MICRORNA-153 TARGETING OF KCNQ4 CONTRIBUTES TO VASCULAR DYSFUNCTION IN HYPERTENSION

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Abstract

Aims: Kv7.4, a voltage dependent potassium channel expressed throughout the vasculature, controls arterial contraction and is compromised in hypertension by an unknown mechanism. MicroRNAs (miRs) are post-transcriptional regulators of protein production and are altered in disease states such as hypertension. We investigated whether miRs regulate Kv7.4 expression.

Methods and Results: In renal and mesenteric arteries of the spontaneously hypertensive rat (SHR), Kv7.4 protein decreased compared to the normotensive rat (NT) without a decrease in KCNQ4 mRNA, inferring Kv7.4 abundance was determined by post-transcriptional regulation. *In-silico* analysis of the 3' UTR of KCNQ4 revealed seed sequences for miR26a, miR133a, miR200b, miR153, miR214, miR218 and let-7d with QPCR showing miR153 increased in those arteries from SHRs that exhibited decreased Kv7.4 levels. Luciferase reporter assays indicated a direct targeting effect of miR153 on the 3' UTR of KCNQ4. Introduction of high levels of miR153 to mesenteric arteries increased vascular wall thickening and reduced Kv7.4 expression/Kv7 channel function compared to vessels receiving a non-targeting miR, providing a proof of concept of Kv7.4 regulation by miR153.

Conclusion: This study is the first to define a role for aberrant miR153 contributing to the hypertensive state through targeting of KCNQ4 in an animal model of hypertension, raising the possibility of the use of miR153-related therapies in vascular disease.

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Introduction

Primary hypertension is a major risk factor for cardiovascular disease which continues to be the leading cause of death globally and is predicted to dominate mortality trends in the future.[1] The hypertensive state is precipitated by decreased luminal diameter brought about by increased arterial contractility, lack of response to endogenous vasorelaxants and vessel wall remodelling.[2-5] Among the possible modulators of arterial contractility, the Kv7 channels encoded by KCNQ genes regulate vascular smooth muscle contractility in rodent and human arterial beds, with Kv7.4 and Kv7.5 playing a key functional role in regulating vascular tone directly and contributing to endogenous vasodilatations.[3,4,6-15] Recently we discovered that Kv7.4 channel activity is downregulated in aorta, mesenteric, renal and coronary arteries from spontaneously hypertensive rats (SHR) and mesenteric arteries from Angiotensin II-infused mice, which was associated with a reduction in total Kv7.4 protein.[3,4,6,7] Interestingly, only in aorta from SHRs was a reduction in KCNQ4 mRNA detected and cerebral arteries from SHRs exhibit no Kv7 dysfunction.[3,6,15] These observations suggest that Kv7.4 abundance is determined by post-transcriptional mechanisms in an artery-specific manner.

MicroRNAs (miRs) are powerful post-transcriptional regulators of gene expression.[16,17] These small (20–25 nt) noncoding RNAs bind to a target recognition site (‘seed sequence’) in the three prime untranslated region (3’UTR) of mRNA transcripts leading to mRNA degradation and/or inhibition/activation of protein translation depending on complementarity of the miR with the target mRNA.[18,19] Several studies have determined the importance of miRs in vascular smooth muscle cell (VSMC) function in health and disease [20-23] but no study has examined the impact of miRs on vascular Kv7.4. Since miRs are heavily implicated in various vascular diseases and given the importance of Kv7 channels in the vasculature, this

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study investigates whether miRs have a regulatory role on Kv7.4 and identifies that miR153 targets KCNQ4, which may contribute to vascular dysfunction in hypertension.

Materials and Methods

Detailed experimental protocols are available in the online-only Data Supplement.

Animals

Male Normotensive (NT) Wistar rats and Spontaneously Hypertensive Rats (SHRs; Charles River, U.K.; 147±6 mmHg- mean BP in a cohort of 12 week SHRs), 12-16 weeks of age (175-225g), were killed by cervical dislocation in accordance with the UK Animal (Scientific Procedures) Act 1986. All animals were culled by Schedule 1 methods and therefore no approval from a local or university ethics review board was required. This investigation conforms to Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

in-silico analysis for prediction of miR targets

Candidate miRs that potentially target the 3' untranslated region (3'UTR) of KCNQ4 were identified using the established miR target prediction algorithm, TargetScan6.2 and mirDIP, a miR data integration portal.

Transfection of miR mimics into Mesenteric or Middle Cerebral Arteries

Synthetic RNA molecules, miR153, miR133a and non-targeting control (NTC) miR (Active Motif, La Hulpe, Belgium) were designed to mimic endogenous mature miR153, miR133a or act as a non-targeting control, respectively. NTC miR has no known homology to human (or

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rat) gene sequences and acts as a negative control. Whilst miR153 acts as a functional equivalent to endogenous human miR153, the sequence is 100% conserved in rat. 12nM miR153 (+ miR133a for cotransfection studies) or NTC miR were transfected into mesenteric or middle cerebral arteries from NT rats using *Trans*IT-X2 and OptiMEM® I Reduced-Serum Medium according to manufacturers' protocol (Mirus Bio LLC, Madison, USA). 24 hours post transfection, arteries were used in functional/staining studies or harvested for RNA or protein for QPCR or western blot, respectively.

Synthesis of KCNQ4 3'UTR Luciferase Reporter, miR153 binding site mutants and Transfection into HEK293 cells

A fragment of the KCNQ4 mRNA 3'UTR (NM_0047000.3) containing 4 predicted binding sites for miR153 was synthesized and subcloned into pLightSwitch_3'UTR luciferase expression reporter vector, downstream of the *Renilla* luciferase reporter gene (GoClone), by SwitchGear Genomics (Active Motif, La Hulpe, Belgium). Site specific mutagenesis of 2 predicted miR153 binding sites were engineered in pLightSwitch-KCNQ4_3'UTR vector using the QuickChange II site-directed mutagenesis kit (Agilent Technologies, California, USA), according to manufacturer's instructions. Deletion of seed sequences corresponding to site 1 (Δ site1, 8 nucleotides) and site 4 (Δ site4, 7 nucleotides) were obtained using specific pairs of primers designed using the online QuikChange Primer Design Program (www.agilent.com/genomics/qcpd). Effective deletion of site1 and site 4 regions was verified by direct sequencing. The pLightSwitch_3'UTR luciferase expression reporter vector containing no insert (EMPTY_3UTR, Active Motif, La Hulpe, Belgium) was used as positive control for transfection to allow assay normalisation. The following groups were set up for transfection of HEK293 cells using *Trans*IT-X2: (1) WT KCNQ4 3'UTR + miR153, (2) WT KCNQ4 3'UTR + NTC miR, (3) Δ site1 KCNQ4 3'UTR + miR153, (4) Δ site1 KCNQ4

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3'UTR + NTC miR (5) Δ site4 KCNQ4 3'UTR + miR153, (6) Δ site4 KCNQ4 3'UTR + NTC miR (7) WT KCNQ4 3'UTR, (8) EMPTY_3'UTR, (9) *Trans*IT-X2. 24 hours post transfection, luciferase activity was measured using the LightSwitch Assay Reagent kit (LS010, Active Motif, La Hulpe, Belgium) designed for use with all GoClone reporter plasmids.

RNA extraction, reverse transcription and Quantitative Polymerase Chain Reaction (qPCR)

The miRNeasy Mini Kit (Qiagen, Manchester, U.K.) was used for effective purification of miR and total RNA from dissected rat arteries. Samples were reverse transcribed using the miScript II RT Kit (Qiagen, Manchester, U.K.) with HiFlex Buffer to allow miR as well as mRNA to be quantified from the same sample. Measurements of miR or mRNA levels were performed by qPCR using the miScript SYBR Green PCR Kit (Qiagen, Manchester, U.K.) with the CFX96 Real-Time PCR Detection System (Bio-Rad, Hertfordshire, U.K.).

Western Blot

Artery protein lysates were probed after transfer to polyvinylidene fluoride (PVDF) membrane (Millipore, Hertfordshire, UK) with a primary antibody diluted in blocking buffer either overnight at 4 °C or for 1 hour at room temperature. Primary antibodies used were rabbit anti-Kv7.4 (1:200; sc-50417; Santa Cruz, Texas, USA) and mouse β-Actin (1:5000; A1978; Sigma Aldrich, Dorset, UK).

Functional studies

Morphological properties of mesenteric arteries from NT rats and SHRs as well as NT mesenteric arteries treated with NTC miR or miR153 were studied using Pressure Servo System PS/200 (Living Systems Instrumentations, Burlington, USA). The structural

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properties of the segments and responses to the Kv7 activator, S1 (NeuroSearch A/S, Ballerup, Denmark) were recorded and acquired with DMK 41AU02 Monochrome Industrial Camera (Imaging Source, Bremen, Germany) hosted by a PC running MyoVIEW II software (Danish Myo Technology, Aarhus, Denmark). Data were analysed using MyoVIEW II and MicroCal Origin 6.0 (MicroCal Software, Northampton, USA). Isometric tension was recorded on NT mesenteric or middle cerebral arteries transfected with either mir153 or NTC miR in a wire myograph (Danish Myo Technology, Aarhus, Denmark). Mesenteric vessels were pre-constricted with 1μM U46619 (Sigma Aldrich, Dorset, UK) and a concentration effect curve to the Kv7.2-7.5 activators ML213 (HelloBio, Bristol, UK), ICA-069673 (HelloBio, Bristol, UK), the β –adrenoceptor agonist isoprenaline (Sigma Aldrich, Dorset, UK) or the Kv7.1-specific activator RL-3 (Tocris, Bristol, UK) as well as a relaxation response to 1μM nicardipine (Sigma Aldrich, Dorset, UK) were obtained. Relaxant responses to S1 or ML213 were also assessed in middle cerebral arteries transfected with miR153 or NTC and pre-constricted with 100nM U46619. Data were recorded and analysed using LabChart® 7 (ADInstruments, Dunedin, New Zealand).

Histology

Mesenteric artery branches transfected with NTC miR or miR153, were embedded in OCT, frozen rapidly and cryosectioned at approximately 7 μm. Slices then were fixed with ice-cold acetone and stained using an Elastica van Gieson staining kit or successively stained with Hematoxylin and Eosin (Merck, NJ, USA). After dehydration, slices were covered with DPX mounting solution and stored for further use. An Axioplan 2 Upright microscope with attached Axio Cam Digital CCD camera (Carl Zeiss Ltd, Cambridge, UK) was used for imaging.

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Statistical Analysis

The data were expressed as mean±SEM (standard error) with N representing the number of animals and n, the number of experiments. Comparisons of data were accomplished by one-way ANOVA or two-way ANOVA followed by Bonferroni multiple comparisons test; student's t-test or paired t-test as appropriate. The differences between means were considered significantly different when $P<0.05$.

Results

Kv7.4 protein is downregulated in hypertensive arteries

Previous findings on Kv7.4/KCNQ4 arterial expression in hypertensive animals have been obtained in different arteries in various separate studies [3,4,6,7,15]. Initial experiments in the current study therefore sought to corroborate these previous results but within one study to allow novel comparisons to be made. Interrogation of protein lysates from HEK293 cells stably expressing Kv7.4, with a Kv7.4 antibody produced reactive bands corresponding to the theoretical molecular weight of Kv7.4 that were not present in untransfected HEK293 cells (n=3, Figure S1). Similar reactive bands were identified in protein lysates from thoracic aortic, mesenteric, renal and middle cerebral arteries from normotensive (NT) rats and SHRs (Figure 1A). Kv7.4 expression was decreased in thoracic aortic (59.45±15.69%, N=8, P=0.02) mesenteric (27.33±8.32%, N=8, P=0.04) and renal arteries (36.14±11.96%, N=6, P=0.03) from SHRs compared to NT rats (Figures 1B-D). In contrast, there was no difference in Kv7.4 protein expression in middle cerebral arteries from SHR and NT animals (N=3, p=0.21, Figure 1E).

Quantitative polymerase chain reaction was undertaken to assess whether the decrease in Kv7.4 protein in SHRs correlated to a reduction in KCNQ4 mRNA. In thoracic aorta from

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SHRs there was a 1.35-fold decrease in KCNQ4 mRNA expression (N=4, P=0.03, Figure 1F) whereas for mesenteric arteries, KCNQ4 mRNA increased 13.35 fold (N=3, P=0.02, Figure 1G) in SHRs compared to NT rats. There was no difference in mRNA levels between SHRs and NT rats in renal arteries (N=4, P=0.29, Figure 1H) or middle cerebral arteries (N=3, P=0.17, Figure 1I). Overall, these data show that Kv7.4 is compromised in different arteries from SHRs that is not consistently associated with a change in transcript level.

miR153 increases in SHR mesenteric, renal and thoracic aortic arteries compared to NT rats

TargetScan6.2 and mirDIP were used to identify candidate miRs targeted at the 3'UTR of KCNQ4 (Figure S2). This analysis revealed putative seed sequences in KCNQ4 for miR-26a, 214, 133a, 200b, 153 and 218 and let-7d. Among them, miR153 showed the highest probability of consensus targeting. Analogous miR153 seed sequences were not detected in the 3' UTR of KCNQ5 or KCNQ1, the other KCNQs highly expressed in the vasculature.[8]

The working hypothesis is that an increase in miR expression in SHR arteries results in Kv7.4 degradation. Figure 2 shows that whilst the expression of miR26a, miR200b, miR214, miR218 and let-7d was not altered in arteries from SHRs compared to NT, a significant increase of miR153 was recorded in SHR mesenteric artery (8.65, N=6, $p<0.0001$), renal artery (2.08, N=6, $p<0.0001$), and thoracic aorta (2.80, N=6, $p<0.0001$). A 1.35-fold increase was also observed for miR133a in SHR aorta (N=6, P=0.006). Interestingly, miR153 was higher in the plasma from SHRs compared to NT plasma (N=6, P=0.14, Figure S3). In contrast, there was no change in the expression of any miRs in middle cerebral arteries from SHRs compared to NT rats (Figure 2D, N=5, all p values were not significant). Consequently

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miR153 expression paralleled hypertension-induced changes in Kv7.4 levels across the arteries under study.

miR153 is confirmed to interact functionally with KCNQ4 3'UTR

A luciferase reporter was engineered to contain a fragment of the KCNQ4 mRNA 3'UTR encompassing 4 predicted binding sites for miR153 (wild-type, WT); or deletion of seed sequences corresponding to site 1 (Δ site1) and site 4 (Δ site4, Figure 3A). HEK293 cells cotransfected with WT KCNQ4 3'UTR and miR153 produced a decrease in luciferase activity compared to those cotransfected with KCNQ4 3'UTR and the non-targeting miR control (NTC miR, n=4, P<0.01, Figure 3B); which was eliminated when the 2 predicted binding sites were deleted (Δ site1, n=3, P=0.72 and Δ site4, n=4, P=0.76); indicating miR153 interacts functionally with the 3'UTR of KCNQ4.

Addition of a synthetic miR153 to NT mesenteric arteries mimics an SHR-like phenotype

In all the arteries studied, SHR mesenteric arteries showed the greatest fold increase in miR153 compared to NT rats hence we focussed on mesenteric arteries from NT rats for our transfections studies with a miR153 mimic. Addition of miR153 to mesenteric arteries from NT rats resulted in a large increase in miR153 levels compared to arteries from the same animal transfected with NTC miR (N=11, P=0.04, Figure 4A). In arteries transfected with miR153, KCNQ4 mRNA expression increased ~2 fold (N=4, P=0.02, Figure 4B) whilst Kv7.4 abundance decreased by 75.43±0.15% (N=3, p<0.0001, Figure 4C) compared to NTC miR transfected vessels, which mirrored the changes recorded for SHRs shown in Figure 1. Cotransfection of NT mesenteric arteries with miR153 and miR133a showed a marked decrease in KCNQ4 (N=4, *P*<0.05, Figure S4A) but not KCNQ5 (N=4, P=0.34, Figure S4B), compared to NTC miR transfected vessels.

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Perfusion myograph experiments showed that transfection of mesenteric arteries from NT rats with miR153 produced morphological changes to the artery that approximated a SHR-like phenotype (Figure 5A-C and Tables S1-S2, n=10-12; N=7-10). In all experiments, arteries with similar outer wall dimensions were used and transfection with NTC miR had no effect on wall morphology (Figure 5, Tables S1 and S2). However, transfection with miR153 for 24h produced a significant increase in the media/lumen ratio that approached the values obtained in arteries from SHRs (Figure 5A, B, Tables S1 and S2). Average wall thickness was also enhanced in miR153 transfected (31.0±1.0 μm) and SHR (37.0±1.4 μm) mesenteric arteries compared to NTC miR transfected vessels (24.9±1.1 μm, $P<0.01$) or NT vessels (22.9±1.3 μm, $P<0.001$), respectively (Figure 5A, C, Tables S1 and S2) with Figure 5Di indicating an increase in the *tunica media* of miR153 transfected vessels compared to the control but no increase in cell number (Figure 5Dii; 49±2 versus 47±3 nuclei per slice for NTC miR or miR153 transfected NT mesenteric arteries respectively, n=12-13, $P=0.621$). Consequently, a short term increase of miR153 in NT mesenteric arteries produced morphological changes analogous to arteries from SHRs.

In further perfusion myograph studies application of the Kv7 activator, S1 caused a decrease in the media/lumen ratio in NTC miR transfected NT vessels (0.162±0.013 to 0.125±0.013, $N=4$, $P<0.01$) but had no effect on miR153 transfected mesenteric arteries (0.195±0.012 ± S1 vessels, $N=4$, $P=0.391$, Figure 6A). Isometric tension recordings of these miR153 transfected vessels also showed decreased relaxation to two structurally different Kv7 activators, ML213[24] ($N=8$, $P<0.01$, Figure 6B), which was similarly lacking in arteries from SHRs ($N=3$, $P<0.05$ Figure 6B); and ICA673 ($N=5$, P $P<0.0001$ or $P<0.01$, Figure 6C). A similar trend in the relaxation response to the β–adrenoceptor agonist isoprenaline was also observed in miR153 transfected mesenteric arteries ($N=6$, $P>0.05$, Figure 6D). Neither the relaxant effect of 1μM nicardipine (86.98±0.68 versus 90.53±2.68 for NTC miR or miR153

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transfected NT mesenteric arteries respectively, N=4-5, P=0.3) and the Kv7.1-specific activator, RL-3[25] (N= 4-8, Figure S5A) nor vessel constriction by the thromboxane A2 analog U46619 (N= 4-8, Figure S5B) were affected by miR153 over-expression. Consequently, increasing miR153 levels exogenously to NT MA mimics functional characteristics of mesenteric arteries from SHRs. Interestingly, in cerebral arteries where responses to Kv7.2-5 activators are unaffected in SHRs[15] and no rise in miR153 was detected, transfection with exogenous miR153 produced a marked impairment in the relaxant response to S1 and ML213 (N=4-5, $P<0.01$, $P<0.001$ respectively, Figure 6E).

Discussion

This study is the first to provide evidence for a role for miRs in the regulation of Kv7.4 in the vasculature and raises the possibility that this post-transcriptional regulation is a contributing factor of vascular dysfunction. As there is considerable evidence that Kv7 channels regulate vascular smooth muscle contractility and contribute to the vasodilator response of many endogenous agents[3,4,6-15,24,26-28] the present study implicates miR153 as a determinant of vascular tone and potentially a novel therapeutic target for the treatment of hypertension.

In accordance with previous findings[3,4,7] we observed a decrease in Kv7.4 protein expression in mesenteric and renal arteries from SHRs that was not associated with a decrease in KCNQ4 mRNA. This suggests a post-transcriptional regulator, such as miRs, dictate Kv7.4 abundance. Indeed the expression profile of miRs predicted to target KCNQ4 revealed an upregulation of miR153 in arteries from SHRs compared to NT rats. Moreover, in cerebral arteries, where there was no difference in Kv7.4 protein or mRNA expression between NT and SHR and no change in Kv7 activity functionally,[15] there was no increase in miR153 or any of the miRs analysed. Hence the miR153 expression profile correlates closely to the

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Kv7.4/KCNQ4 expression profiles for the four arteries in hypertension. The reason for this variability between arteries is difficult to define in detail since the nature of cell specific regulation by miRs continues to be investigated but it provides a mechanism to tailor cellular responses to individual cell types. We propose that miR153 represses translation of KCNQ4 mRNA rather than by transcript degradation as KCNQ4 levels did not decrease in renal or mesenteric arteries. In fact, KCNQ4 expression increased markedly in mesenteric arteries from SHRs or after miR153 transfection, which is probably due to the suppression of a negative feedback loop altering transcription factors.[29] In aorta from SHRs, miR133a as well as miR153 increased but in this artery the decreased Kv7.4 protein expression was associated with a decrease in mRNA. Interestingly, we reduced KCNQ4 transcript levels in NT mesenteric arteries by co-transfecting miR153 with miR133a providing support for our hypothesis that in the aorta a rise in miR133a affects KCNQ4 transcription whereas the rise in miR153 seen in the aorta, renal and mesenteric arteries leads to impaired translation of KCNQ4 mRNA. Mir133a has been implicated in modulation of the VSMC phenotype in general and specifically in the regulation of VSMC proliferation in mouse aorta.[23,30] However, as miR133a was unchanged in other hypertensive vessels that displayed reduced Kv7.4 expression, and miR153 was consistently upregulated in these vessels, we focussed on defining the impact of miR153 on Kv7.4 in hypertension.

Having identified a rise in miR153 expression correlated with a reduction in Kv7.4 we established that miR153 has a direct targeting effect on the 3' UTR of KCNQ4 using a luciferase reporter assay. Furthermore, we showed that addition of synthetic miR153 to mesenteric arteries from NT rats caused an up-regulation of KCNQ4 mRNA, decreased Kv7.4 protein expression and attenuated relaxations to structurally different Kv7 activators similar to that seen in arteries from SHRs (current study and [3,4,7,15]). A shift in the relaxation response to the β –adrenoceptor agonist isoprenaline was also observed in miR153

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transfected mesenteric arteries although not significantly, which may reflect the complex intracellular signalling involved. Interestingly, transfection of miR153 impaired the relaxation induced by Kv7 activators in the cerebral artery, which showed no change in miR153 levels between normotensive and hypertensive rats, further suggesting that a rise in miR153 downregulates Kv7.4 protein function. It is worth stressing that the rise in miR153 produced by transfection with a synthetic mimetic far exceeds levels seen in arteries from SHRs. However, we have no knowledge about the regulation of miR153 in arteries in response to hypertensive cues and the data are a good proof of concept. The striking similarity in Kv7 characteristics between arteries from SHRs and those from NT rats transfected with miR153 provide strong support for miR153 regulation of Kv7.4. Furthermore, it is possible that in SHRs, miRs exert their effect in a time-dependent manner. Thus to obtain a similar effect in our in vitro system, after only 24h, we need a very high concentration of miR153.

Remarkably, our pressure myography data showed that media/lumen ratio and average wall thickness were significantly increased in miR153 transfected vessels compared to NTC miR transfected vessels, showing a tendency of shifting the morphological characteristics of native normotensive mesenteric arteries towards an SHR phenotype within 24h after transfection. The increase in average wall thickness (from 24.9 ± 1.1 µm to 31.0 ± 1.0 µm) produced by miR153 transfection is equivalent to the possible appearance of just one extra layer of smooth muscle cells. Vascular remodelling[31,32] involves several cell types including a phenotypic switch of VSMCs between a differentiated, contractile state with a low proliferation rate to a dedifferentiated, synthetic state in which VSMCs are more proliferative.[33,34] Studies with vascular smooth muscle-specific knockout of Dicer, show miRs are crucial for VSMC development, differentiation, and contractile function.[20-22,35] Importantly, widespread loss of miR expression in these knockout animals caused decreased blood pressure attributed to a

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reduced contractile tone and media thickness in aorta.[36] miR153 has also been linked to proliferation in venous samples after mechanical injury.[37] However, H&E staining did not reveal an increase in cell number in the tunica media suggesting the wall thickening stems from deposition of collagen, fibronectin, and other components of the extracellular matrix similar to the remodelled arterial structures in SHRs[31, 32] or there is an increase in cell size. The mechanisms linking Kv7.4 dysfunction and changes in arterial morphology and responsiveness will be the focus of future studies.

It should be emphasized that miR153-induced KCNQ4 downregulation is only a contributory component in the multifactorial disease of hypertension. MiR153 is known to affect targets other than KCNQ4[37-40] although TargetScan6.2 predicted miR153 to only target the 3'UTR of KCNQ4 and not KCNQ5 or KCNQ1, the other KCNQs highly expressed in the vasculature. Moreover, KCNQ1 and KCNQ5, are down regulated by other miRs namely miR1/133 and miR190 respectively.[41,42] Nevertheless, the parallel change in miR153 and Kv7.4 is clear raising the possibility that correction of the miR153 upregulation that leads to the aberrant Kv7 channel function/expression, vasoconstriction and vascular wall thickening, through the use of miR sponges,[43] chemical inhibitors[44] and antagomirs[45] as a potential treatment for hypertension. Furthermore, a rise in miR153 was detected in the blood of SHRs suggesting that this may present a novel diagnostic marker for hypertensive scenarios. However, investigation of antagomirs on *ex vivo* arteries from SHRs should be approached with caution since we have preliminary data indicating a 'loss' of the SHR phenotype occurs in arteries incubated in DMEM for 24-72hours (Figure S6A-C). Interestingly, we observed a decreased trend of miR153 in mesenteric arteries from SHR upon incubation in DMEM for 48h, possibly explaining the loss of Kv7.4 functional impairment observed in the incubated vessels (Figure S6D).

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Increased vascular tone, a relative resistance to vasorelaxants and vascular remodelling underlie vascular disease. Our data define a role for aberrant miR153 underlying this hypertensive state through targeting of KCNQ4 in an animal model of hypertension. Research into vascular Kv7 channels is in its infancy and therefore it is not possible at the moment to determine how much regulation of Kv7.4 channels by miRs contributes to post-transcriptional control globally. Further research is required to determine the full spectrum of miR153 target genes, possible side effects and a therapeutic strategy/delivery for the correction of miR153 upregulation but the potential use of miR153 related therapies in clinical vascular disease is certainly worth exploring further.

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Conflict of Interest

None declared.

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Figure Legends

Figure 1: Kv7.4 protein expression is decreased in SHR mesenteric, renal and thoracic aortic arteries compared to NT rats with a decrease in KCNQ4 expression in SHR thoracic aortic arteries compared to NT rats. **A** Representative Western blot for Kv7.4 expression in total protein lysates from normotensive (NT) rats and spontaneously hypertensive rats (SHR): thoracic aorta (TA), mesenteric artery (MA), renal artery (RA), and middle cerebral artery (MCA). β-actin bands indicate protein loading controls. **B-E** Mean (±SEM) pixel densities of Kv7.4 bands normalized to their respective β-actin bands for NT and SHR **B**, thoracic aorta, **C**, mesenteric artery, **D**, renal artery, and **E**, middle cerebral artery from at least 3 separate protein isolations (N=3-8). **F-I** Quantitative polymerase chain reaction analysis with $2^{-\Delta\Delta Cq}$ of relative abundance of KCNQ4 of the SHR compared with the NT rat in **F**, thoracic aorta, **G**, mesenteric artery, **H**, renal artery and **I**, middle cerebral artery from at least 3 separate RNA isolations (mean±SEM, N=3-4). **P*<0.05 according to paired Student's *t* test.

Figure 2: miR153 increases in SHR mesenteric, renal and thoracic aortic arteries compared to NT rats. Quantitative polymerase chain reaction analysis with $2^{-\Delta\Delta Cq}$ of relative abundance of miR26a, miR214, miR133a, miR200b, miR153, miR218 and let-7d of the spontaneously hypertensive rat (SHR) compared with the normotensive (NT) rat in **A**, mesenteric artery, **B**, renal artery, **C**, thoracic aorta and **D**, middle cerebral artery from at least 5 separate RNA isolations (mean±SEM, N=5-6). *****P*<0.0001, ***P*<0.01 according to Student's *t* test.

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Figure 3: miR153 targets KCNQ4 3'UTR

A Schematic representation of human KCNQ4 mRNA 5'UTR, coding sequence, and 3'UTR with miR153 binding sites highlighted. Red, blue, light blue and green squares in the 3'UTR indicate the predicted binding sites for miR153 (site 1, 2, 3 and 4, respectively). Seed sequences corresponding to site1 (red), site 2 (blue), site3 (light blue) and site 4 (green) are highlighted in the grey boxes showing the detailed nucleotide sequence of KCNQ4 3'UTR. Positions of the seed sequences are indicated above with the same colour scheme. **B** Mean luciferase activity in HEK293 cells of KCNQ4 3'UTR wild-type (WT), with deleted site 1(Δ site1), and with deleted site4 (Δ site4), co-transfected with miR153. Data are normalised to HEK293 cells transfected with KCNQ4 3'UTR only and expressed as % of HEK293 cells transfected with the same plasmids in the presence of non-targeting control (NTC) (mean±SEM, n=3-4). $^{**}P<0.01$ according to paired Student's *t* test (miR153 vs NTC for each plasmid).

Figure 4: Transfection of NT mesenteric arteries with miR153 leads to upregulation of KCNQ4 expression and downregulation of Kv7.4 expression

Quantitative polymerase chain reaction analysis with $2^{-\Delta\Delta Cq}$ of relative abundance of **A**, miR153 or **B**, KCNQ4 in normotensive mesenteric arteries transfected with non-targeting miR control (NTC) or miR153; data are normalised to NTC-transfected normotensive mesenteric arteries (mean ±SEM, N=11 and N=4, respectively). $^{*}P<0.05$ according to paired Student's *t* test. **Ci** Mean pixel densities of Kv7.4 bands normalised to their respective β-actin bands for normotensive mesenteric arteries transfected with NTC or miR153 from 3 separate protein isolations (N=3). $^{****}P<0.0001$ according to ratio paired *t* test. **Cii** Representative Western blot for Kv7.4 expression in total protein lysates from normotensive mesenteric arteries transfected with NTC or miR153. β-actin bands indicate control for protein loading.

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Figure 5: Addition of miR153 to mesenteric arteries of NT rats leads to vascular wall thickening

A Examples of raw CCD camera images (left) and corresponding automatic digitizing of outer and inner diameters (right) of native rat mesenteric artery segments pressurised to 60 mmHg for normotensive mesenteric artery (NT MA), non-targeting miR control (NTC miR) transfected artery, miR153 transfected artery (miR153) and spontaneously hypertensive rat mesenteric artery (SHR MA). **B** Mean (±SEM) media/lumen ratios and **C** mean (±SEM) media thickness of NTC miR compared to miR153 transfected normotensive mesenteric arteries; and mesenteric arteries of NT rats compared to SHRs (n=10-12; N=7-10). ***P<0.001 or **P<0.01 according to one-way ANOVA followed by Bonferroni multiple comparisons test. **D** Representative images of (i) Elastica van Gieson staining or (ii) Hematoxylin and Eosin staining (dark blue indicates nuclei, n=12-13) of NTC miR (left image) and miR153 (right image) transfected mesenteric artery segments; scale bars: 35 µm.

Figure 6: Addition of miR153 to mesenteric arteries or middle cerebral arteries of NT rats leads to a reduced response to Kv7 activators

A Mean (±SEM) media/lumen ratios of non-targeting miR control (NTC miR) transfected normotensive mesenteric arteries compared to S1 treated NTC miR transfected vessels; and miR153 transfected mesenteric arteries compared to S1 treated miR153 transfected normotensive mesenteric arteries (N=4). **B** Mean (±SEM) percentage relaxation to 1µM ML213 from isometric tension recordings in NTC miR compared to miR153 transfected normotensive mesenteric arteries; and in mesenteric arteries of normotensive (NT MA) rats compared to spontaneously hypertensive rats (SHR MA) (N=3-8). *P<0.05, **P<0.01 according to one-way ANOVA followed by Bonferroni multiple comparisons test. Isometric

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tension recordings of relaxation to increasing concentrations of **C** the Kv7 activator ICA-069673, **D** the β –adrenoceptor agonist isoprenaline in normotensive mesenteric arteries transfected with mir153 or NTC miR. Each point represents the mean of 4-8 animals ±SEM. ****P<0.0001, **P<0.01 according to two-way ANOVA followed by Bonferroni multiple comparisons test. **E** Mean (±SEM) percentage relaxation to 100nM S1 or 100nM ML213 from isometric tension recordings in NTC miR compared to miR153 transfected normotensive middle cerebral arteries (N=4-5). ***P<0.001, **P<0.01 according to two-way ANOVA followed by Bonferroni multiple comparisons test.

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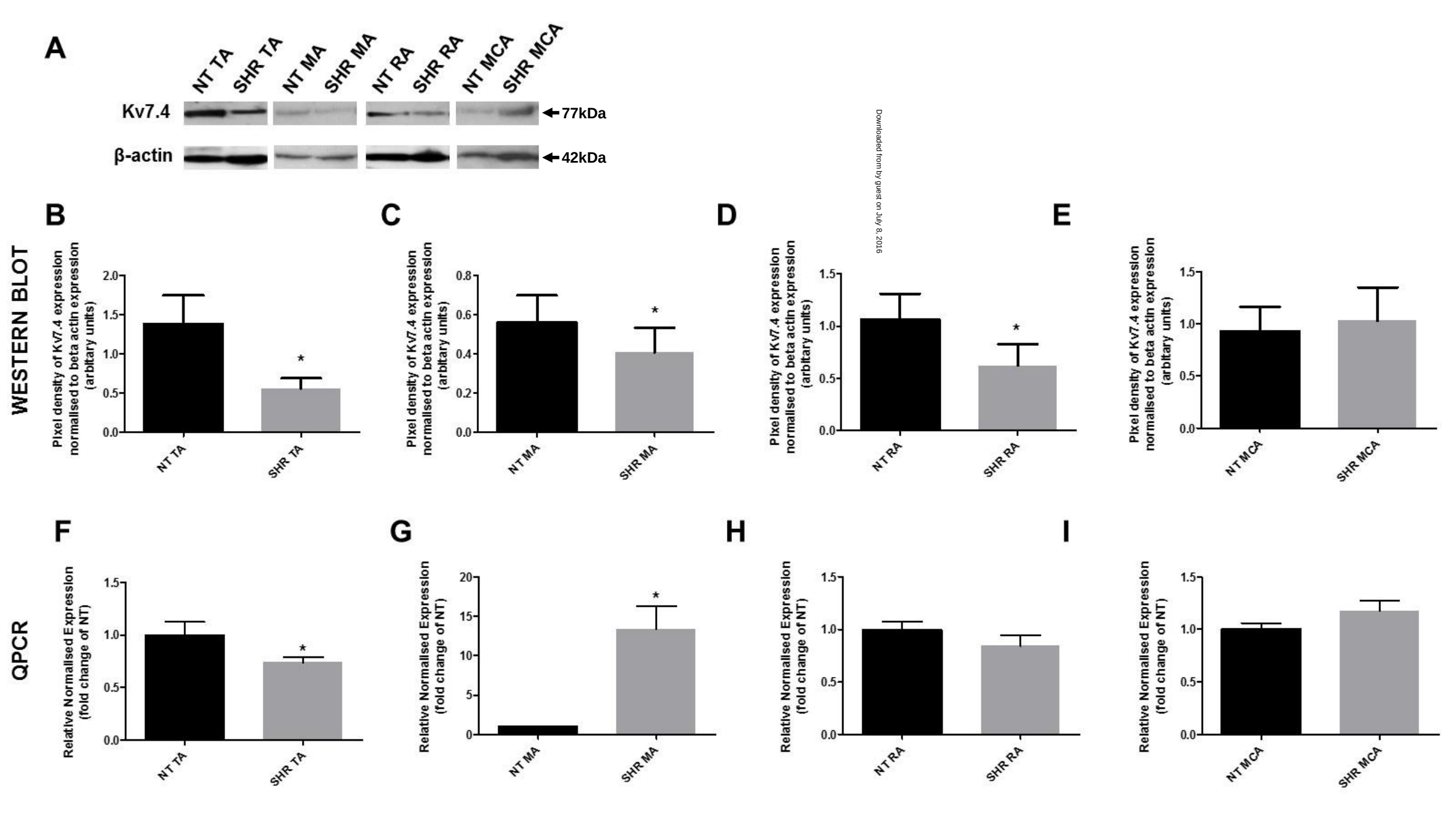

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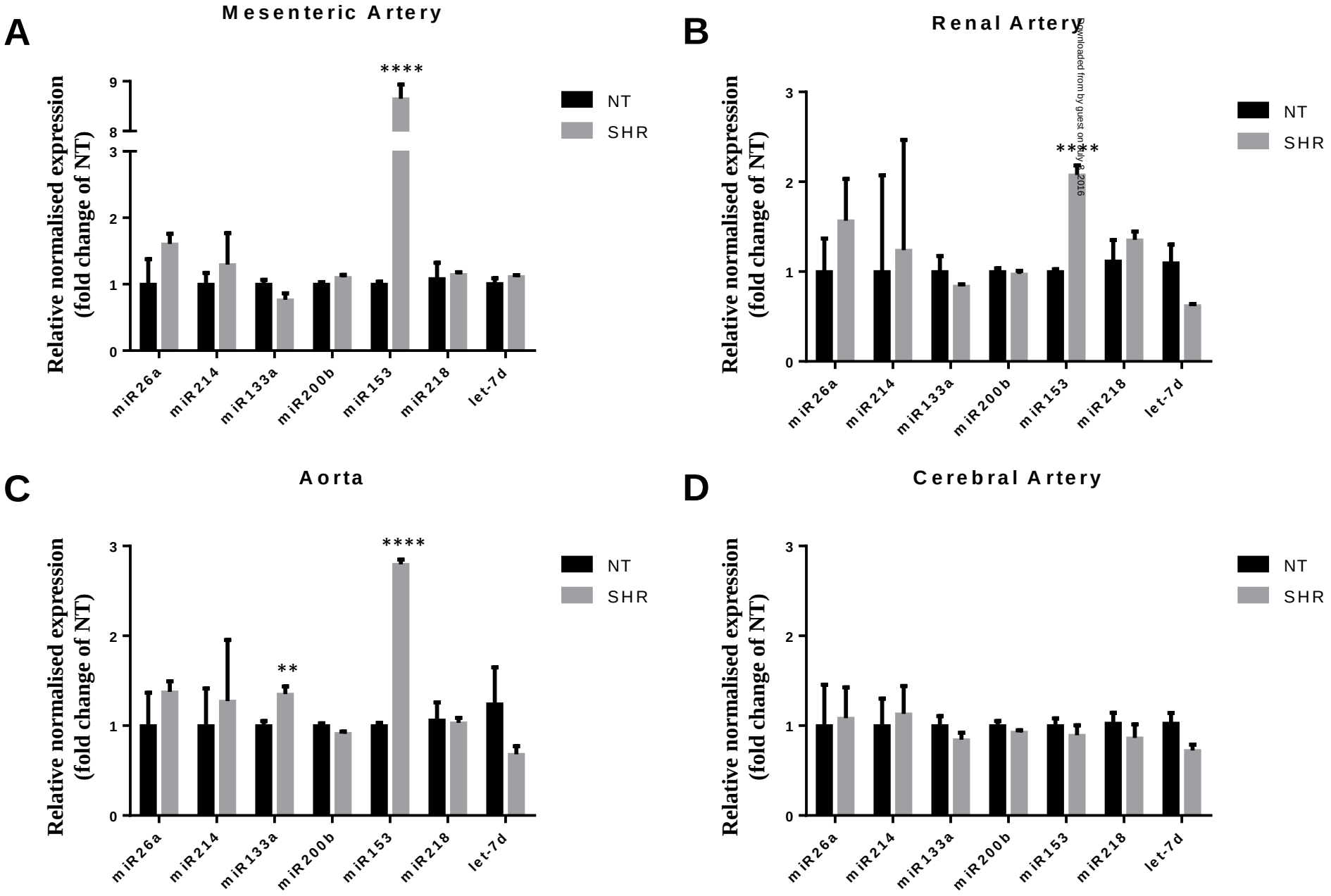

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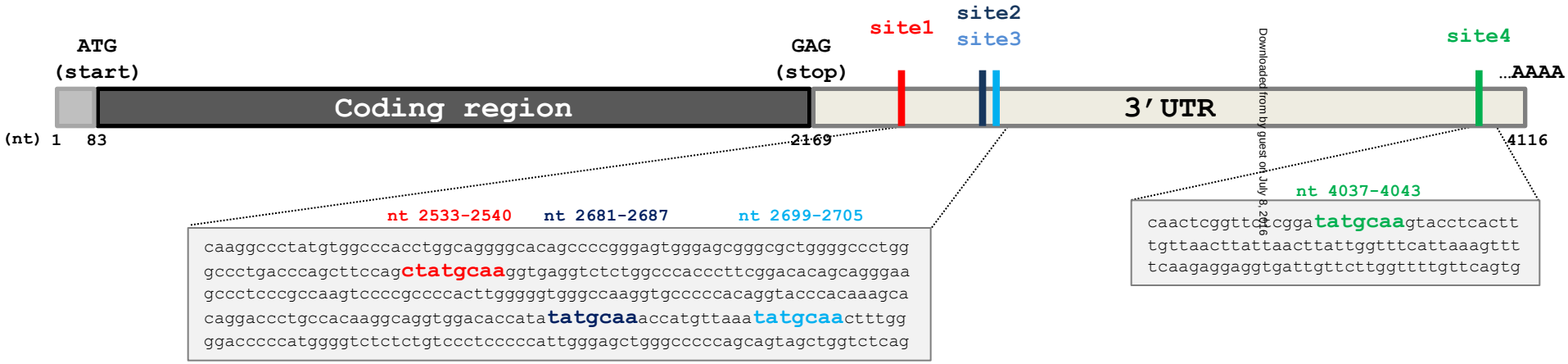
Human KCNQ4 mRNA (NM_004700.3)
ATG
(start)
GAG
(stop)
site1
site2
site3
site4
...AAAA
Coding region
3′UTR
(nt) 1
83
2169
4116
nt 2533-2540
nt 2681-2687
nt 2699-2705
caaggccctatgtggcccacctggcaggggcacagccccgggagtgggagcgggcgctggggccctgg
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nt 4037-4043
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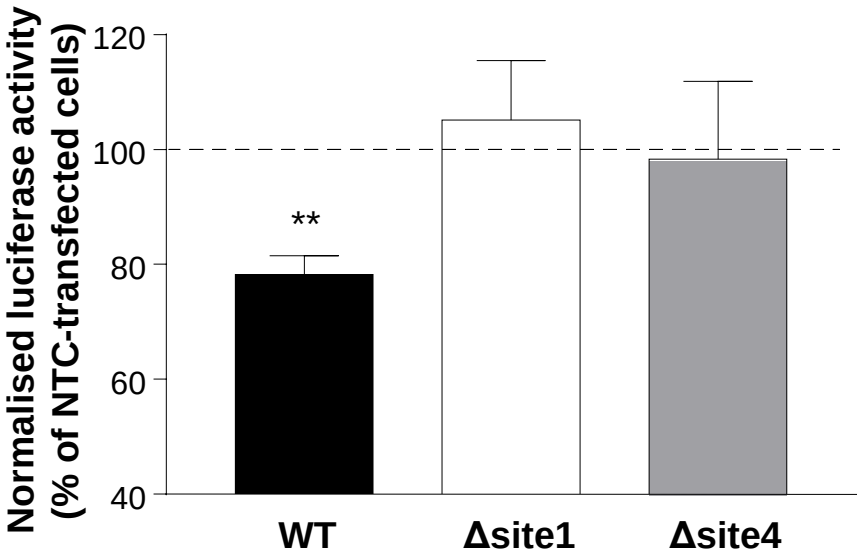
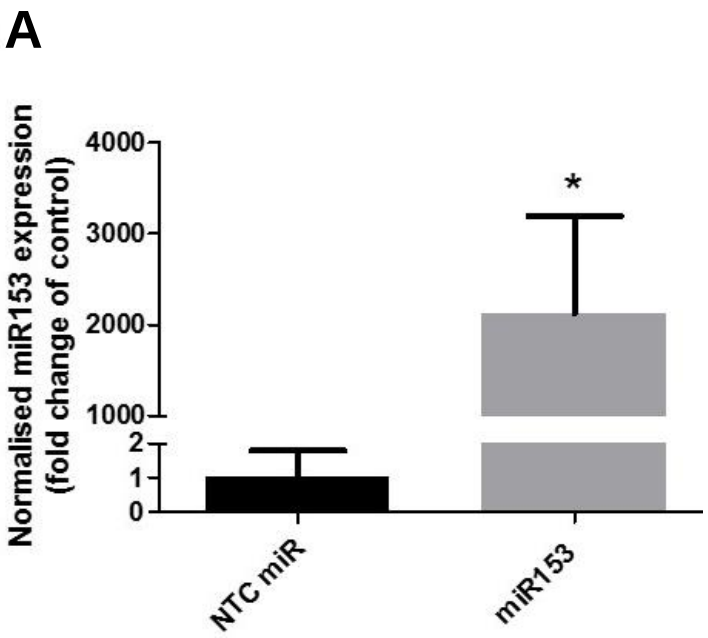
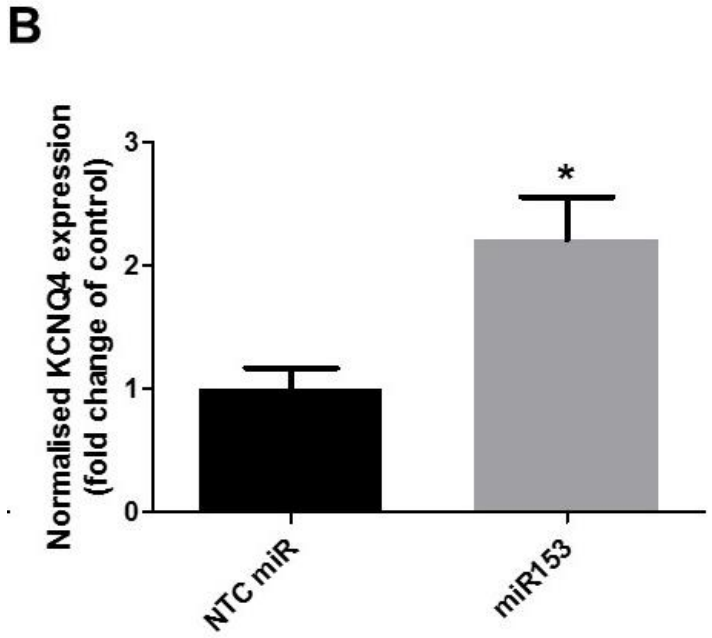
B


Normalised luciferase activity
(% of NTC-transfected cells)
120
100
80
60
40
**
WT
Δsite1
Δsite4





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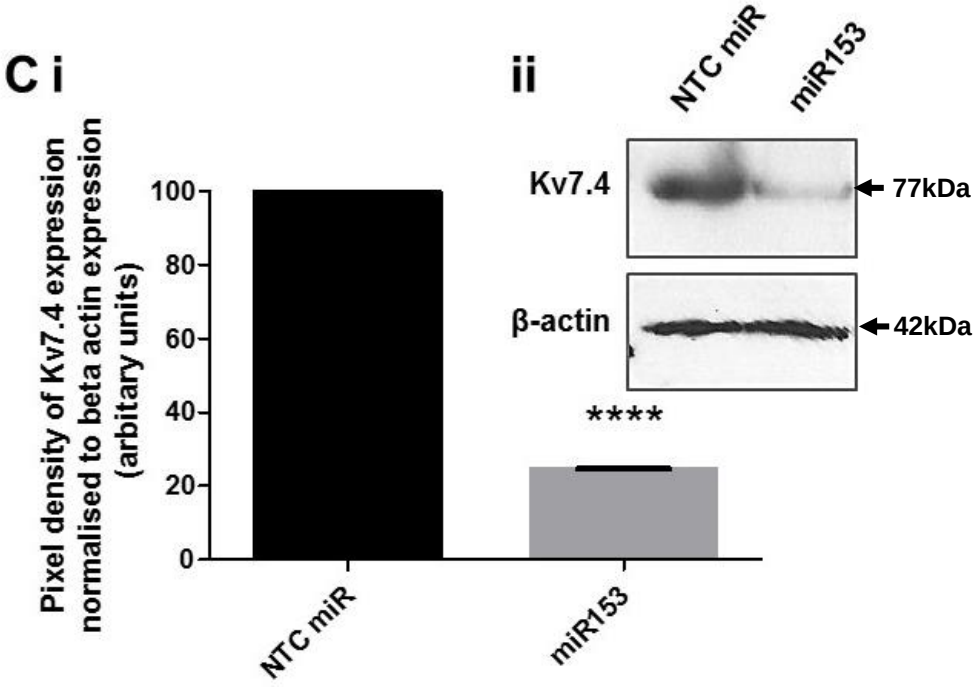

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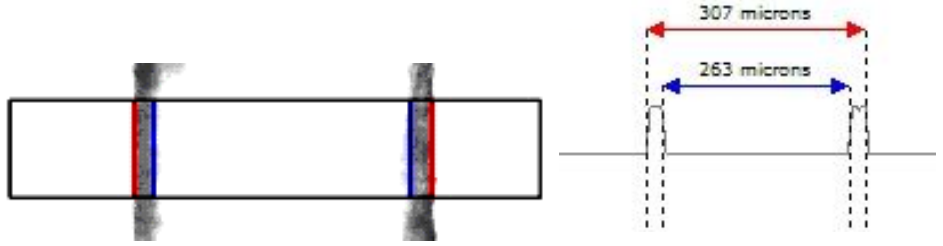
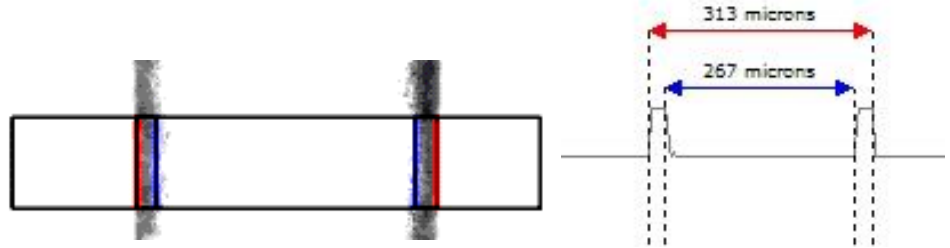


NT MA
M/L ratio = 0.167
av.w.th. = 22.0 µm



NTC miR
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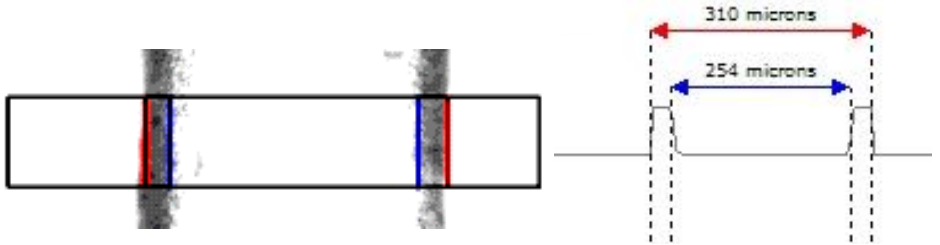
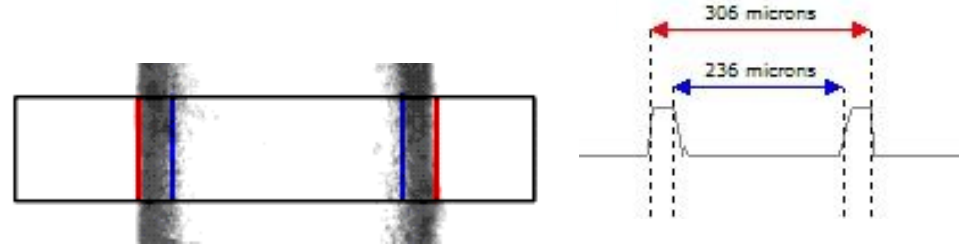
av.w.th. = 23.0 µm

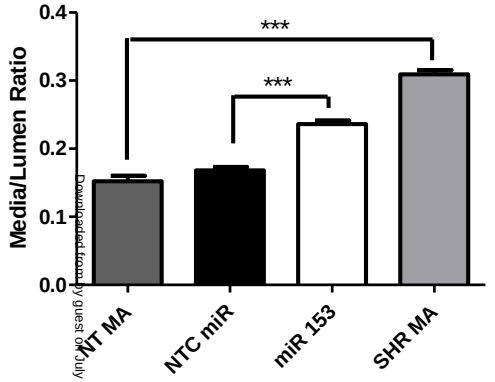


miR 153
M/L ratio = 0.220
av.w.th. = 28.0 µm



SHR MA
M/L ratio = 0.297
av.w.th. = 35.0 µm

B



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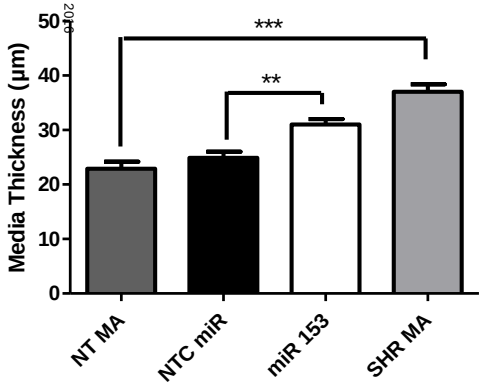

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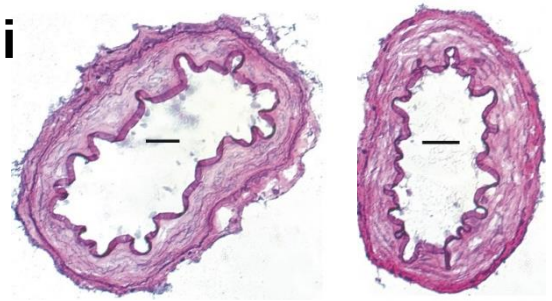

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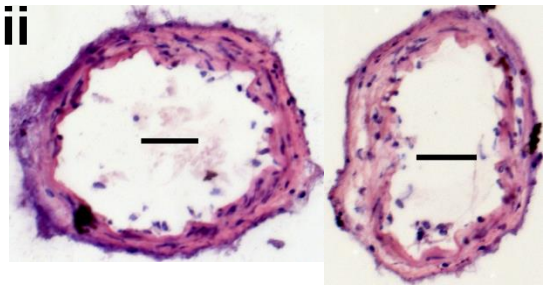

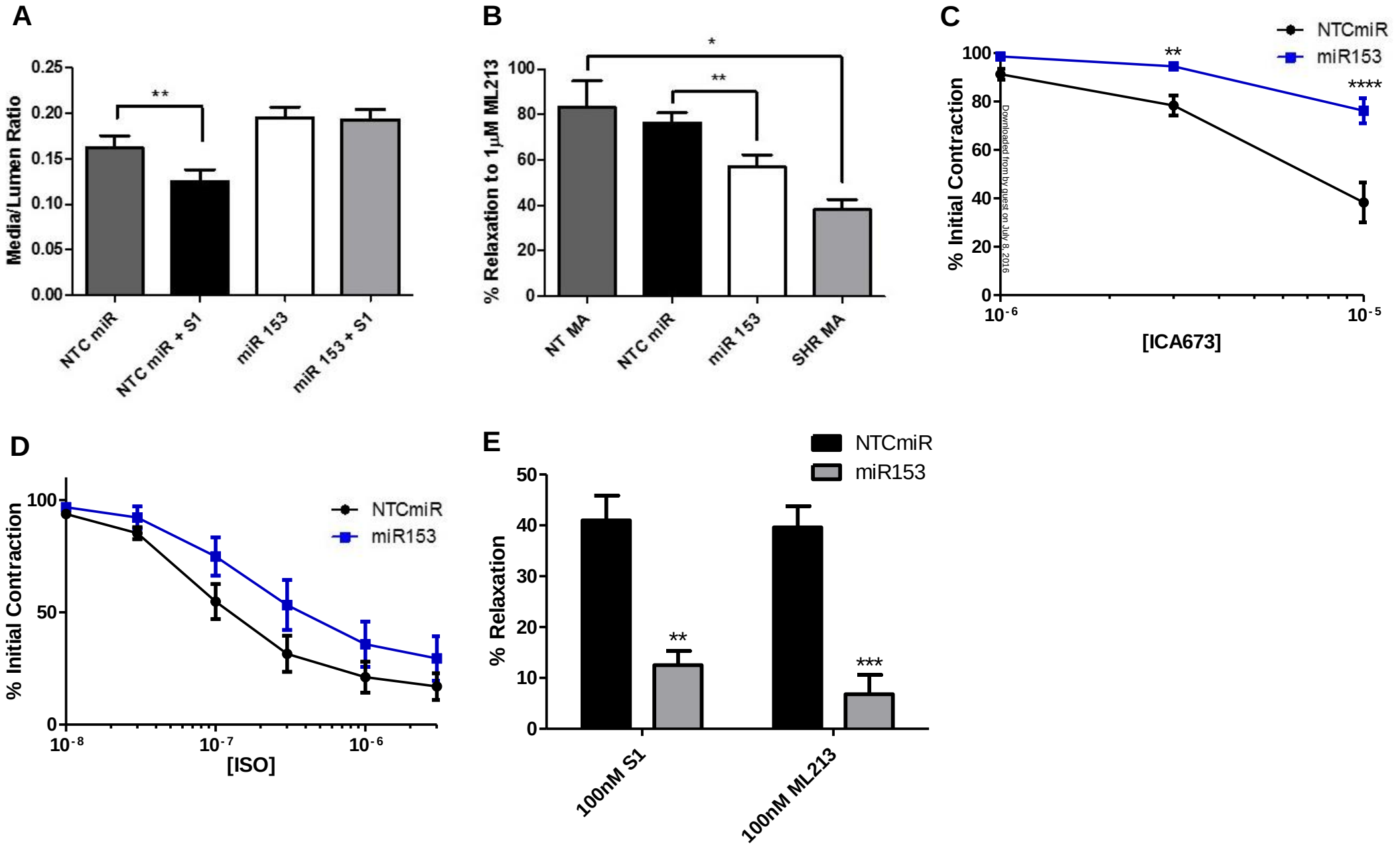
A
Media/Lumen Ratio
0.25
0.20
0.15
0.10
0.05
0.00
**
NTC miR
NTC miR + S1
miR 153
miR 153 + S1
B
% Relaxation to 1μM ML213
100
80
60
40
20
0
*
**
NT MA
NTC miR
miR 153
SHR MA
C
NTCmiR
miR153
% Initial Contraction
100
80
60
40
20
0
**
****
10-6
10-5
[ICA673]
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D
NTCmiR
miR153
% Initial Contraction
100
50
0
10-8
10-7
10-6
[ISO]
E
NTCmiR
miR153
% Relaxation
50
40
30
20
10
0
**
***
100nM S1
100nM ML213