Tissue Engineering of Smooth Muscle under a Mechanically Dynamic Condition

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Abstract In order for engineered tissues to find clinical utility, the engineered tissues must function appropriately. However, smooth muscle (SM) tissues engineered *in vitro* with a conventional tissue engineering technique may not exhibit contractile functions, because smooth muscle cells (SMCs) cultured *in vitro* typically revert from a contractile, differentiated phenotype to a synthetic, nondifferentiated phenotype and lose their ability to contract. SMCs *in vivo* typically reside in mechanically dynamic environments. We hypothesized that cyclic mechanical stretch induces the features of SMCs in *in vitro* engineered tissues to be similar to those of SMCs in native tissues. To test the hypothesis, aortic SMCs were seeded onto elastic, three-dimensional scaffolds and cultured *in vitro* under a cyclic mechanical stretching condition for 4 weeks. A significant cell alignment in a direction parallel to the cyclic stretching direction was found in the SM tissues exposed to cyclic stretching. The cellular alignment and alignment direction were consistent with those of native vascular SM tissues, in which SMCs *in vivo* align in the radial direction (parallel to stretching direction). In control tissues (SM tissues engineered without stretching), cells randomly aligned. The expression of SM α-actin and SM myosin heavy chain, phenotypic markers of SMCs in a contractile state, was upregulated in the stretched tissues by 2.5- and 2.0-fold, respectively, compared to SMCs in the control tissues. The cellular features of alignment and contractile phenotype of SMCs in the SM tissues engineered under a mechanically dynamic environment could allow the engineered SM tissues to exhibit contractile functions.

Tissue engineering offers the possibility of creating functional new tissues that may replace lost or malfunctioning organs or tissues [10]. This approach may provide an alternative to organ and tissue transplantation therapies, both of which suffer from a severe limitation of supply [8, 12]. In the tissue engineering approach, cultured cells are seeded onto a suitable scaffold, and either allowed to develop into a new tissue *in vitro*, or transplanted into a patient to create a new tissue that replaces the functions of the lost or deficient tissue [5]. Smooth muscle (SM) is a functionally critical component of a variety of cardiovascular, gastrointestinal, and urinary tissues. Any attempt to engineer these tissues must include the development of functional SM. Importantly, to utilize engineered tissues clinically, the engineered tissues must function appropriately.

To engineer functional tissues, the transplanted cells must be provided with microenvironments that are similar to those of the native tissues *in vivo* during the process of new tissue development *in vitro* [5]. For tissue engineering of functional SM, mechanical stretch may be a crucial microenvironmental factor. Smooth muscle cells (SMCs) in SM-containing tissues (e.g., blood vessels, intestines, and bladder) *in vivo* are constantly exposed to cyclic mechanical stretch. Many studies have shown that mechanical stretch significantly regulates the phenotype of SMCs in two-dimensional culture systems [1, 4, 14, 16]. Recently, we have demonstrated that cyclic mechanical stretch enhanced

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the development and function of engineered smooth muscle (SM) tissues [6].

In the present study, we tested the hypothesis that cyclic mechanical stretch induces the phenotype of SMCs in *in vitro* engineered tissues to be similar to that of SMCs in native tissues *in vivo*. SMCs *in vivo* normally align in a specific direction and exist in a contractile or differentiated state, which is critical for contractile functions of SM [9]. *In vitro* cultured SMCs, however, do not align in a specific direction and often lose the contractile phenotype [2, 17]. To test the hypothesis, aortic SMCs were seeded onto elastic, three-dimensional scaffolds and subjected to cyclic mechanical stretch. In control experiments, SMCs on scaffolds were cultured without mechanical stretch. Subsequently, we examined the cellular alignment and the expression of SM α-actin and SM myosin heavy chain, phenotypic markers of SMCs in a contractile state [17], in the engineered SM tissues.

MATERIALS AND METHODS

Scaffold Fabrication

Scaffolds were fabricated from poly(glycolide-co-ε-caprolactone) (PGCL) using a previously described solvent casting and particulate leaching method [13]. The copolymer was generously provided by Biomaterials Research Center in Korea Institute of Science and Technology, Seoul, Korea. The copolymer contained an equal molar ratio of glycolide and ε-caprolactone, and had a molecular weight of 121,000. NaCl particles (100–200 μm), which served as porogens, were added to a PGCL solution in chloroform (5% w/v), and mixed homogeneously. The polymer-salt mixture was cast onto glass plates. The solvent was then allowed to evaporate for 48 h, and residual solvent was removed by drying under vacuum for 24 h. The resulting PGCL/salt composite membranes were immersed in distilled deionized water with shaking for 3 days to leach out the salt, resulting in the formation of porous scaffolds. Prior to use, the scaffolds were sterilized with ethylene oxide gas.

Scaffold Characterization

Scaffold morphology was studied with a scanning electron microscope (SEM, Hitachi, Tokyo, Japan). Samples were coated with gold using a sputter-coater (Eiko IB3, Tokyo, Japan), and the microscope was operated at 15 kV to image samples.

Dynamic mechanical tests (n=3) were performed using a custom-made mechanical stretch apparatus (Fig. 1), as previously described [7]. The scaffolds were subjected to cyclic stretch at an amplitude of 5% of initial scaffold length and at a frequency of 1 Hz in PBS solution at 37°C.
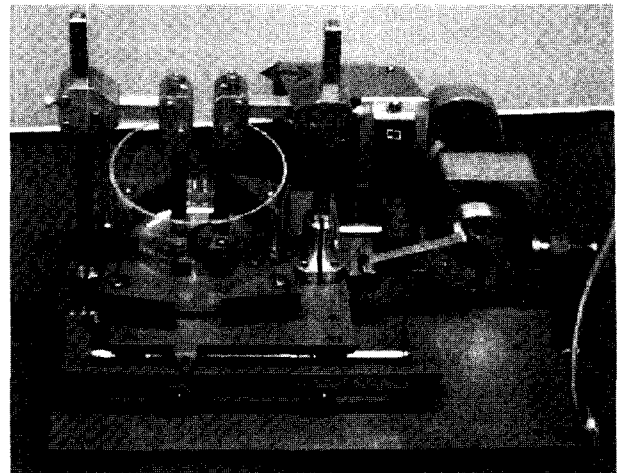
Fig. 1. Apparatus utilized to subject scaffolds to cyclic mechanical stretch.

Scaffolds were immersed in PBS or medium and clamped in the tissue culture chamber (C). The scaffolds were subjected to cyclic stretching by periodical movement of a crank back and forth as an eccentric disk (D) that was driven by a motor and connected to the crank rotated.

We chose these cyclic strain amplitudes and frequencies because it has been reported that these conditions are similar to those experienced by SM tissues *in vivo* [9]. The elastic properties of the scaffolds were determined by measuring the permanent deformation of the scaffolds following repetitive stretching.

Smooth Muscle Cell Isolation and Culture

SMCs were isolated from rat aortas according to the previously published technique [15]. In brief, the descending aortas of 3.5-kg female New Zealand white rabbits, after the removal of endothelium, adventitia, fat, and connective tissue, were cut into multiple small pieces and incubated under agitation with an orbital shaker (60 rpm) for 90 min at 37°C in a sterile conical flask containing an enzymatic dissociation buffer. This buffer contained 0.125 mg/ml elastase (Sigma, St. Louis, MO, U.S.A.), 1.0 mg/ml collagenase (CLS type I, 204 units/mg, Worthington Biochemical Corp., Freehold, NJ, U.S.A.), 0.250 mg/ml soybean trypsin inhibitor (type 1-S, Sigma), and 2.0 mg/ml crystallized bovine serum albumin (BSA, Gibco, Gaithersburg, MD, U.S.A.). Following the complete dissolution of the matrix, the resultant cell suspension was filtered through a 100 μm Nitex filter (Tetko, Inc., Briarcliff Manor, NY, U.S.A.) and centrifuged at 200 ×g for 5 min. The pellet was resuspended in growth medium consisting of Medium 199 (Gibco) supplemented with 10% (v/v) FBS (Gibco), 2 mM L-glutamine (Gibco), 100 units/ml penicillin (Gibco), and 0.1 mg/ml streptomycin (Gibco), and cultured in tissue culture flasks (25 cm^2). SMCs passaged twice were used in this study.

Seeding of Cells onto Scaffolds and Subsequent Culture under Cyclic Stretching Condition

To seed SMCs onto PGCL scaffolds (40×10×2 mm), 0.8 ml of a SMC suspension containing 2.0×10^{7} cells was injected into the scaffolds in tissue culture dishes. After 1, 2, and 6 h, 0.5, 1, and 30 ml of the culture medium was added to each scaffold, respectively. The seeded polymer scaffolds were maintained in a humidified 5% CO_2 atmosphere for 2 days with the medium changed every day. SMCs in the scaffolds were subjected to cyclic stretch *in vitro* using a custom-made cyclic stretching unit in a humidified incubator with 5% CO_2 at 37°C (Fig. 1). The seeded scaffolds were subjected to cyclic strain at a frequency of 1 Hz (1 cycle per second) and an amplitude of 5% of initial scaffold length for 4 weeks. As a control, seeded scaffolds were fixed at only one end of the clamps and moved back and forth at the same frequency and amplitude as the mechanical stretching conditions.

Cell Alignment Analysis

Cell alignment in engineered SM tissues was determined by SEM examinations. Specimens were fixed in 1% (v/v) glutaraldehyde and 0.1% (v/v) formaldehyde for 30 min and 24 h, respectively, dehydrated with a graded ethanol series, and dried. The dried samples were mounted on aluminum supports and sputter coated with gold. A scanning electron microscope was operated to image samples. More than 300 cells in 10 SEM photographs were examined for cell alignment.

Western Blot Analysis

To obtain total proteins, engineered tissues were crushed manually and dissolved in a lysis buffer (pH 7.4) containing 25 mM Tris, 0.4 M NaCl, 0.5% (w/v) SDS, and protease inhibitors [10 mg/ml aprotinin (Sigma), 10 mg/ml leupeptin (Sigma), 5 mg/ml pepstatin (Sigma), and 0.5 M phenylmethylsulfonyl fluoride (Sigma)]. Tissues were vortexed, boiled for 5 min, and centrifuged. To determine sample volumes representing the same amounts of total proteins from each tissue sample, supernatants were removed and analyzed by spectrophotometrically measuring absorbance at 280 nm. Protein samples were mixed with Laemmli sample buffer, loaded and separated by SDS-PAGE on 10% resolving gels for SM α-actin. Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL, U.S.A.). Transfer was carried out at a constant voltage of 100 V in a cold room for 1 h in a buffer system (5.03 g Tris base, 14.5 g glycine, 0.5 g SDS, and 200 ml methanol in 1 l). Membranes were blocked with 5% skim milk in TBS-T (Tris buffered saline with 0.1% Tween-20, pH 7.6) for 1 h and probed with mouse anti-SM α-actin antibody (DAKO, Carpinteria, CA, U.S.A., 1:100 dilution with TBS-T) and mouse anti-SM myosin heavy chain antibody (DAKO, 1:100) for 1 h at

room temperature. Membranes were washed, probed with 1:1,000 diluted horseradish peroxidase conjugated anti-mouse IgG secondary antibody (Sigma) for 1 h and visualized by an enhanced chemiluminescence system (Intron Biotechnology, Inc., Sungnam, Korea). Luminescence was recorded onto X-ray film (Fuji Super RX, Fujifilm Medical Systems, Tokyo, Japan). Band intensity was analyzed by imaging densitometer (Model GS-690, BioRad, Hercules, CA, U.S.A.). Bands were quantified densitometrically by scanning films into Scion Image (NIH Image, Scion Corporation, Maryland, U.S.A.), and analyzed (n=3).

RESULTS AND DISCUSSION

Appropriate scaffolds were first prepared as a three-dimensional cell culture substrate for tissue engineering of SM under a cyclic mechanical stretch condition. Scaffolds fabricated from PGCL (Fig. 2) were chosen as a three-dimensional cell culture substrate in this study. These scaffolds are biodegradable and biocompatible [3], which is important for new tissue formation following cell seeding and culture. In addition, these scaffolds were found to have elastic mechanical properties, which is a prerequisite for subjecting the cell-seeded scaffolds to cyclic mechanical stretch. The elastic properties were evaluated by measuring recovery after cyclic stretching of PGCL scaffolds. PGCL scaffolds were subjected to cyclic loading at 5% amplitude and 1 Hz frequency in PBS (pH 7.4) at 37°C for 4 weeks using a custom-made apparatus (Fig. 1). The scaffolds displayed no permanent deformation.
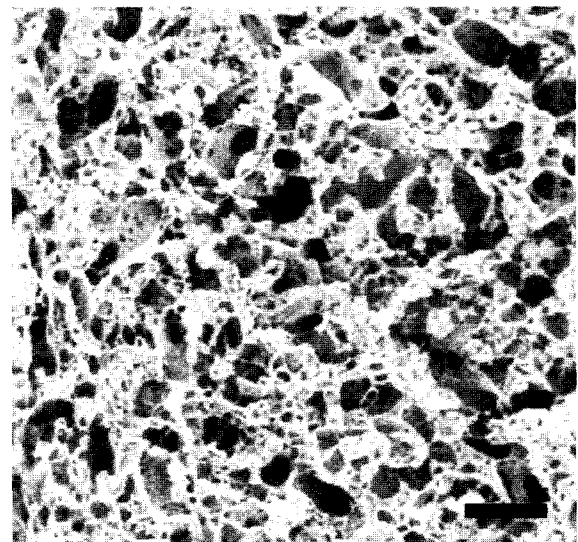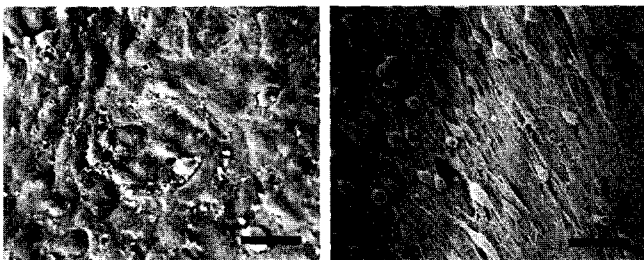

Fig. 2. SEM photomicrograph of PGCL scaffold. The porous, three-dimensional scaffold allows for cell adhesion and subsequent three-dimensional tissue formation.
The size bar indicates 50 μm.
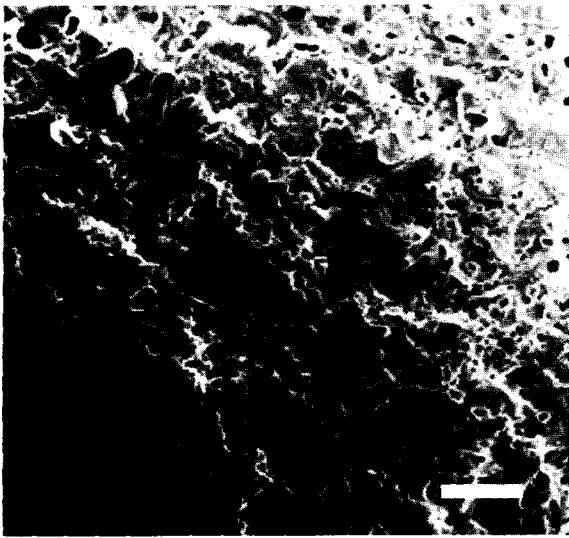
Fig. 3. SEM photomicrograph of SMCs following seeding onto PGCL scaffold for 24 h.
The size bar indicates 50 µm.

Next, we investigated the effects of cyclic mechanical stretch on SMC alignment in engineered tissues. Seeded SMCs adhered well to PGCL scaffolds (Fig. 3). Subsequently, SMCs cultured in PGCL scaffolds were exposed to cyclic mechanical stretch for 4 weeks using a custom-made bioreactor (Fig. 1), and SEM examination indicated that the application of cyclic mechanical stretch induced a significant cell alignment, as compared with control tissues (SM tissues engineered without stretching) (Fig. 4). The cell alignment direction was in parallel to the cyclic mechanical stretch direction. The vascular SMC alignment and alignment direction in this study were consistent with those of native vascular SM tissues, in which SMCs align in the radial direction (parallel to stretching direction) [2, 9]. The induction of SMC alignment in this study was also consistent with previous results in which SMCs in two-dimensional culture systems were exposed to cyclic mechanical stretch, and the alignment direction in two-

Fig. 4. SEM photomicrographs of SMCs on surface of SM tissues engineered with PGCL scaffolds and subjected to no stretch (a) or cyclic mechanical stretch (b) for 4 weeks.
The cyclic stretching direction is indicated with an arrow. The size bars indicate 20 µm.

SM α-actin

SM myosin heavy chain
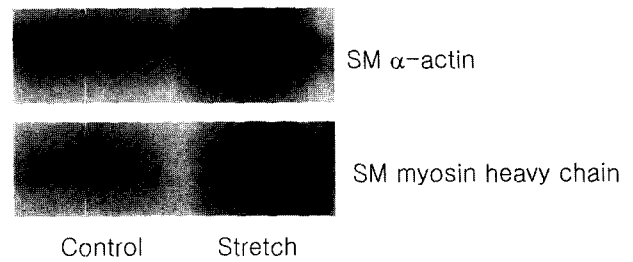
Control   Stretch

Fig. 5. Western blots of SM α-actin and SM myosin heavy chain in SM tissues engineered with PGCL scaffolds and subjected to cyclic mechanical stretch (stretch) or no stretch (control) for 4 weeks.

dimensional culture systems was nearly perpendicular to the stretching direction [2]. In our previous study, histological examination showed that cyclic strain induced the alignment of SMCs present in the inner parts of engineered tissues in a direction parallel to the stretching direction [6].

The effects of cyclic mechanical stretch on the phenotype of vascular SMCs in engineered tissues were next assessed. SMCs in tissues engineered under a cyclic stretch condition exhibited an increased expression of SM α-actin and SM myosin heavy chain (Fig. 5), which are phenotypic markers of SMCs in a contractile phenotype [17]. Densitometric examination indicated that the expression of SM α-actin and SM myosin heavy chain was 2.5 and 2.0 times greater, respectively, in tissues exposed to cyclic stretching than in control tissues. This result suggests that cyclic mechanical stretching induces a differentiated, contractile phenotype in SMCs in engineered tissues. This is consistent with our previous results that SMCs in tissues engineered under cyclic strain contained cellular organelles of contractile phenotype, as analyzed by transmission electron microscopy, while cells in control tissues exhibited the properties of the synthetic, nondifferentiated phenotype [7].

Engineered tissues must be functional in order to be utilized clinically, and mechanical stimulation may be critical for tissue engineering of functional SM. An important function of the SM element in many tissues (e.g., blood vessels and intestines) is to contract in order to regulate blood pressures or transfer fluids. In the present study, mechanical stimulation induced cellular alignment and contractile phenotype in SMCs in engineered tissues, which may allow the engineered SM tissues to exhibit contractile functions. Cell alignment in tissues would make tissue contraction feasible, because the contraction of randomly aligned cells in tissues would not result in tissue contraction (Fig. 6). In order for SMCs to contract, the cell phenotype must be a differentiated, contractile phenotype. SMCs *in vivo* normally exist in a contractile or differentiated state, whereas *in vitro* cultured SMCs lose their ability to contract [17]. Mechanical stimulation, to which SMCs *in vivo* are exposed, has been found to be a strong regulator of SMC phenotype and crucial for the

No cell alignment
Synthetic phenotype

(a)

No
stretching

(b)

contraction

Cyclic
stretching

Cell alignment
Contractile phenotype
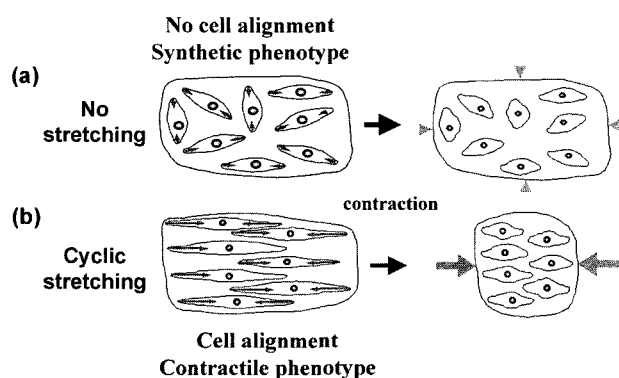
Fig. 6. Proposed model of the effect of cyclic mechanical stretching on the contractile function of tissue-engineered SM. The present study demonstrated that exposing SMCs in engineered tissues to cyclic mechanical stretching converted the cells to a differentiated, contractile phenotype and induced cellular alignment. This would allow the engineered SM tissues to exhibit contractile functions. In contract, in vitro cultured SMCs in engineered tissues without mechanical stimulation normally exist in a relatively nondifferentiated, synthetic phenotype and do not align, resulting in no contraction of the SM tissues.

maintenance of SMCs in a differentiated state [11, 14]. The SM tissues engineered without mechanical stimulation (control tissues) in this study had no cell alignment and decreased feature of contractile phenotype, thus resulting in no contraction of these tissues (Fig. 6). We are currently conducting experiments to investigate the effects of cellular alignment and contractile phenotype on contractile functions of engineered SM tissues.

In conclusion, tissue engineering of SM under a mechanically dynamic condition resulted in cellular features that resembled those of SMCs in SM tissues in vivo. Specifically, SMCs in tissues engineered under cyclic stretching exhibited cellular alignment and upregulated expression of SM α-actin and SM myosin heavy chain. These cellular features could result in contractile functions of the engineered SM tissues. SM tissues in vivo reside in mechanically dynamic environments. Therefore, in order for engineered SM tissues to exhibit the functions of the native SM tissues, it might be necessary to provide the microenvironments of native SMCs in vivo during the in vitro tissue engineering process.

Acknowledgment

REFERENCES

1. Birukov, K. G., V. P. Shirinsky, O. V. Stepanova, V. A. Tkachuk, A. W. A. Hahn, T. J. Resink, and V. N. Smirnov. 1995. Stretch affects phenotype and proliferation of vascular smooth muscle cells. *Mol. Cell. Biochem.* **144:** 131-139.

2. Dartsch, P. C. and H. Hämmerle. 1986. Orientation response of arterial smooth muscle cells to mechanical stimulation. *Eur. J. Cell. Biol.* **41:** 339-346.

3. Gilding, D. K. 1981. Biodegradable polymers, pp. 209-232. In D. F. Williams (ed.), *Biocompatibility of Clinical Implant Materials*, CRC Press, Boca Raton, FL, U.S.A.

4. Kanda, K. and T. Matsuda. 1993. Behavior of arterial wall cells cultured on periodically stretched substrates. *Cell Transplant.* **2:** 475-484.

5. Kim, B. S. and D. J. Mooney. 1998. Development of biocompatible synthetic extracellular matrices for tissue engineering. *Trends Biotechnol.* **16:** 224-230.

6. Kim, B. S., J. Nikolovski, J. Bonadio, and D. J. Mooney. 1999. Cyclic mechanical strain regulates the development of engineered smooth muscle tissue. *Nat. Biotechnol.* **17:** 979-983.

7. Kim, B. S. and D. J. Mooney. 2000. Scaffolds for engineering smooth muscle under cyclic mechanical strain conditions. *J. Biomech. Eng.* **122:** 210-215.

8. Kim, S. K., S. H. Yu, J. H. Lee, J. Y. Lee, A. Rademacher, D. H. Lee, and J. K. Park. 2001. Effect of collagen concentration on the viability and metabolic function of encapsulated hepatocytes. *J. Microbiol. Biotechnol.* **11:** 423-427.

9. Ku, D. N. and C. Zhu. 1993. The mechanical environment of the artery, pp. 1-23. In B. E. Sumpio (ed.), *Hemodynamic Forces and Vascular Cell Biology*, R.G. Landes Company, Austin.

10. Langer, R. and J. P. Vacanti. 1993. Tissue engineering. *Science* **260:** 920-926.

11. Lehoux, S. and A. Tedgui. 1998. Signal transduction of mechanical stresses in the vascular wall. *Hypertension* **32:** 338-345.

12. Lee, D. H., J. H. Lee, J. E. Choi, Y. J. Kim, S. K. Kim, and J. K. Park. 2002. Determination of optimum aggregates of porcine hepatocytes as a cell source of a bioartificial liver. *J. Microbiol. Biotechnol.* **12:** 735-739.

13. Mikos, A. G., A. J. Thorsen, L. A. Czerwonka, Y. Bao, R. Langer, D. N. Winslow, and J. P. Vacanti. 1994. Preparation and characterization of poly(L-lactic acid) foams. *Polymer* **35:** 1068-1077.

14. Reusch, P., H. Wagdy, R. Reusch, E. Wilson, and H. E. Ives. 1996. Mechanical strain increases smooth muscle and decreases nonmuscle myosin expression in rat vascular smooth muscle cells. *Circ. Res.* **79:** 1046-1053.

15. Rothman, A., T. J. Kulik, M. B. Taubman, B. C. Berk, C. W. J. Smith, and B. Nadal-Ginard. 1992. Development and characterization of a cloned rat pulmonary arterial smooth muscle cell line that maintains differentiated properties through multiple subcultures. *Circulation* **86:** 1977-1986.

16. Sumpio, B. E., A. J. Banes, W. G. Link, and G. Jr. Johnson. 1988. Enhanced collagen production by smooth muscle cells during repetitive mechanical stretching. *Arch. Surg.* **123:** 1233-1236.

17. Thyberg, J. 1996. Differentiated properties and proliferation of arterial smooth muscle cells in culture. *Int. Rev. Cytol.* **169:** 183-265.