mir-17–92 Cluster Is Required for and Sufficient to Induce Cardiomyocyte Proliferation in Postnatal and Adult Hearts

Jinghai Chen, Zhan-Peng Huang, Hee Young Seok, Jian Ding, Masaharu Kataoka, Zheng Zhang, Xiaoyun Hu, Gang Wang, Zhiqiang Lin, Si Wang, Willam T. Pu, Ronglih Liao, Da-Zhi Wang

Rationale: Cardiomyocytes in adult mammalian hearts are terminally differentiated cells that have exited from the cell cycle and lost most of their proliferative capacity. Death of mature cardiomyocytes in pathological cardiac conditions and the lack of regeneration capacity of adult hearts are primary causes of heart failure and mortality. However, how cardiomyocyte proliferation in postnatal and adult hearts becomes suppressed remains largely unknown. The miR-17–92 cluster was initially identified as a human oncogene that promotes cell proliferation. However, its role in the heart remains unknown.

Objective: To test the hypothesis that miR-17–92 participates in the regulation of cardiomyocyte proliferation in postnatal and adult hearts.

Methods and Results: We deleted miR-17–92 cluster from embryonic and postnatal mouse hearts and demonstrated that miR-17–92 is required for cardiomyocyte proliferation in the heart. Transgenic overexpression of miR-17–92 in cardiomyocytes is sufficient to induce cardiomyocyte proliferation in embryonic, postnatal, and adult hearts. Moreover, overexpression of miR-17–92 in adult cardiomyocytes protects the heart from myocardial infarction-induced injury. Similarly, we found that members of miR-17–92 cluster, miR-19 in particular, are required for and sufficient to induce cardiomyocyte proliferation in vitro. We identified phosphatase and tensin homolog, a tumor suppressor, as an miR-17–92 target to mediate the function of miR-17–92 in cardiomyocyte proliferation.

Conclusions: Our studies here identify miR-17–92 as a critical regulator of cardiomyocyte proliferation, and suggest this cluster of microRNAs could become therapeutic targets for cardiac repair and heart regeneration. (Circ Res. 2013;112:1557-1566.)

Key Words: cardiomyocyte proliferation ■ cell cycle ■ heart disease ■ miR-17–92 ■ myocardial infarction ■ PTEN

The adult mammalian heart has limited capability to regenerate itself after the loss of mature cardiomyocytes because of a variety of pathological conditions such as myocardial infarction. It is generally conceived that postmitotic cardiomyocytes in adult hearts exit from the cell cycle and stop cell proliferation.1,2 However, the hearts of adult zebrafish can undergo cardiac regeneration without scar formation after resection of ventricle, primarily through cardiomyocyte proliferation.3,4 Intriguingly, a recent report demonstrated that surgical resection of the ventricular apex in newborn mice stimulates the proliferation of cardiomyocytes and repairs the damaged heart, but the mouse heart loses this regenerative potential within 7 days of its postnatal life and it is not clear how the regenerative potential is lost in the adult hearts.5 To date, the molecular mechanism and regulatory pathways that control adult cardiomyocyte proliferation and cardiac regeneration remain largely unknown.

MicroRNAs (miRNAs) are a class of ~22 nt noncoding RNAs that regulate the expression of protein-coding genes post-transcriptionally. More than 1000 human miRNAs have been identified, however the biological functions of many of them remain unknown. miR-17–92 cluster was initially reported as a human oncogene and named oncomir1.6,7 Numerous reports have documented the expression of miR-17–92 in variety of human cancers and disorders.8–12 Genetic studies demonstrated that miR-17–92 is indispensable for mouse development and cell proliferation, and miR-17–92 mutant mice die postnatally, displaying defects in lung, hearts,
and others. We hypothesized that miR-17–92 regulates the proliferation of cardiomyocytes. In this study, we tissue-specifically overexpress or delete the miR-17–92 cluster in cardiomyocytes in transgenic or knockout mice and found that miR-17–92 participates in the regulation of cardiomyocyte proliferation in embryonic, postnatal, and adult hearts.

**Methods**

Cell culture, quantitative RT-PCR analyses, Western blot analyses, histologic assessments, and immunohistochemistry were performed according to routine protocols. Details of Materials and Methods are provided in the Online Data Supplement.

**Statistics**

Values are reported as means±SEM unless indicated otherwise. The 2-tailed Mann-Whitney U test was used for comparing 2 means (Prism, GraphPad). Values of P<0.05 were considered statistically significant.

**Results**

**miR-17–92 Is Required For Cardiomyocyte Proliferation in Embryonic and Postnatal Hearts**

We crossed the miR-17–92Ko/ko mice with Nkx2.5-Cre mice, in which the expression of Cre recombinase is under the control of the cardiac-specific Nkx2.5 gene, to delete miR-17–92 in embryonic, postnatal, and adult hearts (Online Figure 1A). We found that cardiac-specific miR-17–92 mutant mice (named miR-17–92 cKO) are slightly underrepresented (20.3%) at weaning age, suggesting that cardiac-specific deletion of miR-17–92 resulted in partial embryonic lethality (Online Figure 1B). We confirmed that the expression of miR-17–92 miRNAs was significantly reduced in the hearts of mutant mice (Online Figure II).

The hearts of postnatal miR-17–92 cKO mice seem to be smaller than that of their littermate controls (Figure 1A). We examined the proliferation of cardiomyocytes in miR-17–92 cKO hearts, using immunostaining for phosphorylated histone H3 (pH3), which marks mitosis, and we found that there is less proliferation of cardiomyocytes in postnatal hearts of miR-17–92 cKO mice (Figure 1B and 1C; Online Figure III). Quantitative analyses confirmed substantial decrease in total numbers of pH3-positive cardiomyocytes in miR-17–92 cKO hearts (Figure 1D). We also observed a decrease in cardiomyocyte proliferation in miR-17–92 heterozygous hearts (Figure 1D). We asked whether loss of miR-17–92 affected the survival of cardiomyocytes. We performed terminal deoxynucleotidyl transferase dUTP nick end labeling assay to measure apoptosis and observed no change in terminal deoxynucleotidyl transferase dUTP nick end labeling signals in postnatal hearts of miR-17–92 cKO mice when compared with controls (Online Figure IV).

Most miR-17–92 cKO mice survived to adulthood, and we next investigated miR-17–92 loss-of-function phenotype in adult hearts. There was no obvious difference in the gross cardiac morphology of the miR-17–92 cKO and their littermate control mice (Online Figure VA). The heart weight to body weight ratio was not altered in miR-17–92 cKO mice (Figure 1E). However, there seemed to be a compensatory cardiomyocyte hypertrophy in these hearts (Online Figure VB). We used Langendorf perfusion method to isolate cardiomyocytes from adult hearts. Freshly isolated adult cardiomyocytes were stained with α-actinin to label cardiomyocytes; pan-cadherin to recognize desmosomes. Reconstructed confocal images clearly indicate that the majority of isolated cardiomyocytes were intact (Figure 1F). Quantitative analysis indicated a substantial decrease in the total numbers of cardiomyocytes in the hearts of miR-17–92 cKO mice (Figure 1G and 1H). Quantitative measurement of the size of isolated adult cardiomyocytes showed that the size of cardiomyocytes was increased in the heart of miR-17–92 cKO mice (Figure 1I), consistent with the idea that increased size of cardiomyocytes compensates for the reduction of total numbers of cardiomyocytes in mutant hearts. Terminal deoxynucleotidyl transferase dUTP nick end labeling assays detected no difference in apoptosis in the heart of 8-month-old miR-17–92 cKO mice when compared with controls (Online Figure IV). We examined the cardiac function, using echocardiography, and found decreased in ventricle wall thickness, increased in ventricle systolic diameter, and decreased in cardiac function in miR-17–92 cKO mice when compared with their littermate controls (Online Figure VI; Online Table I). Together, these results indicate that miR-17–92 is required for cardiomyocyte proliferation and normal cardiac function in postnatal and adult hearts.

**miR-17–92 Is Sufficient to Induce Cardiomyocyte Proliferation in Embryonic and Postnatal Hearts**

Having demonstrated that miR-17–92 is required for cardiomyocyte proliferation in embryonic and postnatal hearts, we next tested whether overexpression of miR-17–92 was sufficient to induce cardiomyocyte hypertrophy. We generated cardiac-specific conditional transgenic mice to overexpress miR-17–92 in the heart. We first bred the floxed miR-17–92 knock-in (KI) allele, in which a loxP-flanked Neo-STOP cassette was inserted upstream of the bicistronic human miR-17–92 cluster and knocked into the Rosa26 locus (named miR-17–92 KI), with the Nkx2.5-Cre mice to achieve the overexpression of miR-17–92 in embryonic and postnatal hearts (Online Figure VII). The hearts of cardiac-specific miR-17–92 transgenic mice (named miR-17–92TGloxP) were dramatically enlarged (Figure 2A). Histological section revealed that the ventricle wall was substantially thickened in the hearts of miR-17–92 TGloxP mice (Figure 2A). The heart was hyperplasia and trabeculas were highly condensed and packed (Figure 2A, bottom). There was no evidence of cardiomyocyte hypertrophy, and the size of cardiomyocytes is comparable between miR-17–92 TGloxP and control mice. The increase of cardiomyocyte numbers resulted from an increase in cardiomyocyte proliferation. We used...
pH3 to mark proliferating cardiomyocytes and found that overexpression of miR-17–92 is sufficient to enhance cardiomyocyte proliferation in both embryonic and postnatal hearts (Figure 2B–2D; Online Figure VIII). Concordantly, there was a significant increase in heart/body weight ratios in miR-17–92 KO mice (Figure 2E).

Next, we generated transgenic mice to overexpress miR-17–92 predominantly in postnatal and adult cardiomyocytes. We bred miR-17–92 KI mice with α-MHC-Cre transgenic mice, in which the expression of Cre recombinase is directed by the cardiac-specific α-MHC (Myh6) promoter, to generate cardiac-specific miR-17–92 transgenic mice (named...
miR-17–92 TGMHC). We observed about 2 to 5 folds higher miR-17–92 expression in transgenic hearts (Online Figure IX). Most miR-17–92 TG MHC mice survived to adulthood (Online Figure X). The hearts of the miR-17–92 TG MHC mice were substantially enlarged (Figure 3A), and the heart/body weight ratio was significantly increased in these mice (Figure 3B). Cardiac-specific overexpression of miR-17–92 increased the thickness of ventricle wall in transgenic mice (Figure 3C; Online Table II). Quantitative measurement of cardiomyocyte cell size and cell number of the miR-17–92 TGMHC hearts demonstrated a substantial increase in the cell number in the heart of miR-17–92 TG MHC mice, whereas the size of cardiomyocyte was not changed (Online Figure XI). We isolated cardiomyocytes from adult hearts using Langendorf isolation method and determined that there was substantial increase in total cardiomyocyte numbers in miR-17–92 TG MHC hearts (Figure 3D and 3E). Intriguingly, we found that there is an increase in total numbers of mono-nucleus cardiomyocytes and decrease in binuclei cardiomyocytes in miR-17–92 TG MHC hearts (Figure 3F; Online Figure XII). Consistent with the increase in the cell numbers, the proliferation of cardiomyocytes, marked by pH3, was enhanced in miR-17–92 TG MHC hearts (Figure 3G and 3H; Online Figure XIII). Increased cell proliferation in miR-17–92 TG MHC hearts was further confirmed by 5-ethynyl-2'-deoxyuridine (EdU) incorporation and quantification (Figure 3I and 3J). Finally, we stained cardiomyocytes with aurora B to detect cytokinesis. We observed an increase in aurora B signals in postnatal days 5 and 15 hearts of the miR-17–92 TGMHC mice (Online Figure XIV). Z-stack confocal images confirmed that positive aurora B signals are located in cardiomyocytes (Online Figure XIV).

miR-17–92 Induces Cardiomyocyte Proliferation in Adult Hearts and in Response to Injury

The above data indicate that overexpression of miR-17–92 was sufficient to induce cardiomyocyte proliferation in embryonic, neonatal and adult hearts. To further determine the function of miR-17–92 in the proliferation of postmitotic cardiomyocytes in adult hearts more definitely, we used an inducible system to overexpress miR-17–92 in cardiomyocytes of 4-month-old mice. We bred the miR-17–92 KI mice with the α-MHC-MerCreMer transgenic mice in which the Myh6
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promoter directs the expression of a tamoxifen-inducible Cre recombinase in cardiomyocytes. We induced miR-17–92 overexpression in adult cardiomyocytes by activating tamoxifen-inducible Cre recombinase (see Materials and Methods). We confirmed cardiac-specific overexpression of members of the miR-17–92 cluster in the hearts of miR-17–92 transgenic mice (named miR-17–92 \( \text{TG}^{\text{MCreMer}} \)) after tamoxifen administration (Online Figure XV). The hearts of miR-17–92 \( \text{TG}^{\text{MCreMer}} \) mice were substantially larger than that of the littermate controls after miR-17–92 overexpression (Figure 4A). Tissue sections revealed an increase in wall thickness and left ventricle dimension (Figure 4A).

**Figure 3.** miR-17–92 induces cardiomyocyte proliferation in postnatal and adult hearts. A. Gross morphology of hearts of 2-month-old control and miR-17–92 \( \text{TG}^{\text{MHC}} \) mouse (top). Hematoxylin and eosin staining of transverse sections of 2-month-old control and miR-17–92 \( \text{TG}^{\text{MHC}} \) hearts (bottom). Bars=1 mm. B. Heart weight (HW) to body weight (BW) ratios of 1-, 2-, and 4-month-old, wild-type, heterozygote, and homozygote miR-17–92 \( \text{TG}^{\text{MHC}} \) mice. n=5 for each group. C. Echocardiography analyses of cardiac function of 40-day-old miR-17–92 \( \text{TG}^{\text{MHC}} \) mice and their control littermates. n=3 for each group. D. Morphology of freshly isolated adult cardiomyocytes from hearts of miR-17–92 \( \text{TG}^{\text{MHC}} \) and control mice. ACTN1 marks rod-shaped cardiomyocytes (bottom). Bars=250 μm. E. Quantification of total isolated adult cardiomyocytes from 2-month-old hearts of miR-17–92 \( \text{TG}^{\text{MHC}} \) and control mice. n=3 for each genetic group. F. Distribution of isolated adult cardiomyocytes with 1, 2, or 3 and more nuclei from 2-month-old hearts of miR-17–92 \( \text{TG}^{\text{MHC}} \) and control mice. G. Immunohistochemistry of pH3 on transverse sections of 15-day-old control and miR-17–92 \( \text{TG}^{\text{MHC}} \) hearts. ACTN1 marks cardiomyocytes and DAPI labels nuclei. Wheat germ agglutinin (WGA) staining marks cell surface (white). White boxes are enlarged in insets. H. Quantification of percentages of pH3-positive cardiomyocytes of 15-day-old control and miR-17–92 \( \text{TG}^{\text{MHC}} \) hearts. n=4 for each genetic group. I. Immunohistochemistry of 5-ethylidene-2-deoxyuridine (EdU) incorporation on transverse sections of 15-day-old control and miR-17–92 \( \text{TG}^{\text{MHC}} \) hearts. Cardiac troponin T (cTNT) marks cardiomyocytes and DAPI labels nuclei. WGA staining marks cell surface (white). J. Quantification of percentages of EdU-positive cardiomyocytes of 15-day-old control and miR-17–92 \( \text{TG}^{\text{MHC}} \) hearts. n=3 for each genetic group. **P<0.01.

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We examined whether overexpression of miR-17–92 in adult cardiomyocytes could induce cell proliferation. Using EdU incorporation assay, we found marked increase in the EdU incorporation in the cardiomyocytes of 6-month-old miR-17–92 TGMerCreMer hearts (Figure 4B; Online Figure XVI). We isolated cardiomyocytes from the hearts of miR-17–92 TGMerCreMer and control mice after tamoxifen administration. The arrow points to EdU-positive signal in cardiomyocytes. ACTN1 marks cardiomyocytes; DAPI labels nuclei (bottom; bar=20 μm). C, Quantification of total isolated adult cardiomyocytes from hearts of 6-month-old miR-17–92 TGMerCreMer and control mice after tamoxifen administration. n=3 for each group. D, Immunohistochemistry of pH3 on sagittal sections of 6-month-old miR-17–92 TGMerCreMer and control hearts after tamoxifen administration. The arrow points to pH3-positive signal in cardiomyocytes. cTNT marks cardiomyocytes; DAPI labels nuclei. Bars=11 μm. E, Quantification of percentages of pH3-positive cardiomyocytes in 6-month-old miR-17–92 TGMerCreMer and control hearts after tamoxifen administration. n=3 for control group. n=6 for miR-17–92 TGMerCreMer group. **P<0.01 between genetic groups. F, Echocardiography analyses of cardiac function of miR-17–92 TGMerCreMer mice and their control littermates after myocardial infarction (MI) and tamoxifen administration. n=10 for miR-17–92 TGMerCreMer group and 8 for control group. G, Representative images of series of transverse sections of 6-month-old miR-17–92 TGMerCreMer and control mice after MI and tamoxifen administration. Sirius red/fast green collagen staining marks myocardium (green) and scar (red). Bars=1 mm. H, Quantification of the size of scar in the hearts of miR-17–92 TGMerCreMer (n=4) and control mice (n=5) after MI and tamoxifen administration. *P<0.05 between genetic groups. I, Immunohistochemistry of EdU on sagittal sections of 6-month-old miR-17–92 TGMerCreMer and control mice after tamoxifen administration. White boxes are enlarged in insets and arrows point to EdU-positive signal. ACTN1 marks cardiomyocytes; DAPI labels nuclei. Bars=50 μm. FS indicates fractional shortening.
and control mice to determine the total numbers of cardiomyocytes and found that overexpression of miR-17–92 substantially increased total cardiomyocyte number in adult hearts (Figure 4C). Quantitative measurement of the size of isolated adult cardiomyocytes showed that the size of cardiomyocytes was reduced in the heart of miR-17–92 TGMerCreMer mice (Online Figure XVII). To further confirm the cardiomyocyte proliferation in the miR-17–92 TGMerCreMer hearts, we used pH3 to mark proliferating cardiomyocytes and found that overexpression of miR-17–92 is sufficient to enhance cardiomyocyte proliferation in adult hearts (Figure 4D and 4E).

To test whether this cluster of miRNAs is involved in the regulation of cardiomyocyte proliferation and cardiac repair in response to injury, we introduced myocardial infarction (MI) by coronary artery occlusion (Online Figure XVIII). MI resulted in massive cardiomyocyte death, cardiac hypertrophy, fibrosis, and cardiac remodeling. We found that overexpression of miR-17–92 in adult cardiomyocytes modestly protected the heart from MI-induced injury (Figure 4F). There is an increase of cardiac function, as measured by echocardiography and documented as fractional shortening, in miR-17–92 TGMerCreMer mice when compared with controls (Figure 4F and 4G; Online Table III). Quantification confirmed the decrease in the size of scar in the hearts of miR-17–92 TGMerCreMer mice after MI (Figure 4H). Using EdU incorporation assay, we found marked increase in the EdU incorporation in the cardiomyocytes of border zone of miR-17–92 TGMerCreMer hearts (Figure 4I). We asked whether apoptosis is affected in miR-17–92 transgenic hearts. Terminal deoxynucleotidyl transferase dUTP nick end labeling assays showed no difference in apoptosis between miR-17–92 transgenic and control hearts in response to MI injury (Online Figure XIX). Similarly, we treated both miR-17–92 transgenic and control mice with doxorubicin, a cancer drug which causes heart failure as side effect, to induce stress. We found that overexpression of miR-17–92 modestly induces cardiomyocyte proliferation on doxorubicin treatment.

miR-17–92 Is Sufficient to Induce Neonatal Cardiomyocyte Proliferation In Vitro

To test whether members of this cluster of miRNAs play a similar role in cardiomyocyte proliferation in vitro, we transfected neonatal rat cardiomyocytes with mimics or inhibitors of each member of the miR-17–92 cluster and assayed cardiomyocyte proliferation.17,18 We used cardiomyocytes isolated from postnatal day 1 (P1) hearts, in which cardiomyocytes were still undergoing active proliferation.7 After it was transfected with miR-17–92 mimics (Figure 5A) or inhibitors (Figure 5B), the cell culture was then incubated with EdU to label DNA synthesis and cell proliferation. Indeed, we found that miR-17–92 mimics, especially miR-19a/b family, potently induce cardiomyocyte proliferation (Figure 5A and 5C). Conversely, inhibition of members of the miR-17–92 cluster, in particular miR-19a/b, substantially reduced cardiomyocyte proliferation, evidenced by the decrease of EdU signal and the reduction of total cardiomyocyte numbers when compared with controls (Figure 5B and 5D). We applied aurora B staining to detect cytokinesis in these cells (Figure 5E). Quantitative analysis confirmed a significant increase in aurora B signal after miR-19a/b treatment (Figure 5F). Furthermore, we treated postnatal day 4 (P4) cardiomyocytes, in which cell proliferation starts to diminish, with miR-17–92 mimics, and found that miR-17–92 mimics, miR-19a/b in particular, significantly induced EdU incorporation (Online Figure XXA). This observation is further confirmed by quantitative analyses (Online Figure XXB). We examined the expression of cyclin-dependent kinase 1, a highly conserved serine/threonine kinase involved in cell-cycle progression,19 in miR-17–92 mimic-treated cardiomyocytes. Consistent with the view that miR-17–92 induced cardiomyocyte proliferation, we found that miR-17–92 induced the expression of cyclin-dependent kinase 1 in cardiomyocytes (Figure 5G).

Next, we isolated neonatal cardiomyocytes from miR-17–92 KI mice. Cultured cardiomyocytes were infected with Ad-cTNT-Cre to induce the overexpression of miR-17–92 (Figure 5H). We found that overexpression of miR-17–92 in mouse neonatal cardiomyocytes, but not the cells treated with the control Ad-lacZ, dramatically enhanced the incorporation of EdU, indicating an increase in cardiomyocyte proliferation (Figure 5H and 5I). Together, our data demonstrate that miR-17–92 mimics induce, whereas miR-17–92 inhibitors reduce cardiomyocyte proliferation in vitro and ex vivo.

miR-17–92 Represses Phosphatase and Tensin Homolog to Induce Cardiomyocyte Proliferation

We tested the expression of putative miR-17–92 targets that are known to play a role in cell proliferation.28 We reasoned that the expression of these targets should be inversely correlated with the expression of miR-17–92, which is decreased in the hearts of miR-17–92 TG mice and increased in the hearts of miR-17–92 KO mice. Indeed, we found that the expression of several targets was elevated in the hearts of miR-17–92 KO mice (Figure 5J) and repressed in the hearts of miR-17–92 TG mice (Figure 5K). We focused on phosphatase and tensin homolog (PTEN), a tumor suppressor and a member of family of protein tyrosine phosphatases,21–23 which was most dramatically altered in the hearts of miR-17–92 transgenic and mutant mice (Figure 5J and 5K). PTEN was reported a direct target of miR-19a/b,24 the most potent member of the miR-17–92 cluster to induce tumor growth24 and to promote cardiomyocyte proliferation in our study (Figure 5A–5D). Deletion of PTEN led to axon regeneration in central neural system, further highlighting the role of PTEN in cell proliferation and regeneration.25 We asked whether PTEN could mediate the function of miR-19a/b in cardiomyocyte proliferation, and more specifically, we tested whether overexpression of PTEN could suppress miR-19a/b-induced cardiomyocyte proliferation. We overexpressed PTEN in neonatal rat cardiomyocyte, using a modified RNA approach.26 We achieved dose-dependent overexpression of PTEN protein in transfected cells (Online Figure XXI). Overexpression of PTEN completely abolished miR-19a/b-induced cardiomyocyte proliferation (Figure 5L and 5M).

Discussion

In this report, our genetic studies using miR-17–92 knock-out and transgenic mice, together with results of in vitro cell culture, demonstrated that members of the miR-17–92 cluster are required for and sufficient to induce cardiomyocyte
proliferation. We found that PTEN is one of the miR-17–92 targets that mediate the function of this cluster of miRNAs, at least in vitro in cultured cardiomyocytes, to regulate cardiomyocyte proliferation.

In sharp contrast to embryonic cardiomyocytes, which exhibit strong proliferative activity, the rate of cardiomyocyte proliferation and turnover in adult hearts is very low, and it is generally conceived that adult hearts retain very limited (if any) potential for regeneration. As a consequence, the intrinsic renewal rate is insufficient to reverse cardiomyocyte loss and to restore cardiac function under pathophysiological conditions.27,28 Numerous attempts have been made to overcome...

Figure 5. miR-17–92 regulates cardiomyocyte proliferation and represses the expression and function of phosphatase and tensin homolog (PTEN). A, Primary neonatal (P1) rat cardiomyocytes were transfected with indicated microRNA (miRNA) mimics or control mimics and cells were incubated with 5-ethyl-2′-deoxyuridine (EdU; 10 μmol/L). One day later, cultures were fixed and stained with antibodies for EdU. cTNT marks cardiomyocytes. DAPI stains nuclei. Arrowheads point to EdU-positive cardiomyocytes. Bars=70 μm. B, P1 rat cardiomyocytes were transfected with indicated miRNA inhibitors or control inhibitor, and cells were incubated with EdU. One day later, cultures were fixed and stained with antibodies for EdU. cTNT marks cardiomyocytes. DAPI stains nuclei. Arrowheads point to EdU-positive cardiomyocytes. Bars=70 μm. C, Quantification of percentages of EdU+ cardiomyocytes in cultured neonatal rat cardiomyocytes after being treated with miRNA mimics or control. D, Quantification of percentages of EdU+ cardiomyocytes in cultured neonatal rat cardiomyocytes after being treated with miRNA inhibitors or control. E, P1 rat cardiomyocytes were transfected with miR-19a/b mimics or control mimic. One day later, cultures were fixed and stained with antibodies for aurora B (green). cTNT (red) marks cardiomyocytes. DAPI (blue) stains nuclei. Arrowheads point to aurora B positive signal. Bars=50 μm. F, Quantification of percentages of aurora B positive cardiomyocytes in cultured neonatal rat cardiomyocytes after being treated with miR-19a/b mimics or control. *P<0.01. G, Quantification RT-PCR (qPCR) analyses of CDK1 expression in cultured neonatal rat cardiomyocytes after being treated with miRNA mimics or mimic control. H, Neonatal mouse cardiomyocytes were isolated from miR-17–92TG/TG mice and cultured in the presence of serum-free medium and EdU (10 μmol/L). After transduced with Ad-cTNT-Cre (or Ad-lacZ in control), cell proliferation is determined by EdU incorporation. cTNT marks cardiomyocytes. DAPI stains nuclei. Arrows point to EdU-positive cardiomyocytes. Bars=70 μm. I, Quantification of percentages of EdU+ cardiomyocytes in cultured neonatal mouse cardiomyocytes isolated from miR-17–92TG/TG and control mice. J, qPCR analyses of the expression of putative miR-17–92 targets the hearts of 20-day-old miR-17–92 KO and control mice. K, qPCR analyses of the expression of putative miR-17–92 targets the hearts of 15-day-old miR-17–92 TGMHC and control mice. L, P1 rat cardiomyocytes were transfected with miR-19a/b mimics, control mimics, modify RNA for PTEN (modi-PTEN), or both miR-19a/b mimics and modi-PTEN and cells were incubated with EdU. One day later, cultures were fixed and stained with antibodies for EdU (green). cTNT (red) marks cardiomyocytes. DAPI (blue) stains nuclei. Arrowheads point to EdU-positive cardiomyocytes. Bars=150 μm. M, Quantification of percentages of EdU+ cardiomyocytes in cultured neonatal mouse cardiomyocytes after being treated with miR-19a/b mimics, control mimics, modify RNA for PTEN (modi-PTEN), or both miR-19a/b mimics and modi-PTEN. **P<0.01.
this hurdle, and one of the approaches is to induce cell-cycle activity in the surviving cardiomyocytes. Previous reports indicate that targeted overexpression of members of the cyclin D, cyclin D2 in particular, is sufficient to induce cardiomyocyte cell-cycle activity in adult hearts, resulting in improved cardiac function on myocardial injury. Despite the fact that we know the critical role of the cell-cycle regulators in cardiomyocyte proliferation, the molecular pathways that diminish adult cardiomyocyte proliferation remain largely unknown. Our studies reported here, using genetic approaches, demonstrated that miRNAs are previously unidentified regulators of cardiomyocyte proliferation. Interestingly, a recent study reported that family of miR-15 inhibits cardiomyocyte proliferation in a manner that inhibition of it increases myocyte proliferation in adult hearts after myocardial infarction. The potential of applying miRNAs to reconstitute lost cardiomyocytes in injured hearts could be of considerable therapeutic value for human cardiovascular disease.

Whereas miR-17–92 is required for normal animal development, cell proliferation, and tumor growth, individual members of the miR-17–92 cluster seem to possess distinct functions. For example, miR-17 was shown to reduce cell proliferation. Transgenic overexpression of miR-17 resulted in growth retardation in animals. It was recently reported that overexpression of miR-92a blocks angiogenesis in vitro and in vivo. Additional studies found that miR-19 is a key component of the miR-17–92 cluster to induce cell proliferation and oncogenic growth. Our results demonstrate that miR-19a/miR-19b are sufficient and required for neonatal cardiomyocyte proliferation in vitro, consistent with the view that miR-19 is a key component of the miR-17–92 cluster in controlling cell proliferation. In the future, it will be important to determine whether miR-19a/miR-19b promote cardiomyocyte proliferation in vivo, in particular in postmitotic adult cardiomyocytes.

We found that PTEN is a functional target of miR-17–92 cluster. More specifically, we showed that PTEN mediates the function of miR-19a/b in the regulation of cardiomyocyte proliferation. PTEN is a tumor suppressor, which was previously shown to be repressed by miR-17–92 during tumor growth. Genetic deletion of PTEN in the heart led to hypertrophic growth. Additional investigation revealed that loss of PTEN protects the heart from the development of pathological hypertrophy and heart failure under biomechanical stress, further highlighting the critical involvement of PTEN in cardiac function and disease. Our studies link the function of miRNAs and PTEN in cardiomyocyte proliferation. Future studies will be important to illustrate how the miR-17–92–PTEN axis participates in cardiac regeneration.

In summary, our findings uncovered that miR-17–92 plays a key role in the regulation of cardiomyocyte proliferation in embryonic, postnatal, and adult hearts, implying the therapeutic potential of miR-17–92 in human cardiomyocyte proliferation, cardiac repair, regeneration, and related disorders.

Acknowledgments

We thank Dr Bernhard Kuhn for advice and stimulating discussion, and Dr John Mably for critical reading of the article and discussion.
Loss-of-function studies show that miR-17–92 is required for cardiomyocyte proliferation and resulted in an increase of cardiomyocyte numbers in transgenic mice. Circ Res. 2005;96:110–118.


Novelty and Significance

What is Known?

- Loss of cardiomyocytes in adult hearts is one of the most common causes of heart failure.
- Adult mammalian hearts have limited capacity to regenerate.
- microRNAs are a class of small noncoding RNAs that regulate a variety of biological processes.
- It has been shown before that miR-17–92 regulates cell proliferation and tumorigenesis.

What New Information Does This Article Contribute?

- miR-17–92 is a key regulator of cardiomyocyte proliferation.
- Loss-of-function studies show that miR-17–92 is required for cardiomyocyte proliferation.
- Gain-of-function studies demonstrate that miR-17–92 is sufficient to induce cardiomyocyte proliferation in embryonic, postnatal, and adult hearts.

- Phosphatase and tensin homolog is one of the key targets of miR-17–92, which mediates cardiomyocyte proliferation.

The adult heart is primarily composed of terminally differentiated, mature cardiomyocytes that exit the cell cycle. The molecular mechanism that controls the cardiomyocyte proliferation and cardiac regeneration is not fully understood. We hypothesized that microRNAs are key regulators of cardiomyocyte proliferation. Herein we report that genetic deletion of miR-17–92 in cardiomyocytes reduced cardiomyocyte proliferation and total numbers of cardiomyocytes in the heart. Conversely, transgenic overexpression of miR-17–92 in cardiomyocytes stimulated cardiomyocyte proliferation and resulted in an increase of cardiomyocyte number. We found that miR-17–92 directly inhibited phosphatase and tensin homolog, a tumor suppressor, in cardiomyocytes. These findings suggest that miR-17–92 could be a potential therapeutic target for myocardial infarction and heart failure.
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Circ Res. 2013;112:1557-1566; originally published online April 10, 2013;
doi: 10.1161/CIRCRESAHA.112.300658

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supporting Information

Materials and Methods

All experiments with mice were performed according to protocols approved by the Institutional Animal Care and Use Committees of Boston Children's Hospital. miR-17-92\textsuperscript{flox/flox}, miR-17-92\textsuperscript{TG/TG}, Nkx2-5\textsuperscript{Cre/+}, aMHC-Cre\textsuperscript{4}, aMHC-MerCreMer\textsuperscript{5} were described previously.

Cardiac-specific knockout of miR-17-92 cluster in mice
The miR-17-92\textsuperscript{flox/+} mice harbor an allele of loxP-flanked miR-17-92 cluster. miR-17-92\textsuperscript{flox/flox} mice were crossed with Nkx2-5\textsuperscript{Cre/+} mice, in which the expression of Cre recombinase is controlled by the endogenous promoter of cardiac-specific marker gene, Nkx2-5, to generate the miR-17-92\textsuperscript{F/+};Nkx2-5\textsuperscript{Cre/+} offsprings. The miR-17-92\textsuperscript{flox/+};Nkx2-5\textsuperscript{Cre/+} mice were then crossed back to miR-17-92\textsuperscript{flox/flox} mice to obtain the miR-17-92 conditional (cardiac-specific) knockout (cKO) mice (miR-17-92\textsuperscript{flox/flox};Nkx2-5\textsuperscript{Cre/+}).

Cardiac-specific overexpression of miR-17-92 cluster in mice
miR-17-92\textsuperscript{TG/+} mice harbor the miR-17-92 transgene targeted to the Gt(ROSA)26Sor locus. The miR-17-92 transgene has a loxP-flanked Neo-STOP cassette preventing transcription of the downstream human miR-17-92 cluster. When bred to mice that express Cre recombinase, the resulting offspring will have the STOP cassette deleted in the Cre-expressing tissue resulting in ectopic expression of the miR-17-92 cluster. miR-17-92\textsuperscript{TG/+} mice were crossed with Nkx2-5\textsuperscript{Cre/+} mice and αMHC-Cre mice, respectively, to obtain the miR-17-92\textsuperscript{TG/+};Nkx2-5\textsuperscript{Cre/+} offsprings and miR-17-92\textsuperscript{TG/+};αMHC-Cre offsprings. miR-17-92 cluster is cardiac-specific overexpressed in these offsprings. miR-17-92\textsuperscript{TG/+} mice were crossed with αMHC-MerCreMer (MerCreMer) to obtain miR-17-92\textsuperscript{TG/+};MerCreMer offsprings for inducible cardiac-specific overexpression of miR-17-92 cluster. In order to achieve the induction of overexpression, tamoxifen was administrated in these mice. EdU was administered intraperitoneally at 5 mg per g of body weight (adult), 6 times for continuous day.

Myocardial infarction
Myocardial infarction (MI) is induced by ligation of left anterior descending coronary artery. Two months old miR-17-92-TG\textsuperscript{MerCreMer} mice and control mice were performed coronary artery ligation or sham surgery. For surgery, mice are...
anesthetized with isoflurane (3% isoflurane for induction, 2% isoflurane for maintenance). The chest is shaved and cleaned with alcohol. A suture is placed around the front upper incisors and pulled taut so that the neck was slightly extended. The tongue is retracted and held with forceps, and a 20-G catheter is inserted into the trachea. The catheter is then attached to the mouse ventilator via a Y-shaped connector. Ventilation is performed with a tidal volume of 225 µl for a 25 g mouse and a respiratory rate of 130 breaths per min. 100% oxygen is provided to the inflow of the ventilator. The chest is opened through a left parasternal incision, and the heart exposed at the left 3rd-4th intercostal space. Chest retractor is applied to facilitate the view. The pericardium is opened, and ligations made on the left anterior descending coronary artery (LAD) using 8-0 silk sutures (Ethicon). The lungs are slightly overinflated to assist in removal of air in the pleural cavity.

**Measurement of cardiac function by echocardiography**

Echocardiographic measurements were performed on mice using a Visual Sonics Vevo® 2100 Imaging System (Visual Sonics, Toronto, Canada) with a 40 MHz MicroScan transducer (model MS-550D). Mice were anesthetized with isoflurane (2.5% isoflurane for induction and 0.5% for maintenance). Heart rate and left ventricular (LV) dimensions, including diastolic and systolic wall thicknesses, LV end-diastolic and end-systolic chamber dimensions were measured from 2-D short-axis under M-mode tracings at the level of the papillary muscle. LV mass and functional parameters such as percentage of fractional shortening (FS%) and left ventricular volume were calculated using the above primary measurements and accompanying software.

**Modified RNA (ModRNA)**

The modified RNA (ModRNA) experiment was performed essentially as described\(^6\). Briefly, the “universal” ModRNA backbone was generated by modifying previously described pcDNA3.3-TOPO-cMyc ORF plasmid containing T7 promoter and optimized 5’ and 3’ untranslated regions (pcDNA3.3-TOPO-T7-5’UTR-cMyc-3’UTR,). The pcDNA3.3-TOPO backbone with 5’/3’UTRs (pcDNA3.3-TOPO-5’3’UTRs) was amplified by long-range PCR (PrimeStar high fidelity DNA polymerase, Takara). Two unique restriction sites for AscI and NheI were incorporated into the PCR products, in order to create the 5’ and 3’ sticky ends. The ORF of PTEN cDNA was PCR amplified and first cloned into the pcDNA3-N-Flag vector. The fusion Flag-PTEN ORF was then amplified using forward primer containing Nhel site and reverse primer with AscI site, and sub-cloned into the pcDNA3.3-TOPO-5’ and 3’UTRs backbone to generate the pcDNA3.3-TOPO-T7-5’UTR-Flag-PTEN-3’UTR.
Cardiomyocyte isolation and culture
Neonatal rat and mouse cardiomyocytes were prepared as previously described\(^7\). Briefly, Neonatal rat and mouse cardiomyocytes were isolated by enzymatic disassociation of one day-old or four day-old (P1 or P4) neonate hearts with the Neonatal Cardiomyocyte Isolation Kit (Cellutron, Baltimore MD). Cardiomyocytes were plated differentially for 2 hours to remove fibroblasts. Cells were plated on 1% gelatin coated plates in medium containing 10% horse serum and 5% fetal calf serum (FCS). After 24 hours of plating, cells were changed into serum-free medium overnight. Then, 100 nM of microRNA mimic duplex or 200 nM microRNA hairpin inhibitors of miR-17-92 cluster members and negative control oligonucleotide (Dharmacon) were transfected into cardiomyocyte by using Lipofectamine RNAiMAX (Invitrogen) transfection reagent. After 6 hours transfection, the cultures were changed to serum free medium for mimic experiments and changed to 1% FCS medium for inhibitor experiments. EdU (5-ethynyl-2'-deoxyuridine, Invitrogen) was added, 24 hours later, cells were fixed and harvested for Q-PCR analyses and immunohistochemistry analyses.

Adult mouse cardiomyocyte were isolated using a previously described procedure\(^8\) with minor modifications. Briefly, following perfusion and digestion of the heart with collagenase II (Worthington Biochemical Corp, Lakewood, NJ), dissociated cells (myocytes and non-myocytes) were sedimented by gravity. The bottom layer is rich in adult cardiomyocyte for cell counting and staining.

Quantitative RT-PCR
Total RNAs were isolated using Trizol Reagent (Invitrogen) from cell or tissue samples. For quantitative RT-PCR detecting the expression of protein-coding gene, 2.0 µg RNA samples were reverse-transcribed to cDNA using random hexamers and MMLV reverse transcriptase (Invitrogen) in 20 µl reaction system. In each analysis, 0.1 µl cDNA pool was used for quantitative PCR. For quantitative RT-PCR detecting the expression of miRNAs, 10 ng RNA samples were reverse-transcribed to cDNA by using TaqMan® MicroRNA Reverse Transcription Kit (ABI). In each analysis, 1.5 µl cDNA pool and TaqMan® MicroRNA Assays were used for quantitative PCR. For target gene expression, Real time PCR was performed with SYBR Green detection. All qPCR experiments were performed on the Applied Biosystems 7500 Real-Time PCR System.

Histology and immunostaining
Mouse hearts were dissected out, rinsed with PBS and fixed in 4% paraformaldehyde
(pH 8.0) overnight. After dehydration through a series of ethanol baths, samples were embedded in paraffin wax according to standard laboratory procedures. Sections of 5 µm were stained with Haematoxylin and Eosin (H&E) for routine histological examination with light microscope. To determine infarct size, hearts were fixed in 4% PFA, dehydrated and embedded in paraffin. Then the embedded paraffin blocks were cut through from apex to base. The first 10 sections (10 µm thickness each) of every 100 sections were used. Sections were stained with Sirius Red-Fast Green. Infarct size was calculated according to the formula: \[ \text{length of coronal infarct perimeter (epicardial and endocardial)} / \text{total left ventricle coronal perimeter (epicardial and endocardial)} \] \times 100^9.

Immunofluorescence was performed on paraformaldehyde (PFA)-fixed, paraffin-embedded heart sections. After de-paraffinization, re-hydratation and heat-induced epitope retrieval, sections were incubated with antibodies. To identify mitosis and cytokinesis, we used rabbit anti phospho-Histone H3 (pH3, 1:400, Millipore, cat # 06-570) and rabbit anti Aurora B (1:50, Abcam cat # ab2254) antibodies, respectively. Mouse anti a-actinin (ACTN1, 1:250, Abcam, cat # ab9465) and mouse anti-cardiac troponin T (cTNT, 1:500, a generous gift from Dr. Jim Lin of University of Iowa) were used to mark the cardiomyocytes. Nuclei were visualized with 4', 6'-diamidino-phenylindole (DAPI, Invitrogen, 1:5000). Goat anti-rabbit AlexaFluor 488 and goat anti-mouse AlexaFluor 594 secondary antibody (1:400, Invitrogen) were used to be visualized under microscopy. Quantitative data were obtained by measuring co-localization of DAPI (nuclear staining) with pH3 in the cardiomyocyte area and co-localization of aurora B in the cardiomyocyte area. EdU was detected with Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Cat # C10337, Invitrogen). Imaging was performed on a Nikon TE2000 epifluorescent microscope with deconvolution (Volocity; Perkin-Elmer) or on an Olympus FV1000 confocal (FV1000, Olympus).

Immunostaining was also performed on freshly isolated adult mouse cardiomyocytes and cultured neonatal mouse/rat cardiomyocytes. For cardiomyocyte staining, cells were fixed with 3.7% PFA and then permeabilized with 0.5% Triton/PBS. Cells were blocked in 5% goat serum and then incubated with indicated antibodies. To verify intact cardiomyocytes after isolation from adult mouse hearts, we stained desmosomes with a rabbit anti pan-cadherin (Sigma, Cat# C3678, 1:500) antibody as described 10. Imaging was performed on a Nikon TE2000 epifluorescent microscope with deconvolution (Volocity; Perkin-Elmer) or on an Olympus FV1000 confocal (FV1000, Olympus).

**TUNEL assays**
Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assays were performed on paraffin sections to detect apoptotic cardiomyocytes ApopTag® Plus In Situ Apoptosis Fluorescein Detection Kit was used (Cat # S7111 Millipore) according to the manufacturer’s procedure. Positive control slides containing unstained rat mammary glands were performed TUNEL assay as well. The cardiomyocytes were counter-stained with cTNT and DAPI.

Western Blot Analysis
Samples from cultured cells were homogenized and incubated in Cell Extraction Buffer (Invitrogen) with protease inhibitors cocktail (Sigma, Saint Louise, MO) and 1mM PMSF (phenylmethylsulfonyl Fluoride) on ice for 15 minutes. The lysates were centrifuged at 13,000g for 10 minutes at 4°C. Samples were mixed with Laemmlli buffer containing 5% β-mercaptoethanol and were evenly loaded onto SDS-PAGE gels. Proteins were transferred to PVDF membranes. Membranes were incubated overnight at 4°C with primary antibodies, Flag (1:1000, sigma), β-tubulin (1:10,000, Sigma) overnight at 4°C and then washed three times with TBST buffer before adding secondary antibody in 5% BSA. Specific protein bands were visualized by using ECL (Invitrogen) reagents.

Statistics
Values are reported as means ± SEM unless indicated otherwise. The 2-tailed Mann-Whitney U test was used for comparing 2 means (Prism, GraphPad). Values of P<0.05 were considered statistically significant.

Supporting Information References:
4. Oka T, Maillet M, Watt AJ, Schwartz RJ, Aronow BJ, Duncan SA, Molkentin JD. Cardiac-specific deletion of Gata4 reveals its requirement for hypertrophy,


Online Figure I

(a) Strategy of cardiac-specific knockout of miR-17-92 cluster in vivo. (b) Genotyping results of weaning age mice from intercrossing of miR-17-92\(^{\text{floxfloxflo}}\) and miR-17-92\(^{\text{floxfloxflo}}\);Nkx2.5\(^{\text{Cre/+}}\) mice.

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<th>miR17-92(^{\text{floxflo}});Nkx-Cre</th>
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Online Figure I. Generation of cardiac-specific miR-17-92 mutant mice.
Online Figure II

Reduced expression of members of the miR-17-92 cluster in the hearts of cardiac-specific knockout mice. The expression of members of miR-17-92 cluster in 3-week-old heart samples of miR-17-92^{flox/flox}, miR-17-92^{flox/+};Nkx2-5-Cre^{+/-}, and miR-17-92^{flox/flox};Nkx2-5-Cre^{++} mice was determined by quantitative RT-PCR. N of each genotype was indicated.
Online Figure III. Cardiac-specific knockout of miR-17-92 reduced cell proliferation in mouse heart. Immunohistochemistry of sagittal sections of hearts from 2 day-old (P2) wild type, heterozygote and mutant miR-17-92 cardiac-specific KO mice detected the Phospho-Histone H3 positive cells (green dots). Bar=100 μm.
Online Figure IV. Cardiac-specific knockout of miR-17-92 did not induce abnormal cell apoptosis in the heart. Heart sections from 5 days and 8 months old miR-17-92 cardiac-specific KO mice (miR-17-92\(^{fl/fl}\);Nkx2.5-Cre) and their control littermates (miR-17-92\(^{fl/fl}\)) were performed TUNEL staining to detect cell apoptosis. The heart sections were also co-stained with antibodies detecting cTNT for cardiomyocytes (red) and DAPI for nuclei (blue). TUNEL staining positive control section from ApopTag Peroxidase In Situ Apoptosis Detection Kit was included. No abnormal positive TUNEL nuclei (bright green dots) were observed from heart section of both genotypes. Bars=32 μm (5days) and Bars=16 μm (8 months).
Online Figure V

(a) Haematoxylin and Eosin (H&E) staining of transverse sections of hearts from 10 months old wild type, heterozygote and mutant miR-17-92-KO mice. Bar = 1 mm. (b) Cross section area of cardiomyocyte in adult hearts of miR-17-92^{flox/flox} and miR-17-92^{flox/flox};Nkx2.5^{Cre/+} mice was measured. More than 2000 cardiomyocytes were measured from 4 hearts of each genotype.

Online Figure V. Increased cardiomyocyte size in miR-17-92 cardiac-specific knockout mice. (a) Haematoxylin and Eosin (H&E) staining of transverse sections of hearts from 10 months old wild type, heterozygote and mutant miR-17-92-KO mice. Bar = 1 mm. (b) Cross section area of cardiomyocyte in adult hearts of miR-17-92^{flox/flox} and miR-17-92^{flox/flox};Nkx2.5^{Cre/+} mice was measured. More than 2000 cardiomyocytes were measured from 4 hearts of each genotype.
Online Figure VI. Reduced cardiac function in cardiac-specific miR-17-92 mutant mice. Echocardiography of cardiac function of 6-month-old miR-17-92\textsuperscript{flox/flox};Nkx2-5\textsuperscript{Cre/+} (cKO) mice and their control littermates. N of each genotype was indicated. *: P<0.05; **: P<0.01. FS: Fractional shortening; LVID\textsubscript{d}: Left ventricular end diastolic internal dimension; LVID\textsubscript{s}: Left ventricular end systolic internal dimension; LVPW\textsubscript{d}: Left ventricular end diastolic posterior wall dimension; LVPW\textsubscript{s}: Left ventricular end systolic posterior wall dimension; LV Vol\textsubscript{d}: Left ventricular end diastolic volume.
Online Figure VII

(a) Strategy of cardiac-specific overexpression of miR-17-92 cluster in vivo. (b) Genotyping results of weaning age mice from intercrossing of miR-17-92\(^{TG/TG}\) and miR-17-92\(^{TG/+}\);Nkx2-5\(^{Cre/+}\) mice.

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<td>miR17-92(^{TG/+});Nkx-Cre</td>
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<td>miR17-92(^{TG/TG})</td>
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<td>21.9%</td>
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<td>miR17-92(^{TG/TG});Nkx-Cre</td>
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<tr>
<td><strong>Total</strong></td>
<td>146</td>
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Online Figure VIII

Cardiac-specific overexpression of miR-17-92 increased cell proliferation in embryonic and neonatal mouse hearts. Immunohistochemistry of sagittal sections of hearts from embryonic day (E) 16.5 (a) and transverse sections of hearts from postnatal day 4 (P4) (b) wild type and mutant miR-17-92 cardiac-specific overexpression mice detected the Phospho-Histone H3 positive cells (green dots). Bar=500 μm.
Online Figure IX. Increased expression of members of the miR-17-92 cluster in the hearts of miR-17-92 transgenic mice. The expression of members in miR-17-92 cluster in 2-month-old heart samples of miR-17-92<sup>TG/TG</sup>, miR-17-92<sup>TG/+;αMHC-Cre</sup> and miR-17-92<sup>TG/TG;αMHC-Cre</sup> mice was determined by quantitative RT-PCR. N of each genotype was indicated.
Online Figure X

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<th>Genotypes</th>
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<th>miR17-92&lt;sup&gt;TG/+&lt;/sup&gt;, αMHC-Cre</th>
<th>miR17-92&lt;sup&gt;TG/TG&lt;/sup&gt;</th>
<th>miR17-92&lt;sup&gt;TG/TG&lt;/sup&gt;, αMHC-Cre</th>
<th>Total</th>
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<td>Number</td>
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<td>59</td>
<td>58</td>
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<td>Percentage</td>
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<td>27.8%</td>
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Online Figure X. Generation of transgenic mice to overexpress miR-17-92 in the heart in postnatal stage. Genotyping results of weaning age mice from intercrossing of miR-17-92<sup>TG/TG</sup> and miR-17-92<sup>TG/+</sup>,αMHC-Cre mice.
Online Figure XI. Cardiac-specific overexpression of miR-17-92 does not change the size of cardiomyocyte. (a) Transverse sections from hearts of 2-month-old miR-17-92\textsuperscript{TG/TG} and miR-17-92\textsuperscript{TG/TG};\textalpha\textsubscript{MHC-Cre} mice were stained with Wheat Germ Agglutinin (WGA) to show the cross section area of cardiomyocyte. Measurement of the cross section area of cardiomyocyte show no significant (ns) difference between two genotypes. (b) Similar staining and measurement were also performed on 4-month-old heart samples and no significant difference is found between two genotypes. More than 2000 cardiomyocytes were measured from at least 3 hearts of each genotype. Bar=100 μm.
Online Figure XII.

Online Figure XII. Morphology of freshly isolated adult cardiomyocytes from hearts of miR-17-92-TG<sup>MHC</sup> and control mice. Phase contrast images (upper panels) show the morphology of rod shaped cardiomyocytes. DAPI staining marks nuclei (middle panels). Merged images were shown in the lower panels. Arrows point to elongating and dividing nuclei. Bars = 250 µm.
Online Figure XIII

miR-17-92$^{TG/TG}$

miR-17-92$^{TG/TG}$
Online Figure XIII. Cardiac-specific overexpression of miR-17-92 induced cardiomyocyte proliferation in postnatal mouse hearts. (a) Representative image (left panels) of immunohistochemistry showed the Phospho-Histone H3 positive non-cardiomyocytes in 15-day-old control heart sample (miR-17-92\textsuperscript{TG/TG}). Higher magnification images in the boxed region are shown in right panels. (b) Representative image (left panels) of immunohistochemistry showed the Phospho-Histone H3 positive cardiomyocytes in 15-day-old miR-17-92\textsuperscript{TG/TG};\alpha\text{MHC-Cre} heart sample. Higher magnification images in the boxed region are shown in right panels. Bar=100 μm.
Online Figure XIV. Cardiac-specific overexpression of miR-17-92 induced cardiomyocyte proliferation in postnatal mouse hearts. (a) Representative XYZ-axis 3D confocal microscopy images showing the cytokinesis in 15 day-old heart sections from miR-17-92^{TG/TG};α-MHC-Cre mice using Aurora B antibody (Green). The heart sections were also co-stained with antibodies detecting cTNT for cardiomyocytes (red) and DAPI for nuclei (blue). (b) Quantification of positive Aurora B staining from 5 days and 15 days old miR-17-92^{TG/TG};α-MHC-Cre and control miR-17-92^{TG/TG} mice. **P<0.01 Bars = 4 μm.
Online Figure XV. Induced overexpression of miR-17-92 in the heart. The expression of members of miR-17-92 cluster in 5-month-old heart samples after tamoxifen administration (4 months after tamoxifen administration) in miR-17-92^{TG/TG} and miR-17-92^{TG/TG};αMHC-MerCreMer (MerCreMer) mice was determined by quantitative RT-PCR. N of each genotype was indicated.
Online Figure XVI

Cardiac-specific overexpression of miR-17-92 induced DNA synthesis in adult mouse hearts. Immunohistochemistry showed the EdU incorporation on sagittal sections of hearts of 6 month control (miR-17-92^{TG/TG}) and miR-17-92^{TG/+};MerCreMer mice after tamoxifen-induction of miR-17-92 overexpression and Edu administration. Bar=300 μm.
Online Figure XVII

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<th>Short axis (μm)</th>
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<tr>
<td>miR-17-92\textsuperscript{TG/TG}</td>
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<td>miR-17-92\textsuperscript{TG/TG}; MerCreMer</td>
<td>5158±260 *</td>
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<td>42.7±1.5 **</td>
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Online Figure XVII. Quantitative measurement of the size of freshly isolated adult cardiomyocytes from hearts of miR-17-92-TGMerCreMer and control mice. One hundred individual cells from three different hearts were analyzed per group. ** P<0.01 between genetic groups.
Online Figure XVIII. Scheme of experimental procedure to introduce myocardial infarction (MI), tamoxifen and EdU injection and echocardiography measurement.
Online Figure XIX. Low frequency of cardiomyocyte apoptosis in the hearts of miR-17-92Tg+/+;MerCreMer mice after MI. Apoptosis was detected by TUNEL staining in the heart of 2 months old mice. Apoptotic cardiomyocytes were very rare and not appreciably different between control mice and miR-17-92Tg+/+;MerCreMer mice after myocardial infarction (MI). TUNEL staining positive control section from ApopTag Peroxidase In Situ Apoptosis Detection Kit was included. No abnormal positive TUNEL nuclei (bright green dots) were observed from heart section of both genotypes. Bars = 14 μm.
Online Figure XX

a

Control-mimic  miR-17-mimic  miR-19a-mimic  miR-20-mimic  miR-92-mimic

EDU  cTNT

EDU  cTNT

EDU  cTNT

EDU  cTNT
Online Figure XX. Overexpression of miR-17-92 induced the proliferation of P4 neonatal rat cardiomyocyte. (a) Proliferating neonatal rat cardiomyocytes were determined by detecting the EDU positive cardiomyocyte with immunochemistry. The boxed areas in upper panels are enlarged in lower panels. Bar=60 μm in upper panels; Bar=15 μm in lower panels. (b) Quantification of the percentage of EDU positive cardiomyocyte in each experimental group.
Online Figure XXI. Overexpression of PTEN in neonatal rat cardiomyocyte using modified RNA. The overexpression of flag-tagged PTEN protein in modified RNA transfected neonatal rat cardiomyocyte was detected by western blotting.
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<td><strong>Control</strong></td>
<td><strong>cKO</strong></td>
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<td>(N=6)</td>
<td>(N=6)</td>
<td>(N=6)</td>
<td>(N=5)</td>
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**Online Table I.** Echocardiography analyses of cardiac function of different aged cardiac-specific miR-17-92 knockout mice (cKO, miR-17-92^{flox/flox};Nkx2-5^{Cre/+}) and their control littermates. *: P<0.05; **: P<0.01. FS: Fractional shortening; LVID;d: Left ventricular end diastolic internal dimension; LVID;s: Left ventricular end systolic internal dimension; LVPW;d: Left ventricular end diastolic posterior wall dimension; LVPW;s: Left ventricular end systolic posterior wall dimension; LV Vol;d: Left ventricular end diastolic volume.
Online Table II

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<tbody>
<tr>
<td>IVS;d (mm)</td>
<td>0.562±0.061</td>
<td>0.652±0.027</td>
</tr>
<tr>
<td>IVS;s (mm)</td>
<td>1.038±0.078</td>
<td>1.139±0.020</td>
</tr>
<tr>
<td>LVID;d (mm)</td>
<td>3.504±0.664</td>
<td>3.493±0.344</td>
</tr>
<tr>
<td>LVID;s (mm)</td>
<td>1.585±0.249</td>
<td>1.977±0.336</td>
</tr>
<tr>
<td>LVPW;d (mm)</td>
<td>0.592±0.024</td>
<td>0.738±0.037**</td>
</tr>
<tr>
<td>LVPW;s (mm)</td>
<td>1.112±0.075</td>
<td>1.294±0.038*</td>
</tr>
<tr>
<td>EF (%)</td>
<td>86.15±0.70</td>
<td>75.33±7.62</td>
</tr>
<tr>
<td>FS (%)</td>
<td>54.56±1.54</td>
<td>43.53±6.57*</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>61.21±14.53</td>
<td>78.03±12.11</td>
</tr>
<tr>
<td>LV Mass (Corrected, mg)</td>
<td>48.97±11.63</td>
<td>62.43±9.68</td>
</tr>
<tr>
<td>LV Vol;d (uL)</td>
<td>52.91±22.93</td>
<td>51.14±11.82</td>
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<tr>
<td>LV Vol;s (uL)</td>
<td>7.22±2.82</td>
<td>12.82±5.18</td>
</tr>
<tr>
<td>Heart Rate (BMP)</td>
<td>542±26</td>
<td>528±2</td>
</tr>
</tbody>
</table>

**Online Table II.** Echocardiography analyses of cardiac function from 40 days old of miR-17-92<sup>TG/TG</sup> and miR-17-92<sup>TG/TG</sup>; α-MHC-Cre mice. N of each genotype was indicated. **: P<0.01; *: P<0.05; FS: Fractional shortening; LVID;d: Left ventricular end diastolic internal dimension; LVID;s: Left ventricular end systolic internal dimension; LVPW;d: Left ventricular end diastolic posterior wall dimension; LVPW;s: Left ventricular end systolic posterior wall dimension; LV Vol;d: Left ventricular end diastolic volume.
### Online Table III

Echocardiography analyses of cardiac function from miR-17-92Tg\(^*\)MerCreMer and control mice after 2 months of myocardium infarction surgery. N of each genotype was indicated. *: P<0.05; FS: Fractional shortening; LVID;d: Left ventricular end diastolic internal dimension; LVID;s: Left ventricular end systolic internal dimension; LVPW;d: Left ventricular end diastolic posterior wall dimension; LVPW;s: Left ventricular end systolic posterior wall dimension; LV Vol;d: Left ventricular end diastolic volume.

<table>
<thead>
<tr>
<th></th>
<th>Control (N=8)</th>
<th>miR-17-92Tg(^*) MerCreMer (N=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS;d (mm)</td>
<td>0.597±0.099</td>
<td>0.578±0.081</td>
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<tr>
<td>IVS;s (mm)</td>
<td>0.721±0.105</td>
<td>0.685±0.142</td>
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<tr>
<td>LVID;d (mm)</td>
<td>4.732±0.753</td>
<td>4.311±0.546</td>
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<tr>
<td>LVID;s (mm)</td>
<td>3.676±1.009</td>
<td>2.983±0.592</td>
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<tr>
<td>LVPW;d (mm)</td>
<td>0.661±0.108</td>
<td>0.770±0.094*</td>
</tr>
<tr>
<td>LVPW;s (mm)</td>
<td>0.912±0.146</td>
<td>0.946±0.151</td>
</tr>
<tr>
<td>EF (%)</td>
<td>45.77±15.79</td>
<td>58.74±9.74*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>23.31±8.95</td>
<td>31.16±6.39*</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>114.56±23.44</td>
<td>107.94±26.73</td>
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<tr>
<td>LV Mass (Corrected, mg)</td>
<td>91.65±18.75</td>
<td>86.35±21.38</td>
</tr>
<tr>
<td>LV Vol;d (uL)</td>
<td>107.35±43.55</td>
<td>85.39±26.31</td>
</tr>
<tr>
<td>LV Vol;s (uL)</td>
<td>63.12±46.63</td>
<td>36.56±19.64</td>
</tr>
<tr>
<td>Heart Rate (BMP)</td>
<td>582±29</td>
<td>614±48</td>
</tr>
</tbody>
</table>