Epigenetic Histone Modification and Cardiovascular Lineage Programming in Mouse Embryonic Stem Cells Exposed to Laminar Shear Stress

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Abstract—Experimental evidence indicates that shear stress (SS) exerts a morphogenetic function during cardiac development of mouse and zebrafish embryos. However, the molecular basis for this effect is still elusive. Our previous work described that in adult endothelial cells, SS regulates gene expression by inducing epigenetic modification of histones and activation of transcription complexes bearing acetyltransferase activity. In this study, we evaluated whether SS treatment could epigenetically modify histones and influence cell differentiation in mouse embryonic stem (ES) cells. Cells were exposed to a laminar SS of 10 dyne per cm\(^2\)s\(^{-1}\), or kept in static conditions in the presence or absence of the histone deacetylase inhibitor trichostatin A (TSA). These experiments revealed that SS enhanced lysine acetylation of histone H3 at position 14 (K14), as well as serine phosphorylation at position 10 (S10) and lysine methylation at position 79 (K79), and cooperated with TSA, inducing acetylation of histone H4 and phosphoacetylation of S10 and K14 of histone H3. In addition, ES cells exposed to SS strongly activated transcription from the vascular endothelial growth factor (VEGF) receptor 2 promoter. This effect was paralleled by an early induction of cardiovascular markers, including smooth muscle actin, smooth muscle protein 22-α, platelet-endothelial cell adhesion molecule-1, VEGF receptor 2, myocyte enhancer factor-2C (MEF2C), and α-sarcomeric actin. In this condition, transcription factors MEF2C and Smad homolog protein 4 could be isolated from SS-treated ES cells complexed with the cAMP response element-binding protein acetyltransferase. These results provide molecular basis for the SS-dependent cardiovascular commitment of mouse ES cells and suggest that laminar flow may be successfully applied for the in vitro production of cardiovascular precursors. (Circ Res. 2005;96:501-508.)

Key Words: shear stress ▪ flow ▪ embryonic stem cell ▪ chromatin ▪ differentiation

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the formation of an abnormal cardiac chamber and valve formation.11 These alterations resemble those of the defective cardiac phenotype of MEF-2C knockout mice.8 Therefore, this experimental evidence and the fact that SS mediates epigenetic histone modifications in adult endothelial cells12 prompted us to investigate the effect of laminar SS on ES cells chromatin remodeling, gene expression, and differentiation. The results of this work show that ES cells exposed to SS remodel their chromatin structure and express specific cardiovascular and vascular markers. These data provide the first evidence of a role for SS as an in vitro determinant of angioblast-like/cardiovascular precursors in a mouse differentiation model2,3 and allow to envisage the application of laminar flow for the in vitro production of differentiated cardiovascular cells.

Materials and Methods

Cell Culture, SS Experiments, and Cell Treatments

Murine ES cells (ES D3) were purchased by LGC Promochem and were adapted in culture without feeder layer in DMEM with pyroidine/HCl (GIBCO), supplemented with 4.5 g/L glucose, 4 mmol/L glutamate, 0.1 mmol/L 2-mercaptoethanol (Sigma), 10% ES Cult FBS, and 10 ng/mL LIF (Stem Cells Technologies). For SS experiments, ES cells were deprived of LIF and plated onto superfibronectin (10 μg/mL; Sigma)-coated plates 24 hours before SS exposure in a cone-plate apparatus.13 Trichostatin A (TSA; 32 nmol/L; Sigma) was added to complete medium without LIF immediately before SS exposure. SB203580 (10 μmol/L) or control solvent was added to ES cell medium without LIF and supplemented with 2% FBS 30 minutes before 60 minutes of SS exposure and after an overnight starvation of ES cells in culture medium without LIF, plus 0.1% FBS.

Matrigel Assay

ES cells were exposed to SS for 24 hours or kept in static culture; thereafter, they were trypsinized and plated on matrigel-coated plates (Becton Dickinson) in complete medium as described previously.14

Western Blots

Cells were lysed in 1× Laemmli buffer and boiled for 10 minutes. Total extracts were centrifuged for 10 minutes at 14 000 rpm at 4°C. Nuclear histones were detected as described previously.12 Anti-histone antibodies were purchased from Upstate Biotechnology. Anti-VEGF receptor 2 (VEGFR2), anti-PECAM, anti-MyoD, and anti–endothelial NO synthase (eNOS) antibodies were from Transduction Laboratories. Normalization of protein loading was obtained either with anti-H1 antibody or anti-GRB-2 antibody. All the antibodies were used according to manufacturer instructions.

Determination of Acetyltransferase Activity

ES cells were exposed to SS for 60 minutes. Protein extracts and acetyltransferase activity were obtained as described previously.12

Macroray Screening

Atlas Mouse 1.2 array (Clontech) was probed with cDNA from ES cells exposed to SS for 12 hours or kept in static conditions. RNA was extracted with TRIZOL reagent (Invitrogen). Reverse transcription was performed using Superscript II and 18-mers Oligo-dT (Invitrogen). Polymerase chain reactions (PCRs) were performed using AmphyTaq DNA-Polymerase (Perkin–Elmer) in 1.5 mmol/L magnesium chloride and 0.2 mmol/L dNTPs. Primer concentration was 1 μmol/L. Primer sequences were: transforming growth factor-β (TGF-β)-activated kinase (TAK): fwd 5′-cag gat gal cga agg gc-3′; rev: 5′-ctg tga atc aag gct ttg gg-3′; NeuroD1: fwd: gat gca gag aag aaa gag ga-3′; rev: 5′-pac caa att ggt aag gag ct-3′; glial cell missing: fwd: 5′-cgc gag acc agg ctg taa aa-3′; rev: 5′-agt act tct tga gga ttc ggc-3′; MEF-2C: fwd: 5′-agc cgg aca aac tca gag ac-3′; rev: 5′-tat tcc tct gca gaa agg gc-3′; T-box gene 3 (TBX-3): fwd: 5′-tgct atg gta ctc aag aac tgc-3′; rev: 5′-gaa cgg cgg ctg gta ctt cat-3′; forkhead homolog-2 (FKH-2): fwd: 5′-ctg cgg aag tct aaa tgt cg-3′; rev: gat ggt tga aag tgg ga-3′; insulin-like growth factor-2 (IGF-2): fwd: 5′-aag gtc ccc gtt tct gtt-3′; rev: 5′-tct tgt gtt ctc gta aag tgc ga-3′; and GAPDH fwd: 5′-agc aca gtc gcat acc ac-3′; rev: 5′-ctc acc acc ctg ctc gta-3′.

Amplifications were performed in a GeneAmp 2004 Thermacycler (Perkin–Elmer) using the following programs: 1 cycle 5′-94°C, 5′-Tann; 35 cycles 1′-72°C, 30′-94°C, 1′-Tann; 1 cycle 7′-72°C. Annealing temperatures (Tann) were: 55°C for FKH-2, thrombin receptor, angiogenin, NeuroD1, TAK, and GAPDH; and 60°C for TBX-3, IGF-2, glial cell missing, and MEF-2C. Amplified transcripts were loaded on a 2% agarose gel.

RNase Protection Assay

RNA was extracted with TRIZOL reagent (Invitrogen) according to manufacturer instructions. RNase protection assay (RPA) was performed using RiboQuant RNase Protection Assay System (Pharmingen).

MEF-2C probe was obtained by amplifying murine MEF-2C coding region from nucleotide 1186 to nucleotide 1492 using the following oligonucleotides: fwd 5′-ctagcgcgctcgagctccagtc-3′; rev 5′-ctccctgctgccgacgtgc-3′. Amplification was performed with Platinum Pfx DNA Polymerase (Invitrogen) according to manufacturer instruction in a GeneAmp 2400 Thermalcycler using the following program: 1 cycle 5 minutes-94°C, 5 minutes-55°C; 30 cycles: 2 minutes-72°C, 30 s-94°C, 1 minute-55°C; 1 cycle: 10 minutes-72°C. PCR product was cloned in TOPO vector (Invitrogen) and subcloned in pBluScript plasmid. The construct was linearized with BanHI gel-purified, and ethanol precipitated. Labeling was obtained by using the in vitro transcription kit (Pharmingen) according to manufacturer instructions. Protected bands were resolved on a 5% polyacrylamide/UREA gel.

Transient Transfections and Luciferase Assays

Transient transfections were performed using Lipofectamine Plus reagent (Invitrogen), and luciferase assays were performed using the Luciferase Assay Kit (Promega) as described previously.15

Stable Transfections and FACS Analysis

Stable clones were obtained by electroporating 107 cells with 20 μg of −442/+297GFP reporter construct in 0.8 mL of PBS. Electroporation parameters were: 250 V, 200 μF, and 960 μA. Cells were selected using 250 μg/mL of G418 (Life Technologies). FACS analysis was performed as described previously.15

Pull-Down Assay

Nuclear extracts and pull-down assay were performed as described previously.12 MEF2C consensus binding site was: 5′-gatgcctctaaataacagtctgc-3′. Smad4 consensus binding site was: 5′-tggagacggacaaagaaagacaggatggacagca-3′. MEF2C, Smad4, and CREB-binding protein (CBP)/p300 were detected by Western blot using anti-MEF2C, anti-Smad4 and anti-CBP antibodies (Santa Cruz Biotechnology) according to manufacturer instructions.
Figure 1. ES cells undergo chromatin remodeling during SS treatment. Top panels show representative Western blots. At the bottom of each figure, the densitometric analysis of 3 independent experiments is reported. A, A 4-fold increase in histone H3 phosphorylation on S10 under SS conditions is shown at the 180-minute time point. TSA treatment enhanced this phenomenon (~6-fold at 180-minute time point and 8-fold at 8 hours). B, Phosphoacetylation of histone H3 increased 5-fold in 30 minutes of SS to decline between 60 and 180 minutes. TSA treatment delayed and sustained phosphoacetylation of H3 until 180 minutes. C, Acetylation of histone H3 is rapidly induced between 30 and 60 minutes by SS. TSA sustained acetylation until 8 hours. D, SS induced acetylation of histone H4 (4- to 5-fold) between 30 and 60 minutes during TSA treatment. E, SB203580 reduced SS-dependent enhancement H3 phosphorylation and acetylation. OD indicates optical density. F, SS induced methylation of histone H3 (4- to 5-fold) on K79 between 30 and 60 minutes. TSA reduced the overall methylation level of histone H3, although SS still induced H3 K79 methylation. G, A 2-fold increase in endogenous HAT activity after 60 minutes of SS exposure.
Results

SS Enhances Histone Modifications in Mouse ES Cells: Involvement of p38 Mitogen-Activated Protein Kinase

Previous work performed in adult endothelial cells\textsuperscript{12} showed that the presence of active transcription complexes, bearing HAT activity, correlated with the SS-dependent post-translational modification of histones. To investigate whether these modifications occurred in mouse ES cells, ES cells were exposed to a laminar SS of 10 dyne per cm\(^2\)/s \textsuperscript{1} from 30 minutes to 8 hours or kept in static culture in the presence or absence of the HDAC inhibitor TSA. Cell extracts were analyzed by Western blotting. Methylation, phosphorylation, and phosphoacetylation of histone H3 are known to occur early in response to extracellular stimuli.\textsuperscript{16-18} We found that SS enhanced histone H3 phosphorylation on serine 10 (S10) within 30 minutes (Figure 1A). TSA treatment decreased the level of H3 phosphorylation, suggesting that this histone was likely phosphoacetylated in ES cells as seen previously in adult endothelial cells.\textsuperscript{12} In fact, the presence of histone H3 simultaneously phosphorylated on S10 and acetylated on K14 was detected after 30 minutes of SS exposure (Figure 1B). In presence of TSA, histone H3 phosphoacetylation was reduced at 30 minutes; however, it was sustained for up to 180 minutes of SS treatment (Figure 1B). The effect of SS on histone H3 K14 and histone H4 acetylation was also examined. In our experimental conditions, SS-dependent histone H3 acetylation on K14 was strong at 30 and 60 minutes of treatment. On TSA and SS treatment, the level of K14 acetylation was reduced at 30 and 60 minutes but was still detectable at the 8-hour time point (Figure 1C). Notably, as seen previously in adult endothelial cells, SS enhanced H4 acetylation in presence of TSA (Figure 1D) and the p38 inhibitor SB203580 abrogated SS-induced H3 phosphorylation on S10 and acetylation on K14 (Figure 1E), indicating that also in ES cells, histone modifications are strictly dependent on SS signaling pathways.

Previous studies suggested that the level of methylated histone H3 on lysine at position 79 (K79) is a marker of active chromatin regions.\textsuperscript{16,17} We found that SS enhanced K79 methylation on histone H3 with a peak at 60 minutes. Although TSA treatment reduced the level of SS-induced K79 methylation, nonetheless, the overall level of histone methylation on this specific residue was increased compared with static controls (Figure 1F). Moreover, the overall acetyltransferase activity was enhanced in ES cells under SS conditions (Figure 1G). Notably, SS failed to induce chromatin remodeling and HAT activation in nonvascular mouse NIH-3T3 cells (data not shown). These results indicate that mouse ES cells are sensitive to SS becoming susceptible to the activation of chromatin remodeling.

SS Enhances Transcription From VEGFR2 Promoter

Previous work demonstrated that in adult endothelial cells, HAT molecules are required for activation of VEGFR2 promoter.\textsuperscript{15} To correlate SS effect on histone acetylation with the expression of vascular genes in ES cells, cells were

Immunofluorescence

ES cells were exposed to SS for 24 hours or kept in static conditions. Cells were immediately fixed in 4% paraformaldehyde solution for 10 minutes at room temperature, washed 3× for 5 minutes with PBS, and blocked for 1 hour in PBS containing 8% BSA. Cells fields were incubated with primary antibodies in PBS containing 1% BSA O/N, at +4°C. Plates were washed twice for 5 minutes with PBS and incubated with α-rabbit–tetramethylrhodamine B isothiocyanate, mouse–fluorescein isothiocyanate, and goat–fluorescein isothiocyanate secondary antibodies for 1 hour at room temperature in the dark. After 3 washes in PBS for 5 minutes, fields were incubated with 1 μg/mL Hoechst, 20 minutes at room temperature in the dark, and then washed 3× with PBS for 5 minutes. A total of 100 μL of DAKO cytomation-mounting fluorescent medium was used. Staining was visualized with an Axioplan 2 microscope; pictures were obtained by using an Axiocam and analyzed with a KS 300 3.0 acquisition software (Zeiss).

Statistical Analysis

Results were analyzed by 1-way ANOVA. Post hoc tests according to the Student-Newman–Keuls method were used to assess statistically significant differences among different groups. A value of \( P<0.05 \) was considered statistically significant.

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\( \text{A} \)

\( \text{B} \)

\( \text{C} \)
transfected with a −442/+297 VEGFR2luc promoter construct and exposed to SS in the presence or absence of TSA. As shown in Figure 2A, SS enhances TSA-induced luciferase activity. This result was confirmed using stable ES cell clones expressing the green fluorescent protein (GFP) under control of the same VEGFR2 promoter region (Figure 2B). In fact, SS and TSA enhanced the number and the fluorescence intensity of ES–GFP cells. The requirement of TSA to fully
induce VEGFR2 promoter activation underlies the importance of histone acetylation to achieve gene expression in ES.

SS Enhances the Expression of Cardiovascular Markers in ES Cells

The results obtained by transfection analysis and the evidence that mechanical stimuli may have profound effects on cardiomyocytes and heart formation in zebrafish embryos prompted us to investigate whether cell-specific markers were modulated by SS. Expression of cardiovascular markers in undifferentiated ES cells was evaluated after 24 hours of SS treatment. Figure 3A shows that in presence (top right) or absence (top left) of SS, ES cell shape did not visibly change, thus indicating that ES cells, at their early stage of differentiation, are unable to adapt their morphology in presence of biomechanical stimuli. However, SS-treated ES cells plated on matrigel organized in tubular-like structures, becoming visible after 3 to 6 hours from plating (bottom right). Control cells kept in static culture were predominantly organized in round clumps (bottom left). In this condition, the endothelial cell markers VEGFR2 and PECAM were expressed by ES cells exposed to SS, as revealed by immunocytochemistry and Western blot analysis (Figure 3B and 3E). Interestingly, cardiac and smooth muscle markers α-SA, MEF2C, smooth muscle actin (SMA), and SM22α were also upregulated (Figure 3C through 3E). The expression of all markers tested was retained at for least 24 hours after replacing ES cells in static conditions (Figure 3B through 3D). However, PECAM

Figure 4. Laminar SS modulates cardiovascular/mesodermic genes and activates transcriptional complexes in mouse ES cells. A, RT-PCR validation of 7 genes reproducibly upregulated in 5 independent macroarray experiments in ES cells exposed to SS. The top shows the picture of PCRs loaded on a 2% agarose gel; bottom shows the densitometric analysis of labeled spots on Atlas array (data not shown). THRrec indicates thrombin receptor; ANG-1, angiogenin. B, Left panel shows a RPA for MEF2C transcription performed on ES cells after 12 hours of SS exposure. SS increased mRNA levels of MEF2C. Right panel shows Western blot of DNA bound complexes. In static ES cells, there was no detectable MEF2C activation, regardless the presence of CBP. Under SS conditions (16 hours), MEF2C was activated and this was paralleled by an increase in CBP protein levels. B, Western blot on DNA-bound complexes by using a specific oligonucleotide containing a recognition sequence for Smad transcription factor family members. SS activated a Smad4/CBP complex in ES cells. These experiments were performed 4x with similar results.
and SM22α were found downregulated at the 72-hour time point (Figure 3B and 3C). On the contrary, control cells showed a barely detectable expression of the same markers at the 24-hour time point. Notably, during these experiments, the skeletal muscle–specific marker MyoD remained undetectable in static or SS condition (Figure 3F). Moreover, by a macroarray (data not shown) and RT-PCR screening (Figure 4A), genes belonging to the cardiovascular/mesodermal lineage were found upregulated in ES cells exposed to SS. Specifically, MEF-2C,20 thrombin receptor,21 TBX-3,22,23 FKH-2,24 angiogenin,25 IGF-2,26 and TAK27 were reproducibly positive. On the contrary, 2 neurospecific genes NeuroD128 and glial cell missing29 were not modulated. Altogether, these data indicate that SS stimulates expression of lineage-specific markers, possibly underlying activation of a cardiovascular differentiation program.

### Laminar SS Promotes MEF2C and Smad4 Association With CBP/p300

The transcription factor MEF-2C is highly important for cardiovascular development.8–10 RPAs showed that SS treatment upregulates MEF2C expression in ES cells (Figure 4B, left). In this condition, DNA pull-down experiments indicate that SS induced formation of active MEF2C/CBP complexes (Figure 4B, right). Because SS activates Smad proteins,30,31 we performed a pull-down assay to detect SS-dependent nuclear translocation and HAT association of Smad4 transcription factor. Remarkably, the presence of active a Smad4/CBP complexes was observed in nuclear extracts obtained from SS-treated ES cells (Figure 4C), indicating that SS may contribute to the formation of transcription complexes important for ES cell differentiation into cardiovascular precursor cells.

### Discussion

Previous work demonstrated that laminar SS modulates histones acetylation and promotes chromatin remodeling, providing molecular basis for its effect on gene expression in adult endothelial cells.12 In this report, we show that SS activates molecular pathways leading to histone modifications, transcription complex activation, and expression of vascular and cardiovascular markers in undifferentiated ES cells, acquiring the phenotype of angioblast-like cells and cardiomyocyte precursors. SS activates transcription factors including MEF-2C and Smad4 in a complex form with CBP/p300 HAT, recapitulating some events occurring during cardiac formation and vascular development.8–10 In fact, Smad4, which is functionally involved in the TGF-β–dependent embryonal angiogenesis32 and directly activated by SS in adult endothelial cells,30 may also form complexes with MEF2C.7 Members of the Smads family have also been demonstrated to be directly involved in cardiac development and cardiomyocyte differentiation.27,33 Although it remains unexplored whether the SS-dependent activation of transcription complexes leads to chromatin remodeling in the promoter region of specific target genes, this process is likely to have an important role in the angioblast-like cardiomyocyte differentiation of ES cells. Previous observations showing that the loss of hemodynamic forces impairs cardiogenesis of developing embryos31,34 strongly support this possibility. After LIF removal, cultured ES cells undergo spontaneous in vitro differentiation leading to the formation of, among other cell types, cardiovascular precursors.2,3,19 This process normally occurs in several days;3 however, in our experimental conditions, exposure to SS accelerates the onset of cells expressing cardiovascular markers that became detectable after 24 hours of SS treatment. In this condition, ES cells exposure to SS significantly raises the intracellular level of histone acetylase activity, suggesting that those molecular mechanisms leading to the remodeling of chromatin may also be involved in the activation of the cardiovascular differentiation program. However, these results raise the question whether SS-dependent effect on ES cell differentiation is a temporary consequence of cell exposure to shear forces or it may be a stable long-term outcome. In our experimental conditions, most of the cardiovascular markers induced by hemodynamic forces were still retained after 72 hours of SS withdrawal (Figure 3). Indeed, this observation suggests that laminar SS may induce a long-term lineage-specific commitment in ES cells. Therefore, although the formation of the vascular tree occurs in absence of blood flow, SS may act as an important factor for the differentiation of cardiovascular precursors during the maturation of embryonal vascular structures as well as the vascular remodeling that may occur in an adult organism. Nevertheless, further in vitro and in vivo investigations are required to elucidate this point. In conclusion, our results indicate that hemodynamic forces alter the chromatin state of undifferentiated mouse ES cells and activate specific transcription complexes, gathering the onset of a vascular/cardiovascular differentiation program (Figure 5).
5). Although the evidence of a direct link between SS-dependent histone modification and activation of a genetic program leading to the differentiation or maturation of angioblast/cardiovascular precursors is still missing, laminar flow application to protocols for directing the cardiovascular commitment of stem cells may provide a useful tool to improve the in vitro production of cells suitable for molecular studies and genetic manipulation.

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