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**Abstract**

Following myocardial infarction (MI), the adult heart has minimal regenerative potential. Conversely, the neonatal heart can undergo extensive regeneration, and neovascularisation capacity was hypothesised to contribute to this difference.

Here, we demonstrate the higher angiogenic potential of neonatal compared to adult mouse cardiac endothelial cells (MCECs) *in vitro* and use this difference to identify candidate microRNAs (miRs) regulating cardiac angiogenesis after MI. MiR expression profiling revealed miR-96 and miR-183 upregulation in adult compared to neonatal MCECs. Their overexpression decreased the angiogenic potential of neonatal MCECs *in vitro* and prevented scar resolution and neovascularization in neonatal mice after MI. Inversely, their inhibition improved the angiogenic potential of adult MCECs, and miR-96/miR-183 knock-out mice had increased peri-infarct neovascularisation.

In silico analyses identified anillin (*ANLN*) as a direct target of miR-96 and miR-183. In agreement, *Anln* expression declined following their overexpression and increased after their inhibition *in vitro*. Moreover, *ANLN* expression inversely correlated with miR-96 expression and age in cardiac ECs of cardiovascular patients. *In vivo*, ANLN-positive vessels were enriched in the peri-infarct area of miR-96/miR-183 knock-out mice.

These findings identify miR-96 and miR-183 as regulators of neovascularisation following MI and miR-regulated genes such as anillin as potential therapeutic targets for cardiovascular disease.
Introduction

Myocardial infarction (MI) is a major threat to human health and contributes to substantial morbidity and mortality worldwide (1, 2). Conventional treatments aim to promote reoxygenation of the infarcted myocardium, for example through interventions such as coronary artery bypass surgery or percutaneous coronary intervention, with the goal of limiting infarct expansion and therefore progression to heart failure (3-5). While a regenerative response is absent in the adult mammalian heart, recent studies have demonstrated that the neonatal mouse and human hearts possess substantial regenerative capacities (6-9). However, this so-called ‘regenerative window’ closes rapidly after birth. Notably, new blood vessel growth by angiogenesis was identified as one of the five hallmarks of regeneration (10). Thus, the left ventricular (LV) apical resection model of cardiac regeneration in the neonatal mouse heart showed that new blood vessel formation precedes cardiomyocyte repopulation of the regenerating area (11). Accordingly, the neonatal mouse should be useful to identify key regulators of neovascularisation following MI and provide potential therapeutic targets to promote neovascularisation and therefore, cardiac repair following MI in the adult.

MicroRNAs (miRs) regulate many biological processes, including vascular development and angiogenesis (12), and an increasing number of miRs have been shown to either target specific genes involved in angiogenesis or to be modulated by pro- or anti-angiogenic stimuli, including miR-221/222, miR-126 and the miR-17/92 cluster (13). Besides, miRs expression is known to be altered in the setting of MI (14), and some miRs, such as miR-590 and miR-199a, induce cardiac repair by promoting cell cycle re-entry of adult cardiomyocytes and inducing their proliferation (15). Furthermore, other miRs, such as the miR-15 family, are up-regulated in the neonatal mouse heart where they repress several cell cycle genes and contribute to cell cycle arrest, thus limiting cardiac regenerative capacity (16). Interestingly, inhibition of the miR-15 family at early stages of life increases cardiomyocytes proliferation.
and cardiac repair (7). However, the precise role and therapeutic potential of specific miRs in cardiac repair remains poorly investigated. Moreover, miRs are known to act as inhibitory regulators of gene expression by binding to complementary mRNA transcripts (17). Still, their target genes in the context of post-MI neovascularisation have not yet been identified.

Here we demonstrate that neonatal cardiac endothelial cells (ECs) have a greater pro-angiogenic potential than adult cardiac ECs. We further show that neonatal cardiac ECs have a specific miR signature that includes miR-96 and miR-183 as novel potential therapeutic candidates, and we identify the cytoskeletal regulator Anillin (ANLN) as a target of both miR-96 and miR-183. These findings raise the possibility that miR-96/miR-183 and their targets induce a more neonatal-like phenotype in adult cardiac ECs that may be exploited therapeutically to improve post-infarction neovascularisation.
Results

Neonatal and adult cardiac endothelial cells possess different angiogenic phenotype. To assess whether neonatal and adult MCECs have different angiogenic capacities, MCECs from C57BL/6N mice aged P1 (within the regenerative window - later referred as neonatal MCECs) and 4 weeks (after the regenerative window - later referred as adult MCECs) were used. As shown in Fig 1A, neonatal MCECs had higher cell-cell contact and enhanced cell-surface adhesion in comparison to adult MCECs, as assessed with the electric cell-substrate impedance sensing (ECIS) system. Compared to adult MCECs, neonatal MCECs also had an accelerated migration rate, as evaluated with the ECIS-based automated cell migration assay (Fig 1B), and a higher proliferation rate in culture, measured as 5-ethynyl-2'-deoxyuridine (EdU) incorporation (Fig 1C). When seeded at a high density (2x10^4 cells/cm^2), both adult and neonatal MCECs form appropriate tube-like structures after 6 hours in culture (Fig 1D). However, neonatal but not adult MCECs formed extensive tube-like structures after 24h in culture when seeded at a lower density (1x10^4 cells/cm^2) (Fig 1E). Taken together, these data indicate that neonatal cardiac ECs possess greater angiogenic potential than adult cardiac ECs.

The miR signature is different in neonatal and adult MCECs. The greater angiogenic potential of neonatal versus adult MCECs may be the result of several factors. For example, recent findings demonstrate that miRs regulate cardiovascular development, regeneration and angiogenesis (12, 18). We, therefore, investigated whether a change in miR expression might regulate the angiogenic switch from neonatal to adult cardiac ECs. Expression profiling of 150 miRs in neonatal (P1) and adult (W4) MCECs (Fig 2A) showed that all members of the miR-183 cluster, comprised of miR-96-5p, miR-182-5p and miR-183-5p, were upregulated in adult MCECs compared to neonatal MCECS (Fig 2B). In agreement with this finding, miR-183 and miR-96 are highly enriched in ECs according to the FANTOM5 miRNA
Atlas (19). Interestingly, the expression of these miRs in primary MCECs (CD31+/CD45-) collected within (P1), at the end (P7) and after (P12 and 8 weeks) the regenerative window increased gradually over time (Supplementary Fig 1A). These findings raised the possibility that miR-183 and miR-96 are linked to the decreased angiogenic potential of adult MCECs compared to neonatal MCECs.

Overexpression of miR-96 and/or miR-183 reduces the angiogenic capacity of neonatal MCEC in vitro. To understand whether the miR-183 cluster regulates the angiogenic potential of MCECs we manipulated the expression levels of miR-183 cluster in neonatal MCECs using synthetic oligonucleotides. While mimic-mediated miR-183 cluster overexpression in neonatal MCECs did not affect their migration rate (Supplementary Fig 2A), it reduced their proliferation rate (Fig 2C and Supplementary Fig 2B) and prevented the formation of capillary-like structures in Matrigel (Fig 2D and Supplementary Fig 2C). Overexpression of the individual members of the cluster showed that miR-96 and miR-183, but not miR-182, reduced the proliferative rate and the formation of the tube-like structure of neonatal MCECs (Fig 2C and 2D). These findings suggest that miR-96 and miR-183 regulate the angiogenic potential of MCECs.

Overexpression of miR-96 and miR-183 impairs angiogenesis and scar resolution following MI in neonatal mice. We next used the neonatal mouse model of MI to investigate the effect of miR-96 and miR-183 overexpression in vivo. Following induction of MI, miR-96 and miR-183 mimics were injected into the peri-infarct area. To confirm the feasibility of miR mimic delivery into the neonatal mouse heart after MI, we initially injected a combination of miR-96 and miR-183 mimics at a total dose of 200 pmol (20). However, the survival of pups at 24h was dramatically reduced after miR-96 and miR-183 injection compared to control
mimic (Fig 3A). We, therefore, decreased the dose to 50 pmol, which did not compromise survival rates compared to the control group (Fig 3B).

At day 1 following MI, RT-qPCR revealed a significant increase in expression of both miR-96 (Supplementary Fig 3A) and miR-183 (Supplementary Fig 3B) in freshly isolated MCECs between the miR-96/miR-183 and control mimic injected groups. Expression of miR-183, but not miR-96, was also significantly increased in cardiomyocytes (Supplementary Fig 3A and 3B). At 3 weeks post-MI, mice injected with 50 pmol miR-96/miR-183 had increased retention of scar tissue within the LV wall compared to the control group (Fig 3C and 3D). Notably, the retention of scar tissue was associated with decreased vascularisation around the fibrotic tissue (Fig 3E and 3F). Together, these data indicate that overexpression of miR-96 and miR-183 inhibits the angiogenic response and scar resolution in the neonatal mouse heart after MI.

MiR-96 and miR-183 exert their vascular effect by targeting Anillin. To identify candidate target genes for repression by miR-96 and miR-183, we performed an in silico analysis of 3’ untranslated (UTR) mRNA and identified genes that are predicted to be targets of both miRs. We identified as common candidate pathways for miR-96 and miR-183 the Hippo pathway, the PI3K-AKT-FOXO signalling pathway, and the regulation of the actin cytoskeleton (Supplementary Fig 4). Among the target genes in this pathway, anillin was predicted as the direct target of both miR-96 and miR-183 by bioinformatics platforms (Fig 4A). A luciferase reporter gene assay in HEK293 confirmed that miR-96 and miR-183 bound to the 3’-UTR sequence of ANLN, and binding was impaired by a mutation in a predicted binding site of the ANLN 3’-UTR (Fig 4B). Consistent with a role for miR-96 and miR-183 in targeting anillin, their overexpression decreased Anln mRNA levels in neonatal MCECs (Fig 4C). On the other hand, inhibiting miR-96 and miR-183 in adult MCECs increased the expression of Anln
(Fig 4D). Together, these findings identify that miR-96 and miR-183 regulate several pro-angiogenic pathways and identify anillin as one of the direct targets of miR-96 and miR-183.

Inhibiting the miR-183 cluster improves the angiogenic capacity of adult MCECs. Since overexpression of the miR-183 cluster was found to impair the angiogenic properties of neonatal MCECs, we asked whether inhibition of the miR-183 cluster on adult MCECs may have the reciprocal effect. Inhibiting all members of the miR-183 cluster in adult MCECs improved their rates of migration (Fig 5A and 5B), proliferation (Fig 5C and Supplementary Fig 5A) and their ability to form capillary-like structures (Fig 5D and Supplementary Fig 5B). Moreover, inhibiting miR-96 and miR-183 improved adult MCECs proliferation and tube-like formation, whereas miR-182 inhibition did not affect these measures (Fig 5C and 5D). Together, these findings confirm that miR-96 and miR-183 inhibit the angiogenic potential of MCECs and suggest that their expression in adult MCECs impairs their regenerative capacity.

miR-96 and miR-183 inhibition induces neovascularisation in the adult mouse after MI.

To investigate whether miR-96 and miR-183 impair angiogenic properties of adult MCECs after MI, we induced MI by permanent occlusion of the left anterior descending coronary artery in adult miR-96/miR-183 global knock-out (KO) mice and wild type (WT) littermates.

While cardiac function and fibrosis content were unchanged (Supplementary Table 1), both capillary and arteriole densities were increased in the peri-infarct of miR-96/miR-183 KO mice compared to WT controls (Fig 6A, 6B and 6C) at 2 weeks after MI.

Notably, the peri-infarct area of miR-96/miR-183 KO mice showed an increased number of vessels expressing the miR-96/miR-183 target ANLN when compared to that of WT mice (Fig 6D). In addition, Anln overexpression in adult MCECs in vitro increases the formation of
capillary-like structures (Fig 6E). This finding confirms that miR-96 and miR-183 could control the expression of pro-angiogenic target genes in MCECs during MI.

**The miR-183 cluster in human cardiac ECs (HCECs).** To determine whether the miR-183 cluster regulates gene expression also in human cardiac ECs (HCECs), we isolated CD31+ CD45- cells from cardiac biopsies of human subjects aged <1 year up to 61 years of age (Supplementary Table 2 and Supplementary Fig 6A-D). While the expression of both miRs was low in HCECs from neonates and infants up to 1 year of age, it increased in HCECs from 8-10-year-old children and remained elevated in HCECs isolated from adults (Fig 7A). In contrast, ANLN expression was higher in HCECs in children up to 5 years and decreased with age (Fig 7A). Correlation analysis showed a significant inverse correlation between levels of ANLN and miR-96 and the age of the patients (Pearson coefficient r=-0.6311, p=0.0278). The expression of both miR-96 and miR-183, therefore, increased with age in primary HCECs while the expression of their target ANLN decreased.

Next, we investigated whether manipulation of miR-96 and miR-183 could also affect the regenerative potential of HCECs. For this experiment, we obtained commercial HCECs from adult donors, termed HCMECs (see Methods). Whereas mimic-mediated overexpression of miR-96 and miR-183 in these cells reduced the formation of capillary-like structures (Fig 7B and Supplementary Fig 7A) and the proliferation rate (Fig 7C and Supplementary Fig 7B), dual miR-96 and miR-183 inhibition increased both parameters, (Fig 7D and 7E and Supplementary Fig 7C and 7D).

Together, these findings agree with those obtained for MCECs and suggest that the miR-96 and miR-183 pathway is conserved between mouse and human. Manipulation of these miRs may therefore be a suitable therapeutic target to improve the angiogenic potential of cardiac ECs in human subjects after MI, although likely in combination with other therapeutic approaches that modulate inflammation and stimulate cardiomyocyte regeneration.
Discussion

While it has been clear that the angiogenic mechanisms occurring during embryonic and adult life are different (21), the cellular and molecular pathways controlling this switch are relatively poorly understood. Embryonic and neonatal ECs possess a specific transcriptional profile (22), and it is becoming increasingly clear that life stage also influences their phenotype with 'younger' ECs generally thought to possess greater angiogenic capacity (23). Here, we first set out to characterise the phenotypic changes between neonatal and adult primary cardiac ECs.

We observed that neonatal MCECs presented with increased proliferation rates and enhanced migratory capacities when compared to adult MCECs which resulted in higher angiogenic properties.

MiRs are inhibitory regulators of gene expression, which are implicated in the regulation of vascular development (24) and post-natal angiogenesis (25). An increasing number of miRs have been shown to target specific genes involved in angiogenesis or to be modulated by either pro- or anti-angiogenic stimuli (12). We, therefore, investigated whether the phenotypical switch between neonatal and adult MCECs correlated with changes in the expression of miRs. Here we have identified the miR-183 cluster as a regulator of this phenotypical switch. Indeed, overexpression of those miRs in neonatal MCECs decreased their angiogenic capacities giving them an adult-MCECs-like phenotype. Importantly, we identified that miR-96 and miR-183 were the cause of this effect, whereas the other member of the family, miR-182, did not seem to influence MCECs phenotype.

Angiogenesis is one of the five hallmarks of cardiac regeneration in neonatal mice alongside remuscularisation, electromechanical stability, resolution of fibrosis, and immunological balance (10). Indeed, angiogenesis preceded cardiomyocyte repopulation of the regenerating area in an apical resection model of cardiac regeneration (11).
Understanding the mechanisms underpinning re-vascularisation of the neonatal heart in the context of cardiac regeneration is therefore critical to the development of new therapies to improve repair after MI and hamper the progression to heart failure. We, therefore, sought to determine whether manipulation of the miR-183 cluster could be used to modify outcome following MI in the neonatal mouse.

Intra-cardiac injection of miR-96 and miR-183 following induction of MI in neonatal mice resulted in their increased expression in MCECs, which was associated with a reduction of capillary density. Together with our in vitro observations, these results suggest that miR-96 and miR-183 can also manipulate MCECs’ phenotype in vivo. A failure of the neonatal heart to resorb scar tissue was also observed. Whilst this may be secondary to the reduced capillary density, we cannot rule out that it may be resulting from miR-96 and miR-183 overexpression in other cell types, e.g. cardiomyocytes. Hence, given the upregulation of miR-96-183 in both ECs and cardiomyocytes after delivery, their specific relevance in the observed phenotype cannot be distinguished. Moreover, both ECs and cardiomyocytes miR-96-183 expression may well have a role in each cell type and to mediate crosstalk between these cell types.

To investigate the therapeutic potential of miR-96 and miR-183 further, we performed converse in vitro and in vivo experiments in adult mice. We observed that inhibiting both miRs was able to improve the angiogenic capacities of adult MCECs in vitro, indicating that these miRs may play central roles in regulating the phenotypical switch between neonatal and adult MCECs. These observations were also recapitulated in human cardiac ECs, highlighting the translatability of this pathway. Of therapeutic importance, we were able to show that, a global KO of the miR-183 cluster was associated with increased vascularisation of the peri-infarct zone following MI. However, this was not associated with improved function in our 2-week study. In line with this, there is a disconnect and delay between
improved neovascularisation and the subsequent improvement in cardiac function and scar formation, which happens later, in patients following MI (26).

To understand the full potential of miR-183 cluster inhibition as a therapeutic target, future studies should look past two weeks after MI and focus on the progression to heart failure.

It has been proposed that stimulating angiogenesis may benefit cardiac repair (27). However, our observations show that stimulating angiogenesis is not sufficient to provide the desired functional improvement of cardiac function, likely because other processes would have to be co-stimulated, including cardiomyocyte proliferation and maturation as well as a beneficial inflammatory environment.

Successful characterisation of miR activity relies on identifying the best miR targets matching the observed phenotype and disease (28). Using a bioinformatic approach and an in vitro and in vivo validation, we have identified anillin as the target of miR-96 and miR-183. Anillin is an actin-binding protein that is highly conserved across multiple species and associated with the cell cycle and specifically the completion of cytokinesis (29). To analyse the role of cytokinesis during cardiac development and MI, Hesse et al. generated an ANLN-eGFP reporter mouse for the investigation of ANLN in the cell cycle dynamics in the heart (30), showing nuclear localisation of ANLN-eGFP during embryonic heart development. Anillin was found to act as a mitotic marker in pluripotent stem cells and importantly marked endoreduplication in myocardial injury (30). Moreover, anillin is also a bona fide marker for cardiomyocyte binucleation, enabling to unequivocally discern such events from cardiomyocyte division in vitro and in vivo (30).

Recently, transgenic mice expressing an ANLN-eGFP construct under the control of the endothelial-specific Flt-1 promoter have been generated for the monitoring of EC proliferation and cytokinesis (31). ANLN-eGFP signals overlap mainly with Ki67+/PECAM+ during vascular development, showing a decline in ECs proliferation between E9.5 to E12.5, therefore supporting the importance of anillin in ECs proliferation during vessels
development but also its possible role in regulating EC sprouting in miR-96-183 KO mice after MI.

In addition, it is conceivable that Anln upregulation in miR-96/miR183 KO mice might therefore also affect arterial smooth muscle cells (SMCs) and thus increase arterial density through its binding to F-actin and non-muscle myosin (32). Specifically, the molecular interactions between F-actin and non-muscle myosin II govern the contraction of the cytoskeleton to modulate cell shape, division and migration in SMCs during arteriogenesis (33, 34) However, further experiments would be required to investigate this question.

These findings provide valuable insights into the basic biology underpinning angiogenesis in the neonatal infarcted heart and elucidate novel and fundamentally critical molecular mechanisms that govern post-MI angiogenesis during cardiac regeneration. Thereby, our findings highlight the importance and functional role of the miR-183 cluster in ECs biology in re-establishing a neonatal-like regenerative phenotype in adult cardiac ECs. More, miR-96 and miR-183 represent putative therapeutic targets in MI.
**Methods**

*MCECs.* MCECs were either purchased from Cell Biologics (Chicago, IL, USA), or isolated in-house from postnatal C57BL/6N mice. In-house MCECs were obtained from pooled hearts of mixed-sex C57BL/6N mice aged between P1 and 8 weeks. Briefly, hearts were enzymatically digested using a neonatal heart dissociation kit in combination with the gentleMACS™ dissociation kit (Neonatal Heart Dissociation Kit, 30-098-373, Miltenyi biotec) to obtain a single-cell suspension of all cardiac cells. Cardiomyocytes and fibroblasts were removed using a 40 µm cell strainer. CD31+/CD45− ECs were then obtained using microbeads (CD31 Microbeads mouse: 130-097-418 Miltenyi biotec; CD45 Microbeads mouse: 130-052-301, Miltenyi biotec) and the Miltenyi biotec MACS sorting technology according to the manufacturer’s instruction. Immediately after isolation, cells were either cultured in Complete Mouse Endothelial Cell Medium with growth factors supplements (PeloBiotech, Germany) with 10% fetal bovine serum or used from passage 2 to 5. Commercial MCECs from Cell Biologics (Cat. #C57–6024) were isolated from 1 day-old (P1) and 4 weeks-old (4W) C57BL6 mice (35, 36). Cells were from pooled hearts of mixed-sex donors, cultured on gelatin-coated plates and maintained in Complete Mouse Endothelial Cell Medium with growth factors supplements (PeloBiotech, Germany) with 5% fetal bovine serum and used from passage 2 to 5, according to the manufacturer’s recommendations. MCECs were used for functional assays (as detailed below) and miR PCR arrays.

To analyse the miR-96 and miR-183 expression in MCECs and cardiomyocytes after miR-96-183 injection, the protocol described above was applied; however, cardiomyocytes were collected after ECs depletion and cultured in a 6-well plate with DMEM 10% FBS for 2h to allow fibroblasts to attach to the plate. Cardiomyocytes were then collected from the media by centrifugation.
HCECs. HCMECs were purchased from PromoCell. HCMECs were isolated from a healthy male subject (49 years of age) and were cultured in EGM-2 (EBM-2 added with growth factors and other supplements, PromoCell) with 10% fetal bovine serum and used between passage 2 and 4. Additionally, primary CD31+/CD45- HCECs were obtained from Prof. Massimo Caputo, University of Bristol (REC reference: 15/LO/1064; IRAS ID: 156551). Cardiac biopsies from the right ventricle (RV) of patients with a diagnosis of Tetralogy of Fallot/Pulmonary atresia undergoing surgery were collected, and HCECs were isolated using the Neonatal Heart Dissociation kit and the Miltenyi biotec MACS Technology (Miltenyi biotec). Immunocytochemistry for the endothelial markers Von Willebrand Factor and CD31 was performed to characterise HCECs in culture. Briefly, cells were fixed in 4% buffered paraformaldehyde and permeabilized with 0.1% TritonX before incubation with primary antibody for CD31 (1:100; clone JC70A, GA610, Dako) and Von Willebrand Factor (1:100, GA527, Dako). AlexaFluor-labelled secondary antibodies (1:1000, Molecular Probes/Invitrogen) were employed to detect primary antibodies. Staining with 4′,6-diamidino-2-phenylindole (1:2000, DAPI; Vector Laboratories, Inc.) was used to identify nuclei. Images were obtained using a Zeiss Axio Observer.Z1 microscope with Zen Blue software (Zeiss).

**Cell transfection.** MCECs and HCMECs were transfected with 25 nM of miR-96, miR-182 and miR-183 miRIDIAN mimics and inhibitors (or controls) (Dharmacon) using Lipofectamine 2000 Reagent (Invitrogen). Individual miR transfections were performed using 25 nM of mimics or inhibitors; co-transfection experiments using 2 (miR-96 and miR-183) or 3 (miR-96, miR-182 and miR-183) miRs were performed using the total dose of 25 nM of either mimics or inhibitors.

**ECIS system.** Commercial neonatal and adult MCECs were plated on the ECIS chip array (8W1E or 8W10E) in order to assess cell-cell and cell-surface adhesion properties, as well
as their migration rate. Adhesion and migration were additionally evaluated in commercial neonatal MCECs transfected with miR-96 and miR-183 mimics (and controls) and commercial adult MCECs after transfection with miR-96 and miR-183 inhibitors (and controls). The migration speed was calculated in μm/hr, Rb and alpha as reported in Giaever and Keese (37).

*EdU proliferation assay.* Proliferation assay was performed on MCECs and HCMECs transfected with miR mimics, miR inhibitors, or controls using the Click-iT EdU AlexaFluor 555 imaging kit (ThermoFisher). At 48 hours after transfection, ECs (4 × 10^3 cells/well) were seeded in 96-well plates and incubated with EdU (10 μmol/l) for 24 hours. Cells were fixed with buffered 4% PFA (Sigma) in PBS for 15min at RT and stained following manufacturer's instructions. Nuclei were stained with DAPI. Experiments were performed in triplicate. Cells were analysed at a 400X magnification and the percentage of EdU+ cells was determined.

*Matrigel assay.* MCECs or HCMECs were seeded in six-well plates (1.5 × 10^4 cells/well) and transfected with miR mimics, miR inhibitors, or controls for 48 hours, then trypsinized and plated in a flat-bottomed 96-well plate coated with growth factors-enriched Matrigel (Matrigel Matrix, Basement Membrane, BD Biosciences). All cells were seeded at the density of 1 × 10^4 cells/well; Matrigel on adult MCECs was additionally performed seeding the cells at the density of 2 × 10^4/well. Endothelial network formation was quantified at 6, 12 and 24 hours in randomly captured microscopic fields (magnification 40X) by counting the length of vascular-like structures.

*RNA extraction miR expression profiling and analysis.* Total RNA was extracted using miReasy kit (Qiagen). Real-time quantification to measure miRNA was performed with the TaqMan miRNA reverse transcription kit and miRNA assay (ThermoFisher). MiRNA
expression was normalized to the U6 small nucleolar RNA (snRU6). For mRNA analysis, cDNA was produced using QuantiTect Reverse transcription kit (Qiagen). Expression was normalized to 18S ribosomal RNA. Real-time qPCR was used to measure the expression of miR-96 (assay ID: 000186; ThermoFisher), miR-182 (assay ID: 000597, ThermoFisher) and miR-183 (assay ID: 002269; ThermoFisher), snRU6 (assay ID: 001973; ThermoFisher). ANLN and 18S rRNA pre-optimized primers were obtained from Sigma (KiCqStart Primers). miR expression profiling of 150 miRs was performed using mouse miScript miRNA PCR arrays (96 well format; Qiagen) and SYBR Green-based real-time PCR analysis, using Roche LyghtCycler real-time PCR system. The relative expression was calculated using 2-\(\Delta\Delta^{Ct}\) method (38). The Web-based miScript miRNA PCR array data analysis tool was used to analyse the real-time PCR data (Qiagen).

Animal work. C57BL/6N mice bred in-house from stock originally obtained from Harlan (UK) and aged between P1 and 10 weeks. MiR-96/miR-183 KO mice (Mirc40; EMMA ID EM:10856; international strain designation C57BL/6N-Atm1Brd Mirc40\(^{tm1Hmpr/WtsiOulu}\) were obtained from the University of Oulu (Finland) - European Mouse Mutant Archive (EMMA).

Neonatal and adult mouse models of MI. Female miR-96/miR-183 KO mice and C57BL/6N (controls) aged 8 to 10 weeks were used for adult MI studies. MI in adult mice was induced as previously described (39). Briefly, anaesthetized mice (Isoflurane) were orally intubated and artificially ventilated using a Minivent mouse ventilator (Harvard Apparatus, Kent, UK). The chest was opened through an incision in the intercostal space and MI was induced by permanent ligation of the proximal left anterior descending (LAD) coronary artery by using a 7.0 Mersilene suture (Ethicon, USA). The surgical wound was sutured, and animals were allowed to recover.
Both male and female C57BL/6N mice aged P1 were used for neonatal MI studies. MI was induced by ligation of the LAD coronary artery in P1 mice as described in (8, 40). Briefly, anaesthesia was induced by a combination of hypothermia and breathable (Isoflurane) anaesthetic. The animal was secured onto an ice pack for the duration of the procedure to maintain adequate anaesthesia. A 5 mm skin incision was placed over the left thorax above the 5th rib, the skin was blunt dissected, and a small opening was created at the 5th intercostal space to visualise the left pulmonary lobes and heart. The LAD was identified and ligated (9-0 Ethilon, Ethicon). Using a 32G insulin syringe 5 µL containing 200 pmol or 50 pmol of either miR mimics or control were injected within the LV wall below the ligation. The ribs, pectoral muscles, and skin were closed (8-0 Prolene, Ethicon). Analgesia was administrated with one drop of 1/50 bupivacaine (Marcain® Polyamp Steripack 0.25%) in saline onto the wound. The entire litter was tattooed for identification purposes and returned to the nest.

**In vivo high-resolution ultrasound.** Cardiac functional parameters of adult mice were assessed at baseline (1 day before induction of MI) and at 2 weeks. Briefly, anaesthesia was induced using isoflurane in medical O₂ at concentrations: 2-3%. Isoflurane was used for anaesthesia maintenance at around 2%. Rectal temperature was monitored (36.5 – 38°C) with a Physitemp RET-3 probe. Thoracic hair was removed using commercially available electric shavers and depilatory cream. Conductive ultrasound gel (Parkers Lab) was then applied to each paw to record ECG signals. Pre-warmed Aquasonic® 100 ultrasound transmission gel was applied onto the abdomen avoiding bubble formation. Image acquisition was performed on a Visualsonics Vevo® 770 ultrasound biomicroscope. Cardiac function was assessed using standard parasternal long axis (PLAX) images of the LV in M-
mode and ECG-gated kilohertz visualisation (EKV) B-mode. Functional measurements were extracted using the Vevo® 770 software.

**Histological evaluation in the mouse heart.** Fresh heart samples were fixed in 10% formalin for 24h and subsequently placed in 70% ethanol for at least 24h prior to embedding in paraffin. Paraffin-embedded samples were cut into sequential 4 µm thick sections on superfrost slides (VWR). Slides were deparaffinised and rehydrated. Histological analysis was performed on picrosirius red-stained sections. Capillary and arteriole densities were determined using fluorescent microscopy on sections stained with Alexa 488-conjugated isolectin-B4 (1:100, I2141, Molecular Probes), Cy3-conjugated α-vascular smooth actin (1:200, C6198, Sigma) and Anillin (1:100, ab154337, Abcam).

**Bioinformatics prediction and pathway analysis of miR-183 cluster target genes.** Computational prediction of miR-183 cluster target genes was done using a published algorithm TargetScan 7.2 (http://www.targetscan.org). MirPath (http://snf-515788.vm.okeanos.grnet.gr/) was used to perform miRNA pathway analysis.

**Luciferase assay.** Luciferase assay has been performed as previously described (41). ANLN 3’UTR vector was from the LightSwitch™ 3’UTR Reporter GoClone® Collection (Active Motif). Primers for 3’ UTR mutation at the position 110-116 are as follows: forward 5’-CGAAAGGGTTTaataattTATTCACTACGTA -3’ and reverse 5’-TACGTAGTGAATAatattAAACCCTTTCCG -3’. Mutation was performed using GeneArt mutagenesis system (ThermoFisher). Luciferase constructs were transfected into HEK293T cells together with miR-96 and miR-183 mimics or p-SV-β-gal control vector. Cells were cultured for 48h and assayed with the Luciferase and β-Galactosidase Reporter Assay.
Systems (Promega). Luciferase values were normalized to protein concentration and β-galactosidase activity.

**Lentiviral vectors.** Lentiviral vectors pLV: ANLN-eGFP and control pLV: eGFP were purchased from Cyagen™. Production and purification of lentiviral particles were performed at Edinburgh University Core facilities. Cardiac ECs were transduced with 10 M.O.I of lentiviral particles in full media containing 8 μg/ml polybrene overnight. Transduced cells were FACS sorted to have a selected population expressing ANLN-eGFP and eGFP as control.

**Statistics.** Statistical analysis was performed using GraphPad Prism 5 software. Data are expressed as mean ± SEM. Student’s unpaired t-test was used for comparison of 2 groups. For comparison among more than two groups, a 1- or 2- way ANOVA with post-hoc Bonferroni multi-comparison test. A P value of <0.05 was interpreted to denote statistical significance.

**Study approval.** All animal experiments were approved by and performed in accordance with the University of Edinburgh Animal Welfare and Ethical Review Body, and the UK Home Office. Experimental procedures involving human subjects were performed in accordance with the Declaration of Helsinki and were approved by the responsible ethics committees. Research on clinical samples was performed in agreement with the Human Tissue Act (HTA). Written informed consent was received from participants prior to inclusion in the study. (REC reference: 15/LO/1064; IRAS ID: 156551).

**Author contributions.** AC and MM developed and designed the research study. RFPC, MiV, MaV, SJ, DI, DM, BC, AT, AC and MM conducted the experiments and acquired data.
MC provided human samples. CR, DEN, GAG, AHB edited the manuscript. RFPC, AC and MM wrote and edited the manuscript, with contribution from all authors.

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Figure 1. Morphological and functional differences between neonatal and adult MCECs.
(A) Graphs showing increased cell-cell contacts and cell-surface adhesions of neonatal mouse cardiac endothelial cells (MCECs) compared to adult MCECs. (B) Representative impedance curve and quantification showing accelerated migration of neonatal MCECs (red line – red bar) compared to adult MCECs (black line – white bar). (C). Representative microphotographs and bar graphs showing the higher percentage of EdU proliferating cells in neonatal versus adult MCECs after 24hrs in culture. EdU* cells are shown in red and indicated by arrows, DAPI* nuclei in blue. (D) Matrigel experiment with MCECs seeded at high density (2 x 10^4) show that adult MCECs are able to form tube-like structures at 6hrs in culture properly but demonstrate that the length of tube-like structures formed by the same number of neonatal MCECs is higher. (E) Representative microphotographs and bar graphs showing that whereas 1 x 10^4 neonatal MCECs form proper tube-like structures at 24hrs, adult MCECs barely form tube-like structure at the same time-point. Scale bar = 50 μm. B – n =3; A, C, D and E - n = 4/group. *p < 0.05, **p < 0.01 vs adult MCECs (Student’s t-test). Data are expressed as mean ± SEM.
Figure 2. Mimic-mediated overexpression of the miR-183 cluster reduces the angiogenic potential of neonatal MCECs. (A) Volcano plot of the significantly differentially regulated miRs in adult mouse cardiac endothelial cells (MCECs) compared to neonatal MCECs. (B) Real-time PCR showing up-regulation of miR-96-5p, miR-182-5p and miR-183-5p in cultured adult MCECs compared to neonatal MCECs. Cultured neonatal MCECs were transfected with either miR-96, miR-182 or miR-183 (at 25 nM), or with a combination of all mimics (all at 25 nM – miR-183C) or control mimic(s). (C) Bar graphs showing that the number of EdU+ proliferating cells decreases after overexpression of the miR-183 cluster, miR-96 or miR-183 in neonatal MCECs. (D) Bar graphs showing that overexpression of the miR-183 cluster, miR-96 or miR-183 in neonatal MCECs prevent the formation of tube-like structures. B, C – n = 3/group; D – n = 4/group; B: *p < 0.01 vs adult (Student t-test) C-D: *p < 0.05, **p < 0.01 vs control mimic (One-way ANOVA with Bonferroni post-hoc test). Data are expressed as mean ± SEM.
Figure 3. Mimic-mediated overexpression of miR-96 and miR-183 prevents scar tissue resolution and neovascularization of the neonatal mouse heart after MI. (A-B) Survival curves show that whereas all neonatal mice injected with a combination of miR-96 and miR-183 mimics at the dose of 200 pmol died within 24h after MI and mimic injection (A), the mortality rate of mice injected with miR-96 and miR-183 mimics at the dose of 50 pmol was similar to that of control mimic injected mice (B). Representative microphotographs (C) and bar graphs (D) (n = 6 control mimic, n = 8 miR-96-183 mimic) showing differences in fibrosis deposition (in red, assessed by picrosirius red staining) between neonatal infarcted hearts injected with either control mimics or mirR-96 and miR-183 mimics. Scale bar = 1000 μm. Representative microphotographs (E) and bar graphs (F) (n = 4 control mimic, n = 6 miR-96-183 mimic) showing the reduced capillary density in the heart of neonatal mice subjected to MI and injected with mirR-96 and miR-183 mimics compared to control injected mice. Capillaries are stained by isolectin-B4 (green fluorescence), and arterioles are stained by isolectin-B4 (green fluorescence) and α-smooth muscle actin (red fluorescence). Scale bar = 250 μm. Inset: Scale bar = 50 μm. *p < 0.05 vs control mimic (Student’s t-test). Analyses were performed at 21 days after MI. Data are expressed as mean ± SEM.
Figure 4. Anillin (ANLN) is a direct target of miR-96 and miR-183. (A) Prediction of anillin as a target gene of miR-96 and miR-183 by TargetScan. (B) Luciferase assay for ANLN 3’-UTR (n= 3/group). Luciferase activity at 48h post-co-transfection of HEK293T cells with both miR-96 and miR-183 or control mimics and ANLN 3’UTR or ANLN 3’UTR mutated (mut). Relative gene expression of Anln after miR-96 and miR-183 overexpression (C), or inhibition (D) in mouse cardiac endothelial cells (MCECs). (n = 4/group). **p < 0.01 vs control mimic or anti-miR (Student’s t-test). Data are expressed as mean ± SEM.
Figure 5. Inhibition of the miR-183 cluster improves the angiogenic potential of adult MCECs. (A) Graphs showing inhibition of the miR-183 cluster improves migration of adult mouse cardiac endothelial cells (MCECs -green line) compared to adult MCECs transfected with control mimic (black line). (B) Bar graph showing increased migration rate of adult MCECs after miR-183 cluster inhibition. (C) Bar graphs showing a higher number of EdU+ cells (increased proliferation) in adult MCECs after inhibition of the miR-183 cluster or miR-96 or miR-183. (D) Bar graphs showing increased tube-like structures formation in adult MCECs transfected with miR-183 cluster or miR-96 or miR-183 inhibitors. (C – n = 3/group; B and D – n = 4/group). *p < 0.05, **p < 0.01 vs control anti-miR (Student t-test or One-way ANOVA with Bonferroni post-hoc test). Data are expressed as mean ± SEM.
Figure 6. Inhibition of miR-96 and miR-183 increase neovascularisation in the adult mouse after MI.

(A) Bar graphs show the increased number of capillaries (n = 5/group) and (B) small arterioles (n = 6 WT, n = 7 miR-96/183 KO) in the peri-infarct of miR-96/183 KO mice at 14 days post-MI. (C) Representative microphotographs showing capillaries (stained by isoelectin-B4; green fluorescence) and arterioles (stained by isoelectin-B4 - green fluorescence - and α-smooth muscle actin – red fluorescence). Scale bar = 250 μm. Inset: Scale bar = 50 μm. (D) Bar graphs and representative microphotographs show the increased percentage of ANLN positive vessels in the peri-infarct of miR-96/183 KO mice at 14 days post-MI (n = 6/group). ANLN is in red fluorescence, ECs are stained by isoelectin-B4; green fluorescence. Scale bar = 20μm. *p < 0.05 vs WT (Student’s t-test). (E) Adult mouse cardiac endothelial cells (MCECs) were transduced with lentiviral vectors LV-eGFP or LV-ANLN-eGFP and seeded on Matrigel. Representative Matrigel assay images and quantification as total tubule length (n = 5/group). Scale bar = 100μm. **p<0.01 vs LV-eGFP (Student’s t-test). Data are expressed as mean ± SEM.
Figure 7. Effect of miR-183 cluster manipulation on HCECs. (A) Real-time qPCR showing an age-dependent increased expression of miR-96 and miR-183 and decreased expression of ANLN in human cardiac endothelial cells (HCECs) obtained from cardiac biopsies of human subjects. Commercially available human cardiac microvascular endothelial cells (HCMECs) from adult subjects were used to test the effect of miR-183 cluster manipulation in human cardiac ECs. (B) Bar graphs showing that overexpression of miR-96 and miR-183 prevents the formation of capillary-like structures and (C) reduces the percentage of EdU+ cells in HCMECs. On the other hand, inhibition of miR-96 and miR-183 increases the length of tube-like structures (D) and promotes proliferation (E) of cultured HCMECs. n = 4/group. **p < 0.01, *p < 0.05 vs control (Student’s t-test). Data are expressed as mean ± SEM.


