Design and Evaluation of an *In Vivo*, Repeatable, and Real Time Reporter for Myofibroblast Cell Fate as a Surrogate for Cardiac Fibrotic Remodeling

Kevin Shi

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Jennifer Davis

Kelly Stevens

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Abstract

Design and Evaluation of an *In Vivo*, Repeatable, and Real Time Reporter for Myofibroblast Cell Fate as a Surrogate for Cardiac Fibrotic Remodeling

Kevin Shi

Chair of the Supervisory Committee:

Jennifer Davis

Pathology & Bioengineering

Heart disease is the leading cause of death in the United States accounting for 1 in every 4 deaths [“Heart Disease Facts”]. More than half of heart disease deaths are attributed to coronary heart disease which often leads to congestive heart failure costing the United States $30.7 billion annually [“Heart Disease Fact Sheet”]. Myofibroblasts lead coronary heart failure to heart disease through long term adverse fibrotic remodeling [Van Den Borne et al]. After myocardial infarction, fibroblasts transdifferentiate into activated myofibroblasts that orchestrate matrix remodeling through deposition of collagen, cytokine secretion, and contractility forming a fibrotic scar. While this is beneficial in acute wound healing, prolonged presence of the fibrotic scar interrupts proper heart function, increases cardiac workload, and encourages further fibrotic buildup resulting in heart failure. Research on elucidating the regulatory pathways involved in transdifferentiation is occurring slowly and is made difficult without proper cell specific reporting. Currently, methods to track myofibroblast activity are indirect and limited. Immunohistochemistry is the gold standard for measuring cardiac fibrosis in *in vivo* studies yet is limited by invasiveness, quantifiability, and inability for longitudinal study. MRI has recently been able to quantify and track fibrosis with high temporal and special resolution through protein targeted gadolinium contrast agents, however, this method is expensive and indirect [Caravan et

al]. Here we perform literature and bioinformatic survey to investigate potential transcriptional activators in myofibroblast specific gene promoters and show initial *in vitro* support for a genetic luciferase reporter for myofibroblast cell fate driven by chimeric combinations of promoters from genes involved in carrying out fibrotic remodeling as a surrogate for cardiac fibrosis. The addition of double 9x tandem repeat enhancer sequences greatly boosted luciferase expression while maintaining reporter specificity to TGFb treatment and fibroblast cell type. Ultimately, a reporter of this kind will enable real time longitudinal study, reduce animal waste, and has potential applications in gene therapy.

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Introduction

In response to cardiac injury such as myocardial infarction or chronic hypertension, fibroblasts transdifferentiate into myofibroblasts which orchestrate cardiac fibrotic remodeling [Davis et al (2014), Seguro et al, Souders et al]. Myofibroblasts are functionally characterized by their elevated extracellular matrix secretion and contractility are necessary to form fibrotic scar. While formation of scar is critical for acute wound stabilization, especially in response to myocardial infarction where myocardium turns ischemic and necrotizes, perpetual activation of myofibroblasts and failure to regress scar disrupts electrical and mechanical processes in the remaining myocardium leading to chronic cardiac failure. Currently, little is known about the pathobiology behind sustained myofibroblast activity. However, the scientific community has recently started to pay more attention to stromal cells such as fibroblasts and myofibroblasts as further evidence for their importance in tissue physiology and cancer emerges [Powell et al, Souders et al].

Research on the cardiac myofibroblast is currently limited, despite the attention, in part due to the lack of a direct, repeatable, *in vivo* reporter for the cell type. Current methods to study myofibroblast activity – and therefore cardiac fibrosis – are wasteful, expensive, indirect, or incapable of longitudinal reporting. These methods include tissue histology and immunohistochemistry, positron emission spectroscopy (PET), single photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI). Histology and immunohistochemistry require tissue resection or sacrifice of the animal. These techniques are highly disruptive to the system being studied, incapable of producing time dependent results, and encourages wasteful use of animal models. Furthermore, a histology or immunohistochemistry stain capable of directly measuring myofibroblasts in the tissue section currently does not exist. PET, SPECT, and MRI all are limited to measuring the extent of fibrosis rather than myofibroblast cell fate through targeted contrast agents or probes. A PET probe for collagen type I was recently developed ^{68}Ga-CBP8 and validated in bleomycin induced pulmonary fibrosis mouse models [Désogère et al]. For SPECT, 99mTc-sestamibi 2-methoxy-isobutyl isonitrile (MIBI) uptake by myocardium can be used to infer percent fibrosis through a linear relationship developed by Maes et al. MRI can be used to detect fibrosis through EP-3533, a gadolinium based contrast agent that targets collagen [Caravan et al, Helm et al].

Direct genetic transgene reporters are currently the gold standard for identifying bonafide myofibroblasts *ex vivo* as defined as TCF21 lineage traced resident fibroblasts expressing alpha smooth muscle actin (Fu et al, 2018). Fu et al demonstrates the use of mice with tamoxifen inducible Cre knocked into the TCF21 locus crossed with mice containing LoxP flanked eGFP in the Rosa26 locus to identify resident fibroblasts of proepicardial lineage. Alpha smooth muscle actin staining colocalization with eGFP positive cells indicates bonafide activated myofibroblast while POSTN positive TCF21 lineage cells are quiescent resident cardiac fibroblasts.

Some groups have attempted to utilize firefly luciferase bioluminescence for direct real time reporting of either myocardial hypertrophy or myofibroblast cell fate [Andoh et al, Davis et al (2012), Hu et al, Molkentin et al, Piras et al, Wilkins et al, Seo et al]. To best drive cell type or myocardial fibrosis specific expression of luciferase, various promoters from genes and transcriptional activators critical in myofibroblast transdifferentiation or function have been leveraged including: alpha 2 smooth muscle actin (ACTA2), interleukin-6 (IL6), and periostin (POSTN), alpha myosin heavy chain (aMHC), and regulator of calcineurin 1 (PP2B)/nuclear factor of activated T cells (NFAT). Efficacy of these reporters in studying myofibroblast transdifferentiation is highly limited due to insufficient myofibroblast cell type specificity, particularly in *in vivo* contexts, since these genes are expressed in extraneous cell types. Thus, these reporters lack sufficient specificity to confidently conclude their expression levels are a robust surrogate for fibrotic tissue remodeling.

Here we show initial *in vitro* support for mitigating off target luciferase expression through chimeric promoter combinations. By combining transcriptional drivers from multiple genes "characteristically upregulated" by myofibroblasts into a single promoter to drive luciferase expression, we hope to create a promoter that integrates queues from multiple myofibroblast specific signals. This promoter would better recapitulate a distinct transcriptional program unique to myofibroblasts thereby reducing noise and bolstering signal fidelity to cell fate and fibrosis. To find suitable genes that are "characteristically upregulated" we identified several genes involved in contractility (ACTA2), extracellular matrix secretion (collagen 1 alpha 2 (COL1A2) and fibronectin (FN1)), and cell motility (POSTN). Literature review and bioinformatic cross reference revealed key regions in the promoter regions of these genes utilized by myofibroblast transcriptional programs. Furthermore, literature review and bioinformatic screening indicated that serum response factor (SRF) and NFAT were key

regulators of transdifferentiation [Davis et al (2012), Davis et al (2014), Molkentin et al, Qiu et al, Tomasek et al]. Thus, we created firefly luciferase PGL3 plasmids driven by ACTA2 and COL1A2 minimum promoters and evaluated luciferase expression in NIH 3T3 mouse fibroblast cells treated with profibrotic agonist transforming growth factor beta (TGFB). Adding 9x tandem SRF and NFAT to the COL1A2 minimum promoter significantly boosted the luciferase expression while maintaining specificity for transdifferentiation

Results

Literature and bioinformatic survey

To determine enhancers and promoter regions that would boost luciferase expression, literature and bioinformatic survey was performed. Literature review revealed many suitable markers for fibroblasts including alpha smooth muscle actin, periostin, collagen type I, discoidin domain receptor 2, vimentin, fibroblast activation protein, integrin beta-1, fibronectin and many others [Krenning et al, Davis et al (2012)]. Ultimately, we looked most into ACTA2, POSTN, FN1, and COL1A1 and COL1A2 due to their importance in myofibroblast wound repair function.

GPMiner found a novel potential SRF binding site in the alpha smooth muscle actin minimum promoter

Promoter mining in GP Miner within the smooth muscle alpha actin promoter range (-893/+51) revealed a potential SRF binding site (GT**CCCTATATGG**TT) at location -122/-109. SRF is well known to bind conserved CArG box motifs with the consensus sequence (CC(A/T)$_3$GG) which is bolded [Sun *et al*, 2006; West *et al*, 1997, Benson *et al*, 2011]. Leveraging its central role of SRF in myofibroblast transdifferentiation, we utilized this sequence in 9x tandem as the SRF enhancer sequence for the chimeric reporter.

Transcription factor mining in the promoter regions of ACTA2, POSTN, FN1, and COL1A reveals 16 common transcription factors

Utilizing various bioinformatics tools including the Eukaryotic Promoter Database and GPMiner, we tried to identify enhancers that would increase promoter specificity to activated cardiac myofibroblasts. Starting approximately 2000 bp upstream of the transcription start site of alpha smooth muscle actin, periostin, and fibronectin, and collagen type I, we identified 16 common transcription factors (TFs) of which we performed further literature analysis to

investigate any potential to be master regulators for myofibroblast fate (Table 1, Figure 1). Many of these TFs do not appear to be closely implicated in cardiac myofibroblast transdifferentiation, although notably the STAT family of TFs and PAX2 are both involved in endothelial to mesenchymal transdifferentiation of renal tubular cells to myofibroblasts during kidney fibrosis [Nightingale et al, Li et al]. Furthermore, MZF1 has been shown to associate with FHL3 in downregulating FcεRI, a high-affinity IgE receptor [Takahashi et al]. FHL3 is a protein in the LIM protein family which are also known to be regulators in TGFB induced EMT into myofibroblast acting through the SMAD2/3 pathways [Ding et al, Järvinen et al]. The connection between MZF1, LIM domain proteins, and cardiac myofibroblast transdifferentiation in response to TGFB has not yet been established. The majority of these common transcription factors identified by GPMiner appear to be broadly implicated in many transcriptional programs.

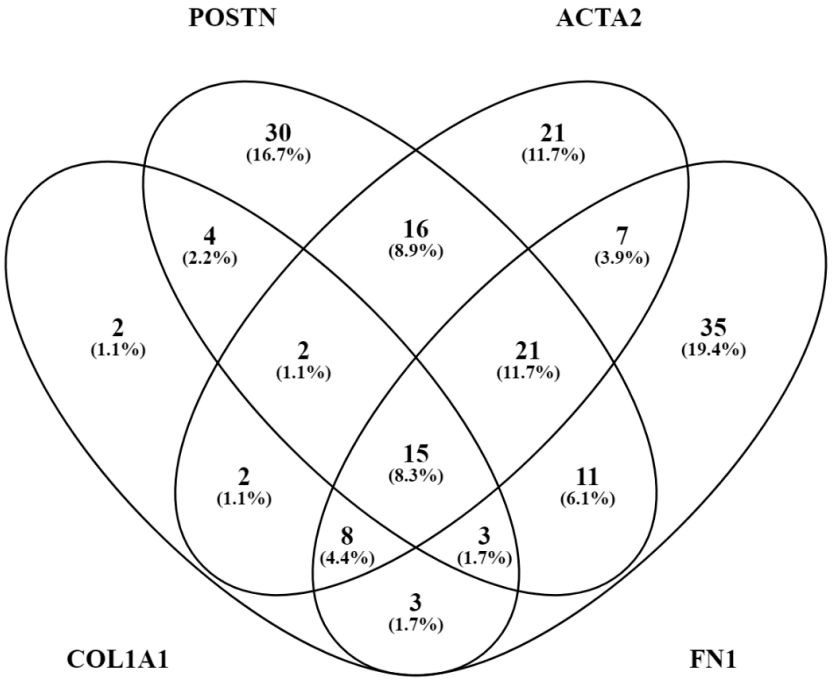


Figure 1. Venn diagram of the common transcription factors between ACTA2 (-2000/+51), COL1A1(-1000/+100), FN1(-2000/+100) and POSTN(-2500/+100) found by GPMiner.

Table 1. Sixteen common transcription factors between the ACTA2, COL1A1, FN1, and POSTN cis-regulatory region found by GPMiner.

Transcription Factor	Key notes
AP2REP 01	Kruppel like factor (AP2) repressor
AR Q6	Androgen receptor
AREB6 04	zinc finger e box binding homeobox, may play a role in IL2 transcriptional repression
ETS Q6	broad transcription factor
MYB (Q3, Q5 01, Q6)	essential in hematopoiesis
MZF1 01	Myeloid zinc finger - shown to be n-Cadherin activator - shown to complex with FHL3 which is a SMAD2/3/4 regulator
NF1 Q6	Neurofibromin, negative regulator for Ras path
PAX2 02	Regulates EMT for renal tubular cells
STAT3 02	activated by IFNs, EGF, IL5, IL6, HGF, LIF, BMP2
STAT5A 04	activated by IL2, IL3, IL7, GM-CSF, erythropoietin, thrombopoietin
STAT6 Q1	Mediator protein in IL4 signal
TATA 01	TATA box – transcriptional initiator
TBP Q6	TATA binding protein
TEF1 Q6	Known transcriptional activator of alpha smooth muscle actin

Minimum promoters to drive the promoter were chosen based off of the number of EBOX motifs present near the transcription start site

We then searched for basal helix loop helix (bHLH) transcription factor EBOX binding motifs in the promoter regions of ACTA2, COL1A2, POSTN, and FN1 (Table 2) using EMBL-EBI EMBOSS Needle global alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html). Many bHLH TFs are known to be key regulators in myofibroblasts including scleraxis, TCF21, and MyoD [Acharya et al, Hecker et al, Espira et al, Davis et al (2012), Davis et al (2015)]. bHLH bind to the following highly conserved motif called an E-box: 5’ – CANNTG – 3’ [Chaudhary et al]. Global alignment of the E-box motif with these four promoters revealed that ACTA2 contained 2 within the region 259 bp

upstream and 51 bp downstream of the transcription start site (TSS). COL1A2 contained three E-box domains between -574/+59 and FN1 contained one domain between -1000/-30. Of note, the COL1A2 -419/-414 E-box site encodes 5' CAGGTG 3' which is scleraxis binds to with high affinity [Espira et al]. The POSTN region -2924/-2119 alone was previously shown to drive greater luciferase expression than the POSTN 3.9 kb whole promoter in Schwann cells [Lindsley et al] and contained four E-box sites. The POSTN region -423/-30 contained 2 E-box sites. The ACTA2 and COL1A2 minimum promoters were chosen to drive luciferase expression due to the proximity of the EBOX sites to the transcription start site. ACTA2 was chosen over POSTN for two reasons. First, ACTA2 is critical to myofibroblast contractility and is the current gold standard to identify activated myofibroblasts in immunohistochemistry. Second, the functions of the myofibroblast can be grouped into contractility and extracellular matrix component secretion. Choosing the ACTA2 minimum promoter leverages the contractile component while the COL1A2 minimum promoter leverages the secretory component.

Table 2. Number, location, and sequence of E-box sites in ACTA2, COL1A2, POSTN, and FN1 promoter regions

Promoter	# of EBOX sites	Location	Sequence
ACTA2 -259/+51	2	-258/-253 -57/-52	CAGTTG CAAGTG
COL1A2 -574/+59	3	-437/-432 -419/-414 -121/-116	CAAGTG CAGGTG CAGCTG
POSTN -2924/-2119	4	-2917/-2912 -2532/-2527 -2477/-2472 -2242/-2237	CAGCTG CAGCTG CAGATG CAGTTG
POSTN -423/-30	2	-319/-314 -298/-293	CAACTG CACCTG
FN1 -1000/-30	1	-514/-509	CAGCTG

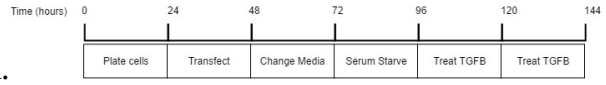
Design/construction

From the literature review and bioinformatic screen, we built several modular promoter building blocks consisting of the ACTA2 -259/+51 promoter, COL1A2 -574/+59 promoter, SRF enhancer, and NFAT enhancer. The basis sequences for the 9x tandem NFAT and SRF are found in table 3. While the SRF sequence was taken from the GPMiner transcription factor screen described above, the NFAT sequence was taken from [Wilkens et al] where a 9x tandem repeat NFAT enhancer driven luciferase reporter was created to measure calcineurin/NFAT coupling in cardiac hypertrophy models. Figure 2 B diagrams the restriction cloning sites of each designed promoter element while Figure 2 C diagrams the plasmid constructs built and evaluated *in vitro*. The restriction sites of the promoter elements were designed to be modular such that the COL1A2 promoter could be inserted upstream of the ACTA2 promoter and both enhancers could be used in a single construct in either spatial orientation.

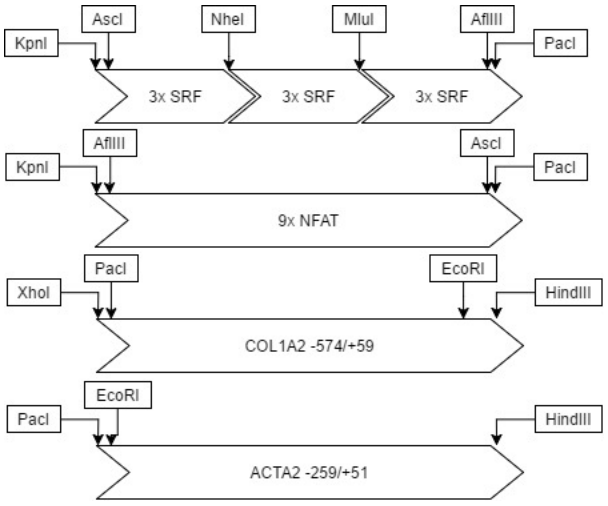
Table 3. NFAT and SRF sequences used for 9x Tandem enhancer repeat construct modules

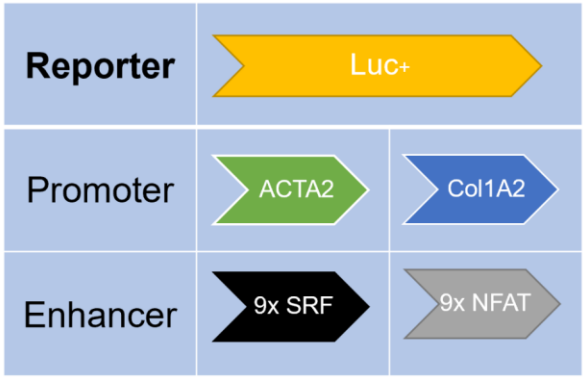
Enhancer	Sequence
NFAT	5’ TGGAAAATT 3’ (sense)
SRF	5’ GTCCCTATATGGTT 3’ (sense)

A.



B.





C.

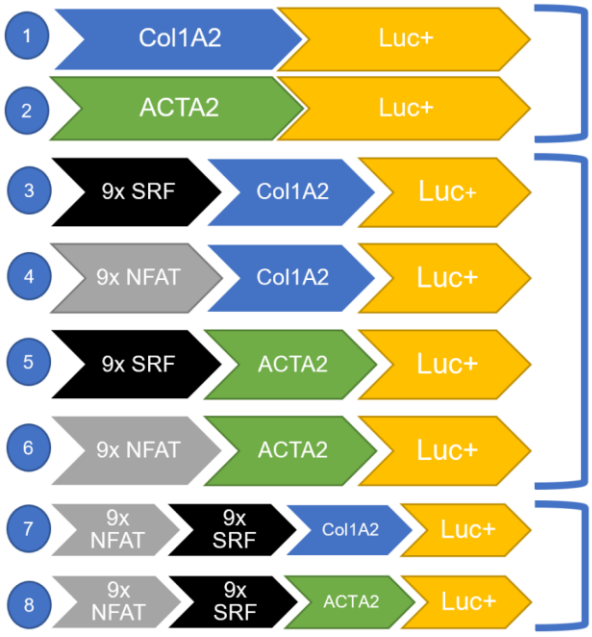


Figure 2. Luciferase assay schema. A) Time course for construct transfection. B) Diagram of enhancer and promoter modules with locations of restriction cloning sites. 5' is on the left and 3'

is on the right. C) Diagram of constructs evaluated *in vitro*. Luc+ refers to luciferase coding region in PGL3 of which the promoter designs were cloned into.

In vitro validation

The constructs enumerated in Figure 2 were evaluated *in vitro* through transient transfection into NIH3T3 immortalized embryonic fibroblasts, mouse embryonic fibroblasts (MEFs), and FVB mouse aortic smooth muscle cells. Luciferase signal in response to two profibrotic agonists, transforming growth factor beta and angiotensin II, was compared to background signal without agonist treatment. A constitutively active cytomegalovirus (CMV) driven luciferase reporter was utilized as a theoretical signal maximum while pMax-GFP was used as a negative control.

Double tandem enhancers significantly boost luciferase output while maintaining low background expression

The alpha smooth muscle actin promoter driven luciferase reporter appeared to perform as well as the constitutively active CMV luciferase reporter but lost signal strength with addition of the 9x tandem SRF and NFAT enhancers in favor for increased signal specificity. In contrast, the COL1A2 minimum promoter alone performed comparatively well to the CMV reporter, but addition of the 9x tandem repeat enhancers boosted signal strength at the expense of signal specificity to the activated phenotype. For both the ACTA2 and COL1A2 promoters, addition of the 5'- 9x NFAT + 9x SRF - 3' chimeric enhancer combination boosted signal to be comparable or greater than the base minimum promoter activity. Signal specificity saw a minimal loss for the ACTA2 construct but gain for the COL1A2 construct. To ensure that the luciferase expression was due to transdifferentiation, an anti-alpha smooth muscle actin Western blot was performed, however, the luciferase assay buffer was incompatible to the BCA assay and protein loading was not controlled for (Supplemental figure 1). Normalization through band quantification was attempted, but the alpha smooth muscle actin lanes for TGFb+ treatment wells were saturated, and the quantification was not representative of the true amount of protein present. The constructs were also evaluated in mouse embryonic fibroblasts against AngII treatment to validate the expression profile in a different fibroblast type and resulted in a slight statistically insignificant increase with AngII (not shown).

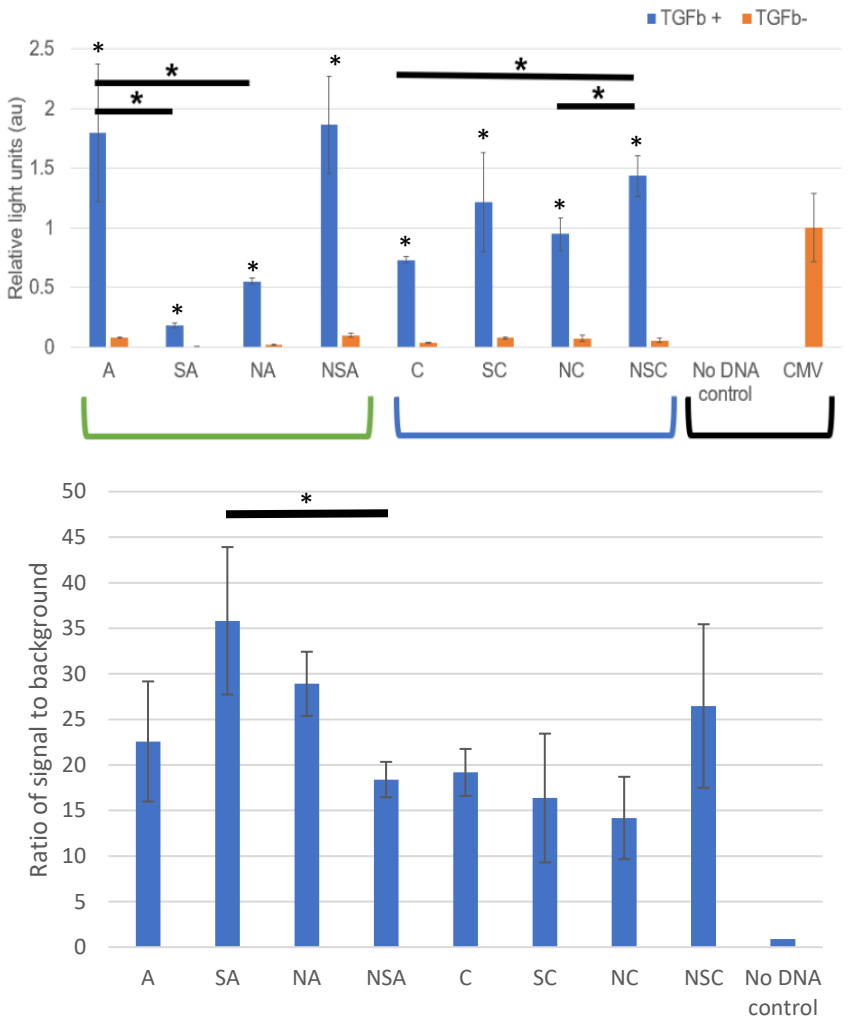


Figure 3. *Double tandem enhancers significantly boost luciferase output while maintaining low background expression.* TOP) Luciferase expression by the chimeric reporters in response to TGFb treatment normalized to CMV driven luciferase. This assay was performed in NIH 3T3 mouse fibroblasts BOT) Ratio of luciferase expression in TGFb+ groups over TGFb- groups to represent signal to noise or reporter specificity to the activated state of the myofibroblast. N = 3 and * indicates a statistically significant difference in the mean of $p < 0.05$ as calculated by two tailed Welch's t test.

Myofibroblast reporter minimally activates in vascular smooth muscle cells

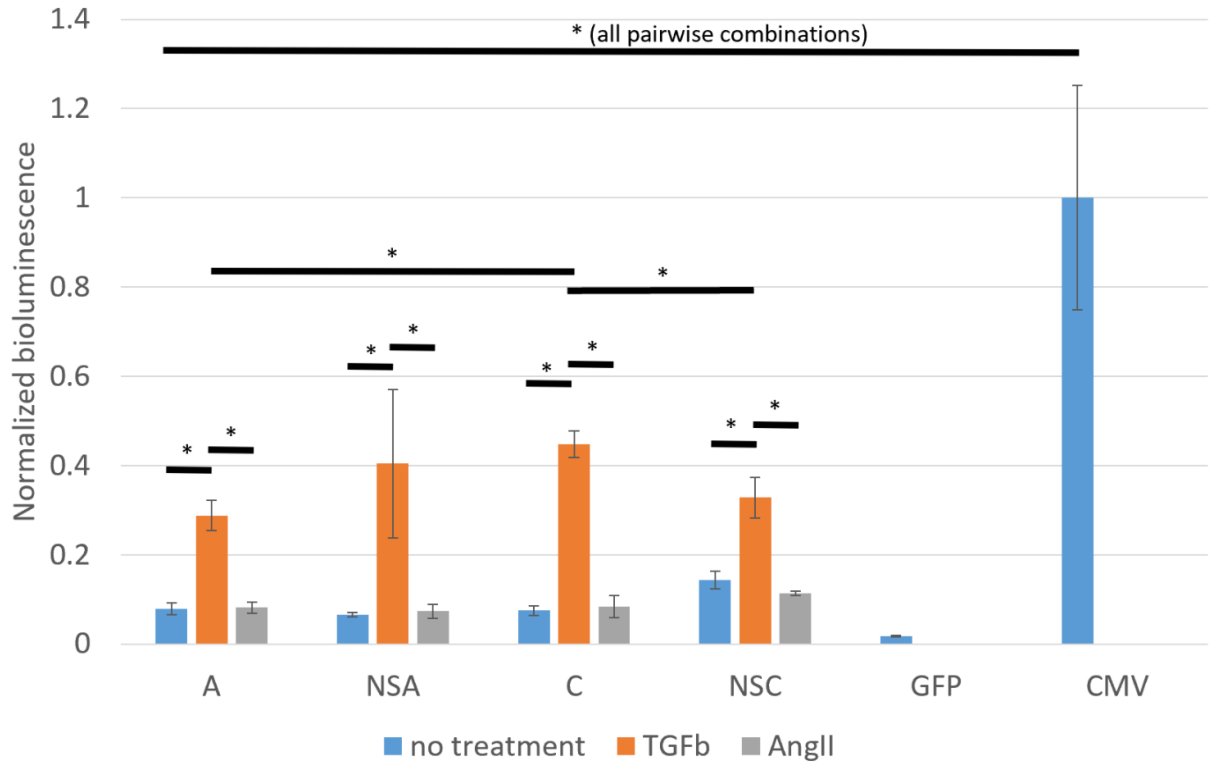


Figure 4. Myofibroblast reporter minimally activates in vascular smooth muscle cells. Vascular smooth muscle cells transfected with the reporter constructs were treated with TGFb or AngII in triplicate. The graph shows the composite results from two experimental replicates. * indicates a statistically significant difference in means, $p < 0.05$, as calculated by a two tailed Welch's t test.

To validate the specificity of the signal to the fibroblast, the double 9x tandem repeat SRF/NFAT enhanced ACTA2 and COL1A2 luciferase reporters were evaluated in FVB mouse aortic vascular smooth muscle cells. Figure 4 illustrates the combination of two replicates of luciferase assays performed in triplicate groups. The performance of the reporters relative to CMV varied between the two experiments, however, the TGFb treated groups consistently had higher expression than the AngII and non-treated groups. One of the experimental replicates had significantly less CMV luciferase activity which skews the composite graph seen in Figure 4 to illustrate greater relative luciferase activity. The bioluminescence values for the constructs were consistently low, but one experimental replicate experienced less transfection efficiency or more cell death than the other.

Discussion

Results of the GPMiner survey are potentially interesting for further investigation. Finding a primary regulator for the myofibroblast phenotype may be highly promising to leverage in a myofibroblast specific reporter. However, of the 17 common transcription factors, only MZF1, PAX2, and STAT family proteins were linked to renal myofibroblast transdifferentiation and none were clearly linked to cardiac myofibroblasts. Because myofibroblasts are highly transient, heterogeneous, and differ depending on organ or tissue context, these results may ultimately be insubstantial. Furthermore, the effectiveness utilizing bHLH binding domains as a predictor for promoter efficacy is unsubstantiated as the COL1A2 minimum promoter had one additional E-box motif compared to ACTA2 yet produced less luciferase expression. Efficacy of promoters should be evaluated more comprehensively for transcriptional repressors in addition to activators. One potential area of investigation may be utilizing tandem repeats of E-box motifs as a chimeric enhancer.

On the other hand, leveraging regulators in the myofibroblast transdifferentiation transcriptional program is nonetheless highly promising for creating a high-fidelity chimeric promoter to drive myofibroblast cell specific expression of luciferase. *In vitro* evaluation shows initial promise of chimerically combining tandem enhancers with minimum promoters from characteristically upregulated genes. The boost in luciferase expression compared to CMV for TGFB treated groups resulting from addition of the double 9x tandem SRF/9x NFAT chimeric enhancer suggests that leveraging binding domains of known transcriptional regulators for transdifferentiation will help reporters better recapitulate the transdifferentiation transcription program compared to relying on myofibroblast gene minimum promoters alone. Therefore, chimeric promoter driven PGL3 constructs may be able to better report activation of myofibroblasts specifically since luciferase driven by ACTA2 or COL1A2 promoters alone will report any cell type that upregulates those genes. This result is promising for reporting *in vivo* where nonspecific reporting majorly hinders single gene promoter driven luciferase reporters for myofibroblasts.

The results of the luciferase assay performed in NIH 3T3 seen in Figure 3 suggests that the transcription factor complexes from binding to the single 9x tandem SRF or the single 9x tandem NFAT enhancer were decreasing transcription efficiency for the transcriptional complex recruited by the ACTA2 minimum promoter. Meanwhile, the same enhancer sequences had the opposite

effect for the COL1A2 minimum promoter transcriptional complex. Generally, transcription efficiency is regulated on many levels, but cis- and trans- up-regulatory elements make transcriptional complexes thermodynamically more stable therefore making complex recruitment and transcription initiation more favorable. The choice of SRF and NFAT as the tandem enhancers was motivated both by the known importance of SRF and NFAT in myofibroblast transdifferentiation but also by their presence as cis- regulatory elements found in the minimum promoters of ACTA2, COL1A, FN1, and POSTN. We thought that including more binding sites of these up-regulators may increase the binding frequency of the transcription factors to drive more expression in response to transdifferentiation signals that act through SRF or NFAT. However, the spatial location of the transcription factors determines their effect on the transcriptional complex. It is possible that the addition of the 9x tandem SRF enhancer to the ACTA2 minimum promoter antagonized the existing SRF transcription factor binding within the minimum promoter region causing the down expression of luciferase. The addition of the 9x NFAT upstream to the 9x SRF then rescued the expression levels, though it is unclear how at this point. Without any way to computationally model or calculate how additional recruitment of one or more transcription factors may affect the transcriptional complex stability, it is also unclear how much both uncontrolled and controlled variables have influenced these results.

The specificity of the reporters for myofibroblasts remains unclear with *in vitro* results. It is possible that the TGFb treatment induced endothelial to mesenchymal transition in the aortic vascular smooth muscle cells thereby triggering upregulation of alpha smooth muscle actin or collagen 1A. However, alpha smooth muscle actin expression is typical for vascular smooth muscle cells to begin with. Furthermore, collagen 1A is excreted by vascular smooth muscle cells in early stages of vascular development to build the elastic components of vessels. We would expect to see more luciferase expression in the minimum promoter only constructs if the addition of the double 9x tandem SRF/NFAT chimeric enhancer truly increases signal specificity. This is not supported by the results, thus further investigation in more cell types *in vitro* as well as *in vivo* experiments are warranted to pick apart the chimeric reporter off target activity.

The lack of significant luciferase upregulation compared to background from AngII treatment both in the fibroblasts is unexpected. Angiotensin II, a well-known profibrotic agonist, promotes fibroblast transdifferentiation through a G coupled protein receptor, AT1, and utilizes the downstream mediator SRF. TGFb induced transdifferentiation also works through SRF *via* the

non-canonical p38 MAPK pathway. Therefore, it is strange that TGFb is producing a robust signal but not AngII. This could be due to downregulation of components of the AngII signaling cascade or compromised AngII reagent. Other laboratory members were also having similar problems with AngII induced myofibroblast transdifferentiation, so compromised reagent is more likely to be the main cause of the lack of luciferase signal and visual transdifferentiation phenotype observed throughout the AngII treatments. TGFb and AngII were hypothesized to produce a strong signal with addition of the chimeric SRF/NFAT enhancer due to SRF mediated upregulation TRPC6 of the calcium induced calcineurin-NFAT transdifferentiation signal cascade. Thus, TGFb and AngII signaling should utilize not only the SRF binding sites but also the NFAT binding sites if the calcium-calcineurin-NFAT pathway is active as expected *in vitro*.

Study limitations and future directions

Initial promising *in vitro* results in NIH 3T3 immortalized embryonic fibroblasts warrants further investigation of reporter performance in more *in vitro* studies as well as *in vivo* cardiac injury models. These studies were all performed in 6 well tissue culture plates with cells from a variety of sources. Of note, the results in the NIH 3T3 were unable to be replicated in a different line of NIH 3T3 cells ordered from the ATCC after the initial stock of cells were depleted. Immortalized cell lines are known to undergo large genetic drift through many passages. Thus, the newer batch of NIH 3T3 cells could have been significantly different from the initial cells on the level of transcriptome and so forth, partially explaining the reproducibility of the results. Moreover, a longitudinal *in vitro* study is warranted as the reporter is designed to boast longitudinal reporting capacity *in vivo*. Ultimately, *in vivo* cardiac injury models will provide the most robust characterization for the reporters by comparing luciferase expression to extent of fibrosis. The results could also be further validated against transgenic reporting of alpha smooth muscle actin positive TCF21 lineage fibroblasts which are considered to be bonafide myofibroblasts.

Methods and Materials

Cell maintenance

NIH 3T3, MEFs, and vascular smooth muscle cells were cultured in Dulbecco's Modified Eagle Medium () with 10% Fetal Bovine Serum (FBS), penicillin, and streptomycin in 10 cm tissue culture petri plates (Falcon) and stored in an incubator at 37 degree Celsius and 5% carbon dioxide.

Cells were passaged every other day at 200,000 cells per milliliter. In passaging, the old media was aspirated and the plate was washed twice with 5 mL phosphate buffered saline (PBS). The cells were incubated in 1.5 mL trypsin for 2 minutes. The plate was then tapped gently and cell suspension was confirmed under microscope. Fresh media, 8.5 mL, was added to the trypsin and mixed via pipetting. Cells and media were transferred to a conical tube. In a new tissue culture plate, 3 mL of cells was mixed with 7 mL of fresh media.

Transfection and luciferase assay

NIH T3T cells were plated on 6-well tissue culture plates (Falcon) at 200,000 cells per well in DMEM 10% FBS and penicillin/streptomycin. Once cells were 75-90% confluent (approximately 24 hours later), transfection was performed with Lipofectamine 3000 (ThermoFisher Scientific) with 6 µg of DNA per well, 8 µL of Lipofectamine, and 6 µL of P3000. Media was changed after 24 hours then all cells were serum starved in 2% FBS media for <24 hours. TGFβ was added to transdifferentiation treatment groups and after 24 hours, cells were harvested with luciferase assay lysis buffer and stored at -80 degrees Celsius for later luciferase assays. Assays were performed by mixing 20 µl thawed cell lysis solution with 180 µL 1x Luciferin luciferase assay buffer in a black 96-well microplate. Fluorescence was measured immediately after mixing with an EnVision 2104 Multilabel Reader (PerkinElmer) using Wallac EnVision Manager software.

For MEFs and vascular smooth muscle cells, the luciferase assay protocol described above was used, but the transfection agent was substituted with 2 µL of XtremeGene HP™ (Roche) Transfection Agent and 2 or 4 µg of plasmid.

Double transfections of reporter plasmids with pMaxGFP plasmid (Amaxa) was performed for either all wells or one well of the no treatment control to control for transfection efficiency.

Molecular cloning

Custom promoter and enhancer constructs were synthesized from GeneWiz (Seattle, WA) in pUC57-KAN plasmids, reconstituted into competent DH5α E.coli cells, and stored in glycerol stock at -80 degrees Celsius. Stock pUC57 plasmid with constructs were obtained by inoculating 200 mL LB with glycerol stock and 1x kanamycin and incubating overnight. DNA was isolated through Maxi-prep (E.Z.N.A Plasmid Maxi Kit, Omega). Promoters and enhancers were removed from pUC57 through overnight restriction digest and gel purified (QIAquick Gel Extraction Kit,

Qiagen). Isolated DNA was kept at -20 degree Celsius until use. Promoters were ligated into plasmid at 1:1 and 1:2 ratios. Ligated DNA was transformed into DH5α competent cells, stored as glycerol stock at -80 degree Celsius, mini-prepped (Qiaprep Spin Miniprep Kit, Qiagen), and checked for successful ligation through agarose gel electrophoresis and restriction digest.

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Explanation of statistics

To see if the difference between the means of the luciferase expression for the TGFB treatment groups in the 9x SRF and 9x NFAT COL1A2 trial, a two-tailed independent two-sample student's t-test was performed. Because the number of samples between 9x SRF, 9xNFAT, and CMV as well as their variances differed, to find statistical significance in the difference in means, a two tailed Welch's t-test was performed for all statistical comparisons.

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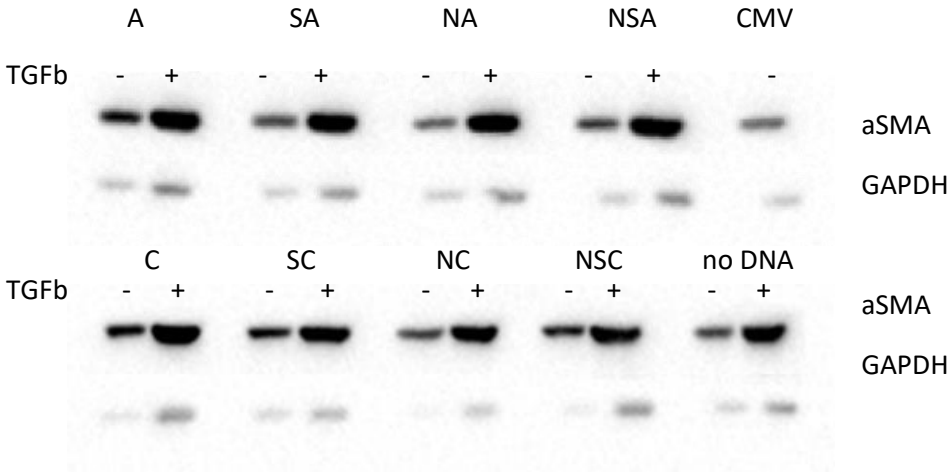

Supplemental figure 1. Western protein blot for the NIH 3T3 luciferase assay corresponding to Figure 3. The western was not protein normalized prior to loading due to incompatibility of the luciferase assay lysis buffer with the BCA assay. Band quantification was not viable due to saturation of the ACTA2 lanes in TGFb+ treatment groups.