Cyclic strain inhibits switching of smooth muscle cells to an osteoblast-like phenotype[1]

JANETA NIKOLOVSKI,* BYUNG-SOO KIM,† AND DAVID J. MOONEY*,§,‡,[2]

*Department of Biomedical Engineering, ‡Chemical Engineering, §Biologic and Materials Sciences, University of Michigan, Ann Arbor, Michigan, USA; and †Department of Chemical Engineering (B-SK), Hanyang University, Haengdang-dong, Seongdong-gu, Seoul, Korea

SPECIFIC AIMS

To identify whether the expression of osteopontin by smooth muscle cells is mechanically regulated and, more generally, whether the mechanical environment of smooth muscle cells (SMCs) regulates a phenotypic switch to a pattern of gene expression more typical for bone and inducive of calcification.

PRINCIPAL FINDINGS

1. Rat aortic SMCs express osteopontin (OPN) within a tissue-engineered model system and expression increases over time in culture

OPN mRNA levels increased over time in cells cultured on standard tissue culture flasks. However, 2-dimensional (2-D) cell culture is a poor representation of the natural tissue environment of SMCs, which is 3-dimensional (3-D), mechanically dynamic, and involves the interaction of multiple cell types. To determine whether up-regulation of OPN resulted solely from the 2-D culture conditions, 3-D SM tissues were engineered by seeding and culturing smooth muscle cells in type I collagen sponges. Cells within the collagen adhered, proliferated, and produced their own extracellular matrix to form 3-D tissues. Immunohistochemical analysis revealed significant OPN deposition around cells within engineered tissues, and OPN mRNA levels increased with time in a similar manner as observed in 2-D culture.

2. Mechanical strain inhibits osteopontin, CBFA-1, and matrix gla protein expression in SMCs

OPN expression in control tissues (maintained in a bioreactor with no strain application) increased over time (**Fig. 1*A***), as noted in standard culture conditions. However, tissues exposed to chronic cyclic strain (7%, 1 Hz) maintained low and fairly constant OPN levels, leading to unstrained tissues expressing between 2.7- and 4.1-fold the OPN mRNA of strained tissues after 5 wk and 5 months, respectively (Fig. 1*A*). A small but reproducible increase in mRNA levels in strained vs. control tissues was found, however, at the earliest time point (2 days). Western analysis of tissue lysates similarly revealed a 2.2-fold decrease in OPN protein deposited in strained vs. nonstrained tissues long-term (after 15 days). A panel of genes typically recognized as markers for osteoblast differentiation was also studied to determine whether the up-regulation of OPN in static conditions was indicative of a larger scale phenotypic change of SMCs to a bone-like pattern of gene expression. First, mRNA levels of CBFA-1, a transcription factor controlling osteopontin expression and normally associated with osteoblast differentiation, were quantified in strained vs. nonstrained engineered tissues. CBFA-1 mRNA levels followed a similar temporal profile as OPN, as expected for a transcription factor and the gene it regulates. Strained tissues maintained fairly constant mRNA levels, but unstrained tissues exhibited higher message levels for the 5 wk duration of the experiment (Fig. 1*B*). The expression of matrix gla protein (MGP), another protein implicated in the calcification process, was next investigated. Expression of MGP followed a similar pattern to that of OPN, as levels were up-regulated over time in culture in control tissues, and this effect was again largely eliminated with application of cyclic strain (Fig. 1*C*). The effect of cyclic strain on long-term levels of OPN, MGP, and CBFA-1 mRNA in strained tissues were compared and all were found to be similarly lower than control/unstrained tissues (Fig. 1*D*).

3. Mechanical strain regulates tissue mineralization

Engineered tissues were analyzed to determine whether the noted changes in gene expression were paralleled by changes in cell phenotype leading to mineralization. The activity of alkaline phosphatase, an enzyme involved in mineral formation and an established osteoblast differentiation marker, was determined in strained vs. unstrained engineered tissues. There were no differences between strained and control tissues at the initial times analyzed, but at 5 wk the alkaline

[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.02–0459fje; to cite this article, use *FASEB J.* (January 2, 2003) 10.1096/fj.02–0459fje

[2] Correspondence: Department of Chemical Engineering, University of Michigan, 2300 Hayward Ave., 3074 H. H. Dow, Ann Arbor, MI 48109-2136, USA. E-mail: mooneyd@umich.edu

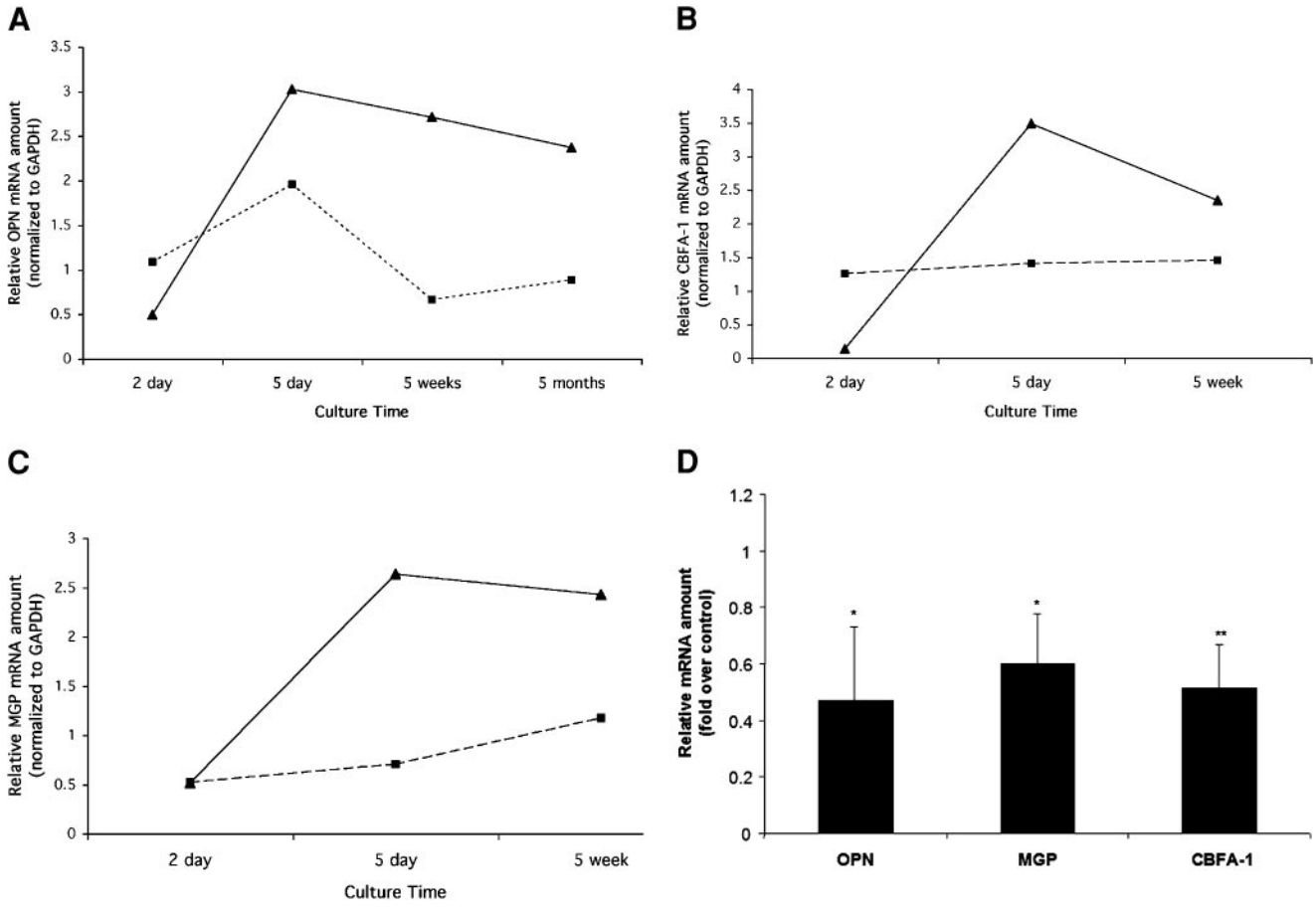

Figure 1. Expression of a number of bone-associated genes are down-regulated over time with exposure to chronic cyclic strain. Quantification of osteopontin mRNA expression in tissues engineered with (dashed lines) or without (solid lines) exposure to strain (*A*). Representative experiment showing CBFA-1 (*B*) and matrix gla protein (MGP) (*C*) mRNA expression in smooth muscle tissues subjected to cyclic strain (dashed lines) or no strain (solid lines) over time. Northern blot analysis from multiple experiments run from 20 to 35 days; values for mRNA levels are represented normalized to control, nonstrained tissue mRNA levels for each gene (*D*), $*P < 0.05$, $**P < 0.1$ compared with nonstrained tissues.

phosphatase activity in control tissues was 3.5-fold greater than in tissues exposed to cyclic strain (**Fig. 2*A***). Not surprisingly, the changes in SMC phenotype resulting from long-term cyclic strain led to significant variations in the total calcium deposited within tissues. Exposure to strain for 5 wk caused a significant decrease in the amount of calcium deposited in strained tissues compared with control tissues (Fig. 2*B*).

CONCLUSIONS AND SIGNIFICANCE

Vascular calcification is a common health problem and thought to be an actively regulated process similar to osteogenesis. Mechanical stress and strain are important extracellular stimuli that regulate the phenotype of vascular smooth muscle cells (SMCs) and play an important role in the development of hypertension and atherosclerosis. However, there is little previous evidence implicating strain in modulating the calcification process regulated by SMCs. As demonstrated in these studies, the mechanical environment of SMCs regulates a phenotypic switch to a pattern of gene expression more typical of bone, and thus relevant to calcification. Using a 3-D smooth muscle tissue model system, the expression of a variety of markers implicated with various stages of bone differentiation and calcification (OPN, CBFA-1, MGP, alkaline phosphatase) were found to be up-regulated over time in statically cultured SMCs, but chronic cyclic mechanical stimulation down-regulated their expression and played a protective role in calcification.

Smooth muscle cells grown in static culture (2-D and 3-D) were found to up-regulate the expression of markers associated with bone differentiation over time. The observation that these genes are expressed by vascular cells is not a new one, as recent evidence has implicated a similar phenotypic transition associated with calcification within SMC cultures, noted by increases in CBFA-1, OPN, osteocalcin, and alkaline phosphatase, and decreases in smooth muscle lineage markers SM22 and alpha smooth muscle actin. Similarly, a calcifying cell line has been isolated from aortas expressing OPN, alkaline phosphatase, and bone mor-

phogenetic protein 2, further providing proof for the existence of cells demonstrating a bone-like phenotype within the vasculature.

The studies outlined here are, however, the first to demonstrate the modulation of expression of these bone-associated proteins in smooth muscle by long-term application of strain. Cyclic mechanical strain suppresses bone-associated gene expression and played a protective role in calcification in this system. Some previous studies clearly document that application of strain can have other significant effects on SMC phenotype (e.g., cell alignment, protease expression) in engineered tissues, but did not examine expression of bone-associated genes. Our findings with OPN are, however, supported by a previous study demonstrating that short-term pressure modulates expression of OPN in 2-D cultured human aortic smooth muscle cells. Similarly, an up-regulation of OPN and MGP was demonstrated over time when aortic rings were excised form rats and grown in static culture. The relevance of a mechanically controlled SMC phenotype switch, as demonstrated in this study, is supported by previous findings that demonstrate an up-regulation of bone-associated genes in the neointima of injured or atherosclerotic arteries or in other situations that result in an altered mechanical environment of SMCs.

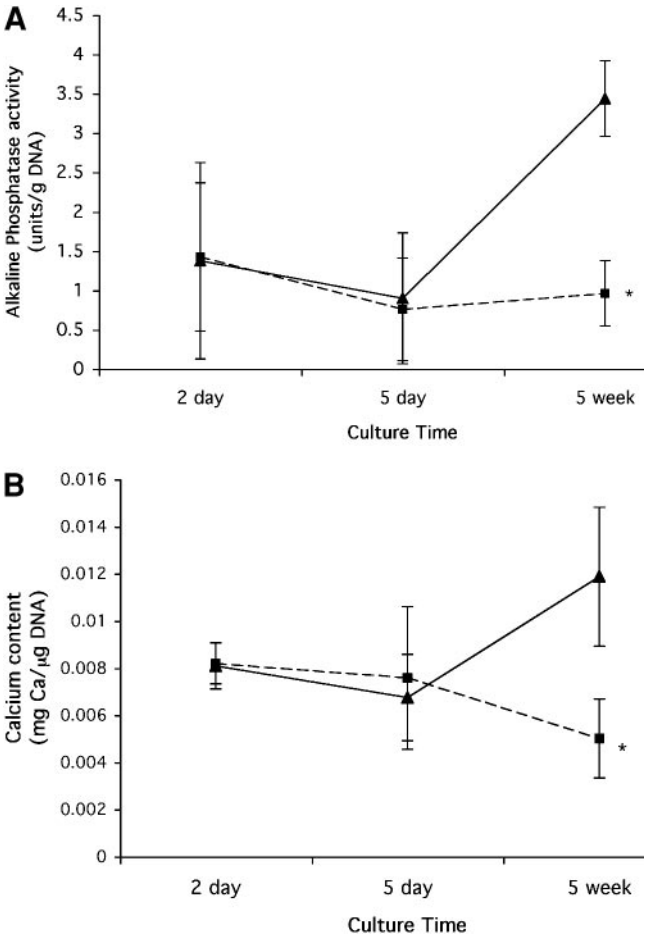

Figure 2. Quantification of alkaline phosphatase activity (*A*) and total calcium (*B*) in smooth muscle tissues engineered with (dashed lines) or without (solid lines) exposure to cyclic strain. Values represent mean (n=3) and SD. *$P < 0.05$ compared with nonstrained tissues.

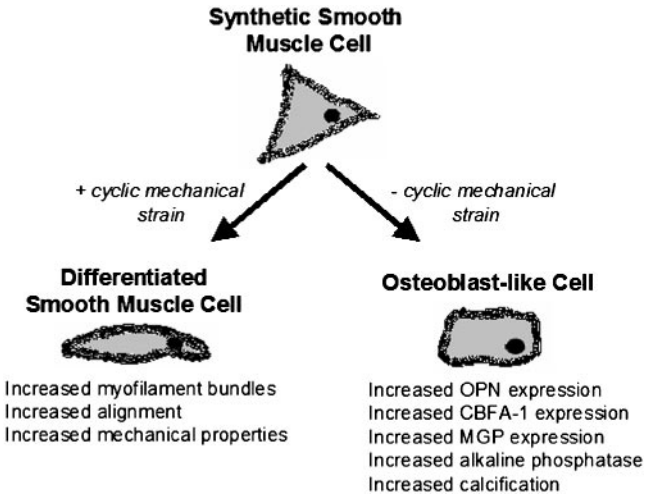

Figure 3. Proposed model of the effect of long-term cyclic mechanical strain on the phenotype of smooth muscle cells. Previous studies have demonstrated that exposing cells in engineered tissues to appropriate mechanical strain conditions converts the cells to a more differentiated phenotype, indicated by increased expression of myofilament bundles and increased cellular alignment. The current study demonstrates that tissues grown without the influence of strain increase their expression of genes indicative of an osteoblast phenotype and calcify to a greater extent.

Altogether, the results of this study lead to a model outlining the effects of mechanical strain on SMC phenotype that suggests SMCs undergo a phenotypic conversion to a more osteoblast-like pattern of gene expression without an appropriate mechanical environment (e.g., exposure to cyclic mechanical strain) (**Fig. 3**). However, exposure to an appropriate strain regimen maintains the SMC phenotype as supported by past findings of enhanced cell and matrix organization and increased myofilament bundles in these engineered smooth muscle tissues. This model may aid in interpreting and preventing the calcification of vascular tissues. These data also provide support to the plasticity of the smooth muscle phenotype and its regulation by environmental factors such as mechanical strain. Finally, this report indicates the utility of engineered tissues for basic biology studies as one can controllably build in the desired features of normal tissues (e.g., 3-D architecture) into a highly defined model system. FJ