Citation: BoneKEy Reports **3**, Article number: 560 (2014) | doi:10.1038/bonekey.2014.55

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ORIGINAL ARTICLE

Enhanced osteoblastogenesis in three-dimensional collagen gels

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Growth and differentiation of osteoblasts are often studied in cell cultures. *In vivo*, however, osteoblasts are embedded within a complex three-dimensional (3D) microenvironment, which bears little relation to standard culture flasks. Our study characterizes osteoblast-like cells cultured in 3D collagen gels and compares them with cells in two-dimensional (2D) cultures. Primary rat osteoblasts and MC3T3-E1 cells were seeded within type I collagen gels, and differentiation was determined by mineral staining and gene expression analysis. Cells growing in 3D gels showed positive mineral staining and induction of osteoblast marker genes earlier than cells growing in 2D. A number of genes, including osteocalcin, bone sialoprotein, alkaline phosphatase and dentin matrix protein 1, were already highly upregulated in 3D cultures 24 h after seeding. The early expression of osteoblast genes was dependent on the 3D structure and was not induced in cells growing on collagen-coated dishes in 2D. Comparison of thymidine incorporation between cells in 3D and 2D cultures treated with agents that induce proliferation—transforming growth factor β, platelet-derived growth factor and lactoferrin—showed a much greater response in 3D gels. Cells in 3D cultures were also much more sensitive to inhibition of proliferation by the protein kinase inhibitor imatinib mesylate. The 3D collagen gels better represent the physiological bone environment and offer a number of technical advantages for the study of osteoblasts *in vitro*. These studies have additional practical implications as 3D collagen gels are considered as a scaffold material in regenerative medicine for the repair of bone defects.

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Introduction

The current understanding of cellular bone biology is based on studies in several *in vitro* and *in vivo* experimental model systems. Typically, investigation of the effects of an agent on osteoblast growth and function begins by testing it *in vitro*, as osteoblasts cultured *in vitro* can recapitulate the life cycle of the osteoblast *in vivo*. *In vitro* assays are frequently employed to investigate the effects of factors on the commitment of mesenchymal stem cells to the osteogenic lineage, on proliferation and differentiation into mature osteoblasts that can lay down mineralized extracellular matrix, and on cell death due to apoptosis. The assays routinely used to investigate osteoblast biology *in vitro* are carried out in two-dimensional (2D) cultures, in which cells adhere to plastic or glass surfaces. However, the properties of 2D cultures are very different from the environment that osteoblasts are exposed to within the tissue *in vivo*.

Collagen is a major component of the extracellular matrix of many tissues, and, in bone, type I collagen constitutes 90% of the organic matter of the bone matrix.[1] Cell culture within type I collagen gels was first described by Elsdale and Bard.[2] Type I collagen gels potentially provide an excellent culture system for osteoblasts, as the cells grow within a three-dimensional (3D) structure made of the most abundant bone matrix protein, conditions that are much more closely related to the *in vivo* environment of the osteoblast than the traditional 2D cultures. It has been shown that culturing cells in 3D collagen gels affects cell shape, cell–cell interactions and response to soluble factors.[1,3]

Collagen gels are also being considered in regenerative medicine of the musculoskeletal system as potential biomimetic bone replacement materials for use in cases of bone loss by trauma or disease.[4] Collagen I provides a tissue-derived natural polymer; it is biocompatible and biodegradable and its

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Received 1 April 2014; accepted 12 June 2014; published online 6 August 2014

mechanical and biological properties can be controlled and varied as required.[3,5] As a scaffold for bone regeneration, collagen can be used alone or in combination with various natural polymers and minerals that provide better representation of the extracellular environment, including hyaluronic acid-modified chitosan/collagen/hydroxyapatite composites[6] and collagen/hydroxyapatite scaffolds.[4]

Although a number of studies have investigated osteoblasts cultured in 3D collagen gels, they mostly used microscopy for visual analysis of the osteoblast phenotype and alkaline phosphatase activity as a measure of osteoblast function.[1,3,7–9] The aim of the current study was to directly compare osteoblasts growing in parallel in 3D collagen gels and in 2D on plastic and to comprehensively characterize the effects of these growth environments on the expression of osteoblast marker genes, on cell differentiation and on responses to factors that affect the rate of cell proliferation.

Results

MC3T3-E1 cells cultured in 3D collagen gels mineralize the extracellular matrix earlier than do cells in 2D cultures

Differentiation of the osteoblastic cell line MC3T3-E1 was studied in 2D cultures on plastic and in 3D cultures in collagen gels. Cells were transferred to osteoblast differentiation medium on day 3, when the 2D cultures were confluent. Sparse mineralization was first visible on day 17 in the 2D cultures (**Figure 1a**), whereas the 3D cultures were strongly positive by day 14 (**Figure 1b**). The percentage of mineralized area was determined by image analysis (**Figures 1c,d**). Although the overall level of mineralization cannot be accurately compared between the two culture systems because of the limitations of analysis of 2D images of 3D structures, our results indicate that differentiation was accelerated in the 3D collagen gels.

Primary osteoblasts cultured in 3D collagen gels show increased expression of osteoblast genes

In order to further characterize osteoblasts in 3D collagen gels in comparison with cells growing in 2D cultures, primary rat osteoblasts cultured in parallel in the two systems were harvested at different time points during the 24-day differentiation assay, and gene expression was determined by real-time PCR. As shown in **Figure 2**, a number of osteoblast marker genes had much higher levels of expression in 3D cultures, in particular at the earlier time points. Thus, on day 3 and day 7 the expression level of the late-osteoblast marker, osteocalcin (*Bglap*), was >100-fold higher in 3D compared with 2D cultures. Other late markers, bone sialoprotein (*Ibsp*) and dentin matrix protein 1 (*Dmp1*), showed a similar pattern with more modest differences between levels of expression in 2D and 3D cultures. Alkaline phosphatase (*Alpl*) expression was also increased in 3D cultures on days 3 and 7, whereas collagen type I alpha 1 (*Col1a1*) was only slightly elevated in 3D cultures. Of the two osteoblast transcription factors tested, osterix showed more than 10-fold increase in 3D cultures at the early time points, whereas Runx2 expression was about twofold higher in 3D than in 2D. Osteoblasts cultured in 3D had higher levels of expression of *Tnfsf11* (RANKL) than did cells in 2D, but *Tnfrsf11b* (osteoprotegerin) levels were similar in the two culture systems. The increased expression levels of osteoblast genes on day 3 (**Figure 2**) suggested that these genes were induced early in the 3D cultures, prior to the change to differentiation medium. To test this possibility, gene expression was studied in primary rat osteoblasts cultured in maintenance medium for 24 h. As shown in **Figure 3**, activation of osteoblast genes in the 3D collagen gels was evident as early as 24 h after seeding the cells. Eight of the nine genes tested showed increased levels of expression in the 3D cultures, whereas the level of osteoprotegerin expression was similar in 2D and 3D cultures.

Induction of osteoblastic genes in cells cultured in 3D collagen gels is not reproduced in cells cultured on collagen-coated plates

To distinguish between the effect of the 3D structure of the collagen gels, and that of collagen itself, primary rat osteoblasts were seeded on plastic, on collagen-coated plates (2D collagen) and in 3D collagen gels, and gene expression was tested 24 and 72 h after seeding. The expression of the late-osteoblast markers *Bglap*, *Dmp1* and *Ibsp* showed clearly that the induction of these genes was dependent on the 3D configuration of the gels (**Figure 4**), as cells growing on collagen-coated plates had very similar levels of expression to cells growing on uncoated plastic.

Primary osteoblasts in 3D cultures respond to factors that affect proliferation with higher sensitivity than do cells in 2D cultures

Proliferation of primary rat osteoblasts growing in 2D on plastic or in 3D collagen gels was determined by measuring thymidine incorporation into cells that were seeded at similar densities. Growth factors that induce osteoblast proliferation—Lactoferrin,[10] TGF-β[11] and PDGF[12]—induced a dose-dependent increase in thymidine incorporation into the cells in both culture systems (**Figures 5a–c**). However, the proliferative effects were much greater in cells growing in 3D collagen gels than in cells in 2D cultures. Treatment of the primary osteoblast cultures with the tyrosine kinase inhibitor imatinib mesylate, which inhibits osteoblast proliferation,[13] demonstrated that the cells in the 3D collagen gels are more sensitive to the inhibitory effect (**Figure 5d**). The effect of imatinib mesylate was detectable at lower doses in the 3D system, and in each of the concentrations tested the inhibition of proliferation was significantly greater in 3D cultures than in the 2D controls.

Discussion

Our study characterized the growth and differentiation of primary osteoblasts and MC3T3-E1 cells growing in 3D collagen gels. Accelerated differentiation occurred in cells seeded in 3D collagen gels in comparison with 2D cultures on plastic, as indicated by the earlier mineralization and high expression of osteoblast marker genes *Bglap, Dmp1, Ibsp, Alpl, Col1a1, osterix* and *Runx2* in the 3D cultures. Mineralization was clearly visible in the 3D cultures on day 14, whereas in the 2D cultures low levels were first visible on day 17. In contrast to determining the time point when mineralization first appeared, the overall level of mineralization could not be directly compared between the two culture systems by the image analysis method used here. For accurate quantitative analysis of matrix mineralization by osteoblasts in 3D cultures, methods such as microcomputed tomography or staining of histological sections are required.

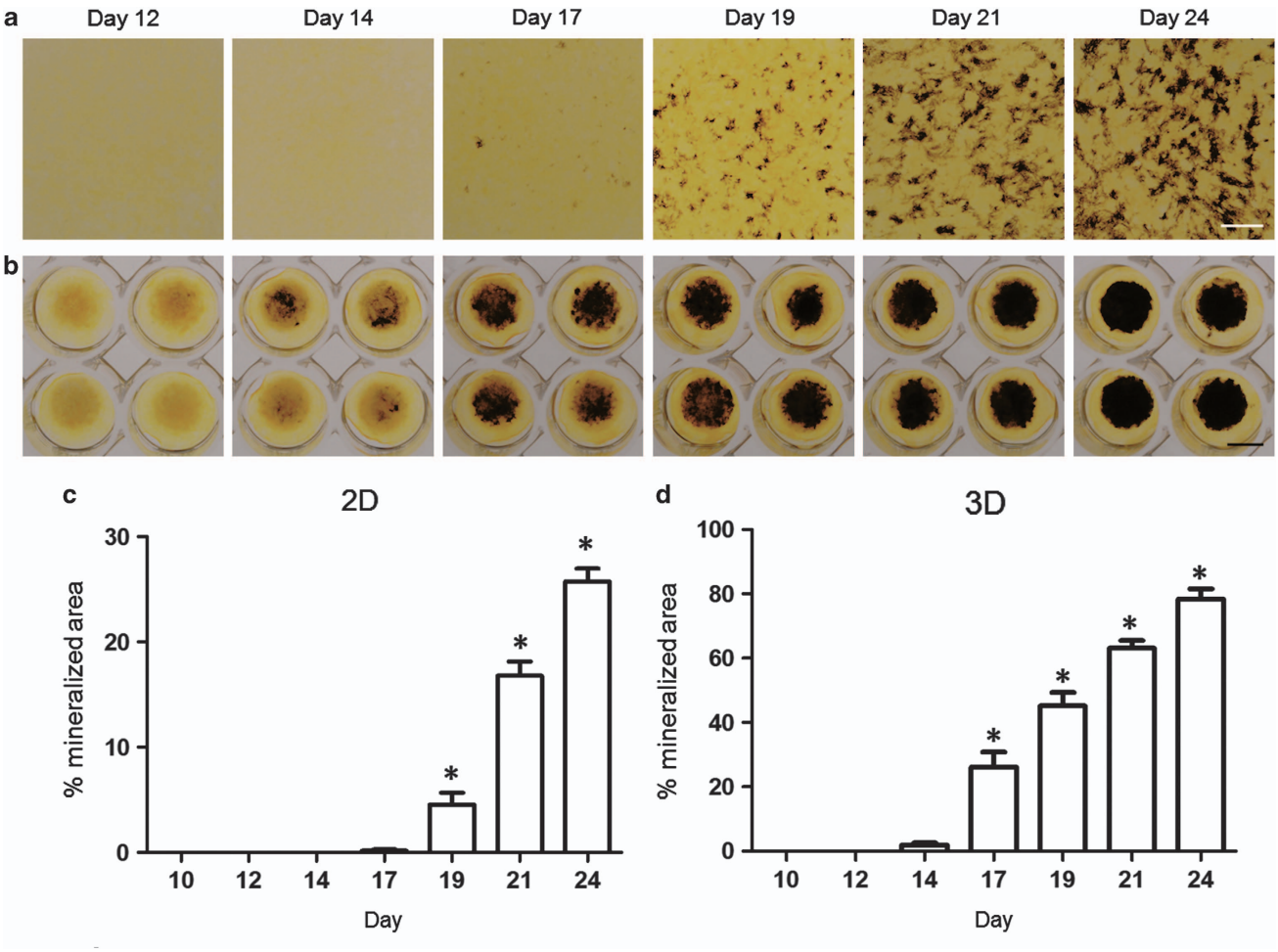

Figure 1 Mineralization occurs earlier in 3D cultures. MC3T3-E1 cells cultured in mineralizing conditions were fixed and stained with von Kossa at the indicated time points. Images from representative wells of 2D cultures (**a**) and sets of wells in 3D cultures (**b**) are shown. Scale bars represent 5 mm. Image analysis was used to quantify the 2D mineralized area and the percentage mineralization is shown for 2D (**c**) and 3D (**d**) cultures. Results are expressed as mean ± s.e.m.; $^{*}P<0.01$ vs day 10.

Osteoblastic gene expression was already markedly elevated in cells growing in 3D cultures 24 h after seeding in maintenance medium. At earlier time points, at 2, 4 or 6 h after seeding, the levels of osteoblast gene expression were similar in cells growing in 2D and 3D collagen cultures (data not shown). In a number of previous studies,[3,8,14,15] collagen gels were formed first and osteoblasts were seeded on top of the gels, allowing for the analysis of cell migration into the gels. Interestingly, while osteoblasts migrated into the gels and formed a 3D cell network, fibroblasts seeded on top of the gels migrated through them and formed a monolayer on the plastic underneath.[7] In our experiments the cells were mixed with the collagen solution before it set as a gel, and hence the cells settled directly into the 3D environment. An inherent methodological difficulty in the study of cells in 2D vs 3D cultures is the fact that the cell densities in the two growth environments are not directly comparable. The densities used in our study were based on preliminary experiments that tested various cell densities and identified those that appeared to provide the best match between the two culture systems. The seeding density for the collagen gels allowed for initial proliferation of the cells within the gels. Additional parameters that varied between the 2D and 3D cultures may have potentially contributed to the accelerated osteoblast differentiation in 3D cultures: (1) cell shape: in 3D gels the cells were able to assume a more *in vivo*-like morphology in comparison with the flat, adherent osteoblasts on the surface of the tissue culture dishes; (2) cell–cell interactions: the 3D configuration allows for more extensive cell–cell interactions; and (3) cell–matrix interaction, specifically the presence of collagen I. Comparison of gene expression in osteoblasts growing in the 3D collagen gels with cells cultured in 2D on collagen-coated plates excluded the possibility that the collagen itself induced the early gene expression, leaving the spatial properties and the cell–cell interactions as the likely factors that promote osteoblast differentiation.

Although most *in vitro* studies of osteoblast growth and function are routinely performed in tissue culture dishes in 2D, 3D cultures not only provide a better representation of the *in vivo* environment but also offer a number of technical advantages. Here, we showed that osteoblasts in 3D cultures are more sensitive to factors that modify proliferation rate and that differentiation is accelerated in 3D collagen gels. Therefore, when testing the effects of various factors on osteoblasts, less of the factor is required in 3D in comparison

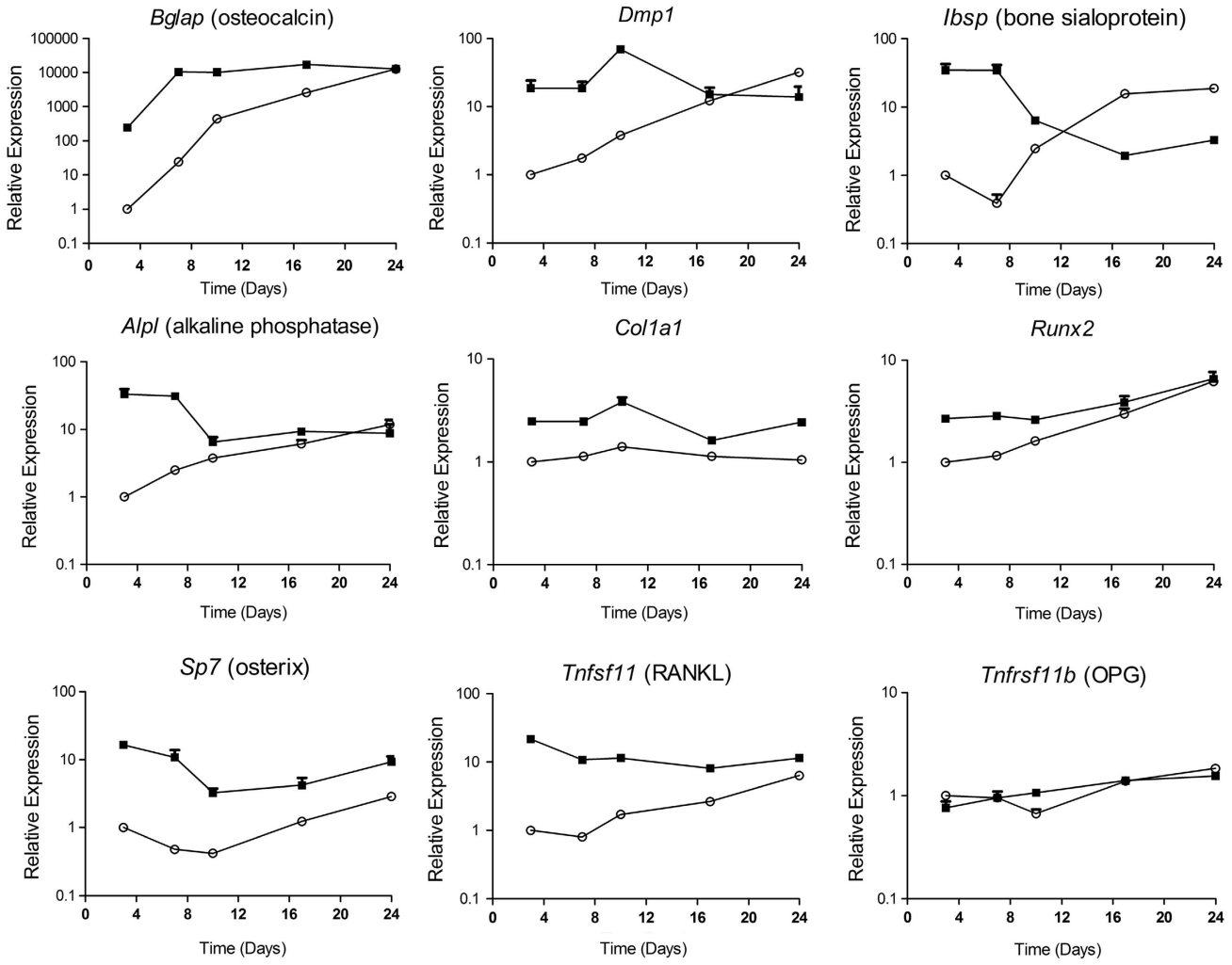

Figure 2 Gene expression in cultures of differentiating primary rat osteoblasts. Relative expression levels of nine genes were determined by multiplex real-time PCR, with the target genes normalized to the endogenous control, 18S ribosomal RNA. The results are presented relative to the expression of each gene in the 2D cultures on day 3, which is the day the 2D cultures reached confluence and cells were transferred to mineralization medium. The 2D cultures are represented by open circles and 3D cultures by black squares. The graphs show mean values ± s.d. of two independent experiments. Analysis by two-way ANOVA showed a significant effect of culture conditions (2D vs 3D) for eight of the genes ($P<0.01$), whereas osteoprotegerin (OPG) showed no significant difference.

with 2D. The lower concentrations required for the proliferation assays are likely to better represent the physiological concentrations that are active in the *in vivo* environment. Moreover, in studies of overexpression or knockdown of gene products, transient expression might prove to be sufficient in 3D cultures because of the accelerated differentiation, and stable transfections for long-term studies might not be required. In our study, thymidine incorporation indicated that the basal rate of proliferation within the collagen gels was lower than that of the cells on plastic. This difference might have contributed to the increased sensitivity of the cells in 3D to the factors that affect proliferation.

The 3D cultures described here could be used in the future for the investigation of more advanced stages of osteoblast differentiation, including the differentiation of osteoblasts into osteocytes. Advanced stages of osteoblast differentiation *in vitro* have been studied by a number of groups that used similar 3D cultures. In a recent study, MC3T3-E1 cells and primary mouse osteoblasts cultured on top of type I collagen gels for up to 35 days migrated into the gel, lay mineral in the collagen and differentiated into osteocyte-like cells that were present within lacunae.[15] Similarly, MC3T3-E1 cells cultured in collagen gels for up to 56 days formed bone-like tissue, with calcified, newly synthesized collagen type I matrix and osteocyte-like cells embedded within the matrix.[7] In addition, osteocyte-like cells developed in a different 3D system, where primary human osteoblasts grown in monolayers were mechanically folded and formed collapsed 3D structures, in which mineralized matrix and osteocyte-like cells with elongated cell process were visualized after 47 days.[16] Human primary osteoblasts were also cultured for 60 days on 3D dense scaffolds made of 40 mg ml^{-1} collagen, which provide a surface that resembles the osteoid. Transmission electron microscopy was used to demonstrate cell–cell interactions through tight junctions, but, although cytoplasmic extensions were shown to penetrate the dense collagen matrix, the cells themselves were aligned on top of the surface and did not penetrate the matrix.[14]

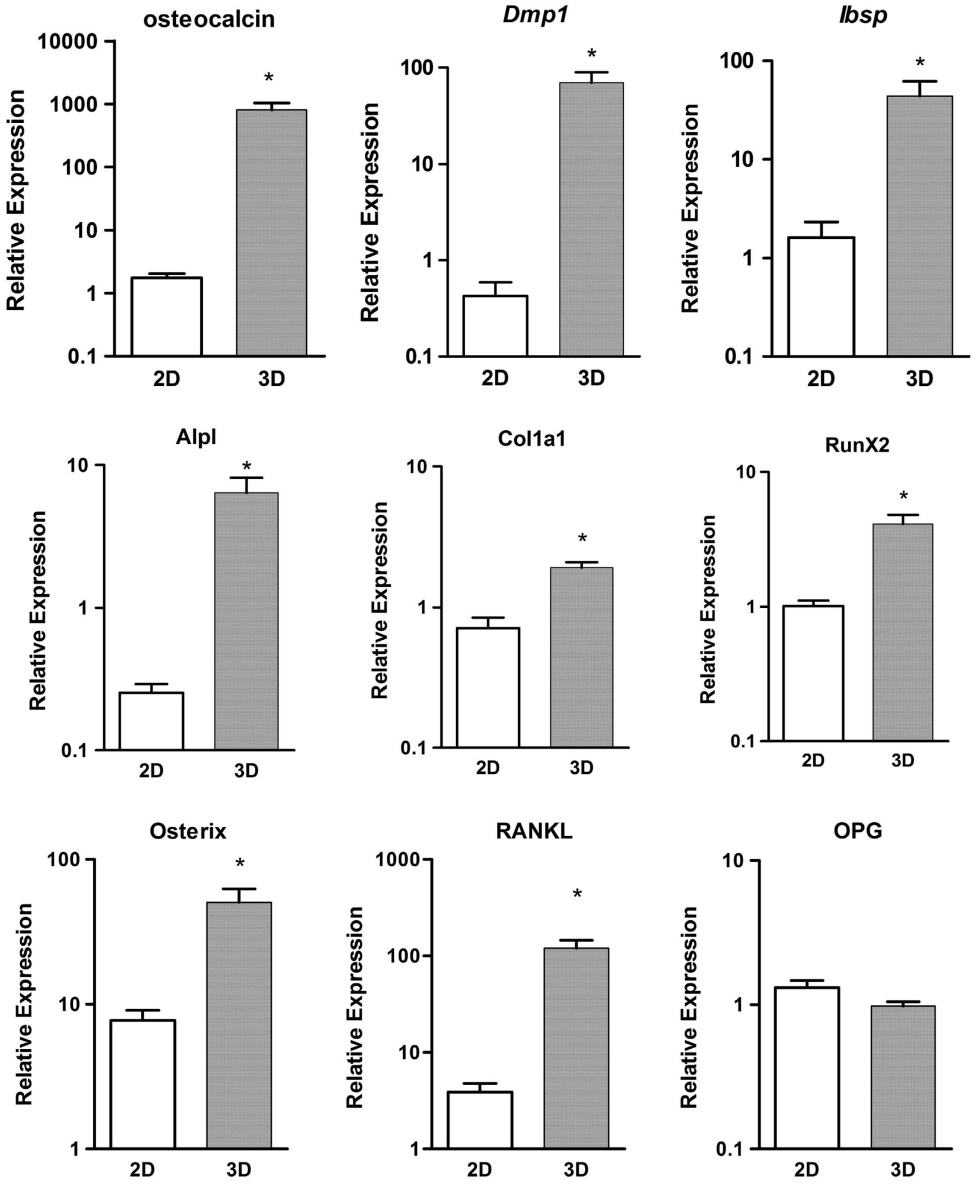

Figure 3 Gene expression in primary rat osteoblasts cultured in maintenance medium. Cells were seeded in 2D on plastic (white bars) and in 3D collagen gels (gray bars). RNA was extracted 24 h after seeding, and the relative expression levels were determined by multiplex real-time PCR with 18S ribosomal RNA as endogenous control. Data are presented as means ± s.d.; $*P<0.05$ vs 2D.

Our findings are relevant to recent work involving the use of scaffolds in bone defects. Scaffolds of natural or synthetic materials, with or without osteogenic cells cultured in them prior to their introduction into the area of bone defect, have an excellent potential for use in the support of bone regeneration. Bone tissue is capable of self-regeneration following injury, provided the defect is below a certain critical size. Autologous bone grafts are being used for the repair of large bone defects that occur as a consequence of fracture, tumor, infection and congenital deformity, but harvest of bone for grafting is often associated with donor site morbidity, and only a limited quantity of bone can be used.[3,17] Tissue engineering is considered a promising alternative approach for the repair of bone defects, aiming to restore both the structural integrity and the normal remodeling activity of bone. The development of effective scaffolds is crucial for the successful engineering of new bone tissue. The scaffolds have to provide an osteoinductive environment for cells to migrate, proliferate, differentiate and promote new bone formation, as well as provide mechanical support during the bone regeneration.[17] Tissue engineering for restoration of bone is also considered for maxillofacial use for the regeneration of alveolar bone after trauma, tumor resection and periodontal infection.[18] A scaffold made of a combination of an absorbable collagen sponge carrier and recombinant human bone morphogenetic protein-2 (INFUSE Bone Graft), which has been shown to induce bone formation, is currently Food and Drug Administration (FDA)-approved as a bone graft substitute.[19]

Our study characterized the growth of osteoblastic cells in 3D collagen gels and in 2D cultures on plastic, directly comparing osteoblast differentiation and matrix mineralization over a 24-day period. One of our novel findings is that

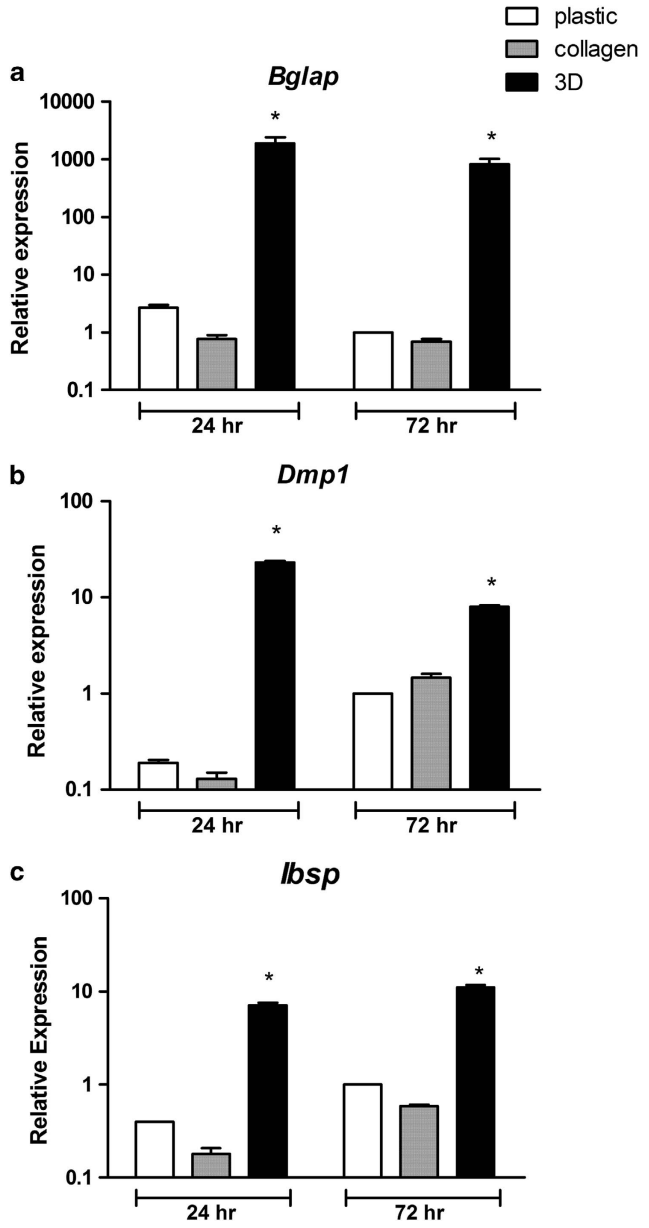

Figure 4 Gene expression in primary rat osteoblasts cultured on plastic, on collagen-coated plates and in 3D collagen gels. Cells were seeded in 2D on plastic (white bars), on collagen-coated plates (gray bars) and in 3D collagen gels (black bars) and RNA was extracted 24 h and 72 h after seeding. The expression levels of the genes were determined by real-time PCR, using 18S ribosomal RNA as endogenous control. Data are presented as means ± s.d.; $^*P < 0.05$ vs cells on uncoated plastic at the same time point.

genes that are usually considered late-osteoblast markers (*Bglap*, *Ibsp*) are induced very rapidly in 3D cultures, and are already upregulated 24 h after seeding. The detailed analysis that demonstrated enhanced sensitivity of cells in 3D cultures to a number of agents that affect cell proliferation is also novel, and identifies the collagen gel cultures as a useful tool for the study of osteoblast growth *in vitro*. Further investigations will focus on the morphology of osteoblastic cells cultured in 3D collagen gels and will aim to elucidate the molecular mechanisms involved in the accelerated differentiation.

Materials and Methods

Reagents

Culture media, fetal calf serum (FCS) and antibiotics were purchased from Life Technologies (Gibco, Carlsbad, CA, USA). Bovine serum albumin was from Immuno-Chemical Products Ltd (Auckland, New Zealand) and rat collagen type I from BD Biosciences (San Jose, CA, USA). L-ascorbic acid-2-phosphate (A2P), β-glycerophosphate and transforming growth factor β were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Bovine lactoferrin was isolated and purified as previously described.[10] Platelet-derived growth factor was from R&D Systems (Minneapolis, MN, USA) and imatinib mesylate was supplied by Novartis Pharma AG (Basel, Switzerland).

Cell culture

All protocols involving use of animals have been approved by the University of Auckland Animal Ethics Committee. Primary osteoblasts were isolated from calvarias of E20 male and female Wistar rats (University of Auckland Vernon Jansen Unit) as previously described[10] and maintained in 10% FCS/Minimum Essential Media (MEM) with 5 μg ml^{-1} A2P. Murine pre-osteoblastic MC3T3-E1 subclone 4 cells were purchased from ATCC (Cryosite Distribution, Lane Cove, NSW, Australia, method of authentication STRS analysis) and maintained in 10% FCS/MEM and 1 mM sodium pyruvate. Cells were used between passage numbers 15 and 22. For 3D collagen cultures, rat collagen type I was neutralized with 1 M NaOH (0.023 × collagen volume) and diluted to a final concentration of 3 mg ml^{-1}. Cells suspended in culture medium were seeded in 50 μl collagen gels at a density of 2×10^5 cells ml^{-1} (10^4 cells per gel). Gels were allowed to set at 37 °C for 1 h before addition of culture medium. Cells seeded on plates coated with 0.15 mg ml^{-1} collagen solution were used in the experiments that compared gene expression in cells in 2D and 3D collagen cultures.

Differentiation assay

MC3T3-E1 cells were trypsinized and seeded in six-well plates at a density of 5×10^4 cells per well in maintenance medium. Once confluent, medium was changed to 15% FCS/αMEM + 50 μg ml^{-1} A2P + 10 mM β-glycerophosphate (day 3). Collagen gel cultures were seeded in 48-well plates as described above and media changes performed in parallel with the 2D cultures. Cells were fixed in 10% formalin at the time points indicated and stained for mineral using von Kossa stain. Cultures were visualized under an Olympus SZ61 Stereo microscope (Olympus Australia Pty Ltd, Notting Hill, VIC, Australia) and photographed with an Olympus DP72 digital camera (Olympus Australia Pty Ltd). 2D mineralized area was determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Experiments were repeated three times, with 3–8 wells per group.

Proliferation assay

Primary rat osteoblasts were seeded in 5% FCS/MEM with 5 μg ml^{-1} A2P in 48-well plates in either culture medium or collagen gels at a density of 10^4 cells per well. After 24 h, cells were growth arrested in MEM, 5 μg ml^{-1} A2P and 0.1% bovine serum albumin for 24 h, and then fresh medium, experimental compounds and [^{3}H]-thymidine were added for 24 h. At the end

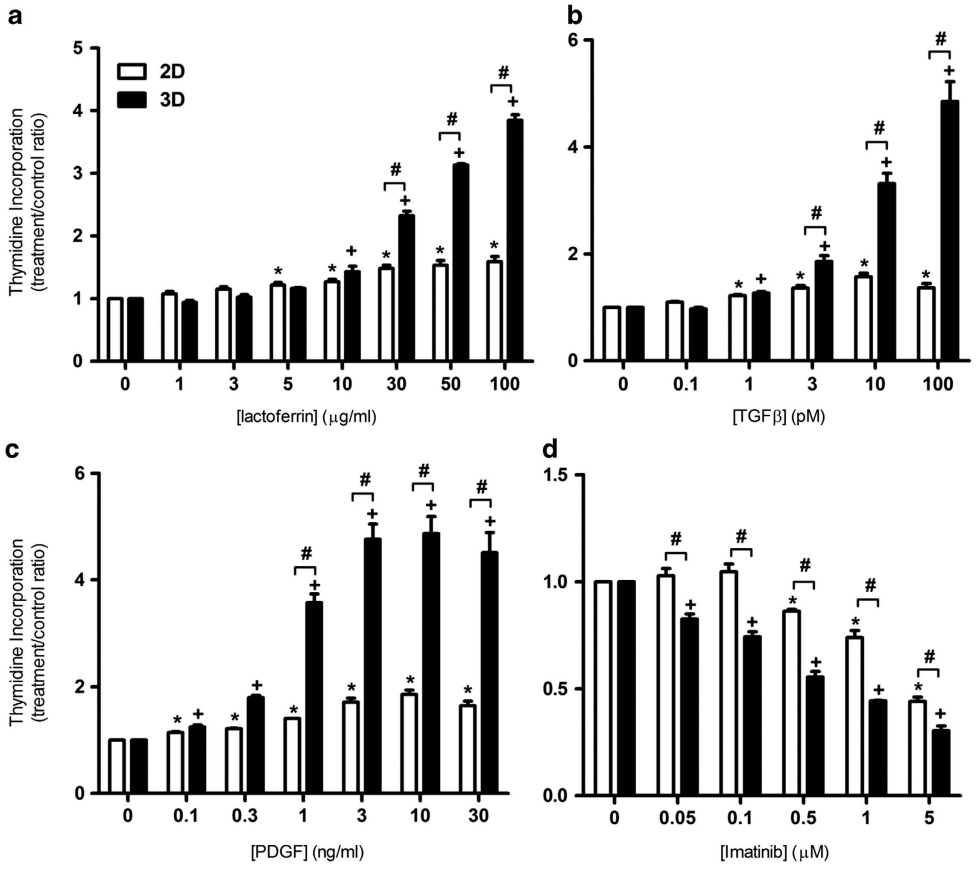

Figure 5 The response of primary osteoblasts to factors that affect proliferation is enhanced in 3D cultures. [^{3}H]-Thymidine incorporation was measured in primary rat osteoblasts cultured on 2D plastic (white bars) and in 3D collagen gels (black bars). The cells were treated with increasing concentrations of the osteoblast-active factors lactoferrin (**a**), transforming growth factor β (TGFβ) (**b**), platelet-derived growth factor (PDGF) (**c**) and imatinib mesylate (**d**). The graphs present the means ± s.e.m. from three to four pooled biological replicates. $^*P<0.05$ vs 2D control; $^+P<0.05$ vs 3D control; $^\#P<0.05$ vs 2D at the corresponding concentration.

of the experiment thymidine incorporation was quantified. Each group had six wells and experiments were performed 3–4 times.

RNA extraction and real-time PCR

RNA was extracted from 2D cultures using the RNeasy mini kit (QIAGEN Pty Ltd, Melbourne, VIC, Australia). For 3D cultures, collagen gels were dissolved in RLT buffer (QIAGEN Pty Ltd) and incubated at 55 °C with 0.2 mg ml^{-1} proteinase K (Life Technologies) for 15 min. Thereafter, 0.8 volumes of 80% ethanol were added and RNA extraction was performed with the RNeasy mini kit. Genomic DNA was removed from all RNA preparations with the RNase-free DNase set (QIAGEN Pty Ltd). All cultures harvested from day 14 onwards were treated with 150 μg ml^{-1} collagenase type 2 (Sigma-Aldrich) for 30 min before collection. Complementary DNA was synthesized with Superscript III (Life Technologies) and used for multiplex real-time PCR in ABI PRISM 7900HT Sequence Detection Systems (Life Technologies). Primers and probe sets were purchased as TaqMan Gene Expression Assays (Life Technologies). All probes used to detect target genes were labeled with FAM (Life Technologies), and the 18S ribosomal RNA endogenous control probe was VIC (Life Technologies) labeled. The ΔΔCt method was used to calculate the relative levels of expression. Each experiment was performed three times and real-time PCR was repeated at least twice in duplicate for each experiment.

Statistical analysis

Data were analysed using GraphPad Prism (v5.04; GraphPad Software, San Diego, CA, USA). The Mann–Whitney test, one-way analysis of variance (ANOVA) with *post hoc* Tukey's or Dunnett's tests and two-way ANOVA with *post hoc* Bonferroni's test were used to analyze the data as appropriate for each experimental design.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

The work was supported by a grant from The Health Research Council of New Zealand.

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