Nuclear envelope proteins modulate proliferation of vascular smooth muscle cells during cyclic stretch application

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Cyclic stretch is an important inducer of vascular smooth muscle cell (VSMC) proliferation, which is crucial in vascular remodeling during hypertension. However, the molecular mechanism remains unclear. We studied the effects of emerin and lamin A/C, two important nuclear envelope proteins, on VSMC proliferation in hypertension and the underlying mechano-mechanisms. In common carotid artery of hypertensive rats in vivo and in cultured cells subjected to high (15%) cyclic stretch in vitro, VSMC proliferation was increased significantly, and the expression of emerin and lamin A/C was repressed compared with normotensive or normal (5%) cyclic stretch controls. Using targeted siRNA to mimic the repressed expression of emerin or lamin A/C induced by 15% stretch, we found that VSMC proliferation was enhanced under static and 5%-stretch conditions. Overexpression of emerin or lamin A/C reversed VSMC proliferation induced by 15% stretch. Hence, emerin and lamin A/C play critical roles in suppressing VSMC hyperproliferation induced by hyperstretch. ChIP-on-chip and MOTIF analyses showed that the DNAs binding with emerin contain three transcription factor motifs: CCNGGA, CCMGCC, and ABTTCCG; DNAs binding with lamin A/C contain the motifs CVGGAA, GCCGCYGC, and DAAGAAA. Protein/DNA array proved that altered emerin or lamin A/C expression modulated the activation of various transcription factors. Furthermore, accelerating local expression of emerin or lamin A/C reversed cell proliferation in the carotid artery of hypertensive rats in vivo. Our findings establish the pathogenetic role of emerin and lamin A/C repression in stretch-induced VSMC proliferation and suggest mechanobiological mechanism underlying this process that involves the sequence-specific binding of emerin and lamin A/C to specific transcription factor motifs.

mechanobiology | emerin | laminA/C | specific-binding sequence | transcription factors

The cyclic stretch caused by the rhythmical distention and relaxation of the arterial wall during the cardiac cycle is an important factor in the regulation of vascular modeling and remodeling (1, 2). There is growing evidence that mechanical cyclic stretch modulates the functions (e.g., apoptosis, proliferation, and migration) of vascular smooth muscle cells (VSMCs) in the media of the arterial wall (2) and that chronically elevated cyclic stretch stimulates VSMC functions to mediate vascular remodeling during hypertension (3, 4).

It has been shown that there are various mechano-sensors in the vascular cell membrane, including lipids (5), glycocalyx (6), and proteins such as integrins (7), G proteins and G protein-coupled receptors (8), receptor tyrosine kinase (9), and Ca^{2+} channel (10) and intercellular junction proteins (2, 11). In recent years, it has been suggested that nuclear envelope (NE) proteins, a hallmark of eukaryotic cells, participate in the mechano-transduction networks. Our previous proteomic analysis revealed that lamin A/C, one kind

number of diseases, such as cardiomyopathy and muscular dystrophy (14, 15, 18). Because our previous work revealed that lamin A/C might contribute to the vascular remodeling in response to shear stress (12), we hypothesized that NE proteins might be mechanical-sensitive molecules that participate in the VSMC functions induced by cyclic stretch.

In the present study, we investigated the effects of emerin and lamin A/C on VSMC proliferation in response to hypertension and studied the mechanobiological mechanisms involved. The results provided insights into the roles of these two important INM proteins in the transcriptional regulation of VSMC proliferation.

Results

Hypertension Represses the Expression of NE Proteins in Vivo. After 1 wk of abdominal aorta coarctation, the operated rats exhibited a marked level of hypertension (mean arterial pressure 161.7 ± 14.9 mmHg in hypertensive rats vs. 96.9 ± 9.9 in sham controls, $P < 0.01$), a marked increase of cell proliferation in the media of common carotid arteries (Fig. 1*A*), and a significant suppression of emerin and lamin A/C expression in the common carotid arteries as compared with the sham-operated controls (Fig. 1*B* and Fig. S1).

The increased cyclic mechanical stretch in hypertension has been shown to play an important role in VSMC proliferation (19). We hypothesized that cyclic stretch may modulate VSMC proliferation by inducing the expression of emerin and lamin A/C.

Cyclic Stretch Modulates the Expression of NE Proteins and the Proliferation of VSMCs in Vitro. Compared with the group subjected to the normal level (5%) of cyclic stretch (20) for 24 h, a high level (15%) of cyclic stretch (21) significantly increased the proliferation of VSMCs, as shown by BrdU ELISA (Fig. 2*A*).

The effects of different levels of cyclic stretch on the expression of emerin and lamin A/C proteins were studied at 6, 12, and 24 h. Compared with the normal 5% cyclic stretch, the 15% cyclic stretch decreased the expression of emerin (at 12 and 24 h but not at 6 h) and lamin A/C (at all time points: 6, 12, and 24 h) (Fig. 2*B*).

Compared with 5% cyclic stretch, 15% cyclic stretch decreased the mRNA level of emerin at the 24-h time point but not at 6 and 12 h. The 15% cyclic stretch showed no significant effect on mRNA level of lamin A/C at any of the time points (6, 12, and 24 h) (Table S1). The proteasome inhibitor MG-132(R) ($C_{26}H_{41}N_3O_5$) (10 μM) was used to detect possible lamin A/C and emerin degradation during the application of cyclic stretch. We found that MG-132(R) reversed, at least partially, the repression of emerin and lamin A/C protein expression induced by 15% cyclic stretch (Fig. S2). These results suggest that pathological (15%) cyclic stretch may increase the degradation of lamin A/C and emerin and that the repressed levels of these two NE proteins may be important in the VSMC hyperproliferation induced by hyperstretch.

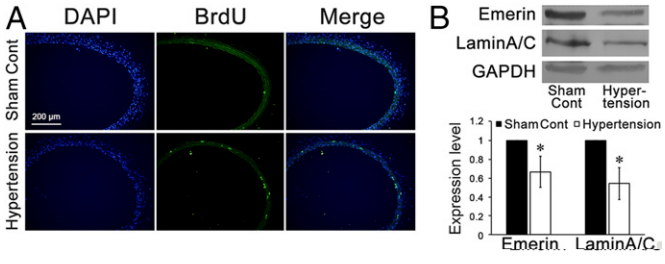

Fig. 1. In situ proliferation and expression of emerin and lamin A/C in the common carotid artery of hypertensive rats and sham-treated controls. (*A*) Immunofluorescence staining against BrdU revealed that after 1 wk of abdominal aorta coarctation, VSMC proliferation in the media of common carotid arteries increased markedly. (*B*) The expression of emerin and of lamin A/C was repressed significantly in the common carotid arteries of the aorta-coarctation–induced hypertensive rats. GAPDH was used for normalization. Values are expressed as mean ± SD. *$P < 0.05$ vs. the sham-treated control ($n = 5$).

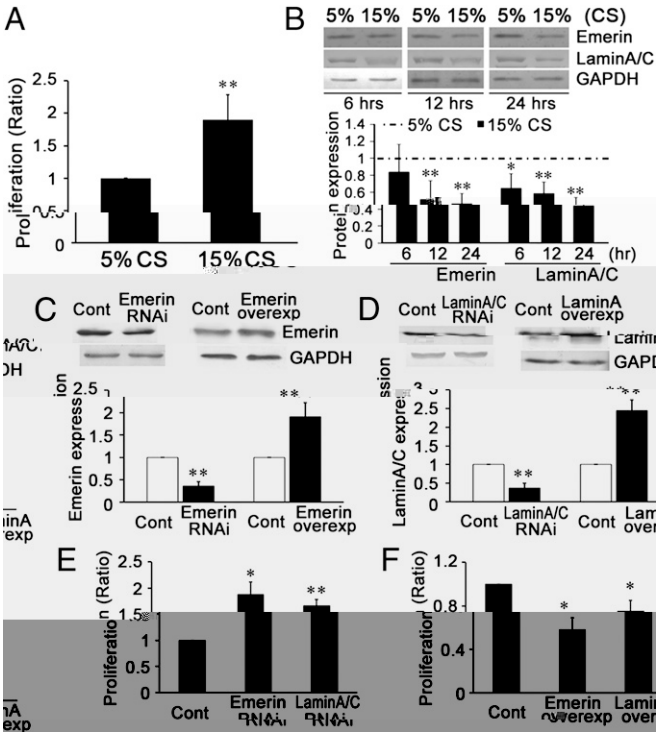

Fig. 2. Cyclic stretch (CS) modulated the expression of NE proteins emerin and lamin A/C, which participate in the stretch-induced proliferation of VSMCs in vitro. (*A*) A 15% cyclic stretch increased VSMC proliferation in comparison with a 5% cyclic stretch. (*B*) A 15% cyclic stretch decreased the expression of emerin and lamin A/C in comparison with a 5% cyclic stretch. (*C*) Under static conditions, target siRNA transfection significantly decreased and plasmid transfection significantly increased the expression of emerin. (*D*) Under static conditions, target siRNA transfection significantly decreased and plasmid transfections significantly increased the expression of lamin A/C. (*E*) Under static conditions, specific RNAi of both emerin and lamin A/C increased VSMC proliferation. (*F*) Up-regulated expression of emerin and lamin A/C decreased VSMC proliferation. For Western blots, GAPDH was used for normalization. Values are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$ vs. the respective control (Cont) ($n = 5$).

To assess the roles of these NE proteins in modulating VSMC proliferation, the expression of emerin or lamin A/C under static conditions was repressed by specific siRNA transfection (Fig. 2 *C* and *D* and Fig. S3 *A* and *B*). The results indicate that the proliferation of VSMCs under static conditions is increased significantly by the repression of either emerin or lamin A/C (Fig. 2*E*). Transfection of a plasmid overexpressing lamin A increased the expression of both lamin A and lamin C (Fig. S3*C*), and plasmids overexpressing emerin and lamin A significantly decreased VSMC proliferation (Fig. 2*F*).

These results indicate that a pathological (15%) level of cyclic stretch (21) decreases the expression of emerin and lamin A/C and increases the proliferation of VSMCs.

Cyclic Stretch Modulates the Proliferation of VSMCs via NE Proteins in Vitro. To confirm the effects of emerin and lamin A/C on cyclic stretch-induced VSMC proliferation, VSMCs were transfected with emerin or lamin A/C target siRNA (Fig. 3*A* and Fig. S4*A*) and then were subjected to normal (5%) cyclic stretch. The results show that, compared with negative control, transfection of either emerin- or lamin A/C-target siRNA significantly increased

VSMC proliferation under 5% cyclic stretch (Fig. 3*B*). In contrast, transfection with a plasmid overexpressing emerin or lamin A (Fig. 3*C* and Fig. S4*B*) reversed the effect of high (15%) cyclic stretch on VSMC proliferation (Fig. 3*D*).

These results suggest that the NE proteins emerin and lamin A/C are crucial mechano-responsive molecules participating in the modulation of VSMC proliferation. The increase in VSMC proliferation induced by the decreased expression of emerin and lamin A/C in response to pathologically increased cyclic stretch may be involved in the pathogenesis of vascular remodeling during hypertension.

ChIP with Microarray Analysis Revealed the Binding Between NE Proteins and Chromatin. To demonstrate the possible mechanism by which NE proteins regulate VSMC proliferation, ChIP with microarray (ChIP-on-chip) and bioinformatics analyses were used to detect the DNA-binding ability of emerin and lamin A/C and to demonstrate whether this binding affects transcription factors related to proliferation. DNA extractions from VSMCs were incubated with emerin or lamin A/C antibody, and the pulled-down DNA was analyzed by CpG Island Plus Refseq Promoter arrays. The results reveal that 1,030 DNA segments were immunoprecipitated with emerin (Table S2), and 1,046 DNA segments were immunoprecipitated with lamin A/C (Table S2).

The primary functions of the immunoprecipitated DNA segments were classified by Gene Ontology (GO) annotations using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v. 6.7 (https://david.ncifcrf.gov/summary.jsp). Fig. 4 shows that the DNA segments immunoprecipitated with emerin or lamin A/C are involved in different functional categories. The top five prominent functional categories for DNA segments immunoprecipitated with emerin were phosphoprotein (232 DNA segments), cytoplasm (122 DNA segments), acetylation (103 DNA segments), nucleotide-binding (81 DNA segments), and zinc (68 DNA segments) (Table S3); the top five prominent functional categories for DNA segments immunoprecipitated with lamin A/C were phosphoprotein (249 DNA segments), cytoplasm (124 DNA segments), nucleus (121 DNA segments), acetylation (102 DNA segments), and nucleotide-binding (81 DNA segments) (Table S3).

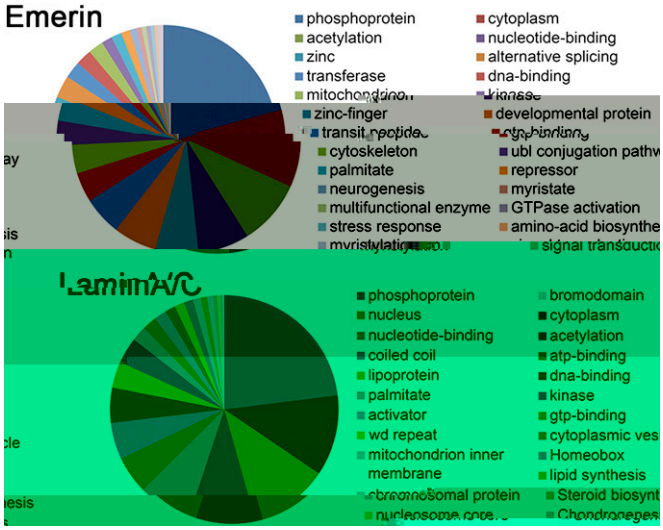

Fig. 4. GO annotations show the primary functional categories of the DNA segments immunoprecipitated with emerin or lamin A/C (Table S2).

These results suggest that both emerin and lamin A/C can bind with DNA segments, but the cellular functions involved differ somewhat.

Motifs of Transcription Factors Enriched in DNA Segments Binding with NE Proteins. To assess whether the binding of DNA segments with emerin or lamin A/C is sequence specific, we used Multiple Em for Motif Elicitation (MEME) (22) and Discriminative Regular Expression Motif Elicitation (DREME) (23) web service to visualize the locations of matches to the promoters of transcription factor (24). The specific motifs in the DNA segments binding with emerin were CCNGGA, CCMGCC, and ABTTCCG (Fig. 5*A*), and those binding with lamin A/C were CVGGAA, GCCGCYGC, and DAAGAAA (Fig. 5*B*). Table S4 shows the transcription factors revealed by the ChIP-on-chip assay and includes the sequence-specific motifs in their promoter regions.

These results suggest that emerin and lamin A/C bind to specific DNA segments, including the promoter regions of transcription factors. To detect whether this sequence-specific binding is involved in VSMC proliferation, Ingenuity Pathway Analysis (IPA), ChIP-quantitative PCR (qPCR), and Protein/DNA array were used to detect the binding of emerin or lamin A/C to the promoter regions of specific transcription factors.

Binding of Emerin and Lamin A/C with the Promoter Regions of Transcription Factors Participates in VSMC Proliferation. Using IPA software (content version: 14197757) (www.ingenuity.com/products/ipa), we investigated the functions of the transcription factors whose promoter regions were immunoprecipitated with emerin or lamin A/C (24). Six transcription factors related to emerin and four related to lamin A/C were involved in VSMC proliferation (Table S4). We then detected the role of altered emerin and lamin A/C expression in the activation of these transcription factors.

Using the Protein/DNA array, we detected the activation of 10 transcription factors revealed by ChIP-on-chip and motif analyses (Fig. 5*C*). The activation of four transcription factors related to VSMC proliferation showed a tendency to increase when emerin and lamin A/C were repressed by siRNA transfection.

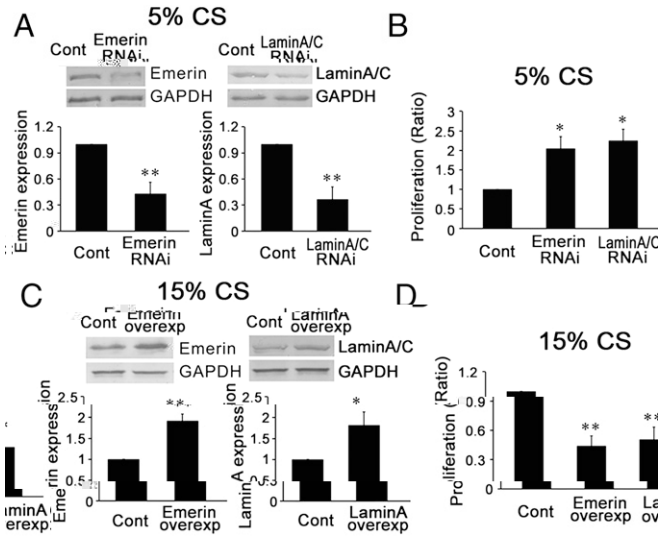

Fig. 3. Cyclic stretch modulates the proliferation of VSMCs via NE proteins in vitro. (*A*) The expression of emerin or lamin A/C was repressed by target siRNA transfection during the application of 5% cyclic stretch. (*B*) Emerin- or lamin A/C-target siRNA transfection increased the proliferation of VSMCs. (*C*) The expression of emerin and of lamin A/C was increased by plasmid transfection. (*D*) Transfection with plasmid overexpressing emerin and lamin A decreased VSMC proliferation under 15% cyclic stretch. For Western blots, GAPDH was used for normalization. Values are expressed as mean ± SD. $*P < 0.05$, $**P < 0.01$ vs. the respective control ($n = 6$).

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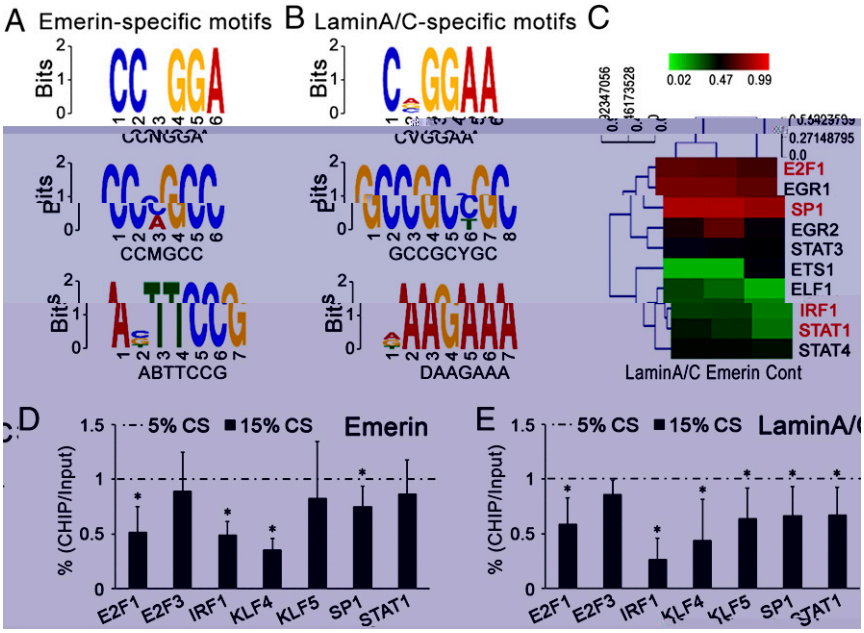

Fig. 5. Motifs of transcription factors enriched in DNA segments binding with emerin or lamin A/C. (*A*) The specific motifs in the DNA segments immunoprecipitated with emerin. (*B*) The specific motifs in the DNA segments immunoprecipitated with lamin A/C. (*C*) Emerin or lamin A/C-target siRNA modulated the activations of transcription factors in Table S4. The red font indicates the factors involved in the proliferation of smooth muscle cells. (*D* and *E*) After immunoprecipitation with emerin (*D*) or lamin A/C (*E*), qPCR detected the ChIP levels of promoter regions of transcription factors related with "proliferation of (vascular) smooth muscle cells" in Table S4 after cyclic stretch was applied for 24 h. Values are expressed as mean ± SD ($n = 4$).

Cyclic Stretch Alters the Ability of Emerin and Lamin A/C to Bind to the Promoter Region of Transcription Factors. After different level of cyclic stretch were applied for 24 h, DNA extracts from the VSMCs were incubated with emerin or lamin A/C antibody, and the immunoprecipitated DNA segments were analyzed by qPCR to quantify the promoter regions of transcription factors related to VSMC proliferation (Table S4).

The results showed that, as compared with 5% cyclic-stretch, 15% stretch decreased the binding of emerin to the promoter regions of E2F1, IRF1, KLF4, and SP1 and decreased the binding of lamin A/C the to the promoter regions of E2F1, IRF1, KLF4, KLF5, SP1, and STAT1 (Fig. 5 *D* and *E*).

Increased Expression of NE Proteins Represses VSMC Proliferation in Vivo. To demonstrate further the possible protective effects of emerin and lamin A/C on VSMC proliferation during hypertension, their overexpression was induced by the transcutaneous injection of lentiviruses around the common carotid artery of hypertensive rats in vivo (Fig. 6 *A* and *B*). The lentivirus of lamin A significantly increased the expression of both lamin A and lamin C (Fig. 6*B* and Figs. S3*C* and S4*C*). In comparison with the negative control, emerin and lamin A/C overexpression significantly decreased cell proliferation in the media of the common carotid arteries of hypertensive rats (Fig. 6*C*).

Discussion

The nuclear lamins form a mesh-like network of intermediate filaments localized mainly at the INM and play a major role in maintaining the mechanical stability and shape of the nucleus (13, 25). There are four lamin isoforms in mammalian cells: A-type lamins, including lamin A and lamin C, which are splice variants encoded by *LMNA*, and B-type lamins, including lamin B1 and B2 encoded by *LMNB1* and *LMNB2*, respectively (25, 26). Lamin B1 and B2 are essential proteins that are expressed in all cells throughout development (4, 8, 26), whereas lamin A and C are expressed only in differentiated cells. The differential expression

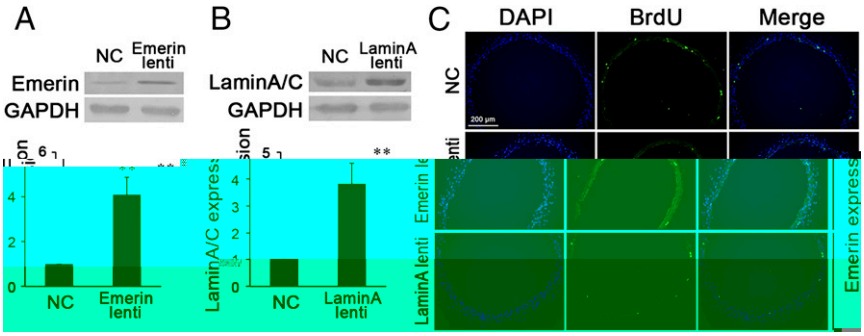

Fig. 6. (*A* and *B*) Local injection of emerin (*A*) or lamin *A* (*B*) lentiviruses (lenti) significantly increased the expression of emerin or lamin A/C in common carotid arteries the hypertensive rats. (*C*) Immun0(hon)3ert.97r2/T151Tf0Tc3.6340Tdc dttain2(lam4210(gainst.5351(BrdUam420Tve)-3651ert)-10that.535)-2in2ommafte

patterns of lamins suggest that lamin A/C may be important for inducing or maintaining the differentiated state. Recently, the differential expression of lamin A/C has been detected in cells subjected to different kinds of mechanical stimuli and has been shown to be involved in differential cellular functions. Harada et al. (27) reported that differentially expressed lamin A/C can modulate cell migration and survival in human mesenchymal stem cells and two cancer cell lines. Swift et al. (13) found that lamin A, but not lamin B, is changed by matrices of different stiffness and participates in the differentiation of mesenchymal stem cells induced by the stiffness of the culture matrix. Consistent with the results reported here indicating that cyclic stretch may post-translationally regulate lamin A/C, a 40-kDa lower band of lamin A/C (Fig. S4) indicates cleavage of lamin A/C that is regulated by adhesion and cell mechanics, as shown by Buxboim et al. (28). Furthermore, our current study demonstrates that, in addition to lamin A/C, the expression of emerin in VSMCs also is modulated by mechanical cyclic stretch and subsequently induces VSMC proliferation.

Emerin acts as an anchor of NE to INM and binds to lamin A/C at the nucleoplasm (26). The mutations in *EMD* (encoded emerin, located on chromosome X in humans and mice) and *LMNA* (encoded lamin A and lamin C, located on chromosome 1 in humans and chromosome 3 in mice) are related to similar diseases characterized by progressive skeletal muscle weakening, abnormal fat deposition, premature aging, and complex cardiac syndrome, including dilated cardiomyopathy and life-threatening irregular heart rhythms (29, 30). Embryo fibroblasts cultured

700-bp fragments. A chromatin aliquot was incubated overnight with 5 μg anti-emerin antibody (Abcam), anti-lamin A/C antibody (Abcam), or control rabbit IgG (rIgG) (Abcam). Antibody-coupled magnetic beads (Invitrogen) were added, washed, and eluted. DNA fragments associated with emerin, lamin A/C, or control antibodies were eluted and purified using the One-Day Chromatin Immunoprecipitation Kit (Millipore). Input genomic DNA was obtained through similar elution and purification procedures.

RefSeq Promoter Array and MOTIF Assay. For the ChIP-on-chip assay, samples were hybridized with the CpG Refseq Prom MeDIP Array (NimbleGen), which covers 15,287 promoters (the design region is about −3,880 bp to +970 bp of the transcription start site). For each ChIP-on-chip dataset, the sequences for the summit region (201 bp) spanning 100 bp up- and downstream from the summit of each peak were retrieved. The top 500 sequences with the highest peak score were selected for MOTIF analysis based on MEME (22) and DREME (23).

Statistical Analysis. Each experiment was performed at least in triplicate, and all values are expressed as mean ± SD. The Student's *t* test was used to compare two groups, and multiple comparisons among the groups were performed using one-way ANOVA and the Student–Newman–Keuls test for post hoc comparisons. A value of $P < 0.05$ was regarded as statistically significant.

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