HERP1 Inhibits Myocardin-Induced Vascular Smooth Muscle Cell Differentiation by Interfering With SRF Binding to CArG Box

Hiroshi Doi, Tatsuya Iso, Miki Yamazaki, Hideo Akiyama, Hiroyoshi Kanai, Hiroko Sato, Keiko Kawai-Kowase, Toru Tanaka, Toshitaka Maeno, Ei-ichi Okamoto, Masashi Arai, Larry Kedes, Masahiko Kurabayashi

Objective—Myocardin is a coactivator of serum response factor (SRF) required for vascular smooth muscle cell (VSMC) differentiation. HERP1 is a transcriptional repressor, which is abundantly expressed in vascular system and is known to function as a target gene of Notch. However, the role of HERP1 in the pathogenesis of vascular lesions remains unknown. The present study characterizes the expression of HERP1 in normal and diseased vessels, and tests the hypothesis that HERP1 inhibits SRF/myocardin-dependent SMC gene expression.

Methods and Results—Immunohistochemistry revealed that HERP1 and myocardin expression was localized to SMC in the neointima of balloon-injured rat aorta and in human coronary atherosclerotic lesions. Expression of both HERP1 and myocardin was elevated in cultured SMCs compared with medial SMC. Overexpressed HERP1 inhibited the myocardin-induced SMC marker gene expression in 10T1/2 cells. HERP1 protein interfered with the SRF/CArG–box interaction in vivo and in vitro. Immunoprecipitation assays showed that HERP1 physically interacts with SRF.

Conclusions—HERP1 expression was associated with the SMC proliferation and dedifferentiation in vitro and in vivo. HERP1 may play a role in promoting the phenotypic modulation of VSMCs during vascular injury and atherosclerotic process by interfering with SRF binding to CArG-box through physical association between HERP1 and SRF. (Arterioscler Thromb Vasc Biol. 2005;25:2328-2334.)

Key Words: HERP1 • myocardin • serum response factor • smooth muscle cells

Phenotypic modulation of vascular smooth muscle cells (VSMCs) from contractile to synthetic forms plays a pivotal role in the pathogenesis of vascular diseases including atherosclerosis and restenosis after angioplasty.1 It is well-established that VSMC phenotype is regulated by a complex array of local environmental cues including humoral factors, cell-cell and cell-matrix interactions, inflammatory stimuli, and mechanical stresses. Such complex stimuli downregulate cell-cell and cell-matrix interactions, inflammatory stimuli, and mechanical stresses. Such complex stimuli downregulate a number of genes required for the contractile phenotype in synthetic VSMCs. These include smooth muscle myosin heavy chain (SM-MHC), SM22α, caldesmon, and calponin. Because the genes encoding these proteins are differentially expressed depending on the proliferative state of VSMCs, transcription factors regulated by numerous stimuli are responsible at least in part for the distinct pattern of gene expression seen in synthetic VSMCs.

There is mounting evidence that most SMC marker proteins such as SM-MHC and SM22α are controlled by serum response factor (SRF), which binds to a sequence known as a CArG box and recruits a potent coactivator, myocardin, for SMC differentiation.2 When myocardin is ectopically expressed in nonmuscle cells, it can induce SMC differentiation.2–3 Most importantly, mouse embryos deficient for myocardin show no evidence of vascular SMC, indicating myocardin as a necessary and sufficient factor for SMC differentiation in vivo.4 These observations, in conjunction with downregulation of SMC marker genes in synthetic VSMC, led us to speculate that myocardin expression might be downregulated in synthetic VSMCs. As yet, however, it is not clarified whether reduced expression of SMC marker genes in synthetic VSMC results from downregulation of myocardin expression.

Although Notch signaling is required during angiogenesis and in vascular homeostasis, the mechanisms by which Notch regulates vascular function remain to be elucidated. In vertebrates, receptors, ligands, and other components of Notch signaling are expressed in vasculature.5 Mutations of genes involved in Notch pathway in mice lead to abnormalities in
many tissues including the vascular system. Human diseases such as Alagille syndrome and CADASIL, which show abnormalities in the cardiovascular system, are caused by mutations of the Notch ligand Jagged-1 and the receptor Notch-3, respectively. Such findings clearly demonstrate a crucial role of the Notch pathway in vascular development and homeostasis. Recently, several studies have reported with some controversial findings that expression level of several Notch components was significantly affected after vascular injury, suggesting that Notch pathway also plays a role in the pathogenesis of vascular diseases.

We and others have recently identified HERP family (for HES-related repressor protein, also referred to as Hesr, Hey, HRT, CHF, and gridlock) that is predominantly expressed in cardiovascular system. Some members of HERP family are proved to be direct downstream targets of Notch and acts as transcriptional repressor. Among them, HERP1 and HERP2 play a crucial role in vascular development in vivo because double knockout of the HERP1 and HERP2 genes in mice resulted in embryonic death with a global lack of vascular remodeling. Mutant singly deficient for gridlock, HERP1 homologue of zebrafish, also showed disturbance of assembly of the aorta. Of particular note, Notch signaling including multiple target genes generally functions as negative regulator of differentiation in various cells. These findings, along with induction of Notch components in injured VSMCs, or synthetic VSMCs, strongly suggest that Notch target genes, HERP1 and HERP2, are also induced in synthetic VSMCs and play a critical role for development of vascular disease as negative regulator of VSMC differentiation.

The present study describes a series of experiments that have explored the role of HERP1 in the phenotypic modulation of VSMC. Our in vitro analyses along with the immunohistochemical study showed that HERP1 plays an important role in modulating VSMC phenotypes, and this was caused by the ability of HERP1 to interfere with SRF binding to CArG box by physically associating with SRF. We propose that Notch-HERP pathway is one of the complex stimuli to modulate VSMC phenotypes, and that the stage of VSMC differentiation is determined by positive regulator myocardin and negative regulator HERP1.

**Materials and Methods**

The Materials and Methods section can be found in an online supplement available at http://atvb.ahajournals.org.

**Results**

**Differential Expression Pattern of HERP1, HERP2, and Myocardin in Adult Tissues**

Although HERP1 and HERP2 are abundantly expressed in developing cardiovascular system, their expression in adult tissue has not been clearly illustrated. Northern blot analysis showed that human HERP1 was abundantly expressed in heart and less strongly expressed in skeletal muscle, whereas human HERP2 was easily detected in other tissues, as well as in heart (Figure 1A). In the cardiovascular system, both HERP1 and HERP2 transcripts were abundantly expressed in aorta as well as heart (Figure 1B). In adult rats, HERP1 mRNA was expressed in several organs including heart, but most strongly in aorta. HERP2 expression in lung was much higher than that in other tissues such as heart and aorta (Figure 1C). Expression of myocardin was also tested in rat multiple organs (Figure 1C). Myocardin was markedly expressed in heart and gastrointestinal tract, but only weakly in aorta, which is in contrast to previous study reporting that in humans, myocardin expression was much stronger in aorta than in heart. These findings suggest that expression of HERP1, HERP2, and myocardin is differentially regulated in adult tissues.

**Induction of HERP1 and Myocardin Expression in Neointima After Balloon Injury**

We examined whether expression of HERPs and myocardin are affected in neointima after balloon injury. Immunohistochemistry of 14-day balloon-injured rat aortas and control vessels revealed that whereas only a few cells were positive for HERP1 in medial layer in sham-operated aorta, HERP1 staining was colocalized with SMα-actin–positive cells in neointima (Figure 2A). HERP1-positive cells also present in the thin layer of media adjacent to adventitia. Because many ligands and receptors for Notch are induced in neointima after vascular injury, strong staining for HERP1 in neointima implicates that HERP1 is induced as a downstream target gene of Notch. In contrast to HERP1, HERP2 was barely stained in aorta from both sham-operated and balloon-injured rats, suggesting cell type-specific expression of HERP family members (data not shown). Unexpectedly, myocardin, a
positive regulator for VSMC differentiation, was clearly detected in the neointima, which is characterized by synthetic phenotype of VSMC. These observations allowed us to speculate that function of myocardin is antagonized by certain factor(s) such as HERP1, a target gene of Notch, which functions in most cases as negative regulator for differentiation.

Expression of HERP1 and Myocardin in Human Coronary Atherosclerotic Lesions
To examine the expression of HERP1 and myocardin in human atherosclerotic lesions, we next double-stained human coronary atherectomy tissues. We confirmed that the tissues contained SMC and endothelial cells, revealed by SMα-actin and von Willebrand factor expression, respectively (data not shown). As shown in Figure 2B, HERP1-positive cells almost colocalized with cells stained positive for SMα-actin and myocardin. These findings suggest that both HERP1 and myocardin are coexpressed in VSMCs, and play a role in the development of vascular disease.

Myocardin and HERP1 Transcripts Are Induced in Cultured Rat Aortic SMC
To determine the expression of the myocardin and HERP1 genes in vitro, we performed Northern blot analysis using total RNA from rat aorta, media of aorta, and cultured rat aortic SMC (RASMC). As shown in Figure 3, HERP1 gene transcripts were significantly increased in cultured RASMC when compared with that in aorta and in media of aorta, which also supports the notion that HERP1 may negatively regulate VSMC differentiation. HERP2 induction was not detected (data not shown). Of particular note, gene transcripts of myocardin were also increased in cultured RASMC. These data led us to postulate that stage of VSMC differentiation may be determined by balance of expression levels between myocardin and certain negative regulator(s) including HERP1.

HERP1 Inhibits Induction of Myocardin-Dependent SMC Marker Genes
To examine whether HERP1 affects myocardin-induced VSMC differentiation, we compared expression of several SMC marker genes such as SM-MHC and SM22α in 10T1/2. As reported,2,3 myocardin strongly induced smooth muscle markers, SM-MHC and SM22α (Figure 4A, lane 3). When HERP1 was simultaneously expressed, however, induction of these markers by myocardin was dramatically decreased (Figure 4A, lane 4). Importantly, HERP1 did not affect the mRNA levels of SRF (Figure 4A) and protein expression of myocardin (Figure 4B). Immunostaining further revealed expression of myocardin in HERP1-positive cells (data not shown).

Figure 2. HERP1 and myocardin are coexpressed in neointima after vascular injury in α-actin–positive cells of human coronary atherectomy tissues. A, Immunohistochemistry of tissues from sham-operated rat aorta (upper) and balloon-injured aorta (lower). Two weeks after operation, aortas were harvested and stained with hematoxylin-eosin (HE), or indicated antibodies. Note strong induction of HERP1 and myocardin in neointima after vascular injury. Original magnification ×400. Scale bar=20 μm. B, Human coronary atherectomy tissues were double-stained with several combinations of indicated antibodies. Merged images are on the right. Yellow in the merged images indicates overlapping area. Original magnification ×100. Scale bar=200 μm.

Figure 3. Both HERP1 and myocardin transcripts are induced in cultured RASMC. Total RNA was isolated from rat aorta, media of aorta, or cultured RASMCs, and analyzed by Northern blot analysis for HERP1 and myocardin mRNAs. Media (noncultured RASMCs) was prepared from aortic tissue from several rats after removing intimal and adventitial layers by enzymatic digestion and mechanical sweeping with cotton swab. The number of passage times for cultured RASMCs is indicated.
These observations strongly suggest that HERP1 inhibits myocardin-dependent SMC differentiation by abrogating function of myocardin protein, not by myocardin expression. We also observed similar results using cultured RASMC. Additional HERP1 introduced by adenovirus markedly repressed expression of SM-MHC without affecting expression of SRF and myocardin (Figure 4C). Of interest, expression of SM22α, an early marker of SMC differentiation, was marginally altered, suggesting that the stage of SMC differentiation is determined by relative abundance between HERP1 and myocardin.

HERP1 Suppresses Myocardin-Dependent Transactivation of SM-MHC and SM22α Promoter

To test whether HERP1 is able to repress myocardin-dependent transactivation of smooth muscle marker genes, we next performed luciferase reporter gene assays with SM-MHC and SM22α promoters (Figure 5). Overexpressed myocardin strongly transactivated the promoters. However, when HERP1 was expressed at the same time, this induction was dramatically reduced in a dose-dependent manner. Overexpression of HERP1 marginally affected basal transcription of SM-MHC and SM22α promoters, suggesting that inhibition of myocardin-dependent transactivation by HERP1 was not through binding to promoter DNA. We next studied whether other HES–HERP family members also possess the same function as HERP1 does. We observed essentially the same results when HERP2 and HES1 were used. These findings suggest that any HES–HERP member may be able to inhibit myocardin-dependent gene expression in a similar fashion in various cells where one of the HES–HERP members and myocardin are simultaneously expressed.

HERP1 Interferes With SRF Binding to CArG Box Through Physical Association With SRF

It has been reported that myocardin, SRF, and DNA probe containing CArG box form a ternary complex in electrophoretic mobility shift assay (EMSA). Our data described (Figures 4 and 5), along with the ability of myocardin to form the ternary complex, raise the question of whether HERP1 disrupts the ternary complex to inhibit myocardin-dependent SMC differentiation. To address this possibility, we performed EMSA with in vitro-translated SRF, HA–myocardin, and HERP1 proteins. As shown in Figure 6A, SRF alone showed a strong band (lane 2), which was supershifted by anti-SRF antibody (lane 5). Of interest, intensity of SRF-specific band was significantly reduced by additional HERP1 in a dose-dependent manner (lanes 3 and 4), which suggests that HERP1 disrupts interaction between SRF and the DNA probe. Because HERP1 per se did not show any specific band with the probe (lane 1), HERP1 seems to disrupt the interaction by associating with SRF directly rather than competing with SRF to bind the CArG box on the probe. When SRF and myocardin were simultaneously incubated, a new band appeared (Figure I lane 1, available online at http://atvb.ahajournals.org). This new band seems to be ternary complex band of SRF–myocardin–DNA probe because it was abolished by additional HA antibody (lane 3), but not by normal IgG antibody (lane 4). Most importantly, this band also disap-
The Basic Helix-Loop-Helix Domain of HERP1 Is Required for Physical Interaction With SRF and Repression of Myocardin-Induced SMC Gene Transcription

To determine the domains of HERP1 that mediate the interaction with SRF and repress myocardin-induced SMC gene expression, we first performed GST-pull-down assay using various truncated mutants of mouse GST–HERP1 fusion proteins. Among 3 mutants, only the basic helix-loop-helix domain of HERP1 as well as full-length HERP1 directly associated with SRF (Figure IV). Next, we performed luciferase assay using various HERP1 truncated mutants. To allow all the truncated mutants of HERP1 to translocate into nuclei, we used GAL-fusion proteins. As expected, the basic helix-loop-helix domain suppressed myocardin-dependent SMC gene transactivation to the same degree as full-length HERP1 did. However, the OCY region in HERP1 also suppressed myocardin-dependent SMC gene transactivation (Figure V, available online at http://atvb.ahajournals.org). Our data suggest that HERP1 represses SRF–myocardin-dependent SMC differentiation through physical interaction between basic helix-loop-helix domain of HERP1 and SRF, and some mechanisms other than physical interaction may be involved in OCY region-mediated repression of SRF function.

Discussion

Our results indicate that HERP1 and myocardin are colocalized in cultured VSMC, as well as in dedifferentiated VSMC in neointima and human coronary atherosclerotic lesions. These in vivo findings were extended to in vitro experiments whereby exogenous HERP1 markedly and specifically suppressed the myocardin-induced expression of SMC marker genes, such as SM-MHC and SM22α in 10T1/2 cells. This was because of the ability of HERP1 to interact with SRF, thereby interfering with SRF–CArG complex formation.

The observation that HERP1 accumulated in the cells of neointima of the injured artery is consistent with the previous study that CHF1/HERP1-null mice showed decreased neointima formation after wire injury and that proliferative and migratory activity of VSMC lacking CHF1 was decreased.20 Another study reported that HRT1/HERP2 facilitates VSMC growth through suppression of the cyclin kinase inhibitor p21 and promotes cell survival by increased expression of the
anti-apoptotic kinase Akt in stable transformant of HRT1-overexpressed cells.21 Because biochemical characteristics of HERP2 are very similar to that of HERP1,14,15 HERP1 may be involved in cell growth and anti-apoptosis through those mechanisms during neointima formation.

It has been generally accepted that transition from contractile phenotype to synthetic phenotype is associated with upregulation of growth-promoting factors such as egr-1, Id, and c-jun, which are directly or indirectly inhibit the function of differentiation factors.1 Contrary to this concept, it is noteworthy that expression of myocardin, a differentiation-promoting factor, is induced in primary cultured RASMC, in neointima, and in atherosclerotic lesions, all of which are characterized by synthetic VSMC. It is intriguing to speculate that HERP1 may play a role in inhibiting the function of abundant myocardin, which allows the VSMC to proliferate. This assumption is currently undergoing investigation.

Recent studies revealed that coactivator function of myocardin is attenuated or abolished by several molecules by different mechanisms.22–26 In the present study, we clearly showed that HERP1 inhibited myocardin-induced transactivation of SMC-marker genes by physical interaction with SRF, then interfering with SRF–CArG box binding. There are several precedent reports that demonstrate the inhibition of SMC marker gene expression by interference with SRF–CArG interaction. HOP, an unusual homeodomain protein, bound to MADS box of SRF and weakly repressed SRF-dependent transcription by inhibiting SRF–DNA binding.22,27 KLF4, Krüppel-like transcription factor, repressed the expression of SMC marker genes by both downregulating myocardin expression and preventing SRF from associating with SMC gene promoters.24 Although the authors did not find the effect of KLF4 on SRF–DNA binding in EMSA, they observed that overexpression of KLF4 was associated with reduction in SRF binding to CArG containing regions of Smo-actin promoter. Because MADS box is responsible for DNA binding,28 both HOP and KLF4, as well as HERP1, are likely to inhibit SRF–DNA interaction through the interface of DNA binding, or MADS box. In contrast to our results, Proweller et al have recently shown that HERP1 inhibits the ability of myocardin to stimulate SRF-mediated SMC gene expression independent of the inhibition of SRF–CArG interaction.29 Despite the very similar approach to detecting the interference with SRF–CArG interaction including the in vitro-translated proteins, they did not find the inhibitory effects of HERP1 on SRF-CArG interaction in EMSA and chromatin immunoprecipitation assay. The precise reasons for such discrepant results deserve further experiments.

What are the upstream molecules of HERP1 induction in neointima? It is most likely that Notch signaling is the one because of following reasons: (1) HERP1 is a direct target gene of Notch in A10 cells derived from aortic SMC;29 (2) many ligands and receptors for Notch are strongly induced in injured SMC;7 and (3) Notch1-null mutant mice showed vascular remodeling defect with remarkable reduction in expression of both HERP1 and HERP2 in vascular system.16 However, HERP1 induction observed in cultured RASMCs was Notch-independent (data not shown). Recent studies revealed that in several cell lines, HERP/HES expression was also induced by other factors such as transforming growth factor (TGF)-β super family and transcription factor c-jun.30–32 Because TGF–β levels and c-fos expression were increased in balloon injury model,33,34 HERP1 induction in neointima may be caused by those factors. Interestingly, several members of TGF-β super family have been reported to cross-talk with Notch signaling and amplify Notch stimulation.30,31 Given that both Notch and TGF–β seem to be active in neointima, they may synergistically elevate HERP1 expression in neointima. Further studies will be needed to elucidate those signaling upstream of HERP1 induction in neointima.

In summary, we demonstrated that 2 transcription factors, HERP1 and myocardin, which have been shown to independently play critical roles in cardiovascular development, antagonistically affect SRF-dependent SMC gene expression. In addition, we presented that both factors are coinduced in synthetic VSMCs. These findings provide novel insight into the molecular mechanisms of phenotypic modulation of VSMCs that are closely associated with vascular disease and vascular development.

Acknowledgments

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Materials and Methods

Cell Culture

Primary cultures from adult rat aortic smooth muscle cells (RASMC) were prepared as described previously\textsuperscript{35}. RASMC were grown in M199 supplemented with 10% FBS. C3H10T1/2 cells (murine embryonic fibroblasts) and 293T cells were cultured in Dulbecco Modified Eagle Medium supplemented with 10% FBS in a 5% CO\textsubscript{2} atmosphere at 37\textdegree C.

RNA isolation, Northern blot analysis and Reverse Transcriptase (RT)–PCR

Total RNA was isolated from various organs of adult male Wister rats, cultured RASMC, and 10T1/2 cells using the ISOGEN reagent (Nippon gene, Tokyo). Human adult multiple-tissue and cardiovascular Northern blots were purchased from Clontech. Northern blot analysis was carried out as described\textsuperscript{35}. Rat myocardin cDNA for probe of Northern blot was isolated by RT-PCR using the following primers; 5'-ggatgcaccaagcacacctc-3' and 5'-ccaggtgttcctcactgtcg-3'. Probes for HERP1 and HERP2 were described elsewhere\textsuperscript{13}. Semi-quantitative RT-PCR was performed with RT-PCR kit (TAKARA, Japan) according to manufacture’s protocol. The gene-specific primers were the following: GAPDH, 5'-accacagtcacctgccatcaec-3' and 5'-tccaccacccctgttggetgta-3'; myocardin, 5'-ccaaaccaaggtgaagaagctc-3' and 5'-tgtcttaacttgacaccttgag-3'; HERP1, 5'-gacactacctcagattatggc-3' and 5'-egggagcatgggaaaage-3'; SM-MHC, 5'-aggaaaaccaaggtcaaagca-3' and 5'-cctgacatgtggtcacaatc-3'; SM22\textalpha, 5'-tccagtcaccaacagcacaag-3' and 5'-gaattgagccacaccttgccatcg-3'; SRF, 5'-ecagegctgctgactgcctgtggc-3' and 5'-gctgcttcacccttgctgctg-3'.

Animal Models and Human Coronary Atherectomy Tissue

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The procedure was performed according to a guideline set by the Council of Animal Care and Experimentation Committee at Gunma University. Thoracic aorta of adult male Wister rats weighing about 300 g were injured with a 2F balloon embolectomy catheter as described elsewhere\textsuperscript{36}. Two weeks after balloon injury, the animals were euthanized, and thoracic aortas were harvested. Human coronary artery tissues with atherosclerosis were obtained by directional coronary atherectomy from symptomatic patients diagnosed with ischemic heart disease at Gunma University Hospital. This protocol was approved by the Institutional Review Board at Gunma University Hospital, and informed consent was obtained from the patients.

**Immunohistochemistry**

Tissue samples were fixed in 4% buffered formaldehyde and embedded in paraffin. Immunohistochemistry for the tissues were performed with antibodies directed against HERP1\textsuperscript{29}, myocardin (Santa Cruz, sc-21559) and SM\textalpha-actin (DAKO, M0851) by using the CSA kit (DAKO, Carpinteria, CA).

**Plasmid Constructs**

Mouse myocardin cDNA was kindly provided by Dr S. Izumo\textsuperscript{37}. Expression plasmids of HERP1, HERP2, HES1 and SRF have been previously constructed\textsuperscript{15,35}. The following promoter regions were cloned into pGL3 Basic vector (Promega) for luciferase reporter gene assays; nucleotides –1326 to +51 of the mouse SM22\alpha gene and nucleotides –1226 to +47 of the mouse SM-MHC gene\textsuperscript{38}.

**Transfection and Luciferase Assay**

C3H10T1/2 cells were transfected with plasmid DNA by modified calcium phosphate precipitation method as described\textsuperscript{35}. The total amount of DNA per well was kept constant by adding the corresponding amount of expression vector without a cDNA
insert. Forty-eight hours later, dual luciferase assays were performed (Promega). Reporter gene luciferase activities were normalized to the activity of the control renilla luciferase.

**Electrophoretic Mobility Shift Assay (EMSA)**

*In vitro*-translated proteins of HA-myocardin, SRF and HERP1 were prepared with a TNT T7-coupled reticulocyte lysate system (Promega). Nucleotide sequence of the c-fos probe containing CArG box (underlined) was 5’-CTTTACACAGGA-TGTCCATATTAGGACATCTGTCGTCAGGT-3’. EMSA was performed as previously described\(^{19}\). For supershift assay, anti-SRF antibody (Santa Cruz Biotechnology, Inc), anti-HA antibody (Roche, 3F10) and normal IgG antibody (Santa Cruz Biotechnology, Inc) were used.

**Western blot analysis, co-immunoprecipitation and in vitro interaction assay**

Western blot analyses, co-immunoprecipitation and Glutathione S-Transferase (GST) pull-down experiments were carried out as described previously\(^{15}\).

**Chromatin Immunoprecipitation (ChIP) Assay**

The ChIP assay was performed as described\(^{39}\). Cultured RASMC was infected with Ad-empty or Ad-HERP1. After 4 days, cells were cross-linked with 1% formaldehyde for 10 min at room temperature. The cross-linked chromatin was sonicated, and immunoprecipitated with 10 µl of anti-SRF antibody (G20, Santa Cruz Biotechnology) or normal IgG antibody (Santa Cruz Biotechnology). Immunoprecipitated chromatin samples were reverse–cross-linked and purified by Qiaquick PCR Purification Kit (Qiagen). Input DNA and DNA isolated from precipitated chromatin were subjected to conventional PCR using primers flanking the 5’ CArG element from the SM-MHC promoter; 5’-gtactggggtccccataacg-3’ and 5’-tcgaggtctgagctggtcct-3’.
Statistical Analysis

Statistical analyses were performed using Student $t$ test with significant difference determined as $P<0.05$. Data are presented as means ± S.D.
Figure I

**EMSA**

![EMSA experiment](image)

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Figure II

**Co-immunoprecipitation assay**

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Figure III

HERP1

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full length (1-339)
N (1-48)
B (47-116)
OCY (110-339)

Figure IV

GST pull-down assay

SM22 luc.

Relative Luciferase Activity

0.2 0.2 0.2 0.2 0.2

myocardin (μg) 0 0.2 0.2 0.2 0.2

GAL-HERP1 (μg) full length 0.4 - - - -
N - - 0.4 - -
B - - - 0.4 -
OCY - - - - 0.4
Figure Legends

Supplemental figure I.  HERP1 disrupts the formation of the ternary complex of SRF, CArG and myocardin.
EMSA was performed using \textit{in vitro}-translated proteins for SRF, myocardin and HERP1 and a radiolabeled probe containing the c-fos CArG element (upper: short-exposed autoradiogram, lower: long-exposed autoradiogram).

Supplemental figure II.  HERP1 does not interfere with the binding between SRF and myocardin.
Co-immunoprecipitation assays for myocardin and SRF with or without HERP1 were done. 293T cells were transfected with expression vectors for FLAG-myocardin and SRF with or without HA-HERP1. Three days after transfection, immunoprecipitation was performed using normal IgG or anti-FLAG antibody. Bound proteins were separated by SDS-PAGE followed by Western blot analysis with anti-SRF antibody. Protein level from each expression vector in crude extracts was confirmed by Western blot analysis with anti-FLAG, anti-SRF or anti-HA antibodies.

Supplemental figure III.  Diagrams of HERP1 truncated mutants\textsuperscript{15}.
N; the amino-terminal region, B; the basic helix-loop-helix domain, O; the orange domain, C; the carboxy-terminal region, Y; YRPW domain.

Supplemental figure IV.  HERP1 directly interacts with SRF through the basic helix-loop-helix domain.
\textit{In vitro}-translated \textsuperscript{35}S-SRF was incubated with equal amounts of GST-empty, GST-HERP1 (full length) or various truncated mutants of GST-HERP1 fusion proteins for 1 hr at 4 °C. Bound proteins were analyzed by autoradiography after SDS-PAGE.
Diagram of each HERP1 truncated mutants is shown in Figure II.

Supplemental figure V. HERP repression activity resides in the basic domain and OCY domain.

10T1/2 cells were transiently transfected with luciferase reporter plasmids for SM22α with the indicated GAL fusion protein expression vectors. Three days after transfection, cells were lysed and assayed for luciferase activity. *$P<0.05$ relative to myocardin alone. Values represent the mean ± S.D.