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Cooperative Binding of KLF4, pELK-1, and HDAC2 to a G/C Repressor Element in the SM22α Promoter Mediates Transcriptional Silencing During SMC Phenotypic Switching In Vivo

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Rationale: We previously identified conserved G/C Repressor elements in the promoters of most smooth muscle cell (SMC) marker genes and demonstrated that mutation of this element within the SM22α promoter nearly abrogated repression of this transgene after vascular wire injury or within lesions of ApoE−/− mice. However, the mechanisms regulating the activity of the G/C Repressor are unknown, although we have previously shown that phenotypic switching of cultured SMC is dependent on Krupple-like factor (KLF4).

Objective: The goals of the present studies were to ascertain if (1) injury-induced repression of SM22α gene after vascular injury is mediated through KLF4 binding to the G/C Repressor element and (2) the transcriptional repressor activity of KLF4 on SMC marker genes is dependent on cooperative binding with pELK-1 (downstream activator of the mitogen-activated protein kinase pathway) and subsequent recruitment of histone deacetylase 2 (HDAC2), which mediates epigenetic gene silencing.

Methods and Results: Chromatin immunoprecipitation (ChIP) assays were performed on chromatin derived from carotid arteries of mice having either a wild-type or G/C Repressor mutant SM22α promoter-LacZ transgene. KLF4 and pELK-1 binding to the SM22α promoter was markedly increased after vascular injury and was G/C Repressor dependent. Sequential ChIP assays and proximity ligation analyses in cultured SMC treated with platelet-derived growth factor BB or oxidized phospholipids showed formation of a KLF4, pELK-1, and HDAC2 multiprotein complex dependent on the SM22α G/C Repressor element.

Conclusions: Silencing of SMC marker genes during phenotypic switching is partially mediated by sequential binding of pELK-1 and KLF4 to G/C Repressor elements. The pELK-1-KLF4 complex in turn recruits HDAC2, leading to reduced histone acetylation and epigenetic silencing. (Circ Res. 2012;111:685-696.)

Key Words: KLF4 ■ pELK-1 ■ HDAC2 ■ smooth muscle cells ■ acetylation ■ smooth muscle ■ gene transcription ■ transcription factors ■ vascular disease

Smooth muscle cells (SMC) are remarkably plastic and transition from a quiescent contractile state to a proliferative-migratory state during vascular injury and development of atherosclerosis. Collectively, this process is termed “phenotypic switching” and is characterized by the coordinate downregulation of markers of differentiated SMCs including SM22α, smooth muscle myosin heavy chain (SM-MHC), and SM α-actin, gene products required for SMC contraction. SMC phenotypic plasticity probably evolved for optimization of vascular repair after injury, although it is also widely accepted that SMC phenotypic switching plays a key role in development and progression of atherosclerotic lesions and regulation of plaque stability.

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Our laboratory and many others have studied molecular mechanisms and factors that repress SMC differentiation marker gene expression as a means to elucidate processes involved in mediating SMC phenotypic switching. Importantly, these studies have clearly established that SMC phenotypic switching is actively regulated (reviewed in Owens et al). Physiol Rev and is mediated through complex processes including extracellular signal-related kinases (ERK)-dependent phosphorylation of ELK-1, loss of SRF-myocardin (MRTF) binding to CArG boxes in SMC marker gene promoters, and epigenetic silencing processes. Moreover, we have presented multiple lines of evidence that phenotypic switching...
of cultured SMC in response to platelet-derived growth factor (PDGF)-BB, PDGF-DD, and oxidized phospholipids is dependent on the embryonic stem cell (ESC) pluripotency Kruppel-like factor (KLF)4, including (1) expression of KLF4 was increased after treatment of cultured SMC with these factors;8,14,35–38; (2) siRNA-induced suppression of KLF4 inhibited suppression of SMC marker genes;8,38–40; and (3) overexpression of KLF4 in cultured SMC was associated with coordinate downregulation of SMC marker genes and the SMC-specific SRF coactivator myocardin.39,40 and epigenetic silencing of SMC marker gene loci.30,31,33,34 However, the preceding studies were conducted almost exclusively in cultured SMCs that have already undergone extensive phenotypic switching. Moreover, it is clear that these simple in vitro models of SMC phenotypic switching fail to recapitulate complex environmental cues that mediate SMC phenotypic switching in vivo. As such, very little is known regarding mechanisms and factors that regulate SMC phenotypic switching in vivo after vascular injury or in disease models, including atherosclerosis.

A major advance in understanding the mechanisms that regulate SMC phenotypic switching in vivo were studies from our laboratory showing that suppression of the SMC marker gene SM22α after vascular injury or within atherosclerotic lesions of ApoE−/− mice was dependent on a G/C Repressor element located in proximity to SM22α 5′ CArG boxes.41,42 Notably, mutation of the conserved G/C Repressor element did not alter developmental expression of this gene in transgenic mice41 but nearly completely abrogated down regulation of the gene after carotid wire injury43 or within atherosclerotic lesions.42 However, studies failed to identify the transcription factors and mechanisms that regulate the activity of the G/C Repressor element, a regulatory element found within promoters of nearly all CArG-dependent SMC marker genes.

There are a number of transcription factor families capable of binding to G/C rich elements, including Sp1 and Kruppel-like zinc finger transcription factors.43 The Owens laboratory first tested Sp1 and Sp3 as potential G/C Repressor binding factors because they are expressed in SMCs and are induced during PDGF-BB phenotypic switching of cultured SMCs.41,42 Interestingly, Sp1 can bind to the SM22α G/C Repressor in electrophoretic mobility shift assays (EMSA), and siRNA suppression of Sp1 inhibited phenotypic switching in cultured SMCs in response to PDGF-BB. However, we were unable to demonstrate direct binding of Sp1 to SMC marker gene promoters within intact chromatin by Chromatin immunoprecipitation (ChIP) assays after PDGF-BB treatment or in vivo after vascular ligation injury.41,42 Moreover, we subsequently showed that Sp1 dependence of SMC phenotypic switching in cultured SMC was mediated by Sp1-dependent activation of KLF4, whose promoter contains three conserved Sp1 binding sites.45 KLF4 is an attractive alternative candidate given that we have previously shown that it is required for SMC phenotypic switching of cultured SMC.8,30–32,38–40,45 Moreover, we demonstrated increased KLF4 binding to SMC promoters after carotid ligation injury in vivo and that global conditional KLF4 knockout mice showed a transient delay in SMC phenotypic switching after carotid ligation injury in vivo.32 However, we have been unable to show specific binding of KLF4 to the SM22α G/C repressor based on EMSA. Furthermore, the effects of global knockout of KLF4 on SMCs may be mediated indirectly through loss of KLF4 in (1) macrophages where it mediates monocyte to macrophage differentiation46,47,48 and/or (2) in endothelial cells where it has proinflammatory effects including mediating activation of leukocyte adhesion molecules.49,50 Thus, at present, there is no direct evidence indicating that the activity of the SM22α G/C Repressor in vivo during vascular injury is dependent on KLF4 or mediated through direct binding of KLF4 to the G/C repressor element. The present studies test the hypothesis that repression of SM22α expression during SMC phenotypic switching in vivo after carotid ligation is mediated through binding of pELK-1 (downstream activator of the mitogen-activated protein kinase pathway) and KLF4 to the G/C Repressor element. We also hypothesize that the pELK-1–KLF4 complex in turn recruits histone de-acetylases (HDACs) to this gene locus and mediates epigenetic silencing. Finally, given evidence that many SMC marker genes contain G/C Repressor elements, we postulate that these mechanisms contribute to coordinate suppression of multiple SMC genes during SMC phenotypic switching.

Methods

Animal protocol models were approved by the University of Virginia Animal Care and Use Committee. An expanded Materials and Methods section is available in the online-only Data Supplement.
Results

Suppression of SM22α Gene Expression After Carotid Ligation Is G/C Repressor Dependent

We previously demonstrated that mutation of the SM22α G/C Repressor element inhibited repression of this gene during SMC phenotypic switching after carotid wire injury or within atherosclerotic lesions of ApoE−/− Western diet–fed mice. Given evidence that SMC phenotypic switching after carotid ligation is KLF4-dependent, we first determined if suppression of SM22α in this model is also G/C Repressor dependent. Carotid ligations were done in SM22α wild-type (WT) and SM22α G/C Repressor mutant LacZ transgenic mice. X-gal staining was examined 3 days after ligation, a time point we previously demonstrated that there is significant loss of expression of the endogenous SM22α gene in this model. Results showed that mutation of the G/C Repressor nearly abolished downregulation of SM22α after ligation injury (Figure 1A and 1B). The unligated left carotid of both SM22α WT and SM22α G/C Repressor mutant LacZ transgenic mice showed no repression and exhibited SMC restricted expression, indicating that the mutation had no discernible effects on expression in differentiated (nonpheno-

typically modulated) SMC, consistent with our previous observations showing normal developmental expression of the mutant transgene. Taken together, these results, and those of our previous studies demonstrate that the G/C Repressor element is required for downregulation of SM22α gene expression in ALL models of SMC phenotypic switching examined to date. Given the diversity of these models, it is thus likely that G/C Repressor dependent SMC phenotypic switching represents a common transcriptional regulatory pathway for SMC phenotypic switching across highly divergent stimuli. To further test this possibility, we determined the importance of the G/C Repressor element in 2 in vitro models of SMC phenotypic switching, treatment with PDGF-BB, and the proatherogenic oxidized phospholipid POVPC. The SM22α G/C Repressor mutant promoter reporter showed attenuated PDGF-BB and POVPC induced repression as compared with the WT SM22α promoter reporter, although both showed some repression consistent with previous reports that there are also G/C Repressor independent mechanisms operative within these in vitro models, although it remains to be shown they are functional in vivo.
KLF4 Binds the G/C Repressor Element In Vivo After Vascular Ligation Injury

We have previously shown enriched binding of KLF4 to SM22α and other SMC promoters after carotid ligation in vivo. However, there is no direct evidence that this binding was G/C Repressor dependent. To test if KLF4 binding in vivo is G/C Repressor dependent, we performed ChIP assays after carotid ligation in SM22α/H9251 WT and SM22α/H9251 G/C Repressor mutant LacZ transgenic mice. Given limited availability of transgenic mice, and the requirement of 10 mouse carotids for each in vivo ChIP data point, we first determined the kinetics of KLF4 binding to the endogenous SM22α/H9251 promoter region using C57/B6 control mice. Ligated right carotids and control left carotid arteries were harvested at 1, 3, 7, 14, and 21 days after vascular injury. Consistent with our previous results, we observed enhanced binding of KLF4 to the SM22α/H9251 promoter region 1 and 3 days after vascular injury (Figure 2A). Binding was selective since KLF4 was not bound to an intronic sequence located within the SM22α/H9251 promoter (Figure 2B). Further ChIP analyses on WT and G/C Repressor mutant mice 3 days after vascular ligation injury were completed to determine if KLF4 binding is G/C Repressor element dependent. As a control, KLF4 ChIP assays used PCR primers that distinguished the endogenous mouse SM22α promoter versus the rat SM22α promoter-LacZ transgenes (Online Figure I). These experiments are important since we originally cloned KLF4 using a yeast one hybrid method based on its ability to bind to another G/C rich cis element located within 200 base pairs of the G/C Repressor element (the TCE element). The TCE element is located proximal to the first CArG element, whereas the G/C Repressor element is located proximal to the second CArG element (Online Figure IVA), and we have shown that mutation of these elements have profoundly different effects. That is, mutation of the TCE element completely abolished transgene expression in vivo in mice, whereas mutation of the G/C repressor had no effect on transgene expression during development and maturation but abrogated repression during SMC phenotypic switching.

Results of SM22α in vivo ChIP analyses demonstrated enriched binding of KLF4 to the WT but not the G/C repressor mutant SM22α LacZ transgene and the endogenous SM22α promoter in both transgenic strains (Figure 2C). In contrast, Sp3 binding to the SM22α promoter was enhanced after carotid ligation but binding was not altered on the G/C Repressor mutation (Figure 2D and Online Figure III). As such, it is interesting to speculate that Sp3 might represent an alternate G/C Repressor–independent repressor pathway in cultured SMC (Figure 1C and 1D) or in vivo. These in vivo ChIP assays provide clear evidence that enhanced binding of KLF4 to the SM22α promoter after carotid ligation is G/C Repressor dependent. The G/C Repressor is also required for suppression of SM22α during phenotypic switching of SMC after ligation injury (Figure 1), wire injury, and within ApoE−/− atherosclerotic lesions. Taken together, these results provide strong evidence that KLF4 plays an integral role in SMC phenotypic switching.
switching and functions through G/C Repressor–dependent mechanisms. Significantly, the preceding results are the first to provide direct evidence that the functional effects of mutating the G/C Repressor element (ie, virtually abrogating downregulation of SM22α in response to vascular injury or within atherosclerotic lesions) are causally linked to G/C Repressor dependent binding of KLF4.

Previously we showed that KLF4 repressed SMC marker gene expression in cultured SMC through multiple mechanisms, including (1) disruption of SRF and myocardin binding to SMC promoters within intact chromatin39,40,45 and (2) induction of histone modifications associated with formation of heterochromatin and transcriptional silencing.30,31,33,34 However, we have not previously determined if these effects are G/C Repressor dependent, which is critical if we are to establish a causal relationship between KLF4 binding, epigenetic changes, and our observations that mutation of the G/C Repressor abrogated SM22α gene suppression in vivo during SMC phenotypic switching. We first tested if KLF4-induced repression of SM22α in cultured SMCs is G/C Repressor dependent via transient transfection assays using a KLF4 overexpression plasmid plus the WT or G/C Repressor mutant SM22α/LacZ transgenes (Online Figure II). KLF4 binding to the SM22α/LacZ promoter was enhanced 12 hours after treatment of cultured SMC with either PDGF-BB (Figure 3B) or POVPC (Figure 3C), and binding was G/C Repressor dependent. Significantly, we showed that the G/C Repressor mutation itself did not result in decreased SRF binding (Online Figure IVB) and thereby contribute to transcriptional repression, consistent with our observations that expression of the G/C Repressor mutant LacZ transgene is normal in differentiated SMCs in vivo (Figure 1 and Regan et al41). However, SRF binding to the WT SM22α/LacZ promoter and the endogenous SM22α promoter but not the G/C Repressor mutant was dramatically reduced in cultured SMCs...
Element 3 Days After Vascular Ligation Injury

and that KLF4 and pELK-1 physically interact based on coimmunoprecipitation assays and sequential ChIP analysis.16,30,31 pELK-1 also inhibits the interaction between SRF and myocardin by competing for same docking site on SRF as myocardin after PDGF-BB treatment.16 The G/C Repressor and ETS binding site overlap by 3 base pairs within smooth muscle marker genes and we are physically unable to differentiate these sites (Online Figure IVA). Therefore, we sought to determine (1) if there is enhanced binding of pELK-1 to the SM22α promoter after ligation injury in vivo, since previous studies by Wang et al were done exclusively in cultured cells,16 and (2) if pELK-1 binding to the SM22α promoter is altered by mutation of the G/C Repressor. Right carotid ligation injuries were performed in vivo. A, C57B6 mice were subjected to right common carotid injury as mentioned in Figure 2A and then subjected to ChIP analysis for pELK-1. Asterisk indicates significant binding compared with noninjured controls. Results are the average of 3 independent experiments.

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Figure 4. pElk-1 binds to the SM22α promoter 3 days after carotid ligation in vivo. A, C57B6 mice were subjected to right common carotid injury as mentioned in Figure 2A and then subjected to ChIP analysis for pElk-1. Asterisk indicates significant binding compared with noninjured controls. Results are the average of 3 independent experiments. B, SM22 and SM22 G/C mutant LacZ mice were subjected to right common carotid injury and then harvested 3 days after vascular injury; tissues from 10 mice were pooled and then subjected to ChIP assay for pELK-1. In each ChIP IP, qPCR analysis was conducted on both the endogenous and LacZ transgene as indicated in the figure. Asterisk indicates significant binding as mentioned in Figure 2D.

No significant binding compared with noninjured controls. Results are the average of 3 independent experiments.

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expression of the WT promoter but not the G/C Repressor mutant. Marked reductions in pELK-1 binding to the WT SM22α promoter was observed after siRNA induced suppression of pELK-1 (Online Figure XA). Moreover, of major interest, suppression of pELK-1 expression also markedly reduced KLF4 binding, indicating that it is required for binding of KLF4 to the SM22α promoter. Taken together, the preceding data provide direct evidence that mutation of the G/C Repressor attenuates binding of KLF4 and pELK-1, and, taken together with previous results,16,30,31 suggest they act cooperatively to mediate repression of SM22/H9251 during SMC phenotypic switching in vivo and in vitro and that pELK-1 binding precedes KLF4 binding on the G/C Repressor element.

KLF4 and pELK-1 Interact Based on In Situ Proximity PLA Assays

We previously demonstrated that KLF4 and pELK-1 interact based on coimmunoprecipitation assays on homogenates of cultured SMC treated with POVPC.31 To determine if KLF4 and pELK-1 interact within intact cells, we performed in situ DuoLink proximity ligation assays which permit detection of proteins located within approximately 40 nm within individual cells based on staining with secondary antibodies containing complementary single stranded DNA molecules (www.olink.com). Assays were performed in human coronary artery cells due to the availability of higher quality PLA-compatible antibodies for detection of human KLF4 and pELK-1 and because this would allow us to determine if the results seen in our rodent SMC lines also apply to human coronary SMC. Consistent with results of studies in rodent SMC lines, results of ChIP assays in human coronary SMC showed marked enrichment of KLF4 and pELK-1 binding but reduced SRF binding to the SM22/H9251 promoter after treatment with PDGF BB (Online Figure VI). Moreover, results of the PLA assays showed evidence of interaction of KLF4 and pELK-1 after treatment with PDGF-BB (Figure 5B) or POVPC (Figure 5C). This increased interaction was blocked by treatment with the ERK inhibitors U1026 and PD98059, indicating that interaction is dependent on ELK-1 phosphorylation, which is critical for physical interaction of ELK-1 and KLF4.31 Consistent with reduced SRF binding to the SM22α promoter in ChIP assays, PLA results showed reduced interaction of SRF with myocardin after treatment of cultured human coronary SMC with PDGF-BB or POVPC but was retained in cells treated with ERK inhibitors39,40 (Online Figures VII and VIII). To our knowledge, these results are the first to actually show interaction of myocardin and SRF within intact cells and provide further evidence in support of our hypothesis that cooperative interactions of KLF4 and pELK-1 mediate G/C Repressor–dependent transcriptional repression during SMC phenotypic switching.

Hypomethylation of the SM22α Promoter After Carotid Ligation Is G/C Repressor Dependent and Mediated at Least in Part by Recruitment of HDAC2

Previously, we demonstrated that PDGF-BB–induced and POVPC-induced phenotypic switching in cultured SMC was
mediated in part by KLF4 dependent recruitment of HDACs 2, 4, and 5 and subsequent DNA hypo-acetylation of SMC marker gene promoter regions. To determine if similar mechanisms function in vivo, we performed H3 acetylation ChIP assays (Figure 6A) in WT and G/C Repressor mutant SM22α promoter LacZ transgenic mice 3 days after carotid ligation. Results showed marked reductions in H3 acetylation of the endogenous SM22α promoter and WT LacZ transgene but not the G/C Repressor mutant transgene. There was also marked enrichment of HDAC2 to the WT and endogenous SM22α promoter but not the G/C Repressor mutant promoter after ligation (Figure 6B). PDGF-BB or POVPC treatment of cultured SMC was associated with increased HDAC2 recruitment to the SM22α promoter that was also G/C Repressor dependent (Figure 6C and 6D). HDAC5 also showed increased binding in cultured SMC after PDGF-BB or POVPC treatment or in vivo after carotid ligation injury, but its binding was not affected by the G/C Repressor mutation (Online Figure XII). Binding was selective for HDACs 2 and 5, since we saw no evidence for enhanced binding of HDACs 3, 4, or 7 in vitro or in vivo (Online Figure XIII and Salmon and Owens, data not shown). Transient transfection assays of SM22α promoter-reporter genes with HDAC2 demonstrated decreased expression with the WT but not the G/C Repressor mutant (Online Figure V). We also showed increased HDAC2 binding to the SM22α promoter in human coronary artery SMC after PDGF BB and POVPC treatment (Online Figure VI and data not shown). Furthermore, we were able to demonstrate that HDAC2 binding requires both pELK-1 and KLF4 via siRNA knock-downs and MEK/ERK inhibitor experiments (Online Figures IX, X, and XI). Finally, to determine if KLF4, pELK-1, and HDAC2 colocalize to the G/C Repressor element, triple sequential ChIP analyses were performed (Figure 7). Sequential ChIP analyses demonstrated that KLF4, pELK-1, and HDAC2 were present within the same chromatin fragments consistent with formation of a higher-order complex. Significantly, we found that the G/C Repressor element was required for binding all 3 factors (Figure 7A and 7C). Finally, we performed additional ChIP analyses on the SMα-actin, SM-MHC, and c-Fos promoters in injured and noninjured mouse carotid artery samples 3 days after ligation injury to determine if pELK-1–KLF4–HDAC2–dependent transcriptional repression is applicable to multiple SMC genes (Figure 8). Results showed significant enrichment of KLF4, pELK-1, and HDAC2 binding to the SMα-actin and SM-MHC promoters after ligation injury and indicate that the mechanisms identified using the SM22α promoter as a model system are probably applicable to multiple CArG-dependent SMC marker genes. However, direct proof of this would require generation of G/C Repressor mutant promoter reporter transgenic mice for each of these genes.
Herein we provide direct evidence that KLF4 mediates the effects of mutating the G/C Repressor element in suppression of SM22α/H9251 during SMC phenotypic switching in vivo in response to vascular injury (Regan et al41 and Figure 1) and atherogenesis.42 Moreover, we provide evidence for a model wherein pELK-1 binds to the G/C Repressor region of the SM22α/H9251 promoter and in turn recruits KLF4 and HDAC2, ultimately leading to epigenetic silencing of the gene locus mediated at least in part through histone deacetylation (Online Figure XIV). Although the present studies focused primarily on a single SMC marker gene, SM22α, we believe it is highly likely that similar mechanisms contribute to coordinate suppression of multiple SMC marker genes during SMC phenotypic switching both in vivo and in vitro given the following observations. First, we have previously shown that KLF4 overexpression markedly suppresses expression of all SMC marker genes examined to date.40 Second, most SMC marker genes, but particularly CArG-SRF–dependent genes including not only SM22α but also SM-MHC and SM α-actin, contain conserved G/C Repressor and/or ETS elements41,42,61 (Online Figure IVA). However, thus far they have only been shown to be functionally important for SM22α in vivo (Figure 1 and Regan et al41 and Wamhoff et al42) and in vitro41,42 and SM-MHC in vitro.61 Third, inhibition of ELK-1 phosphorylation with MEK or ERK inhibitors has been shown to inhibit SMC phenotypic switching in cultured SMC by many labs,36,30,31 although until the results presented herein there was a lack of clear evidence regarding the contribution of this pathway in vivo. Fourth, we have shown that conditional KLF4 knockout mice show delayed SMC phenotypic switching in vivo,32 although it remains to be determined if this is a direct function of loss of KLF4 in SMC versus loss in other cell types including macrophages and endothelial cells, where it has been shown to regulate transitions in phenotype.46–50 Taken together, results are the first, and to date, the only published studies to our knowledge to identify a direct specific molecular mechanism that mediates SMC phenotypic switching in vivo, although further studies including SMC-specific conditional knockout of KLF4 will be required to conclusively show that cell autonomous KLF4 is required for SMC phenotypic switching in vivo.

An additional unresolved issue is that it was not possible for us to distinguish the relative contributions of the ETS domain versus the G/C Repressor element, given the partial overlap of these elements, and the fact that it has been impossible to show binding of the higher-order pELK-1–KLF4-HDAC2 complex

![Figure 7. Triple Sequential ChIP assays demonstrate that KLF4, pELK-1, and HDAC2 occupy the same piece of chromatin and their binding is attenuated with the G/C Repressor mutation. A, Rat aortic smooth muscle cells were prepared as mentioned previously. After serum starvation, cells were treated with 20 ng/mL PDGF-BB for 12 hours and then subject to ChIP analysis. Immunoprecipitations were performed in the sequence mentioned in the y-axis. B, IgG was used as a negative control. Asterisk indicates significant binding as mentioned in Figure 2D. C, Triple sequential ChIP analyses were performed as mentioned in A with a modification to the sequence of the pull-down as indicated on the y-axis. D, IgG was used as a negative control during the sequence of the pull-down as mentioned previously in B.](image-url)
except within intact chromatin or based on PLA assays as demonstrated herein. However, this may be irrelevant since, of course, mother nature did not dictate that these are indeed functionally distinct, and most importantly our studies provide what we believe is compelling evidence that the G/C Repressor mutation was remarkably specific in its effects on promoter function. For example, the mutation had no discernible effect on expression during development and maturation with identical expression patterns to both the WT SM22α/ H9251 promoter-LacZ transgene and the endogenous gene in our previous studies41 and herein in adult SMC tissues (Figure 1). Consistent with these findings, we saw no effect of the G/C Repressor mutation on SRF binding, but it dramatically reduced KLF4, pELK-1, and HDAC2 binding after ligation injury in vivo, or POIVPC-induced or PDGF-BB–induced phenotypic switching in cultured vascular SMC from mice, rats, and humans. Clearly, further studies will be required to determine the structural determinants of this promoter region that mediate recruitment of the pELK-1–KLF4 multiprotein complex.

A key unresolved question is the mechanism of activation of KLF4 expression in SMC, given this gene is epigenetically silenced in almost all differentiated somatic cells other than epithelial cells.51,52 The KLF4 promoter contains a number of conserved regulatory elements for AP-1, GATA-1, Sp1, NFκB, and HLH factors,53 but, as yet, no studies have directly assessed if these factors regulate KLF4 expression with the exception of our evidence showing that Sp1 bound the KLF4 promoter via ChIP assays.45 Oct4 and other ESC factors may play a role given evidence that these factors reciprocally activate one another in ESCs.54 There are a number of unresolved questions regarding mechanisms responsible for activation of KLF4 in SMC. First, what are the mechanisms and factors that activate expression of

Figure 8. KLF4, pELK-1, and HDAC2 bind α-actin and SM-MHC but not c-Fos in vivo after carotid ligation injury. A, Mice were ligated as mentioned in Figure 2. After ligation, right and left carotids were harvested and subject to ChIP analysis for SRF (A), KLF4 (B), pELK-1 (C), HDAC2 (D), and H4ac (E) binding at the α-actin, SM-MHC, and c-Fos promoters. Asterisk denotes significant decreases in binding over vehicle-treated controls via Student’s t test, with probability value < 0.05.
KLF4 in vivo during vascular injury or disease? Remarkably, despite widespread interest in this gene because of its involvement in production of iPSCs, as yet, no studies have identified sufficient regions of the promoter necessary to drive expression of the gene in vivo in transgenic mice, a prerequisite for studies elucidating mechanisms that induce it with injury-inflammation. Second, what mechanisms are responsible for reversing the stable epigenetic silencing of KLF4 during SMC phenotypic switching? Third, are other ESC factors involved in controlling SMC phenotypic switching? Fourth, do posttranslational modifications such as differential mRNA splicing, or chemical modifications such as sumoylation or acetylation regulate effects of KLF4? The latter is of interest since we have shown that TGFβ-induced expression of SM α-actin in cultured SMC is mediated in part through inactivation of KLF4 through protein sumoylation. In summary, further studies are needed to determine if these factors regulate activation of KLF4 in SMC in vivo and if posttranslational modifications regulate KLF4 function.

In summary, results of the present studies provide direct evidence that KLF4 mediates phenotypic switching of SMCs in vivo during vascular injury and does so through binding to a G/C Repressor element. Moreover, our results support a model wherein there is sequential binding of pELK-1 and KLF4 followed by binding of HDAC2 to epigenetically silence the gene locus. Although the present studies have focused on studies where there is sequential binding of pELK-1 and KLF4 Repressor element. Moreover, our results support a model wherein there is sequential binding of pELK-1 and KLF4 followed by binding of HDAC2 to epigenetically silence the gene locus. Although the present studies have focused on studies where there is sequential binding of pELK-1 and KLF4 Repressor element. Moreover, our results support a model wherein there is sequential binding of pELK-1 and KLF4 followed by binding of HDAC2 to epigenetically silence the gene locus. Although the present studies have focused on studies where there is sequential binding of pELK-1 and KLF4 Repressor element. Moreover, our results support a model wherein there is sequential binding of pELK-1 and KLF4 followed by binding of HDAC2 to epigenetically silence the gene locus. Although the present studies have focused on studies where there is sequential binding of pELK-1 and KLF4 Repressor element. Moreover, our results support a model wherein there is sequential binding of pELK-1 and KLF4 followed by binding of HDAC2 to epigenetically silence the gene locus.

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is Known?**

- Mutating the G/C Repressor element within an SM22a promoter-lacZ transgene virtually abolished downregulation of the transgene after carotid wire injury or within atherosclerotic lesions of ApoE/−/− Western diet–fed mice. Thus, the G/C Repressor element is required for suppression of this smooth muscle cell (SMC) marker during phenotypic switching of this cell in vivo.

- PDGF-BB and oxidized phospholipid-induced phenotypic switching of cultured SMC is KLF4 dependent. KLF4 overexpression induces profound SMC phenotypic switching of cultured SMC. However, there is no direct evidence that KLF4 regulates the activity of the G/C repressor.

- There is no evidence that the functional effects of mutating the G/C repressor in vivo are related to KLF4 or to pELK-1, although we have shown that KLF4 and pELK-1 bind SMC marker gene promoters based on chromatin immunoprecipitation (ChIP) assays after carotid ligation injury in vivo and during phenotypic switching of cultured SMC.

**What New Information Does This Article Contribute?**

- We show that KLF4 binding to the SM22α promoter within intact chromatin is markedly elevated after carotid ligation injury in vivo and is dependent on the G/C Repressor element. These findings provide the first direct evidence that the functional effects of the G/C repressor mutations in abrogating suppression of SM22α during SMC phenotypic switching in vivo are mediated by KLF4.

- Binding of pELK-1 and HDAC2 to the SM22α promoter after carotid ligation injury in vivo is also G/C Repressor dependent. Results of studies in cultured SMC provide evidence that there is sequential binding of pELK-1, KLF4, and HDAC2, with the latter contributing to histone hypo-acetylation, chromatin remodeling, and transcriptional silencing.

Studies provide novel evidence that phenotypic switching of SMC in vivo is mediated, at least in part, by binding of the stem cell pluripotency factor KLF4 to a G/C Repressor cis element contained in the promoter of many SMC marker genes. In addition, studies show that KLF4 binds to SMC promoters in conjunction with pELK-1 and HDAC2 and that the latter mediates histone hypo-acetylation and transcriptional silencing through chromatin-remodeling epigenetic mechanisms. These results are significant in that they are the first to define a specific molecular mechanism that contributes to SMC phenotypic switching in vivo, a process believed to play a critical role in postangioplasty restenosis, and the pathogenesis of atherosclerosis.
Cooperative Binding of KLF4, pELK-1, and HDAC2 to a G/C Repressor Element in the SM22α Promoter Mediates Transcriptional Silencing During SMC Phenotypic Switching In Vivo

Morgan Salmon, Delphine Gomez, Elizabeth Greene, Laura Shankman and Gary K. Owens

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Supplemental Material

Cooperative Binding of KLF4, pELK-1, and HDAC2 to a G/C Repressor Element in the SM22α Promoter Mediates Transcriptional Silencing during SMC Phenotypic Switching In Vivo

Running title: KLF4 binds SM22α promoter via a G/C Repressor site

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Expanded Materials and Methods

Assessment of Transgenic Expression in a Ligation Model of Vascular Injury
Animal protocol models were approved by the University of Virginia Animal Care and Use Committee. Transgenic founder lines were previously described by Regan et al.1. Mice were ligated at the right common carotid right below the bifurcation and three days following ligation mice were euthanized and tissue was prepared as described previously1,2.

Cell Culture, generation of LacZ stable cells line and treatment of stably transfected cells with either PDGF-BB or Phospholipids
Rat aortic smooth muscle were isolated and cultured as previously described3. For stable transfections, cells at passage 8 were plated in all 6 wells of a 6 well plate at 1X10⁵ cells per well; 24 hours later cells were transfected with either 200 ng per well of SM22α WT LacZ plasmid or SM22α Mut LacZ plasmid constructed as described previously1, along with linear hygromycin selection vector. 24 hours following transfection, cells were passaged 1:5 and treated with linear hygromycin. 2 weeks after the initiation of hygromycin treatment, individual colonies had formed. Cells were then trypsinized, passaged, and treated for an additional week with hygromycin. Following 3 weeks of treatment cells were again passaged and a portion of cells were isolated for DNA extraction and PCR to assess stable integration compared to LacZ positive mice [Online figure IIB]. Stably transfected rat aortic smooth muscle cells were plated at 1x10⁴ and were grown to 100% confluency and then switched to serum-free media. 72 hours following serum starvation, rat aortic smooth muscle cells were stimulated by either PDGF-BB (Upstate Biotechnology) or POVPC (Cayman Chemical) or representative vehicle for 12 hours as described previously2,4,5. Transient transfection analyses were performed using SM22α-LacZ (-447/89 bp) and SM22α GC Mut-LacZ generated as previously described1. A single siRNA to rat KLF4 or ELK-1 and a non-targeting control were used as previously described4. A MEK inhibit, U1026 (Calbiochem), or an ERK inhibitor, PD98059 (Cell Signaling Technology), were co-treated at 10mM along with either PDGF-BB or POVPC as described previously8.

Proximity ligation in situ assays
Human coronary SMCs were cultured in Lab-Tek permanox chamber slides (Nunc, Thermo Fisher Scientific). After culture in serum-free medium for 24 hours, cells were treated with PDGF-BB (10ng/ml), POVPC (10µg/ml) for 24 hours. Simultaneously, cells were treated with ERK inhibitors PD98059 (10µM, Cell Signaling Technology) and U0126 (10µM, Calbiochem). Then cells were washed in PBS, fixed in PFA (4%) for 20 min and incubated in absolute methanol for 10 min at -20°C. The proximity ligation assays were performed according to manufacturer’s instructions with minor modifications. Briefly, after incubation with primary antibodies, we applied combinations of corresponding PLA probes (i.e. anti-rabbit PLUS, anti-mouse MINUS PLA probes) for 1 hour at 37°C, as previously described (www.Olink.com). Subsequent ligations and detections using DuoLink II Detection Reagents Orange Kit (OLINK Bioscience) were performed (excitation/emission: 554/579). Blocking, antibody hybridizations, proximity ligations and detections were performed according to recommendations (Duolink IQ, OLINK Bioscience). Antibodies used for the detection of protein–protein interactions were anti-KLF4 (2.5µg/ml; Abcam), anti-ELK-1 (2µg/ml; Santa Cruz Biotechnology), anti-myocardin (2µg/ml; Santa Cruz Biotechnology) and anti-SRF (2µg/ml; Abcam). Preparations were mounted in DuoLink mounting medium (OLINK Bioscience).

In vitro and in vivo ChIP assays
Quantitative chromatin immunoprecipitation assays (ChIP) were performed as described previously2,7,8. Antibodies include rabbit polyclonal KLF4 generated previously in the Owens laboratory in conjunction with Chemicon Laboratories3; rabbit polyclonal Sp3 (Santa Cruz biotechnology); rabbit polyclonal pELK-1 (Cell Signaling); Acetylated H3 and H4 (Cell Signaling), HDAC2, 5, 4, 3 and 7 (Cell Signaling).
Real-time PCR primers were designed as previously described for SM22α and the SM22α intronic region\textsuperscript{8} and the SM22α GC Mut 5’ primer 5’tttcttcctcagc 3’ was used with the 3’ primer from the wild-type SM22α primer.

**Statistics**
Two- and three-way ANOVAs were performed as appropriate. The comparison-wise error rate was set to \( \alpha=0.05 \), with * indicating statistical significance. Experiments were performed three times in triplicate and if there were deviations from this standard of conduct it is mentioned within the figure legends.
Reference List


Online Figure I. Schematic Depicting the procedure used for LacZ transgenic ChIP analysis following carotid ligation injury in vivo.
Harvest carotids 3 days after injury pool
10 carotids per IP
ChIP analysis

Ligate right common carotid below bifurcation

Prime for: 1. Endogenous promoter
2. LacZ transgene(WT)

Prime for: 1. Endogenous promoter
2. LacZ transgene(G/C Mut)
Online Figure II. Validation Assays: A.) Model depicting the procedure used for performing CHIP assays in stable SMC lines derived from stable transfection of SM22 WT and G/C repressor mutant LacZ plasmids into rat aortic smooth muscle cells. B.) Genotyping results of transgenic mice and in vitro stable SMCs lines for the presence of the respective SM22 WT and G/C Repressor mutant LacZ transgenes. C.) Effects of PDGF-BB treatment on expression of SM22 WT and G/C repressor mutant LacZ transgenes following cytokine treatment. Stable cells lines(WT and G/C Repressor Mut) were plated at 1x10^4 and allowed to grow to confluency and then switched to serum free media for three days. Following serum starvation, cells were treatment with 20 ng/mL PDGF-BB for 12 hours and then subjected to Bgal assays and normalized to total cell protein.
A. Rat aortic SMCs

SM22 alpha LacZ stable cell line
SM22 alpha G/C Repressor MT LacZ stable cell line

Serum starve 3 days
Treat with 1. 20 ng/mL PDGF-BB or
2. 5 ug/mL POVPC

ChIP analysis

Prime for:
1. endogenous promoter
2. LacZ transgene(WT)

Prime for:
1. endogenous promoter
2. LacZ transgene(G/C MT)

B.

C.
Online Figure III. Sp3 induced repression of SM22 in cultured SMC is not G/C Repressor dependent A). Rat aortic smooth muscle cells were plated at $1 \times 10^4$. 24 hr later cells were transiently transfected using LT-Mirus with 300 ng of SM22 WT or SM22 G/C Rep MT and 200 ng of pcDNA Sp3. 24 hours following transfection, media was removed and replaced and 24 hours later cells were harvested and subject to Bgal and protein assays. Results are the average of three independent experiments performed in triplicate. * indicates significant down-regulation as compared to vehicle treated controls B). C57B6 mice were subjected to right common carotid ligation injury and then ten mice each were harvested at 1, 3, 7, 14 and 21 days following vascular injury and then subjected to ChIP analysis for Sp3 (2 ug/mL, Santa Cruz Biotechnology). * indicates significant binding over non-injured controls based upon a three-way anova with a p-value<0.05. C). Rat aortic smooth muscle cells were plated at $1 \times 10^4$, allowed to grow to confluency and then switched to serum free media for three days. Following serum starvation, cells were treated with 20 ng/ml PDGF-BB for 12 hours and then subject to ChIP analysis for Sp3 (2 ug/mL, Santa Cruz Biotechnology). Results are the average of three independent experiments performed in triplicate. * denotes significant binding over controls based upon three-way anova with p-value <0.015 D). Rat aortic smooth muscle cells were plated and serum starved as previously described. Following serum starvation, cells were treated with 5 ug/ml of POVPC for 12 hours and then subject to ChIP and statistical analyses as mentioned in part C.
A. 

![Bar chart showing B-gal/protein units for SM22 and SM22 GC Repressor with empty vector and 200 ng Sp3.](image)

B. 

![Bar chart showing Sp3 Binding IP/Input Fold Enrichment for PDGF-BB 20 ng/ml and vehicle.](image)

C. 

![Bar chart showing Sp3 Binding IP/Input Fold Enrichment for SM22 LacZ trans. and SM22- G/C mut LacZ trans. with vehicle and PDGF-BB 20 ng/ml.](image)

D. 

![Bar chart showing Sp3 Binding IP/Input Fold Enrichment for SM22 LacZ trans. and SM22- G/C mut LacZ trans. with vehicle and POVPC 5 ug/ml.](image)
Online Figure IV. SRF binding is not affected by the G/C Repressor mutation A) Model depicting the relative locations of the various cis elements within the SM22α, α-actin and SM-MHC promoters. B). Rat aortic smooth muscle cells were plated at 1x10^4, allowed to grow to confluency and then switched to serum free media for three days. Following serum starvation, cells were treated with 20 ng/ml PDGF-BB for 12 hours and then subject to ChIP analysis for SRF(2 ug/mL, Santa Cruz Biotechnology). Results are the average of three independent experiments performed in triplicate. * denotes significant binding over control based upon three-way anova with p-value <0.05 C). Rat aortic smooth muscle cells were plated and serum starved as previously described. Following serum starvation, cells were treated with 5 ug/ml of PDGF-BB for 12 hours and then subject to ChIP analysis for KLF4(2 ug/mL, Abcam). Results are the average of three independent experiments performed in triplicate. * denotes significant binding over control based upon three-way anova with p-value <0.05.
A. SM22α and α-actin gene structures showing putative binding sites for CArG and ETS motifs. Gene maps are depicted with -2.5 kb, -4.5 kb, and -4.2 kb orientations. The SRF binding and IP/input fold enrichment are indicated with vehicle and PDGF-BB 20 ng/ml treatments.

B. Graph showing SRF binding IP/input fold enrichment for SM22 LacZ trans. and SM22- G/C mut LacZ trans. with vehicle and PDGF-BB 20 ng/ml treatments. Significant differences are indicated with asterisks.

C. Graph showing KLF4 binding IP/input fold enrichment for SM22 LacZ trans. and SM22- TCE mut LacZ trans. with vehicle and PDGF-BB 20 ng/ml treatments. Significant differences are indicated with asterisks.
Online Figure V. Transient Transfection of KLF4, ELK-1 and HDAC2 down-regulate the SM22 wild-type but not GC Repressor mutant promoter. A) Rat aortic smooth muscle cells were plated at 1x10^4. 24 hr later cells were transiently transfected using LT-Mirus with 300 ng of SM22 wild-type or SM22 G/C Repressor mutant and 200 ng of either pcDNA-KLF4, pcDNA-ELk-1, pCMV-HDAC2 or empty vector. 24 hours following transfection, media was removed and replaced and 24 hours later cells were harvested and subjected to Bgal and protein assays. Results are the average of three independent experiments performed in triplicate. * indicates significant binding over SM22 GC MT versus WT with p-value <0.05.
Online Figure VI. KLF4, pELK-1 AND HDAC2 bind the SM22 promoter following PDGF-BB treatment in human coronary artery cells. A-D). human aortic smooth muscle cells were plated at 1x10^4, allowed to grow to confluency and then switched to serum free media for 24 hrs. Following serum starvation, cells were treated with 10 ng/ml PDGF-BB for 12 hours and then subject to ChIP analysis. Antibodies used were anti-SRF (2 ug/mL, Santa Cruz Biotechnology), anti-KLF4 (2 ug/mL, Abcam), anti-pELK-1 (2 ug/mL, Cell Signaling Technology) and anti-HDAC2 (2 ug/mL, Cell Signaling technology). Results are the average of three independent experiments. * denotes significant binding over control based upon students t-test with p-value <0.05.
A. SRF Binding

B. KLF4 Binding

C. pElk-1 Binding

D. HDAC2 Binding

Vehicle vs. PDGF-BB (10 ng/ml)
Online Figure VII. SRF-myocardin interaction using Proximity Ligation Assay. Human coronary SMCs were treated with PDGF-BB (10ng/ml), or POVPC (10µg/ml) or vehicle for 24h. In parallel, cells were treated with either vehicle or the Erk inhibitors PD98059 (10µM) or U0126 (10µM). The interaction between SRF and myocardin corresponding with red spots is inhibited by treatment with PDGF-BB or POVPC. The addition of Erk inhibitors abolished the effect of PDGF-BB or POVPC. D). Quantitation of number of spots per nucleus. * denotes significant binding over control based upon students t-test with p-value <0.05*. 
Online Figure VIII. Quantification of SRF-myocardin interaction via proximity ligation assay. Quantitation of number of spots per nucleus. * denotes specific decreases in binding over non-treated serum starved smooth muscle cells based on student’s t-test with p-value <0.05.
Number of spots/nucleus

ERK inhibitors

- Vehicle
- PDGF-BB
- POVPC

POVPC
ERK inhibitors - - - + + +
PDGF-BB Vehicle - - +
POVPC - + +
Online Figure IX. pELK-1 binding is not affected by siRNA knock-down of KLF4 but HDAC2 binding is affected by siRNA knock-down with KLF4. A) rat aortic smooth muscle Cells were plated and 24 hours following plating cells were transfected with either si-control or si-KLF4. 24 hours following siRNA transfection, cells were treated with either vehicle or 20 ng/ml PDGF-BB for 12 hours. Cells were harvested and ChIP analysis was performed using an antibody to pELK-1 (Cell Signaling Technology). Results are the average of three independent experiments. * denotes significant binding over control based upon three-way anova with p-value <0.05. B). Cells were plated, transfected, and harvested as mentioned in part A and then ChIP analysis was performed using an antibody to HDAC2(Cell Signaling Technology).
A. 

**pEK-1 Binding**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Endogenous</th>
<th>Transgene</th>
</tr>
</thead>
<tbody>
<tr>
<td>si control+vehicle</td>
<td>7 ± 1</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>si-control+PDGF-BB 20 ng/ml</td>
<td>5 ± 1</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>si-KLF4+vehicle</td>
<td>3 ± 1</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>si-KLF4+PDGF-BB 20 ng/ml</td>
<td>1 ± 1</td>
<td>0 ± 2</td>
</tr>
</tbody>
</table>

B. 

**HDAC2 Binding**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Endogenous</th>
<th>Transgene</th>
</tr>
</thead>
<tbody>
<tr>
<td>si control+vehicle</td>
<td>9 ± 1</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>si-control+PDGF-BB 20 ng/ml</td>
<td>7 ± 1</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>si-KLF4+vehicle</td>
<td>5 ± 1</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>si-KLF4+PDGF-BB 20 ng/ml</td>
<td>3 ± 1</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>
Online Figure X. pELK-1, KLF4 and HDAC2 binding is affected by siRNA knock-down of ELK-1. A) rat aortic smooth muscle Cells were plated and 24 hours following plating cells were transfected with either si-control or si-ELK-1. 24 hours following siRNA transfection, cells were treated with either vehicle or 20 ng/ml PDGF-BB for 12 hours. Cells were harvested and ChIP analysis was performed using an antibody to pELK-1 (Cell Signaling Technology). Results are the average of three independent experiments. * denotes significant binding over control based upon three-way anova with p-value <0.05. B). Cells were plated, transfected, and harvested as mentioned in part A and then ChIP analysis was performed using an antibody to KLF4 (Abcam). C). Cells were plated, transfected, and harvested as mentioned in part A and then ChIP analysis was performed using an antibody to HDAC2(Cell Signaling Technology).
A. KLF4 Binding

<table>
<thead>
<tr>
<th>IP/Input Fold Enrichment</th>
<th>si control+vehicle</th>
<th>si-control+PDGF-BB 20 ng/ml</th>
<th>si-ELK-1+vehicle</th>
<th>si-ELK-1+PDGF-BB 20 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous Transgene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM22 LacZ trans.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM22- G/C mut LacZ trans.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. KLF4 Binding

<table>
<thead>
<tr>
<th>IP/Input Fold Enrichment</th>
<th>si control+vehicle</th>
<th>si-control+PDGF-BB 20 ng/ml</th>
<th>si-ELK-1+vehicle</th>
<th>si-ELK-1+PDGF-BB 20 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous Transgene</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>SM22 LacZ trans.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM22- G/C mut LacZ trans.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
C. **HDAC2 Binding**

- **IP/Input Fold Enrichment**
  - si control+vehicle
  - si-control+PDGF-BB 20 ng/ml
  - si-ELK-1+vehicle
  - si-ELK-1+PDGF-BB 20 ng/ml

### C.1: Endogenous Transgene
- SM22- G/C mut
- LacZ trans.

### C.2: Transgene
- SM22 LacZ
- SM22- G/C mut
- LacZ trans.
Online Figure XI. pELK-1, KLF4, and HDAC2 binding is diminished by the inhibitors U1026 and PD8059. A) Cells were plated at 1x10^4 and allowed to grow to confluency. After growth to confluency, cells were washed and then transferred to serum free media for three days. Following serum starvation, cells were treated with combinations of either vehicle, 20 ng/ml PDGF-BB, 10 uM U1026 or 10 uM PD8059 for 12 hours. Cells were harvested and ChIP analysis was performed for pELK-1(Cell Signaling Technology) binding. Results are the average of three independent experiments. * indicates significant binding using three-way anova with p-value <0.05. B) Cells were treated and analyzed as described in Supplemental Fig 7A with KLF4 (Abcam) used for the immunoprecipitation. C) Cells were treated as described in Supplemental Fig 7A with HDAC2 (Cell Signaling Technology) used for the immunoprecipitation.
A. 

![Graph showing pELK-1 Binding IP/Input Fold Enrichment for Endogenous and Transgene SM22 LacZ and SM22- G/C mut LacZ transgenic samples under different treatments: vehicle, vehicle + U0126, vehicle + PD8059, PDGF-BB 20 ng/ml, PDGF-BB 20 ng/ml + U0126, PDGF-BB 20 ng/ml + PD8059.](image)

B. 

![Graph showing KLF4 Binding IP/Input Fold Enrichment for Endogenous and Transgene SM22 LacZ and SM22- G/C mut LacZ transgenic samples under different treatments: vehicle, vehicle + U0126, vehicle + PD8059, PDGF-BB 20 ng/ml, PDGF-BB 20 ng/ml + U0126, PDGF-BB 20 ng/ml + PD8059.](image)
C.

[Graph showing HDAC2 binding fold enrichment comparison between endogenous and transgene in SM22 LacZ and SM22- G/C mut LacZ transfections under different treatment conditions: vehicle, vehicle + U0126, vehicle + PD8059, PDGF-BB 20 ng/ml, PDGF-BB 20 ng/ml + U0126, and PDGF-BB 20 ng/ml + PD8059. The graph includes error bars and asterisks indicating significance.]
Online Figure XII. HDAC5 binding to the SM22 promoter is not dependent upon the G/C Repressor mutation. A) Rat aortic smooth muscle cells were stably transfected with either SM22 or SM22 G/C Repressor mutant were plated at 1X10^4 and allowed to grow to confluency and then switched to serum free media for three days. Following serum starvation, cells were treated with 20 ng/ml PDGF-BB for 12 hours and then subject to ChIP analysis for HDAC5 (Cell Signaling Technology). Results are the average of three independent experiments performed in triplicate. * denotes significant binding over controls based upon three way anova with p-value <0.001. B) Rat aortic smooth muscle cells were plated and serum starved as previously described in Supplemental Fig 8A. Following serum starvation, cells were treated with 5 ug/ml of POVPC for 12 hours and then subject to ChIP analysis. Results are the average of three independent experiments performed in triplicate. * denotes significant binding over control based upon three way anova with p-value <0.005. C) SM22 and SM22 G/C mut LacZ mice were subjected to right common carotid injury and harvested 3 days after vascular ligation injury, tissues from ten mice were pooled, and then subjected to ChIP assay for HDAC5. In each ChIP IP, qPCR analysis was conducted on both the Endogenous and LacZ transgene as indicated in the Figure. Results are the average of three independent experiments. * denotes significant binding over control based upon students three way anova with p-value <0.003.
Online Figure XIII. HDAC7 does not bind to the SM22 promoter in vitro or in vivo following carotid ligation injury. A) Rat aortic smooth muscle cells were stably transfected and treated as described in supplemental Fig. 12A. Cells were harvested and then subjected to ChIP analysis for HDAC7 (Cell Signaling Technology). Results are the average of three independent experiments performed in triplicate. B) Rat aortic smooth muscle cells were plated, serum starved and treated as described in Supplemental Fig. 12B and then subjected to ChIP analysis for HDAC7. Results are the average of three independent experiments performed in triplicate. C) SM22 and SM22 G/C mut LacZ mice were subject to right common carotid injury and harvested 3 days after vascular ligation injury, tissues from ten mice were pooled, and then subjected to ChIP assay for HDAC7. In each ChIP IP, qPCR analysis was conducted on both the Endogenous and LacZ transgene as indicated in the Figure. Results are the average of three independent experiments.
Online Figure XIV. Model Depicting proposed mechanism of KLF4 and Elk-1 binding via the G/C Repressor element following vascular injury. A. Model depicting binding of SRF and myocardin to smooth muscle marker genes during normal physiological conditions. B. Model depicting sequential binding of pELK-1, KLF4 and HDAC2 to the G/C Repressor element following treatment of PDGF-BB, POVPC or in vivo following carotid ligation. C. Model depicting mutation of G/C Repressor element prevents binding of normal factors during phenotypic switching.
A. Myocardin

SRF

CaRG box

G/C ETS Rep site

CaRG box

SMC gene active

B. Vascular Injury

PDGF-BB/POVPC

U0126, PD8059

1. Myocardin

SRF

pELK

CaRG box

G/C ETS Rep site

CaRG box

2. Myocardin

SRF

pELK

KLF4

CaRG box

G/C ETS Rep site

CaRG box

3. Myocardin

SRF

pELK

KLF4

CaRG box

G/C ETS Rep site

CaRG box

AcH3, AcH4

SMC gene repressed

C. Vascular Injury

PDGF-BB/POVPC

Myocardin

SRF

CaRG box

G/C ETS Rep site

CaRG box

SMC gene active

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