Supplemental Data

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

Zengdun Shi[1] and Don C. Rockey[2]

Supplemental Experimental Procedures

Hepatic Stellate Cell Isolation

The procedures for rat (1) and mouse (2) stellate cell isolation was modified as follows. The liver was perfused sequentially with a series of the following solutions: solution A: L15 containing EGTA (0.5mM) and 5mM Glucose; Solution B: L15 containing $CaCl_2$ (3.8mM) and pronase (1mg/ml, Roche); Solution C: L15 containing $CaCl_2$ (0.38mM) and collagenase type I (0.13mg/ml, Worthington). The digested liver was agitated in solution D (L15 containing pronase (0.2mg/ml) and DNase (0.0375mg/ml) at 37°C for 20 minutes. The resultant cell suspension was washed once with cold GBSS, and resuspended in cold GBSS solution with Accudenz gradients to final concentration 10% (Accudenz, Accurate Chemical & Scientific Co., Westbury, NY). 10ml of the cell mixture was loaded in 15 ml tube with 3 ml of GBSS solution on the top. Following centrifuge at 4,000 rpm at 4°C for 30 minutes, the interface cell fractions between cell mixture and GBSS solution were combined and centrifuged at 1,550 rpm for 7 minutes. The cell pellet was washed once and cells were placed in plastic culture dishes in 199OR medium containing 20% serum (10% horse/10% calf).

RT-PCR

2.5 μg of total RNA was used for cDNA synthesis by reverse transcription kit (Invitrogen) and the cDNAs were subjected to Real-Time PCR analysis according to manufacture's instructions (Applied Biosystems). The resulted PCR products were analyzed by 1.5% agarose gel electrophoresis.

Supplemental Table 1

Primers	Sequences (5'-3')	Use
STAT1	CTACCAGAGTATCTGCCTAGAC CCTCTCAACCTTCCTGACACC	genotyping
Neomycin	CACGACGGGCGTTCCTTGCGCAG CCTGATGCTCTTCGTCCAGATCAT	genotyping
SM α-actin promoter (rat)	GAGGTCCCTATATGGTTGTG TGTCTGGGGAGGCTGAATGC	cloning/ChIP
SRFpro (-787-172) (mouse)	GTGGACCTGTAATGTCGATCACTC AGACATACCGAACTCGCTGCTG	cloning
SRFpro (-543+11) (mouse)	GATGTGACCTCGCAGCCAGAC AGGTATCCCCCAACCCTTC	cloning/ChIP
SM α-actin cDNA (rat/mouse)	ATGTGTGAAGAGGAAGACAG GTGATGATGCCGTGTTCTATCG	cloning/RPA
SRF cDNA (rat/mouse)	TCTCAGGCACCATCCACCAT CCCAGCTTGCTGTCCTATCAC	cloning/RPA
SMMHC cDNA (rat)	GTACAAGGGCAAGAAGAGGC CATCTCATCATCTTGTGCAGC	cloning/RPA
RNase L cDNA (mouse)	GGCATTGAGGACCATGGAGAC CAAGACTCAGCTCTGTATGCC	cloning/expression
RNase L cDNA (mouse)	GAGATGTTGTCAGGGTCCAGC GCAGCTTCCATGAAAGCCGTG	cloning/RPA
SMpro-CArG-B (rat)	GAGGTCCCTATATGGTTGTG CACAACCATATAGGGACCTC	EMSA
SRFpro-SRE2 (mouse)	GCTCGCCATATAAGGAGCGG CCGCTCCTTATATGGCGAGC	EMSA
IRF1pro-GAS (rat/mouse)	GCCTGATTTCCCCGAAATGATGAGGC GCCTCATCATTTCGGGGAAATCAGGC	EMSA
SRFpro-GAS (mouse)	TCACTCTCTTGCTTAAATTTTCTATCC GGATAGAAAATTTAAGCAAGAGAGTGA	EMSA
SRFpro-GAS (mouse)	CTCCACTGTTCCTTTAAGGAGTTGGCT AGCCAACTCCTTAAAGGAACAGTGGAG	EMSA
SMMHCpro CArG-C (rat)	CGACTTCCTTTTATGGCCTGAG CTCAGGCCATAAAAGGAAGTCG	EMSA
SRF mRNA 3UTR (rat)	TGATCCGCCCGCCACCCTGGACAGATG CAGGTGGTTTAGGCTGGCTCTGACAC	cloning
IRF1 promoter (mouse)	CGCTTAGCTCTACAACAGCC GTGAAAGCACGTCCTACCTC	cloning/ChIP
IRF1 promoter (rat)	CGCTTAGCTCTACAACAGCC GTGAGAGCTCTTCCTACCTC	cloning/ChIP
2-5A synthetase 1A (rat)	ATGGAGCAGGAACTCAGGAGC CGTCTGACTTGCCCTTGAGTG	RT-PCR

Supplemental References

1. Rockey, D. C., Maher, J. J., Jarnagin, W. R., Gabbiani, G., and Friedman, S. L. (1992) *Hepatology* **16**(3), 776-784
2. Shi, Z., Wakil, A. E., and Rockey, D. C. (1997) *Proc Natl Acad Sci U S A* **94**(20), 10663-10668

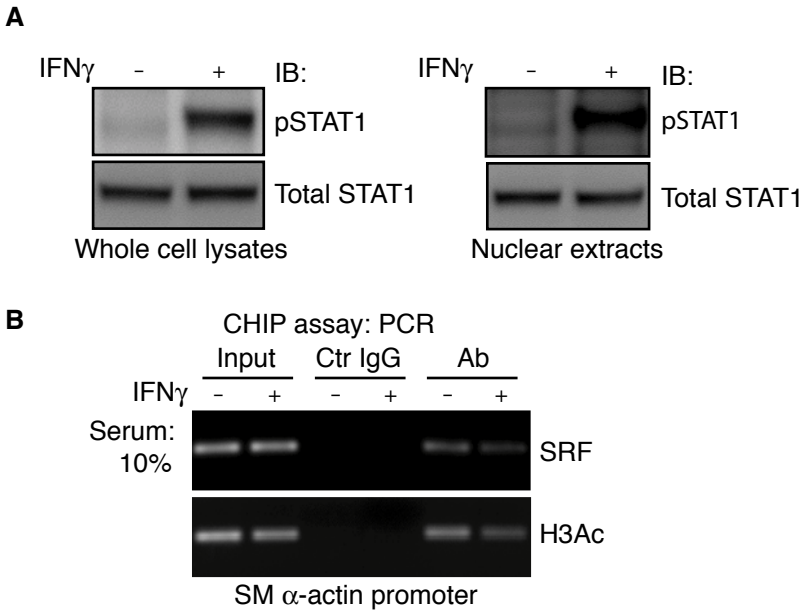
Figure Legends (Supplemental Figures)

Supplemental Figure 1: Signaling through IFNγ-STAT1 pathway reduces SRF binding in SM α-actin promoter *in vivo*. (A) Stellate cells were serum starved (0.1% serum) for 1 day and subsequently exposed to IFNγ (1,000 IU/mL) for 2 hours. Whole cell lysates (left panel) and nuclear extracts (right panel) were subjected to inmmunoblot analyses. (B) Following serum starvation (0.1%) for 1 day, stellate cells were incubated in medium containing 10% serum with or without IFNγ for 16 hours and then subjected to ChIP assay.

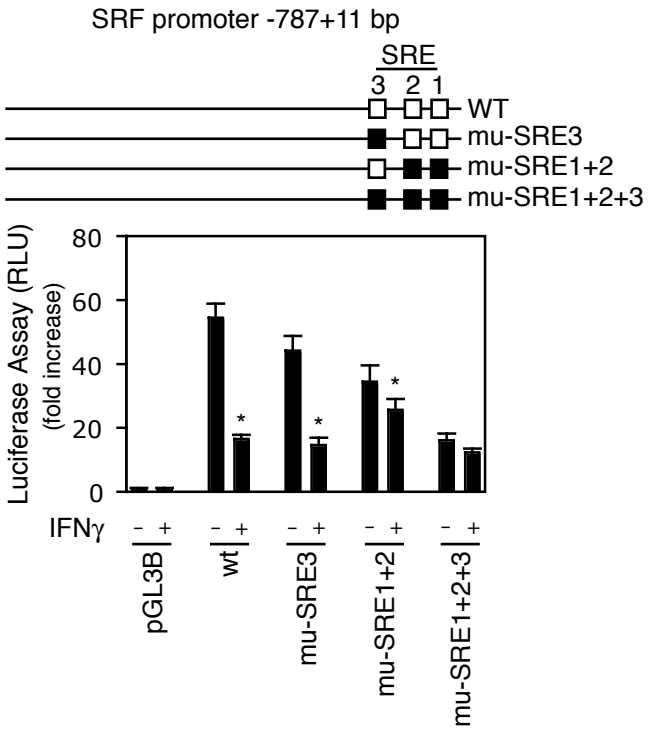
Supplemental Figure 2: SREs in the SRF promoter are responsible for IFNγ responsiveness. The SRF promoter (-787+11bp) and specific mutants are shown in the top panel. Site mutations (black boxes) were introduced into the indicated SREs (wild type SRE1: ccataaaagg, mutant SRE1: atccaaaagg; wild type SRE2: ccatataagg, mutant SRE2: atccataagg; wild type SRE3: gcaaataagt, mutant SRE3: aagcataagt). A pGL3B vector containing the wild type (WT) and different mutant SREs (mu-SRE) were transfected into stellate cells. Stellate cells were incubated in 0.1% serum containing 199OR medium with or without IFNγ for 2 days. Cell lysates were then assayed for luciferase activity (n=3, *$p<0.01$ for +IFNγ vs. -IFNγ, bottom panel).

Supplemental Figure 3: STAT1 does not directly target SRF GAS elements. (A) Stellate cells were serum starved (0.1%) for 1 day and then exposed to IFNγ for 2 hours. Nuclear extracts were prepared and subjected to EMSA to detect binding to GAS elements of the SRF promoter (or the IRF1 promoter, which was used as a positive control). Specific binding of pSTAT1 to GAS elements was confirmed by supershift with anti-pSTAT1 antibody (arrow). (B) and (C) After exposure to IFNγ for 2 hours, stellate cells were fixed with formaldehyde and subjected to ChIP assay. 20% of each sample was subjected to immunoprecipitation with pSTAT1 antibody and immune complexes were subjected to immunoblot with pSTAT1 (B). The remainder of each sample was subjected to immunoprecipitation with pSTAT1 and PCR was performed to amplify GAS containing SRF or IRF1 promoters (C).

Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3