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Serum Response Factor Regulates Expression of PTEN through a Micro-RNA Network in Vascular Smooth Muscle Cells

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Abstract

Objective—Serum response factor (SRF) is a critical transcription factor in smooth muscle cells (SMC) controlling differentiation and proliferation. Our previous work demonstrated that depleting SRF in cultured SMC decreased expression of SMC markers, but increased proliferation and inflammatory mediators. A similar phenotype has been observed in SMC silenced for PTEN, suggesting that SRF and PTEN may lie on a common pathway. Our goal was to determine the effect of SRF depletion on PTEN levels, and define mechanisms mediating this effect.

Methods and Results—In SRF-silenced SMC, PTEN protein, but not mRNA levels were decreased, suggesting post-transcriptional regulation. Re-introduction of PTEN into SRF-depleted SMC reversed increases in proliferation and cytokine/chemokine production, but had no effect on SMC marker expression. SRF-depleted cells showed decreased levels of miR-143, and increased miR-21, which was sufficient to suppress PTEN. Increased miR-21 expression was dependent on induction of FRA-1, which is a direct target of miR-143. Introducing miR-143 into SRF-depleted SMC reduced FRA-1 expression and miR-21 levels and restored PTEN expression.

Conclusions—SRF regulates PTEN expression in SMC through a miR network involving miR-143, targeting FRA-1, which regulates miR-21. Cross talk between SRF and PTEN likely represents a critical axis in phenotypic remodeling of SMC.

Keywords

Smooth muscle cells; serum response factor; PTEN; miR-143; miR-21

Introduction

Cardiovascular-related diseases are the leading cause of death in industrialized nations. Pathological and physiological insults to vessels result in vascular remodeling and intimal hyperplasia, which contribute to the pathogenesis of diseases such as atherosclerosis, restenosis, and pulmonary hypertension. Resident medial smooth muscle cells (SMC) are the major cells involved in vessel remodeling. Under normal physiological conditions SMC display a quiescent, contractile, and differentiated phenotype. However, in response to

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pathological and physiological insults, these cells convert to an activated phenotype characterized by decreased SM-gene expression, increased proliferation and migration, and enhanced production of cytokines/chemokines and extracellular matrix (1). While much effort has been invested into understanding the molecular changes underlying SMC activation, defining repressive pathways actively inhibiting this process is an understudied area of research.

SMC differentiation is defined by a collection of markers most of which are cytoskeletal proteins whose expression is selectively controlled at the transcriptional level (2). However, recent studies have identified a role for non-coding regulatory microRNAs (miRs) in the regulation of SMC differentiation. miRs are 22–23 nucleotides long that target the 3'UTR region of selective RNA transcripts, resulting in inhibition of translation or promotion of mRNA degradation. There is strong evidence that miRs play key roles in SMC development (3). Furthermore, studies have suggested that miR dysregulation is a critical event in the progression of vascular diseases. Collectively, it appears that multiple mechanisms control SMC phenotype, and dysregulation of these processes may play a critical role in the development of activated SMC that are involved in neointimal formation.

Serum response factor (SRF), a member of the MADS family of transcription factors, binds to CArG box elements in the promoter region of targeted genes (4). Studies using conditional knock out models have shown that inactivation of SRF in SMC and cardiac myocytes results in embryonic lethality, demonstrating that SRF is essential for vascular and cardiac development (5). In SMC, SRF directly regulates two classes of genes, SM-markers including smooth muscle α-actin (α-SMA), calponin and smooth muscle myosin heavy chain, as well as immediate early genes such as c-fos. Transcriptional control of these classes of genes involves interactions between SRF and specific co-activators, including members of the myocardin family (6). Recent data indicate that SRF also transcriptionally regulates specific miRs (e.g. miR-143, miR-145) as a mechanism to control SM-gene expression and SMC differentiation (7–9). Using an in vitro approach, we showed that depleting SRF in SMC using specific shRNA constructs resulted in decreased SM-gene expression, but unexpectedly also increased proliferation, migration, and ECM production, which are all hallmarks of activated SMC (10). The effect on proliferation in particular was unanticipated since depleting SRF would be predicted to result in decreased expression of immediate early genes, such as c-fos, leading to decreased proliferation.

Phosphatase and Tensin Homolog (PTEN) is a protein and lipid phosphatase that functions to suppress multiple pathways involved in cellular proliferation, survival, and inflammation (11). The role of PTEN has been well characterized in cancer; however, its role in vascular disease is less well defined. Work by several groups has established that PTEN inactivation results in increased SMC proliferation and decreased cell death (12–14). Furthermore, vascular injury results in SMC PTEN inactivation and is associated with increased neointima formation (15). Recent work by our lab demonstrates that PTEN is a critical regulator of SMC function both in vivo and in vitro. SMC-specific knockout of PTEN in vivo results in a larger neointima in response to vessel wall injury due to increased proliferation and recruitment of inflammatory cells (16). In vitro shRNA-dependent depletion of PTEN promotes an inflammatory SMC phenotype characterized by increased proliferation, decreased SM-gene expression, and increased inflammatory cytokine and chemokine production (17). In addition, work by Ji et al. suggested that miRs, such as miR-21, are differentially expressed by SMC in the setting of vascular injury and that PTEN is a direct target of miR-21 (18). Furthermore alteration of miR-21 in vivo is sufficient to regulate neointima formation.

Since depletion of either SRF or PTEN in SMC in vitro results in similar phenotypes, we hypothesized that an SRF-PTEN signaling pathway existed to regulate SMC phenotype. In this study we report that PTEN protein levels, but interestingly not mRNA levels, are decreased in SRF-silenced cells suggesting post-transcriptional regulation of PTEN. Restoring PTEN levels using viral overexpression was sufficient to reverse the increased proliferation and elevated cytokine production observed in SRF-silenced SMC, but did not result in restoration of SM-specific gene expression. Our studies show that, as expected, miR-143 and miR-145 levels are decreased in SRF-depleted SMC, but miR-21 levels are increased. Furthermore, SRF-dependent regulation of miR-21 occurs transcriptionally through a miR-143- and FRA-1-dependent signaling pathway. Loss of miR-143 in SRF-depleted SMC resulted in FRA-1-dependent induction of miR-21 leading to PTEN loss. Forced expression of miR-143 or depletion of FRA-1 in SRF-depleted SMC reversed the induction of miR-21 and restored PTEN levels. Collectively, we propose that control of SMC de-differentiation by SRF is complex involving direct transcriptional regulation of cytoskeletal proteins as well as indirect regulation of proteins involved in proliferation and inflammation (e.g. PTEN) through miR regulation.

Materials and Methods

An expanded Methods section is available in the Online Data Supplement at http://atvb.ahajournals.org. Cell culture, proliferation, immunoblotting, SRF overexpression and quantitative PCR were performed as previously described (10, 16, 17, 19). Plasmids, transient promoter assays, adenovirus infections, miR quantitation, primer sequences, and statistical analyses are described in detail in the Online Data Supplement.

Results

Decreased PTEN levels in SRF-depleted SMC

Previous work from our laboratory showed that depletion of SRF in aortic SMC decreased SMC-gene expression, and increased proliferation, migration and ECM production, which are hallmarks of a de-differentiated vascular SMC (20). Increased proliferative capacity observed in these cells was particularly surprising, given SRF's known role in regulating immediate early gene (IEG) expression. We postulated that loss of SRF might be regulating an alternative proliferative pathway even in the absence of IEG activation. Recent work from our laboratory showed that loss of PTEN in SMC phenocopied many of the functional changes observed in SRF-depleted SMC, including increased proliferation and cytokine production, and decreased SM gene expression (16, 17). Thus, we hypothesized that SRF regulation of PTEN serves as a mechanism mediating these changes. Stable SMC clones expressing control (U6) or SRF (shSRF2 and shSRF6) shRNA (10) were analyzed for PTEN protein levels under basal conditions. Compared to controls, shSRF2 and shSRF6 expressed lower amounts of SRF and α-SMA (10) and (Figure 1A). Importantly, PTEN was decreased in both SRF-depleted SMC clones compared to control SMC (Figure 1A). To validate that these results are not due to abnormal selection during isolation of shSRF clones, low passage pools of SRF-depleted SMC were generated using a lentiviral shRNA approach (shSRF4). Compared to controls (pGIPz), shSRF4 SMC pools also exhibited decreased PTEN levels similar to SRF-depleted SMC clones (Figure 1B). These cells also exhibited decreased levels of two known downstream targets of SRF, α-SMA and EGR1 (not shown) consistent with what was observed in the shRNA clones.

Increased expression of PTEN is sufficient to reverse proliferative and inflammatory effects of SRF depletion

To determine if PTEN downregulation mediates the functional changes in SRF-depleted SMC, U6 and shSRF2 SMC were transduced with adenoviruses expressing empty vector (EV) or wild type PTEN (PT). PT adenovirus restored PTEN protein levels in SRF-depleted SMC to those observed in control SMC (Figure 1C). By BrdU incorporation, the enhanced proliferation observed in SRF-depleted SMC was decreased by PTEN restoration to levels observed in U6 control cells (Figure 1D).

Transcription profiling revealed that depletion of SRF resulted in induction of several pro-inflammatory chemokines, including MCP-1 and KC (our unpublished data). In agreement with these data, mRNA levels assessed by qRT-PCR were increased for MCP-1 and KC in SRF-depleted SMC compared to controls (Figure 1E,F). PTEN restoration was sufficient to reverse the increase in MCP-1 expression (Figure 1E) and blunt the increase in KC expression (Figure 1F) observed in SRF-depleted SMC. In contrast, decreased α-SMA mRNA and protein expression observed in the setting of SRF depletion was not reversed by PTEN overexpression (Figure 1G,H). These data suggest that SRF-dependent regulation of PTEN mediates the proliferative and inflammatory SMC phenotype observed in response to SRF depletion, despite reductions in IEGs (10).

SRF regulates PTEN through miR-21

To determine if the effects of SRF depletion on PTEN are transcriptionally mediated, we measured PTEN mRNA levels. Unexpectedly, PTEN mRNA in both shSRF clones and pools was elevated compared to their respective controls (Figure 2A,B), suggesting post-transcriptional regulation of PTEN protein levels. One important mechanism of post-transcriptional regulation is through the activity of miRs. To assess the potential role of miRs, SMC were transfected with a construct encoding the 3'UTR of PTEN linked to a luciferase reporter (PTEN 3'UTR-luc). Luciferase activity in both shSRF clones was decreased compared to U6 control cells (Figure 2C); conversely, transient overexpression of SRF in WT SMC resulted in an increase in luciferase activity (Figure 2D). In contrast, inhibition of proteosomal degradation with MG132 inhibitor (Calbiochem) had no effect on PTEN protein levels, but increased protein expression of p21Cip1, which is known to be sensitive to proteosomal degradation (not shown).

These data suggest that silencing of SRF inhibits PTEN expression through a miR-dependent mechanism. While several miRs have been implicated in regulating PTEN, recent studies have demonstrated that miR-21 is a major regulator (21). Ji et al. reported that miR-21 was increased in vascular injury and one of its primary downstream targets in SMC was PTEN (18). Consistent with these findings, overexpression of a miR-21 mimetic decreased expression of PTEN protein (Supplementary Figure 1A). To confirm a role for miR-21 in PTEN regulation, point mutations were introduced into the PTEN 3'-UTR corresponding to the miR-21 MRE (22). In SRF-depleted SMC, a miR-21 inhibitor increased 3-UTR luciferase activity, but failed to alter activity of the miR-21 MRE mutated PTEN 3'-UTR (Supplementary Figure 1B). Consistent with these findings, miR-21 levels were elevated in SRF-depleted SMC clones (Figure 2E) and pools (Figure 2F) compared to their respective controls. In addition, levels of programmed cell death gene 4 (PDCD4), another classical target of miR-21(21), were decreased in SRF-depleted SMC compared to U6 SMC (Figure 2G,H).

SRF negatively regulates miR-21 promoter activity through FRA-1

Since SRF has been shown to regulate several miRs at the promoter level (8, 9), we examined SRF transcriptional regulation of miR-21. Recent work by Fujita et al. (23)

identified the putative promoter region of miR-21 (PPR-miR-21). We cloned this region into a PGL4.12 luciferase reporter (PPR-miR-21-luc) and measured luciferase activity in control and SRF-depleted SMC. Luciferase activity normalized to β-galactosidase was increased in SRF-depleted SMC compared to controls (Figure 3A), consistent with the increased miR-21 levels observed in these cells. Conversely, overexpression of SRF resulted in decreased PPR-miR-21-luc activity compared to control SMC (Figure 3B).

Previous studies identified multiple AP-1 binding sites within the miR-21 promoter region as critical for regulating activity (23). To determine if these AP-1 binding sites are important for SRF regulation of PPR-mIR-21, truncation mutants of the promoter region were generated (PPR-miR-21-F2,F3), and transfected into control or SRF-depleted SMC (Figure 3C). Increases in PPR-miR-21 luciferase activity in SRF-depleted SMC were retained using the PPR-miR-21-F2 mutant, which lacks only one AP-1 site, but were blunted in cells transfected with the PPR-miR-21-luc-F3 mutant, which lacks two of 4 AP-1 sites. A putative SRF binding site has also been identified in the promoter, which is deleted in the F3 mutant. To preclude a direct role for SRF on repression of miR-21 activity, SMC were co-transfected with SRF and either wild type PPR-miR-21-Luc or PPR-mir-21-Luc containing a point mutation in the SRF binding site (S1). Overexpression of SRF decreased PPR-miR-21-Luc activity in SMC co-transfected with both the wild type and SRF mutant luciferase constructs (Figure 3D). Collectively our data suggest that AP-1 binding sites, but not SRF binding sites, within the PPR-miR-21 are necessary for SRF-mediated negative regulation of miR-21 promoter activity.

SRF regulates FRA-1 through a miR-143 dependent regulation

AP-1 transcription factors are composed of c-fos and c-jun family members. Previous work by our lab showed that c-fos protein levels are greatly reduced in SRF depleted SMC (10), which is expected, as c-fos is a direct target of SRF (24). However, c-jun levels are increased in these SRF depleted cells (10). It was recently reported that other c-fos family members, such as FRA-1 and FRA2, interact with c-jun to regulate miR-21 promoter activity (23). Therefore we determined if increased expression of other c-fos family members in SRF-depleted cells could result in formation of heterodimers with c-jun that would increase miR-21 promoter activity in the setting of decreased c-fos. Microarray analysis indicated increased levels of FRA-1 in SRF depleted cells (data not shown). We confirmed this by western blot analysis, showing increases in FRA-1 protein levels in both SRF-depleted clones (Figure 3E) and pools (Figure 3F) compared to their respective controls. A reduction in FRA-1 using siRNA in SRF-depleted SMCs (Figure 3G) blocked miR-21 promoter activity (Figure 3H) and increased PTEN 3'UTR-luc activity (Figure 3I), confirming that FRA-1 is a critical regulator of the miR-21 promoter in the absence of SRF and c-fos.

To define the mechanism by which SRF is regulating FRA-1, we used the microcosm miRNA targeting database (http://www.ebi.ac.uk/). We identified miR-143 as a microRNA that potentially targets FRA-1, but not c-fos. Several groups have shown SRF transcriptionally regulates the miR-143 and miR-145 gene cluster through direct interactions with CArG boxes in their promoter region (8, 9). As expected, levels of miR-143 (Figure 4A,B) and miR-145 (not shown) were decreased in SRF-depleted SMC cells. To determine if miR-143 can directly target FRA-1 we obtained a FRA-1 3'UTR-luc construct and measured expression in response to SRF depletion. SRF-depleted cells exhibited increased luciferase activity while expression of a miR-143 mimetic reversed the effect (Figure 4C), suggesting that FRA-1 is a direct target of miR-143. To confirm regulation by miR-143, we either deleted or introduced point mutations into the putative MRE in the FRA-1 3'-UTR. While a miR-143 mimic decreased activity of the WT 3-`UTR, it failed to alter activity in SMCs co-transfected with either the deletion or the point mutant constructs (Figure 4D).

Concurrently, the increase in FRA-1 protein levels (Figure 4E) and induction of miR-21 (Figure 4F) observed in SRF-depleted cells was also reversed by the miR-143 mimetic. Importantly, these effects were associated with a subsequent restoration of PTEN levels in SRF-depleted SMC to those observed in control SMC (Figure 4G) as well as a functional reversal in the induction of MCP-1 mRNA observed in SRF depleted SMCs (Figure 4H). We conclude that SRF-mediated regulation of miR-143 directly targets FRA-1, leading to repression of miR-21 promoter activity and subsequent release of miR-21 translational repression of PTEN. Inactivation of SRF reverses this process leading to PTEN degradation and the promotion of a proliferative, inflammatory SMC phenotype.

PDGF can regulate the SRF-dependent miRNA network that controls PTEN expression

PDGF is a critical regulator of SMC phenotypic changes in the setting of vascular injury (6). Previous work from our lab showed that PDGF decreased SM-gene expression and this was mediated in part through a re-localization of SRF, which inactivates its transcriptional activity (20). We therefore examined whether PDGF, as a physiological stimulus, also regulates the SRF-dependent miRNA network that controls PTEN expression. Similar to SRF-depleted SMC, PDGF stimulation of early passage SMC resulted in decreased miR-143 transcripts levels (Figure 5A), increased FRA-1 protein levels (Figure 5B), and a complementary increase in miR-21 transcripts levels (Figure 5C). Consistent with the changes in this miR network, PDGF stimulation led to a decrease in PTEN protein levels (Figure 5D). Thus, we conclude that a physiological stimulus such as PDGF that regulates SRF activity may be sufficient to regulate PTEN through an SRF-dependent miR-143-FRA-1-miR-21 network.

Discussion

SRF has been implicated as a critical regulator of two classes of genes: immediate early genes and muscle-specific genes (6, 25). In SMC these genes are associated with enhanced proliferation and promotion of differentiation, respectively. It would therefore be anticipated that inhibition of SRF transcriptional activity would result in de-differentiated SMC due to loss of SM-gene expression, combined with decreased proliferation as a result of loss of IEG gene expression. To model inactivation of SRF, using shRNA approaches, we generated SMC which are silenced for expression of the protein (10). While these cells showed the expected decreases in SM-gene and IEG expression, we made the unanticipated finding that SRF-depleted SMC had higher rates of proliferation and migration. The goal of the current study was to identify the mechanism underlying this enhanced proliferation in the setting of SRF depletion. Here, we describe an alternative proliferative pathway that is activated in the absence of SRF and the downstream classical c-fos dependent proliferative pathway (Figure 6).

Our work implicates PTEN as a downstream effector of SRF, which controls reprogramming of SMC into a proliferative, inflammatory phenotype. Depletion of SRF decreased PTEN expression, while re-expression of PTEN in SRF-depleted SMC reversed the increased proliferation and cytokine/chemokine production observed in the SRF silenced cells. This is the first report to our knowledge linking SRF to PTEN in SMC. In contrast to the effects on proliferation and cytokine/chemokine production, regulation of SM-gene expression was independent of PTEN status in SRF-depleted cells. This is anticipated, since SRF is the primary transcription factor that regulates the expression of many SM-genes through direct binding to multiple CArG boxes in the promoter region of these genes. However, recent work from our lab has shown that PTEN regulates SM-gene expression (17). A potential explanation for this apparent inconsistency is that PTEN may regulate SRF activity through a potential feedback loop to regulate SM-gene expression. In the setting of

constitutive SRF depletion by shRNA, any regulation on SRF by PTEN may be masked; thus reintroduction of PTEN would be predicted to have no effect on SM-gene expression.

Our data also demonstrate that regulation of PTEN expression by SRF is mediated through a microRNA network (Figure 6). Previous work has identified specific miRs whose promoter regions contain CArG boxes, as direct targets of SRF (8, 9). Consistent with these studies we have observed decreased levels of miR-143 in the setting of SRF depletion, and demonstrated that this is critical for regulation of PTEN. Furthermore, SRF depletion results in increased levels of miR-21, which mediates the decreased expression of PTEN. Induction of miR-21 in turn is transcriptionally regulated, and involves increased expression of the transcription factor, FRA-1, through loss of miR-143 repression.

AP-1 transcription factors are heterodimeric proteins composed of fos and jun family members which bind to consensus sites (TRE) in the promoters of target genes. Under conditions of SRF depletion we previously showed a marked decrease in c-fos, and a significant increase in c-jun levels (10). Here we report that loss of miR-143 as a consequence of SRF depletion results in increased expression of the fos family member FRA-1. Importantly, our data demonstrate that SRF regulates transcription factors of the same family in opposite directions and by alternative mechanisms. It is well established that c-fos, which has an SRE in its promoter region, is directly and positively regulated transcriptionally by SRF (24). Interestingly, FRA-1 also has a CArG box within its promoter region, which is required for basal level transcription. However, if the predominant mechanism mediating FRA-1 is through direct SRF-induced transcriptional regulation, then levels should decrease in the setting of SRF depletion similar to c-fos. In fact, FRA-1 levels change in the opposite direction to c-fos, and our data demonstrating alterations in the 3'-UTR strongly support a model in which FRA-1 is a direct target of miR-143. Thus, decreased SRF will result in decreased miR-143 levels, and subsequent release of the negative regulation of FRA-1.

We propose that FRA-1/c-jun heterodimers regulate transcription of miR-21, which in turn decreases PTEN expression. miR-21 has been shown to be increased in the setting of vascular injury, and blocking miR-21 has been shown to reduce neointima progression (18). Several studies including our data here have demonstrated that PTEN is a direct target of miR-21 in SMC. Moreover, an inverse correlation between miR-143 and miR-21 in the setting of vascular injury has been previously reported (8, 18). Our data are consistent with these findings, since both neointimal SMC in vivo and SRF-depleted cells in vitro are characterized by a de-differentiated, proliferative, and inflammatory phenotype. It will therefore be of interest to determine if induction of FRA-1 occurs after injury and mediates miR-21 induction in vivo. In addition to our report, several reports in the cancer field have shown an inverse correlation between miR-143 and miR-21 levels (26, 27). Thus the pathway described here in SMC may have more global consequences by contributing to the modulation of differentiation in other cell types.

While our data strongly support an alternative proliferative and inflammatory pathway induced in the setting of SRF inactivation, it is important to note that others have reported reduced proliferation and migration in siRNA-mediated human coronary artery smooth muscle cells (28). At this time we cannot explain the discrepancies in our findings compared to this recent report, but potential reasons could include differences in cell type (rat aortic versus human coronary artery) or differences in SRF silencing approaches (shRNA versus siRNA). For our study, we analyzed two separate isolates of stable SRF shRNA-expressing SMC clones as well as several individual transient pools of SRF shRNA-expressing SMC and obtained similar results. The novel pathway focusing on an SRF-PTEN axis identified

here was activated in both sets of SRF-silenced SMC and is consistent with our previously published data regarding PTEN regulation of SMC function (17).

Finally, we have demonstrated the significance of this pathway in wild type SMC in response to the physiologic mediator, PDGF. Several groups, including us, have shown that PDGF blunts SRF-dependent SM gene expression likely through inactivation of SRF transcriptional activity. In this context, we showed that PDGF similarly activates the miR-143-FRA-1-miR-21 axis resulting in decreased PTEN levels. Silencing of shSRF is intended to model inactivation of this transcription factor. In spite of extensive publications, the regulation of SRF is still not completely understood. It is likely that activity of SRF is regulated through transcriptional control, as well as post-translational modifications including phosphorylation (29, 30) and SUMOylation (31), and changes in subcellular localization (20). These events are likely to alter the interactions with co-activators such as myocardin family members. It will therefore be critical to define how these regulatory events impact regulation of downstream miRs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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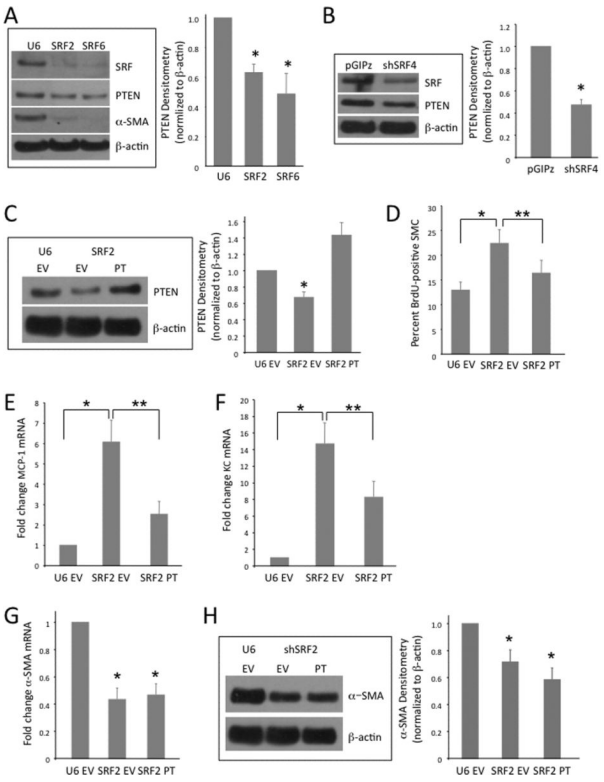

Figure 1. Decreased PTEN Expression Promotes Proliferation and Chemokine Induction in SRF-Silenced SMC

A. Cell lysates prepared from SMC clones silenced for SRF expression (shSRF2, shSRF6) or control shRNA (U6) were immunoblotted for the indicated proteins. **B.** Pools of SMCs infected with a lentivirus shRNA construct against SRF (shSRF4) or control shRNA (pGIPz) were isolated and lysates immunoblotted for the indicated proteins. Shown in both A & B are representative Westerns and fold changes in densitometry measurements±SE from three independent experiments. β-actin was used as a loading control. *P<0.05 vs control shRNA. **C–H.** Control (U6) and SRF-deficient SMCs (shSRF2) were transiently transduced with empty vector adenovirus (EV) or adenovirus encoding wild-type PTEN (PT)(MOI = 100). **C.** Whole cell lysates were analyzed for total PTEN levels. Shown is a representative Western and fold changes in densitometry measurements±SE from three independent experiments. **D.** SMCs were analyzed for BrdU incorporation. Shown is the percent BrdU-positive SMC from 3 independent experiments. **E–G.** qRT-PCR for MCP-1 (**E**), KC (**F**), and SM-α-actin (**G**) mRNAs. β-actin was used for normalization. Shown are fold changes in mRNA copy number±SE from three independent experiments. **H.** Whole cell lysates were analyzed for SM-α-actin protein levels. Shown is a representative Western and fold changes in densitometry measurements±SE from three independent experiments.*P<0.05 vs U6 EV; **P<0.05 vs SRF2 EV.

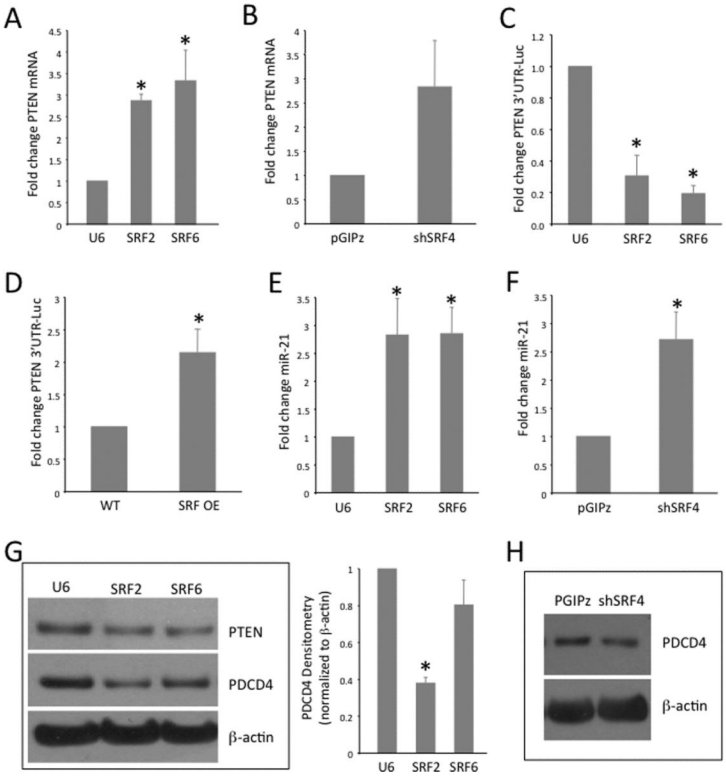

Figure 2. SRF Depletion Increases Expression of miR-21

A&B. qRT-PCR for PTEN mRNA from control (U6 or pGIPz) or SRF-deficient (SRF2, SRF6, shSRF4) SMC clones (**A**) or pools (**B**). Shown are fold changes in mRNA copy number±SE from three independent experiments normalized to β-actin. *P<0.05 vs control shRNA. **C.** Luciferase activity in control and SRF-deficient SMCs transfected with *PTEN* 3′ UTR downstream of a CMV-driven luciferase reporter. Luciferase activity normalized to β-galactosidase was determined; shown are fold changes±SE. *P<0.05 vs U6. **D.** Luciferase activity in wild type SMCs co-transfected with *PTEN* 3′ UTR-luc and either empty vector (WT) or an expression plasmid encoding wild type SRF (SRF OE). Luciferase activity normalized to β-galactosidase was determined; shown are fold changes±SE. *P<0.05 vs WT. **E&F.** qPCR for miR-21 from control or SRF-deficient SMC clones (**E**) or pools (**F**). sno234 was used for normalization of cDNA. Shown are fold changes in miR-21 copy number±SE from three independent experiments. *P<0.05 vs control shRNA. **G&H.** Cell lysates prepared from control and SRF-deficient SMC clones (**G**) or SMC pools (**H**) were immunoblotted for the indicated proteins using β-actin as a loading control. Representative Westerns are shown. Graph in (**G**) shows fold changes in densitometry measurements±SE for PDCD4 from three independent experiments. *P<0.05 vs U6.

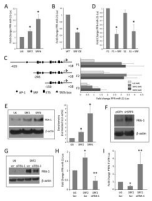

Figure 3. SRF Depletion Regulates miR-21 Promoter Activity

A. Control (U6) and SRF-deficient (SRF2, SRF6) SMCs were transiently transfected with a miR-21 promoter-Luciferase reporter construct (PPR-miR-21-Luc). Luciferase activity normalized to β-galactosidase was determined; shown are fold changes±SE. *P<0.05 vs U6. **B.** Wild type SMCs were co-transfected with PPR-miR-21-Luc and either empty vector (WT) or an expression plasmid encoding wild type SRF (SRF OE). Luciferase activity normalized to β-galactosidase was determined; shown are fold changes±SE. *P<0.05 vs WT SMC. **C.** Control and SRF-deficient SMCs were transiently transfected with full-length PPR-miR-21-Luc (F1) or the indicated truncation mutants (F2, F3). Luciferase activity normalized to β-galactosidase was determined; shown are fold changes±SE from U6 SMC. **D.** Wild type SMCs were cotransfected with full-length PPR-miR-21-Luc (F1) or PPR-miR-21-Luc containing a mutated SRF site (S1) and either empty vector (WT) or an expression plasmid encoding wild type SRF (SRF). Luciferase activity normalized to β-galactosidase was determined; shown are fold changes±SE. *P<0.05 vs WT SMC. **E&F.** Whole cell lysates from control (U6, pGIPz) and SRF-deficient (SRF2, SRF6, shSRF4) SMCs were analyzed for FRA-1 levels. β-Actin was used as a loading control. Shown are representative Westerns; graph in (**E**) shows percent changes in densitometry measurements ±SE from three independent experiments. *P<0.05 vs U6. **G.** Whole cell lysates from control (U6) and SRF-deficient (SRF2) SMCs transfected with scrambled siRNA (scr) or FRA-1-specific siRNA (siFRA) oligonucleotides were analyzed for FRA-1 protein. β-Actin was used as a loading control. Shown is a representative Western. **H.** Control (U6) and SRF-deficient (SRF2) SMCs were transiently co-transfected with PPR-miR-21-Luc and scrambled (scr) or FRA-specific (siFRA) siRNA. Luciferase activity normalized to β-galactosidase was determined; shown are fold changes±SE. *P<0.05 vs U6 scr; **P<0.05 vs SRF2 scr. **I.** Control and SRF-deficient SMCs were transiently co-transfected with *PTEN* 3′-UTR-luc and scrambled (scr) or FRA-1-specific (siFRA) siRNA. Luciferase activity normalized to β-galactosidase was determined; shown are fold changes±SE. *P<0.05 vs U6 scr; **P<0.05 vs SRF2 scr.

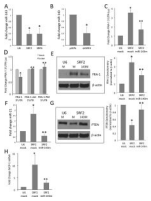

Figure 4. SRF Depletion Increases Expression of FRA-1 through miR-143-dependent regulation

A&B. miR qPCR for miR-143 from control or SRF-deficient SMC clones (**A**) or pools (**B**). sno234 was used for normalization of cDNA. Shown are fold changes in miR-143 copy number±SE from control SMC from three independent experiments. *P<0.05 vs control shRNA. **C.** Control and SRF-deficient SMCs were transiently co-transfected with *FRA-1* 3′-UTR-luc and mock or miR-143-specific mimetic constructs. Luciferase activity normalized to β-galactosidase was determined; shown are fold changes±SE. *P<0.05 vs U6 mock; **P<0.05 vs SRF2 mock. **D.** Whole cell lysates from control and SRF-deficient SMCs transfected with mock or miR-143 mimetic constructs were analyzed for FRA-1 protein. β-Actin was used as a loading control. Shown is a representative Western; graph shows percent changes in densitometry measurements±SE from three independent experiments. *P<0.05 vs U6 mock; **P<0.05 vs SRF2 mock. **E.** miR qPCR for miR-21 from control or SRF-deficient SMC transfected with mock or miR-143-specific mimetic constructs. sno234 was used for normalization of cDNA. Shown are fold changes in miR-21 copy number±SE from three independent experiments. *P<0.05 vs U6 mock; **P<0.05 vs SRF2 mock. **F.** Whole cell lysates from control and SRF-deficient SMCs transfected with mock or miR-143 mimetic constructs were analyzed for PTEN protein. β-Actin was used as a loading control. Shown is a representative Western; graph shows percent changes in densitometry measurements±SE from three independent experiments. *P<0.05 vs U6 mock; **P<0.05 vs SRF2 mock. **G.** qRT–PCR for MCP-1 mRNA from cells treated as in (**F**). β-actin was used for normalization. Shown are fold changes in mRNA copy number±SE from three independent experiments. *P<0.05 vs U6 mock; **P<0.05 vs SRF2 mock.

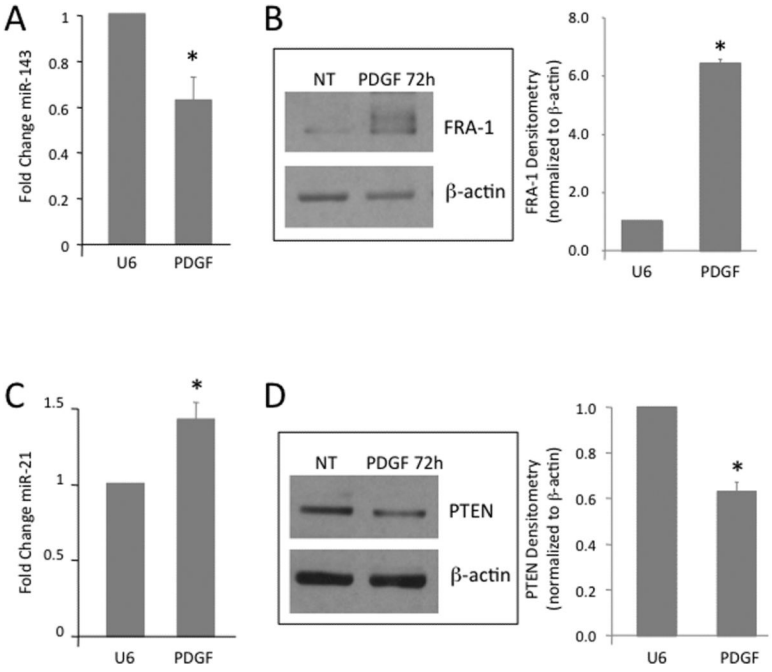

Figure 5. PDGF regulates PTEN expression in SRF depleted cells through the miRNA network

A. miR qPCR for miR-143 from control and PDGF treated (20ng/ml for 72hrs) SMCs. sno429 was used for normalization of cDNA. Shown are fold changes in miR-143 copy number±SE from control SMC from three independent experiments. *P<0.05 vs control SMC. **B.** Whole cell lysates from control and PDGF treated (20ng/ml for 72hrs) SMCs were analyzed for FRA-1 levels. β-Actin was used as a loading control. Shown is a representative Western; graph shows fold changes in densitometry measurements±SE from three independent experiments. *P<0.05 vs control SMC. **C.** miR qPCR for miR-21 from control and PDGF treated (20ng/ml for 72hrs) SMCs. sno429 was used for normalization of cDNA. Shown are fold changes in miR-143 copy number±SE from control SMC from three independent experiments. *P<0.05 vs control SMC. **D.** Whole cell lysates from control and PDGF treated (20ng/ml for 72hrs) SMCs were analyzed for PTEN levels. β-Actin was used as a loading control. Shown is a representative Western; graph shows fold changes in densitometry measurements±SE from three independent experiments. *P<0.05 vs control SMC.

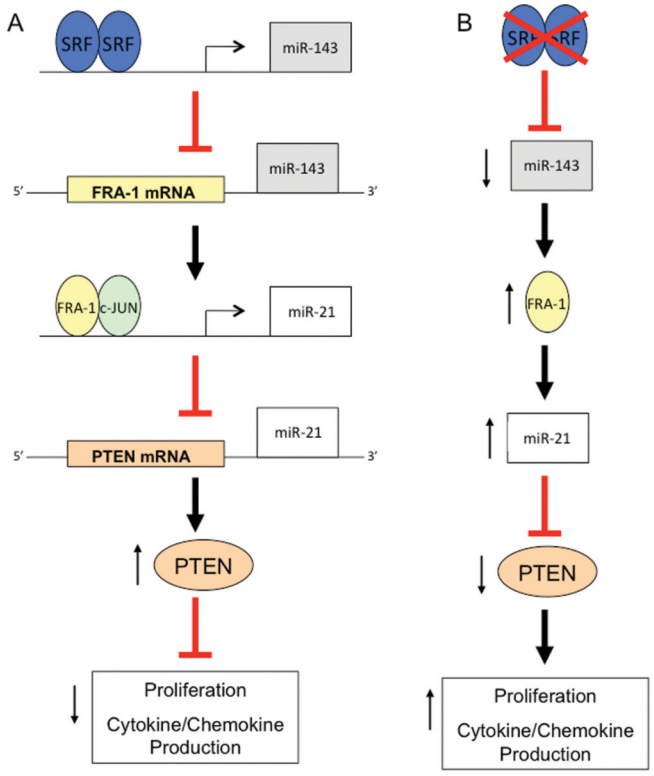

Figure 6. Model of SRF Regulation of PTEN

A. miR-143 is positively regulated by SRF and functions to repress transcription factors (eg. FRA-1) that promote the transcription of microRNAs known to repress PTEN, in particular miR-21. These effects contribute to a quiescent, differentiated SMC phenotype. **B.** Inactivation of SRF results in reduced miR-143 levels, which releases the negative regulation of FRA-1 and leads to the subsequent induction of miR-21. Induction of miR-21 represses PTEN thus promoting a proliferative, inflammatory SMC phenotype