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REGULAR ARTICLES

Phenotypic and proliferative modulation of human mesenchymal stem cells via crosstalk with endothelial cells

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Abstract The purpose of this work was to investigate if a coculture system of human mesenchymal stem cells (hMSC) with endothelial cells (human umbilical vein endothelial cells, HUVEC) could modulate the phenotype and proliferation of harvested MSCs. In addition to previous investigations on the crosstalk between these two cell types, in the present work different relative cell ratios were analyzed for long, therapeutically relevant, culture periods. Moreover, MSCs osteogenic commitment was assessed in a non-osteogenic medium and in the presence of HUVECs through magnetic cell separation, cell quantification by flow cytometry, morphology by fluorescent microscopy, metabolic activity and gene expression of osteogenic markers. Collectively, the present findings demonstrate that, by coculturing MSCs with HUVECs, there was not only the promotion of osteogenic differentiation (and its enhancement, depending on the relative cell ratios used), but also a significant increase on MSCs proliferation. This augmentation in cell proliferation occurred independently of relative cell ratios, but was favored by higher relative amounts of HUVECs. Taken together, this data suggests that HUVECs not only modulate MSC phenotype but also their proliferation rate. Therefore, a coculture system of MSCs and HUVECs can a have a broad impact on bone tissue engineering approaches.

Introduction

Mesenchymal stem cells (MSCs) represent an adherent, fibroblast-like population present not only in bone marrow, but also in a number of other tissues, including blood, adipose tissue, muscle and dermis (Sotiropoulou et al., 2006). In bone tissue engineering approaches, bone marrow MSCs have demonstrated great potential for the treatment of bone loss, fracture non-union and osteogenesis imperfecta (Bajada et al., 2008; Sensebé et al., 2009). The success of bone regeneration strategies is in part based in the reintroduction of *in vitro* expanded cells in a state that guarantees their differentiation into functional bone matrix-producing cells (Hofmann et al.,

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2008). MSCs differentiated into the osteogenic lineage represent thus an appealing source of adult stem cells for bone tissue engineering applications (Moioli et al., 2007).

However, these approaches find limitations in the considerably low percentage of MSCs in adult bone marrow, and the vast numbers required for therapeutic approaches (Sot\iropoulou et al., 2006; Shahdadfar et al., 2005). The identification of mechanisms involved in stem cells self-renewal and differentiation is important for the design of new strategies to expand stem cells in vitro and modulate their phenotype. MSCs fate decision can be regulated by exogenous soluble growth factors, cytokines, hormones and chemicals and by external mechanical forces (Alsberg et al., 2006). Another approach to guide MSCs fate relies in the coculturing with other mature cell populations (Ball et al., 2004). Ball and co-authors demonstrated that MSC are profoundly influenced by other cell types in direct contact (Ball et al., 2004). Among these, endothelial cells (ECs) have been shown to express several factors, such as BMP-2, that induce osteogenic differentiation *in vitro,* when in direct contact with bone marrow stromal cells (Kaigler et al., 2005; Guillotin et al., 2004). Moreover, Villars and colleagues (2002) showed that intercommunication between ECs and osteoblast-like cells is dependent not only on diffusible factors but also on gap junctions (connexin 43) that play a crucial role in cell differentiation, which points to the need of cell-cell contact (Villars et al., 2002; Grellier et al., 2009a). Overall, two main dialogues were identified: a paracrine effect through VEGF, BMP-2, IGF production and a juxtacrine mechanism by gap junctional activity between the two cell types (Grellier et al., 2009b; Clarkin et al., 2008a).

Although several works have been evaluating the influence of a number of parameters for large-scale production of MSCs, such as cell source, age, density, passage number, medium composition, presence of growth factors and serum choice, the concept of using cocultures for MSCs expansion, in particular with ECs, seems to constitute a promising approach for bone regeneration applications and is far from being completely investigated (Sotiropoulou et al., 2006; Shahdadfar et al., 2005; Diascro et al., 1998; Both et al., 2007; Wall et al., 2007; Walsh et al., 2001; Tamama et al., 2006).

The objective of this work was to investigate if a synergistic coculture system of MSCs with endothelial cells could affect the number and state of differentiation of harvested MSCs. In contrast to other papers, in this cell-cell communication topic, the investigation was carried out for a longer time, 3 weeks instead of 1 to 6 days and different ratios of both cell types were considered (Guillotin et al., 2004; Villars et al., 2002; Grellier et al., 2009a; Guillotin et al., 2008; Finkenzeller et al., 2006; Stahl et al., 2004; Wenger et al., 2004; Villars et al., 2000; Xue et al., 2009). Additionally, several media were tested and MSCs osteogenic commitment in a non-osteogenic medium in the presence of ECs was carefully analyzed. The present results demonstrated that, independently of the relative cell ratio used, the coculture system promotes a significant increase in MSCs proliferation and differentiation. Furthermore, these results indicate that, for tissue engineering applications requiring a large number of MSCs committed into the osteogenic lineage, a coculture system with endothelial cells could be advantageous.

Results

Selection of base medium

HUVECs and MSCs behavior was evaluated in several commercial media, including Iscove Modified Dulbecco Medium (IMDM), Medium 199 (M199), Dulbecco's Modified Eagle's medium (DMEM) and a mixture 1:1 of the last two media. The three media present some major differences in terms of composition, (composition provided by the manufacturer). For instance, IMDM and M199 have a higher number of amino acids than DMEM. IMDM has the highest glutamine concentration, followed by DMEM and then M199. Additionally, M199 has the lowest concentrations on vitamins and the other two have a similar composition. In the inorganic salts content, such as calcium, chlorine, iron, potassium, no major differences were found. Regarding other components, IMDM is the only with HEPES (5958 mg/L) and presents a D-Glucose concentration (4500 mg/L) higher than the others (1000 mg/L). Moreover, M199 does not have sodium pyruvate in its composition.

To evaluate the effect of the different media on cell behavior, metabolic activity and protein quantification assays were carried out (Fig. 1). Overall, there was a continuous increase in terms of the metabolic activity and protein content along the time, for both HUVECs and MSCs, in all the media tested. However, the medium that showed the best results was different in both cell types. Considering both assays, for HUVECs the following profile could be outlined: IMDM > M199 = M199 + DMEM > DMEM. For MSCs the following sequence was obtained: M199 + DMEM > DMEM > IMDM = M199. In a coculture approach, the selected medium should take into consideration the behavior of both cell types. Collectively, the described data pointed towards the selection of the combined medium M199 + DMEM, which provided a performance close to the ideal in both situations.

Coculture study: cell quantification, morphology and differentiation

Cocultures were established by seeding the two different cell types in direct contact at a cell ratio of 1:1. Both cell types were cultured (in mono- and coculture) in the selected medium, and the evolution on the number of total cells along the time was analyzed, as depicted in Fig. 2. Cells in coculture exhibited, as a whole, a significant increase in number throughout the culture, reaching from day 14 values significantly higher than any of the monocultures, and continued to proliferate along the last week (Fig. 2A).

When both cell types were analyzed in separate, it could be seen that while the number of MSCs in monoculture significantly increased until day 14th and then remained practically unchanged, the MSCs in coculture (CoMSCs) increased in number throughout the 21 days (Fig. 2C). Moreover, CoMSCs presented higher proliferation rate than MSC in monoculture (at 1.5 $x10^3$ or 3 $x10^3$ cells/cm^2) in the last two weeks of culture. MSCs in coculture increased rapidly, reaching approximately 80% of the cells in coculture after 1 week, and then cells continued to proliferate until the end of the culture, reaching a percentage of 94% (Fig. 2B).

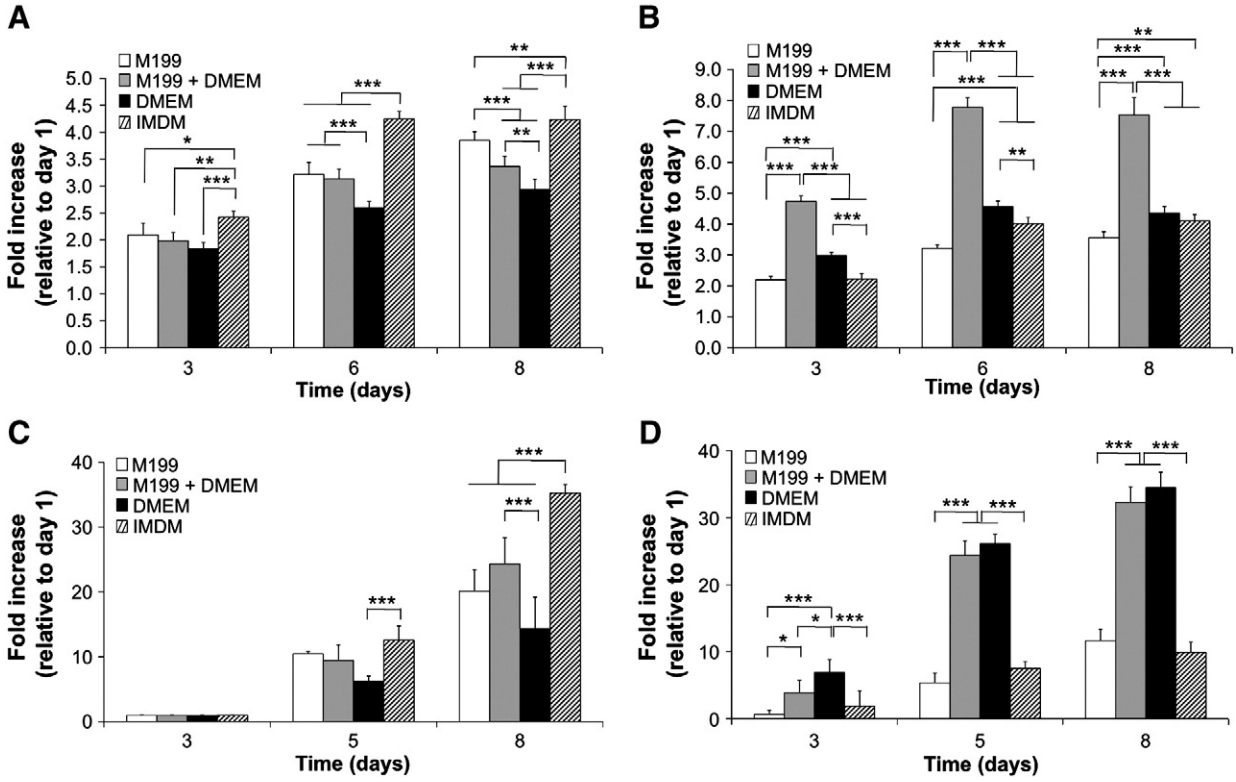

Figure 1 (A,B) Metabolic activity and (C,D) protein concentration of (A, C) human umbilical vein endothelial cells and (B,D) mesenchymal stem cells cultured under four different media: M199, M199 with DMEM, DMEM and IMDM. The initial cell seeding density was the same in all the conditions ($3x10^3$ cells/cm^2). Data are expressed as mean ± standard deviation (n = 3). (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

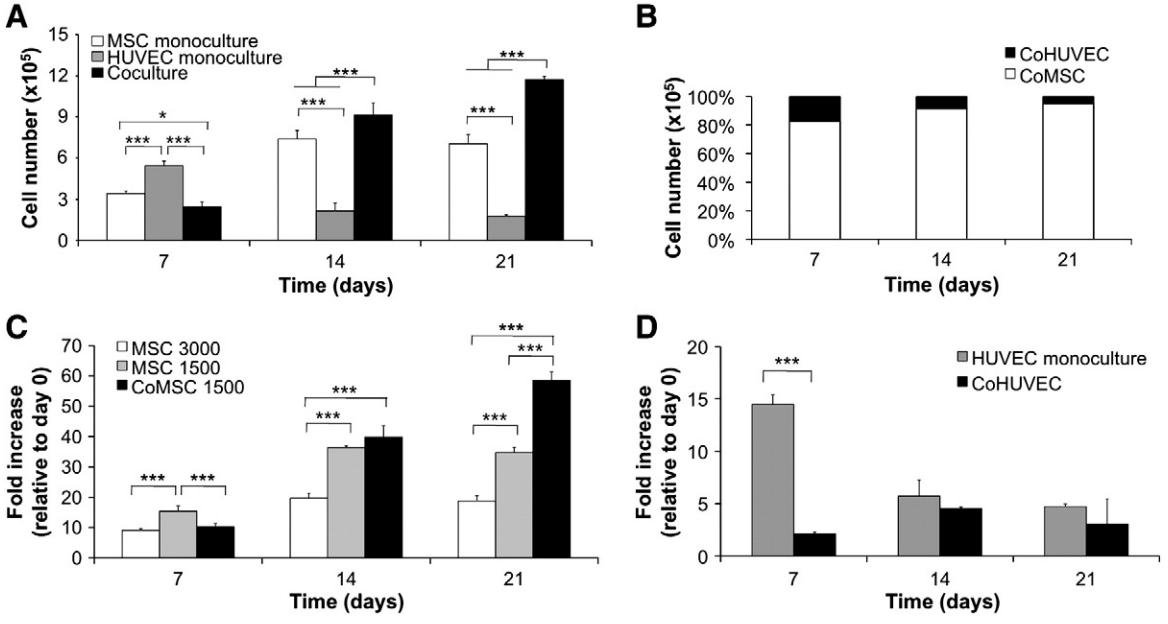

Figure 2 Mesenchymal stem cells (MSC) and human umbilical vein endothelial cells (HUVEC) cultured in mono- and coculture during 21 days. (A) Total cell number of MSC, HUVEC and coculture of both cell types; (B) Relative number of MSC (CoMSC) and HUVEC (CoHUVEC) in the coculture; (C) Fold increase in cell number relative to day 0 of MSC in the coculture (CoMSC 1500; 1500 cells/cm^2 of MSCs with 1500 cells/cm^2 of HUVECs), MSC in monoculture with a cell density of 3000 cells/cm^2 (MSC 3000), and MSC in monoculture with a cell density of 1500 cells/cm^2 (MSC 1500); (D) Fold increase in cell number relative to day 0 of HUVEC in mono- and co-culture during 21 days. Cocultures were established by seeding the two cell types in direct contact at a cell ratio of 1:1 with a final cell seeding density of $3x10^3$ cells/cm^2 and the medium used (mixture 1:1 of DMEM and M199) was the same in all the conditions tested. For monocultures cell density was of $3x10^3$ cells/cm^2 with the exception of MSC 1500. Data are expressed as mean ± standard deviation (n = 3). (* $p \leq 0.05$; *** $p \leq 0.001$).

The HUVECs in monoculture showed higher proliferation rate than the MSCs on the 1st week of culture (Fig. 2A). However, the number of cells significantly decreased thereafter. Although the percentage of HUVECs in coculture (CoHUVECs) progressively declined, cells continued to proliferate during the 2nd week (Fig. 2D).

The cell morphology study, revealed evident differences between cells organization in MSCs mono- and cocultures, as can be depicted in Fig. 3A. MSCs in monoculture appeared randomly distributed at the cell culture surface along the first 2 weeks, revealing, at day 21, a fibroblastic-like shape with stress fibers running in parallel according to cell orientation. In coculture, cells were rearranged in a significantly different manner, forming a kind of tubular-like cellular network. This type of structure becomes less evident along the time, as the cell density rose. At day 21, it was possible to visualize a crisscrossed pattern of actin cytoskeleton with thick stress fibers.

Differences in cells morphology were also evident on the ALP cytochemical analysis (Fig. 3B). However, in cocultures it was possible to detect a much higher amount of ALP positive cells in all time points. For instance, at day 14, the wells from the coculture revealed an almost monochromatic (pink) surface. These results were further corroborated by the ALP colorimetric assay (Fig. 3C). ALP activity, normalized to total protein concentration, reached significantly higher levels in coculture than in monoculture. In monoculture the levels of ALP remained low and constant throughout the culture and always below the values reached in coculture.

As previously described, the two types of cells in coculture were separated and additional differentiation studies were performed using only the MSC fraction. The gene expression profile of several osteogenic markers was analyzed (Fig. 4). The gene profile for ALP and COL I revealed a significant difference between MSC in monoculture and cocultured MSC (CoMSC) (for ALP $p<0.05$ and for type I collagen $p=0.01$). Runx 2 and BSP presented a significant increase after one week of culture in CoMSC. The late-stage osteogenic differentiation marker, OCN, even after 21 days of culture did not show significant differences between MSC and CoMSC.

Effect of different cell ratios on MSC behavior

In order to evaluate the effect of MSCs and HUVECs relative ratios on MSCs behavior, the following combinations were

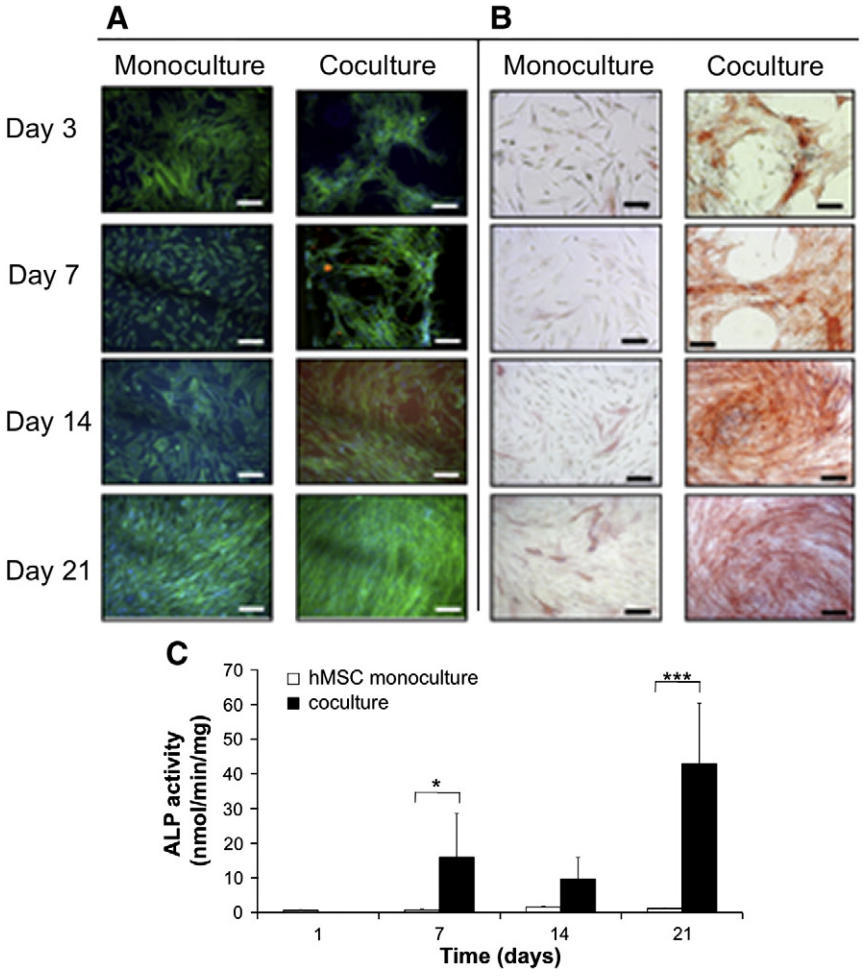

Figure 3 (A) Immunocytochemistry of actin filaments (green) and nuclei (blue) and (B) ALP histochemistry and (C) ALP activity in MSCs cultured alone or cocultured with HUVECs at different time points under basal conditions. Cocultures were established by seeding the two different cell types together at a cell ratio of 1:1 with a final cell seeding density of $3x10^3$ cells/cm^2. Bars correspond to 200 μm. Data are expressed as mean ± standard deviation (n = 3). (* $p \leq 0.05$; *** $p \leq 0.001$).

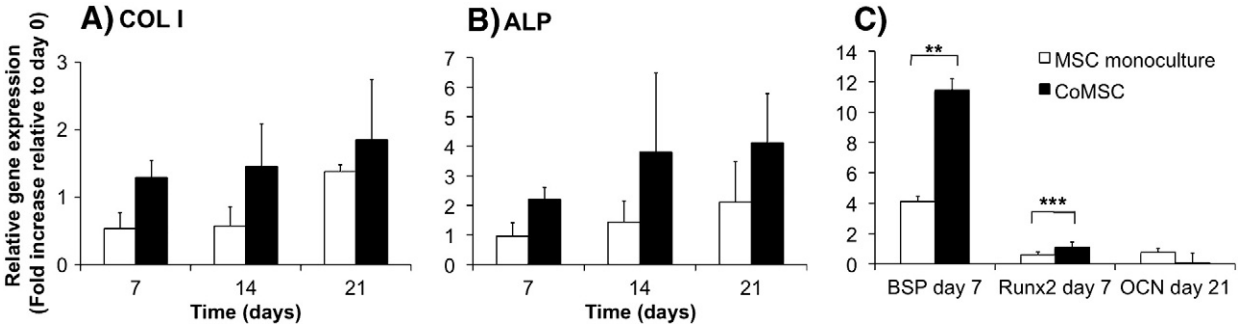

Figure 4 Gene expression levels of osteogenic markers of mesenchymal stem cells (MSC) growing in monoculture and in coculture with endothelial cells (CoMSC) by qPCR. (A) type I collagen (COL I), (B) alkaline phosphatase (ALP) and (C) bone sialoprotein (BSP) and transcription factor Runx2 at day 7 and osteocalcin (OCN) at day 21. To perform gene analysis on CoMSC, cells were separated by magnetic cell sorting using magnetic beads coupled with an antibody against CD31. CoMSC presented significantly higher expression levels for COL I and ALP than MSCs in monoculture (collagen type I $p = 0.01$ and ALP $p < 0.05$). The experiment was performed three times, each in triplicate. (** $p \leq 0.01$; *** $p \leq 0.001$).

tested: 25%HUVEC-75%MSC, 50%HUVEC-50%MSC and 75% HUVEC-25%MSC. MSCs monocultures were used as a control. The influence of endothelial cells on the metabolic activity, proliferation and osteogenic differentiation of MSCs was analyzed.

Concerning metabolic activity (Fig. 5A), the three different ratios presented a significant increase along the first 2 weeks of culture, followed by a plateau, although the MSC monoculture showed the highest fold increase after 1 week. During the 2nd and 3 rd weeks this behavior changed, and cells in coculture reached metabolic activity values significantly higher than the monoculture, at all the ratios tested. Among the different ratios, the combination 50%HUVEC-50%MSC was the one that presented the highest increase, although not significantly different from the others.

The evolution of the number of cells along the time is depicted in Fig. 5B, showing that in all the three ratios tested, the percentage of MSCs increased, while the relative amount of HUVECs decreased. In fact, MSCs were able to proliferate throughout the 21 days of culture and the ratio that showed

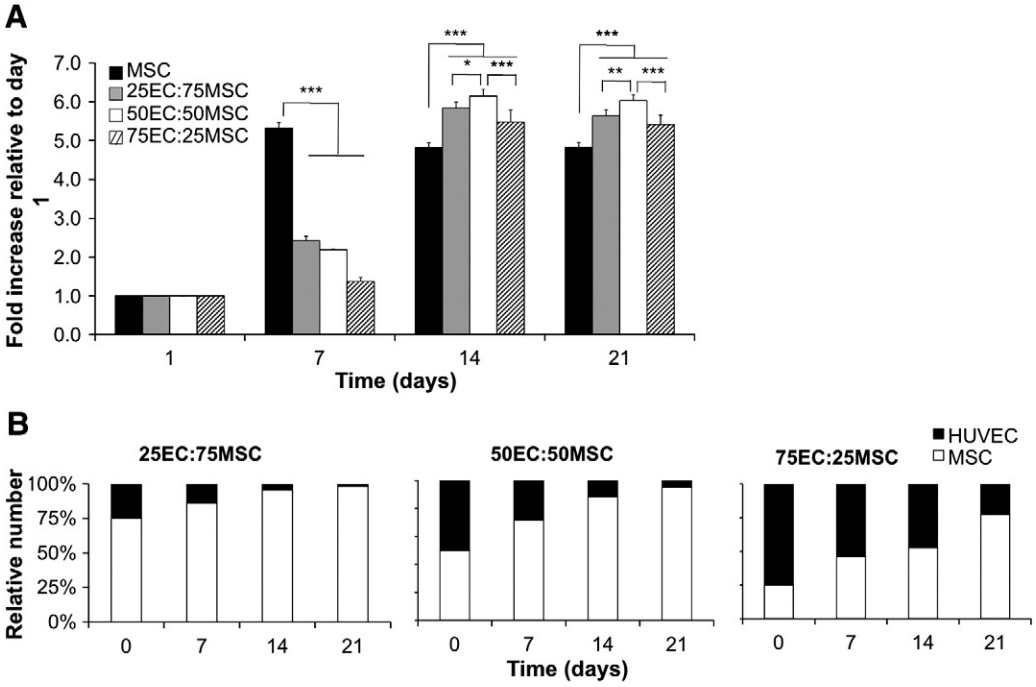

Figure 5 (A) Metabolic activity, relative to day 1, of mesenchymal stem cells (MSC) in monoculture or in coculture with human umbilical vein endothelial cells (HUVEC) and (B) the percentage of both cell types in the coculture during 21 days. Cocultures were tested using different relative cell ratios: 25% HUVEC with 75% MSC (25EC:75MSC), 50%HUVEC with 50% MSC (50EC:50MSC) and 75% HUVEC with 25% MSC (75EC:25MSC). Independently of the cell ratio, cocultures have also the same final density of $3x10^3$ cells/cm^2. Thus, the used percentage for each cell type in the coculture was related to this final value of $3x10^3$ cells/cm^2. Metabolic activity was assessed using the resazurin-based assay and relative quantification was obtained by flow cytometry. Data are expressed as mean ± standard deviation (n = 3).

the highest MSCs proliferation was the one that started with a lower percentage of MSCs (75%HUVEC-25%MSC). Moreover, in this ratio, HUVECs presented the lowest decline.

To analyze MSCs differentiation in the coculture systems, ALP activity was measured weekly for a 3 weeks period, as illustrated in Fig. 6. Under basal conditions (Fig. 6A), MSCs in monoculture kept a similar low value (practically null) throughout the time, without significant variations. However, in the presence of HUVECs, there was an increase on the ALP activity. Although the cocultures showed the highest values, only in the last week they reached values significantly higher than those obtained in monoculture. The ratios 25%HUVEC-75% MSC and 50%EC-50%MSC were the ones resulting in the highest ALP activity values. Under osteogenic conditions, MSCs monoculture showed a significant increase on ALP activity during the 1st week reaching then a plateau (Fig. 6B). In general, in the first 2 weeks of culture, the three relative ratios tested did not present significant ALP differences between them. In the last week there was a significant upregulation in ALP activity in all the cocultures, with values significantly higher than the MSCs monoculture.

When comparing osteogenic with basal conditions, ALP activity values were not very different but were in general slightly higher in the former. Additionally, all the ratios tested achieved significantly higher ALP activity values than the monoculture.

Cytochemical stainings of ALP activity and mineralization nodules are presented in Fig. 7, and provide additional qualitative evidence that cells differentiated along the osteoblastic lineages at all ratios tested.

Discussion

A large number of MSCs committed to the osteogenic lineage is necessary for creating a tissue-engineered implant able to fill bone defects. It is generally accepted that MSCs play a critical role in bone tissue regeneration due to their differentiation capacity into distinct end-stage cell types, immunosuppressive properties and ability to produce a broad spectrum of bioactive macromolecules that are capable of establishing a regenerative microenvironment (Turnovcova et al., 2009). In bone marrow, MSCs represent a very small fraction of all nucleated cells: less than 0.01% (Bartmann et al., 2007; Lee & Park, 2009).

As previously pointed out, this study focused on the use of a MSCs-endothelial cells coculture system as a strategy to modulate MSCs expansion and osteogenic differentiation *in vitro*. Our team is specifically interested in addressing this subject in the context of a minimally invasive bone regeneration strategy using injectable 3D matrices (Evangelista et al., 2007; Grellier et al., 2009c; Bidarra et al., 2010; Fonseca et al., 2011; Munarin et al., 2011). As reviewed by Grellier and colleagues (2009) there are some studies on the reciprocal regulation and functional relationship between bone and endothelial cells which, in turn, may be greatly influenced by the culture conditions (Grellier et al., 2009b).

In a preliminary phase of the present study, and due to its impact in future cell studies, the appropriate medium for the coculture system was established. It is worth mentioning that, due to the dramatic influence that the batch of serum may have on MSCs behavior at different levels (proliferation, differentiation and gene expression) (Shahdadfar et al., 2005), a previous serum batch-selection was made and it was used to supplement all the tested media. Media selection was based on cell metabolic activity, total protein content and optical microscopy analyzes (Fig. 1). The tested media included those routinely used to culture the two cell types in monoculture (DMEM and M199 for MSCs and HUVECs, respectively) and also IMDM, a medium reported in several works for expansion and further coculture of both cell types (Guillotin et al., 2004; Villars et al., 2002; Grellier et al., 2009a; Guillotin et al., 2008; Villars et al., 2000; Meury et al., 2006). In several other studies, the medium selected for the cocultures was the same one used for ECs monocultures (Finkenzeller et al., 2006; Stahl et al., 2004; Wenger et al., 2004; Fuchs et al., 2009a; Fuchs et al., 2007; Fuchs et al., 2009b; Santos et al., 2009; Stahl et al., 2005; Unger et al., 2007; McEwen et al., 2003). Here, the medium selected was a DMEM+M199 mixture (1:1). After one week of culture, the cell performance in the presence of this medium

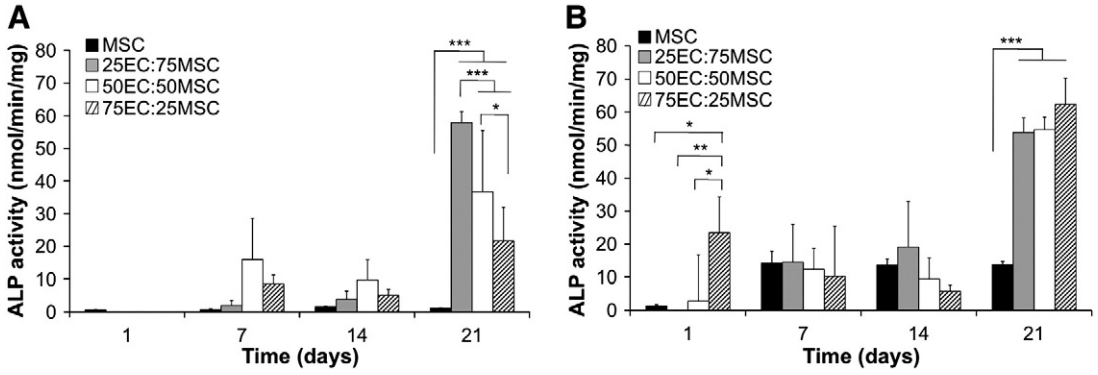

Figure 6 Alkaline phosphatase activity of mesenchymal stem cells (MSC) cultured alone or cocultured with human umbilical vein endothelial cells (HUVEC) at different relative cell ratios under (A) basal conditions and (B) osteogenic conditions. Results are expressed in nmol per min per mg of protein. Cocultures were tested at different relative cell ratios: 25% HUVEC with 75% MSC (25EC:75MSC), 50% HUVEC with 50% MSC (50EC:50MSC) and 75% HUVEC with 25% MSC (75EC:25MSC). Independently of the cell ratio cocultures were also prepared at a total density of $3x10^3$ cells/cm^2, and the percentage for each cell type was calculated with reference to that density. Data are expressed as mean ± standard deviation (n = 3). (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

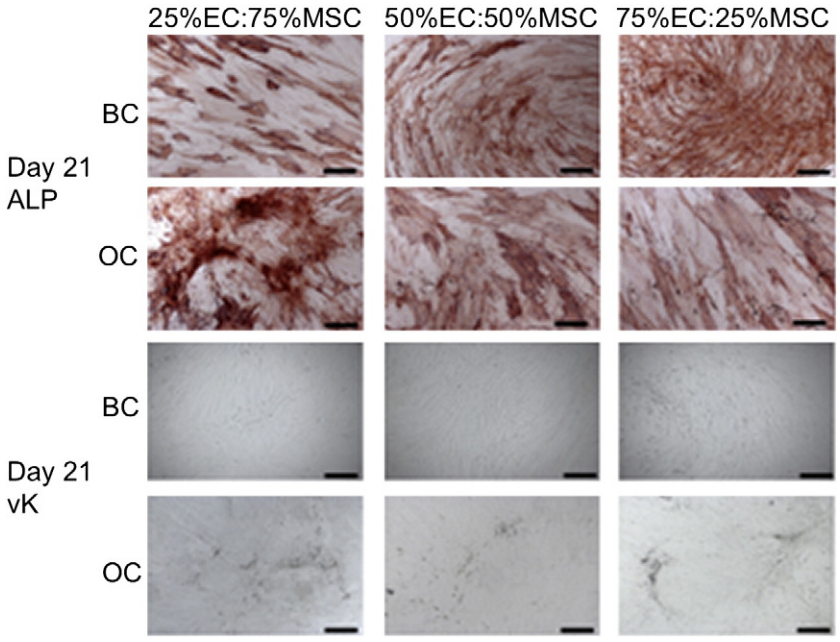

Figure 7 Alkaline phosphatase activity (ALP) and von Kossa (vK) histochemistry in cocultures after 21 days, under basal conditions (BC) and osteogenic conditions (OC). Cocultures were tested at different relative cell ratios: 25% HUVEC with 75% MSC (25EC:75MSC), 50% HUVEC with 50% MSC (50EC:50MSC) and 75% HUVEC with 25% MSC (75EC:25MSC). Independently of the cell ratio cocultures were also prepared at a total density of $3x10^3$ cells/cm^2, and the percentage for each cell type was calculated with reference to that density. Bars correspond to 200 μm.

was closer to the one obtained with DMEM and M199 for MSCs and HUVECs, respectively. Moreover, by choosing this mixed medium, cells in coculture will continue to be in contact with their expansion medium.

Several published studies on this topic provide insights into the relationship between the two cell types but there is still a limited knowledge concerning the proliferation behavior of cells in coculture. For a detailed analysis on cell proliferation in coculture, immunomagnetic beads coupled with an anti-CD31 antibody were used to separate both cell types. This technique allowed confirming that both cell types were proliferating, although with significantly different growth rates, being it higher for MSCs. This difference in cell proliferation could explain the different percentage of both cell types in coculture (Fig. 2). Moreover, MSCs growing in coculture (CoMSC) showed a clearly higher cell growth rate as compared with MSCs in monolayer. On one hand, this result points towards the potential of HUVECs to stimulate MSCs proliferation. On the other hand, MSCs could curtail ECs capability for expansion due to their high proliferative capacity in coculture. Notwithstanding that HUVECs in monoculture presented a higher growth rate than in coculture, CoHUVECs proliferated for a longer period than HUVECs alone. This ruled out the possibility that CoHUVECs may be dying since, in fact, there was an increases in cell number. Noteworthy, in monoculture when endothelial cells are maintained at confluence for an extended period of time, they become tightly packed but show no tendency to overlap or overgrow. Then, when cell density becomes to high, cells start to detach and die (Marin et al., 2001).

Another parameter that could influence cell proliferation was the extracellular matrix produced, since an increase on one of its major components was detected (upregulation of collagen type I). In parallel, a study was perform to validate that the significant increase in CoMSC proliferation was indeed related with HUVECs presence, and not with differences in terms of cell seeding densities (CoMSCs cell seeding density was 1500 cells/cm^2 instead of 3000 cells/cm^2 as the MSC monoculture). For that purpose MSCs were seeded alone at 1500 cells/cm^2, which allow comparing with CoMSC of the same real "age" (having gone through the same cell divisions) during the 21 days of culture (Fig. 2C). Although MSCs at lower cell density presented a higher proliferation rate in the first weak, in the following two weeks CoMSCs continued to exhibit the highest proliferation (Fig. 2C), corroborating the potential of HUVECs to stimulate MSCs proliferation.

It is well established that the cytoskeleton plays important roles in cell morphology, adhesion, growth and signaling. The actin network, one of the three components of the cytoskeleton, is of critical importance in the determination of the mechanical properties of living cells. In this study, it was shown that the actin cytoskeleton changes from an apparently well-organized structure, with long fine fibers running in parallel along the cell axis in MSCs monocultures, to a more random arrangement of the actin cytoskeleton in cocultures (Fig. 3A). Yourek and colleagues observed this type of cytoskeleton changes upon osteogenic differentiation (Yourek et al., 2007). They further stated that this reorganization could be related with the natural reaction of the actin cytoskeleton in bone cells to the shear stress that occurs *in vivo* during bone modeling/remodeling. Grellier et al. (2009a) also observed a similar cell rearrangement in osteoprogenitor cells (HOPs) cocultured with ECs (Grellier et al., 2009a). They further observed HUVECs migration along HOPs and suggested that the

direct contact between the two cell types could stimulate the release of chemotactic factors that, in turn, would stimulate HUVEC migration.

To support the hypothesis that cytoskeleton changes of CoMSC could be related with their osteogenic differentiation in the presence of endothelial cells, further studies were made. Consequently, ALP cytochemistry and activity, and also gene expression analyzes of osteogenic markers, were performed. ALP activity is considered an early osteogenic marker since its expression increases from the beginning of cell differentiation and increases throughout extracellular matrix maturation (Stein et al., 1990). As shown in Fig. 3B there was an increased in the number of ALP stained cells in coculture comparing to monoculture even after only 3 days, suggesting a commitment of CoMSCs into the osteogenic lineage. The ALP activity profiles and gene expression were assessed and both support the previous data (Figs. 3C and 4B). The gene profile of type I collagen, a protein of the extracellular matrix and the main organic component of bone tissue, was also higher in CoMSC (Fig. 4A). In order to further analyze osteogenic commitment of CoMSC, gene expression of two other early markers of osteogenic differentiation, Runx2 and bone sialoprotein (BSP), was evaluated by real time quantitative RT-PCR assay. Runx2, is an earlier transcription factor proven essential for commitment to osteoblatogenesis. BSP is a major noncollagenous extracellular matrix protein in bone and promotes the initial formation of mineral crystals (Ganss et al., 1999; Zhang et al., 2009). Both genes were significantly upregulated, which further confirms the differentiation of CoMSC along the osteoblastic lineage (Fig. 4C). Other gene that was investigated was osteocalcin (OCN) that is a noncollagenous calcium-binding bone protein and is considered a late-stage osteogenic differentiation marker (Ilmer et al., 2009). OCN expression did not increase and, even after 21 days, there was no upregulation. Overall, the genetic profile obtained points towards an osteogenic commitment of MSCs culture in the presence of ECs, but cells were not fully mature.

The effect of the relative HUVECs-MSCs ratio was also investigated. This aspect can be controversial since many different combinations were found in the literature: 4:1 (Santos et al., 2009), 2:1 (Guillotin et al., 2004; Villars et al., 2002; Grellier et al., 2009a; Guillotin et al., 2008; Grellier et al., 2009c; Clarkin et al., 2008b), 1:1 (Hofmann et al., 2008; Kaigler et al., 2005; Finkenzeller et al., 2006; Stahl et al., 2004; Wenger et al., 2004; Fuchs et al., 2009a; Fuchs et al., 2009b; Jones et al., 1995; Wang et al., 1997), 1:1.5 (Fuchs et al., 2007), 6.5:1 (Unger et al., 2007), 3:1 (Villars et al., 2000), 1.5:1 (Ito et al., 2000) of endothelial and osteoblast-like cells, respectively. The majority of these coculture studies have used higher amounts of endothelial cells than bone cells, although the medium composition was often different. Moreover, among these works only one mentions a preliminary cell ratio study to justify the selected ratio used (Unger et al., 2007). Although authors did not show any results, they stated that a ratio of EC: osteoblasts between 5:1 an 10:1 resulted in the presence of both cell types after 1 week of culture, whereas ratios of 1:1, 1:5 and 1:10 resulted in substantial reduction of EC number after the same period. In the present work, a relative cell ratio study was also performed, with an increasing on the relative amount of HUVECs: 25%HUVEC-75%MSC, 50%HUVEC-50%MSC and 75%HUVEC-25%MSC, to investigate how the different ratios could influence cell behavior. Regarding metabolic activity (Fig. 5A), monoculture reaches the maximum at day 7, which is probably related to a higher MSC proliferation. In the coculture, in the following 2 weeks there was a significant increase in metabolic activity, mostly related to a higher CoMSC proliferation, as observed earlier. This one-week difference may be related to the decrease in cell number of ECs in the coculture that was further compensated with CoMSC proliferation (Fig. 5B). The cell quantification assay provided additional information on the behavior of the two cell types at each cell ratio throughout the time (Fig. 5B). As expected, higher initial amounts of HUVECs led to a higher percentage of HUVECs in the coculture at day 21. Additionally, HUVECs relative number decreased throughout time, while MSCs increased using all the three relative cell ratios tested. In short time studies the initial cell seeding may not be essential, although for longer periods the cell ratio in the coculture needs to be carefully evaluated. Concerning ALP activity (Fig. 6A) no major differences were found between the different cell ratios until the last week, where the ratio with higher relative amounts of MSC presented higher ALP activity.

Additionally, cell studies were also performed in the presence of osteogenic media. This allowed assessing the *in vitro* mineralization of MSC in contact with ECs due to the presence of an inorganic phosphate source (beta-glycerophosphate). It was possible to observe mineral deposits in the cell culture of all the ratios (Fig. 7). Moreover, cells in osteogenic conditions showed some changes in cell morphology comparing to cells in basal conditions (Fig. 7). However, regarding ALP activity, no major differences were found between basal and osteogenic conditions in all ratios (Fig. 6).

Collectively, the present findings demonstrate that, by coculturing MSCs with HUVECs, there was not only an enhancement of osteogenic differentiation, but also a significant increase on MSCs proliferation. This augmentation in cell proliferation occurred in the presence of ECs, independently of relative cell ratios, but was favored by higher relative amounts of HUVECs. The main conclusions drawn from this work may be helpful for the development of a strategy for large-scale production of mesenchymal stem cells committed into the osteogenic lineage for tissue engineering applications, where a high amount of cells is required. Further studies are being carried out to assess the relevance of the present findings in an *in vivo* scenario. Previous results from our group provided a clear indication that ECs are also able to induce the commitment of MSCs into the osteogenic lineage *in vivo*. In fact, when both cell types were cocultured within a 3D matrix and then implanted in a rat femoral critical-sized defect, there was a significant increase in mineralization compared with the implantation of MSC alone (Grellier et al., 2009c). Additional studies are being carried out to get further insight into cocultured cell behavior *in vivo*, under different experimental conditions. In case of success, that knowledge would be useful for developing minimally invasive regenerative therapies using injectable 3D matrices as (multi)cell delivery systems (Evangelista et al., 2007; Grellier et al., 2009c; Bidarra et al., 2010; Fonseca et al., 2011).

This work constitutes a step forward in the pursuit for a better understanding of the interaction between these two cell lines, which can easily find application in several other tissue engineering approaches where vascularization is of prime importance. To our knowledge, the current paper showed for the first time that coculturing MSC with HUVECs

significantly increased MSCs proliferation, independently of the relative cell ratio.

Materials and methods

Cell cultures

Human mesenchymal stem cells from bone marrow (MSCs, Lonza) were cultured and expanded in MSCGM (MSC growth medium, Lonza). For the experiments, cells were used at passage 8 and cultured in basal medium: Dulbecco's modified Eagle medium with low glucose (DMEM, Gibco), supplemented with 10% v/v inactivated fetal bovine serum (FBS, Gibco) and 1% v/v penicillin/streptomycin (P/S, Gibco). The culture medium was changed every other day.

Human umbilical vein endothelial cells (HUVECs, ScienCell) were cultured in M199 medium (Gibco), supplemented with 10% of inactivated fetal bovine serum (FBS) (Gibco), 0.1 mg/mL of heparin (Sigma) and 0.03 mg/mL of endothelial cell growth supplement (ECGS) (Sigma-Aldrich). HUVECs were used until passage 8 and harvested on 0.1% w/v gelatin-coated tissue culture. The medium was renewed every 2 to 3 days until the cells reached confluence.

For cocultures, a pre-selection of the most adequate medium was performed. Several commercial media were tested, including Iscove Modified Dulbecco Medium (IMDM), M199, DMEM and a mixture 1:1 of these last two media. Cell behavior was analyzed in terms of metabolic activity and total protein content along the time.

After medium selection, MSC and HUVEC monocultures were seeded at a density of $3x10^3$ cells/cm^2 and cultured for 21 days. For cocultures both cell types were seeded at $1.5x10^3$ cells/ cm^2 (1:1), resulting in a final cell density of $3x10^3$ cells/cm^2. For all the studies the total initial cell density was of $3x10^3$ cells/cm^2. For control experiments MSCs monocultures were seeded with a lower cell density (1500 cells/cm^2, the same MSC density used in the cocultures).

Cell metabolic activity

To measure metabolic activity a resazurin-based assay was performed. This method is based on the conversion of resazurin to resorufin by metabolically active cells that result in the generation of a fluorescent product. At different time points the medium was removed from the wells and resazurin (Sigma) was added in fresh medium at a final concentration of 10% (v/v). Then, cells were incubated at 37 °C for 4 h, after which 200 μL/well were transferred to a 96 well black plate and fluorescence was measured (530 nm_{Ex}/590 nm_{Em}) in a Spectra Max Gemini XS (Molecular Devices).

Protein concentration

The bicinchoninic acid protein assay (BCA, Pierce) was used to detect and quantify the total protein content, as recommended by the manufacturer. Briefly, three samples of cell lysates for each condition, obtained by brief sonication in ice with 1% v/v Trition X-100, were incubated with the bicinchoninic acid (BCA) working reagent for 1 h at 37 °C. Then, the absorbance was read at 540 nm in a microplate reader (SLT Spectra).

Effect of relative cell ratios on MSC behavior

The effect of MSCs and HUVECs relative ratios on MSCs behavior was evaluated by testing the following cell combinations: 25%HUVEC-75%MSC, 50%HUVEC-50%MSC and 75%HUVEC-25%MSC. MSCs monocultures were used as a control and were seeded at a cell density of $3x10^3$ cells/cm^2. Independently of the cell ratio, cocultures were also prepared at the same total density of $3x10^3$ cells/cm^2, and the percentage for each cell type was calculated with reference to that density. The studies were carried on for 21 days.

To induce the formation of mineralized extracellular matrix, studies were performed in the presence of osteogenic stimuli (further on designated as osteogenic medium): basal medium (DMEM and M199 (1:1)) supplemented with 10 mM β-glycerophosphate (Sigma), 10^{-8} M dexamethasone (Sigma) and 50 μg/mL ascorbic acid (Fluka).

Cell separation and quantification

At different time points, cells were detached from the well plate and an aliquot of cell suspension was transferred to a Neubauer chamber for total cell counting.

To quantify and further perform gene analysis on each cell type after coculture, cells were separated by magnetic cell sorting using magnetic beads coupled with an antibody against CD31 (Invitrogen) (Grellier et al., 2009a). For this purpose, cells in coculture were harvested by trypsin and then incubated with anti-CD31 magnetic beads (at a ratio of 10:1 beads/HUVECs) for 30 min at 4 °C under gentle stirring. Afterwards, the supernatant fraction containing the cocultured MSCs (CoMSCs) was separated from the bead fraction, which contained the cocultured HUVECs (CoHUVECs), using a magnet device (Invitrogen).

Cell morphology

To analyze cell morphology, after fixation with 4% w/v paraformaldehyde and permeabilization with 0.1% v/v Triton X-100, cells were stained for F-actin (Alexa Fluor 488 phalloidin; Molecular Probes) and for nuclei (4',6-Diamidino-2-phenylindole dihydrochloride, DAPI; Sigma) and then visualized under an inverted fluorescence microscope (Axiovert 200 M, Zeiss).

Flow cytometry analysis

Flow cytometry studies were performed on MSCs monocultures and on MSCs/HUVECs cocultures at different cell ratios. The percentage of HUVECs in the cocultures was estimated using anti-human CD31 (PECAM-1) antibody labeled with FITC (BD Biosciences). Cell suspensions were stained for 15 min, and for each day and ratio, 10,000 events were analyzed in triplicate on a FACSCalibur flow cytometer (Becton Dickenson). Data processing was performed using FlowJo software 8.7.

Alkaline phosphatase (ALP) activity

For ALP cytochemistry cells were incubated in the dark for 1 h at 37 °C in Naphtol AS-MX phosphate/Fast Violet B salt

(Sigma), as recommended by the manufacturer. After being rinsed with deionized water and air-dried samples were observed under an inverted microscope (Axiovert 200 M, Zeiss).

For quantitative ALP analyses, cell lysates were obtained by treatment with 1% v/v Triton X-100 under brief sonication in ice, and then incubated with the chromogenic substrate 2 mM ρ−nitrophenol phosphate in 0.2 M bicarbonate buffer (pH 10), 0.05% v/v Triton X-100, and 4 mM $MgCl_2$ (Sigma) for 1 h at 37 °C. The reaction was stopped by adding 1 M NaOH and absorbance was read at 405 nm in a microplate reader (SLT Spectra). The amount of product was obtained from a p-nitrophenol standard curve. The enzymatic activity was normalized to total protein concentration, obtained using the bicinchoninic acid protein assay (BCA, Pierce), and expressed as nmol/min/mg protein.

Mineralization

For assessment of mineralization by von Kossa staining cells were fixed with 3.7% v/v formaldehyde in PBS during 15 min. Then, cells were incubated in 2.5 wt-% silver nitrate (Sigma) for 30 min under UV light, followed by incubation in 5 wt-% sodium thiosulfate (Aldrich) for 3 min. Finally, wells were rinsed with deionized water and air-dried prior to observation under an optical microscope.

Phenotype analyses through mRNA expression

Total RNA was extracted from MSCs in mono- and cocultures, at weekly time points, using the Total RNA isolation Kit (Macherey-Nagel), as recommended by the manufacturer. Subsequently, 1 μg of the total RNA was used as template for single strand cDNA synthesis with the Superscript pre-amplification system (Invitrogen) in a 20 μL final volume containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM MgCl2, 10 mM DTT, 0.5 mM of each dATP, dCTP, dGTP and dTTP, 0.5 μg oligo(dT)12-18, and 50 U of reverse transcriptase. After incubation at 42 °C for 50 min, the reaction was stopped at 70 °C for 15 min. After the cDNA synthesis reaction, qReal-Time PCR was carried out in a total volume of 25 μL of a mixture containing 5 μL of cDNA diluted at a 1:80 ratio (corresponding to 3.125 ng of total RNA), 200 nM of each forward and reverse primers and 1x Mesa Green qPCR MaterMix Plus for Sybr Assay (Eurogentec). Primers of ubiquitary ribosomic protein P0 forward: 5′ ATGCCCAGGGAAGACAGGGC 3′; reverse: 5′ CCATCAGCACCACAGCCTTC 3′; type I collagen (COL I) forward: 5′ GGA ATG AGG AGA CTG GCA ACC 3′ and reverse 5′ TCA GCA CCA CCG ATG TCC AAA 3′; alkaline phosphatase (ALP) forward 5′ AGC CCT TCA CTG CCA TCC TGT 3′; reverse 5′ ATT CTC TCG TTC ACC GCC CAC 3′; transcription factor Runx2 forward 5′ GTGCCTAGGCGCATTTCA 3′ and reverse 5′ GCTCTTCTTACTGAGAGTGGAAGG 3′; osteocalcin (OCN) forward 5′ TGAGAGCCCTCACACTCCTC 3′ and reverse 5′ ACCTTTGCTGGACTCTGCAC 3′; bone sialoprotein (BSP) forward 5′ CGATTTCCAGTTCAGGGCAGT 3′ and reverse 5′ CAACACTGGG CTATGGAGAG G 3′. qRT-PCR experiments were run using an iQ5 (Bio-Rad) and analyzed with the iCycler IQ™ software. Relative quantification of gene amplification by qRT-PCR was performed using the cycle threshold (C_t) values and relative expression levels were calculated as follows: 2^(C_t P0gene - C_t gene of interest). >The expression value for each target gene was normalized to the P0 value at each time point. Afterwards, the gene expression at each day was normalized to day 0. For each PCR, samples were analyzed in duplicate and three independent experiments were performed.

Statistical analyses

Statistical analyses were performed using SigmaStat (Statistical Software, STAT32 MFC Application). Statistical significance was assessed by using two way analysis of variance (ANOVA) in conjunction with Tukey's test, pairwase multiple comparison procedures. Differences were considerer statistically significant when p values were lower than 0.05. All data are presented as mean values ± standard deviation.

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