

# **microRNA-352 regulates collateral vessel growth induced by elevated fluid shear stress in the rat hind limb**

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## **SUPPLEMENTAL MATERIAL**

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## Supplementary methods

**Immunofluorescence.** Cryosections were cut at 5  $\mu\text{m}$  thickness and fixed in 4% paraformaldehyde. Following incubation in 0.5% BSA (Solarbio, China) with 0.3% Triton X-100 (SigmaAldrich, MO, USA), the sections were incubated with primary and secondary antibodies (Table S2), followed by Cy2- or Cy3-conjugated streptavidin (Biotrend, Germany). The nuclei were stained with 4',6-diamidino-phenylidole (DAPI, Invitrogen, Life Technologies, OR, USA). The sections were coverslipped and viewed with a Nikon confocal microscope (Nikon, Japan).

Immunostaining of cultured cells was performed following a similar protocol as that described above, except the primary antibody was incubated at 4°C overnight.

Incubation with phosphate-buffered saline (PBS) instead of the primary antibody was performed as negative control to exclude nonspecific binding of the secondary detection system.

**Quantitative measurements.** Quantification of the immunofluorescence intensity of IGF2R, IGF2 and Beclin1 was performed with a Nikon confocal microscope, using the quantitation software EZ-C1 3.70. Briefly, one channel with format 512 and appropriate filters were used. A full range of grey values from black to peak white (0-pixel to 255-pixel intensity level) was set during the entire measurement process. Fluorescence intensity of was expressed as arbitrary units ( $\text{AU}/\mu\text{m}^2$ ).

Quantitative analysis of Ki67 was performed using the confocal microscope. Quantification was performed at 40 $\times$ , and the ratio of the number of Ki67 positive nuclei to the total number of nuclei in the vascular wall was considered as the proliferation index.

**RT-PCR.** Total small RNA was isolated using the mirVana<sup>™</sup> miRNA Isolation Kit (Ambion, Life Technologies, CA, USA), and miRNA expression was validated by quantitative stem-loop PCR technology (TaqMan microRNA Assays, Applied Biosystems, Life Technologies, CA, USA) as previously described<sup>1</sup> (Table S3). The use of target-specific reverse transcription primers and TaqMan hybridization probes allows the specific detection of mature miRNAs. miRNA expression was normalized to the expression of U6.

Total RNA was isolated using TRIzol (Invitrogen, Life Technologies, CA, USA) according to the manufacturer's protocol. Afterwards, 1  $\mu\text{g}$  of total RNA was transcribed to cDNA (Fermentas, Thermo Scientific, Lithuania), and real-time PCR was performed on a mastercycler nexus (Eppendorf, Germany). The expression of 18s RNA served as a loading control. Primer sequences are listed in Table S3.

**microRNA in situ hybridization.** In situ hybridization was performed on 10 $\mu\text{m}$  frozen tissue sections using locked-nucleic acid probes (Exiqon, Denmark) based on a modified version of the protocol described by Heller et al<sup>2</sup>. Sections were thawed for 20 min at room temperature, incubated with protein kinase K (Merck, MA, USA) for 20 min at 37°C, washed for 10 min with PBS and fixed with 4% paraformaldehyde for 10 min. After washing 3 times with PBS, sections were acetylated for 10 min in triethanolamine (Kermel, China) and acetic anhydride (Huihong, China) and washed once. Sections were incubated with

hybridization buffer for 1 h at room temperature; meanwhile, probes (3' and/ or 5'-DIG labelled LNA probes) were mixed with hybridization buffer, heated to 65°C for 5 min, chilled on ice, added to the sections incubated overnight at 54°C. After incubation for 1 h in 50% formamide/ 1×SSC at 54°C, 15 min in 0.2× SSC at room temperature and washed for 15 min in PBS. Sections were blocked with blocking reagent at room temperature for 1 h. For the localization of miR-352 expression in endothelial cells, following anti-DIG-FITC (Roche, Germany) incubation and a wash, the sections were incubated with CD31 (marker of endothelial cells) and secondary antibodies (Table S2), followed by Cy3-conjugated streptavidin (Biotrend, Germany). For the localization of miR-352 expression in smooth muscle cells, anti-DIG-FITC and  $\alpha$ -smooth muscle actin Cy3-conjugated were added for 1h at 37°C. Sections were washed 3 times in PBS with 0.1% Tween-20 (Sigma Aldrich, MO, USA) and 2 times in PBS; and then, they were mounted in DAPI mounting medium. U6 was used as a positive control (Table S4).

**Cell culture.** Rat aorta endothelial cells (RAECs) and endothelial basal medium were purchased from Chiscin Scientific (CHI Scientific, China) and cultured until the fifth passage.

**Transfection.** For transfection with miRNA mimic or miRNA inhibitor (Ribobio, China), RAECs were cultured to 70% confluence before transfection. For overexpression of miR-352, RAECs were transfected with 50 nM miR-352 mimic or mimic ncontrol using Lipofectamine 2000 (Invitrogen, Life Technologies, CA, USA) according to the manufacturer's protocol. For inhibition of miR-352, RAECs were transfected with 100 nM miR-352 inhibitor or miRNA inhibitor Ncontrol using Lipofectamin 2000. For sequences, see Table S5.

For siRNA-mediated gene knockdown, RAECs were cultured to 70% confluence and transfected with IGF2R siRNA (Ribobio, China) and miRNA inhibitor. A scrambled siRNA was used as a control.

**Luciferase reporter assay.** The 1257 bp IGF2R-3'UTR fragment includes a conserved binding site for miR-352. To examine whether miR-352 transcription occurs through IGF2R, RAECs were transfected with miR-352 mimic and miRNA Target IGF2R-3'UTR Vector Plasmid or mutant plasmid (Ribobio, China) in the same final concentrations using Lipofectamine 2000 as stated above (for sequences, see Table S5). At 48 h post-transfection, cell lysates were collected, and the activities of firefly and renilla luciferase were assessed with the Dual-Luciferase Reporter assay system (Promega, WI, USA).

**Western blot.** For Western blot analysis of IGF2R expression, RAECs were lysed in RIPA buffer (CWBio, China) for 20 min on ice. After centrifugation for 10 min at 14,000×g (4°C), the protein content of the samples was quantified using the BCA method. Equal amounts of protein were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Western blots were incubated overnight with antibodies directed against IGF2R;  $\beta$ -actin (CWBio, China) was used as a loading control.

**Tube formation assay.** At 48 h after transfection, RAECs ( $3 \times 10^4$  cells) were cultured

in a 48-well plate (Corning, NY, USA) coated with 100 µl of Matrigel Basement Membrane Matrix (Corning, MA, USA) for 6 h. Images were acquired at 5 randomly chosen microscopic fields. The cumulative tube length and the number of branch points were analysed with AxioVision 4.6 (Zeiss, Germany).

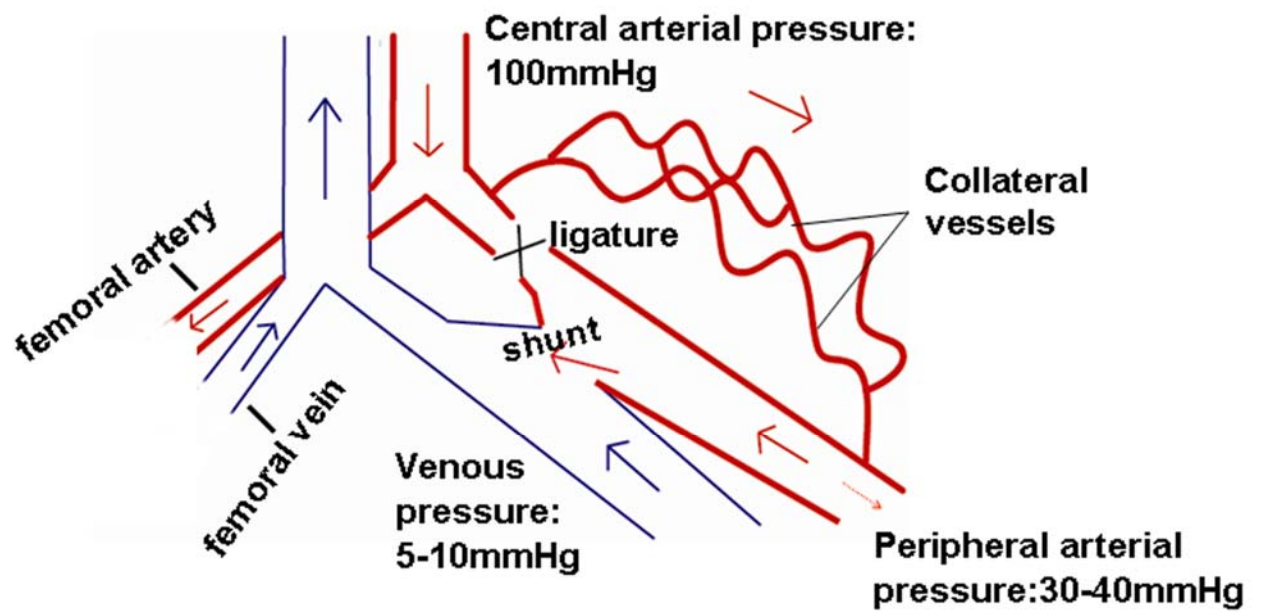
**MTT viability assay.** Assessment of cellular viability was performed at 24h following transfection by measuring the metabolization of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (Solarbio, China). MTT (1 mg/ml) was added to each well and the cells were cultured for an additional 4 h or 24 h, washed with PBS and lysed in dimethyl sulfoxide (DMSO, Solarbio, China). Absorbance was photometrically measured at a wavelength of 490 nm.

**Migration assay.** To evaluate the migration ability of RAECs, the cells were detached with trypsin (0.25% EDTA), harvested by centrifugation at 1,000×g, resuspended in 500 µl of EBM with 1% BSA, counted and placed in the upper chamber of Transwell chambers (5×10<sup>4</sup> cells per chamber, pore size 8 µm, Corning, ME, USA). The chamber was placed in a 24-well culture dish containing complete EBM. After incubation for 48 h, the non-migrated cells on the upper side of the chamber were mechanically removed and the remaining cells on the lower side were fixed with 95% alcohol. For quantification, cells were stained with crystal violet (Kermel, China). Stained migrated cells on the bottom side of the chamber were lysed in 33% acetic acid. Optical density was measured at 570 nm.

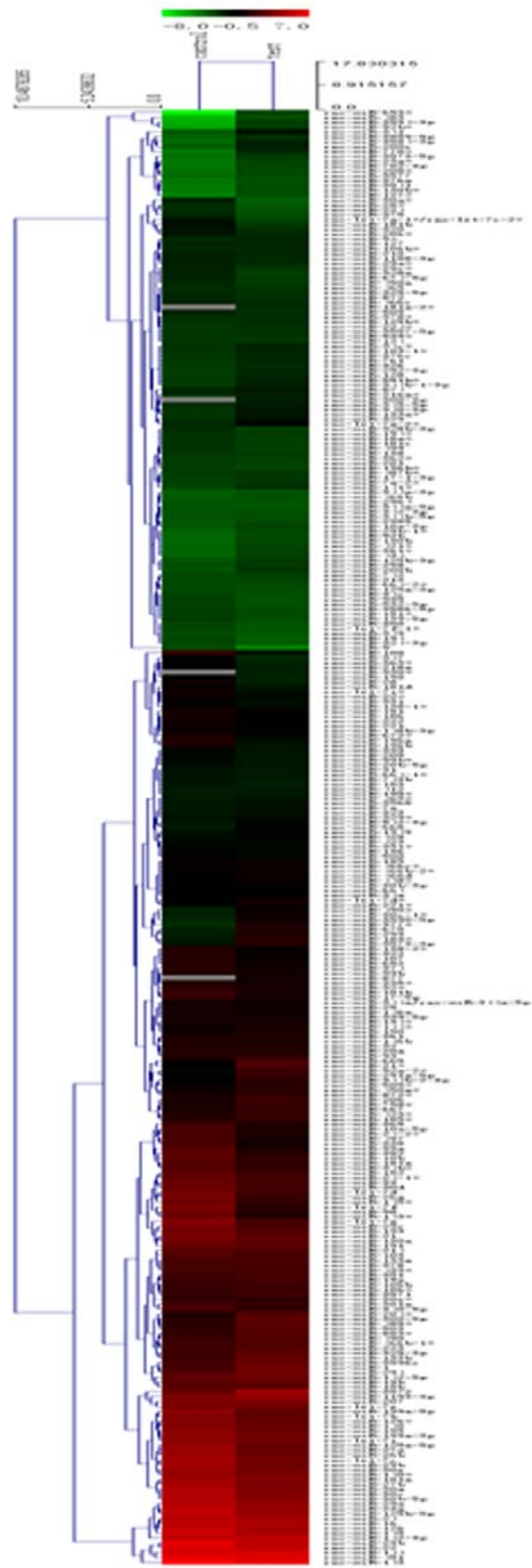
## References

1. Varkonyi-Gasic, E., Wu, R., Wood, M., Walton, E.F. & Hellens, R.P. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. *Plant methods* **3**, 12 (2007).
2. Heller, S., Sheane, C.A., Javed, Z. & Hudspeth, A.J. Molecular markers for cell types of the inner ear and candidate genes for hearing disorders. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 11400-11405 (1998).

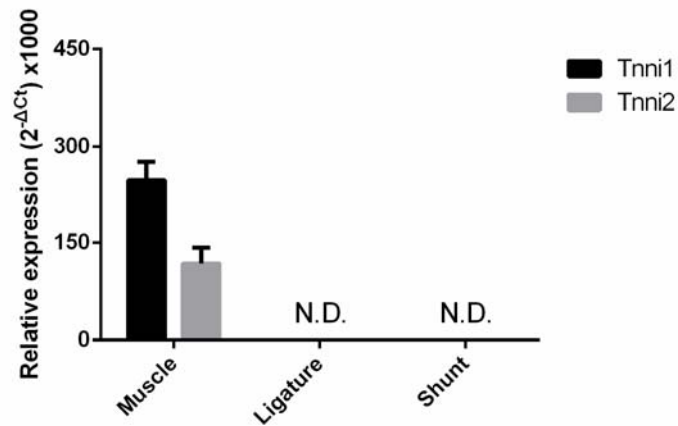
## Supplementary figures



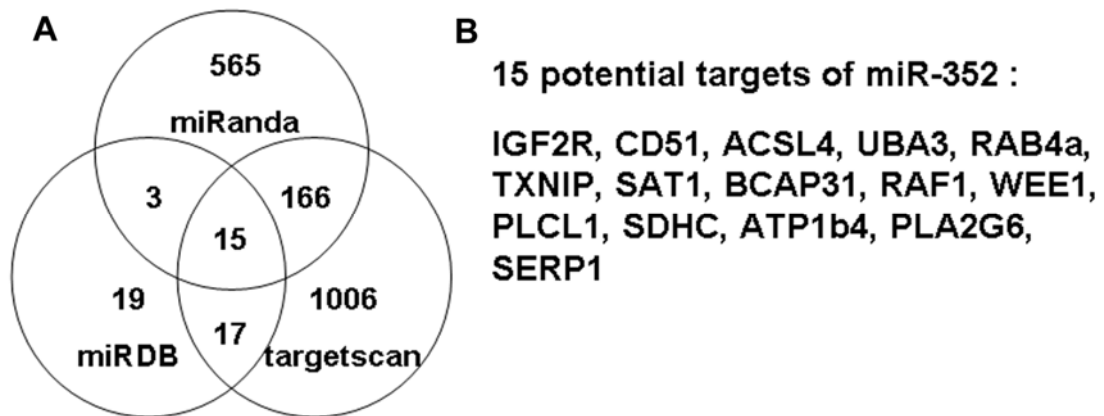
**Figure S1:** Diagram showing the arterio-venous shunt. An arterio-venous shunt was created between the distal stump of the occluded femoral artery and vein. Because of the steep blood pressure gradient along the collateral arteries (arterial pressure at the collateral stem and venous pressure at re-entry), collateral blood flow is increased. Fluid-shear-stress along the collateral artery becomes maximal.



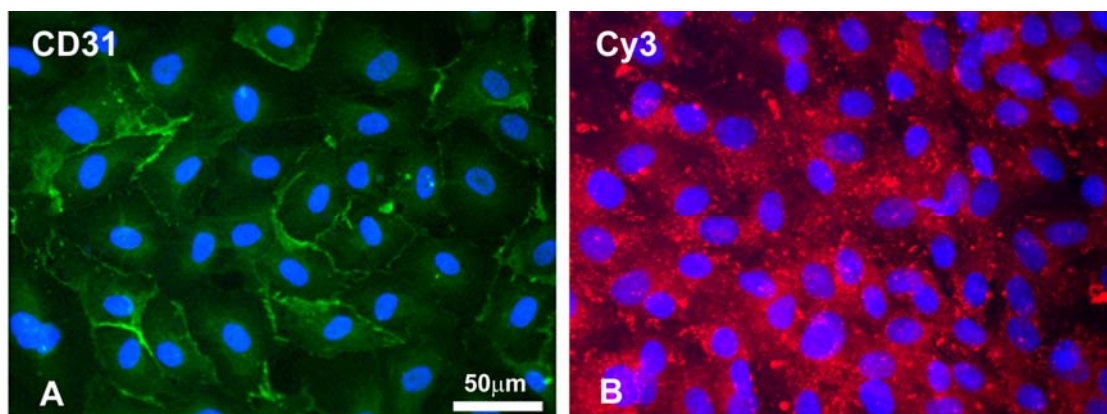
**Figure S2:** Hierarchical clustering for normalized data of miRNAs in all samples. Red indicates high relative expression, and green indicates low relative expression.



**Figure S3:** qPCR analysis of Tnni1 and Tnni2 in collateral vessel samples

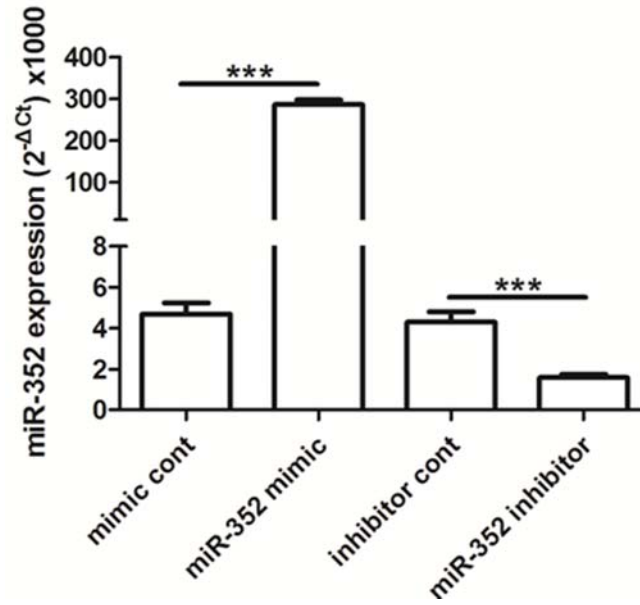


**Figure S4:** miR-352 target genes predicted by all three bioinformatic algorithms. A: Three bioinformatic algorithms were applied; B: the 15 targeted genes.

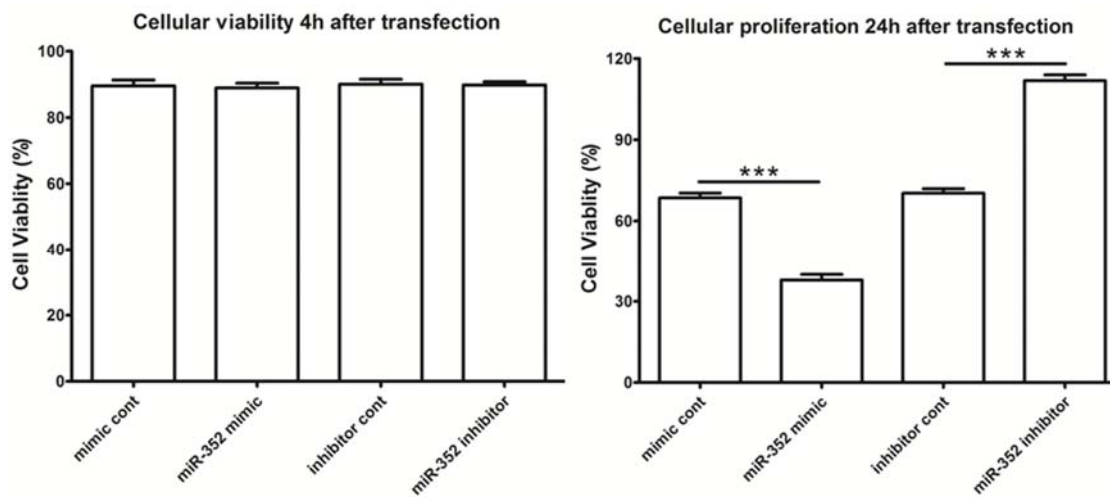


**Figure S5:** Representative micrographs of CD31 (A) and mimic negative control (B) in the RAECs.

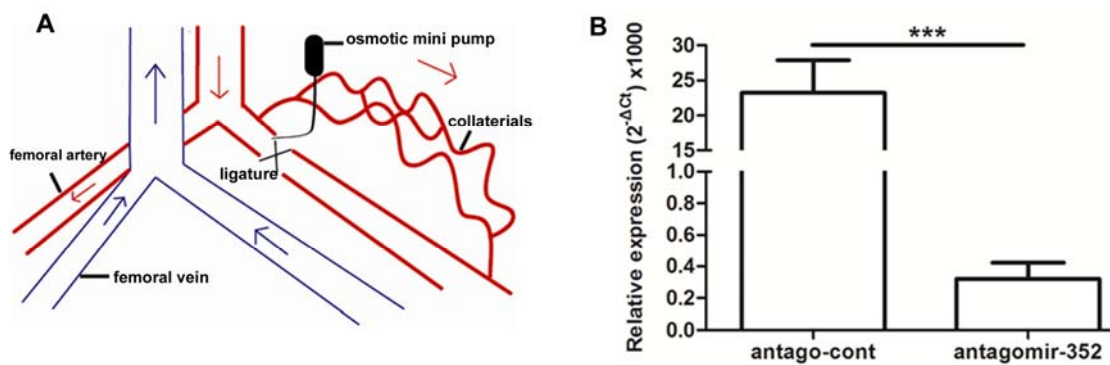




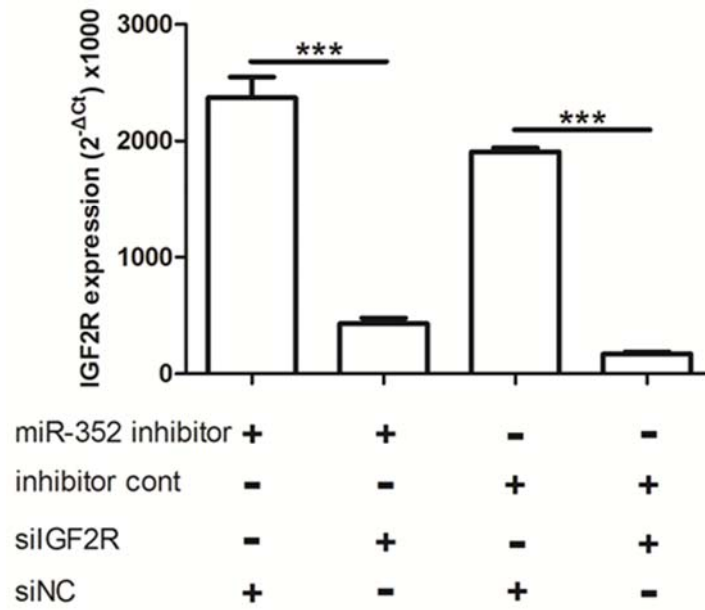
**Figure S6:** miR-352 expression in cultured RAECs.



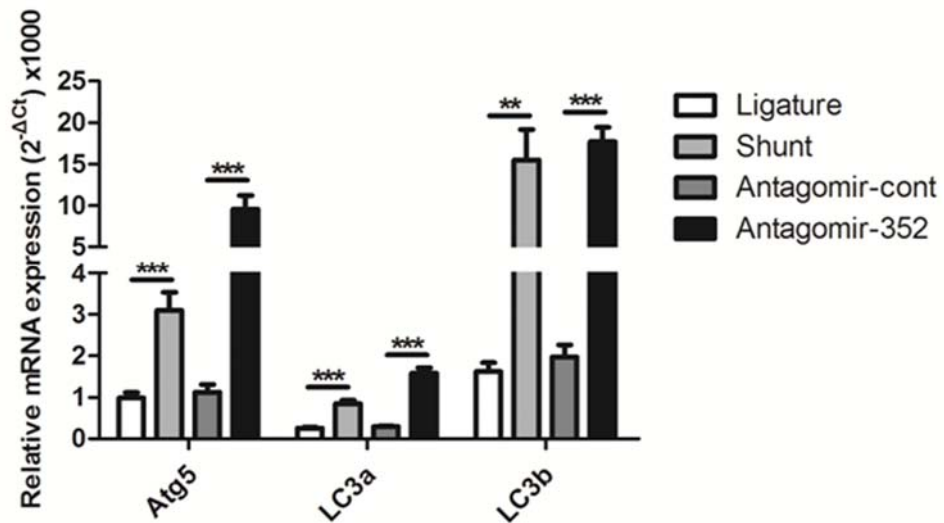
**Figure S7:** The role of miR-352 in endothelial cell viability and proliferation.



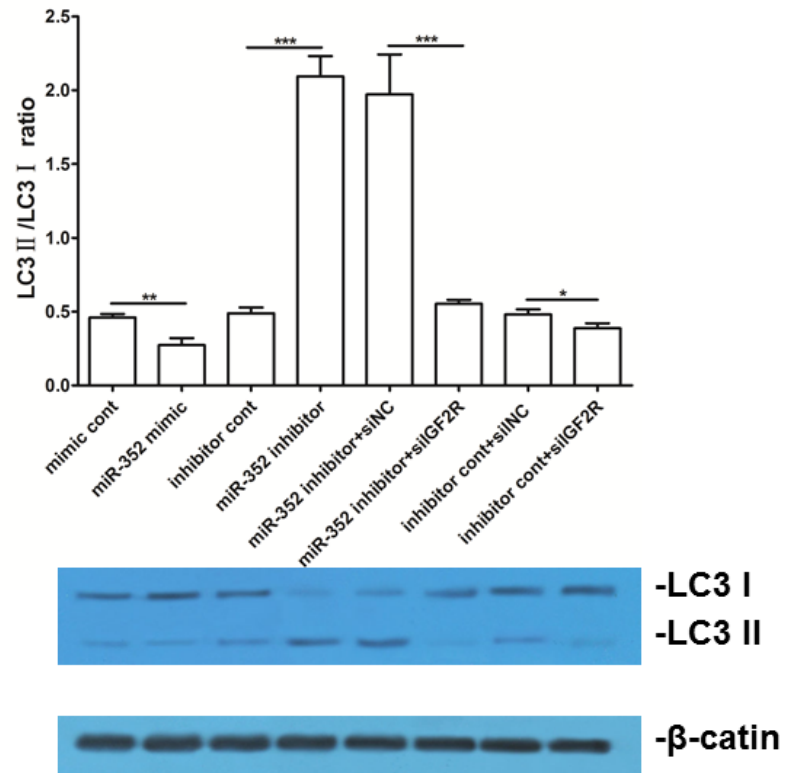
**Figure S8:** Diagram showing the in vivo application of antagomir-352 via osmotic mini pumps and its effect on miR-352 expression in collateral vessels.



**Figure S9:** qPCR showing the effect of siIGF2R on IGF2R expression in RAECs



**Figure S10:** qPCR assay of expression of autophagic markers, LC3a, LC3b and ATG5 collateral vessels in shunt or antagomir-352 treated rats



**Figure S11:** Western blot analysis of the LC3II/LC3I ratio in RACEs treated with miR-352 mimic, miR-352 inhibitor, siIGF2R, or miR-352 inhibitor plus siIGF2R.

## Supplementary tables

**Table S1:** microRNA microarray results

Up-regulated	Fold change AV-shunt vs. control	Down-regulated	Fold change AV-shunt vs. control
rno-miR-653-3p	37.56097561	rno-let-7f-5p	-5.200726271
rno-miR-3594-3p	9.31358885	rno-miR-98-5p	-4.790951973
rno-miR-483-3p	8.414634146	rno-miR-100-5p	-4.56496786
rno-miR-92b-5p	8.096126255	rno-miR-143-5p	-4.399502269
rno-miR-770-3p	5.407129456	rno-miR-30a-3p	-3.848484848
rno-miR-377-5p	4.84735544	rno-let-7a-5p	-3.643793535
rno-miR-341	4.764878049	rno-miR-26a-5p	-3.478532104
rno-miR-21-3p	4.637785208	rno-miR-145-3p	-3.171432554
rno-miR-3584-3p	4.307588076	rno-miR-99a-5p	-3.150019751
rno-miR-675-5p	4.143757751	rno-let-7a-1-3p/rno-let-7c-2-3p	-2.983700643
rno-miR-92a-2-5p	3.808690916	rno-miR-497-5p	-2.888349061
rno-miR-183-3p	3.74730108	rno-miR-9a-5p	-2.880165289
rno-miR-299a-5p	3.626723224	rno-miR-352	-2.873047456
rno-miR-200c-3p	3.593610443	rno-let-7d-5p	-2.854490731
rno-miR-665	3.511737089	rno-miR-674-5p	-2.863621876
rno-miR-291a-5p	3.457928187	rno-miR-28-5p	-2.83042344
rno-miR-3572	3.445658165	rno-miR-29b-3p	-2.78013317
rno-miR-3573-3p	3.261484363	rno-miR-347	-2.776367022
rno-miR-3590-5p	3.227251407	rno-miR-30c-5p	-2.774625087
rno-miR-3573-5p	3.097030753	rno-let-7c-5p	-2.738042055
rno-miR-300-5p	2.87653263	rno-miR-338-3p	-2.725543031
rno-miR-23a-5p	2.866151101	rno-miR-30a-5p	-2.712940006
rno-miR-485-3p	2.803583594	rno-miR-27a-3p	-2.699275761
rno-miR-127-5p	2.780487805	rno-miR-26b-5p	-2.649331845
rno-miR-3559-5p	2.772357724	rno-miR-196a-5p	-2.590468443
rno-miR-30c-1-3p	2.707609756	rno-miR-10b-5p	-2.580960549
rno-miR-702-3p	2.644599303	rno-miR-196b-5p	-2.572727273
rno-miR-204-3p	2.629048381	rno-miR-29c-3p	-2.570647336
rno-miR-130b-5p	2.515243902	rno-miR-199a-5p	-2.56509734
rno-miR-1-3p	2.505729499	rno-miR-125a-5p	-2.54172347
rno-miR-344b-2-3p	2.470711715	rno-miR-23a-3p	-2.495616162
rno-miR-3541	2.454878049	rno-miR-181b-5p	-2.423409923
rno-miR-490-5p	2.373358349	rno-miR-28-3p	-2.420138889
rno-miR-503-5p	2.32738266	rno-miR-24-2-5p	-2.39063622
rno-miR-208-5p	2.309650053	rno-miR-27b-3p	-2.37321744
rno-miR-133b-3p	2.192278644	rno-miR-10a-5p	-2.361033011
rno-miR-376a-3p	2.159428911	rno-miR-30b-5p	-2.305271642
rno-let-7a-2-3p	2.086275937	rno-miR-125b-5p	-2.213879771
rno-miR-883-5p	2.021797788	rno-miR-145-5p	-2.209689889

rno-miR-329-3p	2.021238696	rno-miR-224-5p	-2.198135198
rno-miR-434-3p	2.018792483	rno-miR-190a-5p	-2.170155502
rno-miR-3577	2.012195122	rno-miR-181a-5p	-2.160005478
rno-miR-294	2.010495633	rno-miR-30d-5p	-2.153802865
rno-miR-3596c	2.005032907	rno-miR-30e-5p	-2.152844478
		rno-miR-33-5p	-2.148591392
		rno-let-7b-5p	-2.146867493
		rno-miR-23b-3p	-2.135993695
		rno-miR-375-3p	-2.070707071
		rno-miR-195-5p	-2.042363996
		rno-miR-34b-3p	-2.023000868

**Table S2: Antibodies**

Primary antibody	Host species	Clone ID	Company	label
IGF2R	mouse	2G11	Abcam, UK	-
IGF2	rabbit	-	Abcam, UK	-
Ki67	rabbit	SP6	Vector Lab, CA, USA	-
Beclin1	rabbit	-	Abcam, UK	-
LC3B	rabbit	D11	Cell Signaling Technology, MA, USA	-
CD31	rabbit	EP3095	Millipore, MA, USA	-
$\beta$ -actin	mouse	3E8	CWbio, China	-
Actin-Phalloidin	-	-	Sigma Aldrich, MO, USA	TRITC
Actin-Phalloidin	-	-	Sigma Aldrich, MO, USA	FITC
SMA	mouse	1A4	Sigma Aldrich, MO, USA	Cy3

Secondary antibody	Host species	Cat#	Company	label
mouse IgG	rabbit	610-404-040	Biotrend, Germany	Cy3
rabbit IgG	donkey	611-704-127	Biotrend, Germany	Cy3
mouse IgG	goat	BA-9200	Vector Lab, CA, USA	biotin
mouse IgG	donkey	AP192P	Chemicon, Germany	HRP
rabbit IgG	goat	BA-100	Vector Lab, CA, USA	biotin
rabbit IgG	donkey	AP182P	Chemicon, Germany	HRP

**Table S3:**

TaqMan assay (Applied Biosystems, CA, USA)

Assay name	Assay ID	Company
rno-mir-352	001339	Applied Biosystems
U6 snRNA	001973	Applied Biosystems

PCR-Primers (BGI, China)

Gene	Forward	Reverse	Size(bp)
IGF2R	CACTGTGACCCCTCTGCTGAA	CCAGAGGACACACAGCTGAA	103
IGF2	TAACACCGGCTAGAGCCATCAAC	ACTCAGGCCAAGCGATAGAGACA	102
LC3a	CCAGCACAGCATGGTGAGTGTATC	AGAAGCCGAAGGTTTCTTGGGAG	113
LC3b	TGAATGGGCACAGCATGGTGAGT	CGAACGTCTCCTGGGAGGCATAG	111
ATG5	AGGCTCAGTGGAGGCAACAG	CCCTATCTCCCATGGAATCTTCT	72
Beclin1	CAAATGAATGAGGGCGACAG	TTCTCCAGGTTTTCTGCCAC	131
Tnni1	GACCTGAAGCTGAAGGTGCT	TGGACACCTTGTGTTTGAA	121
Tnni2	GTGCAGGAACTCTGCAAACA	CAGCTCCTTGCTGCTCTTCT	99
RNA 18S	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG	151

**Table S4:** In situ probes (Exiqon, Denmark)

In situ probes	Label	No.	Company
miR-352	5'-DIG and 3'-DIG	41165-15	Exiqon
miR-159	5-DIG	99003-01	Exiqon
U6	5'-DIG	99002-01	Exiqon

**Table S5:**

miRNA (Ribobio, China)

miRNA	Cat #	Company
miR-352 mimic	miR10000610	Ribobio
mimic Ncontrol	miR01101	Ribobio
miR-352 inhibitor	miR20000610	Ribobio
inhibitor Ncontrol	miR02101	Ribobio

siRNA (Ribobio, China)

siRNA	Cat #	Company
si-r-IGF2R	siR1505181136	Ribobio
NControl	siN05815122147	Ribobio