated for 2 days in medium (not containing rhTGF-β1) supplemented with 10^{-8} M Dexametasone (Dex) and 2 mM β- glycerophosphate (β-GP) (Sigma) to help with the osteogenic differentiation. Cultures were fed every third day with fresh medium with or without rhTGF-β1 as appropriate and maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

Adherent culture (ADH) was used like a control. MSC fraction was plated at a concentration of 10^7 cells/75-cm^2 tissue culture flask and maintained in DMEM

containing 10% FBS, 2.5 mM L-Glutamina, 100 U/ml penicillin, 100 µg/ml streptomycin and 1.25 µg/ml fungizone. The culture medium was changed 2 times 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. When culture flasks became near-confluent, cells were detached with 0.25% trypsin containing 1 mmol/L EDTA , and subsequently replated at $5x10^3$ cells/cm^2 for continued passaging.

Flow cytometry analysis of cells

In order to analyze the expression of surface markers characteristic for mesenchymal, endothelial and hematopoietic cells, fluorescence-activated cell sorting (FACS) using specific fluorochrome-conjugated monoclonal antibodies was used. Human cells were washed twice in FACS buffer consisting of 10 mM hepes (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mg/ml bovine serum albumin (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 against CD13, CD29, CD44, CD49a, CD49b, CD73, CD106, CD146, HLA-I, HLA-DR and KDR (BD Pharmigen), CD34, CD45, CD133 and CD271 (Miltenyi Biotech), CD90, CD105 and Stro-1 (R&D Systems) and CD166 (AbD Serotec), or an appropriate isotype control antibody (Sigma). After 30 minutes in the dark on ice, cells were washed again in FACS buffer before flow cytometry analysis. Five hundred thousand events per sample were analyzed on a *MoFlo*® SP1338 (DakoCytomation, Denmark) using Summit software. Cells were gated on forward and side scatter to exclude debris and cell aggregates, and dead cells were excluded by 7-Amino-Actinomycin D (7-AAD, BD Pharmigen) staining.

FACS was used to study the cell cycle as well. $1x10^6$ cells in 1 ml of phosphate-buffered saline (PBS) were fixed for 30 minutes at 4°C with 2ml of methanol. After that, the cell suspension was washed twice with PBS and incubated for up to 30 minutes at 4°C in the dark with 500 µl of staining solution containing propidium iodide (0.1 mg/ml) (Calbiochem), Triton® X-100 (0.1%) and EDTA (3.7 mg/ml) (Sigma) in PBS. Samples were analyzed as described previously.

RT-PCR Analysis

The expression of human Oct-4, Nanog and Sox-2 was semi-quantitatively evaluated for 3D culture at day 10 and 16 by RT-PCR. mRNA extracted from ADH cells was analyzed as a control. Total RNA was isolated using RNeasy total RNA kit (Qiagen), and its quantity and purity was estimated by Nanodrop (Thermo Scientific D-1000). Only samples with an A260/A280 nm ratio of 2.0 were accepted. 200 ng of the total RNA sample was used as a template for first-strand cDNA synthesis by M-MLV reverse transcriptase (Promega, Madison, USA). cDNA was amplified by PCR with the following oligonucleotide primer sets: for Nanog, 5'-GCTTGCCTTGCTTTGAAGCA-3' and 5'-TTCTTGACTGGGACCTTGTC-3'; for Oct4, 5'-GAGCAAAACCCGGAGGAGT-3' and 5'-TTCTCTTTCGGGCCTGCAC-3'; for Sox2, 5'-GCCCCCAGCAGACTTCACA-3' and 5'-CTCCTCTTTTGCACCCCTCCCATTT-3'; for β-actin, 5'-CGCACCACTGGCATTGTCAT-3' and 5'-TTCTCCTTGATGTCACGCAC-3'. The expression of β-actin was used to normalize gene expression levels. All primer sequences were determined through established GenBank sequences. PCR-cycling conditions consisted of initial denaturation at 95°C for 2 minutes; 25 cycles of 94°C for 30 seconds (denaturation), 55°C for 30 seconds (annealing), and 72°C for 30 seconds (extension); and final extension at 72°C for 7

minutes**.** These reactions were carried out in 2720 Thermocycler (Applied Biosystems). PCR products from each sample were analyzed by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide using ChemiDoc™ XRS (Bio-Rad Laboratories).

In vivo implantation and histological study

One million of rat cells were inoculated into demineralized bone matrix (DBM, chambers made of cylinders from bone diaphysis with a capacity of 40 µl in volume) and then implanted subdermally into syngeneic 10-week-old Fisher 344 rats under anesthesia. Chambers, harvested 2, 4 and 8 weeks after implantation, were processed for histology, fixed in Bouin or 4 % paraformaldehyde, dehydrated in ethanol, embedded in paraffin, sectioned at 8 µm thick and the sections stained with picrosirius-hematoxyline (PSH), a technique that shows specificity for collagen I, Alcian blue (AB), that reveals glycosaminaglycans from the cartilage matrix, toluidine blue (TB) for cartilage matrix, Goldner's trichromic that reveals mineralized collagen I fibers, and immunohistochemically for anti-collagen I and II.

Statistics

Means and standard deviations were performed using Sigma Stat software (SPSS Inc., Chicago, IL) with analysis of variance (ANOVA) after the data passed normality and equal variance tests. Comparisons to determine significance between 3D culture and ADH were done with the Student's t-test. Results were considered significantly different at $p>0.05\%$.

RESULTS

Characterization of human bone marrow MSCs from 3D and ADH culture

a) FACS studies

As our group has previously reported, the 3D culture method yielded rounded cells significantly smaller when compared to ADH cells (Table 1, $p<0.05$), evenly distributed and embedded in collagen matrix. Its number suffered a sharp decrease as a consequence of the starvation period for the initial 10 days (Fig. 1). Once normal serum conditions were reestablished, the selected cells proliferated although moderately. Cell cycle studies revealed that only a small fraction of cells are actively engaged in proliferation (approximately 17% at S + G2 + M), while the majority of these cells are standing at the Go/G1 phase (Fig. 2A). In contrast, a high proliferation rate had been shown by ADH cells as 72% at S + G2 + M phases (Fig. 2B).

FACS of ADH culture showed the immunophenotype described for MSCs, where most of the cells expressed CD29, CD44, CD73, CD90, CD105, CD106, CD166, CD271, Stro-1 and HLA-I, and were negative for CD34, CD45 and HLA-DR (Table 2). Nevertheless, the profiles of 3D culture revealed a heterogeneous cell population, where positive cells for hematopoietic (CD34, CD45, HLA-DR), mesenchymal (CD29, CD44, CD73, CD105, CD166, HLA-I, Stro-1) and endothelial markers (CD34, CD133, CD146, KDR) were found (Table 2 and Fig. 3). Comparing 3D cultures at day 10th and 16th, CD45+ and CD166+ populations decreased over time (-19.2%, $p <0.05$ and -37.5%, $p <0.001$, respectively), while there was an increase in the expression of some mesenchymal cell markers such as CD29, CD73 and HLA-I (+4.0%, +2.1%, +9.3%, respectively), and in the CD13+ CD49b+ population (+5.4%, $p <0.001$). In addition, the EPCs-like population

(CD34+/CD133+/CD45-) decreased significantly in favour of a mature endothelial phenotype (CD146+/KDR+, +18.7%, $p < 0.001$).

b) RT-PCR analysis

Pluripotency of embryonic stem cells is controlled by defined transcription factors, such as Oct4, Nanog and Sox2. We analyzed the expression of mRNA for these pluripotent specific markers by RT-PCR and the signal was compared with ADH culture (Fig. 4). Oct4 and Nanog was detected in both 3D culture at day 10 and 16, but was absent in ADH cells. In addition, we evaluated the mRNA levels of other well-described pluripotent marker positively regulated by Oct4: Sox2, which was highly expressed in 3D culture cells.

In vivo implantation and histological study

One million rat bone marrow cells coming from 3D or ADH cultures were implanted subdermally into DBM chambers. They were harvested after 2, 4 and 8 weeks. In addition, and due to the limited cell number obtained in collagen gels, we found interesting to study implants of both types of cells in order to discover a likely synergistic effect. So we also implanted DBM chambers inoculated with 3D and ADH cells in 1:1 proportion.

Histological analysis after 2 weeks revealed that chambers were principally filled with fibrous tissues. Bone did not appear in any case. In the implants containing 3D culture cells, sections stained with PSH and Goldner's trichrome showed a fibrous tissue where there were frequent vascular profiles (Fig. 5A and insert). Implants containing ADH cells showed dense fibrous tissue, but vascularization phenomena were less apparent;

and chondrocyte-like cells located adjacent to the chamber wall were seen (Fig. 5B and insert). Chambers with both types of cells revealed dense tissue condensations with cartilaginous matrices, as demonstrated by type II collagen immunohistochemistry (Fig. 5C and insert).

Four weeks after implantation, chambers appeared full of dense clumps of tissue with various phenotypes, and bone formation was demonstrated in all conditions (Fig. 5D-F). Figure 5D show examples of tissue formed by 3D culture cells where a well-organized trabecular matrix, immunoreactive for collagen I, lined with osteoblast-like cells, regularly scattered blood vessels and marrow spaces were observed. On the other hand, in implants containing ADH cells, abundant cartilaginous occupying large areas of the chambers were found. In these cases, a real process of endochondral ossification with hypertrophic chondrocytes at the ends of the cartilage areas, and new trabecular bone were identified (Fig. 5E). Similar structures were normally found at this time, in implants with 3D and ADH cells (Fig. 5F). Furthermore, it was also common to find large areas occupied by new trabecular bone, whose origin could be different, perhaps intramembranous because no remnants of cartilage matrix were found, even staining with AB or TB (data not shown).

Overall, a further maturation of the osseous tissue was observed at 8 weeks after implantation (Fig. 5G-I). In chambers with 3D culture-derived cells, it became very difficult to distinguish the boundaries between the chamber wall and the new-formed tissues, indicating an active remodeling process. Under these conditions, marrow cavities within bone trabeculae increased in size, being mainly occupied by adipocytes (Fig. 5G). In contrast, in implants containing ADH cells we observed that cartilage areas were totally resorbed, and large numbers of fat cells appeared (Fig. 5H). At this time,

osteoblasts were not seen on the edges of the trabeculae, indicating a final state of maturation, and certain areas of the chambers walls showed regression phenomena. However, some differences were observed following implantation of both types of cells (3D+ADH). In this case, remnants of cartilage and active osteoblasts depositing new matrix on the surface of a pre-existing bone were observed, indicating active osteogenesis after eight weeks of implantation (Fig. 5I).

DISCUSSION

Biomedical researchers have become increasingly aware of the limitations of Petri dishes or any other 2-dimensional structures and have expressed the necessity to develop culture systems that better represent the natural environment of cells in tissues and organs. Accordingly, our group has successfully developed a 3D collagen gel culture system (3,4,5,10) that allows us to study the isolation, expansion and growth factor responsiveness of marrow-derived cells and evaluate the capacity of these cells to undergo differentiation into bone and/or cartilage tissue when implanted *in vivo*.

Previous characterization of 3D culture cell populations using different approaches (DNA content, alkaline phosphatase (ALP) activity, osteocalcin (OC) expression and calcium (Ca) accumulation) revealed marked differences when compared with those receiving treatment or not with rhTGF-β1. Treated cells were found to proliferate more rapidly than control ones and they expressed higher levels of ALP activity, as well as were able to induce Ca precipitation and to exhibit synthesis of OC during the last days of the cultures. Calcium and OC were both absent in untreated cells (10).

In this report we perform the surface markers profile of bone marrow-derived cells, isolated in the described 3D collagen gel culture system and exposed to rhTGF-β1,

using FACS. We analyzed the expression of surface molecules characteristic for mesenchymal, endothelial and hematopoietic lineages.

Despite a significant number of studies, to date there is no specific marker available for the proper identification of MSCs. Although, ISCT established that MSCs must express CD73, CD90 and CD105, others authors recommend Stro-1, CD73, and CD106 as the most useful markers (30). Stro-1-positive population has been shown to be capable of differentiating into multiple mesenchymal lineages, including hematopoiesis-supportive stromal cells with a vascular smooth muscle-like phenotype, adipocytes, osteoblasts, and chondrocytes (20). CD106 (vascular cell adhesion molecule-1, VCAM-1) is expressed on blood vessel endothelial and adjacent cells, consistent with a perivascular location of MSCs (18), and is involved in cell adhesion, chemotaxis, and signal transduction. Moreover, CD106+/Stro-1+ cells show stem cell characteristics such as multipotentiality, expression of telomerase, and high proliferation *in vitro* (26). Likewise, the persistence of CD73 expression throughout culture also supports its utility as a MSC marker. Two monoclonal antibodies (SH-3 and SH-4) against CD73 have been developed with specificity for mesenchymal tissue-derived cells, as do not react with HSCs, osteoblasts, or osteocytes.

Our results showed that immunophenotype of ADH cells correspond with those widely described in the literature for MSCs (11,13,19,22,41); the vast majority of cells expressed CD29, CD44, CD73, CD90, CD105, CD106, CD166, CD271, HLA-I and Stro-1, and were negative for CD34, CD45 and HLA-DR. Moreover, we distinguished some positive cells for these hematopoietic lineage-specific markers in 3D culture. We found positive cells for some mesenchymal molecules (22), such as CD29, CD44, CD73, CD105, CD166, Stro-1 and HLA-I, but not for CD90, CD106, and CD271. Furthermore,

we observed how increased significantly the MAPCs-like cell population (29,44) over time in the 3D cell populations (CD13+/CD49b+/CD34-/CD44-/CD45-/CD90-/CD106-/HLA-I-/HLA-DR-). In contrast, CD166+ cells disappeared at day 16, indicating that cells included in the collagen matrix do not need to express this adhesion molecule (14). On the other hand, endothelial precursor-like cell immunophenotype (CD34+/CD133+/CD45-) found at day 10 decreased significantly in favour of a mature endothelial population (CD146+/KDR+) (51). In recent literature there are discrepancies about the expression of those markers in endothelial progenitors, thus the search for more specific and reliable markers of "true" EPCs is therefore ongoing. CD34 is normally used to isolate EPCs, but its expression is also shared by HSCs and MSCs, therefore should not be used to distinguish between these populations (8,37,45). Similarly, VEGF receptor 2 (VEGFR2, KDR, Flk-1), which is used to identify EPCs, is expressed also on HSC (8,28). In humans, CD133 (AC133) is considered to be a marker associated with more primitive stem cell phenotype than CD34 (50). These CD133+ cells are of great interest because they can differentiate into mature endothelial cells (ECs) and contribute effectively to neovascularization (37,39). In addition, circulating ECs are usually identified by CD146 (46,47). Others markers common to progenitor and mature ECs are the cell surface receptors KDR and Tie2 (7,27,40). As a result, the combination phenotype of CD34/CD133/KDR is now commonly used as a definition for EPCs, since purified populations of CD133+/KDR+ proliferate *in vitro* and can be induced to differentiate into mature adherent ECs (40).

In any case, it is widely recognized that angiogenesis and bone formation are inseparable, both in development and in fracture repair (33,34). Consequently, it is especially important that our 3D culture system is able to select CD34+/CD133+/KDR+

cells, as a strategy to perform tissue engineering composites to promote angiogenesis and to improve bone regeneration.

FACS analysis of cell cycling showed that ADH cells have a distribution according to the parameters of a proliferating population, unlike those of 3D culture cells that are smaller and mainly kept in a quiescent state (only 17% at S + M + G2). It is well documented that an undetectable or low RNA content, as well as small size and low proliferation rate are features of immature and uncommitted cells (17,35). This low proliferation rate, which is a limiting factor for *in vitro* expansion and thereby for use in regenerative therapies, may also be an indication of stemness. While those ADH cells, with high proliferative rate, should correspond to a population of committed mesenchymal progenitors.

For this reason, we evaluated the expression of pluripotent specific markers by RT-PCR. Results showed that Oct4, Nanog and Sox-2 were highly expressed in 3D culture at day 10 and 16, but was absent in ADH cells. Oct4, Sox2 and Nanog target genes, identified in embryonic stem cells (ESCs), are known to overlap substantially, suggesting they collaborate to regulate a common set of genes governing pluripotency, self-renewal, and cell fate determination (56). As Nanog was recognized to be essential for the pluripotent phenotype (16,36), Rodda et al. (2005) highlight the position of the Oct4-Sox2 complex at the top of the pluripotent regulatory network hierarchy (42). However, Oct4 and Sox2 are not sufficient to activate Nanog transcription in differentiated cells, suggesting the additional requirement of stem cell-specific cofactor(s). Thus, induced expression of Oct4, Sox2 and Nanog along or not with other regulatory proteins is enough to reprogram lineage-restricted somatic cell populations.

These cells, which were called iPS (induced pluripotent stem) cells, exhibit the morphology and growth properties of ESCs and express ESC marker genes (49,54,55).

Taken together, these data support the idea that cells in a collagen gel are maintained in a more undifferentiated state, less committed than ADH cells. Furthermore, this 3D culture system isolate, maintains and even allows evolution of cell populations other than undifferentiated MSC.

Histological study of 3D and ADH culture cells, separates or together, placed inside DBM chambers and implanted subdermally into the backs of rat for 2, 4 and 8 weeks, showed significant differences regarding the histogenesis that occurs inside the chambers. One important feature is the formation of significant amounts of blood vessels only in the chambers with cells cultured in the collagen gel, which is clearly observed in sections at 2 weeks of implantation. It is logical to assume that endothelial precursor-like cells that were identified by FACS should be responsible for these angiogenesis phenomena (1,2,48).

Another highlight is the type of tissue formed at 4 weeks. In the case of 3D culture is a well-organized trabecular matrix, lined by osteoblast-like cells, with numerous marrow spaces highly vascularized. This bone tissue seems to have intramembranous origin because no trace of cartilage can be found, such as hypertrophic chondrocytes, cartilage in the process of calcification, or remnants of proteoglycans (12,43). Nevertheless, ADH cells consistently generated abundant cartilaginous tissue in the middle of the chambers and new bone at the edges with features indicating endochondral ossification (43,52). Consequently, when we implanted 3D cells with ADH cells in 1:1 proportion in the same chamber, both types of tissue were observed. Even, a synergistic effect can be detected: a more advanced degree of histogenesis at 2

weeks, with a cartilage tissue itself, active osteoblasts and cartilage matrix after 8 weeks, etc., indicating that the ossification process remained for longer than when implanted both cell types separately.

In conclusion, our results demonstrates that bone marrow-derived cells selected by serum restriction in the presence of rhTGF-β1 in 3D collagen matrix, were a heterogeneous population of uncommitted cells that express pluripotent specific markers *in vitro* and can undergo osteogenic and endothelial differentiation *in vivo*. As these cells clearly have a great potential in cell therapy and show a synergistic effect *in vivo* with conventional adherent cells, and given that large cell numbers are required for clinical feasibility, we propose the implantation of both types of cells as an alternative to improve the repair of bone defects, enhancing MSC effects.

ACKNOWLEDGEMENTS: This work was supported by grants from the Ministry of Science and Innovation (PLE2009-0163, FIS PI10/02529), the Ministry of Science and Technology (BIO2009-13903-C02-01), and the Andalusian Government (P07-CVI-2781, PAIDI BIO-217, PI-0729-2010). Red de Terapia Celular and CIBER-BBN are an initiative funded by the VI National R&D&I Plan 2008-2011, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions, and financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund. The authors wish to thank Carmen Rueda-Martínez for her excellent technical assistance. This study was supported in part by research funding from Fina Biotech to JB, JAA and EG.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

REFERENCES

1. Agbulut; O.; Vandervelde; S.; Al Attar, N.; Larghero, J.; Ghostine, S.; Léobon, B.; Robidel, E.; Borsani, P.; Le Lorc'h, M.; Bissery, A.; Chomienne, C.; Bruneval, P.; Marolleau, J. P.; Vilquin, J. T.; Hagège, A.; Samuel, J. L.; Menasché, P. Comparison of human skeletal myoblasts and bone marrow derived CD133+ progenitors for the repair of infarcted myocardium. J. Am. Coll. Cardiol. 44:458-463; 2004.
2. Amrani, D. L.; Port, S. Cardiovascular disease: potential impact of stem cell therapy. Expert Rev. Cardiovasc. Ther. 1:453-461; 2003.
3. Andrades, J. A.; Han, B.; Becerra, J.; Sorgente, N.; Hall, F. L.; Nimni, M. E. A recombinant human TGF-beta1 fusion protein with collagen-binding domain promotes migration; growth; and differentiation of bone marrow mesenchymal cells. Exp. Cell Res. 250(2):485-498; 1999.
4. Andrades, J. A.; Han, B.; Nimni, M. E.; Ertl, D. C.; Simpkins, R. J.; Arrabal, M. P.; Becerra, J. A modified rhTGF-beta1 and rhBMP-2 are effective in initiating a chondro-osseous differentiation pathway in bone marrow cells cultured in vitro. Connect Tissue Res. 44(3-4):188-197; 2003.
5. Andrades, J. A.; Santamaría, J. A.; Nimni, M. E.; 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. 45(4):689-693; 2001.
6. Arthur, A.; Zannettino, A.; Gronthos, S. The therapeutic applications of multipotential mesenchymal/stromal stem cells in skeletal tissue repair. J. Cell Physiol. 218(2):237-245; 2009.
7. Asahara, T.; Kawamoto, A. Endothelial progenitor cells for postnatal vasculogenesis. Am. J. Physiol. Cell Physiol. 287(3):C572-579; 2004.
8. Asahara, T.; Murohara, T.; Sullivan, A.; Silver, M.; van der Zee, R.; Li, T.; Witzenbichler, B.; Schatteman, G.; Isner, J. M. Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964-967; 1997.
9. Baksh, D.; Zandstra, P. W.; Davies, J. E. A non-contact suspension culture approach to the culture of osteogenic cells derived from a CD49elow subpopulation of human bone marrow-derived cells. Biotechnol. Bioeng. 98(6):1195-1208; 2007.

10. Becerra, J.; Guerado, E.; Claros, S.; Alonso, M.; Bertrand, M. L.; González, C.; Andrades, J. A. Autologous human-derived bone marrow cells exposed to a novel TGF-beta1 fusion protein for the treatment of critically sized tibial defect. Regen. Med. 1(2):267-278; 2006.

11. Beyer, N.; Da Silva, L. Mesenchymal stem cells: isolation in vitro expansion and characterization. Handb. Exp. Pharmacol. 174:249-282; 2006.

12. Bianco, P.; Cancedda, F. D.; Riminucci, M.; Cancedda, R. Bone formation via cartilage models: the "borderline" chondrocyte. Matrix Biol. 17(3):185-192; 1998.

13. Bianco P; Riminucci M; Gronthos S; Robey, P. Bone marrow stromal cells: nature, biology, and potential applications. Stem Cells 19:180-192; 2001.

14. Bruder, S. P.; Ricalton, N. S.; Boynton, R. E.; Connolly, T. J.; Jaiswal, N.; Zaia, J.; Barry, F. P. Mesenchymal stem cell surface antigen SB-10 corresponds to activated leukocyte cell adhesion molecule and is involved in osteogenic differentiation. J. Bone Miner. Res. 13:655-663; 1998.

15. Caplan, A. I.; Dennis, J. E. Mesenchymal stem cells as trophic mediators. J. Cell Biochem. 98(5):1076-1084; 2006.

16. Chambers, I.; Colby, D.; Robertson, M.; Nichols, J.; Lee, S.; Tweedie, S.; Smith, A. Functional expression cloning of Nanog; a pluripotency sustaining factor in embryonic stem cells. Cell 113(5):643-655; 2003.

17. Conget, P. A.; Allers, C.; Minguell, J. J. Identification of a discrete population of human bone marrow-derived mesenchymal cells exhibiting properties of uncommitted progenitors. J. Hematother. Stem Cell Res. 10(6):749-758; 2001.

18. Crisan, M.; Yap, S.; Casteilla, L.; Chen, C. W.; Corselli, M.; Park, T. S.; Andriolo, G.; Sun, B.; Zheng, B.; Zhang, L.; Norotte, C.; Teng, P. N.; Traas, J.; Schugar, R.; Deasy, B. M.; Badylak, S.; Buhring, H. J.; Giacobino, J. P.; Lazzari, L.; Huard, J.; Péault, B. A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3(3):301-313; 2008.

19. Deans, R. J.; Moseley, A. B. Mesenchymal stem cells: biology and potential clinical uses. Exp. Hematol. 28:875-884; 2000.

20. Dennis, J. E.; Carbillet, J. P.; Caplan, A. I.; Charbord, P. The STRO-1+ marrow cell population is multipotential. Cells Tissues Organs 170:73-82; 2002.

21. D'Ippolito, G.; Diabira, S.; Howard, G. A.; Menei, P.; Roos, B. A.; Schiller, P. C. Marrow-isolated adult multilineage inducible (MIAMI) cells; a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. J. Cell Sci. 117:2971-2981; 2004.

22. Dominici, M.; Le Blanc, K.; Mueller, I.; Slaper-Cortenbach, I.; Marini, F.; Krause, D.; Deans, R.; Keating, A.; Prockop, D. J.; Horwitz, E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315-317; 2006.

23. Folgiero, V.; Migliano, E.; Tedesco, M.; Iacovelli, S.; Bon, G.; Torre, M. L.; Sacchi, A.; Marazzi, M.; Bucher, S.; Falcioni, R. Purification and characterization of adipose-derived stem cells from patients with lipoaspirate transplant. Cell Transplant. 19(10):1225-1235; 2010.

24. Fricke, S.; Fricke, C.; Oelkrug, C.; Hilger, N.; Schönfelder, U.; Kamprad, M.; Lehmann, J.; Boltze, J.; Emmrich, F.; Sack, U. Characterization of murine non-adherent bone marrow cells leading to recovery of endogenous hematopoiesis. Cell Mol. Life Sci. 67(23):4095-4106; 2010.

25. Goodell, M. A.; Brose, K.; Paradis, G.; Conner, A. S.; Mulligan, R. C. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J. Exp. Med. 183:1797-1806; 1996.

26. Gronthos, S.; Zannettino, A. C.; Hay, S. J.; Shi, S.; Graves, S. E.; Kortesidis, A.; Simmons, P. J. Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. J. Cell Sci. 116:1827-1835; 2003.

27. Ishikawa, M.; Asahara, T. Endothelial progenitor cell culture for vascular regeneration. Stem Cells Dev. 13(4):344-349; 2004.

28. Isner, J. M.; Asahara, T. Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. J. Clin. Invest. 103(9):1231-1236; 1999.

29. Jiang, Y.; Jahagirdar, B. N.; Reinhardt, R. L.; Schwartz, R. E.; Keene, C. D.; Ortiz-González, X. R.; Reyes, M.; Lenvik, T.; Lund, T.; Blackstad, M.; Du, J.; Aldrich, S.; Lisberg, A.; Low, W. C.; Largaespada, D. A.; Verfaillie, C. M. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41-49; 2002.

30. Kolf, C. M.; Cho, E.; Tuan, R. S. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthritis Res. Ther. 9(1):204; 2007.

31. Krinner, A.; Hoffmann, M.; Loeffler, M.; Drasdo, D.; Galle, J. Individual fates of mesenchymal stem cells in vitro. BMC Syst. Biol. 4:73; 2010.

32. Kucia, M.; Reca, R.; Campbell, F. R.; Zuba-Surma, E.; Majka, M.; Ratajczak, J.; Ratajczak, M. Z. A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia 20(5):857-869; 2006.

33. Li, R.; Stewart, D. J.; von Schroeder, H. P.; Mackinnon, E. S.; Schemitsch, E. H. Effect of cell-based VEGF gene therapy on healing of a segmental bone defect. J. Orthop. Res. 27:8-14; 2009.

34. Matsumoto, T.; Kuroda, R.; Mifune, Y.; Kawamoto, A.; Shoji, T.; Miwa, M.; Asahara, T.; Kurosaka, M. Circulating endothelial/skeletal progenitor cells for bone regeneration and healing. Bone 43:434-439; 2008.

35. Minguell, J. J.; Fierro, F. A.; Epuñan, M. J.; Erices, A. A.; Sierralta, W. D. Nonstimulated human uncommitted mesenchymal stem cells express cell markers of mesenchymal and neural lineages. Stem Cells Dev. 14(4):408-414; 2005.

36. Mitsui, K.; Tokuzawa, Y.; Itoh, H.; Segawa, K.; Murakami, M.; Takahashi, K.; Maruyama, M.; Maeda, M.; Yamanaka, S. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113(5):631-642; 2003.

37. Peichev, M.; Naiyer, A. J.; Pereira, D.; Zhu, Z.; Lane, W. J.; Williams, M.; Oz, M. C.; Hicklin, D. J.; Witte, L.; Moore, M. A.; Rafii, S. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 95:952-958; 2000.

38. Pittenger, M. F.; Mackay, A. M.; Beck, S. C.; Jaiswal, R. K.; Douglas, R.; Mosca, J. D.; Moorman, M. A.; Simonetti, D. W.; Craig, S.; Marshak, D. R. Multilineage potential of adult human mesenchymal stem cells. Science 284:143-147; 1999.

39. Rafii, S.; Lyden, D.; Benezra, R.; Hattori, K.; Heissig, B. Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? Nat. Rev. Cancer 2(11):826-835; 2002.

40. Rafii, S.; Lyden, D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat. Med. 9(6):702-712; 2003.

41. Rasmusson, I. Immune modulation by mesenchymal stem cells. Exp. Cell Res. 312:2169-2179; 2006.

42. Rodda, D. J.; Chew, J. L.; Lim, L. H.; Loh, Y. H.; Wang, B.; Ng, H. H.; Robson, P. Transcriptional regulation of nanog by OCT4 and SOX2. J. Biol. Chem. 280(26):24731-24737; 2005.

43. Scotti, C.; Tonnarelli, B.; Papadimitropoulos, A.; Scherberich, A.; Schaeren, S.; Schauerte, A.; Lopez-Rios, J.; Zeller, R.; Barbero, A.; Martin, I. Recapitulation of endochondral bone formation using human

adult mesenchymal stem cells as a paradigm for developmental engineering. Proc. Natl. Acad. Sci. U. S. A. 107(16):7251-7256; 2010.

44. Serafini, M.; Verfaillie, C. M. Multipotent progenitor cell populations obtained from bone marrow. In: Garcia-Olmo, D.; Garcia-Verdugo, J. M., eds. Cell Therapy. Maidenhead, UK: McGraw Hill; 2008:89-102.

45. Shi, Q.; Rafii, S.; Wu, M. H.; Wijelath, E. S.; Yu, C.; Ishida, A.; Fujita, Y.; Kothari, S.; Mohle, R.; Sauvage, L. R.; Moore, M. A.; Storb, R. F.; Hammond, W. P. Evidence for circulating bone marrow-derived endothelial cells. Blood 92:362-367; 1998.

46. Smadja, D. M.; Mauge, L.; Sanchez, O.; Silvestre, J. S.; Guerin, C.; Godier, A.; Henno, P.; Gaussem, P.; Israël-Biet, D. Distinct patterns of circulating endothelial cells in pulmonary hypertension. Eur. Respir. J. 36(6):1284-1293; 2010.

47. Solovey, A. N.; Gui, L.; Chang, L.; Enenstein, J.; Browne, P. V.; Hebbel, R. P. Identification and functional assessment of endothelial P1H12. J. Lab. Clin. Med. 138(5):322-331; 2001.

48. Stamm, C.; Westphal, B.; Kleine, H. D.; Petzsch, M.; Kittner, C.; Klinge, H.; Schümichen, C.; Nienaber, C. A.; Freund, M.; Steinhoff, G. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet 361:45-46; 2003.

49. Takahashi, K.; Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663-676; 2006.

50. Tondreau, T.; Meuleman, N.; Delforge, A.; Dejeneffe, M.; Leroy, R.; Massy, M.; Mortier, C.; Bron, D.; Lagneaux, L. Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. Stem Cells 23(8):1105-1112; 2005.

51. Untergasser, G.; Koeck, R.; Wolf, D.; Rumpold, H.; Ott, H.; Debbage, P.; Koppelstaetter, C.; Gunsilius, E. CD34+/CD133- circulating endothelial precursor cells (CEP): characterization; senescence and in vivo application. Exp. Gerontol. 41(6):600-608; 2006.

52. van den Bos, T.; Speijer, D.; Bank, R. A.; Brömme, D.; Everts, V. Differences in matrix composition between calvaria and long bone in mice suggest differences in biomechanical properties and resorption: Special emphasis on collagen. Bone 43(3):459-468; 2008.

53. Włodarski, K. H.; Galus, R.; Włodarski, P. Non-adherent bone marrow cells are a rich source of cells forming bone in vivo. Folia Biol. (Praha) 50:167-173; 2004.

54. Yu, J.; Vodyanik, M. A.; Smuga-Otto, K.; Antosiewicz-Bourget, J.; Frane, J. L.; Tian, S.; Nie, J.; Jonsdottir, G. A.; Ruotti, V.; Stewart, R.; Slukvin, I. I.; Thomson, J. A. Induced pluripotent stem cell lines derived from human somatic cells. Science 318(5858):1917-1920; 2007.

55. Zhao, H. X.; Li, Y.; Jin, H. F.; Xie, L.; Liu, C.; Jiang, F.; Luo, Y. N.; Yin, G. W.; Li, Y.; Wang. J.; Li, L. S.; Yao, Y. Q.; Wang, X. H. Rapid and efficient reprogramming of human amnion-derived cells into pluripotency by three factors OCT4/SOX2/NANOG. Differentiation 80(2-3):123-129; 2010.

56. Zheng, C.; Yang, S.; Guo, Z.; Liao, W.; Zhang, L.; Yang, R.; Han, ZC. Human multipotent mesenchymal stromal cells from fetal lung expressing pluripotent markers and differentiating into cell types of three germ layers. Cell Transplant. 18(10):1093-1109; 2009.

Table 1. Cell size and granularity analysis in 3D and ADH culture cells, using FS and SS parameters.

	3D culture cells	**ADH culture cells**
FS	90.55 ± 21.7 *	136.69 ± 18.0
SS	72.36 ± 24.1	65.00 ± 13.3

Values are expressed as the mean ± SEM (n=13). * $p<0.05$ vs. ADH culture cells.

Abbreviations: 3D, three-dimensional; ADH, adherent; FS, forward scatter; SS, side scattter.

Table 2. Statistical analysis of flow cytometry results.

Marker	Adherent culture	3D culture 10 days	3D culture 14 days	3D culture 16 days
CD29	100 ± 0.1	30 ± 1	30.2 ± 0.9	34 ± 3.2
CD44	65.9 ± 19.6	36.7 ± 7.4	20.1 ± 1.9	26.35 ± 4.9
CD73	71 ± 3.3	10.5 ± 2.7	10.1 ± 0.2	12.6 ± 0.3
CD105	70.1 ± 4.1	19 ± 19	15.7 ± 2.3	12.1 ± 2.4
CD106	35.7 ± 2.5	4.8 ± 1.8	2.4 ± 0.9	3.1 ± 1.5
CD166	84.2 ± 3.3	46 ± 3.9	27.5 ± 3.3	8.5 ± 2.4 ***
HLA-I	81 ± 0.5	12.5 ± 4.3	17.8 ± 6.5	21.5 ± 5.2
Stro-1	61.9 ± 5.5	33.1 ± 2.1	38.3 ± 3.3	33.1 ± 2.5
CD13	72.5 ± 3.4	10.8 ± 3.4	15 ± 3	20 ± 5
CD49b	4.6 ± 0.5	3 ± 0.4	3.4 ± 0.4	6.5 ± 1.7
CD13/CD49b	3.4 ± 0.4	1 ± 0.3	3.1 ± 0.2	6.4 ± 0.2 ***
HLA-DR	1.2 ± 0.3	23.6 ± 2.5	21.1 ± 3.2	20.9 ± 5.9
CD34	3.3 ± 0.8	35.7 ± 4.8	23.7 ± 3.5	12.2 ± 3.7
CD45	0 ± 0.5	43.4 ± 3.6	25.9 ± 2	24.2 ± 1.3 *
CD133	ND	64.2 ± 1.9	52.5 ± 0.8	42.9 ± 0.5
CD49a	ND	9.9 ± 0.7	8.2 ± 0.7	7.9 ± 0.2
CD146	ND	1.9 ± 0.1	12.6 ± 0.1	20.2 ± 0.2
KDR	ND	18.3 ± 0.7	25.2 ± 0.3	34.7 ± 0.3
CD146/KDR	ND	1.1 ± 0.1	8.7 ± 0.7	19.8 ± 0.2 ***

Values represent percent of positive cells, and are expressed as the mean ± SEM (n=8).

*** $p<0.001$; * $p<0.05$ vs. 3D culture 10 days.

Abbreviations: 3D, three-dimensional; ND, non data.

FIGURE LEGENDS

Figure 1. Cell morphology in 3D collagen gel and ADH culture. (A-B): Phase contrast images of 3D cultures in the absence (round-shaped isolated cells) and presence of TGF-β1 (cell aggregates) at the end of the amplification period (day 14). (C): Phase contrast image of primary ADH culture. Bars, 200 µm. Abbreviations: 3D, three-dimensional; ADH, adherent; TGF-β1, transforming growth factor beta-1.

Figure 2. FACS analysis of cell cycling after propidium iodide staining. (A): 3D culture cells are standing at the Go/G1 phase (approximately 83%), (B): while ADH cells are actively in proliferation (approximately 72% at S + G2 + M). Abbreviations: 3D, three-dimensional; ADH, adherent.

Figure 3. Immunophenotype profiles of 3D culture cells for hematopoietic, mesenchymal and endothelial markers. Representative FACS analysis at day 10 (A) and 16 (B). Abbreviation: 3D, three-dimensional.

Figure 4. mRNA expression in 3D and ADH cultures by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). 3D cultured cells expressed Nanog, Sox2 and Oct4 while ADH cells did not. Abbreviations: 3D, three-dimensional; ADH, adherent.

Figure 5. Histological sections of DBM chambers with different types of cells after 2, 4 and 8 weeks of *in vivo* implantation. Different types of tissue can be observed, according to cell type implanted and time of implantation. Asterisks represent the walls of the chambers. (A and insert): 3D culture cells at 2 weeks. The chambers were principally filled with fibrous tissue and showed numerous small blood vessels (arrows). PSH (A) and Goldner's trichrome stain (insert). Bars, 500 µm in (A) and 200 µm in insert. (B and insert): ADH culture cells at 2 weeks. Dense fibrous tissue and chondrocyte-like cells (arrows) located adjacent to the DBM chamber wall were observed. Vascularization phenomena were less apparent. PSH (B) and Goldner's trichrome stain (insert). Bars, 500 µm in (B) and 200 µm in insert. (C and insert): 3D and ADH culture cells at 2 weeks. Tissular condensations with cartilaginous matrices were distinguished. PSH stain (C) and type II collagen immunohistochemistry (insert). Bars, 500 µm in (B) and 200 µm in insert. (D): 3D culture cells at 4 weeks. Typical appearance of tissue formed by these cells: a well-organized trabecular matrix, lined with osteoblast-like cells (arrowheads), regularly scattered vessels and marrow spaces. Type I collagen immunohistochemistry. Bar, 500 µm. (E): ADH culture cells at 4 weeks. The chambers were occupied by abundant cartilaginous tissue with hypertrophic chondrocytes at the ends of the cartilage, and new bone formation (star) through endochondral ossification was identified. PSH stain. Bar, 500 µm. (F): 3D and ADH culture cells at 4 weeks. Both type of tissue were observed: large areas of cartilage with trabecular bone formed via an endochondral pathway (star), and new bone tissue whose origin seemed to be intramembranous. PSH stain. Bar, 1 mm. (G): 3D culture cells at 8 weeks. The chamber wall and the new-formed tissues were suffering an active remodeling process. (H): ADH culture cells at 8 weeks. The cartilage areas were

totally resorbed and large numbers of fat cells appeared. Goldner's trichrome stain. Bar, 500 µm. (I): 3D and ADH culture cells at 8 weeks. Remnants of cartilage and active osteoblasts depositing new matrix were observed, indicating active osteogenesis. Goldner's trichrome stain. Bar, 500 µm. Abbreviations: DBM, demineralized bone matrix; 3D, three-dimensional; PSH, picrosirius-hematoxyline; ADH, adherent.