



Effects of a bone-like mineral film on phenotype of adult human mesenchymal stem cells in vitro

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Abstract

Multipotent cell types are rapidly becoming key components in a variety of tissue engineering schemes, and mesenchymal stem cells (MSCs) are emerging as an important tool in bone tissue regeneration. Although several soluble signals influencing osteogenic differentiation of MSCs in vitro are well-characterized, relatively little is known about the influence of substrate signals. This study was aimed at elucidating the effects of a bone-like mineral (BLM), which is vital in the process of bone bonding to orthopedic implant materials, on the osteogenic differentiation of human MSCs in vitro. Growth of a BLM film (carbonate apatite, Ca/P = 1.55) on poly(lactide-*co*-glycolide) (PLG) substrates was achieved via surface hydrolysis and subsequent incubation in a modified simulated body fluid. The BLM film demonstrated significantly increased adsorption of fibronectin, and supported enhanced proliferation of human mesenchymal stem cells (hMSCs) relative to PLG substrates. In the absence of osteogenic supplements hMSCs did not display a high expression of osteogenic markers on BLM or PLG. In the presence of osteogenic supplements hMSCs exhibited greater expression of osteogenic markers on PLG substrates than on BLM substrates, as measured by alkaline phosphatase activity and osteocalcin production. Taken together, these data support the concept that substrate signals significantly influence MSC growth and differentiation, highlighting the importance of carrier material composition in stem cell-based tissue engineering schemes.

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1. Introduction

Several recent studies highlight the potential of multipotent adult cells in regeneration of multiple tissue types, including bone tissue. One particular multipotent cell type isolated from bone marrow, the mesenchymal stem cell (MSC), has received widespread attention due to its relative ease of procurement and ability to differentiate into multiple mesenchymal cell types. Protocols have been developed to induce MSC differentiation down the osteogenic [1,2], chondrogenic [3], adipogenic [2,4], neurogenic [5,6] and myogenic [7]

pathways, and these protocols typically require highly specific culture environments. For example, the presence of TGF- β 3 drives chondrogenic differentiation, but only in micromass culture in which MSCs are exposed to high oxygen tension [2]. Recent studies have focused specifically on identification of soluble signals that induce MSC differentiation into bone-forming cells in vitro, and a variety of these signals (e.g. dexamethasone [1,2], bone morphogenetic protein-2 [8]) have been identified. In addition, strategies based on key inductive soluble signals have now been utilized in vivo to engineer bone tissue regeneration by MSCs [9,10]. Altogether, these results demonstrate the importance of diverse components of the extracellular environment in dictating MSC fate. Identification of these components may aid substantially in design of biomaterials for stem cell-based tissue engineering, particularly in engineering bone tissue regeneration.

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Although, several soluble signals controlling MSC phenotype have been identified, the influence of substrate signals (e.g. extracellular matrix molecules) remains largely unexplored. An understanding of the influence of substrate signals on differentiation is imperative for appropriate design and use of biomaterial carriers for MSC transplantation, and recent work suggests that these effects are substantial. The presence of fibronectin (Fn) or the arg-gly-asp (RGD) peptide on polymer surfaces increases proliferation and spreading of MSCs, and ultimately leads to enhanced osteogenic differentiation in a dose-dependent manner [11]. In vivo differentiation of MSCs down the osteogenic pathway also appears to be highly dependent on substrate composition. MSCs implanted within constructs containing a CaP mineral component (i.e. hydroxyapatite [12], tricalcium phosphate (TCP) [13], collagen/TCP mixtures) form substantial amounts of bone tissue in femoral and cranial bone defects, or in subcutaneous sites 4 weeks post-implantation [9,14–19]. Furthermore, several soluble signals (e.g. TGF- β 1 and - β 3, BMP-2, -4, and -7, dexamethasone) have been reported to drive in vivo bone and cartilage formation by MSCs on CaP substrates, with little or no effect on bone formation by MSCs in the absence of a CaP mineral substrate [15]. Taken together, these studies broadly suggest that CaP-based materials can be effectively utilized, alone or in conjunction with inductive soluble signals, to direct the phenotype of multipotent MSCs in bone tissue engineering applications.

The effects of CaP minerals on MSCs are perhaps not surprising in light of the role of a CaP-based mineral layer in bonding of orthopedic implants to native bone tissue. A thin, bone-like mineral (BLM) film appears to be a prerequisite to bonding of implant materials to native bone tissue [20,21], and has been shown to enhance osteoconductivity of biomaterial implants in vivo [22]. Materials that are not capable of supporting deposition of a BLM layer in vivo (e.g. alumina, untreated titanium) typically do not bond with native bone tissue, and instead become covered with a fibrous capsule [16,23]. One explanation for this phenomenon is that the BLM layer provides a preferred substrate for conduction and differentiated function of resident osteoblasts or committed osteoblast precursors. This explanation is supported by previous studies, which demonstrate that CaP mineral substrates support proliferation and expression of osteoblast phenotype in cultures of osteoblasts or osteoblast precursors [13,24,25]. It has also been proposed that the processes of bone bonding to BLM-coated implants and fibrous encapsulation of non-mineralized implants may each be directed by multipotent fibroblasts derived from bone marrow (i.e. MSCs) [16]. Thus, the presence or absence of a BLM substrate has been considered a potentially important factor in differentiation of bone precursor cells, such as MSCs, into mature osteoblasts.

In the present study a model bone-like mineral film is used to examine the influence of a mineral surface on proliferation and osteogenic differentiation of MSCs in vitro. The MSCs used in this study are a highly homogeneous cell population capable of differentiating down multiple mesenchymal lineages [2], providing a specific platform for studying substrate effects on differentiation. The mineral film, which is similar in structure and composition to human bone mineral [26], serves as a mimic of the BLM layer formed in vivo. We chose poly(lactide-co-glycolide) (PLG) as the base material for mineralization, as it can be readily processed into three-dimensional scaffolds for bone tissue engineering applications [27,28]. Our results demonstrate that MSC proliferation is enhanced on BLM substrates, but the production of osteogenic differentiation markers is reduced, as compared to non-mineralized PLG. These results have implications for design of biomaterials to support MSC transplantation, and suggest that CaP minerals may have a diverse regulatory effect on bone precursor cell phenotype.

2. Materials and methods

2.1. Film preparation and mineralization

PLG pellets with lactide:glycolide=85:15 (inherent viscosity=0.78) were obtained from Alkermes Inc. Films were prepared via a compression molding process in which PLG pellets were placed between two smooth stainless steel plates heated to 200°C, which is above the T_g of 85:15 PLG. The plates were then pressed together at 1.0 MPa for 10 s, followed by release of the pressure and rapid cooling to room temperature. This resulted in smooth, ~250 μ m thick PLG sheets. Circular films (diameter = 15 mm) were then cut out of the sheets to be placed into 24-well tissue culture dishes. A bone-like mineral layer was grown on some polymer films using a previously described approach [26]. Briefly, films were first immersed in 0.5 M NaOH for 60 min to produce a hydrolyzed surface. The hydrolyzed films were then incubated in a 10 ml solution of a modified simulated body fluid (mSBF) for 7 days for mineral growth. The solution was replaced with fresh mSBF daily to ensure adequate ion concentrations for mineral growth. mSBF was prepared by dissolving the following reagents in deionized H₂O: 141 mM NaCl, 4.0 mM KCl, 0.5 mM MgSO₄, 1.0 mM MgCl₂, 4.2 mM NaHCO₃, 5.0 mM CaCl₂, and 2.0 mM KH₂PO₄. The resulting solution was buffered to pH = 6.8 with Tris-HCl, and was held at 37°C for the duration of the mineralization process.

The morphology and atomic composition of mineralized and non-mineralized PLG films were examined via scanning electron microscopy and energy dispersive X-ray analysis. A conductive gold coating was applied

to the surface of each film via sputter coating, and samples were imaged under high vacuum using a Hitachi S-3200 SEM operating at 10–20 kV. An energy dispersive X-ray detector (Noran SiLi detector) was used in conjunction with SEM for elemental analysis of mineral crystals grown on the polymer surface. SEM linescans (average from 5 linescans/substrate type) were used to calculate the relative surface roughness (ratio of arithmetic mean roughness values) of the mineralized substrate versus the non-mineralized substrate.

2.2. Analysis of fibronectin adsorption to cell culture substrates

Adsorption of a model protein, Fn, was measured to gain insight into the influence of surface composition on protein adsorption. Fn labeled with a ^{125}I tracer was obtained from Fisher (Irvine, CA), and non-labeled Fn was from Gibco (Gaithersburg, MD). Mineralized or non-mineralized PLG films ($n=4$) were incubated in PBS containing various total Fn concentrations (50, 140, 1000, 1×10^4 , 1×10^5 ng/ml), with 4 ng/ml of ^{125}I -Fn included in each well as a radioactive tracer. After a 24 h incubation at 4°C the films were incubated in a 10% SDS solution and the solution was boiled to remove the adsorbed Fn. The amount of adsorbed Fn was then counted using a gamma counter, and coating density was calculated by dividing the total mass of adsorbed Fn by the film area.

2.3. Cell seeding and culture

Human mesenchymal stem cells (hMSCs) were purchased from Biowhittaker Inc. at passage 2. These cells are isolated from adult human bone marrow aspirates via density gradient centrifugation (98% homogeneous at passage 2) and were screened for their ability to differentiate into adipocytes (via oil red O staining), chondrocytes (safranin O staining and collagen II immunostaining) and osteoblasts (calcium mineral deposition) by Biowhittaker. Cells were expanded in non-differentiating mesenchymal stem cell growth medium (MSCGM) consisting of MSC Basal Medium (Biowhittaker Inc.) supplemented with 10% fetal bovine serum (selected by Biowhittaker for maintenance of MSC multipotential), 4 M L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen, Carlsbad, CA), and passaged every 7–10 days. Cells between passage 5 and 7 were statically seeded onto the mineralized PLG films, non-mineralized PLG films, or tissue culture polystyrene, allowed to attach overnight, and cultured in MSCGM with or without osteogenic culture supplements (0.1 μM dexamethasone, 10 mM β -glycerophosphate, 50 $\mu\text{g}/\text{ml}$ ascorbic acid, Sigma-Aldrich, St. Louis, MO). To ensure that cells did not migrate off of the mineralized or non-mineralized polymer films onto

the surrounding polystyrene, the tissue culture plates containing films were pre-coated with bovine serum albumin, and the samples were examined daily via light microscopy to confirm that no cells were attached to the tissue culture polystyrene. Cells were seeded at low density (3000 cells/cm²) for examination of attachment and for proliferation assays, and were seeded near confluence (20,000 cells/cm²) for osteogenic differentiation assays to eliminate the potential effect of proliferation on MSC differentiation.

2.4. Analysis of MSC proliferation

At various times after cell seeding (2, 4, 6, 8, and 10 days) proliferation was evaluated via quantification of total DNA in each culture, as described [29]. Briefly, cell layers were washed twice with PBS, then cells were lysed via incubation in a lysis buffer (25 mM Tris-HCl, 400 mM NaCl, 0.5% SDS, pH = 7.4). The lysate was then sonicated and centrifuged. A portion of the resulting supernatant was combined with the Hoescht 33258 dye, and solution DNA was quantified using a fluorometer and standard solutions of calf thymus DNA prepared in parallel.

2.5. Analysis of MSC differentiation

To analyze alkaline phosphatase (ALP) activity quantitatively, cell layers were washed twice with PBS and harvested via exposure to a passive lysis buffer (Promega, Madison, WI). The lysate was sonicated and centrifuged, and the resulting supernatant was assayed for ALP activity by incubating with 50 mM p-nitrophenyl phosphate (PNPP) in an assay buffer (100 mM glycine, 1 mM MgCl₂, pH = 10.5) at 37°C for 45 min. Absorbance was measured at 405 nm and converted to ALP activity using the extinction coefficient for PNPP ($1.85 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). To determine the amount of total DNA in each well, the cell nuclei were disrupted by addition of the aforementioned lysis buffer followed by sonication, centrifugation, and exposure to Hoechst 33258 dye, as described above. We also analyzed production of osteocalcin, the major non-collagenous protein in bone tissue. After various time periods in culture (0, 4, and 8 days) a 50 μl aliquot was removed from the culture medium and analyzed for osteocalcin content using a radioimmune assay (Kit# BT-440, Biomedical Technologies Inc., Stoughton, MA).

3. Results

3.1. Material surface and protein adsorption characteristics

Material properties were first analyzed to ultimately provide insight into surface effects on hMSC proliferation

and differentiation. SEM micrographs demonstrate that the polymer surface is a flat, relatively smooth surface (Fig. 1a), while the mineral substrate has a plate-like nanostructure, similar in morphology to human bone mineral (Figs. 1b,c). The ratio of the mineralized polymer surface roughness to the non-mineralized polymer surface roughness was 20.7, indicating a relatively high mineral surface roughness. Energy dispersive X-ray spectroscopy confirmed that the mineral was a carbonate apatite (Ca/P=1.55), as previously described [26]. Adsorption of Fn was also analyzed, as Fn has been previously described to play an important role in the determination of MSC phenotype [11]. At each level of solution Fn tested, the density of bound Fn on mineralized substrates was significantly higher when compared to non-mineralized substrates (Table 1, $p < 0.05$).

3.2. Mineral substrate promotes hMSC proliferation

We examined surface effects on hMSC proliferation by measuring DNA content on PLG and mineralized PLG substrates, using TCP as a positive control. hMSCs on non-mineralized PLG substrates did not demonstrate significant proliferation until day 6, and then proliferated rapidly from days 6–10 (Fig. 2). In contrast, hMSC proliferation occurred on mineralized PLG from days 2 through 8, and was at the same high level as hMSC proliferation on TCP (Fig. 2). DNA content in the non-mineralized PLG cultures was significantly lower at days 6 and 8 when compared with the mineralized PLG and TCP conditions ($p < 0.05$).

3.3. Mineral substrate inhibits osteogenic differentiation of hMSCs

We next examined the effects of the bone-like mineral substrate on osteogenic differentiation of hMSCs by measuring ALP activity and osteocalcin (OCN) production. In the absence of osteogenic soluble signals (dexamethasone, β -glycerophosphate, and ascorbic acid-2-phosphate) hMSCs did not display upregulation in ALP activity on either of the substrates examined, as expected (Fig. 3a). In the presence of osteogenic supplements hMSCs displayed upregulation in ALP activity on each substrate type, with a peak in ALP activity at day 4 (Fig. 3b). However, there were clear differences in osteogenic differentiation on mineralized vs. non-mineralized PLG substrates. ALP activity was significantly lower in cell populations cultured on mineralized PLG substrates at days 4–12. In each case, ALP activity decreased rapidly after an initial maximum, consistent with the typical temporal pattern of osteogenic differentiation [30]. ALP activity on TCP in the presence of osteogenic supplements was similar to activity on PLG substrates (not shown). hMSCs cultured on mineralized PLG also displayed significantly

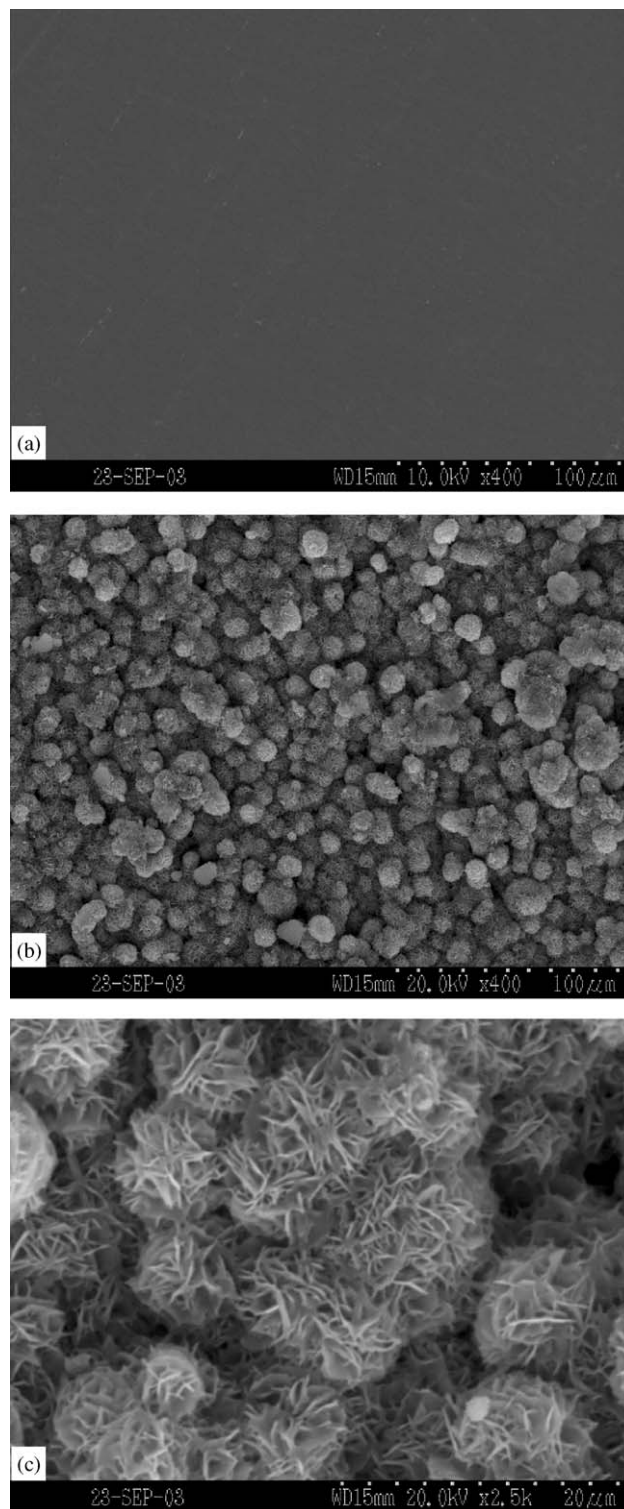


Fig. 1. Electron micrographs of the surface of (a) non-mineralized 85:15 poly(lactide-co-glycolide) (PLG) films, or (b) 85:15 PLG films hydrolyzed and incubated for 7 days in a modified simulated body fluid. (c) Higher magnification image of the mineral film.

decreased production of OCN. The amount of OCN production by MSCs on mineralized PLG was significantly lower when compared to non-mineralized PLG

Table 1
Adsorption of fibronectin to PLG or BLM substrates at various concentrations of soluble fibronectin

Solution [Fn] (ng/ml)	Fn Bound to PLG (ng/cm ²)	Fn Bound to MIN (ng/cm ²)
52.6	0.13±0.03	0.52±0.04
143	0.44±0.07	1.45±0.17
1040	4.4±0.5	11.3±0.2
10,000	27±3	79±8
100,000	212±37	281±23

Values represent means and standard deviations.

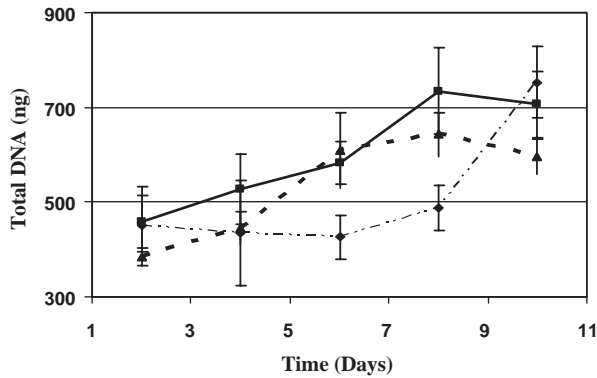


Fig. 2. Total DNA content, as a measure of cell proliferation, following hMSC plating on tissue culture polystyrene (▲), poly(lactide-co-glycolide) (◆), and bone-like mineral (■) films. Values represent means and standard deviations.

on days 4 and 8 ($p < 0.05$) (Fig. 4). The difference in OCN production at Day 0 (24 h after cell seeding) was not significant ($p > 0.05$). Baseline levels of OCN were similar in cell-free cultures containing mineralized PLG (4.7 ± 0.1 ng) and non-mineralized PLG (5.2 ± 1.7 ng) after 24 h in culture, indicating that the differences in OCN levels in the media itself could not be attributed to interactions between soluble OCN and the substrates.

4. Discussion

Previous work indicates that calcium phosphate minerals are osteoconductive [20–22,24] and may provide an excellent carrier for MSC transplantation in bone tissue engineering applications [9,14–19,31,32]. However, recent studies demonstrate varying effects of CaP mineral substrates on MSC differentiation in vitro [33–35]. Here we show that a bone-like mineral substrate promotes hMSC proliferation, and inhibits osteogenic differentiation of hMSCs cultured in osteogenic medium. These results support the concept that substrate signals significantly influence MSC growth and differentiation, highlighting the importance of carrier materi-

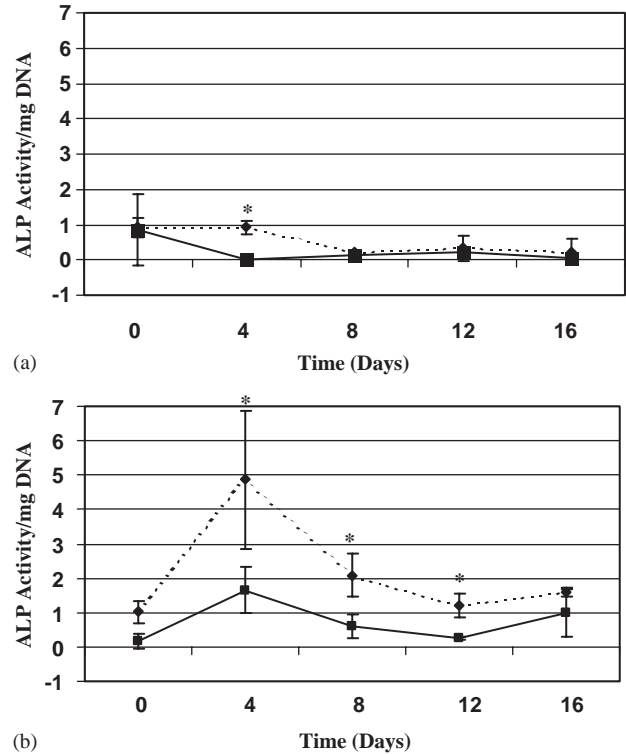


Fig. 3. Alkaline phosphatase activity on poly(lactide-co-glycolide) (◆) and bone-like mineral (■) substrates (a) in the absence of osteogenic culture supplements, or (b) in the presence of osteogenic culture supplements. Values represent means and standard deviations, and * denotes a statistically significant difference ($p < 0.05$).

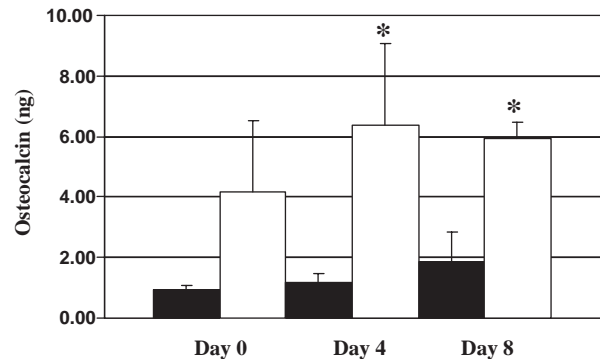


Fig. 4. Osteocalcin production in cultures of hMSCs cultured on poly(lactide-co-glycolide) films (□) or bone-like mineral films (■). Values represent means and standard deviations, and * denotes a statistically significant difference ($p < 0.05$).

al composition in stem cell-based tissue engineering schemes.

The inhibition of MSC differentiation in vitro demonstrated by the bone-like mineral film used in this study is interesting in light of previous studies that have shown striking positive effects of calcium phosphate minerals (e.g. HAP, TCP) on osteogenic differentiation of MSCs in vivo [9,14,15,17–19,31,32]. This contrast can perhaps

be explained by differences in the mineral and cell type used in the various studies, or by more general characteristics of the *in vivo* environment. In previous *in vivo* studies displaying the positive effects of CaP minerals on bone regeneration by MSCs, the carriers were composed of sintered HAP, coralline HAP, or TCP [9,17–19]. The mineral used in the present study is a calcium-deficient, poorly crystalline, carbonated apatite mineral, similar in composition to human bone mineral [26]. Importantly, the crystallinity, crystal size, surface roughness, and dissolution rate of the BLM are distinct from the properties of the mineral phases described in previous *in vivo* studies. Each of these material properties has been shown to substantially influence attachment, proliferation, and/or differentiation of osteoblasts and osteoblast precursor cells on model CaP substrates *in vitro* [35,37–40]. Thus, it is possible that the low crystallinity, small crystal size, high surface roughness, and rapid dissolution rate of the BLM in this study relative to sintered or coralline hydroxyapatite materials could be factors in the inhibitory effect on hMSC differentiation. Another important difference between the present study and previous *in vivo* work is the method of cell procurement. Previous studies have typically used MSCs isolated from bone marrow by selective culturing techniques, which involve selecting for fibroblasts that attach to a substrate and form colonies [12]. The MSCs utilized in the current study were isolated by density gradient centrifugation in addition to selective culturing techniques, and were screened for their ability to differentiate into multiple mesenchymal cell types [2]. Thus, the marrow-derived cells used in previous studies could include significant amounts of committed bone precursor cells, while the MSCs utilized here are of a more homogeneous stem-cell type. It is also possible that the influence of CaP minerals on osteogenesis by MSCs *in vivo* is dependent on environmental factors present *in vivo* and absent in an artificial culture environment.

The negative influence of the CaP mineral film used in this study on osteogenic differentiation of MSCs is surprising, but not unprecedented. Recent *in vitro* studies involving culture of bone marrow-derived precursor cells on mineral substrates have shown inconsistent results. In some previous studies, proliferation is decreased and expression of osteoblast phenotype is increased when bone marrow-derived fibroblasts are cultured on apatite substrates [33,34]. These studies suggest that bonding of calcium phosphate minerals to native bone may be initiated by attachment and differentiation of marrow-derived bone precursor cells. However, other recent studies indicate that apatite surfaces have no significant effect on osteogenic differentiation of marrow-derived precursor cells [35]. Furthermore, some types of calcium phosphate minerals (i.e. TCP, amorphous calcium phosphate) have recently

been shown to inhibit osteogenic differentiation and, in some cases, cause toxicity in cultures of bone precursor cells *in vitro* [33] and in bone defect sites *in vivo* [36]. Therefore, although CaP minerals have potential for use as MSC carriers in bone tissue engineering applications, their effects on osteogenic differentiation remain incompletely understood.

The increased proliferation of hMSCs on the BLM substrate demonstrated here may be due to enhanced adsorption of extracellular matrix proteins. The BLM substrate supported increased Fn adsorption in solutions with Fn concentrations ranging from 50 ng/ml to 100 µg/ml, and the magnitude of Fn adsorption in this study is similar to Fn adsorption to stoichiometric hydroxyapatite and bioactive glasses in previous studies [41]. Fn and the RGD peptide have been shown to enhance proliferation of hMSCs [11] through the $\alpha_5\beta_1$ integrin receptor [42]. The effect of the mineral substrate on proliferation described herein may be similarly mediated by Fn adsorption. Interactions between β_1 integrins and Fn have also been shown to be important in osteogenic differentiation of MSCs [11,42]. However, in the present study the enhanced Fn adsorption to BLM substrates did not increase osteogenic differentiation. The enhanced adsorption of Fn to the BLM substrate could be partially explained by the high surface roughness of the BLM substrate relative to the non-mineralized PLG substrate. The effect of topography on presentation of Fn on the substrate is difficult to quantify due to the inability to predict the fraction of Fn adsorbed that is active and the irregular nature of the mineral film, which precludes micron-scale surface area measurements and could cause Fn to adsorb in regions that are inaccessible to cell surface receptors.

The results of this work have implications for biomaterials design and bone tissue engineering. The materials used in this study were chosen, in part, because they can be readily processed to form three-dimensional, macroporous scaffolds for cell transplantation. We have previously shown that the presence of the BLM layer utilized in the present study within a macroporous PLG scaffold causes a 53% increase in bone tissue regeneration in a rat cranial defect model [43]. In general, the results of the present study demonstrate that substrate signals have a substantial effect on the phenotype of a multipotent cell type, even in the presence of potent osteogenic inductive signals. This result confirms that the composition of the biomaterial carrier for MSC transplantation is likely to be a crucial consideration in cell-based tissue engineering schemes.

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References

- [1] Ter Brugge PJ, Jansen JA. In vitro osteogenic differentiation of rat bone marrow cells subcultured with and without Dexamethasone. *Tissue Eng* 2002;8:321–31.
- [2] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143–7.
- [3] Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* 1998;238:265–72.
- [4] Jaiswal RK, Jaiswal N, Bruder SP, Mbalaviele G, Marshak DR, Pittenger MF. Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. *J Biol Chem* 2000;275:9645–52.
- [5] Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* 2000;61:364–70.
- [6] Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp Neurol* 2000;164:247–56.
- [7] Wakitani S, Saito T, Caplan AI. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle Nerve* 1995;18:1417–26.
- [8] Pereira RC, Delany AM, Canalis E. Effects of cortisol and bone morphogenetic protein-2 on stromal cell differentiation: correlation with CCAAT-enhancer binding protein expression. *Bone* 2002;30:685–91.
- [9] Gundle R, Joyner CJ, Triffitt JT. Human bone tissue formation in diffusion chamber culture in vivo by bone-derived cells and marrow stromal fibroblastic cells. *Bone* 1995;16:597–601.
- [10] Cassiede P, Dennis JE, Ma F, Caplan AI. Osteochondrogenic potential of marrow mesenchymal progenitor cells exposed to TGF-beta 1 or PDGF-BB as assayed in vivo and in vitro. *J Bone Miner Res* 1996;11:1264–73.
- [11] Yang XB, Roach HI, Clarke NM, Howdle SM, Quirk R, Shakesheff KM, Oreffo RO. Human osteoprogenitor growth and differentiation on synthetic biodegradable structures after surface modification. *Bone* 2001;29:523–31.
- [12] Friedenstein AJ, Piatetzky II S, Petrakova KV. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 1966;16:381–90.
- [13] Sun JS, Tsuang YH, Liao CJ, Liu HC, Hang YS, Lin FH. The effects of calcium phosphate particles on the growth of osteoblasts. *J Biomed Mater Res* 1997;37:324–34.
- [14] Krebsbach PH, Mankani MH, Satomura K, Kuznetsov SA, Robey PG. Repair of craniotomy defects using bone marrow stromal cells. *Transplantation* 1998;66:1272–8.
- [15] Krebsbach PH, Kuznetsov SA, Bianco P, Robey PG. Bone marrow stromal cells: characterization and clinical application. *Crit Rev Oral Biol Med* 1999;10:165–81.
- [16] Ohgushi H, Caplan AI. Stem cell technology and bioceramics: from cell to gene engineering. *J Biomed Mater Res* 1999;48:913–27.
- [17] Martin I, Muraglia A, Campanile G, Cancedda R, Quarto R. Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow. *Endocrinology* 1997;138:4456–62.
- [18] Haynesworth SE, Goshima J, Goldberg VM, Caplan AI. Characterization of cells with osteogenic potential from human marrow. *Bone* 1992;13:81–8.
- [19] Ohgushi H, Okumura M, Tamai S, Shors EC, Caplan AI. Marrow cell induced osteogenesis in porous hydroxyapatite and tricalcium phosphate: a comparative histomorphometric study of ectopic bone formation. *J Biomed Mater Res* 1990;24:1563–70.
- [20] Hench LL. Bioceramics: from concept to clinic. *J Am Ceram Soc* 1991;74:1487–510.
- [21] Kokubo T. Recent progress in glass based materials for biomedical applications. *J Ceram Soc Japan* 1991;99:965–73.
- [22] Hench LL, Wilson J. Surface-active biomaterials. *Science* 1984;226:630–6.
- [23] Laing PG, Ferguson AB, Hodge ES. Tissue reaction in rabbit muscle exposed to metallic implants. *J Biomed Mater Res* 1967;1:135–49.
- [24] El-Ghannam A, Ducheyne P, Shapiro IM. Porous bioactive glass and hydroxyapatite ceramic affect bone cell function in vitro along different time lines. *J Biomed Mater Res* 1997;36:167–80.
- [25] Ebara A, Ogata K, Imazato S, Ebisu S, Nakano T, Umakoshi Y. Effects of alpha-TCP and TetCP on MC3T3-E1 proliferation, differentiation and mineralization. *Biomaterials* 2003;24:831–6.
- [26] Murphy WL, Mooney DJ. Bioinspired growth of crystalline carbonate apatite on biodegradable polymer substrata. *J Am Chem Soc* 2002;124:1910–7.
- [27] Lu LC, Mikos AG. The importance of new processing techniques in tissue engineering. *MRS Bull* 1996;21:28–32.
- [28] Crane GM, Ishaug SL, Mikos AG. Bone tissue engineering. *Nat Med* 1995;1:1322–4.
- [29] Cesarone CF, Bolognesi C, Santi L. Improved microfluorometric DNA determination in biological material using 33258 Hoechst. *Anal Biochem* 1979;100:188–97.
- [30] Aubin JE. Bone stem cells. *J Cell Biochem Suppl* 1998;30–31:73–82.
- [31] Krebsbach PH, Kuznetsov SA, Satomura K, Emmons RV, Rowe DW, Robey PG. Bone formation in vivo: comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts. *Transplantation* 1997;63:1059–69.
- [32] Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G. Tissue-engineered bone regeneration. *Nat Biotechnol* 2000;18:959–63.
- [33] Oreffo RO, Driessens FC, Planell JA, Triffitt JT. Growth and differentiation of human bone marrow osteoprogenitors on novel calcium phosphate cements. *Biomaterials* 1998;19:1845–54.
- [34] Oreffo RO, Driessens FC, Planell JA, Triffitt JT. Effects of novel calcium phosphate cements on human bone marrow fibroblastic cells. *Tissue Eng* 1998;4:293–303.
- [35] Deligianni DD, Katsala ND, Koutsoukos PG, Missirlis YF. Effect of surface roughness of hydroxyapatite on human bone marrow cell adhesion, proliferation, differentiation and detachment strength. *Biomaterials* 2001;22:87–96.
- [36] Handschel J, Wiesmann HP, Stratmann U, Kleinheinz J, Meyer U, Joos U. TCP is hardly resorbed and not osteoconductive in a non-loading calvarial model. *Biomaterials* 2002;23:1689–95.
- [37] Chou L, Marek B, Wagner WR. Effects of hydroxyapatite coating crystallinity on biosolubility, cell attachment efficiency and proliferation in vitro. *Biomaterials* 1999;20:977–85.
- [38] Chang YL, Stanford CM, Keller JC. Calcium and phosphate supplementation promotes bone cell mineralization: implications for hydroxyapatite (HA)-enhanced bone formation. *J Biomed Mater Res* 2000;52:270–8.
- [39] Meleti Z, Shapiro IM, Adams CS. Inorganic phosphate induces apoptosis of osteoblast-like cells in culture. *Bone* 2000;27:359–66.

- [40] Ong JL, Hoppe CA, Cardenas HL, Cavin R, Carnes DL, Sogal A, Raikar GN. Osteoblast precursor cell activity on HA surfaces of different treatments. *J Biomed Mater Res* 1998;39:176–83.
- [41] Garcia AJ, Ducheyne P, Boettiger D. Effect of surface reaction stage on fibronectin-mediated adhesion of osteoblast-like cells to bioactive glass. *J Biomed Mater Res* 1998;40:48–56.
- [42] Gronthos S, Simmons PJ, Graves SE, Robey PG. Integrin-mediated interactions between human bone marrow stromal precursor cells and the extracellular matrix. *Bone* 2001;28:174–81.
- [43] Murphy WL, Simmons CA, Kaigler D, Mooney DJ. Bone regeneration via biomineral presentation and induced angiogenesis. *J Dental Res* 2004;83:204–10.