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EXPRESSION OF MINERALIZED TISSUE-ASSOCIATED PROTEINS IS HIGHLY UPREGULATED IN MC3T3-E1 OSTEOBLASTS GROWN ON A BOROSILICATE GLASS SUBSTRATE

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ABSTRACT

Melt- and sol-gel derived bioactive glasses (BGs) are promising bone implant materials with biocompatibility superior to currently used inert titanium and ceramic implants. Recent studies indicate that the addition of boron to BGs may further enhance bone formation. While the beneficial effects of boron on bone formation and maintenance have been recognized, it is still unclear how boron, as an ultra-trace element (<1 ppm concentration), actually stimulates bone formation. We thus tested *in vitro* by examining cell adhesion, proliferation and gene-expression whether MC3T3-E1 bone-precursor cells seeded on borosilicate cover glasses would exhibit signs of differentiation into mature bone cells. As control, cells were seeded on boron-free substrates including a soda-lime silicate glass, 45S5 Bioglass®, a sol-gel derived calcium silicate BG, and tissue culture plastic. Results indicated that MC3T3-E1-cells grown for 17 days on borosilicate cover glasses up-regulated the expression of bone-specific marker proteins more significantly than on any of the other tested boron-free substrates. Since typical borosilicate glasses are expected to dissolve only insignificantly in tissue culture media, our results suggest that either extremely low concentrations of borate ions in solution (well below ppm concentrations) are sufficient to stimulate bone cell differentiation, or more likely that bone-precursor cells are able to 'sense' and react to boron that is present in the substrate. Our results shed new light on the potential role of boron on bone cell differentiation.

INTRODUCTION

Although bone implants date back to 600 A.D., the search for an ideal bone-replacement material continues[1]. In fact, with increasing life expectancy destructive lesions of bones, whether due to disease or trauma, are becoming increasingly common. Some such conditions include benign and malignant bone tumors, bone fractures, hip failure, middle ear deafness and periodontal diseases. In all cases, the ultimate goal is to replace the defective or missing bone tissue with a functioning material that will last a patient's lifetime. Bone implant materials research was revolutionized when Hench[2] discovered Bioglass® in the 1960's. Cells were shown to adhere to this glass of composition $24.5Na_2O$- $24.5CaO$-$6P_2O_5$-$45SiO_2$ (in wt.%). Also it was shown to develop a layer of hydroxyapatite ($Ca_5(PO_4)_3(OH)$) on its surface *in vivo*, the major mineral constituent of bone, making the material bioactive. Next-generation bioactive glasses, or TAMP (tailored amorphous multi-porous) scaffolds with interconnected pores of various size that promote cell adhesion and internal scaffold colonization have now been developed, using novel melt-quench and sol-gel derived techniques.[3,6, 7]. Furthermore, bioactive glasses have been found to resorb over time in the body and are replaced by natural bone; and ions (especially silicon) leaching from bioglass have been attributed to stimulate bone precursor cells to differentiate into mature, calcified matrix secreting osteoblasts[8]. These unique characteristics of bioglasses are not shared by any other currently used bone-replacement material making them superior materials for bone implants.

The element boron exists in low-abundance in the Earth's crust, mainly as water-soluble borate minerals. Borates have low toxicity in mammals (similar to table salt), but are more toxic to arthropods. Boron is an essential micronutrient of plants (<1 ppm concentration), required primarily for maintaining the integrity of cell walls[9]. In mammals, boron was found to play a crucial role in osteogenesis and maintenance of bone[10]. Under conditions of boron deficiency, development and regeneration of bone is negatively influenced.[11-13] However, surprisingly little is known on how this ultra-trace element exerts its beneficial health effects. Boron may interact with steroid hormones, and thus is involved in the prevention of calcium loss and bone de-mineralization[14]. It has also been related to vitamin D function by stimulating growth in vitamin D deficient animals[15]. Surprisingly, no recommended levels of boron have been set by FDA for intake in humans, only upper limits (20 mg/day); and due to the lack of data in humans this limit was extrapolated from animal studies[16]. Despite the lack of functional data, scientists began to evaluate the role of boron on the differentiation of osteoblasts and the formation of bone when added to cell culture media, or bioglass implants. Although somewhat contradicting reports have been published[17, 18], the beneficial effect of boron on osteoblast differentiation and bone formation seems compelling[1922]. We thought to test a potential beneficial effect of boron *in vitro* by seeding MC3T3-E1 osteoblast precursor cells on boron-containing borosilicate coverslip glasses widely used in light microscopic applications. In control experiments, cells were seeded on boron-fee substrates including soda-lime silicate glass, 45S5 and sol-gel derived bioglasses, and tissue culture (TC) plastic. Cell adhesion, proliferation, and potential differentiation were examined on all substrates by analyzing cell morphology, quantitative proliferation, and by evaluating the expression profile of a set of bone-cell specific (RunX2, BSP1, BGLAP) and of other osteoblast-relevant marker proteins (Col1A, ALP, Cx43, GAPDH) known to be upregulated specifically during osteoblast differentiation[23, 24] using quantitative real-time polymerase chain reaction (qRT-PCR) analyses.

MATERIALS AND METHODS

Substrate Materials

The 'Deutsche Spiegelglas' (round, 12 and 18 mm diameter, 0.13-0.17 mm thickness; Carolina, Item. # 633029 and 633033) borosilicate glass was used as the main substrate of this study. It is commonly used as microscope coverslips in biological studies. Its composition was analyzed using a high resolution X-ray photoelectron spectrometer (Scienta ESCA 300). To obtain overall bulk concentration of constituent oxides, the sample was fractured inside the ultra-high vacuum of the spectrometer and analyzed without exposure to ambient. In addition, to compare its effect on bone forming cells, four different boron-free substrates were used: classic 45S5 Bioglass®, a sol-gel derived $30CaO\text{-}70SiO_2$ (in wt.%) glass, commercial soda lime silicate glass slides (VWR), and tissue culture plastic (Falcon). The 45S5 Bioglass® was prepared by the standard melt-quench method as described elsewhere,[25] with batch composition: $24.4Na_2O–26.9CaO–2.6P_2O_5–46.1SiO_2$ (mol%). The procedure for making calcium silicate glass by the sol-gel method is given in reference 4.

Cell Lines, Culture Conditions, and Morphological Analyses

MC3T3-E1 subclone E-4 newborn mouse calvarial bone pre-osteoblasts (ATCC CRL-2593) were cultured under standard conditions in alpha-Modified Eagle's Medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 100 U/ml penicillin/streptomycin in a cell culture incubator at 37˚C, 5% CO_2-atmosphere, and 100% humidity as recommended by the distributor. Cells were subcultured at a ratio of 1:10 once or twice a week on regular tissue culture (TC) plastic when cells reached confluency. For all analyses cells at a low passage number (<20) were seeded on 'Deutsche Spiegelglas' borosilicate cover glasses at a density of 10.000 cells/cm^2 that were

sterilized by flaming in ethanol before being placed into 3.5 cm diameter culture dishes, or 24-well culture plates. Control cells were seeded at comparable densities on soda lime glass (microscope slides cut into 1 cm^2 pieces), discs prepared from 45S5 melt-derived bioglass[25], sol-gel derived bioglass[4, 5, 6, 7], or tissue culture plastic. To replenish nutrients, and dilute out potentially toxic cellular waste products, one-half of the culture medium was exchanged every other day. Cells growing on borosilicate cover glasses and on TC plastic were examined and imaged 1 hour, 2 and 5 days post seeding using an inverted microscope (Nikon TE2000), phase-contrast illumination, a 20x long-distance objective, and a SPOT RT CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI).

Quantitative Cell Proliferation Analyses

Cells proliferating on 4 borosilicate cover slips each for 4, 11, and 17 days, were stained with the blue, live-cell compatible nuclear stain Hoechst 33342 (Molecular Probes/Invitrogen) for 10 minutes at 1 μg/ml (prepared from 10 mg/ml stock in water) at 37˚C. Three representative images per cover glass were acquired using fluorescence illumination and a DAPI filter cube. Immediately after imaging (within 15 minutes after staining) cells were processed for qRT-PCR analyses. Cell nuclei on the acquired images were counted, average cell counts (including standard deviations for the parallel cover glasses) calculated, and graphed over time using Microsoft Excel software.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analyses

The mRNA expression profile for 6 marker proteins (see Results) was analyzed in MC3T3-E1 cells when seeded (day 0), and grown for 4, 11, and 17 days on borosilicate cover glasses. Cells were lyzed and total cellular RNAs were isolated using the SYBR Green Cells-to-Ct kit (Applied Biosystems). mRNAs were reverse-transcribed using oligo-dT primer and Reverse Transcriptase (Superscript III, Invitrogen). Triplicates for 3-4 cover slips per time point, either analyzed separately, or after pooling cell lysates were analyzed on a model 7300 Thermocycler (Applied Biosystems) using sets of custom-designed oligonucleotides that corresponded to the mRNAs encoding the relevant proteins. GAPDH was analyzed in parallel and used as expression reference. Fluorescence signal detection within 35 PCR cycles was considered significant. mRNA level fold-change of relevant proteins on days 4, 11, and 17 was normalized against GAPDH expression, compared to day 0, and plotted using Microsoft Excel software. Cells growing on non-boron containing substrates (described above) were analyzed comparably.

RESULTS

Cell Type and Substrate Selection

To investigate the adhesion, morphology, proliferation, and especially the potential differentiation of precursor cells into mature bone cells when grown on boron-containing substrate, MC3T3-E1 newborn mouse calvarial bone pre-osteoblast cells were chosen[26]. This cell line is used as a generic osteogenic model cell line, since it has been shown to differentiate into mature, calcified matrix secreting osteoblasts under specific favorable conditions, such as the addition of phosphate and ascorbic acid[27], bone-morphogenic protein 2 (BMP-2)[28, 29], or borate ions[19, 22] to the culture medium. German 'Deutsche Spiegelglas'-type, high-quality microscope borosilicate cover glasses were chosen as cell-culture test substrate. In-house X-ray photoelectron spectroscopy revealed a chemical composition consisting of $5.7Na_2O$-$3.7K_2O$-$3.6ZnO$-$1.9TiO_2$-$7.0B_2O_3$-$78.1SiO_2$ (in wt.%).

Adhesion and Morphology of MC3T3-E1 Cells Grown on Borosilicate Cover Glasses

To investigate the adhesion and morphology of MC3T3-E1 pre-osteoblasts on borosilicate cover glasses, cells were harvested by trypsinisation and seeded at low density (~50 cells/mm^2) in 3.5

cm diameter tissue culture (TC) dishes containing, or not containing borosilicate cover glasses. Cells were examined and imaged 1 hour, 2 days, and 5 days post seeding. Representative images are shown in Figure 1. Cells on both substrates adhered and proliferated well, reaching complete confluency within 5 days post seeding (Figure 1, row 3). Cell adhesion occurred slightly faster on TC plastic (Figure 1, row 1), as indicated by a more adherent, spread-out morphology 1-hour post seeding. Cells developed their typical morphology within 1-2 days post seeding, without significant visible morphological differences. However, a slightly more spread-out phenotype with larger, more pronounced lamellipodial extensions was developed on the borosilicate substrate (Figure 1, row 2).

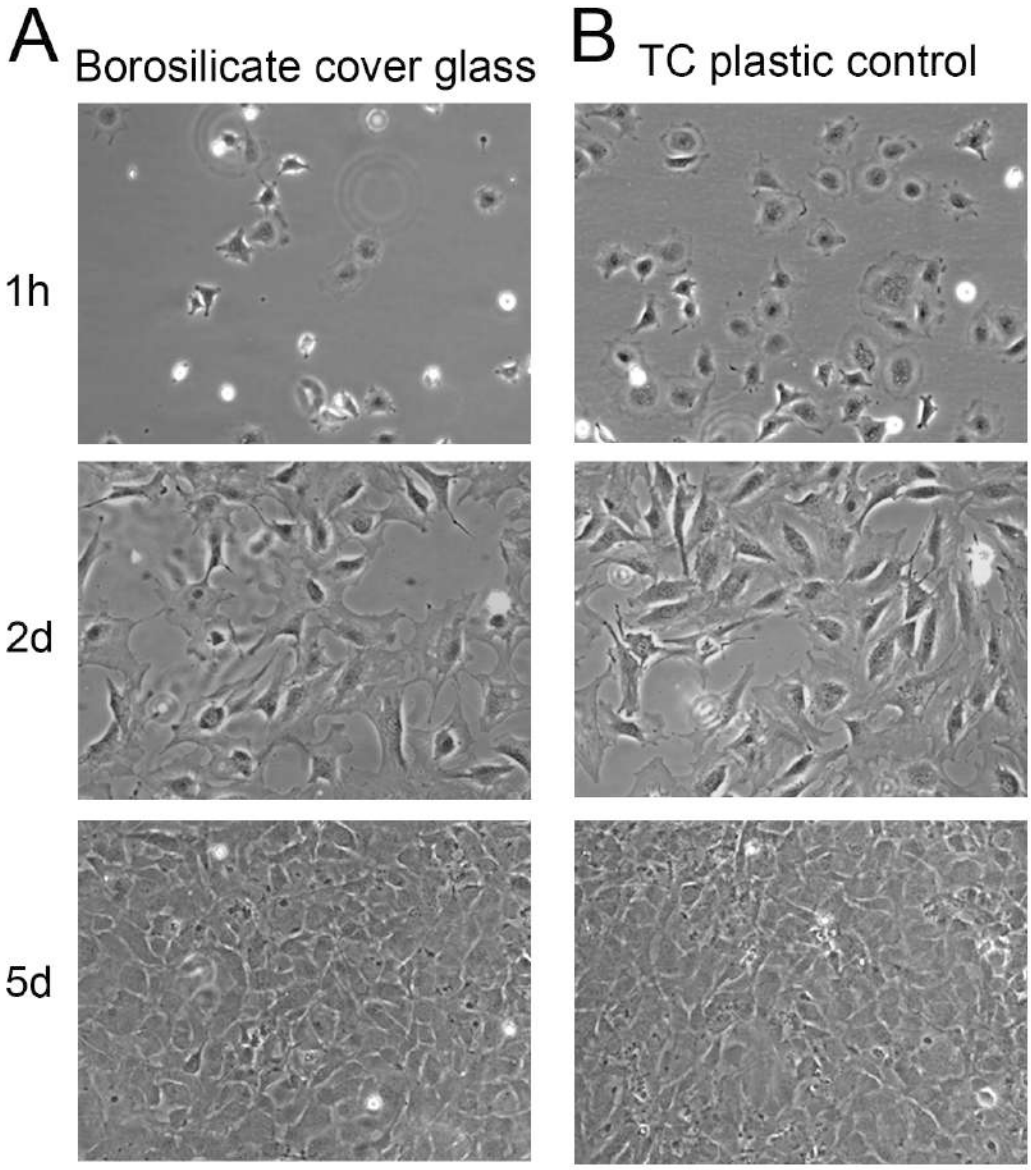

Figure 1: MC3T3-E1 osteoblast precursor cells were seeded (A) on borosilicate cover glasses and (B) on tissue culture (TC) plastic as control. Cell adhesion, morphology, and proliferation were evaluated at indicated times (see text for details).

Proliferation Rate of MC3T3-E1 Cells Grown on Borosilicate Cover Glasses

To quantitatively analyze the proliferation rate of MC3T3-E1 cells growing on borosilicate cover slips, they were stained with the blue, live-cell permeable nuclear stain Hoechst 33342 at

different days post seeding. Representative areas of 3-4 cover slips for each time-point were imaged, cell nuclei counted, and cell numbers were determined and plotted over time. Representative fluorescence images of cells with cell nuclei clearly visible and acquired at days 4, 11, and 17 are shown in Figure 2A. Note the increasing numbers of cell nuclei per imaged area over time. Cell proliferation as determined from the cell-counts is depicted in Figure 2B. Quantitative analysis showed that MC3T3-E1 cells exhibited a fast proliferation rate when growing on borosilicate cover slips, approximately doubling every 30 hours, and exiting logarithmic growth by day 6-7. A comparable fast proliferation rate was determined for MC3T3-E1 cells grown on TC plastic (compare Figure 1, and data not shown).

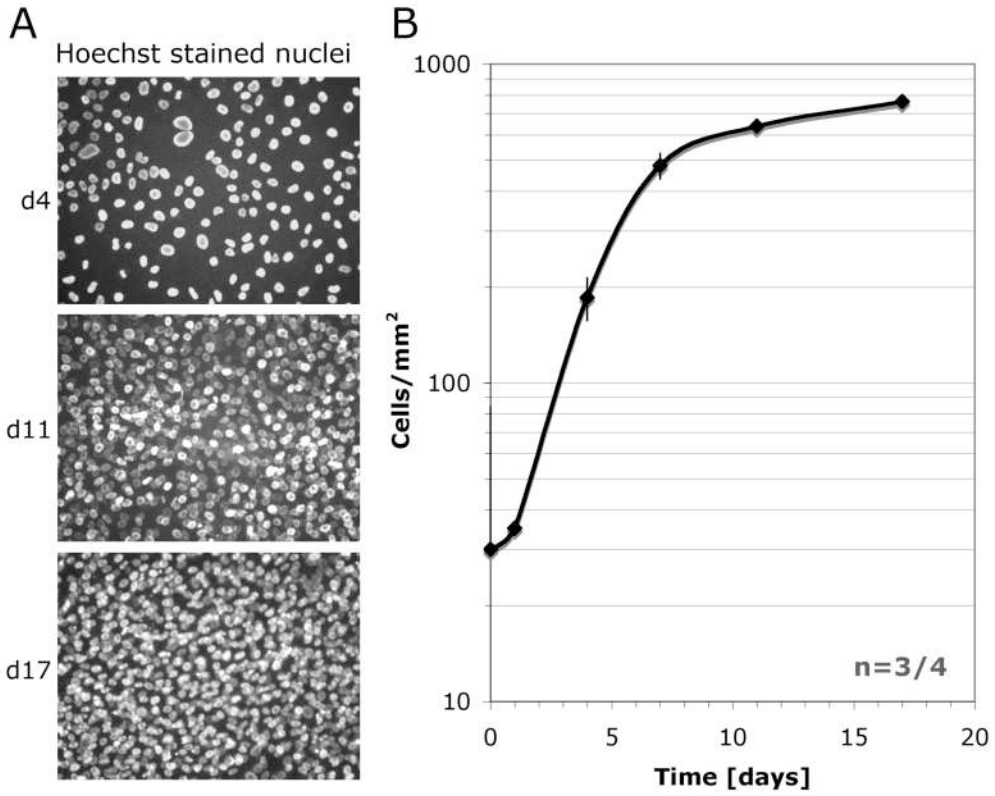

Figure 2: Proliferation of MC3T3-E1 pre-osteoblasts was determined by staining cell nuclei with the fluorescent dye, Hoechst 33342. Images were acquired with a 10x objective, nuclei counted, and cell numbers plotted over time. Representative images taken on days 4, 11, and 17 are shown in (A), a cell growth-curve extrapolated from the cell counts is shown in (B). Cells duplicated app. every 30 hours after experiencing an initial lag period, and before exiting the logarithmic growth phase at around day 6-7 (see text for further details).

Differentiation of MC3T3-E1 Cells Grown on Borosilicate Cover Glasses

To determine whether MC3T3-E1 osteoblast precursor cells would differentiate into mature, calcified matrix secreting osteoblasts when grown for longer time periods on borosilicate cover glasses, Hoechst-stained MC3T3-E1-cells were lyzed and processed for qRT-PCR analyses immediately after staining and imaging (within 15 minutes). Pilot experiments indicated that nuclear Hoechst-staining had no adverse effects on cell lysis, mRNA preparation, reverse transcription, or qRT-PCR analyses. This experimental procedure allowed for direct comparison of cell numbers determined in the proliferation assays with expression profiles detected by qRT-PCR analyses. A set of

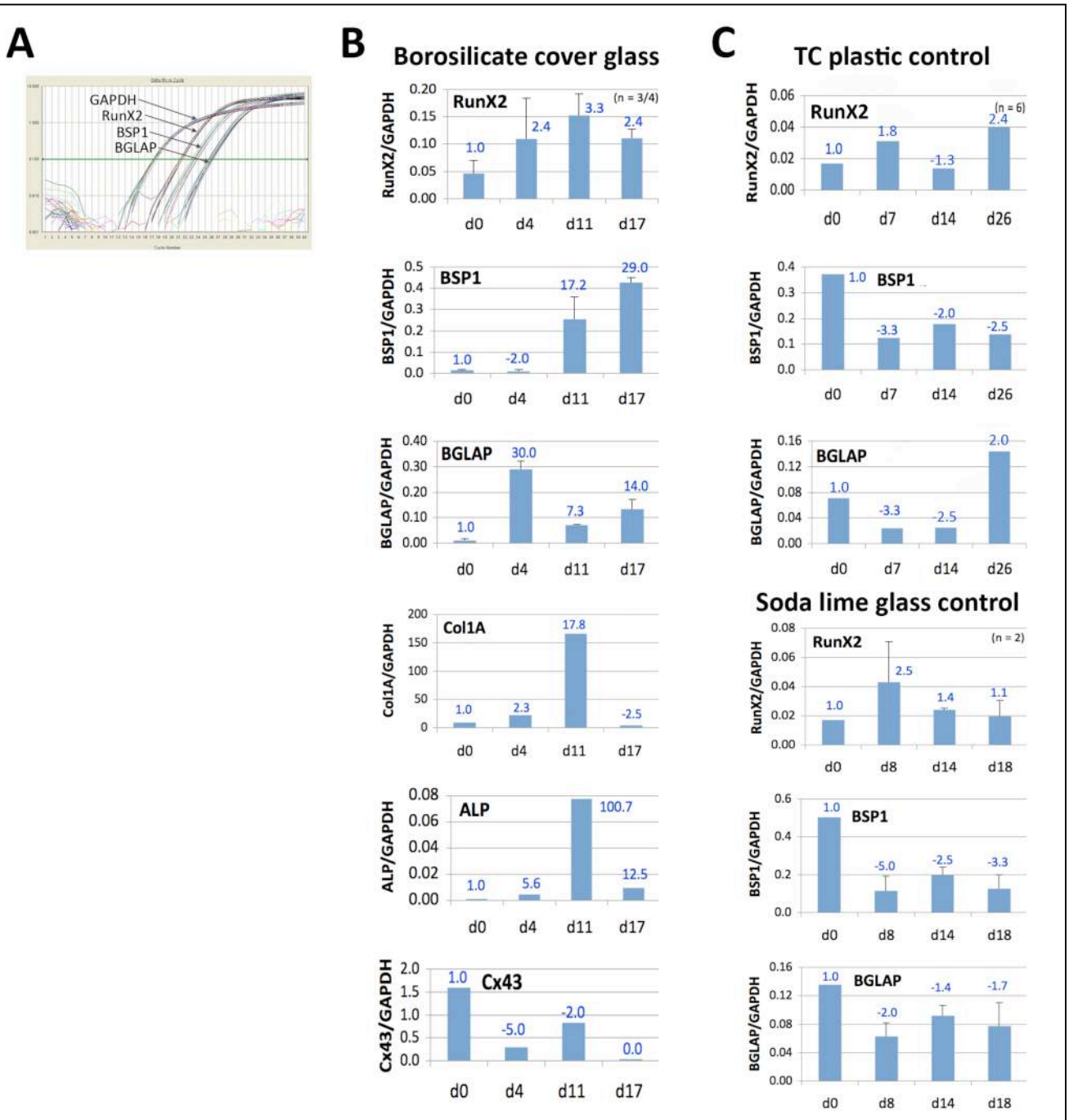

Figure 3: Differentiation of MC3T3-E1 pre-osteoblasts into mature osteoblasts was evaluated by analyzing the expression profile of 3 bone-specific (RunX2, BSP1, BGLAP), and 3 additional osteoblast-relevant proteins (Col1A, ALP, Cx43) known to be up-regulated during osteoblast differentiation by qRT-PCR analyses. (A) A representative set of obtained primary qRT-PCR fluorescence-detection curves of GAPDH, RunX2, BSP1, and BGLAP for the day of seeding (day 0) is shown. (B) Fold changes of RNA levels, normalized against GAPDH expression, for all 6 analyzed proteins of cells grown for 4, 11, and 17 days on borosilicate cover glasses compared to the expression level on day 0 (set to1) are shown (blue/grey numbers above bars). Note the strong up-regulation of most marker proteins on borosilicate cover glasses. (C) MC3T3-E1 cells grown in control on boron-free substrates (TC plastic, soda lime glass) up-regulated marker protein expression only insignificantly (see text for further details).

3 bone-cell specific (RunX2 or Cbfa1, the master transcription factor responsible for bone-cell

differentiation; bone sialoprotein 1/BSP1 or osteopontin/OPN; and osteocalcin or BGLAP, two bone-specific secreted proteins involved in matrix mineralization), and 3 other osteoblast-relevant proteins (collagen 1A/Col1A, a fibril-forming collagen abundantly expressed and secreted by bone, cornea, dermis, and tendon-forming cells; alkaline phosphatase/ALP, a membrane-bound glycosylated enzyme known to be upregulated in bone and other cell types; connexin 43/Cx43, a gap junction forming protein known to be involved in bone-cell differentiation) (for protein nomenclature see GeneCards®; http://www.genecards.org) known to be upregulated specifically during osteoblast differentiation[23, 24] was analyzed and compared to the expression profile of glyceraldehyde 3-phosphate dehydrogenase/GAPDH, an important enzyme involved in carbohydrate metabolism. GAPDH was found to exhibit stable expression levels independent of cellular differentiation stages, and hence is commonly used as a housekeeping control protein.

A representative set of primary qRT-PCR fluorescence-detection curves of RunX2, BSP1, and BGLAP for the day of seeding (day 0) is shown in Figure 3A. Note the close clustering of the curves for each protein indicating highly homogenous parallel samples (n=3/4). Fold change of expression for all 6 analyzed proteins, normalized against GAPDH and compared to the expression level relative to day 0 (set to 1), is shown for days 4, 11, and 17 in Figure 3B. All proteins, except Cx43 expression levels were found to be up-regulated significantly over the 17 day long period (RunX2 = 3.3-fold; BSP1= 29.0-fold; BGLAP = 30.0-fold; Col1A = 17.8-fold; and ALP = 100.7-fold). Only Cx43 expression levels were found to be down-regulated initially by 5.0-fold before recovering by day 11; and this may be attributed to the low cell number shortly after seeding that counteracts gap junction-mediated direct cell-to-cell communication[30]. Notably, the (early) transcription factor differentiation marker, RunX2, was found to be up-regulated early (within 11 days), and less up-regulated later (day 17), while the (late) matrix protein, BSP1, was up-regulated only later (by day 17). The highest up-regulation with over 100-fold was observed for the well-recognized osteoblast differentiation marker, ALP. MC3T3-E1 cells are known to express constitutively large amounts of Col1A, and its mRNA levels were above those of GAPDH as indicated by the higher than one Col1A/GAPDH ratios (Figure 3B). All three secreted extracellular matrix proteins involved in matrix formation and mineralization (BSP1, BGLAP, and Col1A) were up-regulated significantly by 29, 30, and 18-fold (Figure 3B). For none of the non-boron containing substrates (soda lime silicate glass, TC plastic, melt-derived 45S5 or sol-gel derived bioglasses) a similarly significant up-regulation was observed in any of the control experiments that spanned comparable time periods (shown for RunX2, BSP1, and BGLAP of cells grown on TC plastic and soda lime glass (Figure 3C).

DISCUSSION

Taken together, our results derived from this study and presented above indicate that MC3T3-E1 bone precursor cells growing for 17 days on borosilicate cover glasses highly up-regulate the expression of bone-specific and of other osteoblast-relevant marker proteins, suggesting that they began to differentiate into mature calcified matrix secreting osteoblasts on this boron-containing substrate. On none of the non-boron containing substrates (soda lime glass, tissue culture plastic, melt-derived 45S5 or sol-gel derived bioglasses), a similarly significant upregulation was observed when cells were grown for comparable time periods (Figure 3).

Boron has been found previously to stimulate the differentiation of osteoblast precursor and mesenchymal stem cells when added to the culture medium[19, 20], and to enhance bone formation when added to 45S bioglass[21]. This osteogenic effect has been attributed to borate ions in solution that either were added to the culture medium, or leached from the dissolving bioglass after implantation into rat tibias[19, 21]. Boron concentrations used ranged from 2% (by weight added as boron oxide to 45S bioglass)[20], to as low as 0.1 ng/ml (added as boric acid to the culture medium)[19]. Since borosilicate cover glasses are not expected to significantly dissolve in cell culture medium, and in addition, we exchanged one-half of the culture medium every other day, in contrast, in our experiments, a much

lower concentration of soluble boron is expected, suggesting that either much lower concentrations of borate ions in solution (well below ppm concentrations) are sufficient to stimulate bone cell differentiation; or more likely that bone-precursor cells are able to 'sense' and react to boron that is present in the substrate. Cells clearly can detect differences in substrate composition, as for example indicated by the variable adhesion of MC3T3-E1 cells to TC plastic and borosilicate cover glasses described above (Figure 1).

Silicon, another abundant component of borosilicate, soda lime, melt- and sol-gel derived bioglasses is also discussed as a trace element[16], and hence, MC3T3-E1-cell differentiation observed in our study could potentially also be attributed to the effect of this element. However, although soda lime glasses do contain silicon, no significant differentiation of MC3T3-E1 cells was observed on this substrate; nor on a substrate that does not contain boron or silicon (TC plastic) (Figure 3C).

CONCLUSION

In summary, our results shed new light on the potential role of boron on bone cell differentiation, and suggest a beneficial effect of boron when added to bioglass bone-replacement scaffolds. Future experiments should include a careful analysis of the dissolution of boron from borosilicate and boron-containing bioglasses, and of mechanistic aspects that may allow cells to sense boron in solution, as well as in growth-supportive substrates.

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