Published in final edited form as:

Lab Chip. 2013 December 7; 13(23): 4573–4582. doi:10.1039/c3lc50894c.

A Novel System for Studying Mechanical Strain Waveform-Dependent Responses in Vascular Smooth Muscle Cells

Jason Lee^{1,*}, Mitchell Wong^{1,*}, Quentin Smith¹, and Aaron B. Baker¹

¹University of Texas at Austin, Department of Biomedical Engineering, Austin, TX

Abstract

While many studies have examined the effects mechanical forces on vSMCs, there is a limited understanding of how the different arterial strain waveforms that occur in disease and different vascular beds alter vSMC mechanotransduction and phenotype. Here, we present a novel system for applying complex, time-varying strain waveforms to cultured cells and use this system to understand how these waveforms can alter vSMC phenotype and signaling. We have developed a highly adaptable cell culture system that allows the application of mechanical strain to cells in culture and can reproduce the complex dynamic mechanical environment experienced by arterial cells in the body. Using this system, we examined whether the type of applied strain waveform altered phenotypic modulation of vSMCs by mechanical forces. Cells exposed to the brachial waveform had increased phosphorylation of AKT, EGR-1, c-Fos expression and cytoskeletal remodeling in comparison to cells treated with the aortic waveform. In addition, vSMCs exposed to physiological waveforms had adopted a more differentiated phenotype in comparison to those treated with static or sinusoidal cyclic strain, with increased expression of vSMC markers desmin, calponin and SM-22 as well as increased expression of regulatory miRNAs including miR-143, -145 and -221. Taken together, our studies demonstrate the development of a novel system for applying complex, timevarying mechanical forces to cells in culture. In addition, we have shown that physiological strain waveforms have powerful effects on vSMC phenotype.

Keywords

vascular smooth cell differentiation; arterial strain waveform; mechanotransduction; cellular biomechanics; microRNA

Introduction

Within the artery, vascular smooth muscle cells (vSMCs) compose the bulk of the cellular mass of the vascular wall and are exposed directly to pulsatile variations in pressure, leading to cyclic arterial distension and stretch. This dynamic mechanical environment is a powerful regulator of vascular homeostasis and the progression of vascular disease. Mechanical stresses regulate physiological functions such as vasomotor tone¹ and also contribute to pathological disease states by altering the atherogenesis², atherosclerotic plaque rupture³ and vascular hypertrophy/stiffening in hypertension⁴. In addition, in many clinical interventions such as angioplasty and stenting, high levels of mechanical strain to the arterial wall contribute to the formation of restenosis⁵.

Correspondence to: Aaron B. Baker, PhD, University of Texas at Austin, Department of Biomedical Engineering, 107 W Dean Keeton Street, BME 5.202D, C0800, Austin, TX, Phone: 512-232-7114, abbaker@austin.utexas.edu.

*denotes co-first authorship

Disclosures. The authors have filed a patent application on the device described in this work.

Systems for applying mechanical stretch to cells in culture have been used for many years to study the mechanisms of vascular mechanotransduction. Fundamentally, the vast majority of these devices work on the principle of applying mechanical forces to a flexible substrate on which cells can be grown. These systems fall into several categories including those that apply uniaxial stretch through substrate extension, biaxial strain through substrate bending, biaxial strain through out-of-plane circular substrate distention and biaxial strain through in-plane substrate distention (reviewed elsewhere extensively^{6–8}). Among these different configurations, in-plane substrate distention is the only one that produces a uniform strain field. This is essential for controlled studies in which well-defined strains are needed to understand the effect of different types of mechanical stress or to recapitulate the physiological environment accurately. In-plane substrate distention has been induced on cells by forcing a frictionless piston upward through a flexible culture membrane⁹, by applying pneumatic suction around a platen to a similar culture system¹⁰ or by applying biaxial traction to a sheet of flexible culture membranes. These and similar systems have allowed the identification of mechanotransduction pathways responsive to cell stretch in a variety of cell types^{11–14}. In vSMCs, mechanical loading has been shown to activate many signaling pathways^{15–17}, leading to alterations in morphology¹⁸, immediate early gene expression¹⁹, proliferation²⁰, the release of stimulatory growth factors and cytokines^{20, 21} and cell phenotype^{18, 19, 22}.

Within the body, the pressure variations during the cardiac cycle produce a complex time-dependent distention of the artery (arterial strain waveforms) that vary through the different vascular beds in the body^{23–25} and are altered by vascular remodeling due to hypertension or atherosclerosis²⁶. While the effects of mechanical forces on vSMCs are widely recognized¹⁴, the vast majority of studies on vSMC biology take place in the absence of the physiological mechanical environment or under dynamic conditions of a simple sinusoidal waveform of strain. As a consequence, there is a limited understanding of the effects of strain waveform dynamics on vSMC biology independent of the maximum levels of strain.

Here, we present the design and validation of a novel device to apply complex mechanical strains to cells in culture. The system is platform based and, consequently, is easily adaptable to many standard formats including the standard 6-well cell culture plate geometry. The system also incorporates a feedback controlled, true linear motor as the prime mover and thereby provides a means to apply any arbitrary temporal strain profile for simulating the complexity of the in-vivo mechanical environment and systematically testing strain waveform features. The system has been validated to apply uniform strain profiles across the individual wells, addressing the issues of uniformity and repeatability in the multi-well format. We have used the system to examine whether physiological strain waveforms induce an altered biological response in comparison to the sinusoidal waveforms used in many prior studies. Our results support that the nature of the arterial distention waveform, independent of maximum stretch, can profoundly alter its resulting biological effects on vSMC mechanotransduction and phenotypic modulation by mechanical forces.

Methods

Design of multiwell device for applying uniform dynamic stretch

The device consists of three main parts: (1) a culture plate with flexible silicone culture surfaces; (2) a mobile platen containing low-friction, Teflon pistons that apply strain to the silicone membranes; (3) a linear motor with mounts to provide the forced movement of the platen on rails; and (4) a rigid frame that supports the motor, platen and culture plates. Cells were cultured in custom 6-well plates with silicone cell culture surfaces and were mounted to the top of the machine. The system uses a vertically mounted linear motor to push a platen with 36 pistons to displace the silicone culture surfaces and create strain on the cells.

General mode of mechanical strain application

There are several modes for applying strain to silicone membranes with cells in culture. These included in the fluid/pneumatic-based displacement, pin shaped indentation, and glass dome indentation⁶. We selected a low-friction based indentation of the membrane, as this has been shown to apply nearly homogeneous radial and circumferential strains. In the system, a PTFE flange bearing was used as a piston that created strain on the membrane through upward displacement of the fixed silicone membrane (Fig. 1A). The cell culture plates were designed to exactly match the dimensions of a standard 6-well plate (Fig. 1B). This design allows the use of the plates in the standard multipurpose plate readers, microscopes and robotic culture systems. The culture plate consisted of acrylic plate and aluminum base plate that were fastened together by six screws before mounting onto the machine (Fig. 1A). Sandwiched between the plates was a 0.001" thick silicone membrane (Specialty Manufacturing). Two silicone gaskets were used to create a seal and prevent leaking. The silicone membranes were coated with 10 µg/ml type I collagen overnight before cells were seeded into the culture wells. A gas permeable polystyrene lid was used from standard 6-well plates. A custom-mounting jig was used to ensure uniform and consistent tension in the membrane when mounted on the plate.

A platen was used to support the PTFE pistons during the motion and displacement of the membrane within the culture plates. The PTFE pistons were sandwiched between two plates to form a platen with 36 individual pistons (Fig. 1C). The platen was mounted with six, linear bearings and attached to the machine through six case-hardened rods (Fig. 1C,D). These rods supported the vertical motion of the plates and maintained a tight tolerance on the parallel motion of the plate relative to the culture plates and the top plate of the machine. A central mounting hole in the platen allows it to attach to a linear motor placed underneath the plate. When at rest, the entire platen is supported by six springs attached to the rods and held in place with shaft collars. This reduces the static load on the motor and prevents the platen from moving when the motor is turned off.

The prime mover of the system was a linear motor capable of producing a maximal load of 744 N and continuous load of 215 N. The motor is hygienically sealed and is feedback position controlled with incremental encoder output and digital Hall effect sensor. A potential limitation of the linear motor is the excess heat produced from the passage of current through the coils. Heating is a particular challenge in that we wished to design a system that could go into a standard incubator. The typical culture incubator is designed only to heat from room temperature to a desired temperature (eg. 37°C). Consequently, if the motor heats the incubator beyond this range there is no cooling mechanism to deal with the excess heat. The linear motor was encased in a housing that had flow channels within it to allow the circulation of fluid near to the coils of the motor. However, at the force levels required for these studies cooling was not necessary. Structurally, four support rods connected the top and bottom plates of the system. The six motion rails also provided support and stability between the top and bottom plates.

Quantification of the strain on culture membrane

The strain values were calibrated by measuring changes in radial and circumferential ink marks. Distances between dots along the radial axis were measured to find the radial strain. Circumference changes were measured to find circumferential strain. The dots and circles were marked with an industrial grade permanent pen on a silicone membrane using a customized stencil on paper. The membrane with stencil marks were stretched from base 0 to 500 counts at increments of 100 counts on the machine. At every increment, pictures were taken using Nikon D3100 camera mounted on a boom stand. Six pictures were taken per well, resulting in total of 36 pictures. These pictures were converted to TIF file format using

Adobe Photoshop. Next, distances between the dots and the circumference on the TIF images were then measured using MetaMorph software by drawing a line between the dots and circles around the circumference marks from the images. The strains were then found by calculating changes in the distance and the circumference relative to the initial 0 count. From this data, we were able correlate count increments on the machine to the strain applied to the membrane.

Cell culture experiments

Human vascular smooth muscle cells (Lonza) were grown in DMEM supplemented with 10% fetal bovine serum, 2% L-glutamine, and antibiotics. The cells were maintained in culture at 37°C under an atmosphere of 5% CO₂R. The cell culture plates with silicone culture surfaces were assembled and using a UV sterilizer for 30 min of exposure time. Under sterile conditions, the plates were treated with a solution of 10 µg/ml of type-I collagen (Becton Dickenson) for 24 hours. Following collagen coating, the plates were washed three times with PBS and the cells were passaged onto the plates.

Immunofluorescence staining

Following application of mechanical strain, the cells were washed twice with PBS warmed to 37°C. The cells were fixed for 10 min in 4% paraformaldehyde. The cells were permeabilized for 5 min using PBS containing 0.1% Triton X-100. Primary antibodies to paxillin were applied overnight at 4°C in a humidified chamber. The cells were then washed three times with PBS and stained with fluorescently labeled secondary antibody and phalloidin for 90 min at room temperature. The samples were then washed extensively with PBS and coverslipped with anti-fade mounting media. The samples were imaged using an inverted confocal fluorescence microscope (Carl Zeiss). Ten images were taken from each well for each experimental group. For the color intensity a background image was taken and subtracted from the fluorescence intensity levels.

Gene and miRNA expression analysis

Messenger RNA (mRNA) and micro RNA (miRNA) were harvested from the cells following loading using methods previously described^{2, 27}. mRNA and miRNA were isolated from samples using the RNeasy and miRNeasy kit, respectively (Qiagen). Using TaqMan Reverse Transcription Reagents and TaqMan microRNA Reverse Transcription kits (Invitrogen), isolated mRNA and miRNA were reverse-transcribed to create complementary DNA strands. Real time PCR was used to measure mRNA expression using SYBR Green (Applied Biosystems) and the following primers: GAPDH, EGR-1, c-Fos, c-Jun, desmin, calponin1, SM22, and tropomyosin (Table 1). Similarly, the following miRNA expression was measured using TaqMan miRNA Assays (Invitrogen) for RNU48, miR-31, miR-143, miR-145, miR-221, and miR-222.

Immunoblotting and ELISA

Following mechanical loading the cells were assayed by western blotting as previously described²⁸. Briefly, cells were lysed in 20 mM Tris with 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM sodium orthovanadate, 2 mM PMSF, 50 mM NaF, and protease inhibitors. Lysates were run on NuPAGE 10% Bis-Tris Midi gel (Novex) and transferred to nitrocellulose membrane using iBlot Transfer Stack (Novex). The membranes were blocked for 1 hour in 5% non-fat milk in PBS with 0.01% tween-20 (PBST) and exposed to the following antibodies in 4 °C overnight in 1% non-fat milk: anti-phospho AKT, anti-AKT, anti-phospho ERK, anti-ERK, and GAPDH (Cell Signaling Technology). The membranes were washed with PBST, and incubated at room temperature for 2 hours with secondary antibody. The membrane was imaged using chemiluminescence camera (Cell Biosciences)

after treating the membranes with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). The ELISA assay for FGF-2 was performed according to the manufacturer's instructions (R&D Systems).

Statistical Analysis

All results are shown as mean $\pm$ SEM. A two-tailed student t-test was used to compare two groups in the experiments. An ANOVA with Tukey's post hoc test was used to compare multiple groups of continuous variables. $P < 0.05$ was defined as being statistically significant.

Results

Calibration and assessment of strain uniformity

The overall completed system is shown in Fig. 1E. A key design parameter of our device was the application of uniform biaxial strain to the silicone culture membrane. We assessed both the static and dynamic strains that were produced by our system through by tracking dots on the membrane. We tracked the displacement of the dots during the application of load at different static piston displacements (Fig. 1F,G). We also tracked the strain over time during application of for 24 hours with a cycle frequency of 1 Hz and a maximal strain of 10% strain (Fig. 1H). We found that there was strain relaxation in the system over time that reduced the effective applied strain level in spite of constant maximal displacement. The flexibility of our device allowed us to compensate for this change by incrementally increasing the strain over time. This was done by increasing the displacement amplitude over time maintain constant strain on the material. For a 96-hour experiment, we had the amplitude increase linearly between that measured at time zero until reaching the appropriate compensated displacement to obtain the same strain at 96 hours. With this compensation, the new strain profile over 96 hours of cyclic loading maintained a constant maximal strain in the loading cycle throughout the experiment.

Reproduction of pulsatile arterial stretch waveforms

We next examined whether the system was capable of applying complex strain waveforms found in the arterial wall during the cardiac cycle. Arterial distension waveforms vary throughout the vascular tree^{24, 25, 29} and their local effects on vSMC biology in-vivo are unknown. We duplicated arterial distension waveforms measured in human patients for aortic and brachial arteries and scaled them to have a maximal strain of 7.5% and total cycle length of 1 second (Fig. 2). The range of arterial distensions under normal conditions ranges from approximately 2% and 10% strain depending on many factors. Thus, we chose 7.5% as a mid-range value to compare whether the waveform altered the biological response with a constant maximal strain.

Effect of stretching waveform on vSMC mechanotransduction pathways

The vast majority of the mechanistic studies that underlie our current understanding of vascular cell mechanotransduction have examined the effects of cyclic mechanical load on cultured cells using a sinusoidal waveform. To examine whether the strain waveform altered the qualitative and quantitative nature of the response of vSMCs to mechanical forces we applied mechanical stretch to cells in culture using a sine, aortic or brachial waveform. For these waveforms, maximal strain of 7.5% was set to be identical between the groups as well as the frequency for the waveform at 1 Hz.

Mechanical stretch waveform alters the extent of activation of the AKT and ERK pathways

Activation of ERK and AKT signaling pathways has been implicated in induction of early response gene expression²¹ and proliferation in vSMCs following mechanical stretch^{1,3}. We loaded cells for 30 minutes and then examined the activation of these pathways by the cyclic strain waveforms (Fig. 3). All of the waveforms enhanced the phosphorylation of ERK over static baseline levels. Interestingly, the aortic waveform had the most phosphorylation of AKT following stretch but the least ERK activation of the different waveforms, while the brachial waveform had the highest levels of ERK activation following mechanical stretch.

Stretch waveforms have a differential induction of intermediate early response genes and release of FGF-2

We then examined the acute signaling response of vSMCs to the different arterial stretch waveforms. Mechanical strain causes transient increase in immediate-early response genes including early growth response gene-1 (EGR-1), c-Fos and c-Jun¹⁹ which act to increase the pro-inflammatory response of vSMCs including the upregulation of inflammatory growth factors/cytokines and cell adhesion molecules¹⁸ and promotes vSMC proliferation³⁰. We explored the differential response between the sine waveform and the physiological waveforms of the brachial arteries and aorta in their ability to activate initial signal events induced by mechanical stretch. We applied the different waveforms to vSMCs in the system for 30 minutes and assayed for gene expression of EGR-1, c-Fos and c-Jun. We found that the brachial waveform induced both EGR-1 and c-Fos by over three-fold in comparison to cells under static conditions (Fig. 4). In addition, sine waveform caused an increase in EGR-1 expression but the aortic waveform led to a non-significant increase. In addition to the activation of early response genes, mechanical strain has been shown to induce FGF-2 release/production in vSMCs inducing a mitogenic response^{21,31}. We found that the aortic and brachial waveforms induced increased amounts of FGF-2 release, particularly at longer time points (Fig. 5).

Stretch waveforms alter vSMC phenotypic modulation in response to mechanical strain

Cyclic stretch has been shown to increase the expression of contractile differentiation markers in vSMCs^{15,19}. We loaded cells for 24 hours and then examined the expression of several vSMC differentiation markers. Calponin is a key protein in regulating the contraction of vSMCs and is a late marker of vSMC differentiation in development¹⁶. There were increases in calponin for all groups with the greatest increase found in cells exposed to the brachial waveform (six-fold increase in comparison to a three-fold increase for the sine waveform; Fig. 6). Desmin is an intermediate filament and an early marker for muscle cell differentiation²⁰. We found that there was a two-fold increase in desmin expression for cells exposed to the sine waveform. The physiologic waveforms induced desmin more strongly (four-fold for aortic and six-fold for brachial waveform). Transgelin (SM-22) expression was increased for the brachial waveform but decreased for the sine waveform in comparison to the static controls. Tropomyosin, a regulator of actin/myosin interaction during muscle contractions, was increased only in cells exposed to the aortic waveform.

Stretch waveforms differentially regulate focal adhesion and cytoskeletal remodeling in vSMCs

Cytoskeletal rearrangement occurs in response to alterations in vSMC mechanical environment¹⁵. For dynamic mechanical strains, this remodeling leads to the formation of actin stress fibers and focal adhesion complexes. We compared the effects of dynamic strain waveforms on cytoskeletal remodeling and the induction of focal adhesion complex formation. We found that there were nearly two-fold increase in actin stress fibers and

paxillin in brachial waveform compared to the static experimental group. In contrast, we found that aortic waveform showed less actin fiber and focal adhesion formation (Fig. 7).

Mechanical strain-induced miRNA expression is altered by cyclic stretch waveforms

Recent studies have shown that miRNA are essential in stretch-induced differentiation of vSMCs^{32–35}. In particular, miR-143 and miR-145 have been linked to promoting the differentiated state in vascular smooth muscle cells while miR-31 and miR-221/222 has been associated with the dedifferentiated/proliferative state³⁶. For the sine waveform, expression of miR-222 was lowered but the expression of the other miRNAs examined was not affected by mechanical loading (Fig. 8). In contrast, for both the aortic and brachial artery waveforms there were an over 12-fold increase in expression of miR-143 and significant increases in expression of miR-145 and miR-221. For the brachial waveform miR-222 was decreased whereas it was unaffected in the cells treated by the aortic waveforms (Fig. 8).

Discussion

In this work we have created a system that can effectively apply dynamic loading in a format that is amenable to conventional well plate assays and imaging techniques. Using this system we have shown that, normalizing for the frequency of load application and maximal strain, the dynamics of the applied force to vSMC profoundly alter the end effects on signaling, gene expression, cytoskeletal rearrangement and phenotypic state. There are several prior systems, both commercially available and created by academic labs, that can apply mechanical stretch to cells in culture⁶. In comparison to these systems, the device presented here is novel in its incorporation of linear motor as the prime mover for applying mechanical strain. The linear motor and platform design incorporated in our device provides for improved dynamic control and frequency response. This is a key aspect for a system to duplicate complex arterial waveforms such as the brachial waveform used in the study. Even though this waveform repeats at a 1 Hz frequency, the complexity of the wave shape means that it contains high frequency components that are necessary for accurate reproduction of the in-vivo distention waveform. There is a limit to the frequency of loading that can be applied using systems with a silicone membrane both due to the viscoelastic properties of silicone and the frictional interface between the piston and the membrane. However, the silicone membrane systems can propagate fairly high frequency strain provided there is sufficient pretension in the membrane. Our calibration with the brachial and other complex waveforms suggest that the system is able to propagate the high frequency information needed to accurately reproduce these in-vivo waveforms. To our knowledge, the commonly used commercial systems have not been validated for applying these high frequencies and the previous studies on temporal delay would suggest that it may be challenging to produce these waveforms with a pneumatic system. A second novel aspect of the study was the dynamic adaptation of the waveforms to produce constant maximal strain over long periods of time. This calibration essentially removed the reduction in strain levels that occurs due to relaxation of the silicone membrane over time.

The linear motor provides improved dynamic control and also facilitates the system's modularity in allowing mechanical load to be applied in virtually any format by replacing the piston platen and culture plate. We used this flexibility to design a system that recapitulated 6-well plates of the exact geometry as standard culture plates. By modifying the piston and culture plate configuration the system could be modified easily to apply loads in other formats including 24-well or 96-well formats. The system also maintains the advantage of highly uniform biaxial stain application in the region of the piston contact with the membrane⁹ in contrast to methods using pneumatic pressure or other piston designs³. In this regard, the rigid piston-based system may also possess better dynamic properties for

applying strains of varying frequencies than other system types. A pneumatic system has an inherent time delay for the transfer of pressure and the compressibility of air may lead to inaccuracies in complex dynamic loading versus static or slow applied loading. The pneumatic time constant would dictate the ability of the system to apply higher frequency loads and may be a source of deviation of the dynamic strain from the predicted strain from static measure³. Simmons et al have also created a system that uses a similar pneumatic mechanism to stretch membranes over microposts³⁷. For their pneumatic system alone (not accounting for the membrane motion) there was a 1 second delay before the pressure changes stabilizes between a step from 10 kPa to 50 kPa in pressure³ but this would likely be highly dependent upon the dynamics of the particular system configuration. In contrast, our system used a feedback-controlled linear motor in which the primary limitation is the maximal force applied and the optimization of the control loop for producing the defined motions.

Using the system, we examined the effects of strain waveform independent of the maximal strain and the overall period of the mechanical loading cycle. We chose strain waveforms from clinical data to mimic the strains (although with scaled timing and magnitude) that are experienced in different vascular beds. To our knowledge, there have been no previous studies that have examined whether strain waveform shape alters the response of vSMC phenotype to mechanical strain. In our first set of studies, we examined the activation of the ERK and AKT signaling pathways by cyclic mechanical stretch. Both pathways were activated by all of the waveforms but for AKT there was an increase in relative activation for the brachial waveform. Interestingly, this waveform was the only one with a marked increase in EGR-1 following loading. AKT can phosphorylate EGR-1 leading to increased interactions with the alternative reading frame (ARF) protein³⁸. A previous study found that low rates of strain led to a decrease in ERK phosphorylation whereas high strain rates increased its phosphorylation³⁹. This finding would be consistent with our studies as for all three waveforms, the initial strain rate is above the low strain rate used in this study.

The EGR-1 transcription factor is known to be mechanically sensitive and is involved in atherosclerosis and intimal hyperplasia⁴⁰. Several previous studies have shown activation of EGR-1 in vSMCs by mechanical stretch^{19, 30}. These studies used high levels of maximal stretch (15 or 25% strain). Thus, a possible explanation for this difference is that in our studies the maximal level of strain was 7.5% whereas previous studies had used much higher levels of strain. It should be noted that physiologic circumferential strains in the artery do not normally exceed 10% strain^{41, 42}. In our studies we found that EGR-1 was only induced by the brachial waveform and not by the sine or aortic waveforms. Thus, our work adds to the field by demonstrating that the responsiveness of stretch inducible signaling pathways and intermediate early gene expression in vSMCs is sensitive not only to the maximal stretch but the character of the stretch waveform applied. As arterial pulse waveforms are altered with vascular remodeling in hypertension and atherosclerosis^{43, 44}, our work supports that these changes may contribute to altered vSMC behavior in these disease states.

Cyclic mechanical loading has been shown to drive vSMCs into a more differentiated state expressing markers of mature vSMCs^{18, 19, 22}. Our studies now show that the character of the strain waveform applied to the vSMCs alters magnitude and extent of phenotypic modulation induced by mechanical load. Our studies show that the brachial and aortic waveform induced higher levels of mature vSMC markers, FGF-2 release and pro-differentiation regulatory miRNAs. Interestingly, the brachial waveform induced the greatest amount of cytoskeletal rearrangement and AKT phosphorylation. Taken together, these findings illustrate that vSMCs are sensitive to the nature of the mechanical forces applied rather than simply responding to any mechanical load with a binary on/off response. Thus,

our findings give new perspective on the conclusions of many previous experiments using sinusoidal cyclic mechanical loading.

In recent years, microRNAs have been revealed as potent mediators of a variety of vSMC biology, atherosclerosis and intimal hyperplasia^{45–47}. Several studies support that miRNA are essential in stretch-induced differentiation of vSMCs^{32–35}. In particular the miR-143/145 cluster promotes the differentiated state³⁶. There was a striking difference in the regulation of miRNA expression in response to the physiologic strain waveforms in comparison to the sinusoidal loading. Both aortic and brachial waveforms stimulated large increases in miR-143, miR-145 and miR-221 expression. These results are consistent with the increased differentiated phenotype observed in vSMCs exposed to these waveforms suggesting differential mechanical regulation of miRNAs may underlie our findings on vSMC differentiation. While it is tempting to assume a universality to the results obtained from cultures exposed to sinusoidal waveforms of cyclic mechanical stretch, our results would suggest the nature of the loading has a highly potent effect on the signaling, gene expression and functional outcomes of the mechanical loading on vSMC biology.

In vitro experiments, animal studies and human studies have shown that mechanical stresses are powerful determinants in the regulation of arterial function, myocardial remodeling and the differentiation of progenitor cells in the vascular system. In order to more accurately recreate and parameterize the complex in-vivo environment, it is important to have precise control over the temporal stretch profiles that are applied to experiments using cultured cells. The system presented in this work provides a flexible and highly adjustable means to apply stretch to cultured cells by using a linear motor based piston array. The capacity to specify complex strain profiles and alter their character and magnitude independently of other factors enables the systematic characterization of the cells' responses to the mechanical environments representative of virtually any physiologic or disease state. Thus, the results acquired using this device may be valuable in studying cellular regulation and adaptation to mechanical forces in vivo in many biological systems.

Acknowledgments

The authors would like to acknowledge support through the American Heart Association (10SDG2630139) and through the NIH Director's New Innovator Grant (1DP2 OD008716-01).

References

1. Traub O, Berk BC. Arteriosclerosis, thrombosis, and vascular biology. 1998; 18:677–685.
2. Chatzizisis YS, Baker AB, Sukhova GK, Koskinas KC, Papafaklis MI, Beigel R, Jonas M, Coskun AU, Stone BV, Maynard C, Shi GP, Libby P, Feldman CL, Edelman ER, Stone PH. Circulation. 2011; 123:621–630. [PubMed: 21282495]
3. Fukumoto Y, Hiro T, Fujii T, Hashimoto G, Fujimura T, Yamada J, Okamura T, Matsuzaki M. Journal of the American College of Cardiology. 2008; 51:645–650. [PubMed: 18261684]
4. Roman MJ, Pickering TG, Pini R, Schwartz JE, Devereux RB. Hypertension. 1995; 26:369–373. [PubMed: 7635548]
5. Shaw A, Xu Q. Current vascular pharmacology. 2003; 1:41–58. [PubMed: 15320852]
6. Brown TD. J Biomech. 2000; 33:3–14. [PubMed: 10609513]
7. Kim DH, Wong PK, Park J, Levchenko A, Sun Y. Annual review of biomedical engineering. 2009; 11:203–233.
8. Schulz RM, Bader A. Eur Biophys J. 2007; 36:539–568. [PubMed: 17318529]
9. Schaffer JL, Rizen M, L'Italien GJ, Benbrahim A, Megerman J, Gerstenfeld LC, Gray ML. J Orthop Res. 1994; 12:709–719. [PubMed: 7931788]
10. Flexcell I. 1997

11. Wang JH, Thampatty BP. *Biomech Model Mechanobiol*. 2006; 5:1–16. [PubMed: 16489478]
12. Ruwhof C, van der Laarse A. *Cardiovasc Res*. 2000; 47:23–37. [PubMed: 10869527]
13. Silver FH, Siperko LM, Seehra GP. *Skin Res Technol*. 2003; 9:3–23. [PubMed: 12535279]
14. Haga JH, Li YS, Chien S. *J Biomech*. 2007; 40:947–960. [PubMed: 16867303]
15. Numaguchi K, Eguchi S, Yamakawa T, Motley ED, Inagami T. *Circ Res*. 1999; 85:5–11. [PubMed: 10400905]
16. Kawabe J, Okumura S, Lee MC, Sadoshima J, Ishikawa Y. *American journal of physiology. Heart and circulatory physiology*. 2004; 286:H1845–H1852. [PubMed: 15072971]
17. Zhou RH, Lee TS, Tsou TC, Rannou F, Li YS, Chien S, Shyy JY. *Arteriosclerosis, thrombosis, and vascular biology*. 2003; 23:2015–2020.
18. Albinsson S, Nordstrom I, Hellstrand P. *The Journal of biological chemistry*. 2004; 279:34849–34855. [PubMed: 15184395]
19. Albinsson S, Hellstrand P. *American journal of physiology. Cell physiology*. 2007; 293:C772–C782. [PubMed: 17507430]
20. Wilson E, Mai Q, Sudhir K, Weiss RH, Ives HE. *The journal of cell biology*. 1993; 123:741–747. [PubMed: 8227136]
21. Cheng GC, Briggs WH, Gerson DS, Libby P, Grodzinsky AJ, Gray ML, Lee RT. *Circ Res*. 1997; 80:28–36. [PubMed: 8978319]
22. Birukov KG, Shirinsky VP, Stepanova OV, Tkachuk VA, Hahn AW, Resink TJ, Smirnov VN. *Molecular and cellular biochemistry*. 1995; 144:131–139. [PubMed: 7623784]
23. Lehmann ED. *Lancet*. 2000; 355:412. [PubMed: 10665588]
24. Ramsey MW, Goodfellow J, Jones CJ, Luddington LA, Lewis MJ, Henderson AH. *Circulation*. 1995; 92:3212–3219. [PubMed: 7586306]
25. Studinger P, Lenard Z, Reneman R, Kollai M. *Ultrasound in medicine & biology*. 2000; 26:1285–1291. [PubMed: 11120366]
26. O'Rourke MF, Adji A, Namasivayam M, Mok J. *Drugs & aging*. 2011; 28:779–795. [PubMed: 21970306]
27. Baker AB, Chatzizisis YS, Beigel R, Jonas M, Stone BV, Coskun AU, Maynard C, Rogers C, Koskinas KC, Feldman CL, Stone PH, Edelman ER. *Atherosclerosis*. 2010; 213:436–442. [PubMed: 20950809]
28. Baker AB, Ettenson DS, Jonas M, Nugent MA, Iozzo RV, Edelman ER. *Circ Res*. 2008; 103:289–297. [PubMed: 18583708]
29. van Houwelingen MJ, Barenbrug PJ, Hoeberigs MC, Reneman RS, Hoeks AP. *Ultrasound in medicine & biology*. 2007; 33:371–378. [PubMed: 17208354]
30. Zhang W, Yan Q, Zeng YS, Zhang XB, Xiong Y, Wang JM, Chen SJ, Li Y, Bruce IC, Wu W. *Brain research*. 2010; 1359:256–271. [PubMed: 20816761]
31. Sudhir K, Hashimura K, Bobik A, Dilley RJ, Jennings GL, Little PJ. *American journal of hypertension*. 2001; 14:1128–1134. [PubMed: 11724212]
32. Turczynska KM, Sadegh MK, Hellstrand P, Sward K, Albinsson S. *The Journal of biological chemistry*. 2012; 287:19199–19206. [PubMed: 22474293]
33. Song L, Duan P, Guo P, Li D, Li S, Xu Y, Zhou Q. *Archives of biochemistry and biophysics*. 2012; 528:204–211. [PubMed: 23046980]
34. Song J, Hu B, Qu H, Bi C, Huang X, Zhang M. *PloS one*. 2012; 7:e47657. [PubMed: 23082189]
35. Turczynska KM, Bhattachariya A, Sail J, Goransson O, Sward K, Hellstrand P, Albinsson S. *PloS one*. 2013; 8:e65135. [PubMed: 23705032]
36. Liu X, Cheng Y, Yang J, Xu L, Zhang C. *Journal of molecular and cellular cardiology*. 2012; 52:245–255. [PubMed: 22138289]
37. Wentzel JJ, Gijzen FJ, Schuurbijs JC, Krams R, Serruys PW, De Feyter PJ, Slager CJ. *J Biomech*. 2005; 38:1551–1555. [PubMed: 15922767]
38. Yu J, Zhang SS, Saito K, Williams S, Arimura Y, Ma Y, Ke Y, Baron V, Mercola D, Feng GS, Adamson E, Mustelin T. *The EMBO journal*. 2009; 28:21–33. [PubMed: 19057511]

39. McKnight NL, Frangos JA. *Annals of biomedical engineering*. 2003; 31:239–249. [PubMed: 12680722]
40. Khachigian LM. *Circ Res*. 2006; 98:186–191. [PubMed: 16456111]
41. Peterson LH, Jensen RE, Parnell J. *Circ Res*. 1960; 8:622–639.
42. Leone N, Ducimetiere P, Garipey J, Courbon D, Tzourio C, Dartigues JF, Ritchie K, Alperovitch A, Amouyel P, Safar ME, Zureik M. *Arteriosclerosis, thrombosis, and vascular biology*. 2008; 28:1392–1397.
43. Resnick LM, Militianu D, Cunnings AJ, Pipe JG, Evelhoch JL, Soulen RL, Lester MA. *American journal of hypertension*. 2000; 13:1243–1249. [PubMed: 11130766]
44. Wang X, Keith JC Jr, Struthers AD, Feuerstein GZ. *Cardiovascular therapeutics*. 2008; 26:214–223. [PubMed: 18786091]
45. Zampetaki A, Mayr M. *Circ Res*. 2012; 110:508–522. [PubMed: 22302757]
46. McDonald RA, Hata A, MacLean MR, Morrell NW, Baker AH. *Cardiovasc Res*. 2012; 93:594–604. [PubMed: 22065733]
47. Urbich C, Kuehbacher A, Dimmeler S. *Cardiovasc Res*. 2008; 79:581–588. [PubMed: 18550634]

Figure 1. Device for applying mechanical stretch to cells in culture

(A) Mechanical load is applied to cells grown on a flexible silicone membrane through the displacement of an underlying Teflon piston. (B) Top view of plate undergoing mechanical stretch. (C,D) Overall view of system in which a linear motor controls the motion of a platen containing 36 individual pistons that can be used to apply strain to cell cultured in 96-well plates with flexible bottoms. The linear motor can apply arbitrary stretch waveforms (eg. biphasic stretch waves simulating coronary arterial stretch during the cardiac cycle). (E) Photographs of the completed device in a culture incubator. (F) Strain on the membrane as a function of vertical motor displacement. Each 100 counts on the motor index are equivalent to 1 mm of displacement. The error bars at each point represent the variability for 6 wells

within the plate (SEM). (G) Uniformity of the circumferential strain within the well. Plotted is the circumferential strain at three radii from the center of the well. The total well diameter is 35 mm. (H) Alterations in applied strain due to membrane relaxation after 96 hours of cyclic mechanical strain (10% strain, 1Hz loading and temperature of 37°C).

Aortic Waveform

Brachial Waveform

Figure 2. Calibration of strain profiles for dynamic stretch waveforms

Upper graphs represent motor position output of applied waveform and lower graphs are the average of circumferential and radial strains measured from marker displacement.

Figure 3. Activation of signaling pathways by cyclic strain waveforms

The cells were exposed to one of the various strain waveforms at an applied frequency of 1 Hz and maximal strain of 7.5%. The loading was applied for 30 minutes and then the cells were lysed for immunoblotting analysis. Two-way ANOVA statistical test shows significant difference to static, sine, and brachial group denoted with *, †, and ‡, respectively at $P < 0.05$.

Figure 4. Expression of intermediate early genes after application of mechanical load

Vascular smooth muscle cells were exposed to cyclic mechanical stretch of various strain waveforms. The loading was applied at 1 Hz with a maximal strain of 7.5% for 30 minutes. Following loading the mRNA was isolated and assayed using real time PCR. Two-way ANOVA statistical test shows significant difference to static, sine, and brachial group denoted with *, †, and ‡, respectively at $P < 0.05$.

Figure 5. Fibroblast growth factor-2 (FGF-2) concentration in the condition media after application of mechanical load

Vascular smooth muscle cells were exposed to cyclic mechanical stretch of various strain waveforms. The loading was applied at 1 Hz with a maximal strain of 7.5% for 30 minutes, 4 hours, or 24 hours. Following loading, the media was collected and assayed using ELISA. Two-way ANOVA statistical test shows significant difference to static and sine group denoted with * and †, respectively.

Figure 6. Stain waveforms alter gene expression of vascular smooth muscle cell differentiation markers

The cells were exposed to cyclic mechanical strain with 1 Hz frequency of the various waveforms scaled to have a maximal strain of 7.5% for 24 hours. The mRNA was then isolated and the assayed for gene expression using real time PCR. Two-way ANOVA statistical test shows significant difference to static, sine, and brachial group denoted with *, †, and ‡, respectively at $P < 0.05$.

Figure 7. Immunocytochemical staining for paxillin and actin in vascular smooth muscle cells exposed to varying strain waveforms

The cells were treated with the waveforms at a frequency of 1 Hz and maximal strain of 7.5% for 4 hours. Scale bar shows 25 μ m for original images and 5 μ m for magnified images. Two-way ANOVA statistical test shows significant difference to static, sine, and brachial group denoted with *, †, and ‡, respectively at $P < 0.05$.

Figure 8. MicroRNA expression in vascular smooth muscle cells exposed to several strain profiles

The cells were treated with cyclic biaxial strain for 24 hours and then assays for miRNA expression. Two-way ANOVA statistical test shows significant difference to static, sine, and brachial group denoted with *, †, and ‡, respectively at $P < 0.05$.

Table 1

Primers used for Real Time PCR

Gene	Forward Primer	Reverse Primer
EGR-1	CTGACCGCAGAGTCTTTTCCT	GCGGCCAGTATAGGTGATGG
c-Fos	GGGGCAAGGTGGAACAGTTA	GTCTGTCTCCGCTTGGAGTG
c-Jun	TGAGTGACCGCGACTTTTCA	TTTCTCTAAGAGCGCACGCA
GAPDH	AATGGGCAGCCGTTAGGAAA	GCCCAATACGACCAAATCAGAG
Calponin1	CAGCATGGCGAAGACGAAAG	CTGCAGCCCAATGATGTTCC
Desmin	GAAATCCGGCACCTCAAGGA	GATGGGGAGATTGATCCGGC
SM22	AATGATGGGCACTACCGTGG	GGCCAATGACATGCTTTCCC
Tropomyosin	CTCTCAGAAGGCCAAGTCCG	TCAGCTTGTCGGAAGGACC

Abbreviations used were as follows: EGR1 = Early growth response-1; c-Fos = FBJ murine osteosarcoma viral oncogene homolog; c-Jun = Jun proto-oncogene; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase; SM22 = Smooth muscle protein 22.