

Calcium phosphate surfaces promote osteogenic differentiation of mesenchymal stem cells

Petra Müller^a, Ulrike Bulnheim^a, Annette Diener^a, Frank Lüthen^a,
Marianne Teller^b, Ernst-Dieter Klinkenberg^b, Hans-Georg Neumann^b,
Barbara Nebe^a, Andreas Liebold^c, Gustav Steinhoff^c, Joachim Rychly^{a, *}

^a Cell Biology Laboratory, Medical Faculty, University of Rostock, Rostock, Germany

^b DOT GmbH, Rostock, Germany

^c Department of Heart Surgery, Medical Faculty, University of Rostock, Rostock, Germany

Received: May 3, 2007; Accepted: August 9, 2007

Abstract

Although studies *in vivo* revealed promising results in bone regeneration after implantation of scaffolds together with osteogenic progenitor cells, basic questions remain how material surfaces control the biology of mesenchymal stem cells (MSC). We used human MSC derived from bone marrow and studied the osteogenic differentiation on calcium phosphate surfaces. In osteogenic differentiation medium MSC differentiated to osteoblasts on hydroxyapatite and BONITmatrix[®], a degradable xerogel composite, within 14 days. Cells revealed a higher alkaline phosphatase (ALP) activity and increased RNA expression of collagen I and osteocalcin using real-time RT-PCR compared with cells on tissue culture plastic. To test whether material surface characteristics alone are able to stimulate osteogenic differentiation, MSC were cultured on the materials in expansion medium without soluble additives for osteogenic differentiation. Indeed, cells on calcium phosphate without osteogenic differentiation additives developed to osteoblasts as shown by increased ALP activity and expression of osteogenic genes, which was not the case on tissue culture plastic. Because we reasoned that the stimulating effect on osteogenesis by calcium phosphate surfaces depends on an altered cell–extracellular matrix interaction we studied the dynamic behaviour of focal adhesions using cells transfected with GFP labelled vinculin. On BONITmatrix[®], an increased mobility of focal adhesions was observed compared with cells on tissue culture plastic. In conclusion, calcium phosphate surfaces are able to drive MSC to osteoblasts in the absence of osteogenic differentiation supplements in the medium. An altered dynamic behaviour of focal adhesions on calcium phosphate surfaces might be involved in the molecular mechanisms which promote osteogenic differentiation.

Keywords: mesenchymal stem cells • calcium phosphate surface • focal adhesion • osteoblast

Introduction

Tissue engineering of bone based on the combination of multi-potent mesenchymal stem cells (MSC)

and a scaffold represents a new promising approach in bone regeneration to restore bony tissue after extensive loss due to injury or disease [1–5]. Although studies *in vivo*, both animal experiments and clinical trials have shown the usefulness of the implantation of scaffolds with osteogenic progenitor cells to regenerate bone, a number of basic questions remain before application of tissue engineering

*Correspondence to: Prof. Dr. Joachim RYCHLY,
University of Rostock, Medical Faculty
Cell Biology Laboratory, 18057 Rostock, Germany.
Tel.: +49 381 494 5730
Fax: +49 381 494 5739
E-mail: joachim.rychly@med.uni-rostock.de

techniques may become a clinical routine. This concerns the control of stem cell biology in general and mainly the guidance of stem cells by biomaterial surfaces [6, 7]. MSC derived from bone marrow or other sources are able to expand in an undifferentiated state and then to differentiate into osteoblasts or other cells of the connective tissue in appropriate media [8, 9]. In addition to soluble growth and differentiation factors in the cell environment, signals from the extracellular matrix (ECM) contribute to the control of stem cell biology [10, 11]. Although little is known about the mechanisms how the differentiation of MSC is governed by their interaction with the ECM, physical structure and chemistry of a biomaterial surface affect signals from the ECM to direct proliferation and differentiation of osteogenic progenitor cells [12].

Scaffolds or layers of calcium phosphate are widely used as biomaterials in bone tissue engineering. They have osteoconductive and osteoinductive properties, can be used for permanent implants or as biodegradable scaffolds and are mechanically compatible with native bone [13]. Although a number of investigations have shown that calcium phosphate surfaces have a beneficial effect on bone osteogenesis *in vivo*, detailed analyses of the differentiation of hMSC in terms of gene expression on calcium phosphate substrates are rare [4, 14–17]. These data are required to define the characteristics of a scaffold to specifically stimulate bone cell biology. Furthermore, the role of cellular components involved in cell matrix interaction, which can be affected by the characteristics of the material surface in controlling cell differentiation has to be defined. Cell attachment to the ECM is characterized by binding of integrin receptors to molecules of the ECM, like collagen and fibronectin. These receptors and a variety of cytoskeletonally associated proteins form molecular complexes called focal adhesions which are important signalling machineries [18–20]. In addition to the expression and activation of these proteins we speculate that the dynamic interaction of focal adhesions with the underlying substrate is significant for integrin mediated signal transduction and the biological response of the cell. The mobility of focal adhesions in stationary cells is regarded as a prerequisite to assemble the ECM [19]. Previously we have shown that the nature of focal adhesions regarding number, size and mobility depends on the characteristics of the biomaterial surface to which osteoblasts adhere [21].

Here we demonstrate that calcium phosphate surfaces not only enhance osteoblast-specific gene

expression compared with cells on tissue culture plastic, but stimulate osteoblast differentiation of MSC without culture additives for osteogenic progression. Concerning the interaction of cells with the ECM, on a calcium phosphate layer we found a high mobility of focal adhesions, which suggests an appropriate functional activity of cell adhesion components.

Materials and methods

Biomaterials

The following biomaterials were used in the experiments, in general as discs with a diameter of 11 mm.

BONITmatrix[®] (BM) (DOT GmbH): A degradable calcium phosphate xerogel composite which is composed of 87% calcium phosphate (60% hydroxyapatite and 40% β -tricalciumphosphate) and 13% silicon oxide. This material was used as compact disc. BONITmatrix[®] was manufactured by the sol-gel technique [22]. A biphasic calcium phosphate powder, consisting of hydroxyapatite and β -tricalcium phosphate was added to a hydrolysed tetraethoxysilane solution containing HCl as a catalyst. After gelation, the composite was formed and dried at 200°C for 2 hrs. BONITmatrix[®] is a CE Mark product (class III).

Hydroxyapatite (HA): Hydroxyapatite was fabricated in a special process and therefore named BONIT + HA. BONIT + HA is an electrochemically deposited calcium phosphate coating based upon a biomimetic process in which implants are coated in an electrolytic bath with a ~20- μ m thin bioactive layer of calcium phosphate. At the cathodic site brushite (CaHPO_4) · 2H₂O nucleates from the solution, grows and forms thin plates of a length from 10 to 20 μ m. The hydrous brushite phase is then transformed to hydroxyapatite by a chemical treatment in a basic solution (0.5 M NaOH).

Titanium: Titanium of technical purity (grade 2) was used which was polished using SIC wet grinding paper (grit P4000).

For comparison, cells were cultured on tissue culture plastic (TC) (Greiner Bio One, Frickenhausen, Germany) or on Lab-Tec 2-well chambered cover glass (Nalge Nunc International, Naperville, IL, USA).

Cell culture

Human mesenchymal stem cells (hMSC) were isolated from bone marrow which was obtained during median sternotomy. According to a standard protocol, after density gradient centrifugation of the diluted marrow sample ($d = 1.077$ g/l) interface enriched cells were cultured in

expansion medium (EM) in 5% CO₂ and at 37°C for 24 hrs. Adherent cells were harvested and the purity of MSC was checked by the absence of the hematopoietic marker CD34 and their ability to differentiate both to osteoblasts and adipocytes. Cells were grown in cell culture flasks using EM for 14 days before introducing them into the experiments.

In addition, the human osteosarcoma cell line MG-63 was used for experiments. Before seeding the cells on the surfaces, all cells were grown to sub-confluency for 14 days in EM.

The following cell culture media were used during the experiments:

EM: Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) (Gibco Invitrogen, Karlsruhe, Germany); Osteogenic differentiation medium (ODM): DMEM containing 10% FCS and 100 nM dexamethasone (Sigma, Taufkirchen, Germany), 10 mM β -glycerophosphate (Sigma) and 10 μ g/ml ascorbic acid (Sigma). All media contained 1% antibiotic-antimycotic solution.

Before seeding the cells on the different surfaces, cells were grown to sub-confluency, detached with trypsin/EDTA (0.05% trypsin, 0.02% EDTA) (Sigma) and diluted in complete medium at an appropriate density. Discs of the materials were placed in six well cell culture plates, the cells seeded at a density of 10⁴ cells/cm² and cultured in one of the media mentioned above for the indicated times.

Electron microscopy

For scanning electron microscopic analyses, cells on the materials were fixed in 4% glutaraldehyde and post-fixed with 0.5% OsO₄. After dehydration specimens were dried by a critical point dryer and sputtered with gold. Microscopic visualization was performed using a scanning electron microscope DSM 960A (Carl Zeiss, Jena, Germany).

Alkaline phosphatase (ALP) activity

Identical cell numbers were seeded on the materials. For the analyses cells cultured on the substrates were washed in PBS and fixed in 4% paraformaldehyde (PFA) for 5 min. Cells were then incubated with 0.1% naphthol ASMX phosphate and 0.1% fast red violet LB salt in 56 mM 2-amino-2-methyl-1,3-propanediol for 10 min. Quantitative analyses of the staining representing ALP-activity were performed by measuring the fluorescence intensity of ALP (excitation 530 nm/emission 590 nm) using a fluorescence ELISA reader (CytoFluor TM 2350, Applied Biosystems, Darmstadt, Germany). About 100 areas of fluorescence containing about 10 cells were measured and mean value and standard deviation were calculated.

Real-Time PCR

Cells were washed twice in phosphate buffered saline (PBS) and total RNA isolated using the NucleoSpin RNA II Kit with DNAase treatment (Macherey and Nagel, Düren, Germany). After quantifying RNA spectrometrically 500 ng of total RNA was reverse transcribed using superscript II reversion transcription reagents (Invitrogen, Karlsruhe, Germany) for cDNA synthesis. Quantitative real-time PCR assays were performed and monitored in duplicate using an ABI PRISM[®] 7000 Sequence detection system (Applied Biosystems). The PCR reactions contained 2.5 μ l of cDNA in a reaction volume of 25 μ l, 1x Taqman universal master-mix (Applied Biosystems) and 1x assays-on-demand[™] gene expression assay mix for detection of ALP (#Hs00758162_m1ALPL), bone sialoprotein (BSP) (#Hs00173720_m1IBSP), collagen I Coll (#Hs00164004_m1COLA1), osteocalcin OCN (#Hs01587813_g1BGLAP), Runx2 (#Hs00231692_m1RUNX2) (all from Applied Biosystems). Thermocycling conditions were 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Gene expression values were calculated based on the comparative $\Delta\Delta$ CT-method (separate tubes) and normalized to GAPDH (#Hs9999905_m1GAPDH), as reference housekeeping gene.

Transfection of GFP-vinculin and microscopic analyses

MG-63 cells were transiently transfected with a GFP-vinculin-construct (kindly provided by B. Geiger, Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel) using Effectene transfection reagent (Qiagen, Hilden, Germany) as described previously [21].

The microscopic analyses of vinculin labelled focal adhesions in an inverted microscope of the LSM 410 (Carl Zeiss) was described in detail earlier [21]. Briefly, each live cell was scanned in one optical plane and images were taken in intervals of 10 min for 1 hr. Analyses of the number, the length, and the movement of GFP-vinculin containing focal contacts in transfected MG-63 cells were performed with the aid of UTHSCSA Image Tool 3.00 (The University of Texas Health Science Center in San Antonio).

To estimate the mean value of the number of vinculin contacts per cell, focal adhesions in at least 12 cells at three time points were counted for each material surface.

For the analysis of the length of the contacts, the mean value for each contact was obtained by measuring the same contact at three time points. Together, at least 200 contacts were analysed for each material surface.

To calculate the speed of the moving vinculin contacts, the images at three time points were overlapped and the

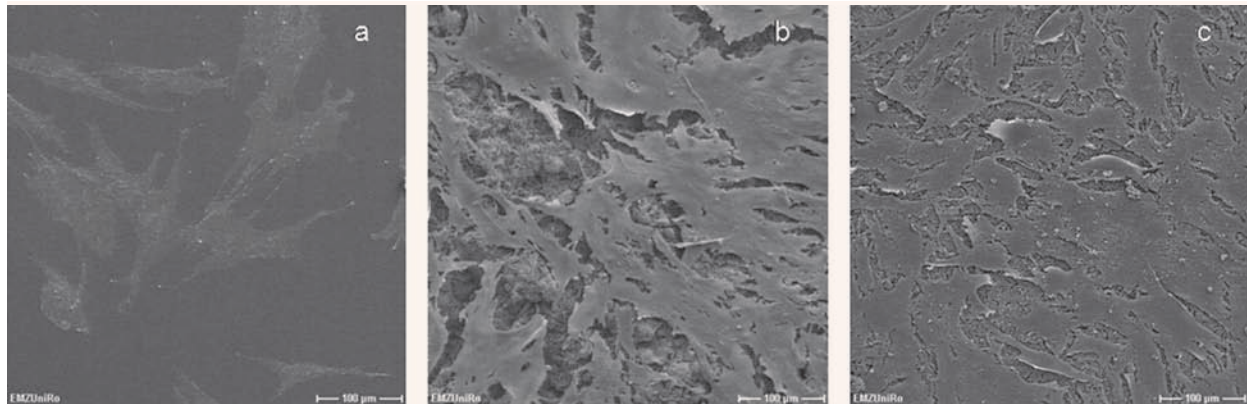


Fig. 1 Scanning electron microscopy of mesenchymal stem cells on tissue culture plastic (a), hydroxyapatite (b) and BONITmatrix[®] (c). On all three surfaces, cells reveal a similar flat morphology.

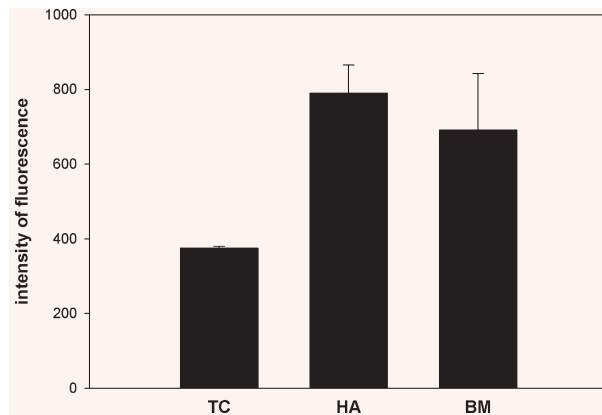


Fig. 2 ALP activity measured in cells on tissue culture plastic (TC), hydroxyapatite (HA) and BONITmatrix[®] (BM) after mesenchymal stem cells were cultured for 14 days in ODM on the different substrates. Results reveal an increased ALP activity on calcium phosphate surfaces.

same contacts at different time points were visualized with different false colours.

Statistical analysis

Statistical analyses were performed by the software SPSS 10.0 and a value of $P < 0.05$ considered as statistically significant using t-test.

Results

MSC cultured on the material surfaces were visualized by scanning electron microscopy to assess sur-

face topography and cell morphology (Fig. 1). Despite differences in the roughness of the surfaces comparing tissue culture plastic, BONITmatrix[®] and hydroxyapatite, the cells revealed a similar flat morphology.

To test the osteogenic differentiation of MSC on the surfaces, we first measured ALP activity after MSC were cultured on tissue culture plastic, hydroxyapatite and BONITmatrix[®] in ODM for 14 days. As demonstrated in Fig. 2, osteogenic differentiation was observed on all three surfaces. Quantitative analyses of the staining for ALP activity revealed an increased ALP activity on the calcium phosphate surfaces compared with tissue culture plastic. To further evaluate osteogenic differentiation on material surfaces quantitative real-time RT-PCR measurements were performed which revealed that both tissue culture plastic and BONITmatrix surfaces were able to stimulate the expression of several osteogenic genes and the transcription factor Runx2 in MSC (Fig. 3). Expression of collagen I after 3 days and osteocalcin after 14 days was increased on the calcium phosphate surface compared with the tissue culture plastic surface. On both days, a slight increase in the expression of the principal osteogenic transcription factor Runx2 was observed on the calcium phosphate surface.

Because on calcium phosphate a significant stimulation of osteoblastic differentiation was observed which exceeded the stimulation by the TCP surface, we next set out to test whether MSC on a calcium phosphate surface in EM without osteogenic differentiation additives are able to differentiate to osteoblasts. Measurements of ALP activity revealed that indeed MSC differentiated to osteoblasts on cal-

cium phosphate substrates in the absence of differentiation additives in the medium (Fig. 4a and b). To confirm this result, we also tested the cell line MG-63 which is often used as a model for osteoblasts. Both in ODM and in the absence of osteogenic differentiation additives, MG-63 cells revealed ALP activity only on calcium phosphate surfaces but not on tissue culture plastic (Fig. 5). Gene expression analyses performed with MSC confirmed the osteogenic differentiation on calcium phosphate without differentiation additives in the medium (Fig. 6). In pure EM, RNA expression of osteogenic genes including Runx2 was detected after MSC were cultured for 3 or 14 days on BONITmatrix®. However, concerning the extent of gene expression when cells were cultured on the calcium phosphate substrate, with the exception of Runx2 we found a higher gene expression in ODM containing soluble differentiation factors compared with EM (Figs. 3 and 6). Concerning the time course of gene expression, both in ODM and EM we observed a similar expression profile. ALP and collagen I were more expressed on day 3, whereas bone sialoprotein and osteocalcin were highly expressed on day 14 (Figs. 3 and 6). Experiments with cells of the MG-63 cell line confirmed the results that a calcium phosphate surface induces gene expression without osteogenic differentiation additives. Both in ODM and in EM, MG-63 cells revealed an increased RNA expression of ALP both after 3 and 7 days on BONITmatrix® compared with cells on tissue culture plastic (Fig. 7).

Because calcium phosphate surfaces promote the osteogenic differentiation of MSC and MG-63 cells both in ODM and EM we reasoned that an altered signal transduction mediated by the interaction of cells with the ECM on these materials stimulates osteogenic differentiation. Using MG-63 osteoblastic cells as model which revealed both an increased activity and RNA expression of ALP on calcium phosphate surfaces we analysed the nature of focal adhesions as principal mediators of integrin mediated signal transduction. Focal adhesions were visualized in live cells by transfection of GFP-vinculin. First we found that number and size of adhesion contacts were reduced in cells on BONITmatrix® compared with cells on tissue culture plastic and also with cells on a smooth titanium surface which represents another widely used biomaterial for implants (Fig. 8a and b). In addition to the number and size of focal adhesions, it is believed that their dynamic interaction with the ECM is a pre-

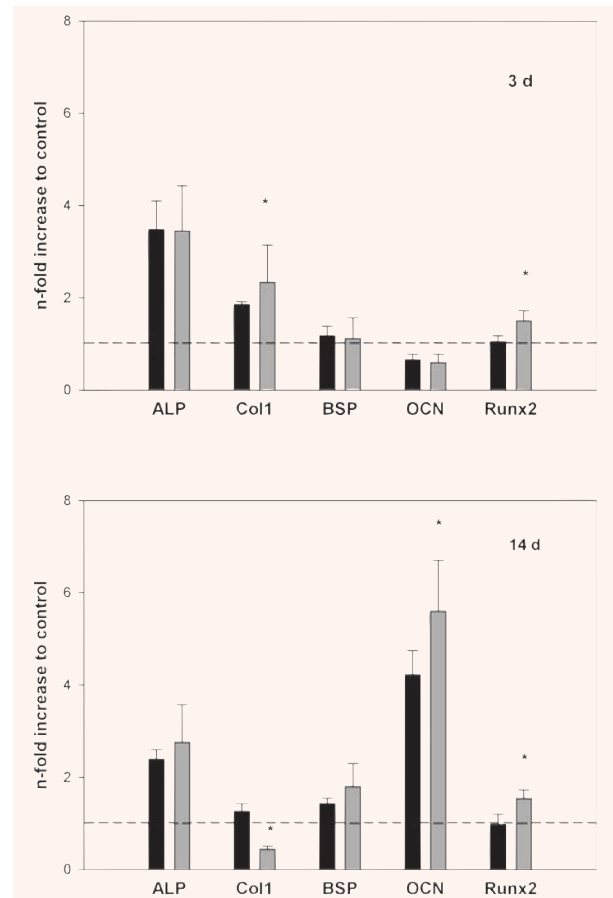


Fig. 3 RNA expression of ALP, collagen I (Col1), bone sialoprotein (BSP), osteocalcin (OCN) and transcription factor Runx2 in cells, after mesenchymal stem cells (MSC) were cultured 3 days and 14 days in ODM on tissue culture plastic (black columns) and on BONITmatrix® (grey columns). Results were related to controls (dashed line) representing MSC cultured on tissue culture plastic in EM. For collagen I (on day 3), osteocalcin (on day 14) and Runx2 (both days) an increased expression on BONITmatrix® was observed. (Asterix indicates statistically significant difference between cells on the two different materials.)

requisite for the functional activity of cells. Therefore, we measured the speed of the movement of focal adhesions within 30 min in stationary cells on the material substrates and found that osteoblasts on the calcium phosphate surface BONITmatrix® revealed a high mobility which exceeded the speed in cells on tissue culture plastic (Fig. 8c). On titanium we found a low mobility of adhesions.

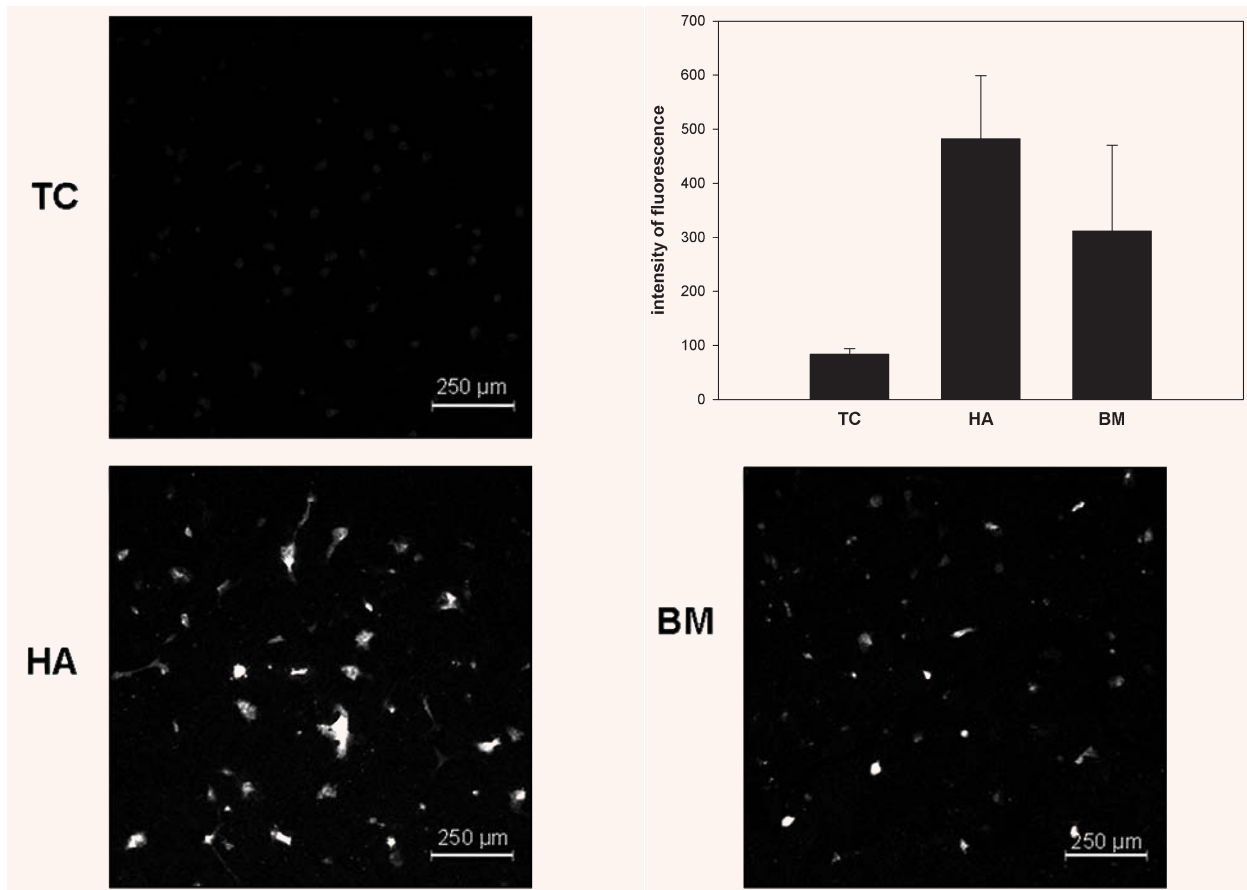


Fig. 4 (a) Fluorescence images of ALP activity in cells on tissue culture plastic (TC), hydroxyapatite (HA) and BONITmatrix[®] after mesenchymal stem cells were cultured in EM on different substrates for 14 days. Note that there was no visible ALP activity in cells on TC. (b) Quantitative analyses of ALP activity in cells on TC, HA and BM. The highest ALP activity was measured in cells on HA.

Taken together, increased capability of calcium phosphate surfaces to promote osteogenic differentiation correlated with lower number and smaller focal adhesions but an increased mobility of focal adhesions.

Discussion

We isolated multi-potent hMSC from bone marrow and demonstrated that these cells were able to differentiate to osteoblasts on different substrates. Calcium phosphate surfaces enhanced the osteogenic differentiation potential. Moreover, on calcium phosphate surfaces, MSC differentiated to osteoblasts also in the absence of osteogenic differentiation factors in the medium, which indicates that these surfaces contain

characteristics that specifically drive the cells toward an osteoblastic phenotype. Because in our experiments the morphology of the cells was similar on tissue culture plastic, hydroxyapatite and BONITmatrix[®] we believe that surface chemistry rather than the topography affected the cell differentiation. The potential implication of our finding on tissue engineering applications is profound. It opens the possibility to control stem cell differentiation *in vitro* as well *in vivo* without application of growth and differentiation factors like bone morphogenic proteins and others. These factors are expensive and it might be difficult to apply them in a suitable mode, whether in solution or in an immobilized form.

Our finding that surface properties specifically stimulate osteogenic differentiation and moreover in

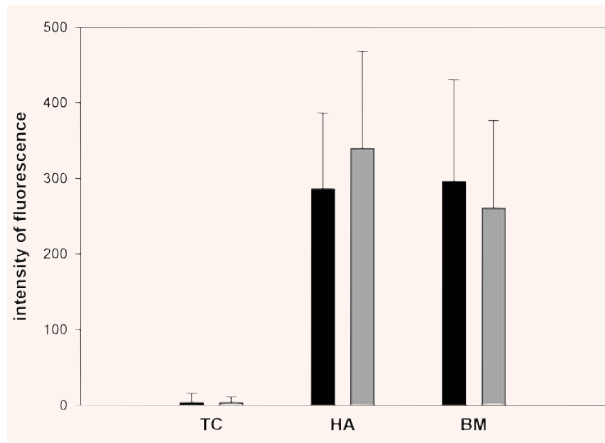


Fig. 5 Quantitative analyses of ALP activity in MG-63 cells on TC, HA and BM after cells were cultured for 7 days in EM (black columns) or ODM (grey columns). ALP activity was only found in cells on calcium phosphate surfaces both in ODM and EM.

the absence of osteogenesis stimulating factors in the medium is supported by investigations on other material surfaces. On an elastin-like polypeptide surface MSC differentiated to chondrocytes without chondrogenic supplements [23]. On silane-modified surfaces the direction of MSC differentiation depended on the presence of distinct chemical groups. NH₂ and SH surfaces promoted osteogenesis, whereas OH and COOH surfaces supported chondrogenesis also in the absence of biological stimuli in the medium [24].

Modulation of MSC differentiation to osteoblasts or other cells of the connective tissue by surface chemistry implies that intracellular signal transduction is driven by changes in the cell–ECM interaction and could replace soluble factors that stimulate cell differentiation. First of all, chemical properties of the surface could induce a selective adsorption of matrix proteins [25]. Non-collagenous matrix proteins, like osteocalcin and bone sialoprotein have calcium and hydroxyapatite binding characteristics and could preferentially be adsorbed on a calcium phosphate surface. A selective effect of matrix proteins on osteogenic differentiation of MSC has been demonstrated [26–28]. Although the data are somewhat contradictory it appears that beside other matrix proteins collagen I, laminin and fibronectin support increase in osteogenesis from MSC [29–31]. The composition of matrix proteins as a substrate for the cells determines the binding and activation of defined integrins.

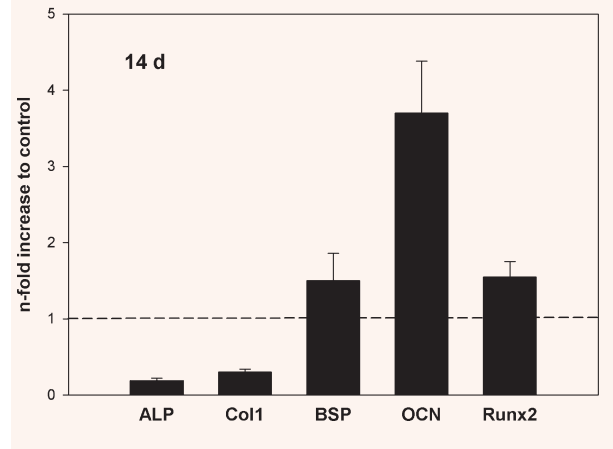
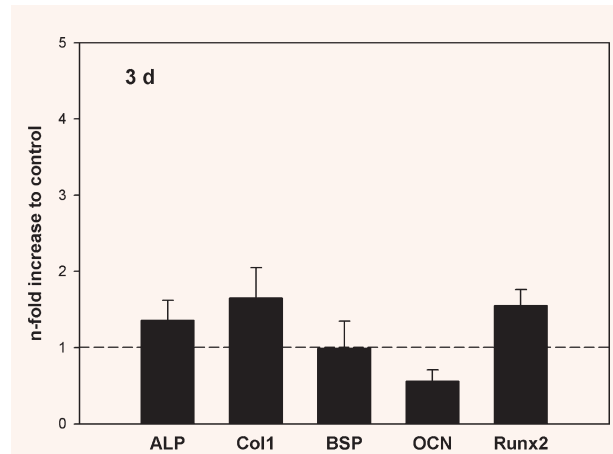


Fig. 6 RNA expression of ALP, Col1, BSP, OCN, and Runx2 after mesenchymal stem cells were cultured for 3 and 14 days in EM on BONITmatrix[®]. Results were related to controls (dashed line) representing cells cultured in EM on tissue culture plastic.

Binding of integrin $\beta 1\alpha 5$ is essential for osteoblast differentiation and mineralization [30]. In addition to a selection of adsorbed proteins by surface characteristics, the structural organization of matrix proteins is controlled by surface chemistry or topography, which may include alterations in the integrin binding domains. It was shown that alteration in the structure of adsorbed fibronectin by surface chemistry affected the integrin binding activity and in consequence modulated osteoblastic differentiation [32].

Concerning the interaction of cells with the ECM we were interested in the nature of focal adhesions.

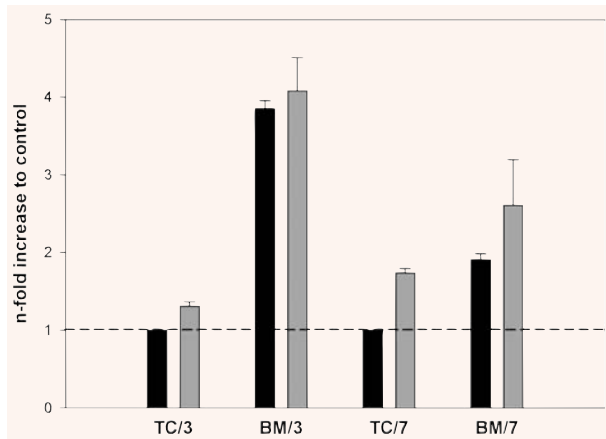


Fig. 7 RNA expression of ALP after MG-63 cells were cultured for 3 days and 7 days on tissue culture plastic (TC/3 and TC/7, respectively) and 3 and 7 days on BONITmatrix[®] (MB/3 and BM/7, respectively). Cells were cultured both in EM (black columns) and in ODM (grey columns). Results were related to controls (dashed line) representing cells cultured in EM on TC. On BM, MG-63 cells revealed an increased expression of ALP.

These are sites integrating ECM with the actin cytoskeleton and mediate signalling [33]. Among the considerable number of proteins that are present and interact in focal adhesions we used GFP labelled vinculin to characterize focal adhesions. We found that cells on BONITmatrix[®] formed small focal adhesions and the number of these complexes was low compared with cells on tissue culture plastic and titanium. It has been shown that addition of ODM in cultures of hMSC provoked a loss of focal adhesions and a remodelling of the cytoskeleton, which suggests a loosely adherent phenotype during osteogenesis [29]. Therefore, a low number and smaller adhesions in cells on the calcium phosphate surface which suggests a low adhesion could be considered as one reason for a promoting effect of the surface to drive osteogenesis. Size and number of focal adhesions could be accompanied by an altered composition of focal adhesion proteins. As shown earlier, surface chemistry modulated the recruitment of talin, paxillin and other proteins to the adhesion structures [34].

Focal adhesions are not static but are dynamic and move in the plane between cell and ECM [35]. This dynamic behaviour in addition to the mobility of the entire cell is required to modulate the ECM. Experiments using time-lapse movies have shown that the fibronectin network undergoes continuous

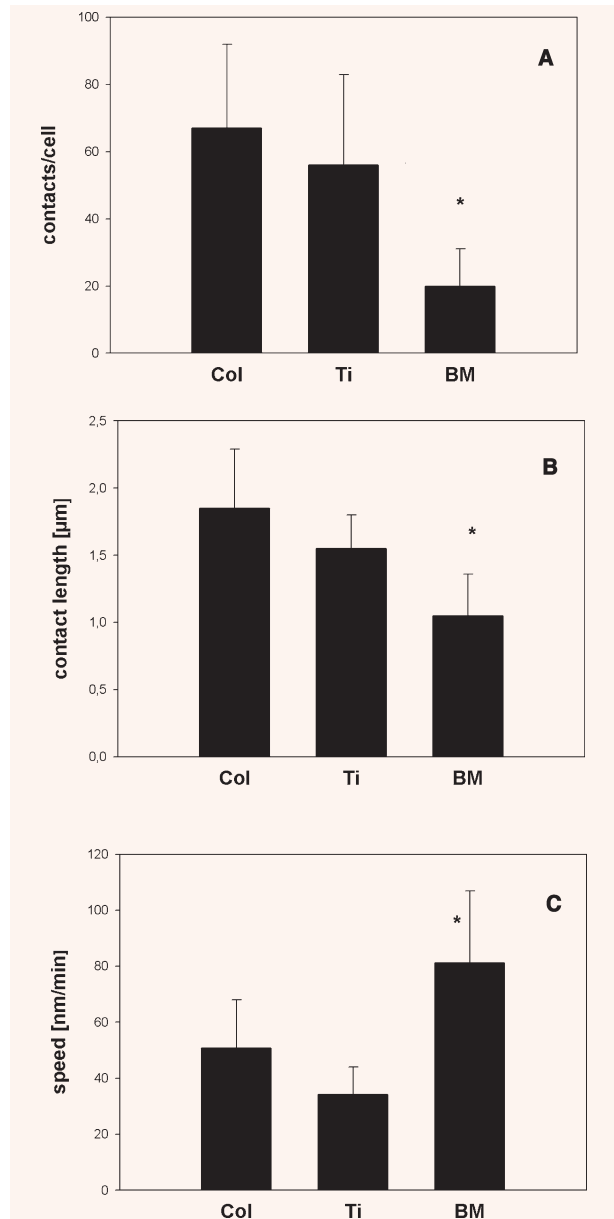


Fig. 8 Analyses of focal adhesions in live MG-63 cells on different materials after transfection of GFP-vinculin. **(A)** Number of focal adhesions per cell: Lower number of focal adhesions in cells on BONITmatrix[®] (BM) compared with cells on collagen coated chamber slides (Col) and on titanium (Ti). **(B)** Size of focal adhesions: Lower size of focal adhesions in cells on BM compared with cells on Col and Ti. **(C)** Speed of the movement of focal adhesions: Increased mobility of focal adhesions in cells on BM compared with Col and Ti, where the lowest movement was observed. (Asterix indicates statistically significant differences between cells on BM compared with cells on the two other materials.)

reorganization that is characterized by fibril stretching, displacements and shunting of fibrillar material [36]. We observed a high mobility of focal adhesions on BONITmatrix[®] compared with cells on titanium and on tissue culture plastic, which indicates an increased capability of cells to dynamically interact with the ECM. This increased mobility of focal adhesions might be advantageous for the osteogenic differentiation of MSC. Because our earlier studies on titanium surfaces have shown that the mobility of focal adhesions did not depend on the topography of the surface [21], the high mobility on BONITmatrix[®] appears to be rather due to the surface chemistry and not to the surface structure.

The molecular mechanisms how the dynamic interaction of cells with the ECM which is mediated by integrins dictates the osteogenic differentiation of MSC are largely unknown. The key molecule of osteogenic differentiation is Runx2 [37, 38]. Runx2 is expressed and activated *via* activation of MAP kinases ERK1 and ERK2 [39]. MAP kinases may be regarded as key signalling proteins, both due to cell stimulation by growth factors and integrin mediated cell adhesion to the substrate. For example, we have previously shown that mechanical stressing of integrin receptors induced MAP kinase activity which depended on the anchorage of signalling proteins to the cytoskeleton [40, 41]. Our analyses of gene expression revealed that Runx2 was equally expressed both by stimulation *via* the calcium phosphate surface alone or in combination with osteogenic soluble factors, whereas expression of osteocalcin, collagen or ALP was higher when the medium was supplemented with biological stimuli. This suggests that integrin mediated signalling modified by the surface characteristics was sufficient to maximally express Runx2, whereas maximal expression of proteins downstream of Runx2 requires further signals from soluble factors.

Regarding clinical applications of bone tissue engineering strategies our data demonstrated that calcium phosphate scaffolds are suitable matrices to promote osteogenic differentiation, when used in *ex vivo* cultures with subsequent implantation into injured bone. The results also suggest that direct implantation of a calcium phosphate material to the site of bone injury would support bone regeneration without application of osteoinductive substances, such as the variety of osteogenic growth factors.

Conclusion

Our results are promising to specifically modify material surfaces for tissue engineering approaches that are able to direct stem cell differentiation to osteogenesis. Calcium phosphate surfaces play a significant role in this concept. Moreover, our results points to a control of the osteogenesis from MSC by material surfaces in the absence of soluble additives. We believe that these possibilities are significant for bone tissue engineering.

Acknowledgements

The work was supported by the Federal Ministry of Education and Research (0313405B) and the Deutsche Forschungsgemeinschaft (NE 560/3-2). We thank Annelie Peters for expert technical assistance. For performing the scanning electron microscopic analyses we thank Ludwig Jonas from the Electron Microscopic Center of the University of Rostock. We are grateful to Burkhard Krüger from the Institute of Pathology, University of Rostock for providing the CytoFluor TM2350.

References

1. **Salgado AJ, Coutinho OP, Reis RL.** Bone tissue engineering: state of the art and future trends. *Macromol Biosc.* 2004; 4: 743–65.
2. **Otto WR, Rao J.** Tomorrow's skeleton staff: mesenchymal stem cells and the repair of bone and cartilage. *Cell Prolif.* 2004; 37: 97–110.
3. **Pountos I, Jones E, Tzioupis C, McGonagle D, Giannoudis PV.** Growing bone and cartilage. The role of mesenchymal stem cells. *J Bone Joint Surg Br.* 2006; 88: 421–6.
4. **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.
5. **Mistry AS, Mikos AG.** Tissue engineering strategies for bone regeneration. *Adv Biochem Eng Biotechnol.* 2005; 94: 1–22.
6. **Baksh D, Song L, Tuan RS.** Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. *J Cell Mol Med.* 2004; 8: 301–16.

7. **Griffith LG, Naughton G.** Tissue engineering—current challenges and expanding opportunities. *Science*. 2002; 295: 1009–14.
8. **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.
9. **Bianco P, Riminucci M, Gronthos S, Robey PG.** Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells*. 2001; 19: 180–92.
10. **Chastain SR, Kundu AK, Dhar S, Calvert JW, Putnam AJ.** Adhesion of mesenchymal stem cells to polymer scaffolds occurs *via* distinct ECM ligands and controls their osteogenic differentiation. *J Biomed Mater Res A*. 2006; 78: 73–85.
11. **Beqaj S, Jakkaraju S, Mattingly RR, Pan D, Schuger L.** High RhoA activity maintains the undifferentiated mesenchymal cell phenotype, whereas RhoA down-regulation by laminin-2 induces smooth muscle myogenesis. *J Cell Biol*. 2002; 156: 893–903.
12. **Lutolf MP, Hubbell JA.** Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat Biotechnol*. 2005; 23: 47–55.
13. **El-Ghannam A.** Bone reconstruction: from bio ceramics to tissue engineering. *Expert Rev Med Devices*. 2005; 2: 87–101.
14. **Cancedda R, Dozin B, Giannoni P, Quarto R.** Tissue engineering and cell therapy of cartilage and bone. *Matrix Biol*. 2003; 22: 81–91.
15. **Kon E, Muraglia A, Corsi A, Bianco P, Marcacci M, Martin I, Boyde A, Ruspantini I, Chistolini P, Rocca M, Giardino R, Cancedda R, Quarto R.** Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. *J Biomed Mater Res*. 2000; 49: 328–37.
16. **Bruder SP, Kraus KH, Goldberg VM, Kadiyala S.** The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J Bone Joint Surg Am*. 1998; 80: 985–96.
17. **Takahashi Y, Yamamoto M, Tabata Y.** Osteogenic differentiation of mesenchymal stem cells in biodegradable sponges composed of gelatin and beta-tricalcium phosphate. *Biomaterials*. 2005; 26: 3587–96.
18. **Wozniak MA, Modzelewska K, Kwong L, Keely PJ.** Focal adhesion regulation of cell behavior. *Biochim Biophys Acta*. 2004; 1692: 103–19.
19. **Geiger B, Bershadsky A, Pankov R, Yamada KM.** Transmembrane crosstalk between the extracellular matrix–cytoskeleton crosstalk. *Nat Rev Mol Cell Biol*. 2001; 2: 793–805.
20. **Wehrle-Haller B, Imhof B.** The inner lives of focal adhesions. *Trends Cell Biol*. 2002; 12: 382–9.
21. **Diener A, Nebe B, Luthen F, Becker P, Beck U, Neumann HG, Rychly J.** Control of focal adhesion dynamics by material surface characteristics. *Biomaterials* 2005; 26: 383–92.
22. **Gerber T, Traykova T, Henkel K, Bienengraber V.** A new sol-gel derived bone grafting material. *Key Eng Mater*. 2002; 218–220: 399–404.
23. **Betre H, Ong SR, Guilak F, Chilkoti A, Fermor B, Setton LA.** Chondrocytic differentiation of human adipose-derived adult stem cells in elastin-like polypeptide. *Biomaterials*. 2006; 27: 91–9.
24. **Curran JM, Chen R, Hunt JA.** The guidance of human mesenchymal stem cell differentiation *in vitro* by controlled modifications to the cell substrate. *Biomaterials*. 2006; 27: 4783–93.
25. **Keselowsky BG, Collard DM, Garcia AJ.** Surface chemistry modulates fibronectin conformation and directs integrin binding and specificity to control cell adhesion. *J Biomed Mater Res A*. 2003; 66: 247–59.
26. **Jadlowiec J, Koch H, Zhang X, Campbell PG, Seyedain M, Sfeir C.** Phosphorylation regulates the gene expression and differentiation of NIH3T3, MC3T3-E1, and human mesenchymal stem cells *via* the integrin/MAPK signaling pathway. *J Biol Chem*. 2004; 279: 53323–30.
27. **Wollenweber M, Domaschke H, Hanke T, Boxberger S, Schmack G, Gliesche K, Scharnweber D, Worch H.** Mimicked bioartificial matrix containing chondroitin sulphate on a textile scaffold of poly(3-hydroxybutyrate) alters the differentiation of adult human mesenchymal stem cells. *Tissue Eng*. 2006; 12: 345–59.
28. **Datta N, Pham QP, Sharma U, Sikavitsas VI, Jansen JA, Mikos AG.** *In vitro* generated extracellular matrix and fluid shear stress synergistically enhance 3D osteoblastic differentiation. *Proc Natl Acad Sci USA*. 2006; 103: 2488–93.
29. **Salasznyk RM, Williams WA, Boskey A, Batorsky A, Plopper GE.** Adhesion to vitronectin and collagen I promotes osteogenic differentiation of human mesenchymal stem cells. *J Biomed Biotechnol*. 2004; 2004: 24–34.
30. **Moursi AM, Globus RK, Damsky CH.** Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation *in vitro*. *J Cell Sci*. 1997; 110: 2187–96.
31. **Klees RF, Salasznyk RM, Kingsley K, Williams WA, Boskey A, Plopper GE.** Laminin-5 induces osteogenic gene expression in human mesenchymal stem cells through an ERK-dependent pathway. *Mol Biol Cell*. 2005; 16: 881–90.

32. **Keselowsky BG, Collard DM, Garcia AJ.** Integrin binding specificity regulates biomaterial surface chemistry effects on cell differentiation. *Proc Natl Acad Sci USA.* 2005; 102: 5953–7.
33. **Zamir E, Geiger B.** Molecular complexity and dynamics of cell-matrix adhesions. *J Cell Sci.* 2001; 114: 3583–90.
34. **Keselowsky BG, Collard DM, Garcia AJ.** Surface chemistry modulates focal adhesion composition and signaling through changes in integrin binding. *Biomaterials.* 2004; 25: 5947–54.
35. **Smilenov LB, Mikhailov A, Pelham RJ, Marcantonio EE, Gundersen GG.** Focal adhesion motility revealed in stationary fibroblasts. *Science.* 1999; 286: 1172–4.
36. **Sivakumar P, Czirok A, Rongish BJ, Divakara VP, Wang YP, Dallas SL.** New insights into extracellular matrix assembly and reorganization from dynamic imaging of extracellular matrix proteins in living osteoblasts. *J Cell Sci.* 2006; 119: 1350–60.
37. **Lian JB, Javed A, Zaidi SK, Lengner C, Montecino M, van Wijnen AJ, Stein JL, Stein GS.** Regulatory controls for osteoblast growth and differentiation: role of Runx/Cbfa/AML factors. *Crit Rev Eukaryot Gene Expr.* 2004; 14: 1–41.
38. **Stein GS, Lian JB, van Wijnen AJ, Stein JL, Montecino M, Javed A, Zaidi SK, Young DW, Choi JY, Pockwinse SM.** Runx2 control of organization, assembly and activity of the regulatory machinery for skeletal gene expression. *Oncogene.* 2004; 23: 4315–29.
39. **Franceschi RT, Xiao G, Jiang D, Gopalakrishnan R, Yang S, Reith E.** Multiple signaling pathways converge on the Cbfa1/Runx2 transcription factor to regulate osteoblast differentiation. *Connect Tissue Res.* 2003; 44 Suppl 1: 109–16.
40. **Schmidt C, Pommerenke H, Durr F, Nebe B, Rychly J.** Mechanical stressing of integrin receptors induces enhanced tyrosine phosphorylation of cytoskeletally anchored proteins. *J Biol Chem.* 1998; 273: 5081–5.
41. **Pommerenke H, Schmidt C, Durr F, Nebe B, Luthen F, Muller P, Rychly J.** The mode of mechanical integrin stressing controls intracellular signaling in osteoblasts. *J Bone Miner Res.* 2002; 17: 603–11.