ESACT Proceedings

Nigel Jenkins
Niall Barron
Paula M. Alves *Editors*

ESACT

Proceedings of the 21st Annual Meeting of the European Society for Animal Cell Technology (ESACT) Dublin, Ireland, June 7-10, 2009

Editors
Prof. Nigel Jenkins
National Institute of Bioprocessing
Research & Training
University College Dublin
Engineering Building, Belfield
Dublin
Ireland
nigel.jenkins@nibrt.ie

Dr. Paula M. Alves
Instituto de Biologia Experimental
e Tecnológica (IBET)
ITQB
Oeiras
Portugal
marques@itqb.unl.pt

Niall Barron
Dublin City University
National Institute for Cellular
Biotechnology
Dublin
Ireland
niall.barron@dcu.ie

ISBN 978-94-007-0883-9
e-ISBN 978-94-007-0884-6
DOI 10.1007/978-94-007-0884-6
Springer Dordrecht Heidelberg London New York

Library of Congress Control Number: 2011933343

Impact of Growth Factors on the Proliferation of Ear Mesenchymal Stem Cells on Porous Microcarriers

Sébastien Sart, Yves-Jacques Schneider, and Spiros N. Agathos

Abstract Mesenchymal stem cells (MSCs) are emerging as an efficient tool for tissue engineering. Nevertheless, due to inefficient protocols for cell recovery, extensive ex-vivo expansion is required. MSCs are primary mechano-sensitive and anchorage dependent cells. Thus, the design of supporting scaffolds and the interactions with their microenvironment appear to be crucial for their efficient propagation in a bioreactor setting. In this work, we show that porous gelatin microcarriers (MC) are an efficient scaffold for MSC expansion. These supports, which contain multiple RGD sequences, allow an accurate spreading of these cells and their propagation in an undifferentiated state is as efficient as on conventional plastic culture dishes. Regarding the microenvironment for optimal cell growth, we observed that among the various standard culture process parameters, growth factor content of the culture medium was directly correlated with the apparent growth rate. Thus, the design of an efficient MSC culture process will require a close monitoring of these molecules, given their association with the percentage of cells in the S-phase of the cell cycle. These two components appear to be complementary for an efficient propagation of ear-derived MSCs (E-MSCs). We further discuss how controlling the bioreactor culture parameters should allow the modulation of the secretory profile of MSCs, thus enabling the generation of adult stem cells for specific therapeutic uses.

1 Introduction

The three main components believed to be essential for tissue regeneration are stem cells, an appropriate scaffold, and growth factors (Prescott et al. 2008). Recently, the use of adult stem cells has emerged as an efficient tool in cell therapy, and an alternative to the use of embryonic stem cells (ESCs). In this field, mesenchymal stem cells (MSCs) have shown high promise for the potential treatment of various diseases (Barry and Murphy 2004). MSCs are consensually defined on the basis of prospective characteristics shared by cells after in vitro culture on plastic dishes. These cells are plastic-adherent, express some non specific fibroblastic membrane

S. Sart (✉)
Université Catholique de Louvain, Institut des Sciences de la Vie, Unit of Bioengineering, Place Croix du Sud 2/19, 1348 Louvain-la-Neuve, Belgium
e-mail: sebastien.sart@gmail.com

N. Jenkins et al. (eds.), *Proceedings of the 21st Annual Meeting of the European Society for Animal Cell Technology (ESACT), Dublin, Ireland, June 7–10, 2009*, ESACT Proceedings 5, DOI 10.1007/978-94-007-0884-6_27,

markers (Sca-1, CD105, CD73, etc.) and are able to be differentiated at least into adipocytes, chondrocytes and osteoblasts (Dominici et al. 2006; Dominici et al. 2009). Nevertheless, due to the nonspecific nature of these markers and relatively low cell recovery, extensive in vitro MSC expansion is needed to obtain enough cells for therapeutic purposes (Sensebe and Bourin 2009).

Various bioreactor configurations have been proposed for the propagation of MSCs. Among them, perfusion bioreactors, microgravity-modeled vessels and stirred bioreactors (Godara et al. 2008) have been found suitable for the propagation of MSCs, especially for the homogenization of mass transport (Li et al. 2009). MSCs are anchorage dependent cells and need scaffolding prior to their incubation in a bioreactor setting. Various polymers have been proposed for MSC tissue engineering applications, including natural (cellulose, chitosan, gelatin, hyaluronic acid, etc.) or synthetic polymers (polyvinyl alcohol, polyethylene glycol, dextran, etc.). Various forms have been proposed including hydrogels, fibrous structures or macroporous supports (Marklein and Burdick 2010). This latter structure, which is analogous to that of microcarriers (MC) used for the propagation of animal cells in bioreactors, appears to be particularly relevant for the expansion of MSCs in a homogeneous agitated environment.

The prerequisite for efficient cell propagation is the formation of focal adhesion complexes that allow an efficient focal adhesion kinase (FAK) autophosphorylation, and a subsequent transcription of cyclin D1, a key regulator of cycle progression and thus growth of the cells (Cox et al. 2006). Focal adhesion also prevents cells from anoikis (Reddig and Juliano 2005). Furthermore, the focal adhesion complexes play a key role in the sensing of the microenvironment. First, the density of adhesion molecules (e.g. RGD sequences) regulates the fate (Connelly et al. 2008) and the proliferation of the cells (Schofer et al. 2009). RhoA phosphorylation (downstream from FAK) and subsequent stress fiber expression is required for osteogenesis (Connelly et al. 2008), whereas RhoA dephosphorylation involves cytoskeleton disruption and promotes adipogenesis (McBeath et al. 2004). Chondrogenesis is inversely dependent on the concentration of adhesion molecules but requires RhoA phosphorylation (Connelly et al. 2008). Substrate rigidity involves modulation of Ca^{2+} signaling, that upregulates phosphorylation of RhoA GTPase (Kim et al. 2009). Moreover, mechanical signals associated with three-dimensional culture are directly sensed by these focal adhesion complexes. First, shear induced by fluid flow is directly associated with a RhoA activation and a subsequent activation of the OSE2 promotor that regulates RUNX2, a master gene governing the osteogenic phenotype (Arnsdorf et al. 2009). Further, mechanical stress appears to regulate chondrogenesis (McBride et al. 2008), putatively through Rac-1 activation (Woods et al. 2007), another Rho GTPase regulated by mechanical stress (Laboureau et al. 2004). Microgravity, on the other hand, inhibits proliferation of human MSCs (hMSCs), represses their osteogenic differentiation and increases their adipogenic differentiation (Dai et al. 2007) through a FAK/RhoA inactivation and a cytoskeletal disruption (Meyers et al. 2005). Under microgravity, NOS2 expression in hMSCs was found to increase as a direct consequence of a RhoA activity, and NO has been shown to negatively impact osteoblast survival (Zayzafoon et al. 2005). Thus for

an efficient propagation of MSCs in an undifferentiated state, cells must be prevented from exposure to shear stress under normal gravity. The design of the MC system must be porous (Shiragami et al. 1993) to shield cells from shear stress, and must incorporate adhesion molecules at a high concentration for accurate adhesion and proliferation. On the other hand culture modes involving a perfusion bioreactor (King and Miller, 2007) or cyclic fed-batch culture (Schop et al. 2008) have been shown to provide a favorable environment for the optimized propagation of MSCs. To design efficient processes of proliferation of MSCs in a bioreactor, it is clear that a better understanding of MSC metabolism is needed. According to Schop et al. (2008, 2009), glucose and glutamine consumption rates of MSCs appear to be low, even at low medium concentrations of these basic nutrients. Moreover, it has been shown that concentration of glucose (either high or low) did not affect proliferation, differentiation potential or trophic factor secretion of human bone-marrow MSCs (BM-MSCs) (Li et al. 2007; Pal et al. 2009; Weil et al. 2009). In addition, trying to infer the growth rate of the cells from glucose consumption presented large discrepancies (Higuera et al. 2009). Hence the content of the medium in basic nutrients does not seem to play a critical role in optimized expansion of MSCs. Thus, we tested whether growth factor content in the medium might play a major role in the proliferation of MSCs in stirred bioreactor cultures. For this we used an alternative model of MSCs extracted from the outer ear (E-MSCs). Such a cell population, which displayed all the in vitro features of MSCs, had been previously shown to be an efficient model for MSC investigations in a bioreactor setting due to their high growth rate compared to BM-MSCs (Sart et al. 2009). As an appropriate support fulfilling the above-mentioned considerations, we used Cultispher-S, a gelatin based MC, in itself a 3D porous structure containing multiple copies of RGD sequences (Rohanizadeh et al. 2008).

2 Materials and Methods

2.1 Primary Ear Mesenchymal Stem Cell Culture

E-MSCs were extracted as previously described (Sart et al. 2009). Briefly, external ears of Wistar rats (*Rattus norvegicus*) were cut and minced. The tissue was digested using a 40 mg/mL collagenase type II (Sigma-Aldrich) solution in DMEM (Lonza) plus 10 % Fetal Bovine Serum (Hyclone). E-MSCs were selected by the plastic anchorage methodology (Gawronska-Kozak, 2004), and cultivated in DMEM plus 10 % FBS (referred to as expansion medium).

2.2 E-MSC Characterization

E-MSCs were characterized by their expression of MSC markers, on the basis of semiquantitative RT-PCR analysis and FACS analysis. Notch-1, Sca-1 and CD73

RNA expression was quantified on passage 0 (P0) E-MSCs according to the methodology described by Sart et al. (2009). Primer sequences are presented in Table 1. FACS analysis on Sca-1 and CD 73 expression was performed as previously described (Sart et al. 2009).

2.3 E-MSC Microcarrier Culture

Cultispher-S (Percell-Biolytica) beads were prepared and cells were seeded as described previously (Sart et al. 2009). Briefly, in a 250-mL spinner flask (ABS), E-MSCs were seeded on these MC using intermittent stirring (3 min agitated at 70 rpm, 30 min static) for 4 h in 50 mL of expansion medium (Sart et al. 2009). After the seeding period, the medium volume was adjusted to 100 mL in a continuous stirring mode (70 rpm).

To count the cells on Cultispher-S carriers, a protocol was adapted from Percell-Biolytica's procedure (Sart et al. 2009). Briefly, a 500 μL aliquot of the culture was harvested. MC were allowed to settle. After washing in PBS and digestion with a trypsin 10X solution (PAA), cells were collected by centrifugation and counted by trypan blue exclusion.

Batch culture was carried out in expansion medium, without medium change during the whole period of cultivation. Cyclic fed-batch culture was carried out in expansion medium and fifty percent of the culture volume was changed every two days. Pulsed culture was carried out as in cyclic fed-batch mode, but for the second medium change, a pulse of serum (Hyclone) plus TGFβ-1 (Sigma) was added: on day 4 of cultivation half of the medium was replaced by a solution of 80% serum plus 2 ng/mL TGFβ1, resulting in a final content of 40% serum and 1 ng/ml TGFβ1. Alternatively, a pulse of FGF2 (Sigma) was also tested, following the same culture conditions: on day 4 half of the medium volume was replaced by a 20 ng/mL FGF2 solution in expansion medium.

Bead-to-bead transfer was carried out as described in Sart et al. (2009): when cells reached confluence, fresh beads were added at a ratio of 1:1. The culture was pursued in expansion medium with intermittent stirring for 4 h (as described above for cell seeding), and subsequently continuous stirring was imposed (70 rpm) until the end of the cultivation.

2.4 Cell Cycle Analysis

Homogeneous samples of 500 μL of culture (beads and cells) were withdrawn from the spinner flask and allowed to settle. Medium was removed and MC were washed with PBS. Next, the PBS solution was replaced by a 10X trypsin solution (PPA), and the beads were digested under gentle agitation.

Cells were recovered by centrifugation, and resuspended in 1 mL PBS solution. Cells were fixed and permeabilized with a 70% ethanol solution at 4°C for 40 min.

Cells were then centrifuged and the pellet was resuspended in a solution containing 20 μg/mL propidium iodide (Sigma-Aldrich) plus 0.5 mg/mL RNAse A (Sigma-Aldrich). Cells were incubated at 37°C for 1 h. Finally, cells were washed in PBS and analyzed using a FACScallibur flow cytometer (BD) and the results were treated using CellQuest software.

2.5 E-MSC Differentiation Potential

2.5.1 Adipogenic Differentiation

The protocol used followed the one of Wang et al. (2006). Briefly, 5×10^6 E-MSC were seeded in a T-75 flask and cultivated in expansion medium until almost confluence was reached. Then expansion medium was switched to a serum-free medium containing DMEM supplemented with a 1X ITS+1 (Sigma) solution and 5 μM ciglitazone (Sigma). Cells were analyzed by Oil Red O staining after a 7-day culture period.

2.5.2 Osteogenic Differentiation

The protocol was adapted from Jaiswal et al. (1996). Briefly, 5×10^6 E-MSC were seeded in a T-75 flask and cultivated in expansion medium until almost confluence was reached. The day after, the medium was switched to differentiation medium consisting of expansion medium plus 50 mM L-ascorbic acid and 100-nM dexamethasone. Medium was changed every two days. On day 11, 2 mM ß-glycerol phosphate was also added, and this amended medium was subsequently changed every 2 days until day 21. Cells expressing alkaline phosphatase were stained using the L83-R5 kit (Sigma-Aldrich), following the manufacturer's procedure (Sart et al. 2009).

2.5.3 Chondrogenic Differentiation

Chondrogenic differentiation was performed as described in Barbero et al. (2003). Briefly, 1×10^6 cells/mL were seeded as a pellet culture, and expansion medium was switched to a differentiation medium, containing DMEM plus ITS+1 (Sigma), 0.1 mM ascorbic acid-2-phosphate, 10^{-7} M dexamethasone, and 10 ng/mL of TGFβ1. Cells were cultivated under these conditions for two weeks. Pellets of cells differentiated into chondrocytes were dehydrated and embedded into paraffin using standard histology protocols. Aggregates were minced down to 7 μm and stained using Alcian Blue (pH 1). With this procedure, the proteoglycans of cartilage differentiated cells were stained (Sart et al. 2009).

3 Results

3.1 E-MSC Characterization

Figure 1a shows that P0 E-MSCs expressed Notch-1 at a high rate. This marker was found to be associated with a progenitor state in articular cartilage cell populations (Hiraoka et al. 2006; Ustunel et al. 2008), and also the Notch-1 positive cells manifest the ability to form colonies (Dowthwaite et al. 2004). Sca-1 and CD73 were found to be expressed in our E-MSCs population. Sca-1 and CD73 expression levels using RT-PCR tended to correlate with the data obtained by FACS analysis showing that respectively 30 and 70% of the cells were positive for these markers (Fig. 1b). These two markers were previously shown to be expressed in bone-marrow and adipose-tissue derived MSCs (Gawronska-Kozak et al. 2007). Moreover, according to Fig. 1c, P0 E-MSCs displayed differentiation potential along the adipogenic, osteogenic and chondrogenic pathways. Thus, these data provide evidence that our ear-derived cells harbored a progenitor state in vitro, with features and differentiation potential of MSCs.

3.2 Influence of the Mode of Culture on E-MSC Growth Extent

Figure 2a, b shows that batch culture attained limited cell growth rate and extent, whereas cyclic fed-batch led to higher cell growth extent and the pulse culture further enhanced this effect (Fig. 2b). In either condition, the same bead concentration was used; therefore clearly the surface area available for cells was never a limiting culture parameter.

In batch culture, cell growth stopped on day 4 (Fig. 2a). Compared to cyclic fed-batch, we noticed a reduced growth rate in this culture mode ($3.79 \times 10^{-2} \pm 2 \times 10^{-3}$ h^{-1} versus $1.8 \times 10^{-2} \pm 1.16 \times 10^{-3}$ h^{-1}). In any case, basic nutrients were not limiting (data not shown), since the overall change in glucose and glutamine concentrations, over the entire culture period, never reached the limit imposed by the expansion medium content of these nutrients (e.g., consumption $\Delta_{glucose}$ Cyclic fed-batch $= 6.98 \times 10^{-1} \pm 1.14 \times 10^{-1}$ g/L; consumption $\Delta_{glucose}$ batch $= 1.65 \pm 4.86 \times 10^{-1}$ g/L).

In Fig. 2b, we observe that in cyclic fed-batch and during the first period of the pulsed cyclic fed-batch culture, growth rate was practically identical ($1.85 \times 10^{-2} \pm 4.96 \times 10^{-3}$ h^{-1}), as expected. However, in the period of pulse that ensued, this growth rate was maintained, whereas growth rate of the simple cyclic fed-batch dropped ($1.9 \times 10^{-2} \pm 3.32 \times 10^{-3}$ h^{-1} versus $9.70 \times 10^{-3} \pm 3.46 \times 10^{-3}$ h^{-1}).

According to Fig. 3, an increase in the growth factor content of the medium using either a pulse of serum plus TGFβ1 or a pulse of FGF2 was followed by an increased final cell density compared to standard cyclic fed-batch (an 1.82 ± 0.16 and 1.68 ± 0.05 fold increase respectively). Thus, clearly, DMEM plus 10% serum appears to be insufficient to provide enough growth factor content leading to high extent of growth. A similar effect was obtained by using bead-to-bead transfer (2.27

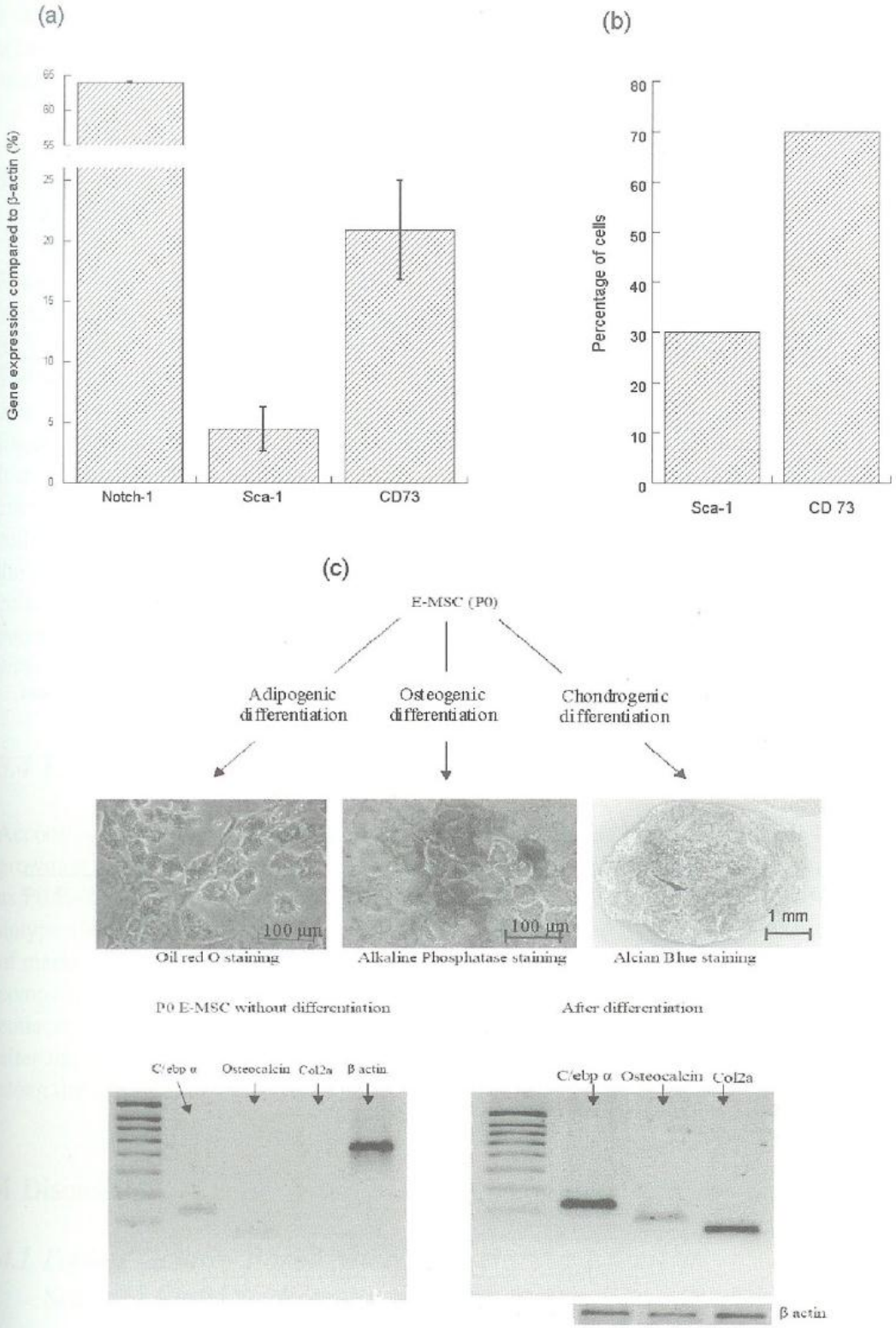

Fig. 1 Ear mesenchymal stem cell characterization : marker expression (RT-PCR analysis) (**a**), FACS analysis (**b**), differentiation potential (**c**)

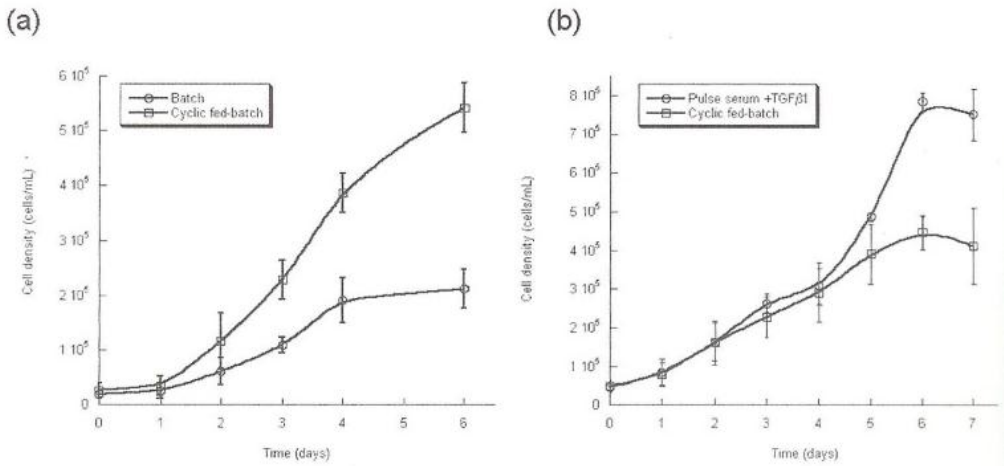

Fig. 2 Effect of mode of culture on E-MSC proliferation : batch versus cyclic fed-batch (**a**), pulsed serum plus TGFβ1 versus cyclic fed-batch (**b**)

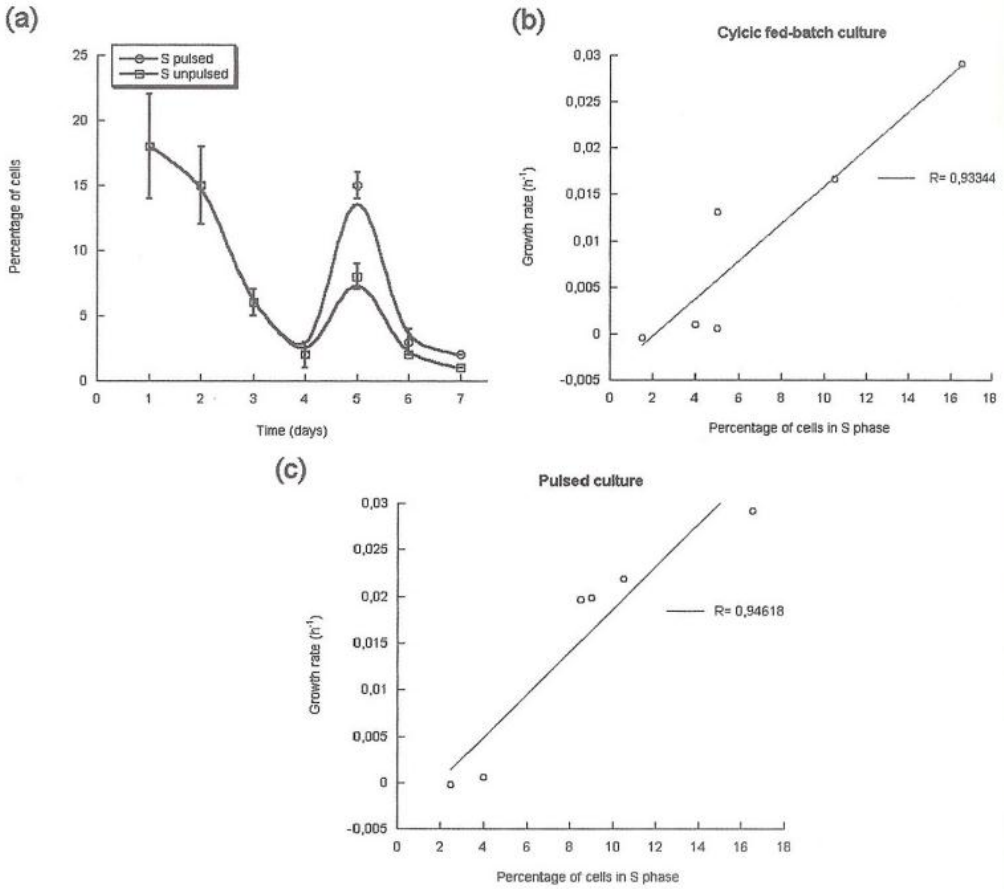

Fig. 3 Cell cycle distribution in S-phase of E-MSCs during cyclic fed-batch and pulsed culture (**a**) and correlation between growth rate and percentage of cells in S-phase (**b**)

±0.08 fold increase in final cell density compared to cyclic fed-batch), but this approach was considerably less efficient, because an extensive culture period was needed (18 days) to reach similar growth extent.

3.3 Influence of the Mode of Culture on E-MSC Cell Cycle Behaviour

Figure 3a shows that in standard cyclic fed-batch culture, the percentage of E-MSCs in the S-phase of the cell cycle declined gradually after the first medium change (day 2). On day 5, after the second medium change, the percentage of E-MSCs in S-phase increased four-fold (from 2 to 8%), and this percentage dropped gradually until the end of the culture. This suggests that the higher final cell density obtained for cyclic fed-batch culture could be related to the additional amount of growth factors provided by the serum of the new fed-batch cycle. To further enhance this effect, we added the pulse of serum together with TGFβ1. According to Fig. 3a, cells followed the same profile, but the pulsing of serum plus TGFβ1 led to double the number of cells in S-phase on day 5 (15%) compared to standard cyclic fed-batch culture (8%). Moreover, the apparent growth rate appears to be correlated with the percentage of cells in S-phase (Fig. 3b, c). Thus, the apparent growth rate is linked with the amount of growth factors provided in the culture medium (Fig. 3a).

3.4 E-MSC Fate After a Pulsed Culture

According to Fig. 4a, after a pulsed culture using 40% FBS plus TGFβ1, E-MSCs grown on gelatin MC displayed at the transcriptional level the same MSC markers as P0 E-MSCs propagated on plastic dishes. Thus, E-MSCs maintained their phenotype after a pulsed culture. Moreover, when we analyzed the profile of expression of marker genes of differentiation on the E-MSCs after the same pulsed culture in comparison with the expression profile of P0 E-MSCs (Fig. 4b), spontaneous differentiation was not much higher than the control cells grown on plastic dishes. Finally, after the above pulsed culture, E-MSCs maintained their differentiation potential along the adipogenic, osteogenic and chondrogenic pathways (Fig. 4c).

4 Discussion

4.1 Porous Gelatin Based Microcarriers: An Efficient Scaffold for MSC Propagation

Porous gelatin MC turned out to be a suitable scaffold for efficient MSC propagation. First, we observed essentially the same efficiency in the propagation of E-MSCs on the Cultispher-S beads compared to plastic dishes (Sart et al. 2009).

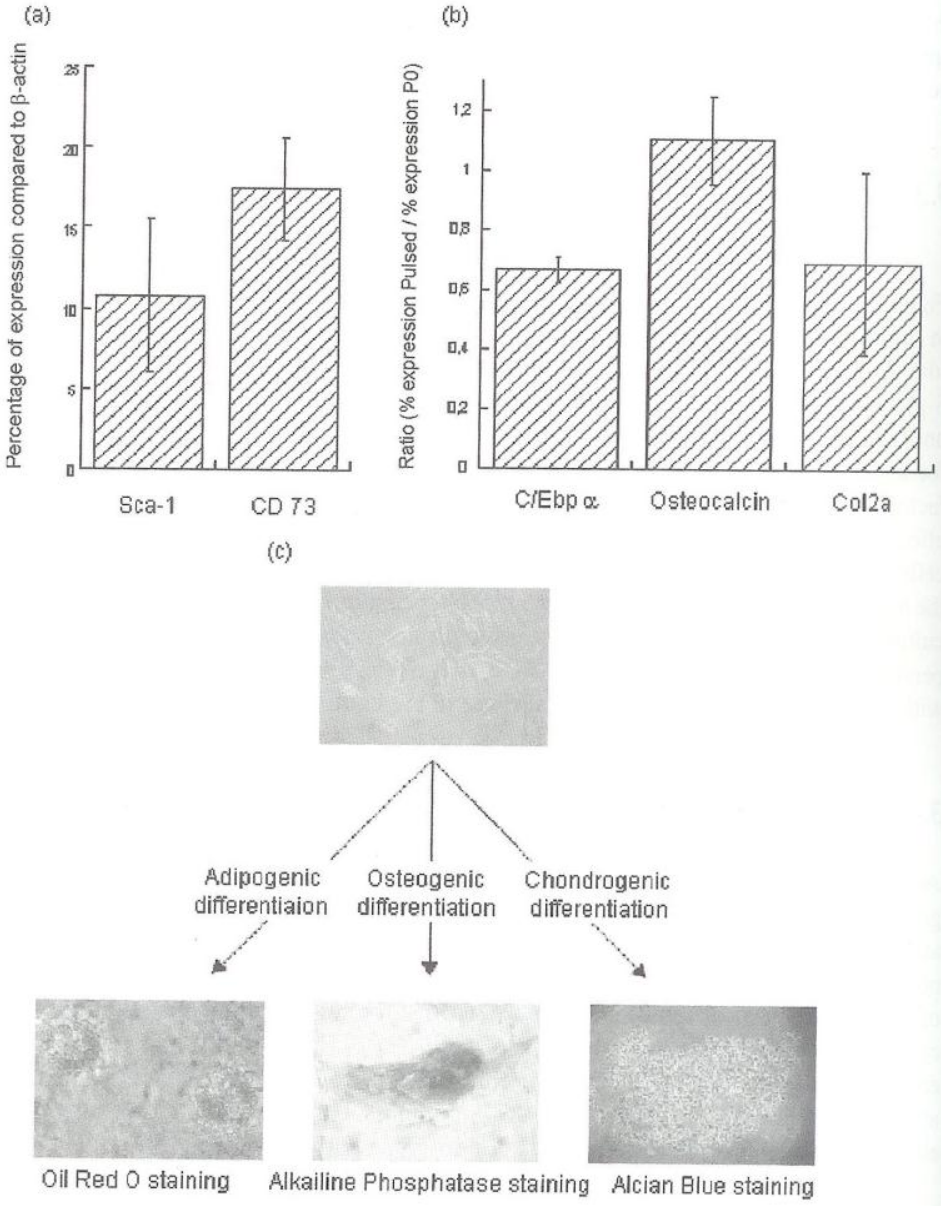

Fig. 4 Fate of E-MSCs after a pulsed culture : marker expression (RT-PCR analysis) (**a**), spontaneous expression of markers of differentiation (**b**), differentiation potential (**c**)

Secondly, according to our data, after submitting the E-MSCs to the optimized culture mode (pulsed serum and growth factors), we did not observe any upregulation in osteocalcin expression compared to the cells' propagation on plastic dishes. This suggests that Cultispher-S supports may be protecting E-MSCs from shear stress, and that presumably this mode of cultivation did not lead to an excess

in adhesion molecules. Thirdly, we observed a slight downregulation of C/ebp α expression. This is consistent with the hypothesis that there was no cytoskeleton disruption when using Cultispher-S, and the spreading of the cells appeared more efficient compared to their propagation on plastic dishes. Finally, we did not observe any upregulation of col2a expression, even when we added TGFβ1 in the culture medium. This suggests that the proper density of adhesion molecules on the carriers helped to prevent chondrogenic commitment.

Moreover, this type of macroporous gelatin-based support has been shown to be suitable for in vivo applications, eliciting an inflammatory reaction and able to be degraded over time, and providing efficient scaffolding for MSC usage in bone tissue engineering (Lippens et al. 2010). Various other kinds of carriers have been tested for the propagation of MSCs in a bioreactor setting. We (Sart et al. 2007) and others (Yang et al. 2007) have shown that there was no increase in BM-MSC and E-MSC numbers using Cytodex-1 or Cytopore-1 and -2 MC cultures. Others (Schop et al. 2008) have also noticed a poor spreading of BM-MSCs on Cytodex-1. This appears to suggest that Cytodex-1 and Cytopore beads, which consist, respectively, of a dextran matrix with substituted N,N-dimethylaminoethyl groups or of a cellulose matrix (Malda and Frondoza, 2006) and neither of them contains intrinsically any adhesion-facilitating chemical structures, do not adsorb enough adhesion molecules for an efficient spreading and growth of MSCs. Other MC materials may be equally problematic. For instance, we (data not shown) and others (Amaral et al. 2005) have shown that chitosan was not conducive to an efficient propagation of E-MSCs or BM-MSCs. As reported by Ragetly et al. (2010), low cell adhesion on chitosan is directly associated with its chemical structure, and, therefore, surface modification of chitosan via crosslinking or by other interventions leading to improved adsorption of serum molecules would be necessary to promote cell attachment to chitosan via indirect cell-matrix interactions. Other molecules (e.g. PLGA) have also shown the need of an association with RGD tripeptide structures for efficient propagation of MSCs (Schofer et al. 2009).

4.2 Considerations on MSC Mode of Culture

The cell cycle distribution of E-MSCs in S-phase appeared to gradually decrease over the culture period (Fig. 3a). Medium exchange had a tendency to restore the percentage of cells in S-phase while pulsing of serum plus growth factors reinforced this effect.

The apparent growth rate was found to be closely correlated with the percentage of cells in S-phase, and this percentage is known to be proportional to the fraction of growth factor-receptor complexes (Lauffenburger and Cozens, 1989). Thus, there is a clear correlation of growth rate with the amount of growth factor(s) present in the culture medium. It is also important to notice that during the pulsed culture period, basal medium (DMEM) was diluted by a factor of 2.5. Thus, the observed beneficial effects of pulsing was not associated with the basic nutrients of the medium, i.e. glucose and glutamine. This is also clearly apparent when the

specific consumption rate of these nutrients is measured in batch and cyclic fed-batch culture. This seems to explain the difficulty to correlate cell growth of MSCs with their glucose consumption (Higuera et al. 2009).

Thus, the design of an efficient culture process for MSCs must take into account that the growth factor content in the culture medium is not constant (Zi and Klipp 2007). A variable or decreasing concentration of growth factors may impair cell growth rate, but its value can be indirectly assessed by measuring the proportion of cells in S-phase.

In this connection, initiation of the S-phase and DNA synthesis require a threshold level of growth factor-receptor complexes. It is important to note that the number of receptors on individual cells can vary up to 1000-fold within a cell population, so that individual cells require different times to reach the threshold value of receptor occupation enabling cellular activation. This unsynchronized start of the proliferative response leads to markedly different growth rates within a cell population (Despa and Despa, 1997). Since in a single cell population the threshold varies considerably, it is not surprising that various MSC populations from different tissues have been reported to display intrinsically different proliferation profiles (Peng et al. 2008).

Therefore, it is becoming progressively clear that, at least in this respect, MSCs are not a tissue-independent universal cell population. Indeed, the gene expression profile of MSCs reflects their tissue of origin, and their cytokine expression may differ, indicating that MSCs originating from different tissues may be suited for specific clinical applications (Horwitz and Prather 2009). Thus, the specific requirements for the efficient propagation of a particular adult stem cell population must be carefully investigated.

5 Perspectives

Optimized bioreactor expansion of MSCs is achievable by maintaining their key in vitro features, particularly in terms of in vitro differentiation potential. This unhampered expansion in the undifferentiated state should be attainable by using appropriate scaffolds and growth factor feeding. As mentioned by Chiquet et al. (2009), there is a cooperation between growth factor and integrin signaling in a "context-dependent signaling". Thus, there appears to be an interplay between integrins and growth factors. Integrin/receptor kinase complexes amplify the signaling of growth factors to MAPK and other pathways involved in cell growth and differentiation. Moreover, physical and functional interactions of shared components allow the crosstalk between growth factor- and integrin-triggered signaling pathways. Because integrins are involved in interactions with the extracellular matrix (ECM) and are thought to be associated with the mecano-sensitivity of MSCs, the association of integrins with growth factor receptors at matrix adhesion contacts allows cells to integrate mechanical with growth factor derived signals, resulting in context-dependent cellular phenomena.

Until recently the therapeutic utilization of MSCs had been thought to be associated with these cells' differentiation and recolonization abilities. Nevertheless, emerging data have characterized their engraftment in vivo as low or even completely absent (Horwitz and Dominici 2008). In fact, the presumed self-renewal of MSCs has not been properly characterized nor has it been convincingly proven in vitro or in vivo (Dominici et al. 2009). MSCs are hypo-immunogenic cells: they harbor low expression of MHC I and no MHC II, even after differentiation into adipocytes, chondrocytes and osteobasts. They have also been shown to not express any of the co-stimulatory molecules CD40, CD80, and CD86. Moreover, MSCs as well as their progeny have been shown to inhibit the proliferation of allogeneic lymphocytes, B cells, natural killer (NK) cells and dendritic cells. The relevant mechanism(s) are not completely understood but may involve secretion of soluble factors, including IL-4 and IL-10 (Chen and Tuan 2008). Additionally, MSCs secrete trophic factors that stimulate the mitosis of local progenitor cells and they appear to be involved in neo-angiogenesis (Caplan 2009; da Silva Meirelles et al. 2008). A part of these trophic factors have been shown to be modulated by culture conditions. In normal adherent culture on plastic dishes MSCs express several bioactive factors, including G-CSF, M-CSF, LIF, IL-6, IL-11 and SCF (Caplan 2009). When the culture conditions are shifted, e.g., upon stromagenesis (incubating cells with IL-α), MSCs modify their trophic factor expression by increasing the secretion of G-CSF, M-CSF, LIF, IL-6, IL-11 and SCF (Caplan 2009). During osteogenesis (incubating cells with dexamethasone), MSCs express at least G-CSF, M-CSF and SCF (Caplan 2009). Shear stress is another parameter found to be a potent regulator of the expression of von Willebrand factor, VEGF and FGF-1 (Park et al. 2007; Wang et al. 2005). In addition, hypoxia modulates the paracrine activity of MSCs, causing upregulation of various secretable factors, among which are important angiogenic molecules such as VEGF and IL-6 (Das et al. 2009). Use of composite scaffolds made of hydroxyapatite and polylactic acid has been recently shown to upregulate VEGF expression (He et al. 2010). Finally, cyclic tensile strain has been shown to downregulate IL-6 expression while increasing IL-8 expression in MSCs (Sumanasinghe et al. 2009). Consequently, the culture process (encompassing the continuous liquid phase, the solid phase on which the MSCs adhere and the forces exerted in their microenvironment) can modify the secretory profile of MSCs, leading to the generation of adult stem cells with unique cytokine expression profiles. In conclusion, cell processing should enable biochemical engineers to optimize the cytokine profile of MSCs for a given clinical application (Horwitz and Prather 2009).

References

Amaral, I.F., M. Lamghari, S.R. Sousa, P. Sampaio, and M.A. Barbosa. 2005. Rat bone marrow stromal cell osteogenic differentiation and fibronectin adsorption on chitosan membranes: the effect of the degree of acetylation. *J Biomed Mater Res A* 75:387–397.

Arnsdorf, E.J., P. Tummala, R.Y. Kwon, and C.R. Jacobs. 2009. Mechanically induced osteogenic differentiation – the role of RhoA, ROCKII and cytoskeletal dynamics. *J Cell Sci* 122:546–553.

Barbero, A., S. Ploegert, M. Heberer, and I. Martin. 2003. Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. *Arthritis Rheum* 48:1315–1325.

Barry, F.P., and J.M. Murphy. 2004. Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* 36:568–584.

Caplan, A.I. 2009. Why are MSCs therapeutic? New data: new insight. *J Pathol* 217:318–324.

Chen, F.H., and R.S. Tuan. 2008. Mesenchymal stem cells in arthritic diseases. *Arthritis Res Ther* 10:223–235

Chiquet, M., L. Gelman, R. Lutz, and S. Maier. 2009. From mechanotransduction to extracellular matrix gene expression in fibroblasts. *Biochim Biophys Acta* 1793:911–920.

Connelly, J.T., A.J. Garcia, and M.E. Levenston. 2008. Interactions between integrin ligand density and cytoskeletal integrity regulate BMSC chondrogenesis. *J Cell Physiol* 217:145–154.

Cox, B.D., M. Natarajan, M.R. Stettner, and C.L. Gladson. 2006. New concepts regarding focal adhesion kinase promotion of cell migration and proliferation. *J Cell Biochem* 99: 35–52.

da Silva Meirelles, L., A.I. Caplan, and N.B. Nardi. 2008. In search of the *in vivo* identity of mesenchymal stem cells. *Stem Cells* 26:2287–2299.

Dai, Z.Q., R. Wang, S.K. Ling, Y.M. Wan, and Y.H. Li. 2007. Simulated microgravity inhibits the proliferation and osteogenesis of rat bone marrow mesenchymal stem cells. *Cell Prolif* 40:671–684.

Das, R., H. Jahr, G. van Osch, and E. Farrell. 2010. The role of hypoxia in MSCs: Considerations for regenerative medicine approaches. *Tissue Eng Part B Rev.* 16:159–168.

Despa, S.I., and F. Despa. 1997. Diffusion model for growth factors – cell receptors interaction. *Biosystems* 44:59–68.

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

Dominici, M., P. Paolucci, P. Conte, and E.M. Horwitz. 2009. Heterogeneity of multipotent mesenchymal stromal cells: from stromal cells to stem cells and vice versa. *Transplantation* 87:S36–S42.

Dowthwaite, G.P., J.C. Bishop, S.N. Redman, I.M. Khan, P. Rooney, D.J. Evans, L. Haughton, Z. Bayram, S. Boyer, B. Thomson, M.S. Wolfe, and C.W. Archer. 2004. The surface of articular cartilage contains a progenitor cell population. *J Cell Sci* 117:889–897.

Gawronska-Kozak, B. 2004. Regeneration in the ears of immunodeficient mice: identification and lineage analysis of mesenchymal stem cells. *Tissue Eng* 10:1251–1265.

Gawronska-Kozak, B., J.A. Manuel, and V. Prpic. 2007. Ear mesenchymal stem cells (EMSC) can differentiate into spontaneously contracting muscle cells. *J Cell Biochem* 102: 122–135.

Godara, P., C.D. McFarland, and R.E. Nordon. 2008. Design of bioreactors for mesenchymal stem cell tissue engineering. *J Chem Technol Biotechnol* 83:408–420.

He, J., D.C. Genetos, and J.K. Leach. 2010. Osteogenesis and trophic factor secretion are influenced by the composition of hydroxyapatite/poly(lactide-co-glycolide) composite scaffolds. *Tissue Eng Part A* 16(1):127–137.

Higuera, G., D. Schop, F. Janssen, R. van Dijkhuizen-Radersma, T. van Boxtel, and C.A. van Blitterswijk. 2009. Quantifying in vitro growth and metabolism kinetics of human mesenchymal stem cells using a mathematical model. *Tissue Eng Part A* 15:2653–2663.

Hiraoka, K., S. Grogan, T. Olee, and M. Lotz. 2006. Mesenchymal progenitor cells in adult human articular cartilage. *Biorheology* 43:447–454.

Horwitz, E.M., and M. Dominici. 2008. How do mesenchymal stromal cells exert their therapeutic benefit? *Cytotherapy* 10:771–774.

Horwitz, E.M., and W.R. Prather. 2009. Cytokines as the major mechanism of mesenchymal stem cell clinical activity: expanding the spectrum of cell therapy. *Isr Med Assoc J* 11: 209–211.

Jaiswal, N., S.E. Haynesworth, A.I. Caplan, and S.P. Bruder. 1996. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J Cell Biochem* 64: 295–312.

Kim, T.J., J. Seong, M. Ouyang, J. Sun, S. Lu, J.P. Hong, N. Wang, and Y. Wang. 2009. Substrate rigidity regulates Ca^{2+} oscillation via RhoA pathway in stem cells. *J Cell Physiol* 218:285–293.

King, J.A., and W.M. Miller. 2007. Bioreactor development for stem cell expansion and controlled differentiation. *Curr Opin Chem Biol* 11:394–398.

Laboureau, J., L. Dubertret, C. Lebreton-De Coster, and B. Coulomb. 2004. ERK activation by mechanical strain is regulated by the small G proteins rac-1 and rhoA. *Exp Dermatol* 13:70–77.

Lauffenburger, D., and C. Cozens. 1989. Regulation of mammalian cell growth by autocrine growth factors: Analysis of consequences for inoculum cell density effects. *Biotechnol Bioeng* 33:1365–1378.

Li, Y.M., T. Schilling, P. Benisch, S. Zeck, J. Meissner-Weigl, D. Schneider, C. Limbert, J. Seufert, M. Kassem, N. Schutze, F. Jakob, and R. Ebert. 2007. Effects of high glucose on mesenchymal stem cell proliferation and differentiation. *Biochem Biophys Res Commun* 363:209–215.

Li, D., T. Tang, J. Lu, and K. Dai. 2009. Effects of flow shear stress and mass transport on the construction of a large-scale tissue-engineered bone in a perfusion bioreactor. *Tissue Eng Part A* 15(10):2773–8273.

Lippens, E., G. Vertenten, J. Girones, H. Declercq, J. Saunders, J. Luyten, L. Duchateau, E. Schacht, L. Vlaminck, F. Gasthuys, and M. Cornelissen. 2010. Evaluation of bone regeneration with an injectable, in situ polymerizable Pluronic(R) F127 hydrogel derivative combined with autologous mesenchymal stem cells in a goat tibia defect model. *Tissue Eng Part A* 16(2):617–627.

Malda, J., and C.G. Frondoza. 2006. Microcarriers in the engineering of cartilage and bone. *Trends Biotechnol* 24:299–304.

Marklein, R.A., and J.A. Burdick. 2010. Controlling stem cell fate with material design. *Advanced Materials* 22(2):175–189.

McBeath, R., D.M. Pirone, C.M. Nelson, K. Bhadriraju, and C.S. Chen. 2004. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell* 6:483–495.

McBride, S.H., T. Falls, and M.L.K. Tate. 2008. Modulation of stem cell shape and fate B: mechanical modulation of cell shape and gene expression. *Tissue Eng Part A* 14:1573–1580.

Meyers, V.E., M. Zayzafoon, J.T. Douglas, and J.M. McDonald. 2005. RhoA and cytoskeletal disruption mediate reduced osteoblastogenesis and enhanced adipogenesis of human mesenchymal stem cells in modeled microgravity. *J Bone Miner Res* 20:1858–1866.

Pal, R., M. Hanwate, M. Jan, and S. Totey. 2009. Phenotypic and functional comparison of optimum culture conditions for upscaling of bone marrow-derived mesenchymal stem cells. *J Tissue Eng Regen Med* 3:163–174.

Park, J.S., N.F. Huang, K.T. Kurpinski, S. Patel, S. Hsu, and S. Li. 2007. Mechanobiology of mesenchymal stem cells and their use in cardiovascular repair. *Front Biosci* 12:5098–5116.

Peng, L., Z. Jia, X. Yin, X. Zhang, Y. Liu, P. Chen, K. Ma, and C. Zhou. 2008. Comparative analysis of mesenchymal stem cells from bone marrow, cartilage, and adipose tissue. *Stem Cells Dev* 17:761–773.

Prescott, R.S., R. Alsanea, M.I. Fayad, B.R. Johnson, C.S. Wenckus, J. Hao, A.S. John, and A. George. 2008. In vivo generation of dental pulp-like tissue by using dental pulp stem cells, a collagen scaffold, and dentin matrix protein 1 after subcutaneous transplantation in mice. *J Endodont* 34:421–426.

Ragetly, G.R., G.J. Slavik, B.T. Cunningham, D.J. Schaeffer, and D.J. Griffon. 2010. Cartilage tissue engineering on fibrous chitosan scaffolds produced by a replica molding technique. *J Biomed Mater Res A* 93(1):46–55.

Reddig, P.J., and R.L. Juliano. 2005. Clinging to life: cell to matrix adhesion and cell survival. *Cancer Metastasis Rev* 24:425–439.

Rohanizadeh, R., M.V. Swain, and R.S. Mason. 2008. Gelatin sponges (Gelfoam) as a scaffold for osteoblasts. *J Mater Sci Mater Med* 19:1173–1182.

Sart, S., Y.J. Schneider, and S.N. Agathos. 2007. Culture of rat bone marrow mesenchymal stem cells on Cultispher-S supports. In: *Cells & Culture, Abstracts of the 20th ESACT Meeting*. Dresden, Germany, June 17–20, 2007. p. 182, Abstract no. P-3.53.

Sart, S., Y.J. Schneider, and S.N. Agathos. 2009. Ear mesenchymal stem cells: an efficient adult multipotent cell population fit for rapid and scalable expansion. *J Biotechnol* 139:291–299.

Schofer, M.D., U. Boudriot, S. Bockelmann, A. Walz, J.H. Wendorff, A. Greiner, J.R. Paletta, and S. Fuchs-Winkelmann. 2009. Effect of direct RGD incorporation in PLLA nanofibers on growth and osteogenic differentiation of human mesenchymal stem cells. *J Mater Sci Mater Med* 20:1535–1540.

Schop, D., F.W. Janssen, E. Borgart, J.D. de Bruijn, and R. van Dijkhuizen-Radersma. 2008. Expansion of mesenchymal stem cells using a microcarrier-based cultivation system: growth and metabolism. *J Tissue Eng Regen Med* 2(2–3):126–35

Schop, D., F.W. Janssen, L.D. van Rijn, H. Fernandes, R.M. Bloem, J.D. de Bruijn, and R. van Dijkhuizen-Radersma. 2009. Growth, metabolism, and growth inhibitors of mesenchymal stem cells. *Tissue Eng Part A* 15:1877–1886.

Sensebe, L., and P. Bourin. 2009. Mesenchymal stem cells for therapeutic purposes. *Transplantation* 87:S49–S53.

Shiragami, N., H. Honda, and H. Unno. 1993. Anchorage-dependent animal cell culture by using a porous microcarrier. *Bioprocess Eng* 8:295–299.

Sumanasinghe, R.D., T.W. Pfeiler, N.A. Monteiro-Riviere, and E.G. Loboa. 2009. Expression of proinflammatory cytokines by human mesenchymal stem cells in response to cyclic tensile strain. *J Cell Physiol* 219:77–83.

Ustunel, I., A.M. Ozenci, Z. Sahin, O. Ozbey, N. Acar, G. Tanriover, C. Celik-Ozenci, and R. Demir. 2008. The immunohistochemical localization of notch receptors and ligands in human articular cartilage, chondroprogenitor culture and ultrastructural characteristics of these progenitor cells. *Acta Histochem Acta Histochem* 110:397–407.

Wang, H., G.M. Riha, S. Yan, M. Li, H. Chai, H. Yang, Q. Yao, and C. Chen. 2005. Shear stress induces endothelial differentiation from a murine embryonic mesenchymal progenitor cell line. *Arterioscler Thromb Vasc Biol* 25:1817–1823.

Weil, B.R., A.M. Abarbanell, J.L. Herrmann, Y. Wang, and D.R. Meldrum. 2009. High glucose concentration in cell culture medium does not acutely affect human mesenchymal stem cell growth factor production or proliferation. *Am J Physiol Regul Integr Comp Physiol* 296:R1735–R1743.

Woods, A., G. Wang, H. Dupuis, Z. Shao, and F. Beier. 2007. Rac1 signaling stimulates N-cadherin expression, mesenchymal condensation, and chondrogenesis. *J Biol Chem* 282:23500–23508.

Yang, Y., F.M. Rossi, and E.E. Putnins. 2007. Ex vivo expansion of rat bone marrow mesenchymal stromal cells on microcarrier beads in spin culture. *Biomaterials* 28:3110–3120.

Zayzafoon, M., V.E. Meyers, and J.M. McDonald. 2005. Microgravity: the immune response and bone. *Immunol Rev* 208:267–280.

Zi, Z., and E. Klipp. 2007. Cellular signaling is potentially regulated by cell density in receptor trafficking networks. *FEBS Lett* 581:4589–4595.