Effects of pH on human bone marrow stromal cells *in vitro*: Implications for tissue engineering of bone

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Abstract: The objective of this study was to address the hypothesis that changes in extracellular pH alter collagen gene expression, collagen synthesis, and alkaline phosphatase activity in bone marrow stromal cells (BMSCs). Potential effects of pH on cell function are of particular importance for tissue engineering because considerable effort is being placed on engineering biodegradable polymers that may generate a local acidic microenvironment on degradation. Human and murine single-cell marrow suspensions were plated at a density of 2×10^4 cells/cm^2. After 7 days in culture, the pH of the culture medium was adjusted to one of six ranges: ≥7.8, 7.5.–7.7, 7.2–7.4, 6.9–7.1, 6.6–6.8, or ≤6.5. After 48 h of exposure to an altered pH, alkaline phosphatase activity and collagen synthesis decreased significantly with decreasing pH. This decrease was two-to threefold as pH decreased from 7.5 to 6.6. In contrast, α1(I) procollagen mRNA levels increased two- to threefold as pH was decreased. The trend in osteocalcin mRNA expression was opposite to that of collagen. Small shifts in extracellular pH led to significant changes in the ability of BMSCs to express markers of the osteoblast phenotype. These pH effects potentially relate to the microenvironment supplied by a tissue-engineering scaffold and suggest that degrading polymer scaffolds may influence the biologic activity of the cells in the immediate environment. © 2002 John Wiley & Sons, Inc. J Biomed Mater Res 60: 292–299, 2002; DOI 10.1002/jbm.10050

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INTRODUCTION

When transplanted in scaffolds that support cell adhesion and differentiation, bone marrow stromal cells (BMSCs) are capable of bone formation[1–4] and can promote the repair or regeneration of osseous defects.[5–8] Results from *in vitro*, ectopic, and orthotopic models, however, show variability in the extent of bone formation and the ability of the new bone to support hematopoiesis.[4,9] This variability implies that the nature of the microenvironment that cells are exposed to, including the transplantation scaffold, is a critical parameter, especially if human cells are used.[3,10]

Biodegradable co-polymers of lactic and glycolic acid (PLGA) have been shown to support osteoblast attachment, growth, and function *in vitro* and *in vivo*.[11–13] These co-polymers hydrolytically degrade into products that are readily incorporated into metabolic pathways and are eventually excreted as CO_2 and H_2O.[14] Therefore, polylactide-co-glycolide co-polymers have generally been deemed biocompatible. Nonetheless, use of degradable scaffolds to support cell proliferation and extracellular matrix synthesis implies a transient microenvironment that may influence cell behavior. Moreover, there are incidences of foreign body reactions and bone resorption after im-

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plantation of PLGA fracture fixation devices.[15–17] It has been theorized that this inflammatory response is associated with the kinetics of polymer degradation and the local capacity of tissues to clear acidic polymeric byproducts.[17,18] Based on these inflammatory reactions, questions about the biologic responses to acidic degradation products have been raised.[19,20]

Growth and development of a variety of cells, including osteoblasts, are linked to regulation of pH and acidity of the extracellular microenvironment.[21–24] The processes of mineralization and repair of skeletal tissues are influenced by the ionic and molecular composition of the extracellular fluids, and it is well known that Ca-P salts are precipitated in a pH-dependent manner.[23,25,26] Therefore, it has been suggested that mineralization and bone repair are tissue pH dependent.[23] On a cellular level, even modest reductions in extracellular pH have an effect on osteoblast function *in vitro*.[27,28] Cellular mechanisms involved in bone formation and resorption may therefore be responsive to the acid-base balance.[28] Further knowledge of how pH affects bone metabolism stems from studies that show that during metabolic acidosis the activity of osteoblasts declines, whereas during metabolic alkalosis osteoblast activity increases.[22,29–33] Likewise, the pH in the immediate microenvironment of osteoblasts as well as the intracellular spaces of differentiating bone varies with progressive stages of osteogenesis and may be linked to the regulation of gene expression.[27,28,31]

In light of the physiological relevance of a reduced pH and the potential relationship to biomaterial-cell interactions, the objective of this study was to address the hypothesis that changes in extracellular pH, such as could be expected within the microenvironment of a tissue engineering scaffold, modulate the expression of genes associated with an osteoblast phenotype in BMSCs. We show that small shifts in extracellular pH lead to significant changes in the ability of BMSCs to express markers of the osteoblast phenotype. These pH effects potentially relate to the microenvironment that bone marrow stromal cells would likely be exposed to, at least transiently, if transplanted within degradable polymers.

MATERIALS AND METHODS

Preparation of BMSC cultures

Human bone marrow was collected from the long bones of patients undergoing reconstructive surgery. All human samples were collected after informed consent in accordance with appropriate University of Michigan Internal Review Board policy. The bone marrow samples were placed in ice-cold α-modified minimum essential medium (αMEM; Life Technologies, Grand Island, NY) with 100 U/mL sodium heparin (Fisher Scientific, Fair Lawn, NJ), were centrifuged at 100 g for 10 min, and the cell pellet was resuspended in fresh αMEM. All preparations were pipetted repeatedly to break up cell aggregates. Subsequently, marrow cell suspensions were passed consecutively through 16.5- and 20.5-gauge needles before culture.

Single-cell marrow suspensions were plated at a density of 5×10^6–5×10^7 nucleated cells per flask. Cells were then cultured at 37°C, 100% humidity, and 5% CO_2 in flasks containing 30 mL of differentiating medium (αMEM, 10% fetal bovine serum, ascorbic acid, dexamethasone, 100 U/mL penicillin, 100 mg/mL streptomycin sulfate, and 1 μg/mL amphotericin). Culture medium was replaced on days 7 and 14. The resulting adherent cells were harvested as follows: cells were washed twice with Hank's balanced salt solution (HBSS), treated with two consecutive applications of trypsin-ethylenediaminetetraacetic acid (EDTA) for 5–10 min each at 37°C, and washed with medium containing serum.

Mouse bone marrow (Balb/c/C57Bl/6) was harvested from the femoral, tibial, and humeral medullary cavities flushed with α-MEM. The entire marrow content of six bones (two each of femur, tibia, and humerus) was plated into 75-cm^2 culture flasks and was cultured under conditions identical to the human BMSCs. We tested three different cell preparations of murine BMSCs and cells from two different human donors. All experiments were performed at least twice for each cell preparation.

pH modification of culture medium

The pH of the culture medium was adjusted to one of six ranges: ≥7.8, 7.5–7.7, 7.2–7.4, 6.9–7.1, 6.6–6.8, or ≤6.5 by titrating with an appropriate volume of either 1.0*N* HCl or NaOH, based on a buffering calibration curve at 37°C. Before resuspending the cells, medium was incubated for 24 h under culture conditions to allow the desired pH ranges to equilibrate. After incubation, a small adjustment in pH was occasionally required to create the desired final pH. BMSCs were plated at a density of 2.0×10^4 cells/cm^2. A total of 36 six-well dishes were used, such that six wells per pH range were used for each assay. In parallel, wells containing only medium buffered to the same pH ranges were maintained. The pH was monitored with a pH meter (model 320, Corning). Readings were made at the time of preparation, after equilibrium, and after aspiration of conditioned medium (24 and 48 h). Medium was changed at 24 h.

Collagen assay

After a total culture period of 48 h, cells were harvested. The following assays were performed on human BMSCs: total DNA content, percent collagen synthesis (PCS), alkaline phosphatase activity (ALP), and mRNA analyses for α1(I) procollagen and osteocalcin (OCN). mRNA levels for α1(I) procollagen were also determined for mouse BMSCs. Alkaline phosphatase and collagen synthesis were quanti-

fied by analyzing cells and medium from each well independently. Cells from wells of similar pH range were pooled for mRNA analyses. The amount of total collagen produced was determined by radiolabeling all synthesized proteins with ^{3}H-proline and selectively digesting the collagenous proteins with collagenase.[34,35] Cells were pulsed with 4 μCi/mL and harvested 6 h later. Proteins were extracted with 1*M* acetic acid containing 1 mg/mL pepsin for 4 h at 25°C. Aliquots of the protein extracted solutions (0.2 mL) were added to 1.5-mL microcentrifuge tubes along with 0.1 mL αMEM and 0.2 mL of 0.02*N* HCL containing 1.25 μmol N-ethylmaleimide, 25 μg purified collagenase, and 0.25 μmol $CaCl_2$. The tubes were incubated at 37°C for 90 min while agitating. Adding an equal volume of 10% trichloroacetic acid (TCA) solution containing 0.5% tannic acid (TA) stopped the reaction. Tubes were placed on ice for 25 min and centrifuged at 150 g for 5 min at 4°C. Each supernatant solution was transferred to vials containing 12 mL of scintillation cocktail. Precipitates were resuspended with 0.5 mL of 5% TCA with 0.25% TA and centrifuged under the same conditions. Each supernatant was added to the corresponding vials, and solutions were counted in a scintillation counter for the presence of radioactive proline. Noncollagenous digestible protein (NCP) and collagenous digestible protein (CDP) were determined. The NCP remaining in the precipitates were resuspended in 1.5 mL of 5% TCA/0.25% TA and counted. The amount of collagen was calculated as follows: % Collagen synthesized = [CDP/(CDP + 5.2 NCP)] × 100 and then normalized to total DNA content.

Measurement of alkaline phosphatase activity

Alkaline phosphatase activity was measured spectroscopically by using standard procedures with p-nitrophenylphosphate as the substrate. Cultures were washed in HBSS and cell layers were harvested in 0.2–1 mL lysis buffer, sonicated for 4 min on ice, and then subjected to three freeze-thaw cycles. Aliquots of 10 μL were incubated with p-nitrophenol phosphate/lysis buffer mix (1:1). The presence of ALP was measured by monitoring light absorbance by the solution at 405 nm. The slope of the absorbance versus time curve was used to calculate ALP activity, which was then normalized to total DNA content. Quantitative data for ALP activity and percent collagen synthesis at each pH were pooled and analyzed via a one-way ANOVA with an all pairwise multiple comparison using a Student-Newman-Keuls test.

RNA extraction and northern blot analysis

Total RNA (12 μg) was separated on a 0.9% agarose-1*M* formaldehyde gel and transferred by capillary action to nylon membranes (Magna NT; MSI). cDNA probes for collagen α1(I) and OCN were labeled with [α^{32}P]dATP by a random-primed oligonucleotide method using T4 polynucleotide kinase (Stratagene, La Jolla, CA) and were hybridized at 54°C. Unincorporated isotope was removed by centrifugation through G-50 Sephadex spin columns (5 Prime-3 Prime Inc., Boulder, CO). Prehybridization, hybridization, and washing conditions were performed by using standard methods.[36] Blots were stripped and reprobed with an [α^2P]dATP-labeled actin cDNA and β emissions from the decaying ^{32}P-labeled probe were quantified by scanning membranes on a β-scan instant imager (Packard Instrument Co., Downers Grove, IL).

RESULTS

Several experiments were performed to determine the effects of pH on bone marrow stromal cells in culture and to address the hypothesis that extracellular pH modulates the ability of BMSCs to express markers of the osteoblast phenotype. Because the extracellular pH was altered just after the cells reached confluence, our experiments defined the effects of pH on BMSCs that completed the proliferation stage, but had not yet progressed through the matrix maturation and mineral deposition stages of *in vitro* differentiation. Shifts in pH over the course of the 48-h experiments were ±0.1–0.3 for both experimental and control groups. Therefore, results are presented as functions of pH ranges.

Although present in a number of other tissues, the expression of ALP is a time-honored marker of osteoblast cell function. In *in vitro* developmental studies, it appears as one of the earliest markers of cells that acquire the ability to form a mineralized matrix. Therefore, expression of ALP activity was used as one of the osteoblast phenotypic markers in our experiments. ALP activity in human BMSCs was influenced by small shifts in pH. After 48 h of exposure to an altered pH, ALP activity in human BMSCs decreased significantly with decreasing pH (Fig. 1).

The osteoblast synthesizes a complex extracellular matrix of which type I collagen is the major constituent. Like ALP activity, collagen synthesis also significantly decreased in human BMSCs exposed to a more acidic medium (Fig. 2). This decrease was two- to threefold as pH decreased from 7.5 to 6.6. In contrast, α1(I) procollagen mRNA levels increased as pH was decreased in both human (Fig. 3) and murine BMSCs (Fig. 4). When the hybridization signals were normalized to actin mRNA, the collagen mRNA levels increased two- to threefold with decreasing pH (Figs. 3 and 4). Duplicate experiments with both human and murine cells from different donors yielded similar results (data not shown).

OCN is a late marker of osteoblast cell function. Although osteocalcin was recently identified in other tissues, it is expressed at high levels in bone-forming cells late in the development of mineralizing cell cul-

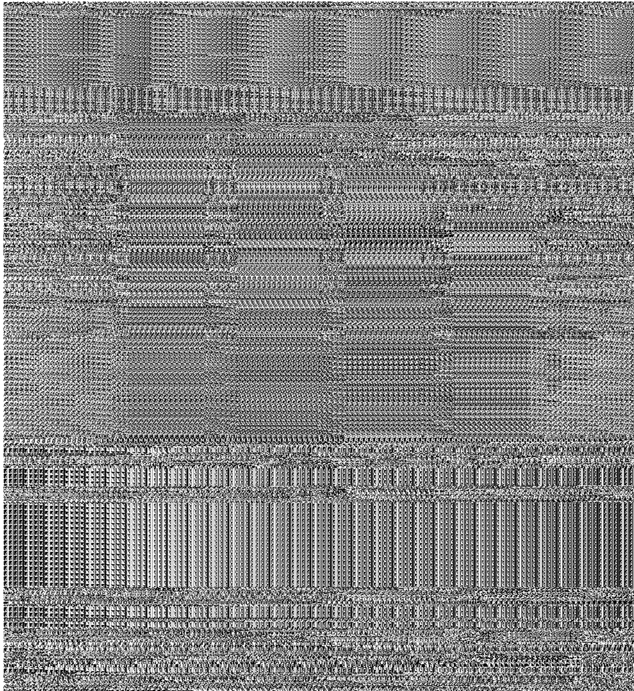

Figure 1. Alkaline phosphatase (ALP) activity normalized to total DNA content in human BMSCs cultured for 48 h in medium buffered to different pH ranges. There was a statistically significant decrease in ALP activity with decreasing pH. ns designates a value that is not significantly different, $p > 0.05$.

Figure 3. Northern blot analysis of human BMSC α1(I) procollagen mRNA levels in response to changes in pH. Lane 1, pH ≥ 7.9; Lane 2, pH = 7.7–7.9; Lane 3, pH = 7.5–7.7; Lane 4, pH = 7.2–7.4; Lane 5, pH = 6.9–7.1; Lane 6, pH = 6.6–6.8. (A) α1(I) procollagen mRNA, (B) ethidium bromide staining of nucleic acids, and (C) actin mRNA. The hybridization signals of each gene were quantified by measuring the radioactive decay of ^{32}P-labeled probes and are represented by the ratio of α(I) procollagen mRNA to actin mRNA levels (Col1A1/Actin).

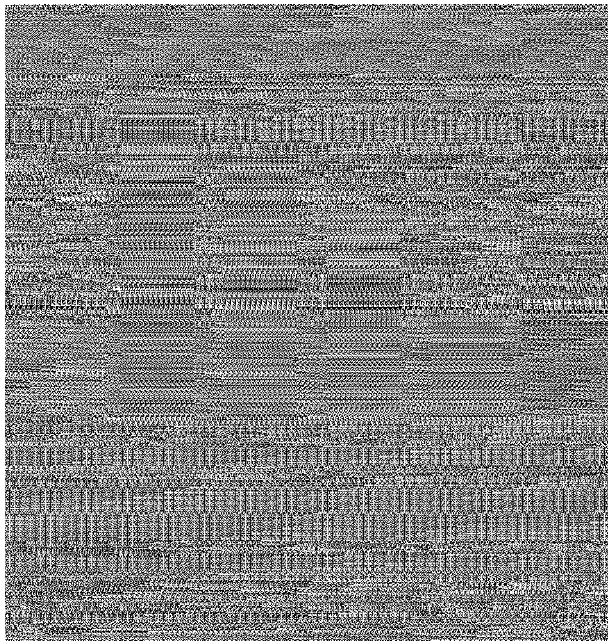

Figure 2. Percent collagen synthesis (PCS) normalized to total DNA content in human BMSCs cultured for 48 h in medium buffered to different pH ranges. There was a statistically significant decrease in PCS with decreasing pH. ns designates a value that is not significantly different, $p > 0.05$.

tures.[37] To observe osteocalcin expression in BMSCs, autoradiographs needed to be exposed for 7 days and therefore led to a higher than normal background. Despite the high background, osteocalcin mRNA levels could be detected. The trend in OCN mRNA expression in human BMSCs (Fig. 5) was opposite to that of procollagen. Osteocalcin was expressed at low levels at superphysiological pH levels and declined rapidly to undetectable levels below physiological pH.

DISCUSSION

Proliferation and differentiation of osteoblasts and the formation of mineralized tissue are linked to pH regulation.[22,23,32] Likewise, the osteogenic capacity of BMSCs also varies depending on the culture environment.[10,38–40] On the basis of the results of this study, it is clear that small shifts in extracellular pH lead to significant changes in the ability of BMSCs to express markers of the osteoblast phenotype. The trends in ALP and collagen synthesis (Figs. 1 and 2) are consistent with previously reported data from osteoblast-like cell lines[28] and cultured human osteoblasts.[27] However, in this study the effects of extracellular pH

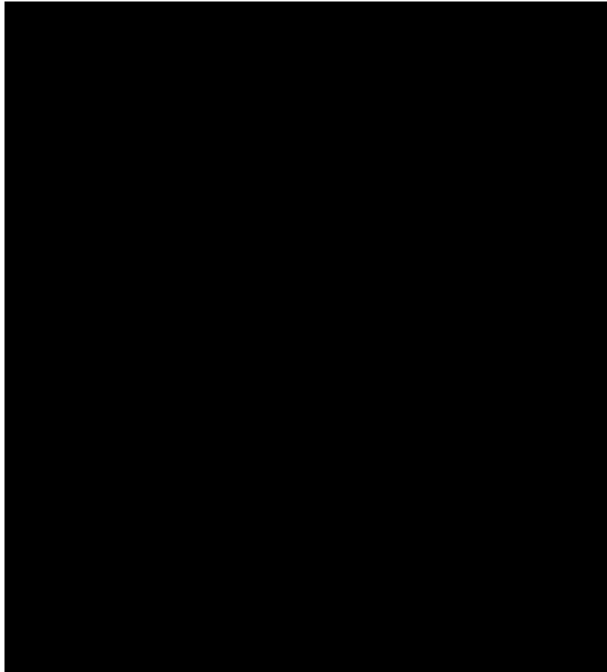

Figure 4. Northern blot analysis of mouse BMSC α1(I) procollagen mRNA levels in response to changes in pH. Lane 1, pH ≥ 7.9; Lane 2, pH = 7.7–7.9; Lane 3, pH = 7.5–7.7; Lane 4, pH = 7.2–7.4; Lane 5, pH = 6.9–7.1; Lane 6, pH = 6.6–6.8. (A) α1(I) procollagen mRNA, (B) ethidium bromide staining of nucleic acids, and (C) actin mRNA. The hybridization signals of each gene were quantified by measuring the radioactive decay of ^{32}P-labeled probes and are represented by the ratio of α1(I) procollagen mRNA to actin mRNA levels (Col1A1/Actin).

on cell function and gene expression were performed on BMSCs, a population of cells that unlike cell lines may serve as an abundant source of osteoprogenitor cells for skeletal tissue regeneration in humans.

The inverse trends between collagen synthesis (Fig. 2) and procollagen mRNA levels (Figs. 3 and 4) were not anticipated. Collagen content has routinely been shown to decrease with decreasing extracellular pH *in vitro*, a result that has been attributed to lower α1(I) procollagen gene expression.[27,28] The trend in osteocalcin expression in human BMSCs with changing pH (Fig. 5) was opposite to that of α1(I) procollagen expression. Osteocalcin mRNA was expressed at low levels at high pH and declined to undetectable levels below physiological pH. In this manner, OCN expression followed type I collagen synthesis and not α1(I) procollagen gene expression. The low level of OCN expression was anticipated because of the relatively short-term culture period used and the fact that cells had not yet reached the mineral deposition stage.[41]

The experiments performed were short-term *in vitro* studies, as were many of the material degradation/pH and cell culture/pH studies that motivated our hypothesis.[20,27,28,31] There is still debate about the role of the body itself in buffering of degradation products in an *in vivo* microenvironment. By analogy to wound healing, degradation-mediated acidity could be important *in vivo*. During wound healing, relatively acidic conditions prevail.[42,43] Low blood flow and poor oxygenation can result in a decreased ability to nourish bone cells and remove metabolic waste products such as lactic acid.[27,44] Conversely, patients with respiratory alkalosis have an increased rate of fracture healing.[45] Therefore, the collective literature indicates that biomineralization and skeletal regeneration are influenced by the ionic composition and pH of the extracellular microenvironment[23,25,26] and that the magnitude of the pH drop in a wound healing microenvironment is equivalent to the range of pH that reduces osteoblast function, as determined in the current experiments and elsewhere.[27,28]

Having demonstrated that small pH shifts significantly affect BMSC function and that these shifts are physiologically relevant, it is important to note that many acid-base effects on the bone microenvironment also potentially relate to the microenvironment supplied by an engineered scaffold. This is of particular importance for tissue engineering because considerable effort is being placed on producing biodegradable polymer scaffolds for localized skeletal regeneration. The magnitude of the pH changes studied here reflect pH shifts that bone cells would likely be exposed to, for at least a short period of time, if seeded on a degradable polymer.[43,46,47] The physiological scenarios in which declining pH leads to acid buildup and reduction in osteoblast function may be analogous to the cellular response to resorbable polymer degradation products. Hydrolysis of poly(α-hydroxyacid)-based polymers is acid catalyzed and accelerates once

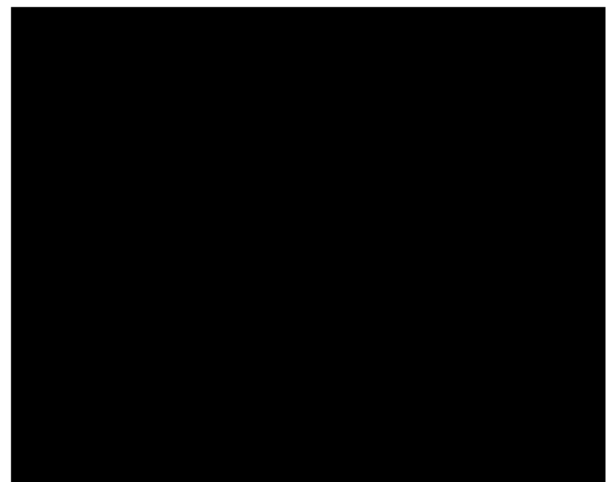

Figure 5. Northern blot analysis of human BMSC osteocalcin (OCN) mRNA as a function of medium pH. Lane 1, pH ≥ 7.9; Lane 2, pH = 7.7–7.9; Lane 3, pH = 7.5–7.7; Lane 4, pH = 7.2–7.4; Lane 5, pH = 6.9–7.1; Lane 6, pH = 6.6–6.8. OCN mRNA levels decrease with decreasing pH, a trend opposite to that of collagen mRNA (Figs. 3 and 4).

started. The pH in the eroding zone of degrading polymers has been measured to be as low a 2.0–6.0.[46–49] Changes in the local microenvironment produced by acidic PLGA degradation products may, therefore, inhibit bone metabolism and impair healing, at least for more dense forms of these materials where diffusion may be restricted by a fluid-constraining microenvironment.[46] To compensate for these potentially adverse biological effects, investigators have begun modifying these co-polymers to neutralize the acidic degradation products. Buffering the microenvironment may be achieved by incorporating basic salts within polymers[48] or by generating composite polymer scaffolds containing a carbonated apatite mineral surface.[50]

Long-term organ culturing on PLGA showed a progressive acidification of the culture medium, reaching a level as low as 4.1, leading to decreased glycolysis, collagen synthesis, and mineral content.[47] Other work on osseous tissue engineering has shown that it is necessary to precondition the surfaces of glasses because ionic leaching leads to a profound and rapid change in cell function and activity.[51] These latter two studies, along with the pH-dependence of biomaterial function,[25,26] indicate that even in the absence of pH effects *in vivo*, the microenvironment of *in vitro* cell seeding can be impacted, thus influencing tissue engineering efficiency. Finally, it should be recognized that pH-dependent effects *in vitro* are still important, especially if tissue engineering applications move toward use of osteoprogenitor or stem cells. Therefore, the results of these studies could impact the *ex vivo* expansion of cells for tissue engineering applications.

The specific cellular and molecular mechanisms responsible for the observations made in this study are not yet fully understood. It is not known whether the pH effects represent an effect on BMSC differentiation or proliferation of a specific subpopulation of cells, or what second messengers may be involved. The inverse trends between α1(I) procollagen gene expression and collagen synthesis suggest an uncoupling of transcription and translation. A similar finding of uncoupling of collagen transcription and translation has been reported for other insults to cultured osteoblasts, such as addition of tumor necrosis factor-α.[52]

A potential mechanism for the observed uncoupling of collagen transcription and translation involves the role of heat shock proteins in collagen biosynthesis. Lower pH weakens the association of heat shock protein-47 (HSP47), a collagen chaperone, with the nascent procollagen polypeptide. Close association of HSP47 is essential for proper folding of procollagen molecules until export to the Golgi apparatus.[53] Procollagen propeptides separated from HSP47 in an acidic environment rapidly degrade, leading to a net decline in collagen synthesis. The association between HSP47 and procollagen propeptide is pH dependent and is disrupted below pH = 6.5.[54–56]

In addition to increasing the H^+ concentration, the addition of HCl to the culture medium results in an increased Cl^- concentration in the extracellular medium. The Cl^- gradient stimulates the HCO_3^-/Cl^- exchanger, which in turn may lower intracellular pH (pH_i).[57–59] The HCO_3^-/Cl^- pump is known to be involved in the regulatory pathways of osteoblasts[22] and has also been shown to reduce intracellular cAMP levels.[21] A reduction in pH_i also changes mitochondrial activity (higher ATP/lower cAMP) and causes release of Ca^{2+} from mitochondria.[60,61] Increased levels of intracellular Ca^{2+} affect signal transduction pathways,[62,63] and, depending on the pathways active in a specific cell population or developmental stage, a variety of physiological responses may be observed.

Although the use of a heterogeneous cell population makes discerning mechanisms of action difficult, this pluripotent cell population reflects the *in vivo* reality for many tissue engineering applications. The pH effects on BMSCs observed in this study potentially relate to the microenvironment supplied by a tissue-engineering scaffold and suggest that degrading polymer scaffolds may influence the biologic activity of the cells in the immediate environment.

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