INTERFERON-γ-MEDIATED INHIBITION OF SERUM RESPONSE FACTOR-DEPENDENT
SMOOTH MUSCLE SPECIFIC GENE EXPRESSION*

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Running head: IFNγ inhibits smooth muscle α-actin via SRF

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Interferon-γ (IFNγ) exerts multiple biological effects on effector cells by regulating many downstream genes, including smooth muscle specific genes. However, the molecular mechanisms underlying IFNγ-induced inhibition of smooth muscle specific gene expression remain unclear. In this study, we have shown that serum response factor (SRF), a common transcriptional factor important in cell proliferation, migration and differentiation is targeted by IFNγ in a STAT1-dependent manner. We show that the molecular mechanism by which IFNγ regulates SRF is via activation of the 2-5A-RNaseL system, which triggers SRF mRNA decay and reduced SRF expression. As a result, decreased SRF expression reduces expression of SRF target genes such as smooth muscle α-actin and smooth muscle myosin heavy chain. Additionally, IFNγ reduced p300 and acetylated histone-3 binding in both smooth muscle α-actin and SRF promoters, epigenetically decreasing smooth muscle α-actin and SRF transcriptional activation. Our data reveal that SRF is a novel IFNγ-regulated gene and further elucidate the molecular pathway between IFNγ, IFNγ-regulated genes, and SRF and its target genes.

IFNγ, a pleiotropic cytokine that is primarily produced by T cells and NK cells, plays a complex and central role in antiviral, antiproliferative, antifibrogenesis, antitumor, and immunomodulatory activities (reviewed in (1,2)). The complexity of such a variety of IFNγ effects appears to be achieved by its signaling to over 200 genes, leading to a highly networked pattern of cell-specific gene regulation (3). In the canonical pathway, through binding to cognate IFN type II receptors, IFNγ initiates a cascade that includes JAK family kinases and the STAT1 family of transcriptional factors to induce STAT1 dependent gene expression, which largely mediate IFNγ’s actions (reviewed in (4)). Among IFNγ-regulated genes, smooth muscle α-actin, a cytoskeleton protein that is critical in smooth muscle cell differentiation (reviewed in (5)), myofibroblast activation (reviewed in (6)), and epithelial-mesenchymal transition (EMT) (7), is negatively regulated by IFNγ (8,9). Although the observation that IFNγ regulates smooth muscle α-actin is well established, the molecular mechanisms underlying IFNγ-induced inhibition of smooth muscle α-actin expression remain unclear.

Regulation of smooth muscle α-actin expression is complex, having been studied extensively in cardiovascular and vascular diseases, particularly in smooth muscle cells (reviewed in (5)). It has been well demonstrated that all most muscle specific genes including smooth muscle α-actin, are SRF target genes. SRF binds to the CArG boxes (CC(A/T)6GG) of the smooth muscle α-actin gene promoter and activates smooth muscle α-actin transcription (reviewed in (10)). Extracellular factors can exert their effects on smooth muscle gene expression through regulating SRF or and SRF cofactors (11). TGFB, a well-characterized cytokine important in smooth muscle cell differentiation and EMT, upregulates smooth muscle α-actin expression in both smooth muscle cell and non-smooth muscle cells, which is mediated through a TGFB responsive element in smooth muscle α-actin gene promoter (12) and elevation of SRF expression (13,14). While IFNγ clearly exhibits an inhibitory effect on smooth muscle α-actin expression in smooth muscle (8) and non-smooth muscle cell types (9), its effects on the smooth muscle α-actin promoter are unknown.
Others and our previous studies have shown that IFNγ inhibits smooth muscle α-actin expression in activated hepatic stellate cells (9), a cell type that undergoes phenotypic transition to a myofibroblast-like cell in a process that is tightly linked to a smooth muscle-specific gene expression program (15,16). Further, IFNγ exerts a protective role in liver fibrogenesis, likely via effects on stellate cells (17). In the present study, we have demonstrated the presence of a novel signaling network linking IFNγ, SRF, and smooth muscle specific genes. We show that the mechanism by which IFNγ regulates SRF is via the 2-5A-RNase L system, which triggers SRF mRNA decay. The degradation of SRF mRNA, in turn, creates to a negative auto-regulatory cycle, which further reduces SRF expression (18) and consequently reduces smooth muscle α-actin and smooth muscle myosin heavy chain (SMMHC). The findings provide novel insight into the molecular mechanism of IFNγ-mediated regulation of smooth muscle specific genes.

EXPERIMENTAL PROCEDURES

Stellate cell isolation, culture and animals: All stellate cells used in the experiments presented were primary cells isolated from normal Sprague Dawley rats or wild type Balb/C or STAT1 deficient mice as described in supplemental data. STAT1 deficient mice (129S6/SvEV, Taconic Farms, Germantown, NY) were backcrossed with Balb/c inbred mice (Taconic) for more than four generations. Genotyping of the mice was performed by PCR as described (17). Cell purity was assessed by examination of morphologic features, vitamin A droplets, and immunohistochemical detection of desmin (characteristic of stellate cells) and was greater than 95% pure in all cases as previously described (15). Stellate cells were cultured for 4 - 5 days (activated stellate cells) before experiments unless indicated otherwise. Animals were cared for and experiments were performed in accordance with National Institutes of Health (NIH) guidelines.

Plasmids: A -125+5bp fragment of the rat smooth muscle (SM) α-actin promoter (SMpro-125) and a -787+11bp fragment of the mouse SRF promoter (SRFpro-787) were cloned from genomic DNA using PCR. PCR primers were designed based on available DNA sequences (GenBank accession number S76011 and AC165445). The sequence of the isolated mouse SRF gene promoter (798bp) has been submitted to GenBank (accession number: EF654102). PCR products were ligated into a pGL3 Basic luciferase reporter vector (Promega) and a series of deletions and mutations were generated. A rat SRF mRNA 3'UTR fragment (1,500bp from the stop codon, GenBank accession number: XM-576514) was cloned from rat stellate cell cDNA and inserted into XbaI site of pGL3 promoter luciferase reporter vector. Mouse RNaseL (GenBank accession number: NM-0118820) cDNAs were cloned into a pcDNA3.1 vector (with a Flag tag). The SMMHC promoter construct was obtained from Dr. White (University of Vermont).

For RPA cRNA probe constructs, 237bp fragments of rat and mouse SM α-actin were subcloned from SM α-actin full-length cDNA (GenBank accession numbers: X06801 and X13297, respectively) into pGEM7Zf(+) (Promega); the rat SMMHC cRNA probe was constructed by cloning a 552bp cDNA fragment (GenBank accession number: XM-001053402) into PCRII vector (Invitrogen); a 156bp fragment of rat SRF cDNA (GenBank accession number: XM-576514) was cloned and ligated into pGEM7Zf(+) (Promega); a 292bp fragment of mouse RNase L cDNA was subcloned from full-length RNase L cDNA and inserted into the XbaI and HindIII sites of pGEM7Zf(+) vector (Promega).

The sequences for all the constructs were confirmed by sequencing (UT Southwestern DNA sequencing core facility). All PCR primers are available in the supplemental data.

Transfection and luciferase assay: Stellate cells were transduced with 2 µg of plasmid DNA using lipofectamine 2000 (Invitrogen). Cells were incubated with 199OR medium containing 0.1% serum with or without IFNγ (1,000 IU/mL, PBL Biomedical) for 2 days and whole cell lysates were assayed using dual-luciferase reporter assay system (Promega). All transfection experiments were performed in triplicate and repeated at least 3 times. Relative light units (RLU) from pGL3 basic luciferase reporter vector (pGL3B) (Promega) were arbitrarily set to 1 and experimental data were presented as fold-increase relative to pGL3B activity. The RLU from pGL3
promoter-SRF mRNA 3’UTR construct was arbitrarily set to 100 and experimental data were presented as percentage (%). decrease. Overexpression of exogenous RNase L- HEK293 cells were grown in DMEM-10% FBS and the expression plasmid harboring Flag-tagged RNase L was transduced using lipofectamine 2000 (Invitrogen) with low-serum (0.5%) medium overnight. The cells were harvested after 24 hours for immunoblot. Wild type RNase L (+/+ ) and RNase L null (-/-) mouse embryo fibroblasts (MEF) were obtained from Dr. Silverman (Cleveland, Ohio).

Immunoblot- Cultured stellate cells were washed three times with cold PBS and proteins were extracted with RIPA buffer containing protease inhibitors (Roche). Cell lysates were incubated on ice for 30 minutes and centrifuged at 4°C for 20 minutes. The supernatant was harvested and protein concentration measured (Bio-Rad). Proteins were subjected to immunoblotting as previously described (19). Anti-SM α-actin, anti-β-actin, anti-flag M2 and anti-α-tubulin were from Sigma; anti-SRF and anti-pSTAT1 from Santa Cruz and anti-STAT1 from BD Transduction Laboratories. Specific signals were visualized using enhanced chemiluminescence (Pierce) and captured with a digital imaging system (Chemigenius2 photodocumentation system, Syngene). Intensities of specific bands were quantified using standard documentation system, Syngene). Intensities of specific bands were quantified using standard software (Gene Tool, Syngene). The level of significance was calculated using a student’s t-test was used for comparison in experiments examining the effect of IFNγ (i.e. +IFNγ vs. -IFNγ). The level of significance was considered to be p < 0.05.

RESULTS

IFNγ-mediated inhibition of smooth muscle α-actin expression is dependent on CArG boxes. We initially examined the effect of IFNγ on smooth muscle α-actin mRNA expression in activated stellate cells. As shown in Figure 1A, the expression of smooth muscle α-actin mRNA was significantly suppressed between 12-24 hours after IFNγ exposure. Concomitant with the effect of IFNγ on smooth muscle α-actin mRNA, smooth muscle α-actin protein levels were also reduced (Figure 1B). To explore the molecular mechanism by which IFNγ exerts its inhibitory effect on smooth muscle α-actin expression, we examined smooth muscle α-actin transcriptional regulation. Constructs harboring a series of rat smooth muscle
α-actin promoter fragments were generated and used to examine promoter activity. In Figure 1C, it is shown that the Smpro-125 construct, which contains CArG-B and A boxes generated a prominent response to IFNγ - a 5-fold reduction of promoter activity compared to control. However, deletion of the CArG-B box (Smpro-109) resulted in marked reduction of promoter activity, which was almost complete after IFNγ exposure. Since CArG boxes are known to be DNA binding sites for SRF, these data suggest that the IFNγ-mediated inhibitory effect on smooth muscle α-actin gene promoter activity is linked to SRF.

To further examine the relationship between CArG boxes and the IFNγ-mediated inhibitory effect on smooth muscle α-actin promoter activity, we generated mutations in CArG-B and CArG-A boxes. Promoter activity for the wild type construct was reduced by 5-fold following IFNγ treatment (Figure 1D). In contrast, mutation of CArG-A or CArG-B boxes or both led to reduced promoter activity and loss of IFNγ responsiveness. These data indicate that smooth muscle α-actin promoter CArG-A and CArG-B boxes are critical in mediating the inhibitory effect of IFNγ on smooth muscle α-actin expression and that SRF is an important intermediate partner.

IfNγ reduces SRF expression and binding to the smooth muscle α-actin promoter. Given previous data indicating that SRF is an essential transcription factor for multiple muscle-specific genes, we postulated that it might be a target of IFNγ. To explore this possibility, we examined known IFNγ signaling pathways in stellate cells. As predicted, IFNγ exposure led to increases in STAT1 phosphorylation (pSTAT1) in both whole cell lysates and nuclear extracts (Supplemental Figure 1A). Next, we found that IFNγ led to a significant reduction in SRF expression in stellate cells (Figure 2A). Serum stimulation increased SRF expression and serum stimulation failed to elevate SRF in the presence of IFNγ (Figure 2B). These data indicate that SRF is a target of IFNγ in stellate cells. Next, we examined whether the IFNγ-mediated inhibition of SRF expression affected smooth muscle α-actin promoter activity. Serum stimulation increased promoter activity compared to 0.1% serum-containing medium (Figure 2C). However, IFNγ abrogated this effect. These data suggest that the IFNγ-induced inhibitory effect on smooth muscle α-actin promoter activity occurs, at least in part, via reduction of SRF in stellate cells.

Since smooth muscle α-actin promoter activity is SRF dependent (Figure 1D), we examined whether IFNγ might inhibit SRF binding activity. In these experiments, a probe containing the CArG-B box of smooth muscle α-actin promoter was utilized. Nuclear extracts from stellate cells exposed to IFNγ had a weaker shifted band compared to control (Figure 2D). Addition of anti-SRF antibody and cold probe demonstrated that the SRF binding was specific. These data suggest that IFNγ reduces nuclear SRF binding in stellate cells. Further, we examined whether IFNγ decreased SRF binding to smooth muscle α-actin promoter CArG boxes in vivo. Following exposure of stellate cells to IFNγ for 16 hours, SRF binding to smooth muscle α-actin promoter CArG boxes was dramatically reduced compared to control (Figure 2E). Interestingly, decreased binding of SRF to smooth muscle α-actin promoter CArG boxes was accompanied by reduced binding of H3Ac, which itself is associated with gene transcriptional activation (21). We also found that IFNγ reduced both SRF and H3Ac binding to smooth muscle α-actin promoter CArG boxes even under serum stimulation (Supplemental Figure 1B). Since p300, a transcriptional coactivator, plays a critical role in gene regulation through histone acetylation and interaction with a variety of transcriptional factors such as STAT1 (22), we examined whether p300 and STAT1 might form DNA-protein complexes in the smooth muscle α-actin promoter in vivo. As shown in Figure 2F (top panel), p300 was found in control, but after exposure to IFNγ, p300 in the smooth muscle α-actin promoter essentially disappeared, consistent with the reduced amount of H3Ac in the smooth muscle α-actin promoter after IFNγ (Figure 2E). In contrast, we could not identify pSTAT1 in the smooth muscle α-actin promoter (Figure 2F, middle panel; as a control, pSTAT1 was readily identified in the IRF1 (interferon regulatory factor 1) gene promoter, bottom panel). Taken together, these data demonstrate that both SRF expression and binding activity to smooth muscle α-actin
promoter CArG boxes were significantly reduced by IFNγ. IFNγ also induced negative epigenetic regulation of smooth muscle α-actin through reducing smooth muscle α-actin promoter histone 3 acetylation.

**IFNγ inhibits SRF expression through its own transcriptional regulation.** To further explore the molecular mechanism by which IFNγ down-regulates SRF, we cloned a 798-bp fragment in the proximal SRF gene promoter region from stellate cells and tested the effects of IFNγ on SRF transcription. SRF promoter activity was dramatically suppressed by IFNγ (Figure 3A). Next, we asked whether IFNγ-mediated suppression of SRF promoter activity could be associated with the serum response elements (SREs) of the SRF gene promoter. As shown in Supplemental Figure 2, all the SREs appeared to be required for maintenance of full SRF promoter activity. Mutation of SRE1 and 2 in the SRF promoter substantially abrogated the inhibitory effect of IFNγ on SRF promoter activity. These data led us to further hypothesize that IFNγ could reduce SRF mRNA levels. As expected, SRF mRNA expression was reduced at all time points after IFNγ exposure compared to control (Figure 3B). However, the most prominent SRF mRNA reduction occurred at the 12-hour time point, which correlated with the most significant reduction in smooth muscle α-actin mRNA (Figure 1A). Given the finding that IFNγ down-regulated SRF mRNA expression, we further hypothesized that SRF levels in stellate cell nuclei would likewise be reduced; in turn leading to reduced SRF binding to its own gene promoter.

To test this postulate, we examined SRF levels in stellate cell nuclear extracts as well as SRF binding activity to SRF promoter CArG boxes in **vitro** and **in vivo**. IFNγ decreased SRF in stellate cell nuclei (Figure 3C). SRF binding to its CArG boxes was reduced following IFNγ exposure (Figure 3D,E). Additionally, H3Ac and p300 were reduced in the SRF promoter after IFNγ exposure. These findings were similar to those found with the smooth muscle α-actin promoter (Figure 2).

We next examined whether two potential gamma activated sites (GAS) (23) in the SRF promoter might modulate SRF expression. As shown in Supplemental Figure 3A, pSTAT1 did not appear to form a DNA-protein complex with either of the two putative SRF GAS elements. Although cross-linked DNA-pSTAT1 complexes could be readily identified in the lysates from cells exposed to IFNγ (Supplemental Figure 3B), specific DNA fragments harboring GAS elements from the SRF gene promoter were undetectable whether exposed to IFNγ or not (Supplemental Figure 3C, upper panel). In contrast, a DNA fragment containing IRF1 GAS element was identified (Supplemental Figure 3C, bottom panel). These data suggest that IFNγ-mediated down-regulation of SRF occurs via pathways other than by direct targeting of SRF gene transcription.

**IFNγ-mediated down-regulation of smooth muscle α-actin and SRF is STAT1 dependent.** Since IFNγ exerts its effects through both STAT1 dependent or independent pathways (24), we studied stellate cells from STAT1+/+ (wild type) and STAT1-/- (knockout) mice. Stellate cells from STAT1+/+ mice (Figure 4A, left) and STAT1-/- mice (Figure 4A, right) exhibited remarkably different promoter activity responses to IFNγ. Smooth muscle α-actin promoter activity was reduced by IFNγ in stellate cells from STAT1+/+ mice but not in those from STAT1-/- mice. Similar results were obtained in experiments with SRF promoter constructs (Figure 4B), in which the inhibitory effect of IFNγ on SRF promoter activity was abrogated in STAT1 deficient stellate cells (Figure 4B, right). The results suggest that IFNγ-induced inhibitory effects on smooth muscle α-actin and SRF promoter activity are both STAT1 dependent.

Given the evidence that STAT1 is required for IFNγ's negative effects on both smooth muscle α-actin and SRF promoter activity, we reasoned that IFNγ-mediated down-regulation of smooth muscle α-actin and SRF gene expression would be abrogated in STAT1 deficient stellate cells. As predicted, SRF and smooth muscle α-actin mRNA expression were reduced in stellate cells from STAT1+/+ mice after IFNγ exposure (Figure 4C). However, smooth muscle α-actin and SRF mRNA levels were not reduced by IFNγ in STAT1 deficient stellate cells. Immunoblot analyses paralleled mRNA findings (Figure 4D). We further examined whether STAT1 is required for IFNγ-mediated inhibition of SRF binding activity and histone 3 acetylation in both smooth muscle
**α-actin and SRF promoters. IFNγ failed to reduce SRF and H3Ac binding activity in both promoters in STAT1 deficient stellate cells (Figure 4E).** Further, we examined pSTAT1 levels in stellate cells from STAT1+/+ and STAT1-/- mice (Figure 4F). pSTAT1 was readily detected in STAT1+/+ stellate cells following IFNγ, but was undetectable in stellate cells from STAT1-/- mice.

**IFNγ induces SRF mRNA degradation in a STAT1-dependent manner.** It is well known that mRNA stability plays an important role in determining levels of gene expression (25). We further examined whether IFNγ might contribute to SRF mRNA degradation in stellate cells. We first cloned the rat SRF mRNA 3' UTR region and examined mRNA decay with a luciferase reporter; IFNγ significantly reduced luciferase activity (Figure 5A) - suggesting that IFNγ likely targets SRF mRNA stability. Next, we examined whether IFNγ might contribute to SRF mRNA degradation in stellate cells. IFNγ led to a persistent decrease in SRF mRNA levels, while SRF mRNA levels in control samples remained stable (Figure 5B). We further examined whether IFNγ enhances SRF mRNA decay under actinomycin D treatment. Cells were exposed to IFNγ for 2 hours to activate the IFNγ signal pathway (Supplemental Figure 1A) and then incubated with actinomycin D. SRF mRNA was decreased at the 3-hour time point compared to controls (Figure 5C). These results suggest that IFNγ is able to activate SRF mRNA degradation machinery in stellate cells.

Given that IFNγ down regulated SRF mRNA expression via a STAT1 dependent pathway, we further hypothesized that deletion of STAT1 would abrogate IFNγ-mediated SRF mRNA decay in stellate cells. As predicated, IFNγ led to degradation of SRF mRNA in stellate cells from STAT1+/+ mice, similar to rat stellate cells (Figure 5D). Notably, IFNγ failed to induce SRF mRNA degradation in STAT1 deficient stellate cells (Figure 5D). These data suggest that IFNγ targets the SRF gene via posttranscriptional STAT1-mediated SRF mRNA degradation.

Furthermore, we explored whether IFNγ might induce smooth muscle α-actin mRNA degradation, which would contribute to the reduction in smooth muscle α-actin expression. Compared to SRF, IFNγ had little effect on smooth muscle α-actin mRNA stability in stellate cells (Figure 5E), suggesting that IFNγ reduces smooth muscle α-actin expression not directly, but by regulation of SRF.

**The 2-5A synthetase-RNase L System mediates IFNγ-induced SRF mRNA decay.** To explore the pathways leading to SRF mRNA decay, we first examined 2-5A system, an RNA degradation pathway that can be induced by IFNs (26). IFNγ increased 2-5A synthetase mRNA levels approximately 5 fold compared to control (Figure 6A). Since 2-5A synthetase generates 2-5A (2'-5'-phosphodiester-linked oligoadenylates) and activates RNase L, we hypothesized that RNase L might also respond to IFNγ stimulation. As shown in Figure 6B, RNase L mRNA levels were robustly stimulated by IFNγ compared to control. Next, we examined whether RNase L targets SRF mRNA by using RNase L-/- MEFs (27). IFNγ reduced SRF mRNA levels in RNase L+/+ MEFs, but failed to reduce SRF mRNA levels in RNase L deficient MEFs (Figure 6C). The results suggested that RNase L plays a critical role in IFNγ-mediated SRF mRNA degradation. We next examined SRF protein levels in RNase L-/- and +/- MEFs following IFNγ exposure. IFNγ led to reduction in SRF expression in wild type but not knockout RNase L MEFs (Figure 6D, top panel), consistent with SRF mRNA levels depicted in Figure 6C.

Since RNase L has been shown to regulate skeletal muscle cell differentiation (28), we postulated that it may also regulate smooth muscle programs. Interestingly, smooth muscle α-actin expression was detected in RNase L-/- MEFs but not in RNase L+/+ MEFs at both protein (Figure 6D, middle panel) and mRNA levels (Figure 6E). Notably, IFNγ-induced inhibition of smooth muscle α-actin expression was abrogated in RNase L null MEFs (Figure 6D,E), which paralleled SRF levels in these cells (Figure 6C,D top panel). Furthermore, overexpression of RNase L led to decreased SRF levels in HKE293 cells compared to the control (Figure 6F). Taken together, these data indicated that SRF is a new molecular target in the IFNγ-induced 2-5A-RNase L pathway, which plays a critical role in IFNγ-induced SRF mRNA degradation.

**IFNγ-induced inhibition of SMMHC mRNA expression links to decreased SRF binding in
**CArG box of SMMHC promoter.** In addition to smooth muscle α-actin, SMMHC is another smooth muscle cell and myofibroblast marker, whose expression, at least in smooth muscle cells, is also tightly controlled by SRF (5). Therefore, we examined whether IFNγ-induced targeting of SRF might affect SMMHC mRNA expression. SMMHC mRNA expression was reduced at all time points following IFNγ exposure (Figure 7A). Promoter analysis indicated that IFNγ-induced reduction of luciferase activity was closely linked to CArG boxes in the SMMHC promoter (Figure 7B). Next, we examined SRF binding activity to the SMMHC promoter CArG box. As predicted, IFNγ caused a reduction in SRF binding to the SMMHC promoter CArG box compared to control (Figure 7C). These data provide further evidence for the prominent effect of IFNγ on SRF (and thus a repertoire of smooth muscle specific gene expression).

**DISCUSSION**

In this study, we have identified a novel target of IFNγ, namely SRF. We have also discovered a novel IFNγ-induced SRF mRNA decay promoting pathway that involves the 2-5A-RNase L system. Together, this pathway makes up a novel signaling network from IFNγ to SRF and smooth muscle protein expression (Figure 7D). In the context of IFNγ biology, our work is consistent with previous studies emphasizing a number of IFNγ-regulated genes. Further, elucidation of such targets is critical to understand IFNγ-mediated biological effects (1,3,29).

Abundant evidence links IFNγ to fibrogenesis, and this cytokine has been proposed as a putative therapy for fibrosis (17,30,31). In the wounding milieu, IFNγ exhibits prominent inhibitory effects on fibroblasts and myofibroblasts, including hepatic stellate cells, liver specific myofibroblasts. Myofibroblasts are characterized by de novo expression of smooth muscle α-actin and excessive production of extracellular matrix, particularly collagen type 1 (32). While the mechanisms for IFNγ-mediated inhibition of collagen type 1 expression have been well described (33), the molecular mechanism by which IFNγ inhibits smooth muscle α-actin expression appears to be different. Specifically, IFNγ’s affect on myofibroblasts appears to be tightly linked to SRF regulation. Here, we have demonstrated that binding activity of SRF to the smooth muscle α-actin promoter (CArG-boxes) is reduced by IFNγ (Figure 1, 2). This occurs as a result of IFNγ targeting SRF (Figure 2). We speculate that IFNγ mediated inhibition of SRF (Figure 2, 3) is likely to be a critical modulator of myofibroblast differentiation in wound healing, since SRF targets the promoters of multiple smooth muscle genes that are expressed in myofibroblasts. In support of this position is our finding that not only did IFNγ inhibit smooth muscle α-actin, but that it also potently inhibited SMMHC promoter activity (Figure 7B). Further, because SRF is also regulated in an apparent feedback loop (18), it is possible that reduction of SRF by IFNγ likely has indirect effects on its own promoter activity (Supplemental Figure 2). Interestingly, reduced SRF binding in the smooth muscle α-actin and SRF promoters was closely linked to decreased p300 and H3Ac binding (Figure 2, 3), which further led to decreased SRF and smooth muscle α-actin expression through IFNγ-induced a negative epigenetic regulation.

Although IFNγ is able to signal via STAT1-independent pathways, the IFNγ-STAT1 pathway likely mediates the majority of IFNγ-induced biological effects, which have been well demonstrated in STAT1 gene knockout animal models (34). Our data are highly consistent with this position, as specifically demonstrated in Figure 4 and 5. The finding that STAT1 deletion completely abrogated IFNγ-mediated SRF mRNA decay (Figure 5) and previous work linking STAT1 to 2-5A synthetase/RNase L (26) led us to explore the possibility that the 2-5A synthetase/RNase L signal pathway could play a role in our system. We found that IFNγ regulated SRF mRNA stability in a 2-5A synthetase/RNase L dependent manner (Figure 6). Thus, our data have also highlighted an additional novel target (i.e. SRF) of the 2-5A synthetase/RNase L system. A surprising finding in our study was that smooth muscle α-actin expression was activated in RNase L deficient MEFs (Figure 6D,E). This finding implicates the 2-5A synthetase/RNase L system in control of smooth muscle gene transcriptional activation through regulation of SRF and/or SRF cofactors. For example, it remains to be
determined whether RNase L also targets the SRF cofactor, myocardin, whose expression was tightly linked to smooth muscle specific gene expression including smooth muscle α-actin and SMMHC (35).

RNase L is a latent endoribonuclease whose activity appears to be tightly regulated by 2-5A. Furthermore, 2-5A is generated by 2-5A synthetase, which is induced by IFNs (36). Importantly, the effects of 2-5A are transient, since 2-5A is unstable due to the activities of phosphodiesterases and phosphatases (37). Such a sensitive regulatory cascade appears to be important for regulating protein synthesis via mRNA degradation in response to exogenous stimuli. In our study, IFNγ-induced 2-5A synthetase and RNase L expression rapidly increased around 12 hours (Figure 6A, B); simultaneously, SRF/smooth muscle α-actin mRNA levels reached their lowest point around 12 hours (3 hours with actinomycin D) and then gradually rebounded (Figures 1, 2, 3, and 5). The phenomenon of SRF mRNA rebound was highly reproducible, and likely reflects a crucial effect of RNase L in regulation of SRF mRNA stability (Figure 6) as well as the complex nature of the SRF mRNA regulatory machinery. Nonetheless, these data integrate IFNγ-STAT1 signaling with the 2-5A/RNase L system, SRF and SRF target genes, and provide a framework for a complicated molecular regulatory network for IFNγ-mediated inhibition of smooth muscle specific gene expression (Figure 7D).

Identification of SRF as a novel target of IFNγ in myofibroblasts has implications not only for wound healing, but also in vascular biology, and perhaps even oncogenesis. It is well appreciated that SRF plays a central role in smooth muscle cell differentiation, which is characterized by expression of a unique repertoire of contractile proteins, such as smooth muscle α-actin and SMMHC. Vascular diseases such as atherosclerosis are characterized by dysregulation of contractile protein expression in smooth muscle cells and it is likely that SRF plays a role in regulation of these proteins (10). Further, SRF expression appears to be linked to cancer invasion/metastasis (38). Thus, although speculative, our data raise the possibility that the IFNγ-SRF signaling pathway identified here could be important in oncogenesis. Finally, the complicated nature by which SRF is regulated in our system and in other studies implies a highly complex regulatory hierarchy, and suggests that efforts to manipulate SRF biologically will be challenging.
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**FOOTNOTES**

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The abbreviations used are: IFNγ, interferon-gamma; SM α-actin, smooth muscle α-actin; SRF, serum response factor; STAT1, signal transducers and activators of transcription 1; H3Ac, acetylated histone3; 3UTR, 3’ untranslated region.

Supplemental data include 3 Figures, 1 Table, and Supplemental Procedures.

**FIGURE LEGENDS**

Figure 1. IFNγ mediated inhibition of smooth muscle α-actin requires CArG boxes. (A) and (B) stellate cells were starved (0.1% serum) for 1 day and subsequently exposed to IFNγ. Cells were harvested and RNA isolated at the indicated times. In (A), Smooth muscle α-actin (SM α-actin) mRNA abundance was measured by RPA as in Experimental Procedures (n=3, *p<0.05 for IFNγ vs. control (-)). In (B), following exposure to IFNγ for 48 hours, cells were harvested and subjected to immunoblotting to detect SM α-actin (β-actin was used as a loading control). (n=3, *p<0.05 for IFNγ vs. control (-)). (C) Luciferase reporter constructs harboring different truncated SM α-actin gene promoter (Smpro) fragments were transduced into stellate cells and promoter activity was assayed (n=3, p<0.01 for IFNγ vs. control). (D) SM α-actin gene promoter CArG B and A boxes were mutated individually or combination. The
resultant luciferase reporter constructs were transduced into stellate cells and promoter activity assayed (n=3, *p<0.01 for IFNγ vs. control).

**Figure 2.** IFNγ-STAT1 pathway reduces SRF expression and binding to CArG Boxes in smooth muscle α-actin promoter. (A) Stellate cells were starved (0.1% serum) for 1 day and incubated with IFNγ for 2 days. Cell lysates were subjected to immunoblotting with anti-SRF antibody. (B) Stellate cells were starved (0.1% serum) for 1 day, then replaced with 10% serum-containing 199OR medium with or without IFNγ for 2 days. Cell lysates were subjected to immunoblotting with anti-SRF antibody. (C) Following transduction with the Smpro-125 luciferase reporter construct, stellate cells were incubated in 0.1% serum-containing medium for 2 days, then the medium was changed to 10% serum-containing medium with or without IFNγ for a further 24 hours. Cell lysates were assayed for luciferase activity (n=3, *p<0.01 for 0.1% vs. 10% serum-containing medium and # p<0.01 for IFNγ vs. control). (D) After incubation with 0.1% serum-containing medium for 1 day, stellate cells were exposed to IFNγ for 2 days and nuclear extracts were prepared for EMSA with a probe containing the CArG-B box of SM α-actin gene promoter. The first lane on the left contains buffer plus labeled probe only (i.e. without nuclear extract). Arrows denote the SRF and probe complex or a supershift complex with SRF antibody. (E) and (F) Stellate cells were starved (0.1% serum) for 1 day and exposed to IFNγ for 16 hours; cells were subjected to ChIP assay as in Experimental Procedures. In (E), data are depicted graphically below (n=3, *p<0.01 for IFNγ vs. control).

**Figure 3.** IFNγ inhibits SRF promoter activity and reduces SRF binding to CArG boxes in the SRF promoter. (A) Stellate cells were transduced with truncated SRF reporter constructs as indicated and then incubated in 0.1% serum-containing 199OR medium with or without IFNγ for 2 days. Cell lysates were assayed to detect SRF promoter activity (n=3, *p<0.01 for IFNγ vs. control). (B) Stellate cells were starved (0.1% serum) for 1 day and exposed to IFNγ at indicated time points. Total RNA was extracted and SRF mRNA levels were measured by RPA. (C) and (D) Stellate cells were starved in 0.1% serum-containing 199OR medium for 1 day and exposed to IFNγ for 2 days. SRF was detected in nuclear extracts by immunoblotting (C) and EMSA (D). The first lane on the left contains buffer plus labeled probe only (i.e. without nuclear extract). Arrows denote SRF and probe complex or a supershift complex with SRF antibody (D). (E) Stellate cells were starved (0.1% serum) for 1 day and exposed to IFNγ for 16 hours. SRF binding activity to its own promoter was examined by ChIP assay. Data are depicted graphically below (n=3, *p<0.01 for IFNγ vs. control).

**Figure 4.** STAT1 is required for IFNγ-induced down-regulation of smooth muscle α-actin and SRF. (A) and (B) Stellate cells from STAT1 wild type (+/+) and STAT1 knockout (-/-) mice were transduced with SM α-actin (A) or SRF (B) reporter constructs as indicated. Cells were incubated in 0.1% serum-containing medium with or without IFNγ for 2 days before harvest (n=3, *p<0.01 for IFNγ vs. control). (C) Stellate cells from wild type and STAT1 knockout mice were serum starved for 1 day and then exposed to IFNγ for 24 or 48 hours. SM α-actin and SRF mRNA levels were measured by RPA. Data were quantitated and are depicted graphically below (n=3, *p<0.01 for IFNγ vs. control). (D) Stellate cells were starved (0.1% serum) for 1 day and incubated with or without IFNγ for 2 days. Cell lysates were immunoblotted with specific antibodies as indicated. (E) Stellate cells from STAT1 -/- mice were serum starved for 1 day and exposed to IFNγ for 16 hours. ChIP assay was performed as in Figure 2E. (F) Genotypes of wild type and STAT1 knockout stellate cells were further verified by immunoblotting.

**Figure 5.** IFNγ induces SRF mRNA degradation but has no affect on smooth muscle α-actin mRNA stability. (A) Following transfection with a SRF mRNA 3’ UTR-luciferase reporter construct, stellate cells were exposed to IFNγ for 2 days and luciferase activity was measured in cell lysates (n=3, *p< 0.05 for IFNγ vs. control). (B) Following serum starvation (0.1%) for 1 day, stellate cells were exposed to IFNγ.
for various periods of time, and total RNA was subsequently extracted and subjected to RPA. Data were quantitated and are depicted graphically below (n=3, *p<0.01 for IFNγ vs. control). (C) Stellate cells were starved (0.1% serum) for 1 day. Cells were exposed to IFNγ for 2 hours and then incubated with actinomycin D (10 µg/ml). Total RNA was subsequently extracted and subjected to RPA. Data were quantitated and are depicted graphically below (n=3, *p<0.01 for IFNγ vs. control). (D) and (E) Stellate cells from wild type (+/+ ) and STAT1 knockout (+/- ) mice were subjected to mRNA decay assay as in (C). Data were quantitated and are depicted graphically (n=3, *p<0.01 for IFNγ vs. control in (C)).

Figure 6. SRF is a novel target gene of IFNγ-induced 2-5A-RNase L system. (A) Stellate cells were serum starved (0.1%) for 1 day and exposed to IFNγ for 24 hours. 2-5A synthetase 1A mRNA expression was determined by RT-PCR (n=3, *p<0.05 for IFNγ vs. control). (B) and (C) RNase L+/+ (B) and RNase L-/- (C) MEFs were serum starved (0.2%) for 1 day and exposed to IFNγ at the indicated time points. RNase L (B) and SRF (C) mRNA expression were measured by RPA. In (C) data were quantitated and are depicted graphically (n=3, *p<0.05 for IFNγ vs. control in RNase L+/+ MEFs). (D) and (E) RNase L+/+ and RNase L-/- MEFs were serum starved (0.2%) for 1 day and exposed to IFNγ for 24 hours. In (D), cell lysates were subjected to immunoblotting with specific antibodies as indicated. Data were quantitated and are depicted graphically (n=3, *p<3.05 for IFNγ vs. control in RNase L+/+ MEFs). In (E), SM α-actin mRNA expression was measured by RPA. (F) HEK293 cells were transfected with a mouse Flag-RNase L expression construct or an empty vector overnight, incubated in 0.2% serum-containing medium for 24 hours and nuclear extracts were subjected to immunoblotting with specific antibodies as indicated. SRF bands were quantitated and shown in the graph on the right.

Figure 7. IFNγ inhibits SMMHC mRNA expression and SRF binding to SMMHC promoter CArG boxes. (A) Stellate cells were serum starved (0.1% serum) for 1 day and exposed to IFNγ at the indicated time points. Total RNA was isolated and SMMHC mRNA expression was measured by RPA. (B) A luciferase reporter plasmid harboring different truncated SMMHC promoter fragments were created as in the top panel. Following transfection, stellate cells were incubated in 0.1% serum-containing medium with or without IFNγ for 2 days. Cell lysates were assayed for luciferase activity (n=3, *p<0.05 for IFNγ vs. control). (C) Stellate cells were serum starved (0.1% serum) for 1 day and exposed to IFNγ for 2 days. Nuclear extracts were subjected to EMSA. Arrows denote shifted bands and supershift with SRF antibody. The first lane on the left contains buffer plus labeled probe only (i.e. without nuclear extract). (D) An overview of the IFNγ SRF signaling pathway is highlighted.
Figure 1

A

IFNγ         -   -   -   +   +    IFNγ
Time (hr)  12  24  48  12  24  48

SM α-actin mRNA

SM α-actin mRNA (% decrease)

IFNγ         -   -   +   +    IFNγ
Time (hr)  12  24  48

Luciferase Activity (RLU)

B

IFNγ         -   -   +   +    IB:

SM α-actin

β-actin

C

CArG-B

CArG-A

WT

B mu

A mu

B+A mu

Luciferase reporter

Luciferase Activity (RLU)

Luciferase Activity (fold increase)

D

-125bp

WT

B mu

A mu

B+A mu

Luciferase Activity (RLU)

Luciferase Activity (fold increase)
Figure 2

A

IFNγ - + IB: SRF 
β-actin

serum (%) 0.1 0.1

B

IFNγ - - + IB: SRF 
β-actin

serum (%) 0.1 10 10

C

Luciferase Activity (RLU) vs Serum (%)

IFNγ pGL3B Smpro-125

+ - -

Serum (%) 0.1 0.1 10 10 +

D

Supershift SRF

Probe CArG-B

SFR Ab Cold probe IFNγ

Input Ctr IgG Ab

SM α-actin promoter

H3Ac

E

IFNγ Input Ctr IgG Ab

SM α-actin promoter

H3Ac

F

Input Ctr IgG Ab

SM α-actin promoter

IRF1 promoter

pSTAT-1

P300
Figure 3

A

B

Time(hr) 6 12 24
IFNγ - + - - + +

C

Luciferase Activity (RLU)
(fold increase)

D

Cold probe - - - - + +
SRF Ab - - + + - -
IFNγ - - + + - -

Supershift

SRF

E

IFNγ Input Ctr IgG Ab
- + - - + +

SRF H3Ac p300

 p300

Input Ab

By guest on March 23, 2020
Figure 4

A

STAT1+/+

Luciferase Activity (RLU) (fold increase)

IFNγ - - + + pGL3B Smpro-125

ST1+/ - - + + pGL3B Smpro-125

B

Luciferase Activity (RLU) (fold increase)

IFNγ - - + + pGL3B Smpro-125

ST1+/ - - + + pGL3B Smpro-125

C

STAT1 IFNγ 24 hr 48 hr mRNA abundance (fold decrease)

IFNγ - - + + + + - - + + + + pGL3B Smpro-125

D

STAT1 IFNγ IB: pGL3B Smpro-125

IFNγ - - + + + + - - + + + + pGL3B Smpro-125

E

IFNγ Input Ctr IgG Ab SM α-actin

IFNγ - - + + + + - - + + + + pGL3B Smpro-125

F

STAT1 IFNγ pSTAT1

IFNγ - - + + + + - - + + + + pGL3B Smpro-125
Figure 5

A. Luciferase activity (RLU) in response to IFNγ.

B. Time course of SRF mRNA abundance in response to IFNγ and IFNα.

C. Western blot analysis of SRF and GAPDH expression under different conditions.

D. Western blot analysis of STAT1 and GAPDH expression under different conditions.

E. Western blot analysis of SM α-actin and GAPDH expression under different conditions.
Figure 6

**A** IFNγ -  +  2-5A synthetase
GAPDH

**B** ExposureTime (hr)
RNase L +/+
GAPDH

**C** SRF
GAPDH

**D** IFNγ -  +  RNase L+/+
RNase L -/-

**E** SM α-actin
GAPDH

**F** Control vector
Flag-RNase L

IB: Flag
SRF
α-Tubulin
Figure 7

A

Time (hours)  6  12  24
IFNγ - + - + - +
SMMHC
GAPDH

B

| luciferase activity (RLU) (fold increase) |
|------------------------------------------|
| IFNγ pGL3B -1620 -1414 -1105 |

C

| CArG-C |
|--------|
| SRF Ab - - - + + |
| IFNγ - - + - + |

D

SRF Ab

SMα-actin
H3Ac
RNase L
2-5A Synthetase

SRF cofactors
pSTAT1
Jak

SMα-actin gene
SRE: 3 2 1
SRF mRNA decay

2-5A Synthetase

SRF
pSTAT1
P300

H3Ac

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