Published in final edited form as:

*Circulation*. 2016 January 19; 133(3): 291–301. doi:10.1161/CIRCULATIONAHA.115.018174.

# MiR-125b is Critical for Fibroblast-to-Myofibroblast Transition and Cardiac Fibrosis

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## Abstract

**Background—**Cardiac fibrosis is the pathological consequence of stress-induced fibroblast proliferation and fibroblast-to-myofibroblast transition (FMT). MicroRNAs have been shown to play a central role in the pathogenesis of cardiac fibrosis. We identified a novel miRNA-driven mechanism that promotes cardiac fibrosis via regulation of multiple fibrogenic pathways.

**Methods and Results—**Using a combination of *in vitro* and *in vivo* studies, we identified that miR-125b is a novel regulator of cardiac fibrosis, proliferation and activation of cardiac fibroblasts. We demonstrate that miR-125b is induced in both fibrotic human heart and murine models of cardiac fibrosis. In addition, our results indicate that miR-125b is necessary and sufficient for the induction of FMT by functionally targeting apelin, a critical repressor of fibrogenesis. Furthermore, we observed that miR-125b inhibits p53 to induce fibroblast proliferation. Most importantly, *in vivo* silencing of miR-125b by systemic delivery of locked nucleic acid (LNA) rescued Angiotensin II-induced perivascular and interstitial fibrosis. Finally, the RNA-sequencing analysis established that miR-125b altered the gene expression profiles of the key fibrosis-related genes and is a core component of fibrogenesis in the heart.

**Conclusions—**In conclusion, miR-125b is critical for induction of cardiac fibrosis and acts as a potent repressor of multiple anti-fibrotic mechanisms. Inhibition of miR-125b may represent a novel therapeutic approach for the treatment of human cardiac fibrosis and other fibrotic diseases.

### Keywords

Cardiac fibrosis; miR-125b; Fibroblast-to-myofibroblast transition (FMT); TGF-β; Angiotensin II; RNA-seq

### Journal Subject Terms

Heart Failure; Remodeling; Hypertension; Gene Expression and Regulation; Growth Factors/ Cytokines

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**Disclosures:** None.

## INTRODUCTION

Cardiac fibrosis is a common pathological hallmark of a wide variety of chronic and acute cardiovascular disorders. It is characterized by the excessive deposition of fibrous extracellular matrix (ECM) proteins in the heart, resulting in myocardial stiffness, cardiac remodeling, and eventual heart failure[1–6]. During cardiovascular insult, Angiotensin II (Ang II) activates transforming growth factor-β (TGF-β) signaling to mediate cardiac fibrosis by accumulation of fibroblasts and fibroblast-to-myofibroblast transition (FMT)[2, 5–11]. TGF-β orchestrates profibrotic signaling by binding to the constitutively active TGF-β receptor II, which in turn phosphorylates TGF-β receptor I kinase (TβR1) to activate Smad-dependent TGF-β canonical pathway[2, 5, 6, 10]. In addition, TGF-β can also signal through non-canonical pathways including ERK1/2, p38 MAPK and PI3K/Akt. These non-canonical pathways can coordinate with the Smad-dependent canonical pathway to induce fibrosis[2, 6, 10]. Furthermore, the negative regulators of the TGF-β pathway including p53 and apelin are well-defined to protect against fibrosis[2, 10, 12–16] .

Numerous pre-clinical and animal model studies suggest that cardiac fibrosis can be prevented by the inhibition of TGF-β[17–20]. However, the success of TGF-β-based anti-fibrotic therapies has been limited due to their adverse effects on immune function[2, 10, 19]. Therefore it is absolutely necessary to target the downstream effectors of TGF-β that play a critical role in the development of cardiac fibrosis. Recent studies have reported that TGF-β regulated microRNAs (miRNAs) play a crucial role in various disease pathologies[21–23]. MiRNAs are short (~ 20–22 nucleotide), endogenous, noncoding single-stranded RNA molecules that negatively regulate gene expression by mRNA degradation or inhibition of mRNA translation. Strikingly, a single miRNA has the ability to regulate multiple mRNA molecules associated with a biological process[24, 25]. Alteration of miRNA levels plays a critical role in various pathophysiological processes in the heart[25–31]. The goal of this study was to identify novel miRNAs that are regulated by TGF-β profibrotic signaling in order to elucidate the mechanism underlying the pathogenesis of cardiac fibrosis.

We have previously reported differential expression of miRNAs during cardiac endothelial-to-mesenchymal transition (EndMT)[21]. In particular, we identified that miR-125b was markedly upregulated in TGF-β-induced EndMT-derived myofibroblast-like cells[21]. Notably, miR-125b is highly conserved across species and is reportedly upregulated in diverse cardiac disorders[32–34]. However, the functional role of miRNAs in the induction of cardiac FMT or cardiac fibrosis has not yet been characterized. In this study, we investigated for the first time, the mechanistic role of miR-125b in cardiac FMT, fibroblast proliferation and cardiac fibrosis. We tested the therapeutic potential of inhibition of miR-125b in an Ang II model of cardiac fibrosis. Using a combination of *in vitro* and *in vivo* studies, we demonstrate that miR-125b is a critical component of profibrotic signaling in the heart. We conclude that TGF-β-induced upregulation of miR-125b results in inhibition of anti-fibrotic genes to promote both proliferation and activation of the cardiac fibroblasts, eventually resulting in cardiac fibrosis.

## METHODS

Refer to Supplemental Materials for expanded and detailed information.

### Human studies

All the protocols and the use of human heart tissues were approved by Northwestern University Institutional Review Board (IRB# STU00012288) and the subjects gave informed consent.

### Animal studies

All the experimental procedures were approved by the IACUC of Northwestern University and were in accordance with Northwestern's guidelines.

### Cell Culture

Normal human cardiac fibroblasts (HCFs) were purchased from Cell Applications, Inc (San Diego, CA). Fibroblasts were maintained in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Carlsbad, CA).

### Overexpression or knockdown of miR-125b

miR-125b mimic (Ambion, Austin, TX) or negative control (Ambion Austin, TX) were used for overexpression of miR-125b. For knockdown experiments, custom-designed 1 μM antagomir-125b (Thermo Scientific, Waltham, MA) or negative control (antagomir-control, Thermo Scientific, Waltham, MA) were used.

### Cell Proliferation Assay

Fibroblast proliferation was determined using MTT Cell Proliferation Assay Kit (ATCC, Manassas, VA) according to manufacturer's instructions.

### Osmotic mini-pump implantation and Ang II infusion to induce cardiac fibrosis

Wild type mice (C57BL/6J) were infused with Ang II (1.6 μg/kg/min; Bachem, Torrance, CA), or a vehicle control (saline). LNA-125b (15 mg/kg) or scrambled LNA (15 mg/kg) (Exiqon, Vedbaek, Denmark) was injected via tail-vein at day 1, 3 and 8 of Ang II infusion.

### Transverse aortic constriction (TAC) surgery

C57BL/6J black mice were subjected to transverse aortic constriction for 28 days as described in Verma *et al.*[35].

### Blood pressure measurements

A non-invasive tail-cuff device (Volume Pressure Recording, CODA, Kent Scientific Corp, CT) was used to measure systolic blood pressure in conscious mice (n=6–8).

### Ki67 staining in the mouse heart

Serial sections of mouse hearts were immunostained for Ki67. An automated TissueGnostics imaging system was used to image the heart sections. Quantification of Ki67 staining in control and treated groups was performed by using HistoFaxs software.

### Histopathology analysis

Masson's trichrome staining was used to stain all forms of collagen in the heart. The fibrotic area was quantified by calculating the percentage of collagen staining (blue staining) using Image-pro analysis (Media Cybernetics Rockville, Rockville, MD).

### Luciferase reporter construct and assay

GLuc/SEAP dual-reporter vector system was employed to assess for miR-125b binding to 3′UTR of apelin.

### RNA-seq analysis

HCFs were transfected with either miR-125b mimic or control mimic (n=3) and RNA was sequenced using Ion Proton™ System platform. Fastq files were analyzed using Tuxedo package and downstream pathway analysis was performed using Ingenuity Pathway Analysis.

### Statistical Analysis

Data are presented as Mean ± SEM. Statistical significance was evaluated using unpaired Student's T-test with Welch's correction for comparing two groups. For comparing three or more groups, means were compared using one-way analysis of variance accounting for differences in variance among the groups. Post-hoc pairwise comparisons were done using the Tukey-Kramer method. Data were analyzed using GraphPad prism 6 or SAS PROC MIXED statistical software, which allows for specification of group specific variances.

## RESULTS

### miR-125b is upregulated in fibrotic human heart and murine model of cardiac fibrosis

Using an unbiased miRNA array approach, we have previously reported that miR-125b expression was significantly enhanced during TGF-β-induced endothelial-to-mesenchymal transition (EndMT)[21]. In addition, Ang II, a potent inducer of cardiac fibrosis, has been shown to enhance miR-125b levels in cardiac fibroblasts[36]. In this study, we directly compared the expression of miR-125b between normal and failing human hearts. Our results demonstrate that miR-125b expression was significantly upregulated in the failing human hearts compared to normal hearts (Fig. 1A). In order to investigate if the failing human hearts exhibited fibrosis, we measured the mRNA levels of various markers of fibrosis including Type I Collagen (Col1), alpha smooth muscle actin (α-SMA), Type III Collagen (Col3), and Plasminogen activator inhibitor-1 (PAI-1). Col 1 is the major ECM component and accounts for most of the collagen (85% to 90%) and Col 3 accounts for only about 5% of the total collagen in the cardiac interstitium[37]. We observed that failing human hearts exhibited a significant increase in mRNA levels of Col1 and α-SMA (Fig. 1B–C). In

addition, mRNA levels of Col 3 and PAI-1 were also elevated in failing human heart tissues (Supplementary Fig. 1A–B, $p > 0.05$). Notably, the failing human heart tissues were disorganized and stained heavily for total collagen as evidenced by Masson's Trichrome staining (Fig. 1D). Additionally, hearts of mice subjected to transverse aortic constriction (TAC) exhibited considerably higher amounts of collagen as compared to sham surgery (Fig. 1E). Next, we evaluated the expression levels of miR-125b in a TAC model of cardiac fibrosis. Consistent with the results in fibrotic human hearts, we observed a modest upregulation of miR-125b expression in the TAC-subjected hearts compared to the sham group (Fig. 1F). As expected, mRNA levels of Col1 and α-SMA were elevated in the hearts of TAC-subjected mice compared to sham (Fig. 1G–H). Although not statistically significant, Col3 and PAI-1 were also upregulated in TAC subjected mice (Supplementary Fig. S1C–D). Collectively, these findings indicate a strong correlation between expression of miR-125b and cardiac fibrosis in both mice and humans.

### miR-125b is upregulated during TGF-β-induced FMT

FMT-derived myofibroblasts are the major source of collagen and other ECM proteins during pathological matrix remodeling[2, 10, 19]. Importantly, persistent TGF-β signaling is the primary mechanism of FMT and fibrosis[2, 10, 19]. We investigated if miR-125b is downstream to TGF-β-induced fibrotic signaling during FMT. The results of qRT-PCR analysis indicated that miR-125b was significantly upregulated in TGF-β-treated fibroblasts compared to vehicle alone, while treatment with SB431542 (a small molecule inhibitor of TβR1) completely protected against TGF-β-induced miR-125b (Fig. 2A, Supplementary Fig. S2A–B). As expected, treatment with TGF-β strongly induced the molecular markers of FMT (α-SMA and Col1) while the inhibition of TβRI by SB431542 treatment prevented upregulation of α-SMA and Col1 (Fig. 2B–C). As shown in Fig. 2D, TGF-β treatment induced a morphology shift in fibroblasts from spindle-shaped morphology to a well-spread myofibroblast-like morphology. In comparison, treatment with SB431542 exhibited no change in morphology upon TGF-β treatment (Fig. 2D). In addition, fluorescence staining demonstrated decreased α-SMA staining in the fibroblasts co-treated with SB431542 and TGF-β as compared to TGF-β treatment alone (Supplementary Fig. S2C). Our findings indicate that miR-125b expression is controlled by the TGF-β-canonical signaling pathway and is upregulated during TGF-β-induced FMT.

### miR-125b is necessary and sufficient for the induction of FMT

To evaluate the functional significance of miR-125b in the activation of FMT, we overexpressed miR-125b in HCFs (Supplementary Fig. S3B). Transfection efficacy in fibroblasts was further confirmed by transfecting cy3-tagged mimic control for 24 hours (Supplementary Fig. S3A). The overexpression of miR-125b induced a myofibroblast-like morphology in fibroblasts (Supplementary Fig. S3C), and upregulated mRNA and protein expression of α-SMA and Col1 (Fig. 3A–C). Next, fibroblasts overexpressing miR-125b were treated with TGF-β in order to mimic the conditions present during FMT-induced cardiac fibrosis. As expected, treatment with TGF-β alone induced a myofibroblast-like morphology (Supplementary Fig. S3C), and upregulated the expression of molecular markers of FMT (α-SMA and Col1) (Fig. 3A–C). Notably, overexpression of miR-125b further augmented TGF-β-induced Col1 expression (Fig. 3B). In addition, the

overexpression of miR-125b further increased mRNA and protein expression of α-SMA (Fig. 3A, 3C). These findings indicate that overexpression of miR-125b is sufficient to induce FMT and potentiates TGF-β-induced FMT.

To investigate if miR-125b is required for the activation of TGF-β-induced FMT, we designed a specific antagomir against miR-125b (antagomir-125b) to knockdown the expression of miR-125b (Supplementary Fig. S4A, S4C). Fluorescence microscopy revealed efficient uptake of cy3-tagged antagomir-125b in fibroblasts (Supplementary Fig. S4B). As shown in Supplementary Fig. S4D, antagomir-125b-treatment prevented induction of myofibroblast-like morphology compared to TGF-β treatment alone. Notably, the knockdown of miR-125b attenuated TGF-β-induced mRNA and protein expression of α-SMA and Col1 (Fig. 3D–F). We conclude that miR-125b is essential for the activation of TGF-β-induced FMT.

## Selective inhibition of miR-125b attenuates Ang II-induced cardiac fibrosis *in vivo.*

Next, we used an Ang II model of cardiac fibrosis to establish the functional significance of miR-125b in the induction of cardiac fibrosis *in vivo*. On account of the vasoconstrictive action of Ang II, we observed an increase in systolic blood pressure in Ang II-infused mice (Supplementary Fig. S5A). As shown in Supplementary Fig. S5B, Ang II-infused animals exhibited an increase in heart weight; however, there was no statistical difference between heart weights of LNA-125b treated and LNA-125b + Ang II infused mice. In addition, the inhibition of miR-125b in the absence of Ang II treatment had no apparent effect on heart weight (Supplementary Fig. S5B). As shown in Fig. 4A, cardiac miR-125b was upregulated by 1.7-fold in Ang II-infused animals ($P = 0.07$). In addition, knockdown of miR-125b by treatment with LNA-125b attenuated Ang II-induced miR-125b expression. Moreover, we observed a significant upregulation of PAI-1 and Col1 mRNA expression in the hearts of Ang II-infused animals (Fig. 4B, 4D). Importantly, these markers were significantly attenuated by LNA-125b treatment (Fig. 4B, 4D). However, Ang II treatment did not upregulate cardiac α-SMA expression either in the presence or absence of LNA-125b (Fig. 4C). In the setting of blunted Col1 production, we observed an increase in the expression of Col3 in Ang II + LNA-125b group as compared to treatment with Ang II (Fig 4D, Supplementary Fig. S6). However, we observed a remarkable reduction in the overall collagen deposition with the treatment of LNA-125b in Ang II-infused mice, as demonstrated by Masson's trichrome staining (Fig. 4E–G). We conclude that Ang II-iinfusion induced both perivascular and interstitial fibrosis (Fig. 4E–G). Most importantly, treatment with LNA-125b significantly prevented Ang II-induced perivascular and interstitial fibrosis (Fig. 4E–G). Together, these results establish for the first time that miR-125b is essential for the induction of Ang II-induced cardiac fibrosis *in vivo*. In accordance, the inhibition of miR-125b attenuates hypertension-induced cardiac fibrosis independent of blood pressure changes.

## miR-125b induces fibroblast proliferation via inhibition of p53

p53 is an important negative regulator of the fibrogenic process[15, 16, 38], and increased expression of p53 is known to suppress fibroblast proliferation[39, 40]. In addition, p53 is a *bona fide* target of miR-125b in various cell types[32, 33]. *In silico* analysis predicted that

miR-125b targets the 3′UTR region of p53 (Fig. 5A). Importantly, overexpression of miR-125b or treatment with TGF-β significantly decreased p53 protein expression during FMT (Fig. 5B). We observed that miR-125b overexpression induced fibroblast proliferation (Fig. 5C–D). To test if the miR-125b-induced proliferation is mediated by p53, fibroblasts were co-transfected with p53 and miR-125b. Although forced expression of p53 prevented fibroblasts proliferation, the overexpression of miR-125b in the presence of overexpressed p53 was sufficient to restore the proliferation capacity of fibroblasts (Fig. 5D). Consistent with this *in vitro* observation, *in vivo* data revealed that while Ki67 staining (a proliferation marker) was significantly increased in Ang II-infused myocardial tissues, however, Ang II treatment did not induce proliferation in the LNA-125b treated group (Fig. 5E–F). Our results indicate that miR-125b plays an important role in fibroblast proliferation and this process is mediated via suppression of p53, a negative growth regulator and anti-fibrotic factor.

### miR-125b functionally targets apelin to augment cardiac FMT

miRNAs can have a robust effect on a single biological pathway by modulating multiple mRNA targets[25, 33]. *In silico* analysis using TargetScan Human v.6.2 predicted that miR-125b targets a putative 3′UTR site of apelin (Fig. 6A), an important repressor of the fibrogenic pathway. Recent studies have reported that apelin is a key suppressor of the Ang II-TGF-β axis, and is protective against heart failure[12–14, 23]. However, the mechanism by which endogenous cardiac apelin signaling is regulated is poorly understood. In order to determine the effect of miR-125b on apelin expression, we overexpressed miR-125b in cardiac fibroblasts both in the presence and absence of TGF-β. The results revealed that mRNA expression of apelin was significantly downregulated upon treatment with TGF-β (Fig. 6B). Importantly, overexpression of miR-125b alone was sufficient to inhibit apelin expression (Fig. 6B). Additionally, *in vivo* studies indicated that cardiac expression of endogenous apelin was highly upregulated in Ang II-infused mice treated with LNA-125b compared to Ang II treatment alone (Fig. 6C) suggesting a potential regulatory mechanism to counteract cardiac stress in response to the inhibition of miR-125b. Notably, Luciferase reporter assay elucidated that miR-125b directly binds to 3′UTR of apelin (Fig. 6D). Taken together, our results indicate a novel finding that miR-125b is an important negative regulator of apelin and thus potentiates the fibrogenic pathway.

To evaluate if apelin is functionally important for the induction of FMT, we tested the effect of knockdown of apelin in cardiac fibroblasts (Fig. 6E). Results indicate that knockdown of apelin upregulated mRNA and protein expression of α-SMA and Col1 (Fig. 6F–H). In addition, the knockdown of apelin followed by treatment with TGF-β exhibited further increase in the protein expression of α-SMA and Col1 (Fig. 6G). Furthermore, fibroblasts transduced with apelin shRNA exhibited myofibroblast-like morphology (Supplementary Fig. S7A). Our findings indicate that overexpression of miR-125b (Fig. 3A–C, Supplementary Fig. S3C) and knockdown of apelin (Fig. 6E–H, Supplementary Fig. S7A) in fibroblasts have a similar effect in the induction of FMT. Although not statistically significant, exogenous treatment of apelin in the presence of TGF-β decreased the mRNA expression of α-SMA and Col1 (Supplementary Fig. S7B–C). Finally, we tested the ability of apelin to prevent miR-125b-induced FMT by treating HCFs with exogenous apelin in the

presence of miR-125b overexpression. Interestingly, there was a blunted response in the FMT markers (α-SMA and Col1) in the presence of apelin treatment (Supplementary S8A–B). Collectively, these findings provide strong evidence that miR-125b activates FMT at least in part via inhibition of apelin, a well-defined repressor of profibrogenic signaling.

### miR-125b regulates multiple gene networks in fibroblasts

A single miRNA can degrade multiple mRNAs in parallel, which may further regulate a different pool of genes resulting in the interaction of diverse gene networks. In order to gain an in-depth understanding of the role of miR-125b in fibrotic gene regulation, we performed a RNA-seq analysis on HCFs both in the presence or absence of miR-125b overexpression. As shown in Fig. 7A–B, about 1120 genes were differentially expressed in the presence of miR-125b mimic. The miR-125b pathway analysis elucidated that various direct targets of miR-125b were downregulated (data not shown). Importantly, the Ingenuity pathway analysis demonstrated significant regulation of TGF-β, p53, and proliferation pathways (Fig. 7C–D, Supplementary Fig. S9A–B). The results also establish that miR-125b regulated various other fibrosis-related processes and pathways (Supplementary Fig. S9A–B). These results confirm that higher than normal levels of miR-125b are pathological and miR-125b is at the center-stage of fibrosis signaling. Finally, the RNA-seq data strongly suggests that miR-125b induces an epistatic regulation of important gene networks and various cellular processes that are indispensable for fibrogenesis (Fig. 7C–D, Supplementary Fig S9A–B).

## DISCUSSION

In the cardiovascular system, miRNAs fine-tune complex molecular signaling networks by downregulating key target proteins involved in a variety of cellular processes[25]. In the past decade, several studies have emphasized the importance of miRNAs in mediating cardioprotective effects and the progression of cardiovascular disease[8, 25, 27, 30, 31, 41, 42]. There is a growing consensus that targeting a single miRNA can form an eloquent strategy for the treatment of complex diseases like cardiac fibrosis. However, identifying novel miRNAs that regulate cardiovascular disorders is still an active area of research. We have previously identified miR-125b as one of the highly upregulated miRNAs during cardiac EndMT[21]. Here we report that miR-125b is pathologically upregulated in FMT-derived myofibroblasts and fibrotic human hearts. We discovered that miR-125b is a direct effector of TGF-β canonical signaling and is indispensable for hypertension-induced cardiac fibrosis. We have further presented substantial evidence supporting that miR-125b augments FMT and cardiac fibrogenesis by targeting p53 and apelin, two potential repressors of the profibrogenic process. Importantly, the RNA-seq analysis unraveled an important finding that miR-125b significantly regulates about 5.57% of all the genes in the human genome.

The prevailing paradigm suggests that cardiac fibrosis is the pathological consequence of persistent activation of TGF-β signaling[1–4, 6, 8]. Beyond activating profibrotic genes, TGF-β functionally impairs the anti-fibrotic proteins, both of which are instrumental for the progression of cardiac fibrosis[2, 3, 6]. In this study, we have discovered that miR-125b is a crucial component of the TGF-β-mediated suppression of the anti-fibrotic cascade. To our

knowledge, this is the first report demonstrating that miR-125b is regulated in response to diverse fibrotic stimulus and plays a functional role in cardiac fibrogenesis.

Activation of multiple key fibrogenic pathways has been shown to contribute to the progression of fibrosis[2, 5, 6, 10, 16, 18, 21, 41, 43, 44]. Of these, proliferation of the resident fibroblasts is one of the major mechanisms of cardiac fibrosis. This notion is supported by a very recent fate mapping study which demonstrates that the proliferation of resident cardiac fibroblasts leads to fibroblast accumulation following pressure overload[44]. Consistent with these findings, we report that fibrotic mouse hearts exhibited increased number of Ki67 positive cells. Notably, the inhibition of miR-125b prevented the increase in number of Ki67 positive cells in an Ang II model of cardiac fibrosis. In addition, our *in vitro* studies emphasize that miR-125b is a strong inducer of fibroblast proliferation via inhibition of p53. These findings are further supported by our observation that forced expression of p53 blunts the ability of cardiac fibroblasts to proliferate. In contrast, miR-125b rescues the anti-proliferative effect of p53 while imparting higher proliferative capacity to fibroblasts. Several studies have reported that in addition to acting as a negative regulator of cell proliferation, p53 also negatively regulates the production of ECM proteins[15, 16, 38, 39]. Additionally, the inhibition of p53 promotes renal fibrosis in response to acute kidney injury[38]. Furthermore, overexpression of p53 attenuates TGF-β-induced collagen synthesis in dermal and lung fibroblasts and is also associated with mesenchymal transition in epithelial cells[15, 45]. The present study along with existing data confirms that suppression of p53 plays an important role in the progression of cardiac fibrosis and this process is controlled by miR-125b.

It is common for miRNAs to have multiple targets in a single biological process[25]. Thus, we also examined other repressors of TGF-β-induced profibrogenic signaling and fibrogenesis. Our results demonstrate a novel finding that apelin is a critical target of miR-125b in the context of cardiac FMT and fibrosis. Apelin has been recently identified as a negative regulator of the Ang II-TGF-β axis[14, 46–48]. Another study has reported that apelin knock-out mice exhibit impaired heart function in response to TAC injury as compared to wild-type TAC[14]. Furthermore, apelin has been shown to have cardio-protective effects by directly antagonizing Ang II signaling[12, 14, 46]. However, the fundamental molecular mechanism underlying the regulation and function of endogenous apelin remained elusive. Here, we provide a novel mechanism highlighting that overexpression of miR-125b as well as TGF-β-induced miR-125b expression suppresses endogenous apelin, thereby promoting myofibroblast transition of cardiac fibroblasts. More importantly, miR-125b directly binds to the 3′UTR of apelin and inhibits apelin expression. This has been supported by the observations that depletion of apelin in cardiac fibroblasts is associated with increased expression of FMT and profibrotic markers at both the transcript and protein levels. This finding is further strengthened by the study elucidating that apelin treatment was sufficient to protect from miR-125b-induced α-SMA levels. Furthermore, administration of LNA-125b significantly increased the level of apelin in the presence of Ang II compared to Ang II alone. Based on these observations we speculate that higher level of apelin is sufficient to blunt Ang II-induced cardiac fibrogenesis. These observations strengthen our notion that miR-125b is an important determinant of cardiac fibrogenesis via downregulation of key repressors of the profibrogenic pathway.

Despite the high incidence of morbidity and mortality related to cardiac fibrosis, no anti-fibrotic therapies are currently available[2, 3, 5, 27]. Although angiotensin converting enzyme inhibitors, angiotensin receptor blockers, and aldosterone synthase inhibitors have shown promise to benefit against cardiac fibrosis, targeting the direct molecular effectors holds the key to developing more potent therapeutic interventions[49]. Recent clinical trials targeting disease-associated miRNAs by an anti-miR approach have highlighted the potential for miRNA-mediated therapeutic intervention[27, 41, 50]. In addition to identifying novel molecular mechanisms associated with cardiac fibrosis, we directly evaluated the therapeutic potential of the inhibition of miR-125b in a murine model of cardiac fibrosis. We examined the changes in cardiac remodeling caused by Ang II by quantifying the extent of perivascular and interstitial fibrosis. Ang II-infused mice exhibited robust collagen deposition in both perivascular and interstitial regions. This increase in fibrosis was markedly attenuated by inhibition of miR-125b as these mice had a marginal increase in the extent of fibrosis as compared to the control group. The capacity of the LNA-125b to reduce the development of cardiac fibrosis suggests that inhibition of miR-125b alone or in combination with other targets may represent a promising therapeutic approach. On the other hand, genetic manipulation of miR-125b may provide further insight into the mechanistic role of miR-125b in cardiac fibrosis. Therefore, we are generating miR-125b knock-out mice to further our understanding on miR-125b in the context of fibrosis. In addition, it is plausible to dissect the salutary effect of inhibition of miR-125b in different models of heart disease.

Although we did an exhaustive study to define the molecular players involved in fibrosis, it was important to capture a composite picture and characterize the entire system than just individual components. It was critical to perform an unbiased whole transcriptome analysis to understand the epistatic regulation of the genes and to evaluate the therapeutic potential of modulating miR-125b. The RNA-seq analysis in human fibroblasts overexpressing miR-125b has highlighted the regulation of various components of both TGF-β pathway and proliferation pathway in parallel. MiR-125b also affected multiple gene networks and biological processes including cell morphology, cellular movement, cell death, connective tissue disorders, cardiovascular disease, cell cycle, and renal disorders etc. In addition to confirming our current findings, the RNA-seq analysis provided more insights into the overall fibrotic pathways. These findings would be instrumental while studying fibrosis in other organs or to assess potential toxic effects when designing a new fibrosis therapeutic intervention.

In conclusion, we have discovered a novel regulator of cardiac fibrosis and a miR-125b-mediated mechanism underlying the pathology of cardiac fibrosis (Fig. 8). We propose that upon cardiac injury, the Ang II-TGF-β axis triggers the expression of miR-125b, consequently suppressing apelin and p53 expression. These events are collectively responsible for the induction of fibroblast proliferation and cardiac FMT, and other fibrotic gene networks in an epistatic manner. Our findings reveal that miR-125b is critical for the synchronization of the fibrotic machinery by fine-tuning multiple fibrogenic pathways.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

The authors are grateful for technical assistance from Alfred W Rademaker, Sol Misener, Nedereh Jafri and Matthew John Schipma and helpful discussions with Mesut Eren and Panagiotis Flevaris. We also extend our thanks to giftofhope (organ and tissue donor network) for providing with normal human heart samples. Author contributions: V.N., A.K.G. and D.E.V conceived the project. V.N. designed and conducted the experiments. V.N, R.R, A.T.P, and S.B.M were involved in the acquisition of data. V.N, A.K.G and D.E.V were in involved in interpretation of the data. S.K.V provided essential tools. V.N., A.T.P, A.K.G and D.E.V were involved in manuscript preparation.

**Funding Sources:** This study was supported by grants from National Institutes of Health (1P01HL108795-01) to Douglas E. Vaughan and American Heart Association (14PRE18840028) to Varun Nagpal.

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## Clinical Perspectives

Fibrosis is the final common pathway of most chronic diseases and contributes to about 45% of all deaths in the developed world. Despite of morbidity and mortality linked to fibrosis, there are no FDA approved drugs for the treatment of fibrosis. This is the first study to report that therapeutic silencing of a single miRNA; miR-125b attenuates hypertension-induced cardiac fibrosis. Importantly, miR-125b is a critical component of the TGF-β-mediated inhibition of antifibrotic cascade. In particular, miR-125b activates both fibroblast proliferation and fibroblast-tomyofibroblast transition, both of which are critical and common cellular events associated with all fibrotic diseases. Thus, this study may serve as a stepping-stone for investigating the salutary effects of inhibiting miR-125b in otherwise fatal fibrotic disorders. RNA-seq analysis provided us with unprecedented insights into gene networks that are regulated by miR-125b. From a drug discovery standpoint, this analysis is instrumental especially to understand the secondary toxic effects and to investigate other possible applications of targeting miR-125b. We believe that the inhibition of miR-125b may serve as a novel therapeutic target for the treatment of cardiac fibrosis and miR-125b inhibitor can be a promising component of cocktail of drugs for development of new anti-fibrotic treatments.

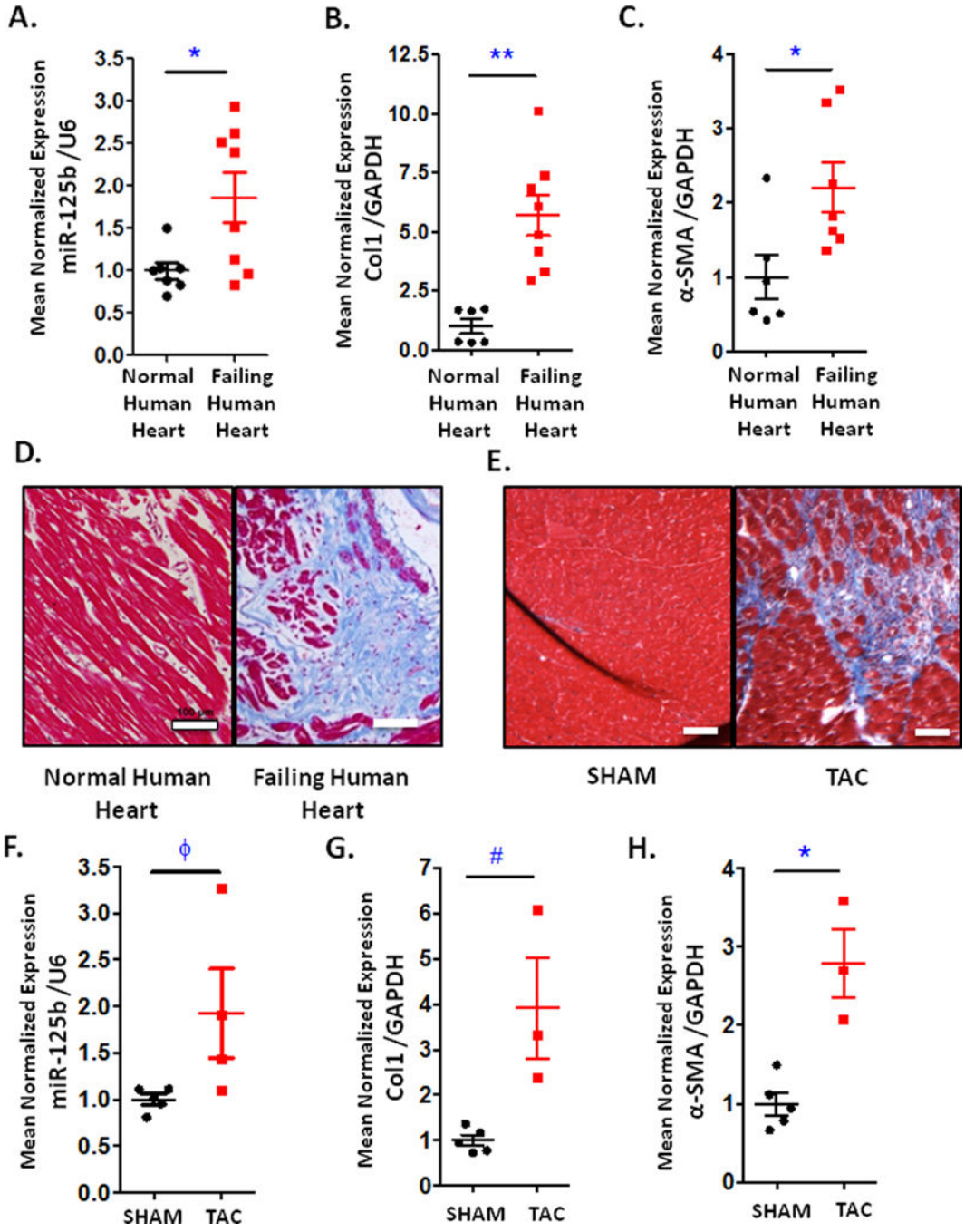


**Figure 1.**

miR-125b expression is induced in both fibrotic human and mouse hearts. The levels of miR-125b (**A**), mRNA expression of Col1 (**B**) and α-SMA (**C**) were measured by qRT-PCR; n=6–8. Representative images of Masson's trichrome staining of the LV sections of normal hearts and failing human hearts (**D**); the hearts from SHAM vs TAC-subjected mice (**E**); n=3–5, Scale bars, 100μm. The levels of miR-125b (**F**) mRNA expression of Col1 (**G**) and α-SMA (**H**) were measured by qRT-PCR. Error bars represent standard error of the mean; n=3–5. *P≤0.05, **P<0.001, $^{\#}$P=0.06, $^{\phi}$P=0.07.

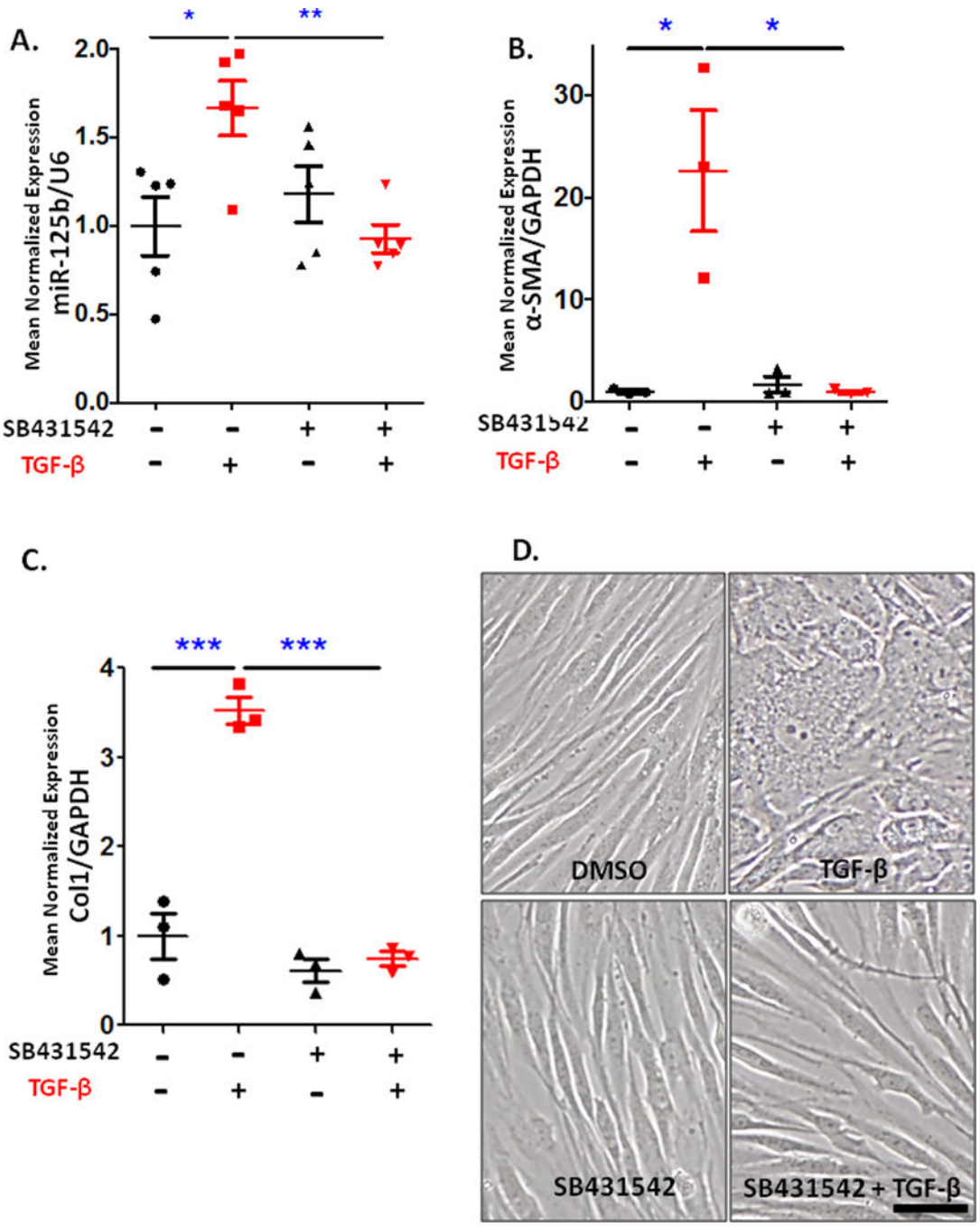


**Figure 2.**

miR-125b is upregulated during TGF-β-induced FMT. The levels of miR-125b (**A**), mRNA expression of α-SMA (**B**), and Col1 (**C**) were measured by qRT-PCR. Bright-field microscopy images representing morphology analysis upon treatment with TGF-β +/− SB431542; Scale bars, 100 μm **(D)** DMSO was used as vehicle control. Error bars represent standard error of the mean; n=3–5. *P≤0.05, **P<0.001, ***P<0.0001, #P=0.06, ns (non-significant).

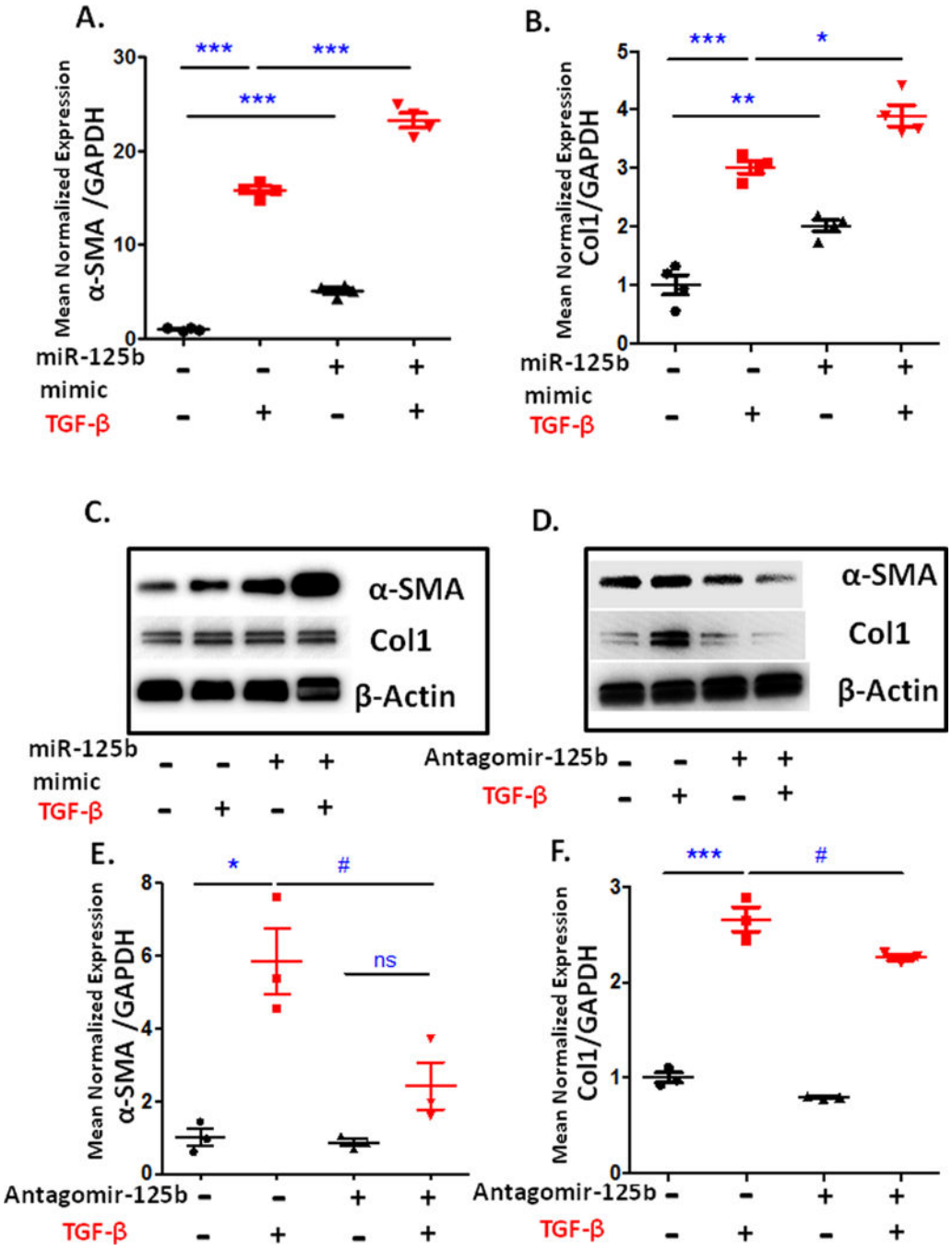


**Figure 3.**

miR-125b is sufficient and necessary for the induction of FMT. The mRNA expression of α-SMA (**A**), Col1 (**B**) and protein expression of α-SMA and Col1 (**C**) in HCFs treated with TGF-β in the presence or absence of miR-125b overexpression. The protein expression of Col1 and α-SMA (**D**), mRNA expression of α-SMA (**E**), and Col1 (**F**) in HCFs treated with TGF-β in the presence or absence of antagomir-125b. mRNA expression was quantified by qRT-PCR analysis, GAPDH mRNA served as an internal control (**A**–**B, E**–**F**). Protein expression was assessed using western blot analysis, β-Actin served as a loading control (**C**–

**D**). Additional negative controls include mimic control (**A**–**C**) and antagomir control (**D**–**F**). Error bars represent standard error of the mean; n=3–4, *P≤0.05, **P<0.001, ***P<0.0001, ns (non-significant).

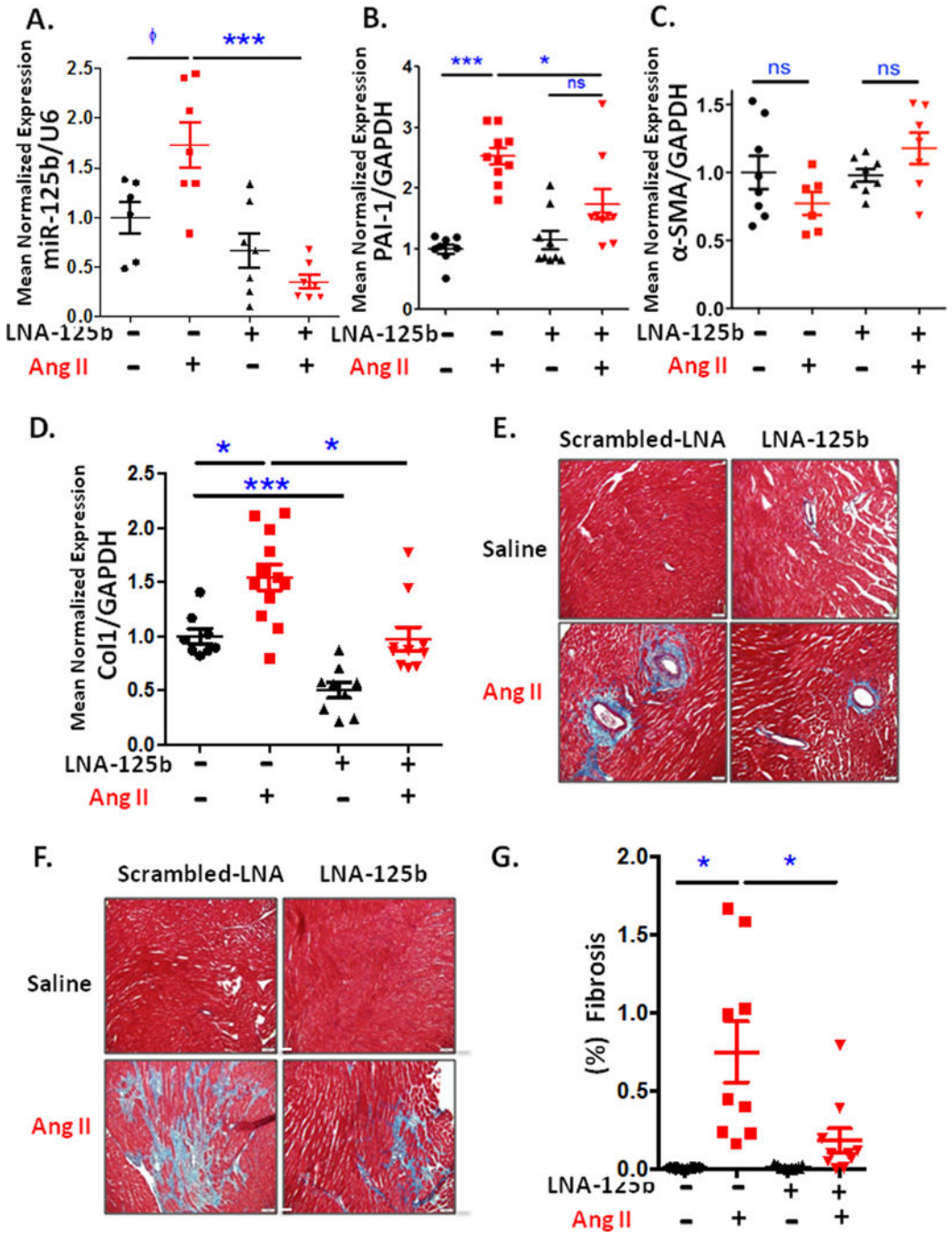


**Figure 4.**

Inhibition of miR-125b protects against Ang II-induced cardiac fibrosis *in vivo.* The levels of miR-125b (**A**), mRNA expression of PAI-1 (**B**), α-SMA (**C**), and Col1 (**D**) were measured by qRT-PCR; Internal controls included U6 snRNA (**A**), and GAPDH mRNA (**B**–**D**); n=6–8 (**A**), n=8–10 (**B**–**D**). Representative images of Masson's trichrome staining in the perivascular region (**E**) and interstitial region (**F**) of the mouse heart. Scale bars, 50 μm. Quantification of the total fibrotic area using Image pro analysis, n=8–10 (**G**). Error bars

represent standard error of the mean; *P≤0.05, ***P<0.0001, ϕP=0.07, ns (non-significant). Scrambled LNA served as a negative control (**A**–**G**).

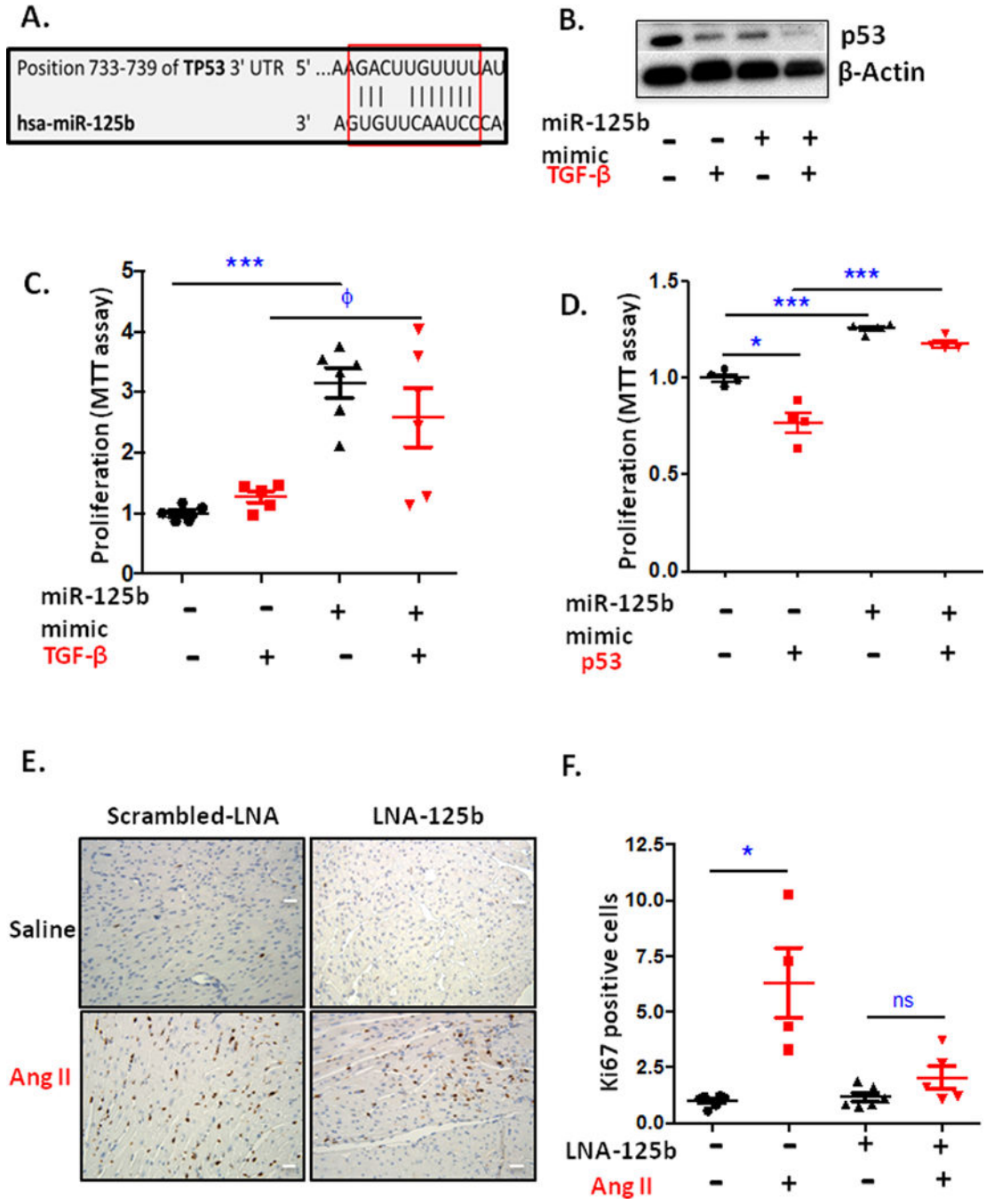


**Figure 5.**

miR-125b regulates fibroblast proliferation. Identification of miR-125b target sites in the 3′ UTR of TP53 using Target scan analysis (**A**). Western blot analysis showing protein expression of p53 in HCFs treated with TGF-β in the presence or absence of mir-125b overexpression; β-Actin served as a loading control and mimic control acted as a negative control (**B**). MTT assay for assessment of proliferation of HCFs overexpressing miR-125b in the presence or absence of TGF-β, (**C**), or p-53 overexpression (**D**). Mimic control acted as a negative control (**C**–**D**), pCMV empty vector served as an additional control (**D**); n=5–6 (**C**),

n=4 (**D**). Immunostaining of Ki67 (proliferation marker) in mouse hearts subjected to AngII-infusion with or without LNA-125 injection; Scale bars, 50 μm (**E**). Quantification of Ki67 positive cells in control and treated groups using HistoFaxs software; n=5 (**F**). Scrambled LNA served as a negative control (**E**–**F**). Error bars represent standard error of the mean; *P≤0.05, ***P<0.0001, ϕP=0.07, ns (non-significant).

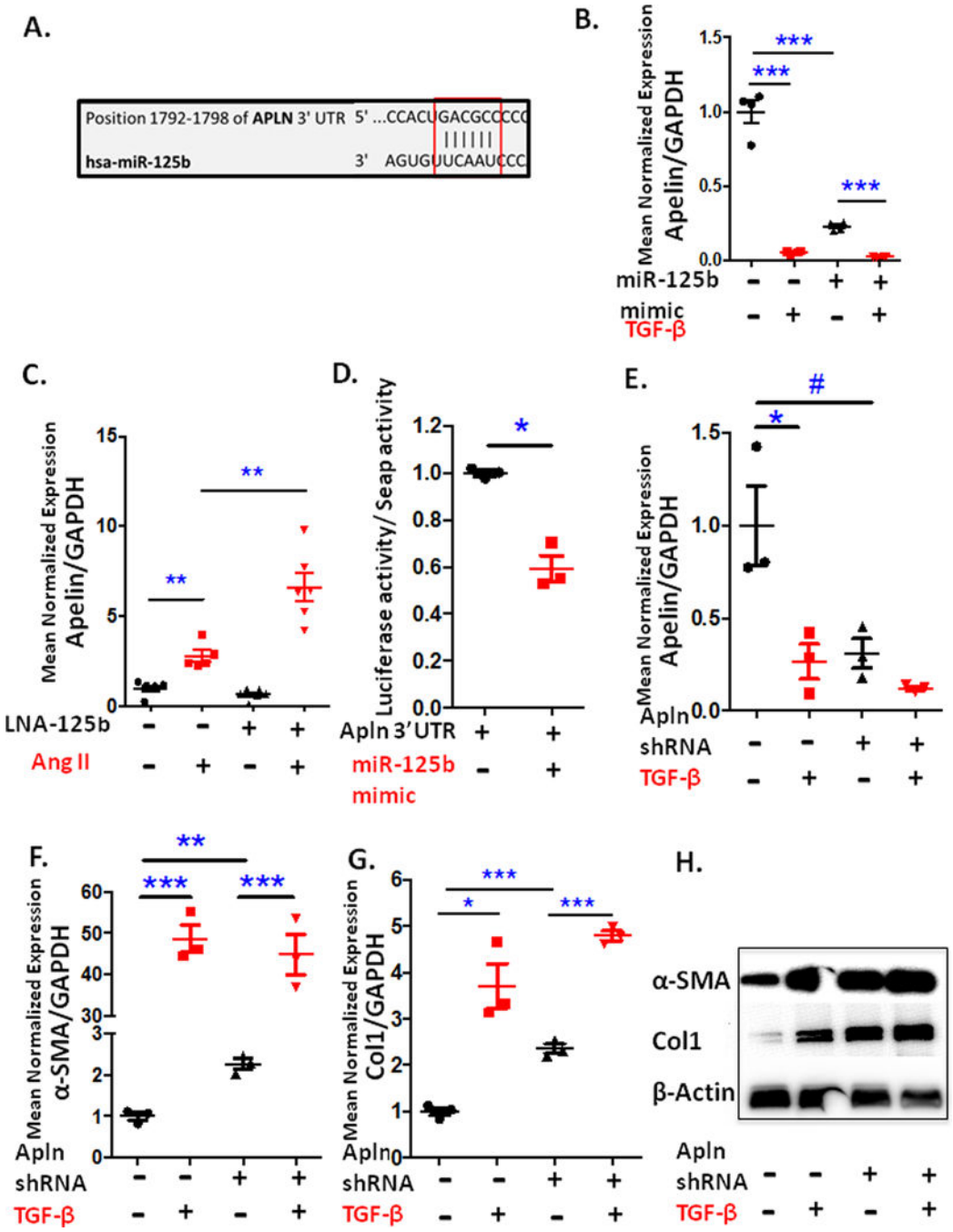


**Figure 6.**

miR-125b functionally targets apelin to induce cardiac FMT. Target scan analysis predicted that 3′UTR of apelin is a direct target of miR-125b (**A**). qRT-PCR analysis showing mRNA expression of apelin in HCFs overexpressing miR-125b in the presence and absence of TGF-β; n=3 (**B**). qRT-PCR analysis showing mRNA expression of apelin in Ang II-infused mouse hearts treated with LNA-125b; Scrambled LNA was used as an internal control;, n=5–6 (**C**). miR-125b mimic significantly decreased the luciferase activity of 3′UTR-apelin luciferase construct; Dual luciferase reporter assay; Seap activity served as internal control; n=3–4. (**D**)

mimic control served as an internal control (**B, D**). qRT-PCR analysis showing mRNA expression of apelin (**E**), α-SMA (**F**) and Col1 (**G**) in HCFs treated with apelin shRNA in the presence and absence of TGF-β; n=3. The level of GAPDH mRNA was used as an internal control (**B**–**C, E**–**G)**. Western blot analysis showing protein expression of α-SMA and Col1 in HCFs treated with apelin shRNA in the presence and absence of TGF-β. β-actin was used as a loading control (**H**). Scrambled shRNA acted as a negative control (**E**–**H**). Error bars represent standard error of the mean; *P≤0.05, **P<0.001, ***P<0.0001, #P=0.06.

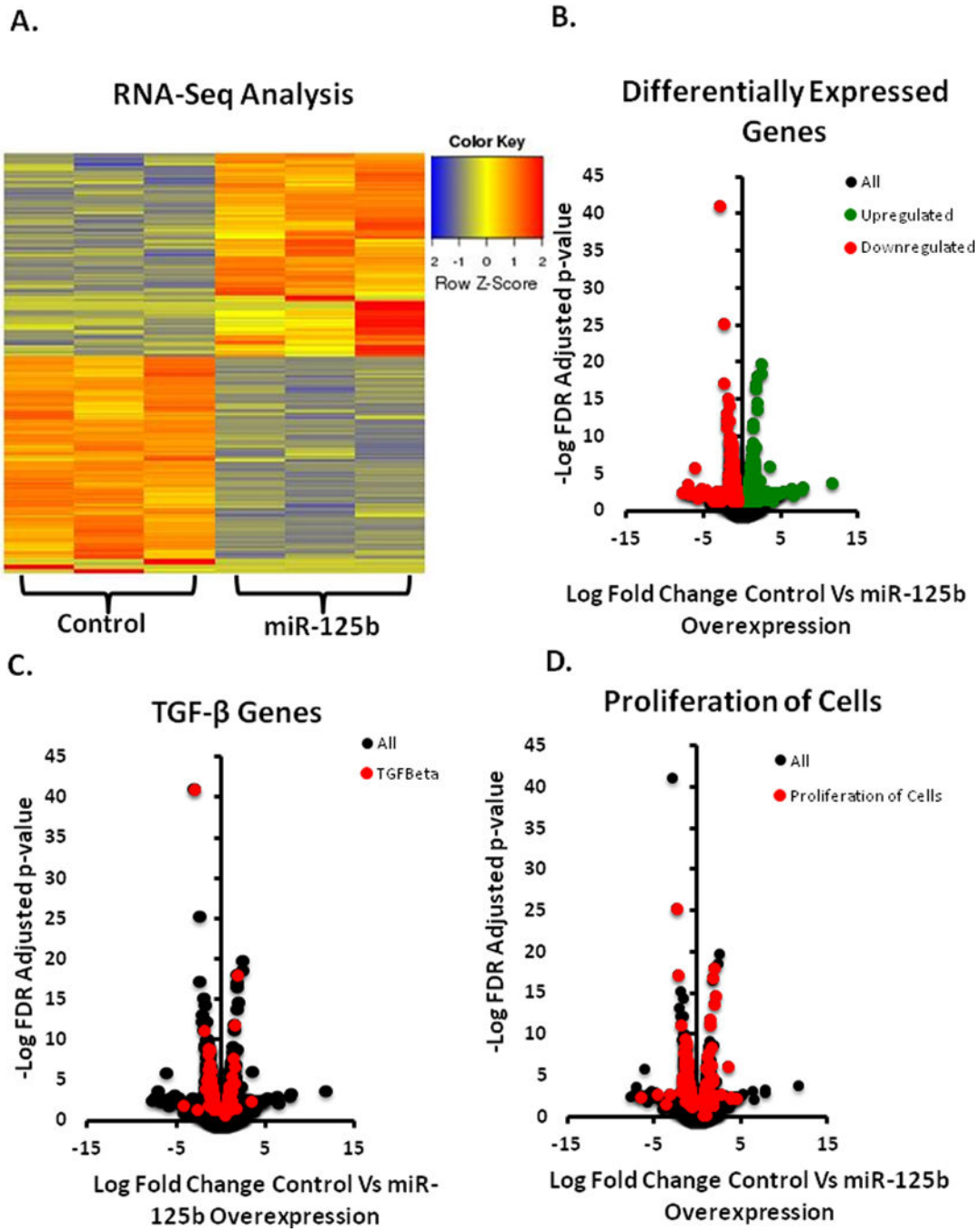


**Figure 7.**

miR-125b and epispastic regulation of fibrosis pathways. Heatmap representation of differential expressed genes (**A**), Volcano plot representation (**B**–**D**) of differentially expressed genes compared to all genes (**B**), regulated genes in the TGF-β network compared to all regulated genes (**C**), differentially expressed genes associated with proliferation pathway compared to all regulated genes (**D**).

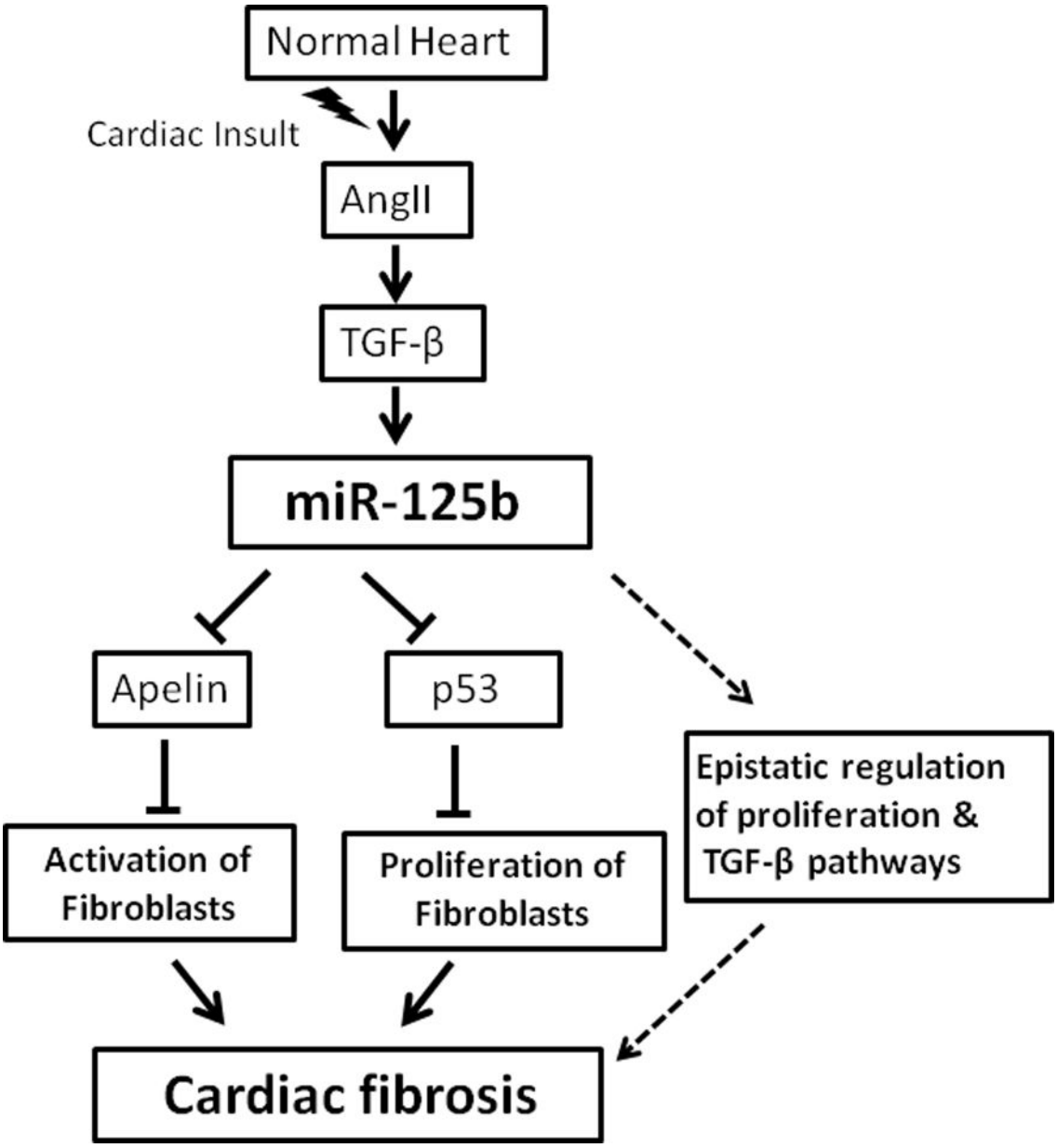


**Figure 8.**

Proposed molecular mechanism of miR-125b driven profibrogenic pathway. Upon cardiac stress, Ang II-TGF-β profibrotic signaling is activated, which in turn results in the induction of miR-125b expression. Increased miR-125b expression is responsible for the suppression of apelin and p53, resulting in augmented FMT, and fibroblast proliferation. Collectively, miR-125b has a concomitant effect on other important cellular processes including epistatic regulation of proliferation and TGF-β pathways, thereby promoting cardiac fibrosis.