

Vascular importance of the miR-212/132 cluster

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Rationale	Many processes in endothelial cells including angiogenic responses are regulated by microRNAs. However, there is limited information available about their complex cross-talk in regulating certain endothelial functions.
Aim	The objective of this study is to identify endothelial functions of the pro-hypertrophic miR-212/132 cluster and its cross-talk with other microRNAs during development and disease.
Methods and results	We here show that anti-angiogenic stimulation by transforming growth factor-beta activates the microRNA-212/132 cluster by derepression of their transcriptional co-activator cAMP response element-binding protein (CREB)-binding protein (CBP) which is a novel target of a previously identified pro-angiogenic miRNA miR-30a-3p in endothelial cells. Surprisingly, despite having the same seed-sequence, miR-212 and miR-132 exerted differential effects on endothelial transcriptome regulation and cellular functions with stronger endothelial inhibitory effects caused by miR-212. These differences could be attributed to additional auxiliary binding of miR-212 to its targets. <i>In vivo</i> , deletion of the miR-212/132 cluster increased endothelial vasodilatory function, improved angiogenic responses during postnatal development and in adult mice.
Conclusion	Our results identify (i) a novel miRNA-cross-talk involving miR-30a-3p and miR-212, which led to suppression of important endothelial genes such as GAB1 and SIRT1 finally culminating in impaired endothelial function; and (ii) microRNAs may have different biological roles despite having the same seed sequence.
Keywords	Angiogenesis • MicroRNAs • Endothelial cells • Sirtuins • Vascular function

Translational Perspective

MicroRNAs are increasingly used as powerful targets in various diseases including vascular disease. Within a decade from the first identification of miRNAs, the field moved to first phase I and II clinical trials using miRNA inhibitors. We here show novel vascular importance of the miR-212/132 cluster. *In vivo*, inhibition of this cluster increased vascularization and angiogenesis. This investigation may translate in the future to valuable miRNA therapeutics to treat patients with ischaemic cardiovascular diseases.

Introduction

MicroRNAs (miRNAs/miRs) are a class of small, non-coding RNAs orchestrating gene expression by inducing messenger RNA (mRNA) transcript degradation or translational inhibition. MicroRNAs regulate many processes in endothelial cells including angiogenic responses. However, information about their hierarchy and cross-talk in regulating certain functions is scarce.

Transforming growth factor-beta (TGF- β) is a key molecule involved in vascular remodelling and functions as an angiogenic modulator^{1–3} in part by shaping miRNA expression in endothelial cells such as induction of miR-21⁴ or silencing of the miR-30 family.⁵ The miR-212/132 cluster is critically involved in cardiac hypertrophy⁶ and miR-132 was described to induce angiogenesis,⁷ although inhibition of miR-132 improves cardiac function during cardiac remodelling.⁶ Here, we investigated the role of this miRNA

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cluster in endothelial cell function and angiogenesis and identified a novel miRNA-cross-talk involving miR-30a and the miR-212/132 cluster critically regulating endothelial cell function.

Methods

Please refer to Supplementary material online.

Statistical analysis

All experiments were performed at least in triplicates and results are presented as average with standard error of the mean (SEM) or standard deviation (SD) unless mentioned otherwise. Statistical analysis was performed using Graphpad Prism. Student's *t*-test was used for comparisons between two groups and ANOVA followed by the Bonferroni multiple comparison test was used for comparison between more than two groups. A probability value of ≤ 0.05 was considered statistically significant.

Results and discussion

Transforming growth factor- β causes miR-30a-mediated derepression of the cAMP response element-binding protein (CREB) binding protein (CBP) leading to activation of miR-212/132 with miRNA-specific consequences for endothelial cell function

We previously demonstrated miR-212/132 to play an important role in the development of cardiac remodelling.⁶ Therefore, we anticipated that expression of these miRNAs may increase after treatment with pro-fibrotic agents such as TGF- β . Indeed, mature

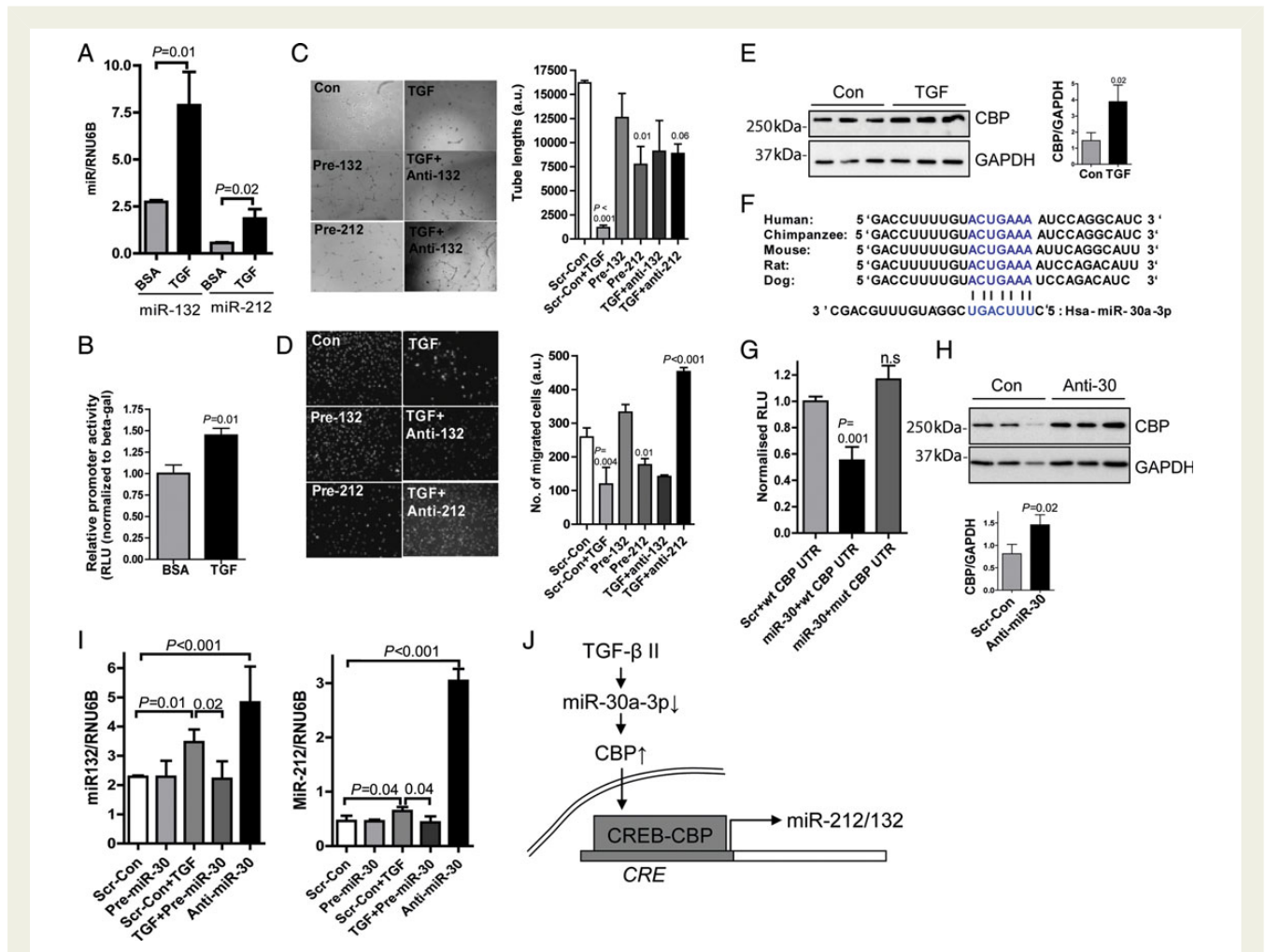


Figure 1 Expression of miR-132 and miR-212 in endothelial cells after transforming growth factor- β treatment (A); transforming growth factor- β -mediated activation of a luciferase reporter gene, which is under control of the miR-212/132 promoter (B); effect of miR-212/132 modulation on tube-forming capacity (C) and Boyden chamber migration (D) of human endothelial cells; cAMP response element-binding protein (CREB) binding protein (CBP) expression in HUVECs after 72 h Transforming growth factor- β treatment (E), cAMP response element-binding protein (CREB) binding protein (CBP) 3'UTR includes a potential binding site for miR-30a-3p (F); miR-30a-3p decreases luciferase activity in a luciferase reporter assay harbouring the potential miR-30a-3p target site in wild-type (Wt) but not in mutated cAMP response element-binding protein (CREB) binding protein (CBP) 3'UTR, (G); effect of miR-30a-inhibition (Anti-30) on cAMP response element-binding protein (CREB) binding protein (CBP) expression (H); effect of miR-30a-3p modulation or transforming growth factor- β treatment on the expression of miR-132 and miR-212 (I). Schematic diagram showing the mechanism of transforming growth factor- β modulating miR-212/132 expression (J). *n* = 3 independent experiments except in (C) and (D) where *n* = 5 per group. Data are represented as mean \pm SD.

miR-212/132 levels were increased after TGF- β treatment in endothelial cells (Figure 1A). By using a luciferase reporter assay, we found that TGF- β directly induces transcriptional activation of both miRNAs rather than enhancing post-translational maturation as described for other miRNAs⁸ (Figure 1B). This was in line with TGF- β -mediated increase in primary (pri)-miR-212/132 levels (Supplementary material online, Figure S1). The detrimental effects of TGF- β treatment on endothelial migration and capillary tube formation could be mimicked by transfection of miR-212, but not miR-132 precursors suggesting different effects of these two miRNAs on endothelial biology despite having the same seed sequence (Figure 1C). In line, inhibition of miR-212, but not miR-132 could restore endothelial migration and capillary tube formation up on TGF- β challenge (Figure 1C and D). Effects of miR-212 appeared to be independent of TGF- β effects on Akt activation or possible modulation of endothelial-to-mesenchymal transition (EndMT) (Supplementary material online, Figure S2).

We next studied underlying regulatory mechanisms in TGF- β -mediated change of the miR-212/132 cluster. The transcription factor cAMP response element-binding protein (CREB), which regulates miR-212/132 transcription,^{9,10} forms a functional complex with the CREB-binding protein (CBP). Transforming growth factor- β

treatment did not change CREB expression (Supplementary material online, Figure S3) but that of the CBP protein (Figure 1E). As availability of CBP is the rate-limiting step for transcription mediated by the CREB-CBP complex,^{11,12} its TGF- β -mediated increase suggests an involvement in the activation of miR-212/132. A bioinformatic search of the CBP 3'UTR identified several putative miRNA-binding sites (data not shown). Although there was no binding site for miR-212 or miR-132, we found an evolutionarily conserved putative target site for miR-30a-3p (Figure 1F). The miR-30 family has been recently shown to have pro-angiogenic properties¹³ and expression of this miRNA family is known to be suppressed by TGF- β .^{5,14} Importantly, global miRNA profiling after TGF- β treatment of endothelial cells has revealed members of the miR-30 family to be repressed by TGF- β treatment, with miR-30a-3p being the strongest down-regulated candidate.⁵ Luciferase reporter assays confirmed targeting of CBP 3'UTR by miR-30a-3p (Figure 1G) and miR-30a-3p inhibition increased CBP protein expression (Figure 1H), while transfection of miR-30a-3p precursors reduced CBP expression (Supplementary material online, Figure S4). We thus hypothesized that TGF- β -up-regulation of CBP (and thus activation of the miR-212/132 cluster) is miR-30a-3p-dependent. Indeed, overexpression of miR-30a-3p blunted TGF- β -mediated miR-212/132 increase, whereas miR-30a-3p

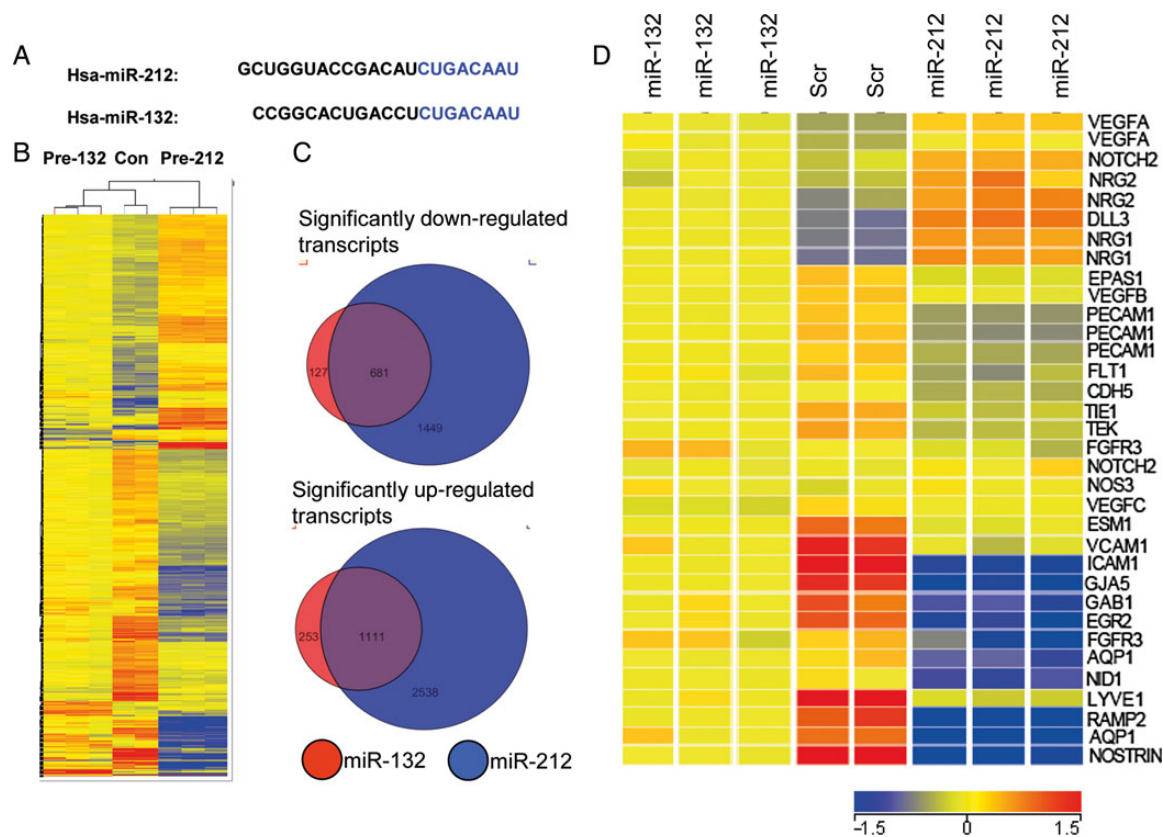


Figure 2 Sequences of mature miR-212 and miR-132 (A); Heat-plots of the effect of miR-212 or miR-132 transfection on mRNA-transcriptome changes in human endothelial cells (Affymetrix GeneChip Human Genome U133 Plus 2.0) (B); Differential effects of miR-212 over miR-132 on the endothelial cell transcriptome (C); Heat map depicting changes in endothelial relevant genes after miR-132, miR-212, or scrambled (Scr)-control transfection in human endothelial cells (D). $n = 3$ experiments/group except for scrambled control group where $n = 2$.

inhibition exaggerated miR-212/132 expression (Figure 1). Similarly, miR-30a-3p transfection suppressed TGF- β -stimulated miR-212/132 promoter luciferase reporter activity while miR-30a-3p inhibition exaggerated activity (Supplementary material online, Figure S5). In summary, TGF- β de-represses CBP via miR-30a-3p leading to CREB-CBP-mediated transcriptional activation of miR-212/132 in endothelial cells (Figure 1).

Differential endothelial effects of miR-212/132 cluster members are caused by auxiliary miRNA-binding sequences

We next aimed to understand the different biological effects of miR-212 and miR-132 on endothelial biology despite having the same seed sequence (Figure 2A). We performed global transcriptome analysis after endothelial transfection of miR-132 or miR-212 precursors. Both miR-212 and -132 detection levels were similarly enhanced after transfection with respective pre-miRs (Supplementary material online, Figure S6). Surprisingly, transfection of miR-212 had a much stronger regulatory effect on mRNA expression patterns than that of miR-132 (Figure 2B); miR-132 significantly down-regulated 808 transcripts, while miR-212 down-regulated 2130 transcripts. Interestingly, 84% of transcripts down-regulated by miR-132 were also significantly down-regulated by miR-212, but a set of 1449 genes was exclusively regulated by miR-212 (Figure 2C), which may

explain the differential effects of miR-212 and miR-132 on endothelial cell behaviour. Thus, miRNAs belonging to the same family may differ in efficacy and have different sets of targets despite having the same seed sequence, suggesting that the canonical seed sequence is not the sole determinant for miRNA-mRNA interactions. When we selected regulated mRNAs with a known correlated function in endothelial cells, almost all mRNAs known to be important for endothelial cell function/angiogenesis were down-regulated by miR-212 (Figure 2D, Supplementary material online, Table S2). 18.9% (77 out of 407) of all bioinformatically predicted targets of miR-212 were significantly down-regulated at least 1.5-fold upon miR-212 over-expression (Supplementary material online, Table S3). More than 80% (17 out of tested 21) of regulated genes could be validated in independent quantitative RT-PCR assays (Supplementary material online, Figure S7). These approaches identified Grb2-associated binder 1 (Gab1), which has binding sites for miR-212/132 (Figure 3A) to be one of the most down-regulated genes. With respect to angiogenesis, Gab1 is an interesting miRNA target as it has been shown to be essential for endothelial migration¹⁵ and postnatal angiogenesis.^{16–18} Consistent with the microarray data, Gab1 transcript and protein levels were stronger down-regulated by miR-212 over-expression than by miR-132 (Figure 3B, Supplementary material online, Figure S7). In line with our observations, a recent study has also reported stronger effect of miR-212 over miR-132 in regulating its target Bcl6 in a luciferase reporter assay.¹⁹

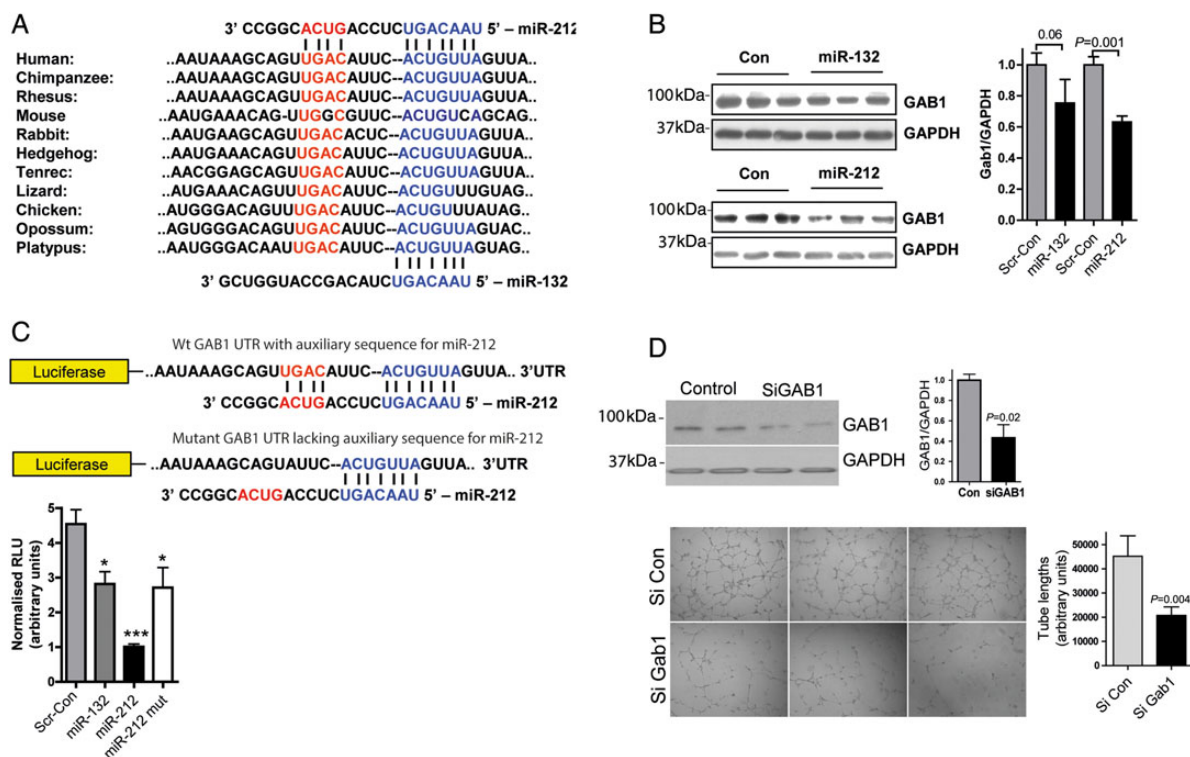


Figure 3 Potential targeting of Gab1 3'UTR by miR-132 and miR-212 (A); effect of miR-132 or miR-212 on GAB1 protein expression (B); changes in luciferase reporter activity after deletion of four nucleotides in Gab1 3'UTR that bind with the auxiliary sequence of miR-212 (C); effect of Gab1 silencing (upper graph) on tube-forming capacity of endothelial cells (D, lower graph). $n = 3$ independent experiments, except (C) where $n = 6$. Data are represented as mean \pm SD.

We next used the bioinformatic score system Context+,²⁰ which effectively models miRNA–target interactions based on 3' pairing, local AU appearance, target site abundance, and seed-pairing stability. Indeed, the Context+ score for miRNA-212 at the Gab1 3'UTR-binding site was three times more favourable than that of miR-132. In addition, despite having the same seed sequence, miR-212 harbours an auxiliary four-nucleotide match (Figure 3C) which may increase stability of miR–target interactions.²¹ To examine this hypothesis, we created a luciferase reporter with a mutant Gab1 3'UTR containing the common miR-212/132-binding site at the canonical seed sequence but lacking the auxiliary-binding region which is specific to miR-212 (Figure 3C). Our data indicate that miR-212 more effectively targets the wild-type Gab1 3'UTR that has both the canonical seed sequence and the auxiliary-binding site (Figure 3C), suggesting that more complete binding of the miRNA to its target may have stronger effect in suppressing protein expression. Similarly, other putative target transcripts of both miRNAs which harbour the auxiliary-binding site for miR-212 (e.g. Anp32a, Dazap2, Itpkb, Large, Lsm11, Mtmr10, Rfx3, Runx1t1, Serp1, and Tusc3) were stronger down-regulated by miR-212 than

by miR-132 (Supplementary material online, Figure S8). Additionally, we tested protein expression of the predicted targets LSM11 and MTMR10 after miR-212 or miR-132 transfection and found that miR-212, which has an additional auxiliary binding in the 3'UTR of these transcripts, suppresses both LSM11 and MTMR10 more effectively than miR-132 (Supplementary material online, Figure S9A and B). In line with this, we found severe impairment in capillary tube-forming capacity after silencing of Gab1 in endothelial cells (Figure 3D). In addition to Gab1, which has been selected from the microarray data, there may be several other miR-212 target genes that are important for endothelial cell function. Such examples include the previously verified miR-212/132 targets such as FoxO3A and Sirt1.^{6,22} The latter is especially important in the context of endothelial cell biology because of its well-established pro-angiogenic effects.^{23–26} Consistent with this, TGF- β silenced Sirt1 expression (Supplementary material online, Figure S9C). Interestingly, overexpression of either Gab1 or Sirt1 rescued tube formation capacity in miR-212 over-expressing endothelial cells (Figure 4). These results highlight the importance of the miR-212 targets Gab1 and Sirt1 in endothelial cells. Sirt1

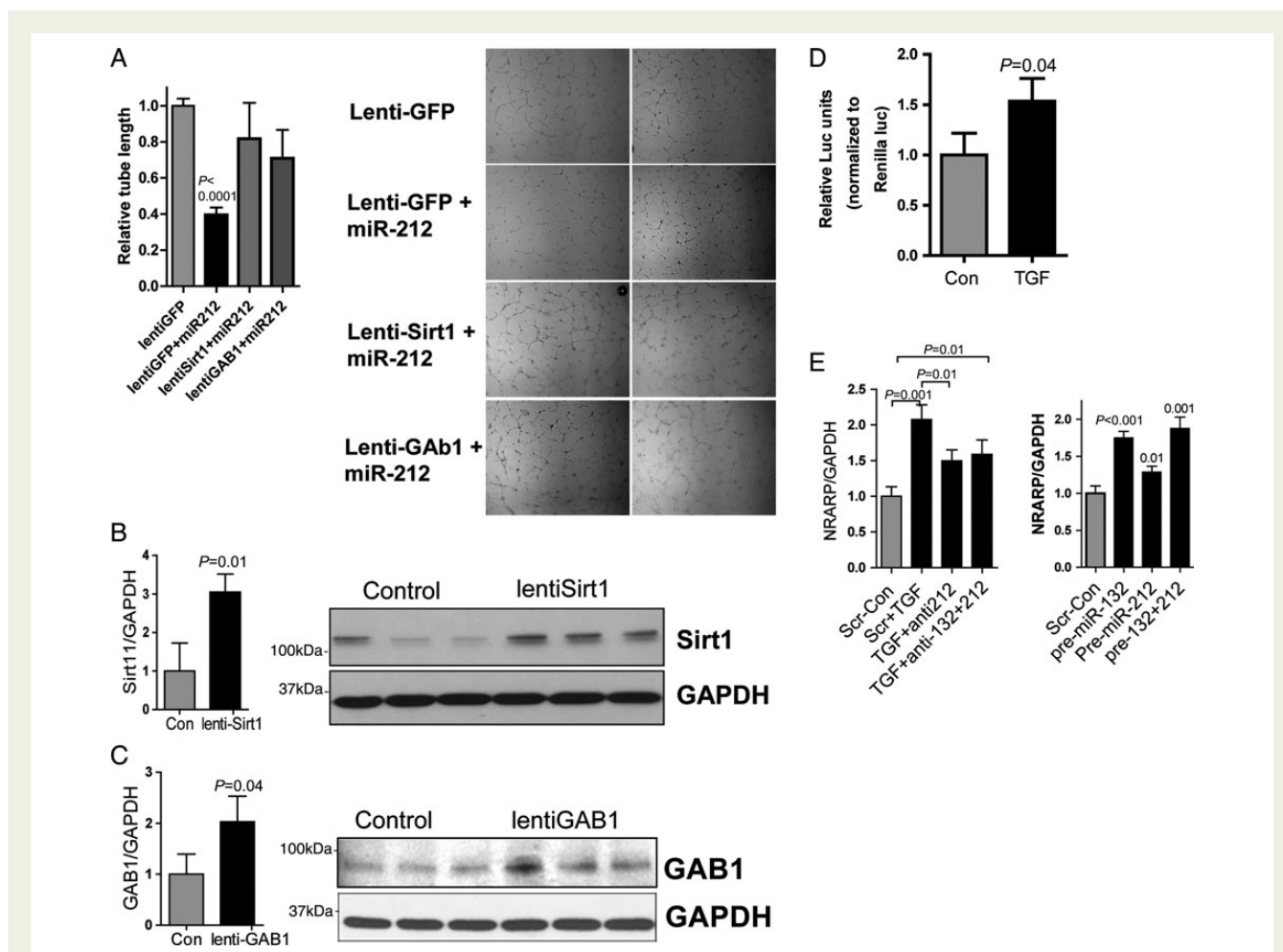


Figure 4 Effect of Sirt1 or Gab1 on tube-forming capacity of endothelial cells transfected with pre-miR-212 (A); validation of lentivirus-mediated Gab1 (B) or Sirt1 (C) overexpression in endothelial cells; effect of transforming growth factor- β on Notch-reporter luciferase activity (D); effect of transforming growth factor- β treatment or miR-212/132 modulation on the expression of a Notch target gene NRARP (E). $n = 3$ independent experiments/group. Data are represented as mean \pm SD.

inhibits Notch signalling which is a negative regulator of angiogenic functions.²⁴ In line, TGF- β significantly enhanced Notch-reporter luciferase activity (luciferase reporter gene under the control of a minimal CMV promoter and tandem repeats of the RBP-Jk transcriptional response element) (Figure 4D). As an endogenous readout for Notch signalling activation, we studied expression of the Notch-regulated ankyrin repeat protein (Nrarp).²⁷ Transforming growth factor- β treatment or miR-212 precursor transfection both up-regulated Nrarp expression. Importantly, inhibition of miR-212 partly normalized TGF- β -induced overexpression of this Notch-activated gene (Figure 4E). These results highlight that TGF- β suppresses Sirt1 and activates Notch signalling in endothelial cells partly via miR-212.

Deletion of miR-212/132 *in vivo* improves endothelial function

Previous studies indicated that endothelial nitric oxide synthase (eNOS) signalling is impaired in endothelium-specific Gab1-KO mice providing a rationale for the endothelial phenotype.¹⁶ Silencing

of Gab1 indeed resulted in decreased eNOS expression in endothelial cells (Supplementary material online, Figure S10). Additionally, the adrenomedullin receptor-regulating protein Ramp2, an important gene for endothelial angiogenesis, was shown to be a modulator of eNOS expression; Ramp2 overexpression in endothelial cells has been described to increase eNOS expression,²⁸ while Ramp2 deficiency is shown to suppress eNOS expression.²⁹ Interestingly, while we show that Gab1 is a direct target of miR-212, microarray and qRT-PCR analyses revealed that Ramp2 is also strongly down-regulated in miR-212 over-expressing endothelial cells (Supplementary material online, Table S2 and S3 and Figure S7). Thus, these two eNOS-modulating genes are directly or indirectly suppressed by miR-212. We thus anticipated that endothelium-dependent vasodilatation would be increased in miR-212/132-deficient animals when compared with controls. Interestingly, while endothelium-independent vasodilatation was comparable in wild-type and miR-212/132 KO animals, endothelium-dependent vasodilatation was significantly increased in miR-212/132 null animals (Figure 5A and B). However, there was no difference in the

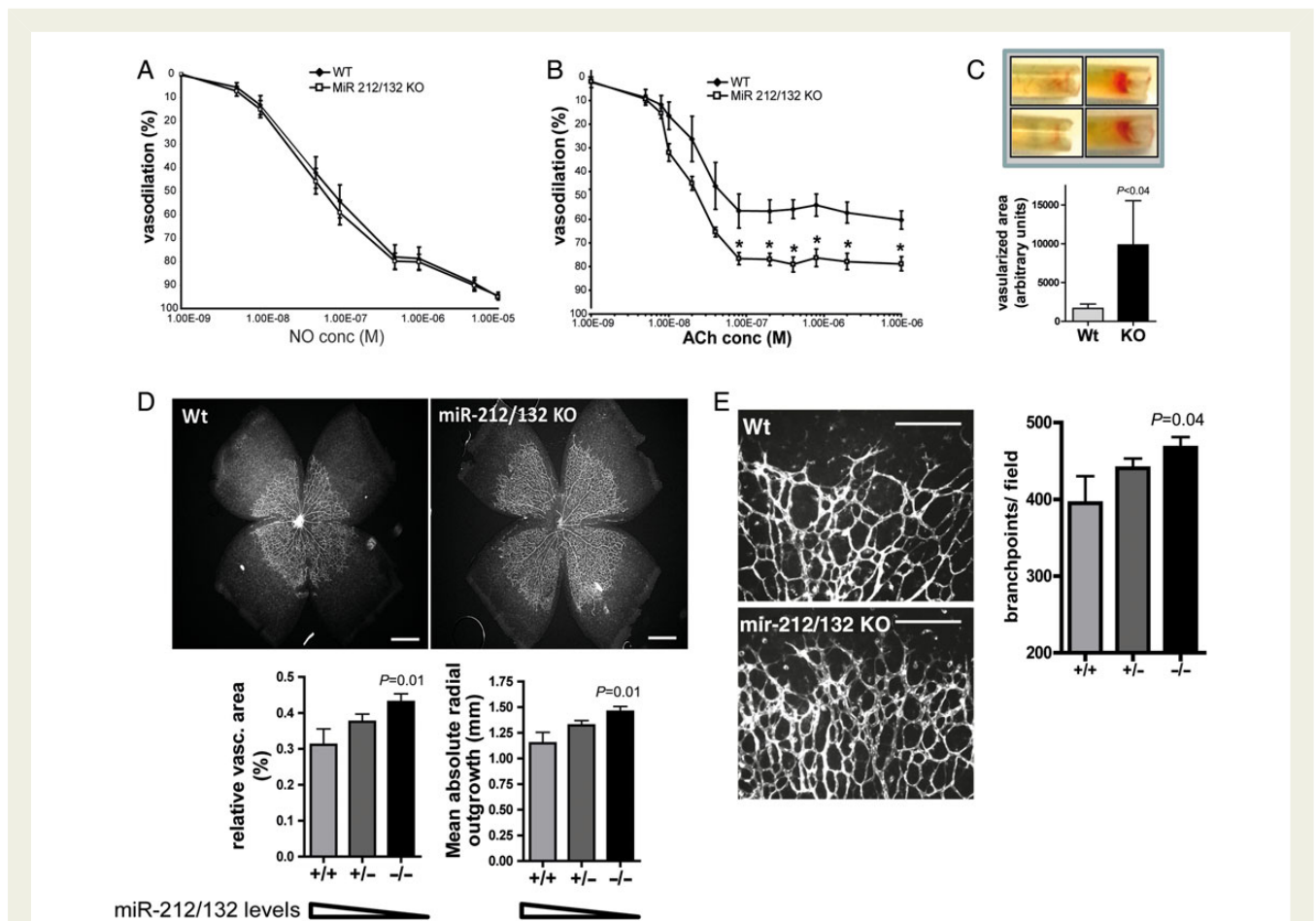


Figure 5 Endothelium-independent (A) and -dependent (B) vasodilatation in wild-type (Wt) and miR-212/132 KO animals; blood vessel density in angioreactors implanted in wt and miR-212/132 KO mice (C); area of retinal vasculogenesis in wt and miR-212/132 null mice at postnatal Day 5 (D). Representative magnification image showing differences in branching points and number of tip cells in postnatal Day 5 (P5) retinas from wt and miR-212/132 KO mice (E). $n = 6$ aortic rings excised from three animals in each group (A); $n = 3$ (C); $n = 6$ retinas in each group harvested from three animals in each group (D and E). Data are represented as mean \pm SD. Wt and KO animals were littermates.

acetylcholine-dependent vasocontractile response when aortic rings were prepared under addition of the NOS blocker L-NAME (Supplementary material online, Figure S11). These results indicate that deletion of miR-212/132 has stimulatory effects on endothelial function.

MiR-212/132 deletion increases capillary formation and angiogenic responses *in vivo*

To study the role of miR-212/132 cluster in angiogenesis, we implanted angioreactors into wild-type and miR-212/132 null mice. We found significantly more invading endothelial cells in angioreactors subcutaneously implanted to mice-lacking miR-212/132 (Figure 5C). To gain more direct insight on the effect of these miRNAs in angiogenesis, we next studied the development of the retinal vasculature *ex vivo*. Retinas were isolated from postnatal day 5 (P5) pups. While the endothelial plexus in WT mice had covered ~40% of the retinal surface, miR-212/132 KO mice demonstrated a dramatic increase in retinal vasculature development, consistent with a general anti-angiogenic role of the miR-212/132 complex. In addition, KO animals displayed significantly higher number of branch points and radial outgrowth in their retinas (Figure 5D and E).

The present study highlights anti-angiogenic features of miR-212 via direct regulation of at least the two target genes *Sirt1* and *Gab1*. Although miR-132 slightly increased endothelial cell migration as described previously in a tumour angiogenesis model,⁷ we did not observe major effects of miR-132 on endothelial cell behaviour in strong contrast to that of miR-212. Our results identify miR-212 as a crucial anti-angiogenic miRNA in endothelial cells which may serve as a potential therapeutic target in diseases with altered vascular density.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: S.G., A.U., K.C., and T.T. filed patents about the miR-212/132 cluster. The other authors report no conflicts of interest.

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CARDIOVASCULAR FLASHLIGHT

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Cardiac injury as a rare cause of cardiogenic shock following polytrauma

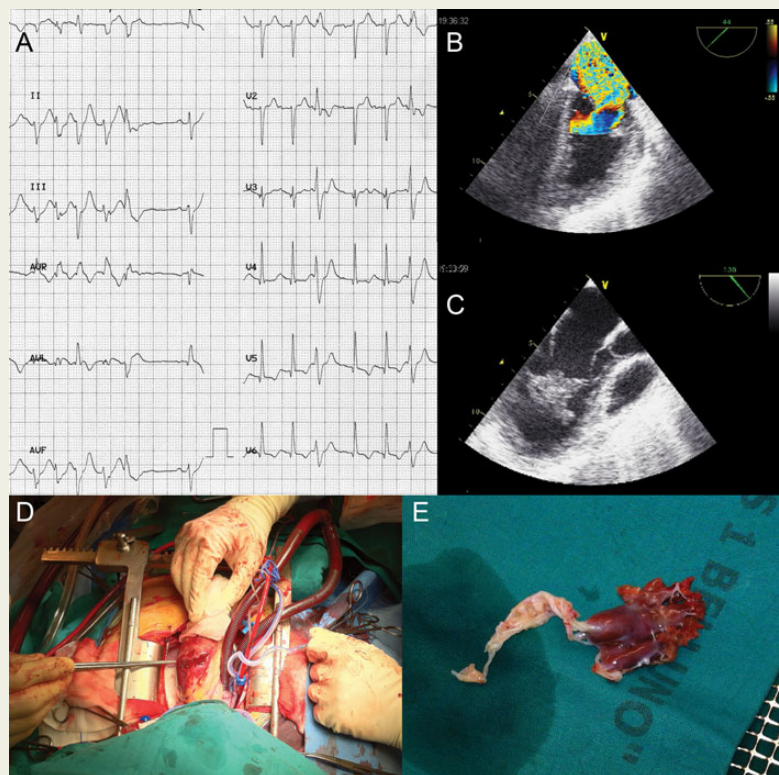
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A 27-year-old man was referred to our emergency department because of polytrauma due to a car accident. Because of airbag inflation, he reported a blunt chest trauma with large pulmonary contusions (70% of lung surface involvement at CT scans). An endotracheal tube was inserted because of severe desaturation due to pulmonary oedema and cardiogenic shock. Patient's ECG showed sinus tachycardia, q waves V3–V4 leads, T-wave inversion V4–V6 leads, frequent isolated ventricular beats with right bundle branch block morphology and right-axis deviation and frequent run of non-sustained ventricular tachycardia (Panel A). The patient underwent transoesophageal echocardiography (TEE). Colour Doppler revealed severe mitral regurgitation jet (Panel B; Supplementary material online, Video S1–S2) secondary to the rupture of the anterolateral papillary muscle (ALPM), which prolapsed into left atrium during ventricular systole (Panel C, Supplementary material online, Figure, Videos S3–S4). After intra-aortic balloon insertion, he was referred to emergency surgery. Macroscopic observation during cardiac surgery displayed left ventricular (LV) wall tear in correspondence of ALPM insertion (Panel D). After mitral valve exposure, complete avulsion of ALPM and part of adjacent myocardium was then observed (Panel E). The patient underwent mitral valve replacement with a biological prosthesis and he showed rapid clinical recovery. At 3-month follow-up, he remained steady in I NYHA class. Traumatic rupture of the papillary muscles is a rare condition after blunt chest trauma; prompt diagnosis and urgent cardiac surgery appear to give a good clinical outcome.

ECG shows sinus tachycardia, q waves V3–V4 leads, T-wave inversion V4–V6 leads, frequent isolated and monomorphic ventricular beats with right bundle branch block morphology, right-axis deviation morphology and frequent non-sustained polymorphic ventricular tachycardia (Panel A). TEE four chambers and dual chambers long-axis view (Panels B and C). Colour Doppler reveals severe mitral regurgitation jet (Panel B) due to complete avulsion of the anterolateral papillary muscle, which prolapses into the left atrium (Panel C). Intra-operative views (Panels D and E). LV wall after dissection of pericardium; epicardial tear corresponds to anterolateral papillary muscle insertion (Panel D), subsequently repaired with a pericardial patch. Anterolateral papillary muscle and surrounding myocardium specimen after mitral dissection (Panel E).



Supplementary material is available at *European Heart Journal* online.