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Aging-associated changes in microRNA expression profile of internal anal sphincter smooth muscle: role of microRNA-133a

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Running Head: MicroRNA-133a and aging IAS smooth muscle

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Abbreviations used in the paper:

IAS, internal anal sphincter; SMC, smooth muscle cells; RI, rectoanal incontinence; IASP, internal anal sphincter pressures; miR, microRNA or miRNA; IPA, ingenuity pathway analysis; qPCR, quantitative reverse transcription (RT)-PCR or qRT-PCR; RhoA/ROCK, RhoA-associated kinase; WB, western blot; ICC, immunocytochemistry; FEP, fibroelastic properties; RGS2, regulator of G protein signaling 2; Myocd, myocardin; SRF, serum response factor; SM22α, smooth muscle marker or transgelin; ACT, actin; FNI, fibronectin; ELN, elastin; COL, collagen; ncRNA, non-coding RNA; miRNA, microRNA; TGF-β, transforming growth factor-β ; ECM, extracellular matrix; ZEB, zinc finger E-box-binding homeobox; Y27632, ROCK inhibitor; U46619, thromboxane A_2 analog; KPS, Krebs physiological solution

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ABSTRACT

A comprehensive –omic, computational, and physiological approach was employed to examine the (previously unexplored) role of microRNAs (miRNAs) as regulators of IAS smooth muscle contractile phenotype and basal tone. MicroRNA profiling, genome wide expression, validation and network analyses were employed to assess changes in mRNA and miRNA expression in IAS smooth muscles from young vs. aging rats. Multiple miRNAs, including rno-miR-1, rno-miR-340-5p, rno-miR-185, rno-miR-199a-3p, rno-miR-200c, rno-miR-200b, rno-miR-31, rno-miR-133a and rno-miR-206 were found to be up-regulated in aging IAS. qRT-PCR confirmed the up-regulated expression of these miRNAs and down regulation of multiple, predicted targets (*Eln, Col3a1, Col1a1, Zeb2, Myocd, SRF, Smad1, Smad2, RhoA/ROCK2, Fn1, Sm22-v2, Klf4, and Acta2)* involved in regulation of SM contractility. Subsequent studies demonstrated an aging-associated increase in the expression of miR-133a, corresponding decreases in RhoA, ROCK2, MYOCD, SRF and SM22α protein expression, RhoA-signaling, and a decrease in basal and agonist (U-46619 (thromboxane A_2 analog))-induced increase in the IAS tone. Moreover, *in vitro* transfection of miR-133a caused a dose-dependent increase of IAS tone in strips, which was reversed by anti-miR-133a. Lastly, *in vivo* perianal injection of anti-miR-133a reversed the loss of IAS tone associated with age. This work establishes the important regulatory effect of miRNA-133a on basal and agonist-stimulated IAS tone. Moreover, reversal of age-associated loss of tone via anti-miR delivery strongly implicates miR dysregulation as a causal factor in the aging-associated decrease in IAS tone, and suggests miR-133a is feasible therapeutic target in aging-associated rectoanal incontinence.

Key words: aging-associated changes, rectoanal incontinence, RhoA/ROCK down-regulation, microRNA-133a

INTRODUCTION

The basal tone in the internal anal sphincter (IAS) smooth muscle (SM) plays a critical role in the rectoanal incontinence (RI) (5, 22), while intrinsic and extrinsic nerves in the IAS play an important role in the rectoanal inhibitory reflex-induced IAS relaxation, and modulation of the basal tone (6). In addition, there are significant data to associate a decrease in the IAS tone with the increase in incidence of RI that occurs with aging in humans (2, 45). Due in part to the lack of adequate knowledge of molecular mechanisms mediating basal IAS tone, there is no satisfactory therapeutic management of RI, or of motility disorders associated with IAS SM dysfunction (5, 34).

Recent studies from our laboratory have focused extensively on uncovering mechanisms regulating basal IAS tone (28, 32), and have ascribed an important role to the RhoA/ROCK pathway in regulating both animal and human IAS basal tone (30, 31). RhoA/ROCK expression and related signal transduction cascade in the IAS SMCs are higher in comparison with that in the adjoining non-tonic SM (28, 31, 36). Activation of RhoA/ROCK causes phosphorylation of myosin-binding subunit of myosin light-chain phosphatase (p-MYPT1) leading to an increase in phosphorylation of regulatory myosin light-chain (p-MLC_{20}) (3, 23, 29), which in turn maintains smooth muscle tone.

In addition to the role of RhoA/ROCK in regulating basal tone, a compromise in the fibroelastic properties (FEP) of the IAS may also play a critical role in the incidence of RI during aging (16, 35, 40). Presently, there are no data on the pathophysiological mechanisms that regulate RhoA/ROCK and related signal transduction, or the FEP of the IAS during aging. It is conceivable that the basal tone and the FEP of the IAS may be regulated by changes in the extracellular matrix (ECM), including collagen and elastin. Recent literature suggests that regulatory mechanisms effecting ECM expression, in multiple systems, are regulated by microRNAs (12, 27).

We hypothesize that dysregulation of microRNAs in aging IAS plays a major role in decrease in the RhoA/ROCK-mediated IAS tone and compromise in the FEP of the IAS. To test this, we performed a genome-wide expression profile of miRNAs and computationally analyzed their target gene networks,

and assessed the roles of different miRNAs in regulating contractility in the IAS from younger vs. older rats. We found that aging significantly up-regulates specific miRNAs in IAS resulting in down-regulation of target genes critical to the basal tone and FEP of the IAS. We identified a significant correlation between the expression pattern of the highly expressed miRs-199a-3p, -31-5p, -133a, -185-5p, -200b-3p and miR-340-5p, known to target genes associated with fibrosis in older vs. young rats {13602, 13723, 13724, 13608, 13725, 13197}. Gain of function analysis of miR-133a in SM strips isolated from rat IAS further suggests that miR-133a negatively regulates contractile protein expression. Finally, we demonstrate that targeting miR-133a with anti-miRs in rat IAS reverses the age-associated loss of IAS tone. In conclusion, our findings identify miR-133a as an important determinant in the mechanistic regulation of IAS tone and that its dysregulation contributes to the decrease in the IAS tone that occurs with aging-associated RI.

MATERIALS AND METHODS

Gene expression microarrays.

mRNA microarrays. All studies were performed using IAS from Fischer rats (F344 of 6, 18, and 26 M old age groups provided by the National Institutes of Aging). The experimental protocols were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University. Microarray analysis was performed as described previously (47). Briefly, mRNA and miRNA fractions were isolated from the purified SMCs from the circular smooth muscle (CSM) layer of the IAS as previously described (36), by using miRVana miRNA Isolation kit following the manufacturer's protocol (8). These studies were performed at Functional Genome Centre of Thomas Jefferson University.

Amplification of cDNA was performed from 50 ng RNA using the Ovation Pico WTA-system V2 RNA amplification system according to NuGen protocol (NuGen Technologies, Inc.). 5 μg cDNAs were fragmented and chemically labeled with biotin to generate biotinylated cDNA using FL-Ovation cDNA biotin module (NuGen Technologies, Inc.).

Affymetrix Gene Chips, rat gene 1.0 and 2.0 ST arrays (Santa Clara, CA), were hybridized with 5 μg fragmented and biotin-labeled cDNA in 220 μl of hybridization cocktail. Target denaturation was performed at 99°C for 2 min and then 45°C for 5 min, followed by hybridization for 18 h. Arrays were then washed and stained using Gene Chip Fluidic Station 450, using Affymetrix GeneChip hybridization wash and stain kit. Chips were scanned on an Affymetrix Gene Chip Scanner 3000, using Command Console Software.

Data were analyzed using GeneSpring software 11.5 (Agilent Technologies, Santa Clara, CA). Heat maps were generated from differentially expressed gene list, which was loaded into Ingenuity Pathway Analysis (IPA) 8.0 software (http://www.ingenuity.com) for biological network and functional analyses.

MicroRNA microarrays. miRNA microarray studies were performed as described previously (33, 43). Briefly, Affymetrix GeneChip miRNA Arrays were hybridized with Flash Tag biotin-labeled total RNA (500 ng) from experimental and control samples in 100 μl hybridization cocktail. Target denaturation was performed at 99^{o}C for 5 min. and then 45^{o}C for 5 min. followed by hybridization at 48^{o}C for 18 hrs. Arrays were washed and stained using Fluidic Station 450, and hybridization signals were amplified using antibody amplification with goat IgG and anti-streptavidin biotinylated antibody, followed by scanning as stated above. MiRNA data were analyzed by Affymetrix MiRNA QC tool and Genespring V 11.5 software using Robust Multichip Average (RMA).

Biological function and pathway analysis. To identify pathways and functions of differentially expressed mRNAs and miRNAs, expression data files were analyzed using IPA software (Ingenuity Systems, Redwood City, CA). Ingenuity functional analysis and canonical pathway analysis were carried out by performing IPA core analysis of log2 fold change in the rat IAS SMCs from young and old age groups.

Identification of miRNA Target datasets and Interactome analysis. We uploaded miRNA and mRNA data in IPA and applied miRNA-mRNA interaction filter. Ingenuity Systems combines TargetScan (http://www.targetscan.org) and TarBase (http://diana.cslab.ece.ntua.gr/tarbase) (1, 46). We matched and paired all possible down-regulated targets to the up-regulated miRNAs in the data from microarrays. These targets sites were based on all experimentally validated miRNA-mRNA interactions reported in literature and predicted target sites on UTR regions of mRNAs.

To further explore the impact of expressed miRNA and mRNAs on different pathways and interacting molecules, and their role in aging IAS SM, we performed Interactomes analysis using IPA. The miRNA-mRNA interacting molecules were imported into the separate IPA software spread sheet, and Interactomes pathways were generated. Networks generated by this program are scored based on the number of Network Eligible molecules. It predicts the cross talk among the down-regulated targets of miRNAs by validated interactions among different proteins reported in literature, and facilitates identification of upstream and downstream molecules that may have direct and indirect impact on the pathophysiology of IAS SM during aging. These direct and indirect interactions among different genes and the role of up-regulated miRNA during aging were represented in oval dendrogram.

Validation of differentially regulated miRNA and mRNA from genome-wide microarray.

Quantitative reverse transcription (RT)-PCR (qRT-PCR or qPCR).

We performed qPCR by using cDNA synthesis kit, SYBR Green RT-PCR Kit (Qiagen) and gene-specific primers (Table 1) synthesized by Integrated DNA technology (IDT). miRNA RT-PCR was performed using miR Universal cDNA Synthesis Kit (Promega, Madison, WI), using miR SYBR Green master mix RT-PCR Kit (Promega, Madison, WI) and specific primers to rat miRNAs (Qiagen).

Immunoblot analysis. Immunoblot analysis was performed as described previously (37). Briefly, total protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were subjected to immunoblot analysis using antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and immunoreactive proteins relative to GAPDH were visualized as described previously (32, 37).

Immunocytochemical (ICC) analysis. Using SMCs from 6, 18 and 26 M old rats, high-resolution laser scanning fluorescence microscopy was performed using a confocal microscope (Carl Zeiss LSM 510 UV META, (Carl Zeiss Microimaging, Thornwood, NY)) and Plan-Apo ×40 oil immersion, numerical aperture 1.0 lens. The images were captured as single acquisitions using Zeiss AIM 4.2 SP1 software (Bioimaging Facility, Kimmel Cancer Center of Thomas Jefferson University) and analyzed by MetaMorph v7.65. The nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI). Images taken at same magnification and intensity for 6 M and 26 M old SMCs were imported into Image J (National Institutes of Health) using LOCI Bio-Formats for quantitation. Calculations of florescence intensity per unit area were made via image J, by randomly selecting 4 different diagonally opposite points across the cell. Area of 5μm^2 was selected at each position; and intensity per unit area was calculated by dividing average intensity by the area. 15 to 20 cells were studied in each group. Texas red-conjugated IgGs from mouse, goat and rabbit were used as background fluorescence intensity controls.

IAS SM Strips preparation and transfection of miR-133a. The IAS SM strips (1 x 10mm) from the circular smooth muscle (CSM) layer of 6 M old rats prepared as described previously (35), pinned flat on sylgard-coated 33mm plates containing 2ml of F12 media were transfected with miRNA-133a and antimiR-133a (0 to 60 nM) using INTERFERin (polyplus, SA, France) transfection reagent following manufacturer's instructions. After 48h, changes in the basal tone and effect of contractile agonist U46619 (that works via RhoA/ROCK activation) were recorded as follows.

Force measurement. The IAS SM strips were transferred into 2-ml muscle baths where force was recorded using force transducers (FORT10, WPI, 108 Sarasota, FL). The strips were continuously perfused with oxygenated Krebs physiological solution (KPS). Initially, 1.0 g of tension was applied and strips were allowed to equilibrate for 60 min, with repeated washing with fresh KPS every 20 min. All force data were monitored using Chart 4.1.2 via a PowerLab/8SPdata-acquisition system (ADInstruments, Colorado Springs, CO) (35). The spontaneously developed basal IAS tone, and its maximal increase and decrease were recorded in response to 1 μM U46619 and 0 Ca^{2+}, respectively in the beginning and at the end of each experiment. Concentration-response curves (CRC) for U46619 (0.1 nM to 10 μM) were examined in the SM strips pretreated for 48h with scrambled miRNA (control), and miRNA-133a, before and after anti-miR-133a.

In vivo studies: recording of intraluminal IAS pressures (IASP) and effect of perianal injection of anti-microRNA. The IASP in 6 MO and 26 MO rats were measured before and 48h after perianal injections of scrambled (control) vs. *in vivo* ready miRNA-133a inhibitor (miRCURY LNA™ Power microRNA inhibitor (Exiqon, Inc., Woburn, MA; 7.5 mg/kg of tissue mass). The IASP was measured using high-fidelity intraluminal manometry catheter assembly via PowerLab/8SP recorder, and analyzed via the software Chart 4 PowerLab (ADInstruments). The catheter assembly was initially introduced into the rectum and then gradually pulled out in a precise step-wise manner via a motorized device till the highest and steady pressures (IASP) were recorded in the high pressure zone of the IAS (7 to 8 mm from the anal verge). The IASP consisted of rhythmic fluctuations superimposed on the steady tone.

Details of adapted procedure for the perianal injections have been described previously (13). For these injections we used microneedle (31-gauge) attached to 300μl insulin syringe.

Both intraluminal manometry and perianal injection procedures were carried out under isoflurane inhalation anesthesia (initially with 5% isoflurane and then maintained with 1 % isoflurane throughout the length of the experiment).

Statistical analyses. miRNA microarray data were verified by a close correlation between qPCR and microarray via linear regression analysis. qPCR data for mRNA and miRNA was replicated further on four to six rats in different experiments. Genes showing gradient of expression in 6 M vs 26 M old rats were selected for miRNA target analysis. Comparison between 2 groups was analyzed using the 2-tailed Student's *t* test; and comparison between multiple groups was made using one-way ANOVA and Newman-Keuls posttest using GraphPad Prism 5.0. Data are presented as the mean ± SEM.

RESULTS

Differential gene expression during aging in IAS smooth muscle. Genome-wide expression profiles via microarray on the total RNA of the IAS SMCs from three groups of rats, 6, 18, and 26 MO employed Affymetrix rat Gene Chips 1.0 and 2.0. These chips probed for 27,342 and 29,489 sets of genes, respectively. 6 M rats served as control or reference point to determine up- and down-regulation of gene transcripts. All unknown transcripts (without known gene name and ID) and transcripts with weak signal intensity (below 50) were ignored. Only the transcripts with noticeable gradient for up or down-regulation between 6, 18 and 26 M age groups were considered. These transcripts were clustered into groups by biological function. We applied hierarchical clustering (Pearson correlation) to the expression profiles of differentially expressed transcripts in the IAS SMCs from younger vs. older rats to determine patterns in the data. These results are presented as a heat map and dendrogram **(**Figure 1A**)**. A striking finding was a significant down-regulation of genes known to be involved in SM contractile phenotype (*Myocd*) and those in the basal tone of IAS (*RhoA*), in the IAS of older vs. younger rats.

Relevant functions and pathways in IAS smooth muscle of aged rat. IPA (25) identified a number of significantly differentially expressed genes in IAS SM during aging. One of the pathways that showed the highest differential gene expression was found to be RhoA/ROCK signaling pathway. Significant IPA canonical pathways and the associated molecules are presented in Supporting **Figure S1**. These include Rho GTPase, Gα12/13, Gαq/Rho signaling in the IAS SMCs of 26 months old (26 MO) rat.

We validated the microarray array results using qPCR, immunoblot and immunofluorescence analyses for 12 selected genes. Data revealed that *Eln, Col3a1, Col1a1, FN1, Zeb2, Klf4, Myocd, Sm22-V2, Smad1, Smad2, RhoA*, and *Act2* mRNA were down-regulated in 26 months old IAS SMC samples. The relative expression of these genes from these samples is shown in Figures 1B (mRNA) and Figure 2B (protein). PCR and WB bands are given in Figure 2A.

Differential down-regulation of these genes plus up-regulation of *Rgs2* in IAS SMCs of 26 M vs. the younger rats was further confirmed by immunofluorescence studies (Figure 3A, B, C). Because initial comparison of mRNA and mi-RNA arrays and validations studies between 6, 18, and 26 month age groups revealed most significant and consistent differences in the 26 M vs. 6 M groups, immunofluorescence and other detailed studies were performed in these age groups only.

Differential miRNA expression during aging in IAS smooth muscle. Affymetrix miRNA Expression Profiling Assay system in IAS from 6, 18 and 26 months rats identified marked differential expression of a number of miRNAs as shown as a heat map in Figure 4A. All mRNA and miRNA microarray data were deposited in the Gene Expression Omnibus (GEO) database and can be accessed as http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE79348.

The correlation between miRNAs microarray and qPCR data was confirmed by regression analysis of low and high-expressing miRNAs (Figures 4B, and 4C, respectively).

Based upon this analysis we selected miRNAs targeting *RhoA* directly, and other signaling molecules regulating RhoA expression indirectly (Figure 4D).

qPCR analysis confirmed that miR-1, miR-133a-3p, miR-185-5p, miR-199a, and miR-200b-3p, were significantly up-regulated in IAS SMCs from 26 MO rats (n = 4; $P < 0.05$; Figure 4E).

Following IPA, we identified that multiple miRNAs may target single mRNA. Predicted and experimentally observed targets from the literature are given in Supporting Figure 2. The analysis indicates that *Smad2, Klf4, Myocd, RhoA* and *Sm22* mRNAs are predicted targets of multiple miRNAs.

Predicted targets of altered miRNAs and Network construction. To assess the interaction between miRNAs and genes, miRNA-Gene-Network was built using IPA. By multiple interactions, miR-133a may lead to down-regulation of multiple genes in the aging IAS SM. Other important microRNA, miR-199a-3p is predicted to affect multiple targets. Interactomes of these miRNAs and genes involved are given in supplementary data (supplementary Figures S1, and S2). The networks were built based on these differentially up-regulated miRNAs in the IAS SMCs from 26MO rat.

miR-133a overexpression and effect on RhoA/ROCK pathway. Transfection (for 72h) of primary SMCs from 6MO rats with miR-133a-3p oligonucleotide caused a significant decrease in the expression levels of RhoA, ROCK2, MLC_{20}, p-MLC_{20}, MYOCD, SRF, and SM22 as determined via immunoblot analyses (Figure 5A,B) (n = 4; $P < 0.05$). These results suggest the involvement of miR-133a in inhibiting regulatory molecules important in IAS tone.

Effect of miRNA-133a on the basal tone of IAS. Given our earlier studies have shown that Rho kinase is the primary determinant of the basal IAS SM tone (31, 32), we investigated whether the decreased expression of RhoA by miRNA-133a is responsible for the changes in the basal tone. We measured the basal IAS tone in the SM strips pre-treated with scrambled or miRNA-133a (both for 48h).

miRNA-133a produced concentration-dependent and significant decrease in the basal IAS tone (Figure 6A). Percent of maximal basal IAS tone following the pretreatment of the strips with 60 nM of

miRNA-133a was 55. 5 ± 4.1 % (*; $P < 0.05$; n = 4). This represents a 45% decrease in the basal IAS tone. The absolute values of basal IAS tone in these experiments in the presence of scrambled vs. 60 nM miRNA-133a-transfected SM strips were 268 ± 32 mg and 149 ± 11 mg, respectively. The decrease in IAS tone caused by miRNA-133a was significantly (**; $P < 0.05$; n = 4; Figure 6A) blocked by its antagomir. Conversely, miRNA-200c had no significant effect on the basal tone.

Effect of miRNA-133a on agonist-induced contraction in IAS. To investigate the effect of miRNA-133a overexpression on agonist-mediated increase in the IAS tone, we compared the effect of scrambled vs. miRNA-133a on the contractile effect of RhoA/ROCK activator U46619 (15, 41).

U46619 increased IAS tone in a concentration-dependent manner. The pretreatment of muscle strips with miRNA-133a (20 and 60 nM) for 48h caused a significant and concentration-dependent right-ward shift in the U46619 CRC (*; $P < 0.05$; n = 6-8). These concentrations of miRNA-1333a were based on the previous smooth muscle studies by Torella et al. (44), and our optimization for the maximal transfection efficiency in rat IAS SMCs and smooth muscle strips using fluorescent-tagged scrambled siRNA (10 to 60 nM) from Qiagen. This shift in the U46619 CRC with miR-133a was attenuated by anti-miR-133a, and CRC obtained during the presence of combined use of the miR + anti-miR (both 60 nM) was not significantly ($P > 0.05$) different from that obtained during scrambled RNA-treated (control) SM strips. These results demonstrate that miRNA-133a reduces the IAS tone not only in the basal state but also in the stimulated state, both involving RhoA/ROCK pathway (Figure 6B).

Rescuing effect of anti-microRNA inhibitor on aging-associated decease in IASP. In agreement with above *in vitro* data, *in vivo* studies revealed significantly (*; $p < 0.0$; n = 3; Figure 6C) reduced IASP in aging rats. To further implicate miR-133a in the age-associated decrease in IASP, studies in Figure 6C demonstrate that perianal injections of anti-miR-133a into IAS significantly rescued the loss of IASP 48h and 72 h post injection both in males and females (**; $P < 0.05$; n = 3 animals of

each age and sex group). These recusant effects as determined at 72 h were found to be statistically significant and similar both in male and female rats.

DISCUSSION

In the present study we performed a high throughput screening involving microarray and the genome-wide transcriptome analysis to gain insight into miRNA-dependent regulatory mechanisms that influence age-related differences in IAS tone. We identified 22 genes that are down-regulated in IAS smooth muscle from older rats, including *Myocd, SRF, RhoA and ROCK2* which are known important regulators of SM differentiation and contractility. In parallel, a comprehensive screening identified eleven microRNAs that are significantly up-regulated in the aging IAS SM. Subsequent bioinformatics analysis combined with pathway analysis and predicted targets led to the conclusion that miR-133a downregulates RhoA/ROCK2, MYOCD and SRF.

The present study also identified significant changes related to age in the SM signaling transduction molecules (RGS2, PRKACA, RhoA/ROCK, PP1R12A, SHIP2), intermediate filaments (VIM, DES), growth and transcription factors (TGFB2, SMADs/ZEBs, KLF4, SIP1/ZEB2), regulators of SMCs differentiation (SRF, MYOCD), certain components of ECM (ELN, COL12A1, COL1A1, FN1), and early markers of SMCs (SM22α, ACT2). (It is noteworthy that not all ECM genes were down-regulated during aging as a significant number of other ECM genes were either up-regulated or unaffected (data not shown)). These data support our previous studies asserting an important role of the RhoA/ROCK pathway in the aging-associated decrease in the IAS tone (35), and also suggest age-related changes in IAS SM phenotype and fibroelastic properties.

Above conclusions were based on the changes in genetic, and miRNAs profiles, as found during microarray studies, using SMCs from rats of three different aging groups, young (6 M), adult (18 M) and aging (26 M) rats. The reason for using SMCs was two-fold. One, previous studies have shown that the majority of the basal tone in the IAS in humans and animals is via the specialized myogenic properties of the SMCs (28, 29, 36). Two, this approach avoids contamination with other phenotypic cells in this area

which would have made interpretation of the data difficult. These changes in the genomic and miRNA profiles as a function of aging were further validated via qPCR, immunoblot, immunocytochemistry analyses, and finally by functional studies, especially focusing on 6 M vs. 26 M old rat IAS SMCs.

MicroRNAs (miRNAs) are well-known small noncoding RNAs that can act as 'master switches' of the genome to regulate diverse cellular pathways involved in the pathophysiology of smooth muscle function {13762, 13728, 13763, 13726, 13649}. Therefore, miRNAs may serve as diagnostic and prognostic markers, and provide important insights into the pathophysiology and therapeutic targeting of SM dysfunctions. Several miRNAs are ubiquitously expressed, while others are-tissue specific, and are enriched in specific tissues. For example, miR-1, miR-133a, miR-133b, miR- 206, miR-208, miR-208b, miR-486, and miR-499 are muscle-enriched miRNAs (4, 12, 24). In the present study, using mRNA and miRNA microarrays followed by bioinformatics analyses, we identified differentially expressed mRNA and miRNAs, in the rat IAS SM.

Earlier studies attempting to understand the regulation of basal IAS tone, and its decrease during aging, have shown the critical role of RhoA/ROCK pathway in animals and humans (29, 30, 32, 35). A number of genomic, proteomic and functional studies have documented miR-133a can target *RhoA* {13197, 13756, 13649}, a major upstream trigger for the downstream signal transduction cascade for the sustained SM tone (23, 29, 32). To determine the effect of miR-133a on SM contractile proteins expression, we overexpressed miRNA-133a in rat IAS SMC. This overexpression decreased the endogenous protein levels of two markers of myogenic determination, myocardin and SRF, whereas inhibition of the endogenous miR-133a by antagomir-133a resulted in the up-regulation of these proteins (data not shown). This is in agreement with previous studies in which manipulating miR-133a levels in other tissue types showed a significant reduction in contractile proteins expression (44) implicating miR-133 as a regulator of muscle differentiation (50).

Myocardin is a master regulator of SMC differentiation and contractile phenotype, and its negative regulation by miRs-143/145, and 204/211 induces SMC synthetic phenotype differentiation (7, 12, 52). The development of SM is a well-coordinated process of cell proliferation, differentiation and migration

that is regulated by evolutionarily conserved networks of myogenic transcription factors (48, 52). The maintenance of a SM contractile phenotype requires a fine balance between the expression and the repression of many genes (19).

The current study demonstrates for the first time that miR-133a is up-regulated in aging IAS SM and its overexpression inhibits the basal tone and agonist-induced contraction. Previous studies have demonstrated that aging reduces the basal IAS tone (35) that has been associated with the down-regulation of RhoA/ROCK2. Although the mechanism of down-regulation of RhoA and ROCK2 in IAS SM during aging is not fully understood, present data suggest that miRNA-133a is an important factor in that regard.

Present data demonstrate a direct relationship between the changes in miRNA and changes in mRNA expression in the IAS SM from different age groups, especially in 26 M vs. 6 M. In addition, miRNA-133a is up-regulated in the IAS from aged rat and the overexpression of miRNA-133a mimics the aging-associated decrease in the IAS tone in basal as well as stimulated state by RhoA/ROCK activator U46619 (15, 41). These findings have significant relevance in the pathophysiology and potential reversal of aging-associated IAS dysfunction, via miRNAs intervention. We speculate that aging-associated RI (especially characterized by the hypotensive IAS), is in part associated with by changes in expression profile of miRs, which may be reversed by the respective antagomirs or miRs. Conversely, down-regulation of miR-133a may lead to anorectal motility disorders characterized by hypertensive IAS (e.g. recurrent anal fissures and hemorrhoids), potentially reversible by overexpression of miR-133a using appropriate oliogmiR. Our data clearly show that reduced IASP observed in aging rats is reversible by anti-miR-133a both in male and female rats.

The present data contribute significantly to our current understanding of the role of miRNAs in the regulation of SM contractility and differentiation (26, 38). However, the effects of aging on the expression of miRNA-133a, and the associated functional consequences, appear to be system-/cell type-dependent. For example, studies have implicated decreases in the levels of miRNA-133a in the arterial SMCs from patients with arteriosclerosis obliterans (ASO), a common occurrence in the aging population

(17). In contrast, aging has been shown to have opposite effects, i.e., an increase in the levels of miRNA-133a in human skeletal muscle (11). Regardless, it is well known that miRNA-133a negatively regulates RhoA; downregulation of miR-133a promotes the proliferation, migration, and contraction, while its upregulation has the opposite effect, by targeting RhoA (9, 17).

In summary, this work identifies the role of miRNAs in the regulation of IAS smooth muscle basal tone and agonist-induced contraction. Data reveal a significant impairment in myogenic factor and contractile proteins expression due to miRNA/mRNA interaction with aging. These data provide strong evidence for dysregulated miRNA as a key factor in the compromise of SM plasticity with age, and in pathogenic mechanisms associated with RI. This constitutes the first study to demonstrate that miRNA-133a and its gene targets are crucial to RhoA signaling pathway in relation to contractility, and IAS SM phenotype in the aging. We speculate that the basal tone and the FEP of the IAS are regulated by changes in the transduction molecules, growth and transcription factors, regulators of SMCs differentiation, ECM components, and early markers of SMCs via microRNA-targeting diverse signaling pathways. Collectively, present data identify miR-133a as a potential target for therapeutic application in aging-associated RI with compromise in the IAS basal tone and biomechanics.

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Table 1. Primers used in this study (listed in alphabetical order)

Primer Sequences			
Accession No.	**Gene Symbol**	**Forward**	**Reverse**
NM_053304.1	COL1A1	GAGCGGAGAGTACTGGATCG	GCAGGGACTTCTTGAGGTTG
NM_032085.1	COL3A1	GAAAAAACCCTGCTCGGAATT	GGATCAACCCAGTATTCTCC
NM_012722.1	Eln	GTGGCTTTCCTGGCTATGGT	CCCTGCTCCTCCAAGATCAC
NM_019143.2	FN1	TCGAGGAGGAAATTCCAATG	CTCTTCATGACGCTTGTGGA
NM_053713.1	KLF4	CGACTAACCGTTGGCGAGAG	CGGGACTCAGTGTAGGGGTA
NM_017343	MLC 20	AAGAGGCCTTCAACATGATCGACCAG	CTCATCCACTTCCTCATCTGTGAAGC
NM_182667.1	Myocardin	CGCCTGTACGGATGAGAGTC	CCCAATGGGGCTGTGAGAAT
NM_053890	MYPT1	GCATCTCGAATCGAGTCTCTGGAG	ACGGCTTCTTCCTATTGTCTTTTCGGC
NM_016802	RhoA	CAGCAAGGACCAGTTCCCAGA	TGCCATATACTGCCTTCTTCAGG
NM_013022	ROCK-II	TAGAAGAACACCTTAGCAGTGAGGTACAAG	GCTGTCCTCTTCTCCAGCTCTACTTTTG
NM_013130.2	Smad1	TTTCAGATGCCAGCCGACAC	ACACCTCTCCTCCGACGTAA
NM_019191.1	Smad2	GGGTGGAGACACCAGTCTTG	CTCCACTGCTGACGGACTTT
NM_003186.3	TAGLN, transcript variant 2	GAAGCCTTCTTTCCCCAGACA	ATCACGCCATTCTTCAGCCA
NM_001033701.1	ZEB2	GGGACAGATCAGCACCAAAT	GACCCAGAATGAGAGAAGCG
NM_017008.4	GAPDH	GTTACCAGGGCTGCCTTCTC	CTCGTGGTTCACACCCATCA

FIGURE LEGENDS

Figure 1. Genome-wide expression profile of mRNA in IAS smooth muscle from younger and older rats. A. Hierarchical clustering: Heat map showing differentially (↑ for increase, and ↓ for decrease) expressed mRNA detected in the IAS SMCs from 26 M old vs. 6 and 18 M old rats. The dendrogram illustrates the clustering tree resulting from hierarchical clustering of gene expression values (involved in SM phenotype, and in contractile biomechanics). **B.** qPCR data for the selected transcripts validating down-regulation of important SM markers (initially observed in the mRNA microarray profile) in aging IAS. These data reveal significant decreases in the expression levels in 26 M vs. 6 M group (*; $P < 0.05$; n = 4; student *t* test) but not in 18 M vs. 6 M ($P > 0.05$).

Figure 2. Aging down-regulates smooth muscle contractile and regulatory protein expression in rat IAS. A. RNA and protein were extracted from 6, 18, and 26 M old rat IAS SMCs and subjected to RT-PCR (left) and immunoblot (right) analysis for the indicated proteins. Representative blots from four independent experiments are presented. **B.** Graph showing significant decrease in the relative expression (*; $P < 0.05$; n = 4; student *t* test) of different proteins (corresponding to the mRNAs described in Figure 1), in 26 M old vs. 6 M old rats as compared with younger rats.

Figure 3. Decreased expression of smooth muscle contractile and regulatory protein in rat IAS. SMC isolated from 6 months vs. 26 MO rat IAS SMCs (**A**) were stained with the indicated antibodies. The images (taken on a Carl Zeiss LSM 510 UV META inverted confocal microscope) (panel **A**) compare the expression of the proteins between 6 and 26 M old rats (bar = 20 μm). **B** and **C** panels show significant decrease in immunofluorescence intensities for different proteins examined (*; $P < 0.05$; n = 4).

Figure 4. Genome-wide expression profile of miRNA in IAS from younger and older rats. A. Heat map showing differentially expressed miRNAs detected in IAS SMCs from 6, 18 and 26 M old rats. Analysis was carried out using a 2-color miRNA microarray. Each column represents results from an independent experiment (6, and 18, and 26 M old rats). Each row corresponds to a single miRNA probe. The dendrogram illustrates the clustering tree resulting from hierarchical clustering of gene expression

values. Panels **B,C,** reveal significant correlation between the signal intensity of miRNA expression from the microarray vs. relative expression of qPCR values, examining low-expressing, and high-expressing miRNAs, respectively. **D.** Expression levels of selected miRNA (in numerical order), shown as average fold change of miRNA in IAS SMCs of 26 vs. 6 M rat. **E.** qPCR data showing significant (*; $P < 0.05$; n = 4) increase in the values of selected miRs validate the microarray data for the selected miRNAs in 26 M vs. 6 M rat IAS SMCs. Data was normalized to U6 RNA; and experiments were performed in triplicates.

Figure 5. A-B Effect of miRNA-133a overexpression on contractile and regulatory proteins' expression in primary IAS SMCs. Panels **A** and **B**, Immunoblots analysis (**A**), and quantitative data (**B**) showing significant decrease (*; $P < 0.05$; n = 4) in the expression levels of RhoA/ROCKII, MYPT1, p-MYPT1, p-MLC_{20}, SRF, myocardin, SM22α, and calponin, following pre-treatment of the cells with miRNA-133a, and anti-miR-133a. The anti-mR blocks the inhibitory effects of miR-133a to the levels not significantly different from controls.

Figure 6. Effect of miRNA-133a overexpression on basal (A), agonist-induced increase in IAS tone (B), and reversal of the decreased intraluminal pressures of IAS (IASP) by anti-miR-133a (C). **A.** miR-133a produces significant and concentration (20 and 60 nM)-dependent decrease in the IAS tone (*; $P < 0.05$; n = 4) as compared with scrambled miR (control) or another miR-200c. Decrease in IAS tone by 60 nM miR-133a is significantly blocked by 60 nM antagomirs (**; $P < 0.05$; n = 4). **B.** miR-133a significantly shifts U46619 CRC of increase in the IAS tone towards right (*; $P < 0.05$; n = 6-8). The latter is blocked by the antagomirs pre-treatment so that U46619 CRC in the presence of 60 nM of miR-133a + antimiR is not significantly different from control ($P > 0.05$; n = 6-8). **C.** Perianal injection of anti-miR-133a significantly (**; $P < 0.05$) rescues the decreased (*; $P < 0.05$) IASP during aging in 26 M rats as compared with their corresponding 6 M both in males and females (n = 6 animals, three males and three females used for each animal group). The rescuing effect was found to be sustained for up to 72 hours following the injection of the anti-miR, both in males and females.

Figure 1

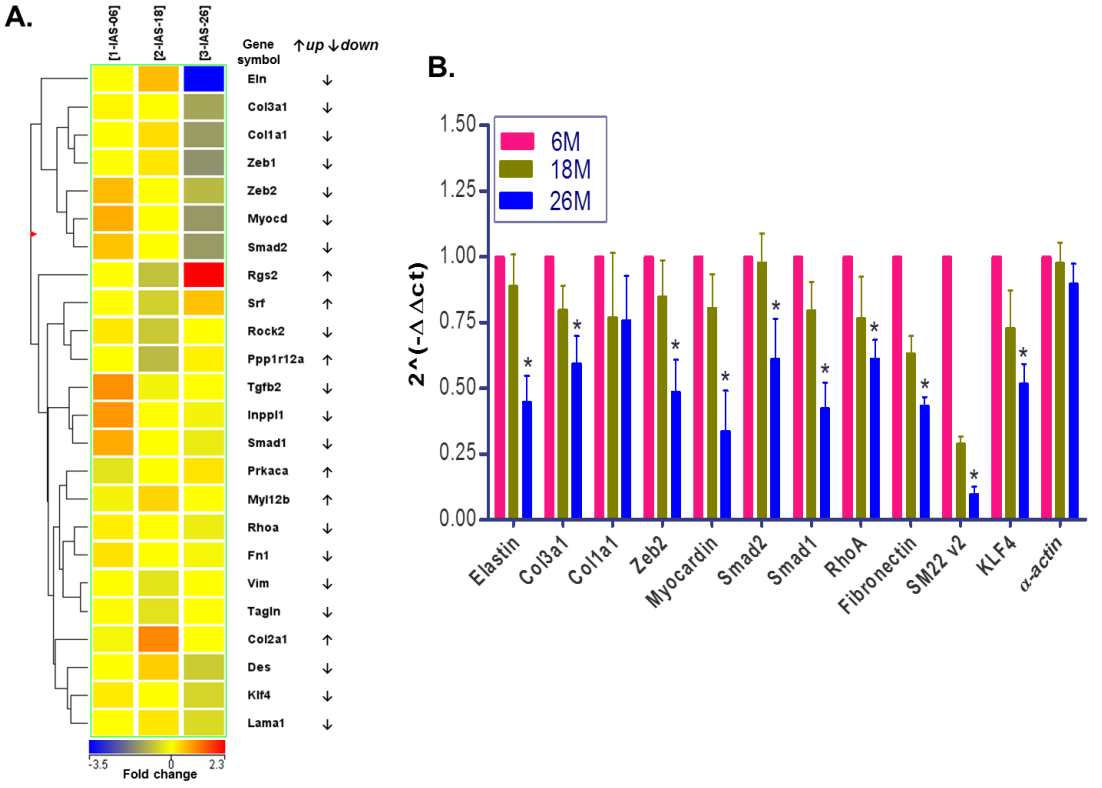

Figure 2

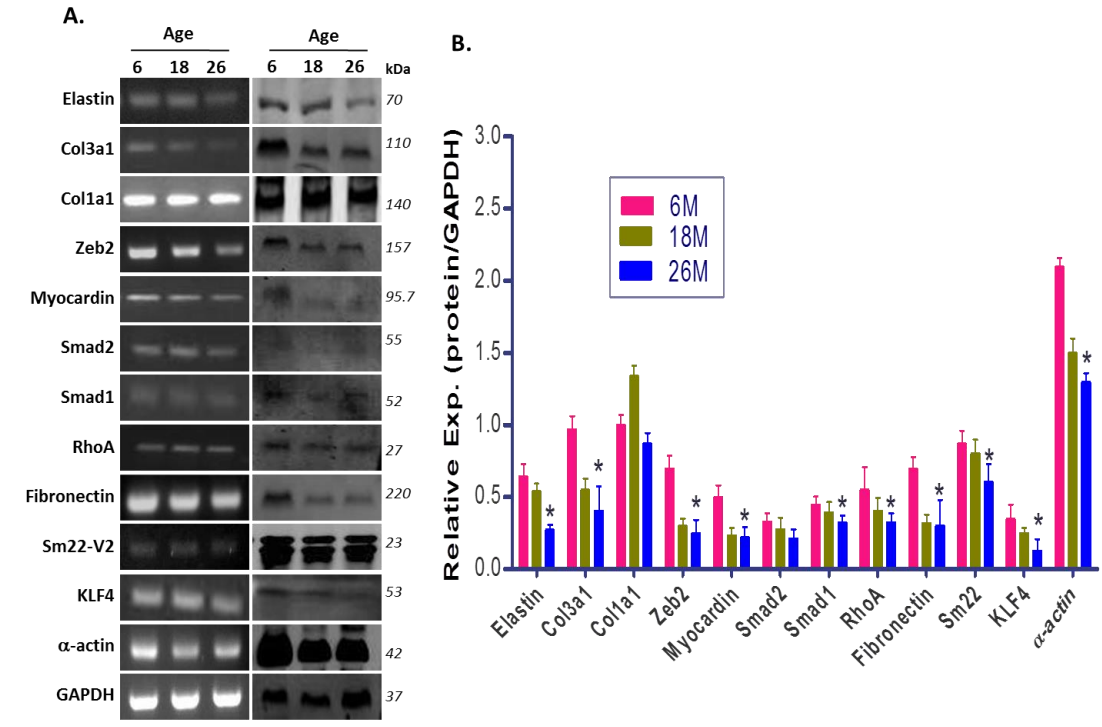

Figure 3

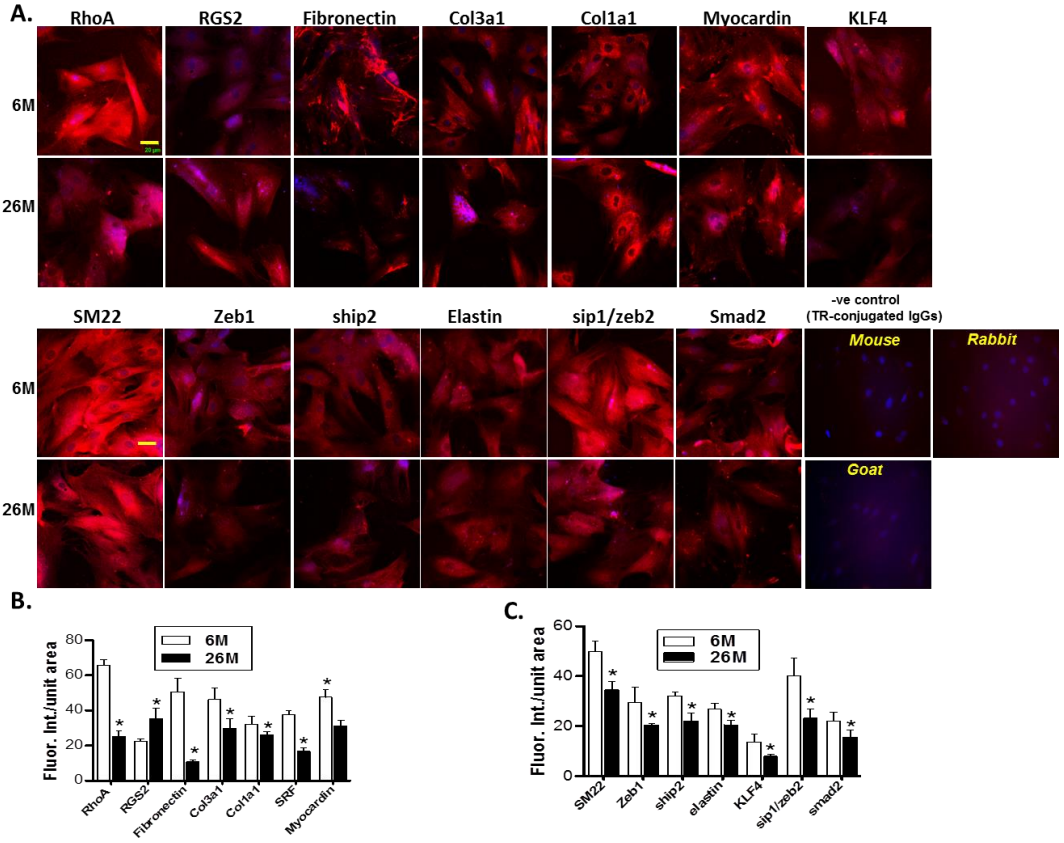

Figure 4

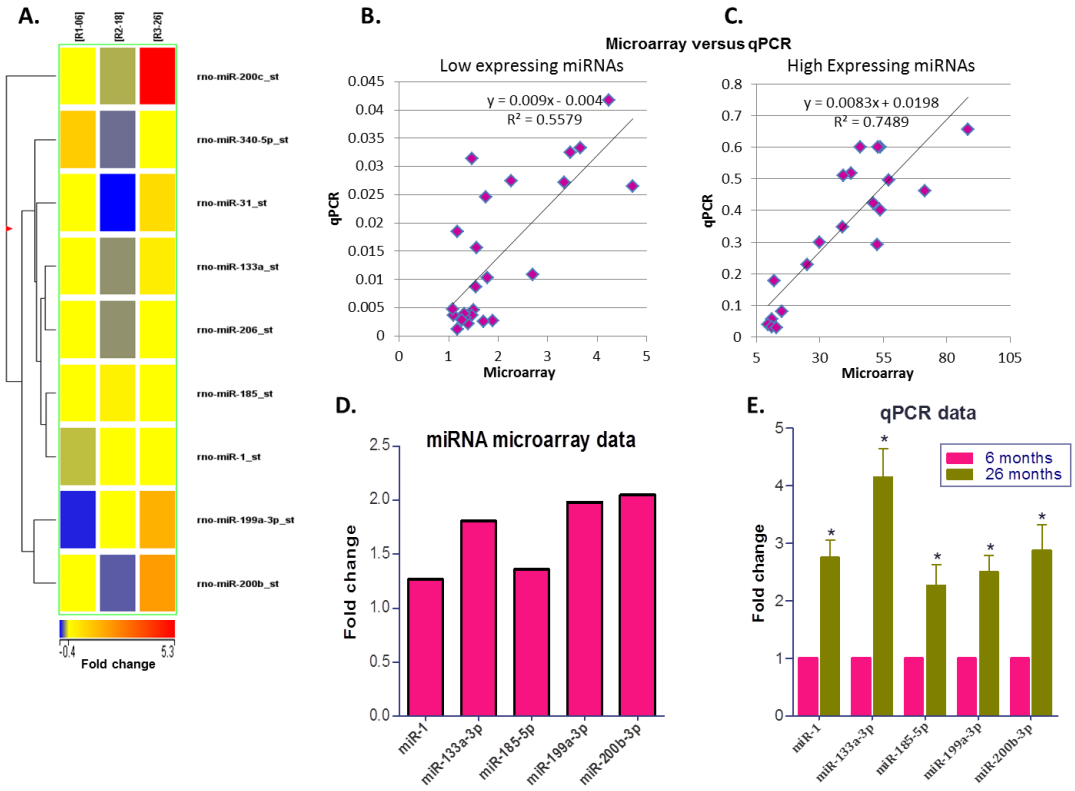

Figure 5

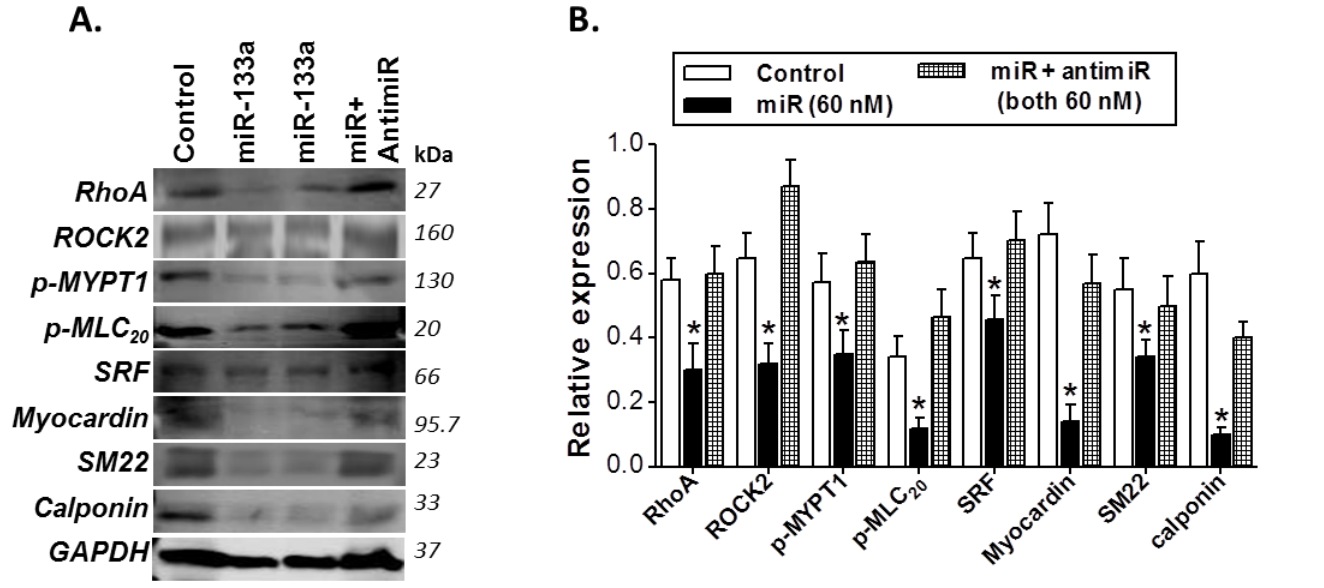
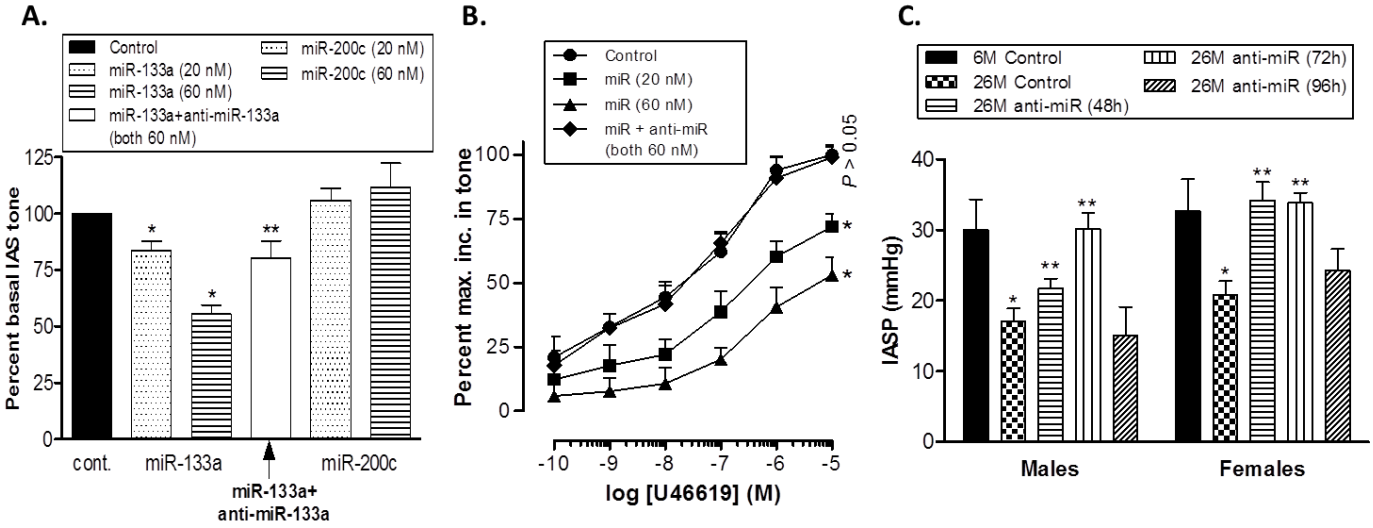
Figure 6