Kruppel-like Factor 4 Abrogates Myocardin-induced Activation of Smooth Muscle Gene Expression*

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Platelet-derived growth factor BB (PDGF-BB) has been shown to be an extremely potent negative regulator of smooth muscle cell (SMC) differentiation. Moreover, previous studies have demonstrated that the Kruppel-like transcription factor (KLF) 4 potently represses the expression of multiple SMC genes. However, the mechanisms whereby KLF4 suppresses SMC gene expression are not known, nor is it clear whether KLF4 contributes to PDGF-BB-induced down-regulation of SMC genes. The goals of the present studies were to determine the molecular mechanisms by which KLF4 represses expression of SMC genes and whether it contributes to PDGF-BB-induced suppression of these genes. Results demonstrated that KLF4 markedly repressed both myocardin-induced activation of SMC genes and expression of myocardin. KLF4 was rapidly up-regulated in PDGF-BB-treated, cultured SMC, and a small interfering RNA to KLF4 partially blocked PDGF-BB-induced SMC gene repression. Both PDGF-BB and KLF4 markedly reduced serum response factor binding to CArG containing regions within intact chromatin. Finally, KLF4, which is normally not expressed in differentiated SMC in vivo, was rapidly up-regulated in vivo in response to vascular injury. Taken together, results indicate that KLF4 represses SMC genes by both down-regulating myocardin expression and preventing serum response factor/myocardin from associating with SMC gene promoters, and suggest that KLF4 may be a key effector of PDGF-BB and injury-induced phenotypic switching of SMC.

Alterations in the differentiated state of the smooth muscle cell (SMC), or phenotypic switching, has been shown to play a key role in the repair of tissue damage and in the development of a variety of major human diseases, including atherosclerosis, restenosis, and asthma (1, 2). A hallmark feature of this phenotype is coordinate repression of the expression of the normal repertoire of genes that distinguish differentiated mature SMC from other cell types. However, the mechanisms that regulate this process are very poorly understood. Indeed, major challenges for the field have been to define regulatory cis-elements within the promoter enhancer regions of SMC marker genes, such as SM α-actin, SM22α, and SM myosin heavy chain, to identify factors that bind and regulate the activity of these regulatory regions and to elucidate environmental factors/cues that influence these regulatory processes in response to tissue injury and/or pathological circumstances. The model that has emerged is that regulation of SMC differentiation is extremely complex and involves constant interplay between environmental cues and the genetic program that controls the expression of virtually all SMC marker genes characterized to date is dependent on CArG box elements and SRF (reviewed by Miano (3)). Moreover, an especially exciting recent discovery was the demonstration (by our laboratory and others (4–8)) that the SMC/cardiac myocyte-restricted SRF co-activator myocardin is required for the expression of multiple SMC marker genes by inducing CArG/ SRF-dependent transcription of SMC genes in SMC and many embryonic stem cells/fibroblasts and, most importantly, that it is necessary for SMC differentiation in vivo. However, so far, it is not clear whether down-regulation of SMC marker genes associated with phenotypic switching is due to the decreased expression of a transcriptional activator, such as myocardin, and/or stimulation of the expression of cell-selective gene repressors.

The most potent and efficacious inhibitor of SMC differentiation identified to date is PDGF-BB (1). Indeed, a series of studies by our laboratory and others demonstrated that treatment of cultured SMC with PDGF-BB was associated with profound selective down-regulation of SMC gene expression through a combination of transcriptional (9) and post-transcriptional mechanisms (10–12). It is of particular significance that we also demonstrated that these effects were not a direct function of the mitogenic properties of PDGF-BB, in that chronic treatment of cultured SMC with PDGF-BB in post-confluent cultures was associated with sustained, but reversible, repression of SMC gene expression in the absence of sustained mitogenesis (11). In addition, we found that PDGF-BB could induce suppression of SMC marker genes at concentrations that were 1–2 orders of magnitude lower than that required to induce maximal mitogenesis (11). Given that PDGF-BB levels are increased dramatically following vascular injury (13, 14), it has been hypothesized, although not yet proven, that PDGF-BB is a key mediator of SMC phenotypic switching in response to vascular injury in vivo. Surprisingly,
however, despite the fact that it has been over a decade since we first demonstrated the differentiation repressing activity of PDGf-BB in cultured SMC, virtually nothing is known as to how it mediates this effect. Of interest, based on a yeast one-hybrid screen for factors that bound to a novel TGFβ control element (TCE) found just’ to a required CArG element within the SM α-actin promoter, we identified a potent repressor of SMC gene expression termed KLF4. This Kruppel-like factor potently inhibited the expression of multiple SMC promoter-reporter genes in co-transfection studies (15). However, KLF4 was not expressed in SMC tissues in vivo based on either in situ (16) or RT-PCR analyses (15) and was expressed only at low levels in vitro (15, 17); and it is unknown whether KLF4 is up-regulated in response to PDGF-BB in vitro or injury in vivo. The goals of the present studies were: 1) to determine molecular mechanisms whereby KLF4 inhibits expression of SMC marker genes, including testing the hypothesis that it interferes with CArG/SRF/myocardin-dependent gene expression and recruitment of SRF to CArG containing regions of SMC genes within intact chromatin and 2) to determine whether KLF4 expression is induced by treatment of cultured SMC with PDGF-BB and/or within phenotypically modulated SMC in vivo following vessel injury.

MATERIALS AND METHODS

Cell Cultures and Stimulation of SMC by PDGF-BB—Rat aortic SMC and NIH3T3 cells were cultured as described previously (17). COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 200 μg/ml L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). SMCs were plated at 1.5 × 10^5/cm² and starved the next day with serum-free medium, ISFM (Dulbecco’s modified Eagle’s medium/F12 supplemented with 200 μg/ml l-glutamines, 6.25 ng/ml sodium selenite, 5 μg/ml transferrin, and 200 μg/ml ascorbic acid). Roughly 16–17 h after starvation, SMCs were treated with 30 ng/ml PDGF-BB (Upstate Biotechnology) or vehicle (10 mM acetic acid and 2 mg/ml fatty acid-free bovine serum albumin) for different time points (18). Treating SMCs with cycloheximide (Sigma) was described previously (19).

Generation of Constructs and Adenovirus—SM α-actin promoter/luciferase reporter constructs with (PPTCCE-Luc) or without (PPLuc) mutation of the TCE and expression plasmids pcDNA-KLF4 were constructed as described previously (17). pcDNA-FLAG-KLF4 was generated by putting a FLAG tag sequence after KLF4 ATG via PCR-based mutagenesis, and the construct was verified by sequencing. pcGNN-SRF and pcGNN-dnSRF were kindly provided by Dr. R. Misra (Medical College of Wisconsin) (20, 21). pcDNA-FLAG-KLF5 was a generous gift from Dr. E. Olson (University of Texas Southwestern Medical Center at Dallas) (22). pcDNA-KLF5 was provided courtesy of Dr. Masahiko Kurabayahi at the Gunma University (23). Adenovirus constructs Ad-FLAG-myocardin and Ad-FLAG-KLF4 were constructed as described previously (4, 17), and adenovirus overexpressing myocardin and KLF4 were amplified and purified by the Gene Transfer Vector Core at The University of Iowa (Iowa city, Iowa). KLF4 siRNA construct (pm-KLF4) was generated by inserting a siRNA-generating sequence against KLF4 after a mouse H1 promoter in the pMighty vector.²

Transient Transfection and Infection—Cultured NIH3T3 cells were transfected with reporter PPI-Luc or PPTCCE-Luc and effectors pcDNA-KLF4, pcDNA-SRF, pcDNA-KLF5, and/or pcDNA-FLAG-myocardin in triplicate using Superfect (Qiagen) according to the manufacturer’s protocol. The transfected cells were incubated 48 h before harvesting. The cultured SMCs were fed with fresh serum-free medium (ISFM), and transfected with PPI-Luc and pm-KLF4 in triplicate using FuGENE 6 according to the manufacturer’s instructions 1 day after plating. Between 20 and 21 h after transfection, the SMCs were treated with PDGF-BB (Upstate Biotechnology) or vehicle for 24 h. The transfected cells were harvested with lysis buffer (Promega). Luciferase activity was measured with luciferase assay substrate (Promega) and was normalized to total protein (Coomassie Plus protein assay reagent, Pierce). Transfections were repeated at least three times, and the relative luciferase activities were presented as mean ± S.E.

² M. H. Hoofnagle and G. K. Owens, unpublished data.

RESULTS

KLF4 Dramatically Repressed SRF/myocardin–induced Activation of SM α-Actin Promoter, and the Repression Could Be Rescued by Overexpression of SRF—Given that KLF4 was originally identified based on its binding to TCEs, which are found adjacent to CArG elements within SMC promoters (19), we hypothesized that KLF4 may act, in part, through direct inhibition of SRF/CArG-dependent transcription. As an initial test of this hypothesis, cultured SMC was co-transfected with myocardin and with KLF4 in conjunction with the SM α-actin...
promoter-luciferase constructs. Consistent with previous reports, myocardin dramatically activated expression of the wild-type SM\(\alpha\)-actin promoter inducing a nearly 600-fold increase (Fig. 1a) as compared with control constructs. Interestingly, although absolute transcription rates were decreased by mutation of the TCE, myocardin-induced activation was unaffected, eliciting a similar enhancement of expression over control groups as seen with the wild-type promoter. The activating effect of myocardin peaked with 0.4 \(\mu\)g of plasmid for both wild-type and TCE mutation promoter. It is of major interest that the results showed that overexpression of KLF4 virtually abolished myocardin-induced activation of the SM\(\alpha\)-actin promoter (Fig. 1a). This effect was concentration-dependent in that increasing the myocardin plasmid concentration relative to the KLF4 plasmid concentration was associated with reduced KLF4-induced repression (Fig. 1b). Also of interest is that the repression of the activation by myocardin could be rescued by wild-type SRF (20) but not by SRF containing a mutated MADS domain (21), which has been shown by Wang et al. (22) to be required for its binding to myocardin (Fig. 1c).

**KLF4 Specifically Bound to SRF in Immunoprecipitation Assays**—The preceding results suggest that KLF4 and SRF may physically interact with each other. To assess this possibility, a series of immunoprecipitation assays were performed. Because no antibody is available that is completely specific for KLF4 and works in immunoprecipitation assays, we constructed a FLAG epitope-tagged KLF4 plasmid (pcDNA-FLAG-KLF4) and an adenovirus with which to overexpress a FLAG-tagged KLF4 (Ad-FLAG-KLF4). In this manner, we could perform both SRF immunoprecipitation assays, test for binding of FLAG-tagged KLF4, or perform KLF4 immunoprecipitation assays and test for the presence of SRF. Results showed that wild-type SRF (but not SRF containing a mutated MADS domain) specifically interacted with KLF4 within COS-7 cells (Fig. 2a). Subsequent experiments were then done to test for interaction between KLF4 and SRF within cell lysates derived from SMC infected by Ad-FLAG-KLF4. Results showed that the endogenous SRF bound to the FLAG epitope-tagged KLF4 in SMCs (Fig. 2b). The specificity of the association was confirmed by reciprocal precipitations with either SRF antibody or FLAG antibody. Taken together, these results provide compelling evidence that SRF bound to KLF4 and that the MADS domain of SRF was required for this binding.

**FIG. 1.** NIH3T3 cells were transiently transfected with \(\sim 2560 \text{ to } +2784\) SM\(\alpha\)-actin promoter/enhancer/luciferase construct with or without mutation of the TGF\(\beta\) control element (TCE) (PPITCE-Luc or PPI-TCE) and expression plasmids pcDNA-myocardin, pcDNA-KLF4 (a and b), pCGN-SRF, and pCGN-dnSRF (c). Cells were harvested 48 h after transfection. Cell lysates were subjected to luciferase assays, and the luciferase activity was normalized to total protein as described under “Materials and Methods.” All experiments were done in triplicate and repeated at least three times. The data are presented as mean ± S.E.
KLF4 in Myocardin-induced Smooth Muscle Gene Expression

PDGF-BB Up-regulated KLF4 Expression in Cultured SMC, and Both PDGF-BB and KLF4 Repressed Myocardin Expression—Results of the current (Fig. 1) and previous studies (15, 17) provide compelling evidence that KLF4 potently represses expression of multiple SMC differentiation marker genes. However, although KLF4 has the capacity to repress SMC genes, there was no direct evidence as to whether it actually contributes to SMC gene expression under either physiological or pathological circumstances. Interestingly, KLF4 is expressed in cultured SMCs that are known to be phenotypically switched (27), but it is not normally expressed in fully differentiated SMC in vivo (15, 16). Because both PDGF-BB and KLF4 potently repress SMC marker genes in vitro (9–12), we hypothesized that the effects of PDGF-BB might be mediated at least in part by the induction of KLF4. Consistent with this, results of real-time RT-PCR analyses showed that treatment of cultured SMC with PDGF-BB was associated with rapid induction of KLF4 mRNA expression. Indeed, expression was increased by 2-fold within 15 min of treatment, peaked at an increase of 5-fold at 45 min, and subsequently decreased to control levels by 8 h (Fig. 3a). Furthermore, treatment of cultured SMC with the protein synthesis inhibitor cycloheximide failed to block PDGF-BB-induced increases in KLF4 expression (Fig. 3b). Of major interest is that PDGF-BB induced marked repression of myocardin (Fig. 3c), with levels decreasing by 75% of levels in untreated control SMC at 4 h. These results suggest that PDGF-BB-induced expression of KLF4 may represent an early and direct effector pathway for PDGF-BB-induced repression of myocardin. In support of this, adenoviral-mediated overexpression of KLF4 dramatically repressed endogenous myocardin mRNA expression in SMCs, as measured by real-time RT-PCR (Fig. 3d).

Suppression of KLF4 Expression by Interference RNA Partially Blocked PDGF-BB-induced Down-regulation of SM α-Actin Promoter Activity—To directly test the role of KLF4 in PDGF-BB-induced repression of SMC marker genes in cultured SMCs, we constructed a unique KLF4 siRNA plasmid with which to inhibit PDGF-BB-induced expression of KLF4. The plasmid contains a mouse H1 promoter driving the expression of siRNA targeted to KLF4. Because an antibody to KLF4 is not available, the efficacy of the KLF4 siRNA plasmid was documented by Western blot analysis of the effects of the KLF4 siRNA plasmid on expression of FLAG epitope-tagged KLF4 in cultured SMC co-transfected with our FLAG epitope-tagged KLF4 expression plasmid. Results showed that expression of FLAG-KLF4 was significantly (although not completely) inhibited by pM-KLF4 as compared with multiple control siRNAs, including an empty vector (pMighty) or pM-Scramble (Fig. 4a). Significantly, PDGF-BB-induced repression of SM α-actin was partially abrogated by transfection with the KLF4 siRNA plasmid (Fig. 4b), suggesting that KLF4 was at least one effector of PDGF-BB-induced transcriptional repression in cultured SMCs. Similar results were obtained with several independent siRNA-targeting constructs, whereas no effect was seen with the scrambled siRNA or with an siRNA targeting EGFP.

PDGF-BB Treatment or Overexpression of KLF4 Virtually Abrogated SRF Binding to the CArG Region of SM α-Actin—Because the MADS domain of SRF was reported to be necessary for DNA binding (28), we tested the possibility that KLF4-induced repression might be the result of the inhibition of SRF binding to CArG elements. Significantly, although KLF4 had no effect on SRF binding in in vitro gel shift assays (data not shown), results of quantitative chromatin immunoprecipitation assays showed that KLF4 overexpression, as well as PDGF-BB treatment, of cultured SMC were associated with marked reductions in SRF binding to CArG-containing regions of the SM α-actin promoter within intact chromatin (Fig. 5, a and b). Importantly, these effects were selective in that SRF binding to the c-fos CArG was unchanged by KLF4 and increased by PDGF-BB. Consistent with these results, our previous transfection studies (18) showed that PDGF-BB treatment was associated with repression of the SM α-actin promoter but activation of the c-fos promoter, whereas KLF4 significantly repressed expression of the SM α-actin promoter (Fig. 5c).

KLF4 Expression Was Markedly Increased in Vivo following Vascular Injury—The KLF4 knock-out mice died at birth due to major defects in skin development, thus precluding direct investigation of their role in SMC phenotypic switching in vivo using conventional knock-out mice. However, as a first step to determining whether KLF4 might contribute to suppression of SMC gene expression associated with phenotypical modulation in vivo, expression of KLF4 was measured in the medium of the rat carotid artery at various time points following balloon embolism-induced vascular injury, which we (1) and others (29, 30) have previously shown results in profound down-regulation of SM α-actin and other SMC marker genes. RNA was extracted from control and injured rat carotids at various time points between 1 h and 14 days and analyzed by real-time RT-PCR. Results showed that KLF4 mRNA expression was dramatically increased within 1 h after injury, remained high for another hour, and began to drop 2 h after injury (Fig. 6). At 24 h post-injury, the expression level of KLF4 was still 2-fold over basal expression but by 48 h had returned to control levels. Because of the lack of specific KLF4 antibody, it is not known what the KLF4 protein levels are throughout vascular injury. It is interesting to note that increases in KLF4 expression preceded detectable repression of myocardin and SM α-actin (1, 31).

DISCUSSION

The present studies provide evidence supporting a model in which KLF4 selectively represses SMC genes by two complementary pathways: 1) profound inhibition of myocardin expression and 2) direct repression of myocardin-induced activation of CArG-dependent SMC genes by selectively decreasing SRF binding to DNA within intact chromatin. Moreover, the results suggest that KLF4 may act as one effector for PDGF-BB-in-
FIG. 3. The effect of PDGF-BB on expression of endogenous KLF4 (a) and myocardin (b) in SMCs was assessed by real-time RT-PCR assays. Total RNA samples were prepared from cultured SMCs treated with PDGF-BB for the indicated time or co-treated with PDGF-BB and cycloheximide for 1 h (c) as described under “Materials and Methods.” Cultured SMCs were infected with Ad-KLF4, Ad-EGFP, or Ad-CMV control viruses for 24 h before cell harvesting for real-time RT-PCR assays (d). RNA samples were reverse-transcribed and subjected to real-time RT-PCR assays. Relative mRNA levels were normalized against GAPDH or 18 S rRNA. Experiments were done with sample triplicate at least three times, and data are presented as mean ± S.E.
duced repression of SM genes and might play a role in SM phenotypical switching associated with vascular injury. These results are of considerable interest in that they are among the first to define molecular mechanisms by which PDGF-BB represses SM gene expression. Although the present studies were focused on the examination of the effects of KLF4 on the earliest known SMC differentiation marker gene SM α-actin, we previously demonstrated that overexpression of KLF4 also is highly efficacious in repressing expression of SM22α and the highly definitive marker of SMC lineage, smooth muscle myosin heavy chain (15, 17). Moreover, it is well established that virtually all SMC-selective differentiation marker genes characterized to date are CArG/SRF/myocardin-dependent (3). As such, it is likely that results of the present studies are relevant to mechanisms that induce coordinate down-regulation of multiple SMC marker genes inresponse to injury and/or PDGF-BB treatment.

It has been shown that KLF4 possesses both activation and repression domains (32). Previous studies demonstrate that KLF4 activate some genes, including keratin 4, but repressed others, including cyclin D1, CYP1A1 (33–35). Although these results are of extensive interest, the precise mechanism by which KLF4 activity is regulated is poorly understood. Moreover, a very intriguing aspect of the current studies is that KLF4 selectively repressed expression of an entire subset of SMC-selective/specific genes, whereas it has previously been shown to activate genes, including p21, p27, and p53 (36). The present studies support a mechanism whereby KLF4 selectively represses SM gene expression by down-regulating expression of the SMC-selective SRF coactivator myocardin (Fig. 3) and simultaneously by directly inhibiting myocardin-induced gene activation (Fig. 1). Moreover, results provide evidence that the mechanism for the latter effect likely involves KLF4 selectively attenuating SRF binding to the CArG region of SM α-actin within intact chromatin (Fig. 5). Because overexpressed myocardin selectively enhances SRF binding to de-generate CArG containing promoters of SM genes (31), it is certainly possible that KLF4 decreases SRF binding to SM α-actin CArG by down-regulating myocardin. However, the fact that we found evidence that KLF4 can bind directly to SRF in co-immunoprecipitation assays (Fig. 2) and that overexpression of wild type but not a SRF lacking the MADS domain, (required for myocardin binding to SRF), suggests that effects are not solely the result of reduced myocardin expression but rather through a combination of this effect and KLF4 decreasing SRF binding by sequestering its DNA binding domain. Of note is that both KLF4 and myocardin selectively control SRF binding to SM genes as compared with c-fos, suggesting that the differential response may be attributed to intrinsic differences between these promoters (1, 3). For example, it is demonstrated that most SMC genes possess multiple copies of CArG boxes, whereas c-fos has only one CArG site (1). Studies by Wang et al. suggest multiple CArG sites are important by showing that disruption of myocardin dimerization causes decreased SMC gene expression (6), possibly explaining why myocardin could not activate c-fos (22). Furthermore, SMC gene CArG has been shown to have weaker binding affinity to SRF than c-fos CArG (37). An angiotensin II-induced homeodomain protein Prx1 has been shown to selectively enhance SRF binding to SM CArG sites (38, 39). Taken together, the emerging model suggested by the present studies is that PDGF-BB represses SMC genes at least in part by up-regulating KLF4 in a cell growth-independent fashion and that KLF4 then acts to repress myocardin expression and to inhibit myocardin-mediated activation of CArG-dependent SMC genes by selectively attenuating SRF binding to DNA within intact chromatin (Fig. 5). Finally, it is possible that KLF4 may also displace myocardin binding to SRF and, in so doing, attenuate SMC gene expression.

There is extensive evidence based on studies with pharmacological inhibitors (1) and PDGF-BB-neutralizing antibodies (40) implicating a key role for PDGF-BB in the development of
myointimal thickening in response to vascular injury in vivo. However, the precise role of PDGF-BB in this process is not clear, but based on extensive in vitro studies it has been postulated to contribute through a combination of effects, including stimulation of SMC growth, migration, and overall phenotypic switching (12, 41). Although results of studies in cultured SMC have identified many candidate genes that may be involved in mediating the effects of PDGF-BB on cell proliferation and migration (42–44), the present studies were the first to provide direct evidence identifying an effector of PDGF-BB-induced repression of SMC differentiation marker genes. Of major interest is that siRNA-induced knockdown of KLF4 ex-

FIG. 6. Real-time RT-PCR assessment of gene expression in the injured rat carotid. Balloon-injured carotid samples (n = 6) were obtained from Zivic Miller Laboratories, Inc. Total RNA was prepared from independent, injured carotids and control vessels at different time points: 1, 2, 6, and 24 h or 1, 3, 7, and 10 days and subjected to real-time RT-PCR analysis for KLF4. mRNA expression was corrected for the 18 S rRNA level in each vessel, and then the injured carotids were normalized to their uninjured contralateral control vessels. Error bars denote standard error of the mean.
pression in cultured SMC resulted in a 2–3-fold increase in SM α-actin promoter expression and partially inhibited PDGF-BB-induced repression of this gene (Fig. 4). Indeed, it is possible that our siRNA results may have underestimated the relative role of KLF4 in this response, because the siRNA was not totally efficacious in blocking the PDGF-BB-induced increase in KLF4. Moreover, it should be noted that, unlike differentiated SMC in vitro, cultured SMC expressed KLF4 even under basal conditions (i.e. without the addition of exogenous PDGF-BB). As such, it is likely that siRNA-induced KLF4 knockdown was incomplete. In addition, it is possible that PDGF-BB may also induce suppression of SMC genes through KLF4-independent mechanisms that remain to be identified. Interestingly, we have recently presented evidence showing that cts-1 or sp1 can inhibit expression of at least some SMC marker genes, although we presented no direct evidence that mediates responses to PDGF-BB (18, 45). Wang et al. (47) also have recently presented evidence that PDGF-BB-induced repression of SM22 was mediated through mechanisms by which ELK-1, a co-factor of SRF involved in the activation of immediate early response genes, competed for binding of myocardin to this promoter (47). However, we have previously shown that ELK-1 does not appear to be involved in the regulation of the expression of SM α-actin (48). Examination of a list of SMC promoters showed that many of them lack the ETS site in the proximity of the CARG site (3, 50). In addition, a major difference between our results and those of Wang et al. (47) is that they did not detect repression of myocardin by PDGF-BB in the A7R5 embryonic SMC culture system employed in their studies. Of interest, it is unknown what SMC subtype A7R5 cells represent, and these cells express T-type voltage-gated calcium channels characteristic of cardiac muscle, not L-type channels normally expressed in native, contractile, differentiated vascular SMCs (49). Even more importantly, we have recently shown that expression of myocardin mRNA is dramatically reduced in injured rat carotid (31), suggesting that our rat aortic SMC culture system employed in their studies. Of interest, it is unknown what SMC subtype A7R5 cells represent, and these cells express T-type voltage-gated calcium channels characteristic of cardiac muscle, not L-type channels normally expressed in native, contractile, differentiated vascular SMCs (49). Even more importantly, we have recently shown that expression of myocardin mRNA is dramatically reduced in injured rat carotid (31), suggesting that our rat aortic SMC culture system may better recapitulate what occurs in vivo.

Consistent with a potential role of KLF4 in suppressing SMC differentiation, the results of previous in situ studies show that KLF4 is normally expressed at high levels in epidermal layer of skin and epithelial cells but is absent from SMC (16). Similarly, we found no evidence of the expression of KLF4 within SMC in vivo based on RT-PCR analysis of vascular and gastrointestinal SMC (15). However, of major interest are results of the present studies that showed that KLF4 is rapidly induced in vascular SMC in vivo in response to vascular injury (Fig. 6), as well as in cultured SMC in response to treatment with the potent mitogen and SMC differentiation repression factor PDGF-BB (Fig. 3). Indeed, in both cases, KLF4 levels increased dramatically with kinetics equivalent to that observed for immediate early response genes, such as c-fos and c-jun (50). Unlike c-fos, no CARG element has been identified within a 1-kb KLF4 promoter region, which confers PDGF-BB-induced activation in vitro (data not shown). Importantly, there is extensive evidence showing that repression of SMC differentiation marker genes is not directly coupled to activation of cell growth (1, 11). For example, although the PDGF-BB-induced growth of cultured SMC is associated with profound coordinate down-regulation of SMC marker genes, this is not the case with growth induced by serum, thrombin, or bFGF (51–53). Taken together, these results suggest that KLF4 may play a key role in concert with early response genes in inducing phenotypic switching of SMC from a quiescent, differentiated phenotype to a proliferative cell capable of contributing to the repair of vascular injury. However, there is no direct evidence that KLF4 plays this role in vivo, and because KLF4 knock-out mice experience early postnatal death because of poorly developed skin (54), a direct test of this hypothesis will require development of SMC-targeted conditional KLF4 knock-out mice.
KLF4 in Myocardin-induced Smooth Muscle Gene Expression

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