J Appl Physiol 104: 766–772, 2008.
First published January 10, 2008; doi:10.1152/japplphysiol.00870.2007.

Cyclic strain induces mouse embryonic stem cell differentiation into vascular smooth muscle cells by activating PDGF receptor β

Nobutaka Shimizu,[1,2]* Kimiko Yamamoto,[1,3]* Syotaro Obi,[1] Shinichiro Kumagaya,[1] Tomomi Masumura,[1] Yasumasa Shimano,[1] Keiji Naruse,[4] Jun K. Yamashita,[5] Takashi Igarashi,[2] and Joji Ando[1]

[1]*Department of Biomedical Engineering and* [2]*Department of Pediatrics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan;* [3]*PRESTO, Japan Science and Technology Agency, Saitama, Japan;* [4]*Department of Cardiovascular Physiology, Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University, Okayama, Japan;* [5]*Stem Cell Research Center, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan*

Submitted 13 August 2007; accepted in final form 22 December 2007

Shimizu N, Yamamoto K, Obi S, Kumagaya S, Masumura T, Shimano Y, Naruse K, Yamashita JK, Igarashi T, Ando J. Cyclic strain induces mouse embryonic stem cell differentiation into vascular smooth muscle cells by activating PDGF receptor β. *J Appl Physiol* 104: 766–772, 2008. First published January 10, 2008; doi:10.1152/japplphysiol.00870.2007.—Embryonic stem (ES) cells are exposed to fluid-mechanical forces, such as cyclic strain and shear stress, during the process of embryonic development but much remains to be elucidated concerning the role of fluid-mechanical forces in ES cell differentiation. Here, we show that cyclic strain induces vascular smooth muscle cell (VSMC) differentiation in murine ES cells. Flk-1-positive (Flk-1$^+$) ES cells seeded on flexible silicone membranes were subjected to controlled levels of cyclic strain and examined for changes in cell proliferation and expression of various cell lineage markers. When exposed to cyclic strain (4–12% strain, 1 Hz, 24 h), the Flk-1$^+$ ES cells significantly increased in cell number and became oriented perpendicular to the direction of strain. There were dose-dependent increases in the VSMC markers smooth muscle α-actin and smooth muscle-myosin heavy chain at both the protein and gene expression level in response to cyclic strain, whereas expression of the vascular endothelial cell marker Flk-1 decreased, and there were no changes in the other endothelial cell markers (Flt-1, VE-cadherin, and platelet endothelial cell adhesion molecule 1), the blood cell marker CD3, or the epithelial marker keratin. The PDGF receptor β (PDGFRβ) kinase inhibitor AG-1296 completely blocked the cyclic strain-induced increase in cell number and VSMC marker expression. Cyclic strain immediately caused phosphorylation of PDGFRβ in a dose-dependent manner, but neutralizing antibody against PDGF-BB did not block the PDGFRβ phosphorylation. These results suggest that cyclic strain activates PDGFRβ in a ligand-independent manner and that the activation plays a critical role in VSMC differentiation from Flk-1$^+$ ES cells.

hemodynamic force; biomechanics; blood vessel

EMBRYONIC STEM (ES) cells derived from the inner cell mass of a blastocyst stage embryo are able to differentiate into the three embryonic germ layers (endoderm, ectoderm, and mesoderm) and are thus able to produce virtually all types of somatic cells (5, 16). ES cells are considered a promising source of seed cells for tissue engineering (22), and a great effort has been made to develop methods of inducing ES cells to differentiate into various specialized cells (1, 14, 25, 31). Yamashita et al. (34) developed a method that uses cell growth factors to induce selective differentiation of ES cells into vascular cells. In this method, undifferentiated mouse ES cells are cultured on type IV collagen-coated dishes, and vascular endothelial growth factor (VEGF) receptor 2 (Flk-1)-positive (Flk-1$^+$) cells are isolated by flow cytometry sorting. Addition of VEGF to the cultures promotes endothelial differentiation, whereas mural cells, including vascular smooth muscle cells (VSMCs) and pericytes, are induced by platelet-derived growth factor-BB (PDGF-BB). The vascular cells derived from Flk-1$^+$ cells have been shown to contribute to the developing vasculature in vivo.

Adult blood vessel cells are known to alter their shape, function, and gene expression in response to fluid-mechanical forces, such as shear stress produced by flowing blood and cyclic strain generated by pulsatile changes in blood pressure (3). The vascular cell responses to mechanical forces are thought to play an important role in sustaining the homeostasis of the circulatory system and in blood flow-dependent phenomena, such as angiogenesis, vascular remodeling, and atherogenesis. Fluid-mechanical forces have recently been shown to control embryonic development and organogenesis: intracardiac fluid forces are essential for the formation of a functional heart in zebrafish embryos (7), and the direction of fluid flow on the node of mouse embryos determines left-right asymmetry in the body plan (19). Moreover, it is now clear that fluid-mechanical forces affect immature and undifferentiated cells, as well as adult cells. Our previous studies (32, 33) showed that shear stress induces selective differentiation by bone marrow-derived endothelial progenitor cells and Flk-1$^+$ ES cells into the vascular endothelial cell (EC) lineage in vitro.

The hemodynamics of the mammalian embryo has recently been analyzed. Jones et al. (10, 11) made quantitative flow measurements during early organogenesis in mouse embryos and detected laminar shear stress levels of between 0 and 5.5 dyn/cm^2 in embryos from 8.5 to 10.5 days postcoitum (dpc) and a heart rate ranging from about 80 to 100 beats/min. According to data obtained from rat and chick embryos, pressure levels in embryos are low, ~1–2 mmHg (8, 17). During the process of embryonic development, ES cells appear to be exposed to shear stress and cyclic strain generated by the beating heart. Cyclic strain and shear stress have both been recognized as important modulators of vascular cell function, including cell proliferation, apoptosis, differentiation, morphology, migration, and the secretion of various macromolecules (12). More recent studies have revealed that cyclic strain

* N. Shimizu and K. Yamamoto contributed equally to this work.

Address for reprint requests and other correspondence: J. Ando, Dept. of Biomedical Engineering, Graduate School of Medicine, Univ. of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan (e-mail: joji@m.u-tokyo.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

 8750-7587/08 $8.00 Copyright © 2008 the American Physiological Society

Downloaded from http://jap.physiology.org/ by 10.220.33.6 on September 6, 2017

affects ES cell differentiation. Schmelter et al. (28) demonstrated that static mechanical strain promotes cardiovascular differentiation by ES cells through the generation of reactive oxygen species. Saha et al. (26), on the other hand, showed that mechanical strain has an inhibitory effect on ES cell differentiation. Thus the role of fluid-mechanical forces in ES cell differentiation seems open to discussion.

In the present study, we investigated whether cyclic strain affects the differentiation of Flk-1$^+$ ES cell and, if so, which cell lineage they differentiate into. Mouse Flk-1$^+$ ES cells cultured on flexible silicone membranes were subjected to controlled levels of cyclic strain and examined for changes in the expression of various cell lineage markers. We also investigated the molecular mechanism involved in the effects of cyclic strain on Flk-1$^+$ ES cell differentiation in terms of PDGF receptor phosphorylation.

MATERIALS AND METHODS

Cell culture. MGZ5 ES cells [gift from H. Niwa (Riken, CDB, Kobe, Japan)] were maintained, differentiated, and cultured as previously described (32). The cells were initially maintained undifferentiated without a feeder layer on gelatin-coated tissue culture dishes in DMEM (IBL, Fujioka, Japan) containing 15% FBS (JRH Biosciences), 10^3 U/ml leukemia inhibitory factor (ESGRO Complete kit; Chemicon), 1× nonessential amino acid (ICN Pharmaceuticals), and 5×10^{-5} mol/l β-mercaptoethanol (Sigma). To initiate ES cell differentiation, trypsinized cells were plated on type IV collagen-coated Petri dishes (BD Falcon) and cultured without leukemia inhibitory factor in α-MEM (GIBCO) containing 10% FBS, 50 U/ml penicillin-streptomycin (ICN Pharmaceuticals), and 5×10^{-5} mol/l β-mercaptoethanol. On *day 4*, Flk-1$^+$ ES cells were isolated by standard immunomagnetic techniques (MACS kit; Miltenyi Biotech) using anti-mouse Flk-1 antibody (Clone Avas 12α1; Pharmingen) and plated in differentiation medium (α-MEM containing 10% FBS, 50 U/ml penicillin-streptomycin, and 5×10^{-5} mol/l β-mercaptoethanol) in silicon chambers. After culture for 3 days, cells became confluent and were used for experiments.

Cyclic strain experiments. Flk-1$^+$ ES cells were exposed to cyclic strain with a uniaxial mechanical strain-loading device, as described previously (30). Briefly, type IV collagen-coated polydimethylsiloxane chambers in which the cells were cultured were fixed in a cyclic strain-loading device (STREX ST-140; Strex, Osaka, Japan). One end of the chamber was firmly attached to the fixed frame, and the other end of the chamber was fixed to the movable frame connected to a motor-driven shaft. The amplitude and frequency of stretching were controlled by a programmable microcomputer, and cyclic strain in the 2–12% range with 1 Hz was used in the present study. The polydimethylsiloxane membrane (32 mm × 32 mm) was uniaxially and uniformly stretched over the entire membrane area, except at both lateral edges (2–3 mm in width), where the strain was slightly lower than the amount applied; i.e., the difference between the lateral edges and other areas was no more than one-tenth of that applied. All experiments were performed at 37°C in a CO_2 incubator.

Immunohistochemistry. Cells were fixed with 4% paraformaldehyde (Sigma), permeabilized with 0.1% Triton X-100 (Sigma), and maintained in 1% normal BSA (Sigma) to block nonspecific protein binding sites. The cells were incubated with monoclonal antibodies against platelet endothelial cell adhesion molecule 1 (PECAM-1; Pharmingen) and then with monoclonal antibody against smooth muscle α-actin (SM α-actin; Sigma). After they were washed, cells were incubated with a secondary antibody (Alexa Fluor 488 goat anti-rat IgG or Alexa Fluor 594 goat anti-mouse IgG; Molecular Probes) at a dilution of 1:500. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma). Stained cells were photographed through a confocal laser scanning microscope (Leica), and all images were imported into Adobe Photoshop as JPEGs for contrast manipulation and figure assembly.

Western blot analysis. Western blot analyses were performed as previously described (32). Briefly, cells were dissolved in lysis buffer containing a 0.1% protease inhibitor mixture (Sigma) and centrifuged at 2.6×10^4 *g* for 30 min. The protein concentration of the lysate was determined with a protein assay kit (Bio-Rad). Equal amounts of protein were dissolved in SDS-PAGE sample buffer, separated by SDS-PAGE, transferred to Immobilon membranes (Millipore), and incubated with antibodies against SM α-actin or smooth muscle myosin heavy chain (SM-MHC; Biomedical Technologies). Anti-mouse PDGF receptor β (PDGFRβ) phosphospecific antibody (pY857; BD Pharmingen) was used for the analysis of PDGFRβ phosphorylation. After they were washed and incubated with horseradish peroxidase-linked anti-mouse or anti-rabbit IgG, immunoreactive proteins were visualized with the enhanced chemiluminescence plus detection system (Amersham) and GS363 molecular imager system (Bio-Rad).

Flow cytometry. Expression of various cell lineage marker proteins was measured by flow cytometry. Cells were detached from the dishes by incubation at room temperature for 15 min in PBS supplemented with 1 mM EDTA (Sigma) and then suspended in PBS with 10% FBS. A total of 200,000 cells were then incubated for 60 min at 4°C with monoclonal antibodies against the EC markers, including the VEGF receptors Flk-1 (Pharmingen) and Flt-1 (Chemicon), and the intercellular adhesion molecules VE-cadherin (Pharmingen) and PECAM-1, the blood cell marker T3 antigen (CD3; Pharmingen), and the epithelial cell marker keratin (NeoMarkers). Next, the cells were incubated for 60 min at 4°C with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) and analyzed by fluorescence-activated cell sorting (Becton Dickinson). Histograms of cell number vs. logarithmic fluorescence intensity were recorded for 20,000 cells per sample. Background fluorescence was obtained from the negative control cells stained with the secondary antibody and subtracted from the mean fluorescence of the specific staining patterns. The expression level of each antigen was expressed as the mean channel fluorescence.

Real-time PCR analysis. Total RNA samples were prepared from cells with ISOGEN (Nippon Gene, Tokyo, Japan), and first-strand cDNAs were generated by using Moloney murine leukemia virus reverse transcriptase (Roche) and RNA primed with oligo(dT) primer. After reverse transcription of the RNA into cDNA, real-time PCR was used to monitor gene expression with a Smart Cycler (Cepheid) according to the standard procedure. PCR was performed with a Takara EX *Taq* R-PCR version (Takara) and the primer pairs shown in Table 1. The temperature profile consisted of initial denaturation for 30 s at 95°C followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, elongation at 72°C, and fluorescence monitoring at 85°C. The specificity of the amplification reaction was determined by performing a melting-curve analysis. Relative quantification of the signals was achieved by normalizing the signals of the different genes to β-actin.

Statistical analysis. All data are expressed as means ± SD. Statistical significance was evaluated by an ANOVA and a Bonferonni's adjustment applied to the results of a *t*-test with software from SPSS. A P value of <0.05 was regarded as statistically significant.

RESULTS

Cyclic strain enhances Flk-1$^+$ ES cell proliferation. The same number of Flk-1$^+$ ES cells were plated in silicon chambers; after the cells became confluent, they were subjected to cyclic strain (4, 8, or 12% strain, 1 Hz) or incubated under static conditions for 24 h. The cells were removed by trypsinization, and a Coulter counter was used to count their number (Fig. 1*A*). Cell number increased in response to cyclic strain, peaked at 8% strain, and leveled off at 12% strain. The

Downloaded from http://jap.physiology.org/ by 10.220.33.6 on September 6, 2017

Table 1. *Oligonucleotide primers used for real-time PCR*

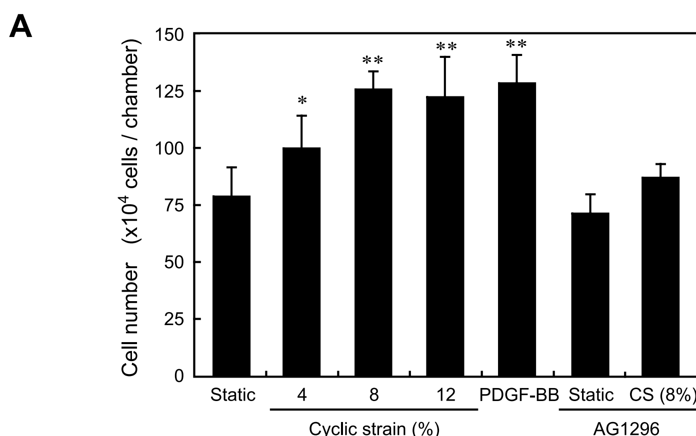
Gene	Primer Sequence	Amplified Fragment Size, bp
SM α-actin	Fwd: 5′-ACGGCCGCCTCCTCTTCCTC-3′	415
	Rev: 5′-GCCCAGCTTCGTCGTATTCC-3′	
SM-MHC	Fwd: 5′-GACAACTCCTCTCGCTTTGG-3′	201
	Rev: 5′-GCTCTCCAAAAGCAGGTCAC-3′	
SM22α	Fwd: 5′-GCAGTCCAAAATTGAGAAGA-3′	507
	Rev: 5′-CTGTTGCTGCCCATTTGAAG-3′	
Flk-1	Fwd: 5′-TCTGTGGTTCTGCGTGGAGA-3′	248
	Rev: 5′-GTATCATTTCCAACCACCCT-3′	
Flt-1	Fwd: 5′-CGGAAGCTCTGATGATGTGA-3′	199
	Rev: 5′-TATCTTCATGGAGGCCTTGG-3′	
VE-cadherin	Fwd: 5′-CTTCCGAATAACCAAGCAGG-3′	369
	Rev: 5′-TACTTGACCGTGATGTTGGC-3′	
PECAM-1	Fwd: 5′-ACATGCCATAGGCATCAGC-3′	305
	Rev: 5′-TCACAGAGCACCGAAGTACC-3′	
β-Actin	Fwd: 5′-GTCGTACCACAGGCATTGTGATGG-3′	493
	Rev: 5′-GCAATGCCTGGGTGCATGGTGG-3′	

Fwd, forward; Rev, reverse; SM, smooth muscle; MHC, myosin heavy chain; PECAM-1, platelet endothelial cell adhesion molecule-1.

increase in cell number at 8% or 12% strain was almost the same as the level induced by a maximally effective concentration of PDGF-BB (23). Flk-1$^+$ ES cells were subjected to cyclic strain (8% strain, 1 Hz) in the presence of the PDGF receptor kinase inhibitor AG-1296, which potently and selectively inhibits signaling of PDGFRα and PDGFRβ as well as of its family member *Kit* (13). AG-1296 almost completely suppressed the cyclic strain-induced increase in cell number, indicating that PDGF receptor activation is involved in the effect of cyclic strain on Flk-1$^+$ ES cell proliferation.

Flk-1$^+$ ES cells that had been cultured under static conditions or that had been exposed to cyclic strain (8%, 1 Hz) or PDGF-BB for 24 h were immunostained for an EC marker, PECAM-1, and a VSMC marker, SM α-actin (Fig. 1*B*). Under static conditions, most of the cells stained positive for SM α-actin (red) and some of the cells stained positive for PECAM-1 (green). When exposed to cyclic strain, the number of PECAM-1-positive cells decreased, whereas the number of SM α-actin-positive cells increased, and their long axis became oriented perpendicular to the direction of strain. Addition of PDGF-BB also decreased the number of PECAM-1-positive cells and increased the number of SM α-actin-positive cells, but it did not cause any change in cell orientation. The percentage of PECAM-1-positive cells determined by flow cytometry was 12.3 ± 0.25% (mean ± SD, $n = 5$) of the static control cells, 2.41 ± 0.35% of the cells exposed to cyclic strain ($P < 0.01$ vs. static control), and 3.25 ± 0.24% of the cells treated with PDGF-BB ($P < 0.01$ vs. static control).

Cyclic strain induces differentiation of Flk-1$^+$ ES cells into the VSMC lineage. Flk-1$^+$ ES cells that had been cultured under static conditions or exposed to cyclic strain (2, 4, 8, or 12%, 1 Hz) for 24 h were examined for changes in expression of various cell lineage markers. When exposed to cyclic strain, expression of the VSMC markers SM α-actin and SM-MHC increased markedly in a dose-dependent manner (Fig. 2, *A* and *B*). By contrast, cyclic strain (8%, 1 Hz, 24 h) significantly decreased the expression of the EC marker Flk-1 and had no effect on the expression of the other EC markers (Flt-1, VE-cadherin, and PECAM-1), the blood cell marker CD3, or the epithelial marker keratin (Fig. 2*C*). The addition of PDGF-BB to static Flk-1$^+$ ES cells had almost the same



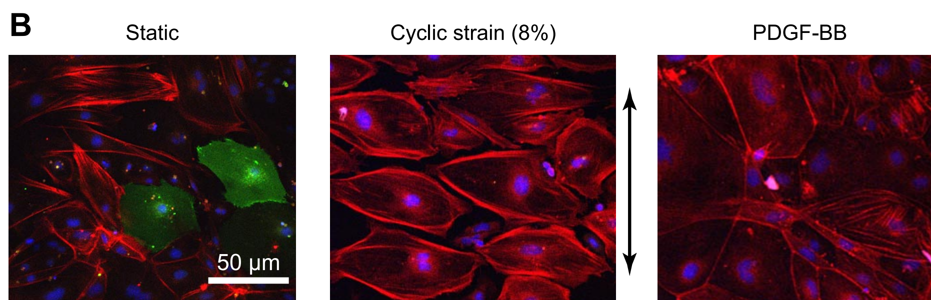


Fig. 1. Effect of cyclic strain on cell proliferation and differentiation. *A*: cell number of Flk-1-positive (Flk-1$^+$) embryonic stem (ES) cells cultured under static conditions or exposed to cyclic strain (4, 8, or 12% strain, 1 Hz) or PDGF-BB (10 ng/ml) for 24 h. Cell number increased in response to cyclic strain. The PDGF receptor β (PDGFRβ) kinase inhibitor AG-1296 (10 μmol/l) blocked the cell proliferation-promoting effect of cyclic strain. CS, cyclic strain. Values are means ± SD of data from 5 separate cell samples. *$P < 0.05$ and **$P < 0.01$ vs. static control. *B*: photomicrographs of immunostained Flk-1$^+$ ES cells cultured under static conditions or exposed to cyclic strain (8%, 1 Hz) or to PDGF-BB (10 ng/ml) for 24 h. Cells were immunostained for the endothelial cell (EC) marker platelet endothelial cell adhesion molecule 1 [PECAM-1 (green)] and a vascular smooth muscle cell (VSMC) marker [smooth muscle (SM) α-actin (red)]. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). Cyclic strain and PDGF-BB increased SM α-actin-positive cells, whereas they decreased PECAM-1-positive cells. Under cyclic strain, SM α-actin-positive cells aligned themselves perpendicular to the direction of the strain. The direction of strain is from *top* to *bottom* (arrow).

Downloaded from http://jap.physiology.org/ by 10.220.33.6 on September 6, 2017

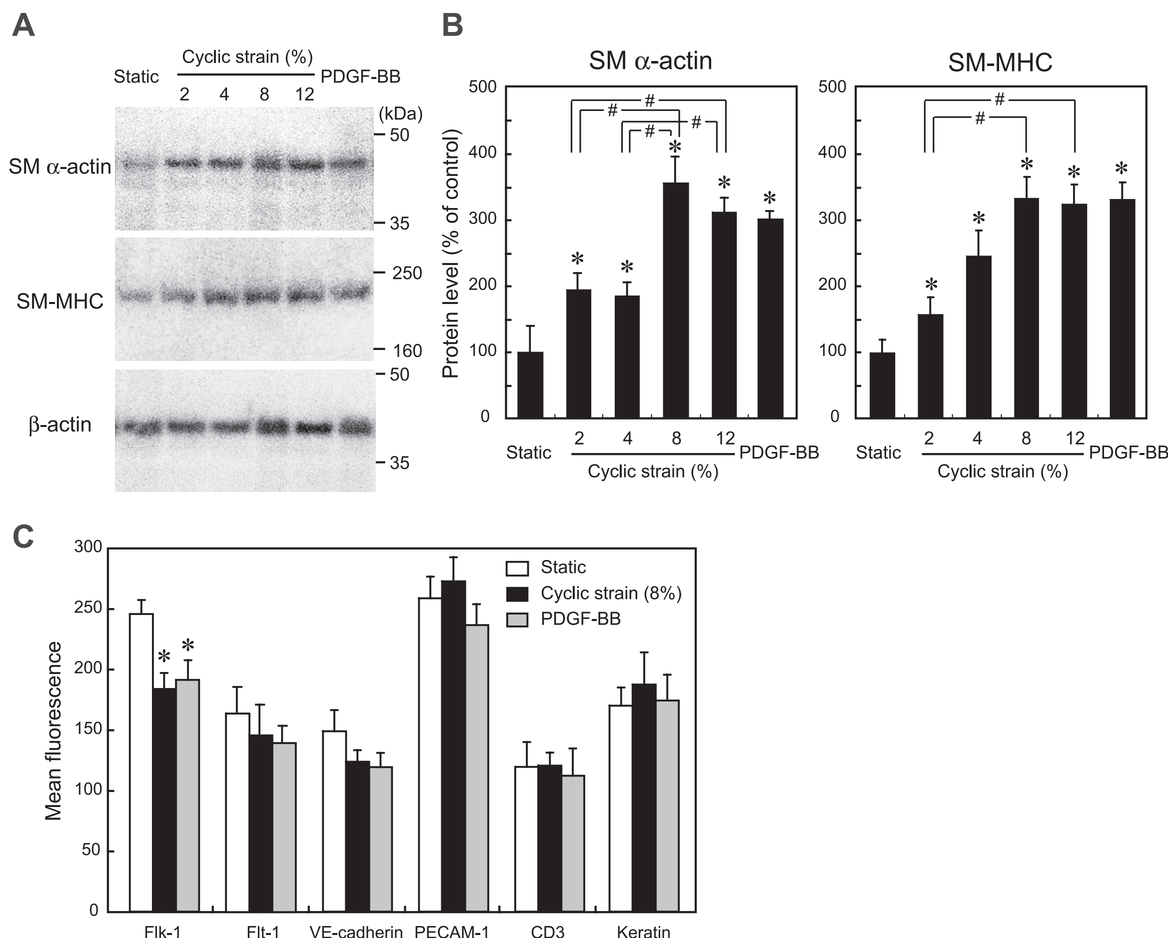


Fig. 2. Effect of cyclic strain on expression of various cell lineage marker proteins. *A*: visualization of bands of the VSMC markers SM α-actin and smooth muscle myosin heavy chain (SM-MHC). Total protein was isolated from Flk-1$^+$ ES cells cultured under static conditions or exposed to cyclic strain (2, 4, 8, and 12%, 1 Hz) or to PDGF-BB (10 ng/ml) for 24 h. After electrophoresis and transfer to a PVDF membrane, the protein was immunoblotted with specific antibody to SM α-actin, SM-MHC, or the cytoskeletal protein β-actin, which was used as a protein loading control. *B*: relative protein levels of SM α-actin and SM-MHC. Cyclic strain dose dependently increased protein expression of both SM α-actin and SM-MHC. Values are means ± SD of 5 different experiments. $^*P < 0.01$ vs. static control. $\#P < 0.01$ between the cells exposed to 2% and 4% strain and those exposed to 8% and 12% strain. *C*: relative protein levels of the EC markers Flk-1, Flt-1, VE-cadherin, and PECAM-1, the blood cell marker CD3, and the epithelial marker keratin. After exposure to cyclic strain (8%, 1 Hz) or PDGF-BB (10 ng/ml) for 24 h, cells were immunostained with specific antibody against each marker and examined for changes in cell surface expression by flow cytometry. Cyclic strain decreased protein expression of Flk-1 but had no effect on expression of Flt-1, VE-cadherin, PECAM-1, CD3, or keratin. Exposure to PDGF-BB had the same effect as cyclic strain. Values are means ± SD of 4 separate samples. $^*P < 0.01$ vs. static control.

effect on expression of these cell lineage marker proteins as 8% strain did.

Gene expression of cell lineage markers was examined by real-time PCR. Cyclic strain markedly increased the mRNA levels of the VSMC markers SM α-actin, SM-MHC, and smooth muscle 22α in a dose-dependent manner (Fig. 3*A*). By contrast, the Flk-1 mRNA levels decreased in response to cyclic strain (8%, 1 Hz), but the mRNA levels of Flt-1, VE-cadherin, and PECAM-1 remained unchanged (Fig. 3*B*). Together, these results indicate that cyclic strain selectively promotes differentiation of Flk-1$^+$ ES cells into VSMCs but not into the EC, blood cell, or epithelial cell lineages.

PDGFRβ is involved in the cyclic strain-induced differentiation of Flk-1$^+$ ES cells. Flk-1$^+$ ES cells were subjected to cyclic strain (8%,1 Hz) for 24 h in the presence or absence of AG-1296 and examined for changes in the expression of SM α-actin and SM-MHC proteins. Cyclic strain markedly increased the expression of SM α-actin and SM-MHC in the absence of AG-1296 but not in its presence (Fig. 4). AG-1296 decreased the basal levels of SM α-actin and SM-MHC, indicating that a slight degree of PDGF receptor phosphorylation occurs even under static conditions. AG-1296 seems to have inhibited both basal and cyclic-strain-induced PDGF receptor phosphorylation. These findings suggest that PDGFRβ activation plays an important role in the cyclic strain-induced VSMC differentiation from Flk-1$^+$ ES cells.

Cyclic strain activates PDGFRβ in a ligand-independent manner. Because the experiments with AG-1296 showed the involvement of PDGFRβ activation in cyclic-strain-induced ES cell differentiation, we investigated whether cyclic strain causes PDGFRβ activation. When Flk-1$^+$ ES cells were exposed to PDGF-BB or cyclic strain, phosphorylation of PDGFRβ occurred within 10 min but was almost completely blocked by AG-1296 (Fig. 5*A*). The cyclic strain-induced PDGFRβ phosphorylation was dose dependent (Fig. 5*B*). Neither neutralizing antibody against PDGF-BB nor against VEGF inhibited the cyclic strain-induced PDGFRβ phosphorylation (Fig. 5*C*). To investigate whether a ligand released by the cells was involved in the

Downloaded from http://jap.physiology.org/ by 10.220.33.6 on September 6, 2017

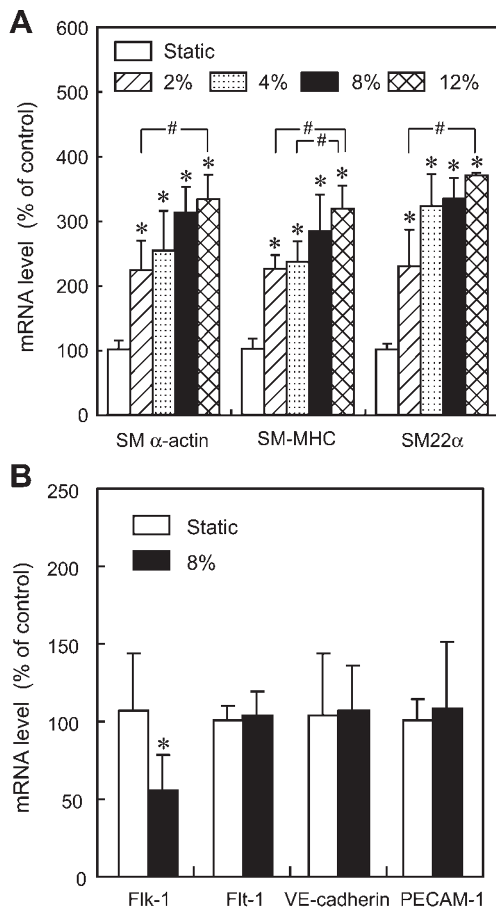


Fig. 3. Effect of cyclic strain on the gene expression of cell lineage markers. Flk-1$^+$ ES cells were exposed to cyclic strain (2, 4, 8, and 12% strain, 1 Hz) for 24 h and examined for changes in gene expression by real-time PCR. *A*: changes in the mRNA levels of VSMC markers. Cyclic strain increased the mRNA levels of SM α-actin, SM-MHC, and smooth muscle 22α (SM22α) in a dose-dependent manner. Values are means ± SD of 3 cell samples. *$P < 0.01$ vs. static control. #$P < 0.01$ between the cells exposed to 2% and 4% strain and those exposed to 8% and 12%. *B*: changes in the mRNA levels of the EC markers Flk-1, Flt-1, VE-cadherin, and PECAM-1. Cyclic strain (8%, 1 Hz, 24 h) decreased the mRNA level of Flk-1 but had no effect on the mRNA level of Flt-1, VE-cadherin, or PECAM-1. Values are means ± SD of 6 cell samples. *$P < 0.01$ vs. static control.

PDGFRβ activation, Flk-1$^+$ ES cells were exposed to conditioned medium obtained from cells exposed to cyclic strain (8%, 1 Hz) for 10 min. However, the conditioned medium did not induce PDGFRβ phosphorylation, and the extracellular ATP scavenger apyrase, the G protein-coupled receptor inhibitor pertussis toxin, and depletion of extracellular Ca^{2+} were incapable of attenuating the PDGFRβ phosphorylation. These results indicate that cyclic strain causes PDGFRβ phosphorylation in a ligand-independent manner and that transactivation of PDGFRβ secondary to activation of ATP receptors or G-protein-coupled receptors or Ca^{2+} influx via ion channels is not involved in the cyclic strain-induced PDGFRβ phosphorylation.

DISCUSSION

The results of this study demonstrated that cyclic strain significantly promotes the proliferation of Flk-1$^+$ ES cells and increases the expression of SM α-actin, SM-MHC, and smooth muscle 22α, which are markers of a differentiated VSMC phenotype (20). Upregulation of VSMC markers by cyclic strain has been observed in other immature cell lines, such as rat bone marrow progenitor cells (6), human bone marrow mesenchymal stem cells (21), and murine embryonic mesenchymal progenitor cells (24). However, this study showed that cyclic strain decreases the expression of Flk-1 but has no effect on the expression of other EC markers, including Flt-1, VE-cadherin, and PECAM-1, the blood cell marker CD3, or the epithelial cell marker keratin. These findings suggest that cyclic strain induces selective differentiation of Flk-1$^+$ ES cells into the VSMC lineage and not into other cell lineages. Our previous study showed that shear stress induces endothelial differentiation by Flk-1$^+$ ES cells, indicating that shear stress and cyclic strain have very different effects on ES cell differentiation (32). It therefore appears that shear stress may inhibit a pathway that leads to ES cell differentiation into smooth muscle cells and that cyclic strain may inhibit a pathway that leads to ES cell differentiation into ECs. Because, under in vivo conditions, ES cells seem to be exposed to both cyclic strain and shear stress, to understand the roles of fluid-mechanical forces in cardiovascular differentiation and devel-

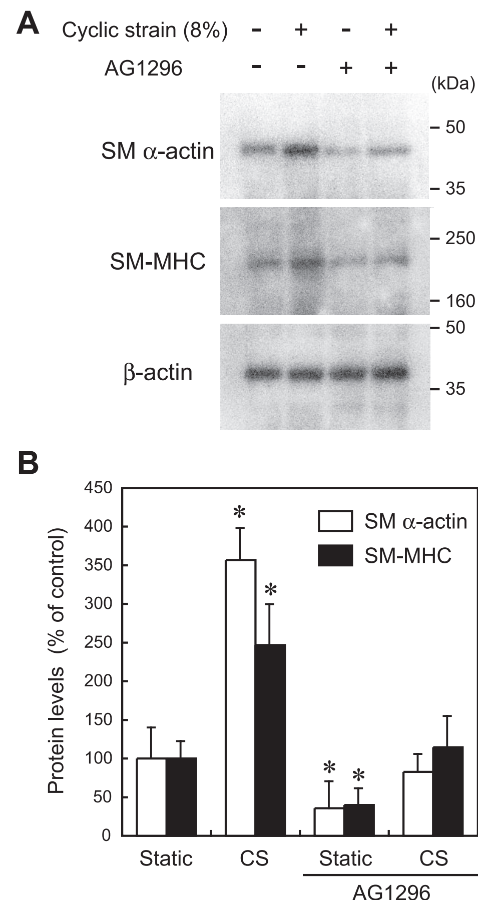


Fig. 4. Effect of the PDGFRβ kinase inhibitor AG-1296 on cyclic strain-induced upregulation of VSMC markers. Flk-1$^+$ ES cells were either cultured under static conditions or exposed to cyclic strain (8%, 1 Hz) for 24 h in the absence or presence of AG-1296 (10 μmol/l). *A*: visualization of bands of SM α-actin, SM-MHC, and β-actin (used as a protein loading control). *B*: histograms of the relative protein levels of SM α-actin and SM-MHC. Cyclic strain (CS) increased protein expression of both SM α-actin and SM-MHC, and AG-1296 almost completely abolished the effects of cyclic strain. Values are means ± SD of 4 cell samples. *$P < 0.01$ vs. static control.

Downloaded from http://jap.physiology.org/ by 10.220.33.6 on September 6, 2017

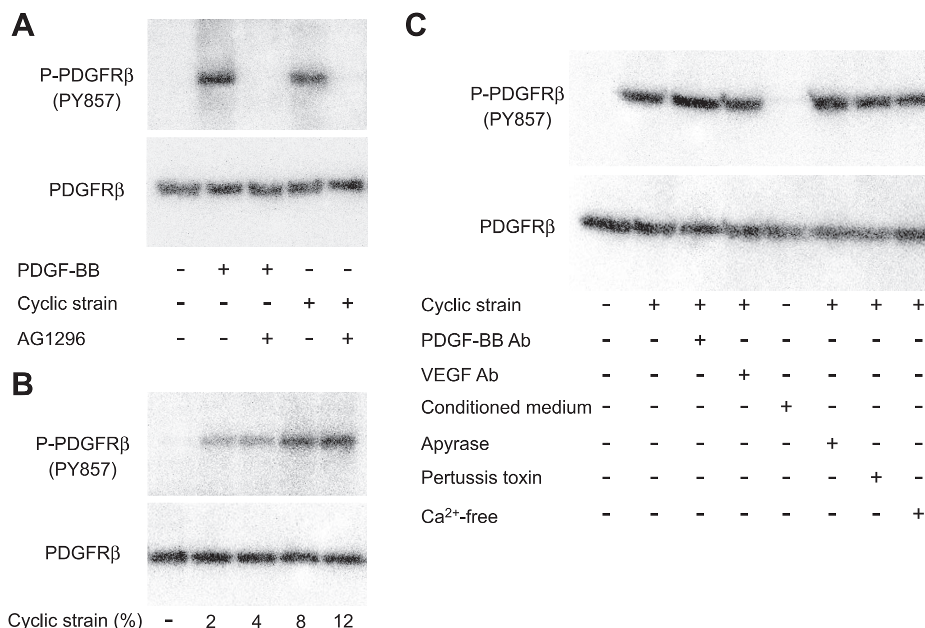


Fig. 5. Effect of cyclic strain on PDGFRβ phosphorylation. *A*: cyclic strain-induced phosphorylation of PDGFRβ. Flk-1^+ ES cells were either cultured under static conditions or exposed to PDGF-BB (10 ng/ml) or cyclic strain (8%, 1 Hz) for 10 min. Tyrosine-phosphorylated proteins associated with anti-PDGFRβ (P-PDGFRβ) were detected by immunoblotting with anti-phosphotyrosine antibody (PY-857). The total amount of PDGFRβ in cell lysates was also determined with anti-PDGFRβ antibody. Cyclic strain and PDGF-BB caused phosphorylation of PDGFRβ, and it was completely blocked by AG-1296 (10 μmol/l). Experiments were repeated 3 times, and similar results were obtained. *B*: dose dependency of cyclic strain-induced PDGFRβ phosphorylation. *C*: effects of various inhibitors on PDGFRβ phosphorylation. None of the neutralizing antibodies against PDGF-BB (1 μg/ml; R&D Systems), VEGF (1 μg/ml; R&D Systems), apyrase (20 U/ml; Sigma), or pertussis toxin (100 ng/ml; Sigma) attenuated the cyclic strain-induced PDGFRβ phosphorylation. Addition of conditioned medium obtained from Flk-1^+ ES cells exposed to cyclic strain (8%, 1 Hz) for 10 min did not cause PDGFRβ phosphorylation. Cyclic strain caused PDGFRβ phosphorylation even after the incubation for 10 min in Ca^{2+}-free HBSS with EGTA (2 mM). Experiments were repeated 3 times, with similar results.

opment in embryo it will be necessary to know the ES cell responses, not only to cyclic strain alone and to shear stress alone but to combinations of the two.

The upregulation of Flk-1^+ ES cell proliferation and expression of VSMC markers by cyclic strain were almost completely blocked by the PDGFRβ kinase inhibitor AG-1296, suggesting that PDGFRβ activation plays an important role in the effects of cyclic strain. Western blot analysis revealed that phosphorylation of PDGFRβ occurs immediately after the application of cyclic strain to Flk-1^+ ES cells. Because cyclic strain has been shown to cause mature ECs and VSMCs to release PDGF-BB, which is the ligand for PDGFRβ (15, 29), we investigated whether Flk-1^+ ES cells release PDGF-BB in response to cyclic strain. The concentration of PDGF-BB in the conditioned medium obtained from the cells exposed to cyclic strain for 10 min was below the limits of detection by ELISA (data not shown), indicating that very little, if any, PDGF-BB release occurred shortly after the onset of cyclic strain. However, because autocrine systems can be active even when no ligands are found in the extracellular medium (4), it is impossible to rule out the possibility based on the results of ELISA that a small amount of PDGF-BB is elicited by cyclic strain and that it acts on adjacent neighbor cells in a highly localized manner as an autocrine or paracrine signal. It was recently reported that VEGF-A binds and activates PDGFRα and PDGFRβ in bone marrow-derived human adult mesenchymal stem cells (2). However, neutralizing antibody specific to PDGF-BB and VEGF did not block the cyclic strain-induced PDGFRβ phosphorylation. In addition, phosphorylation of PDGFRβ did not occur when Flk-1^+ ES cells were exposed to conditioned medium obtained from the cells subjected to cyclic strain for 10 min. From the above findings, the phosphorylation of PDGFRβ induced by cyclic strain does not seem to involve any ligands, including PDGF or VEGF.

The ligand-independent activation of PDGFRβ by cyclic strain seen in Flk-1^+ ES cells is analogous to that observed in adult VSMCs (9). Cyclic strain rapidly induced phosphorylation of PDGFRα in VSMCs, but neither antibodies that bind to all forms of PDGFs nor conditioned medium from VSMCs exposed to cyclic strain blocked cyclic strain-induced PDGFRα activation. It is unclear, however, how cyclic strain activates PDGF receptor in a ligand-independent manner. The following possibilities can be considered. *1*) Cyclic strain mechanically deforms the cell membrane, which may influence PDGF receptor conformation or dimerization, and lead to its phosphorylation. *2*) Other molecules besides PDGF receptor, including other receptors, ion channels, and integrins, may transduce the mechanical stress into chemical signals and lead to PDGF receptor activation via a cross-talking mechanism. This study did not cover the involvement of stretch-activated ion channels or integrins, both of which are known to function as mechanotransducers (18, 27), but the results showed that apyrase, pertussis toxin, and depletion of extracellular Ca^{2+} had no effect on cyclic-strain-induced PDGFRβ activation. These findings suggest that PDGFRβ was not transactivated, at least not via ATP receptors, G protein-coupled receptors, or Ca^{2+} influx. Interestingly, our previous study (32) revealed that shear stress activates Flk-1 in a ligand-independent manner and that the activation of Flk-1 plays a critical role in endothelial differentiation of Flk-1^+ ES cells. Thus cyclic strain and shear stress may act by

Downloaded from http://jap.physiology.org/ by 10.220.33.6 on September 6, 2017

a common mechanism in which growth factor receptors are activated by mechanical forces without ligand binding. Elucidation of the mechanism would lead to a better understanding of mechanotransduction and its role in ES cell differentiation.

ACKNOWLEDGMENTS

The authors thank Yuko Sawada for technical assistance.

GRANTS

This study was supported in part by Grants-in-Aid for Scientific Research on Priority Areas and from the Ministry of Education, Culture, Sports, Science, and Technology and by a research grant for cardiovascular diseases from the Japanese Ministry of Health, Labor, and Welfare.

REFERENCES

1. **Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI.** Embryonic stem cells express neuronal properties in vitro. *Dev Biol* 168: 342–357, 1995.
2. **Ball SG, Shuttleworth CA, Kielty CM.** Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. *J Cell Biol* 177: 489–500, 2007.
3. **Davies PF.** Flow-mediated endothelial mechanotransduction. *Physiol Rev* 75: 519–560, 1995.
4. **DeWitt AE, Dong JY, Wiley HS, Lauffenburger DA.** Quantitative analysis of the EGF receptor autocrine system reveals cryptic regulation of cell response by ligand capture. *J Cell Sci* 114: 2301–2313, 2001.
5. **Evans MJ, Kaufman MH.** Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292: 154–156, 1981.
6. **Hamilton DW, Maul TM, Vorp DA.** Characterization of the response of bone marrow-derived progenitor cells to cyclic strain: implications for vascular tissue-engineering applications. *Tissue Eng* 10: 361–369, 2004.
7. **Hove JR, Koster RW, Forouhar AS, Acevedo-Bolton G, Fraser SE, Gharib M.** Intracardiac fluid forces are an essential epigenetic factor for embryonic cardiogenesis. *Nature* 421: 172–177, 2003.
8. **Hu N, Clark EB.** Hemodynamics of the stage 12 to stage 29 chick embryo. *Circ Res* 65: 1665–1670, 1989.
9. **Hu Y, Bock G, Wick G, Xu Q.** Activation of PDGF receptor α in vascular smooth muscle cells by mechanical stress. *FASEB J* 12: 1135–1142, 1998.
10. **Jones EA, Baron MH, Fraser SE, Dickinson ME.** Measuring hemodynamic changes during mammalian development. *Am J Physiol Heart Circ Physiol* 287: H1561–H1569, 2004.
11. **Jones EA, Crotty D, Kulesa PM, Waters CW, Baron MH, Fraser SE, Dickinson ME.** Dynamic in vivo imaging of postimplantation mammalian embryos using whole embryo culture. *Genesis* 34: 228–235, 2002.
12. **Kakisis JD, Liapis CD, Sumpio BE.** Effects of cyclic strain on vascular cells. *Endothelium* 11: 17–28, 2004.
13. **Kovalenko M, Gazit A, Bohmer A, Rorsman C, Ronnstrand L, Heldin CH, Waltenberger J, Bohmer FD, Levitzki A.** Selective platelet-derived growth factor receptor kinase blockers reverse sis-transformation. *Cancer Res* 54: 6106–6114, 1994.
14. **Kramer J, Hegert C, Guan K, Wobus AM, Muller PK, Rohwedel J.** Embryonic stem cell-derived chondrogenic differentiation in vitro: activation by BMP-2 and BMP-4. *Mech Dev* 92: 193–205, 2000.
15. **Ma YH, Ling S, Ives HE.** Mechanical strain increases PDGF-B and PDGF beta receptor expression in vascular smooth muscle cells. *Biochem Biophys Res Commun* 265: 606–610, 1999.
16. **Martin GR.** Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 78: 7634–7638, 1981.
17. **Nakazawa M, Morishima M, Tomita H, Tomita SM, Kajio F.** Hemodynamics and ventricular function in the day-12 rat embryo: basic characteristics and the responses to cardiovascular drugs. *Pediatr Res* 37: 117–123, 1995.
18. **Naruse K, Yamada T, Sokabe M.** Involvement of SA channels in orienting response of cultured endothelial cells to cyclic stretch. *Am J Physiol Heart Circ Physiol* 274: H1532–H1538, 1998.
19. **Nonaka S, Shiratori H, Saijoh Y, Hamada H.** Determination of left-right patterning of the mouse embryo by artificial nodal flow. *Nature* 418: 96–99, 2002.
20. **Owens GK.** Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev* 75: 487–517, 1995.
21. **Park JS, Chu JS, Cheng C, Chen F, Chen D, Li S.** Differential effects of equiaxial and uniaxial strain on mesenchymal stem cells. *Biotechnol Bioeng* 88: 359–368, 2004.
22. **Pedersen RA.** Embryonic stem cells for medicine. *Sci Am* 280: 68–73, 1999.
23. **Raines EW, Ross R.** Purification of human platelet-derived growth factor. *Methods Enzymol* 109: 749–773, 1985.
24. **Riha GM, Wang X, Wang H, Chai H, Mu H, Lin PH, Lumsden AB, Yao Q, Chen C.** Cyclic strain induces vascular smooth muscle cell differentiation from murine embryonic mesenchymal progenitor cells. *Surgery* 141: 394–402, 2007.
25. **Rohwedel J, Maltsev V, Bober E, Arnold HH, Hescheler J, Wobus AM.** Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. *Dev Biol* 164: 87–101, 1994.
26. **Saha S, Ji L, de Pablo JJ, Palecek SP.** Inhibition of human embryonic stem cell differentiation by mechanical strain. *J Cell Physiol* 206: 126–137, 2006.
27. **Sasamoto A, Nagino M, Kobayashi S, Naruse K, Nimura Y, Sokabe M.** Mechanotransduction by integrin is essential for IL-6 secretion from endothelial cells in response to uniaxial continuous stretch. *Am J Physiol Cell Physiol* 288: C1012–C1022, 2005.
28. **Schmelter M, Ateghang B, Helmig S, Wartenberg M, Sauer H.** Embryonic stem cells utilize reactive oxygen species as transducers of mechanical strain-induced cardiovascular differentiation. *FASEB J* 20: 1182–1184, 2006.
29. **Sumpio BE, Du W, Galagher G, Wang X, Khachigian LM, Collins T, Gimbrone MA Jr, Resnick N.** Regulation of PDGF-B in endothelial cells exposed to cyclic strain. *Arterioscler Thromb Vasc Biol* 18: 349–355, 1998.
30. **Takeda H, Komori K, Nishikimi N, Nimura Y, Sokabe M, Naruse K.** Bi-phasic activation of eNOS in response to uni-axial cyclic stretch is mediated by differential mechanisms in BAECs. *Life Sci* 79: 233–239, 2006.
31. **Vittet D, Prandini MH, Berthier R, Schweitzer A, Martin-Sisteron H, Uzan G, Dejana E.** Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps. *Blood* 88: 3424–3431, 1996.
32. **Yamamoto K, Sokabe T, Watabe T, Miyazono K, Yamashita JK, Obi S, Ohura N, Matsushita A, Kamiya A, Ando J.** Fluid shear stress induces differentiation of Flk-1-positive embryonic stem cells into vascular endothelial cells in vitro. *Am J Physiol Heart Circ Physiol* 288: H1915–H1924, 2005.
33. **Yamamoto K, Takahashi T, Asahara T, Ohura N, Sokabe T, Kamiya A, Ando J.** Proliferation, differentiation, and tube formation by endothelial progenitor cells in response to shear stress. *J Appl Physiol* 95: 2081–2088, 2003.
34. **Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, Naito M, Nakao K, Nishikawa S.** Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* 408: 92–96, 2000.

Downloaded from http://jap.physiology.org/ by 10.220.33.6 on September 6, 2017