Preclinical Development of a MicroRNA-Based Therapy for Elderly Patients With Myocardial Infarction

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ABSTRACT

BACKGROUND Aging populations show higher incidences of myocardial infarction (MI) and heart failure (HF). Cardiac remodeling post-MI leads to progressive impaired cardiac function caused by a disarray of several processes including derailed autophagy. Microribonucleic acids (miRNAs) are known to be key players in cardiovascular disease but their involvement in cardiac autophagy and aging is not well understood.

OBJECTIVES This study sought to identify new miRNA candidates that regulate cardiac autophagy and aging.

METHODS We exploited a high-throughput, fluorescence-activated cell sorting-based green fluorescent protein–LC3 detection method to measure the autophagic flux in cardiomyocytes after transfection of a precursor miRNA library consisting of 380 miRNAs. This was followed by a series of molecular and in vivo studies.

RESULTS Together with additional expression screenings, we identified miR-22 as an abundant and strong inhibitor of the cardiac autophagy process. Cardiac miR-22 expression levels increased during aging of mice as well as in aging neonatal cardiomyocytes in vitro by a P53-dependent mechanism. Inhibition of miR-22 in aging cardiomyocytes in vitro activated autophagy and inhibited cellular hypertrophy. Pharmacological inhibition of miR-22 post-MI in older mice activated cardiac autophagy, prevented post-infarction remodeling, and improved cardiac function compared with control subjects. Interestingly, similar effects were less pronounced in younger mice with significantly lower cardiac miR-22 expression levels. In addition, circulating levels of miR-22 in 154 patients with systolic HF were highly associated with early mortality.

CONCLUSIONS We concluded that miR-22 is an important regulator of cardiac autophagy and a potential therapeutic target, especially in the older myocardium. Finally, circulating miR-22 provides prognostic information for HF patients, highlighting miR-22 as a promising therapeutic and biomarker candidate for cardiovascular disorders. (J Am Coll Cardiol 2016;68:1557–71) © 2016 by the American College of Cardiology Foundation.

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Abbreviations and Acronyms

ATG = autophagy-related gene
FACS = fluorescence-activated cell sorting
GFP = green fluorescent protein
LNA = locked nucleic acid
MI = myocardial infarction

Noncommunicable diseases, notably cancer, diabetes, cardiovascular and neurological disorders, account for 65.5% of deaths (approximately 34.5 million) worldwide (1). Nearly 45% of these deaths are due to cardiovascular disease (CVD), which remains the leading cause of mortality worldwide (1). Myocardial infarction (MI) leading to chronic heart failure (HF) is the most common form of CVD that is prevalent in our society, and its incidence increases dramatically with age. The loss of cardiac tissue and ensuing impaired contractile function due to MI results in cardiac remodeling and cardiac hypertrophy, fibrosis, and altered autophagic activity. In older patients, all these processes are more pronounced (2), although the molecular rationale remains unclear.

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Autophagy, a process that recycles cellular components to maintain cellular homeostasis, is deregulated in age-related disease, like HF (3). Indeed, cardiac-specific deletion of the autophagy gene Atg5 in mice leads to a progressive decline in function with age, together with early appearance of other cardiac aging hallmarks (cardiac hypertrophy and fibrosis) eventually leading to premature death (4). Temporally controlled deletion of Atg5 in adult mice also results in cardiac dysfunction, hypertrophy, disorganized sarcomere, mitochondria aggregation, and accumulation of ubiquitinated proteins (5). Importantly, in the case of MI, accumulation of p62 colocalized aggresomes has been observed in the infarct border zone and in remote areas (6). Mammalian sterile 20-like kinase 1 (Mst1/C0)/ mice do not show aggresome accumulation and pathological left ventricular remodeling post-MI. This phenotype is lost when Mst1/C0−/− are crossed with Beclin−/− mice, which show lower autophagic activity (6). Therefore, the identification of therapeutic targets that modulate autophagy holds great promise as a new treatment approach to cardiac remodeling and HF, especially in older age.

Microribonucleic acids (miRNAs) are a class of noncoding regulatory ribonucleic acid (RNA) molecules of around 22 nucleotides in length; they exert their function by complementary binding via their seed sequence. In the past decade, miRNAs have been identified as critical regulators of cardiac pathophysiology (7–9), but only a few miRNAs regulating cardiac autophagy have been studied in detail (10). miRNA-212/132 was demonstrated to have prohypertrophic and antiautophagic effects in cardiomyocytes via regulation of FoxO3a (8). Additionally, the miRNAs miR-199a and -221 have been reported to be antiautophagic, thus promoting cardiac hypertrophy (11,12).

We conducted a detailed functional study of 380 miRNAs to gain further insight into miRNA-dependent regulation of cardiac autophagy. We identified miR-22 as a strong inhibitor of cardiac autophagy using in vitro and preclinical in vivo models of MI in young and older mice. Likewise, the prognostic importance of circulating miR-22 was tested in a clinical cohort of 198 patients with systolic heart failure.

Methods

Fluorescence-Activated Cell Sorting-Based Autophagy Measurements and Library Screening.

To measure autophagic flux by a fluorescence-activated cell sorting (FACS)-based method, HL-1 cells were seeded in 48 well cell culture plates. The next day, cells were transfected with precursor miRNAs together with Lipofectamine 2000 (Thermo Fisher Scientific, Inc., Waltham, Massachusetts) and transduced together with a green fluorescent protein (GFP)-LC3 adenovirus at a multiplicity of infection of 10; 48 h later, cells were trypsinized and fixed, and GFP intensity was measured with a Guava Easycyte Flow Cytometer (EMD Millipore Corporation, Darmstadt, Germany). Data were analyzed with the FlowJo software (FlowJo LLC, Ashland, Oregon), and mean fluorescence intensity (MFI) was calculated and normalized to the control subjects. miRNA library screening was performed with transfection of an miRNA precursor library (4391437 AMO1T9H Mouse – Pre-miR, Ambion, Thermo Fisher Scientific). Normal medium was changed to starvation medium 8 h prior to the endpoint, and thus flux was measured in response to starvation. Screening was performed in single wells, whereas validation of the screen was done in triplicates.

A detailed Methods section can be found in the Online Appendix.

Results

A FACS-based high-throughput assay measuring GFP-LC3 was used to determine the autophagic flux in the HL-1 cardiac cell line (13). A lower GFP intensity after starvation indicates activation of autophagy, and an accumulation of GFP with thapsigargin, chloroquine, and bafilomycin A1 treatment indicates autophagy inhibition (Figures 1A and 1B, Online Figures 1A to 1D). We performed a functional screen of a miRNA precursor library (380 miRNAs) by using this FACS-based strategy to identify miRNAs that serve as autophagic regulators. We identified several miRNAs that were functioning as inhibitors or
Fluorescence-activated cell sorting (FACS)-based green fluorescent protein (GFP) measurements varied in normal, starved, and thapsigargin-treated HL-1 cells transduced with GFP-LC3 adenovirus seen in both (A) a representative FACS plot and (B) GFP mean fluorescence intensity (MFI). (C) Activators (blue dots) and inhibitors (orange dots) were identified from precursor microribonucleic acid (miRNA) library screening. (D) MiR-22, -9, -204, -141, -200, and -211 were confirmed as inhibitors and miR-93 as an activator of autophagy, verifying the screening results. (E) MiR-22 was identified among the top candidates that regulated autophagy and had relatively higher cardiac expression. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. Ct = cycle threshold; miR = microribonucleic acid.
Higher aggregation of p62 was evident with overexpression of autophagic inhibitors miR-22 and -9 in HL-1 cells (A), reaching significance in both instances (B), also shown by frequency distribution of the p62 aggregated cells (C). (D) The number of autophagosomes detected with GFP-LC3 puncta decreased significantly (E) after overexpression of miR-22 in primary neonatal rat cardiomyocytes; again, the frequency distribution of the calculated GFP-LC3 punctas per cell varied (F). (G) There was a significant difference in accumulation of p62 in neonatal rat cardiomyocytes after overexpression of miR-22. Scale bar = 100 μm. ***p = 0.001.

GAPDH = glyceraldehyde 3-phosphate dehydrogenase; other abbreviations as in Figure 1.
activators of autophagy (Figure 1C, Online Table 1). Interestingly, miRNAs with the same seed sequence often showed similar results (e.g., miR-141/200a or -204/211) (Online Figure 1E). Certain miRNA hits were individually validated (Figure 1D).

Next, we compared the identified autophagic miRNA regulators with the cardiac miRNA expression profile and identified miR-22 to be highly abundant and have a strong autophagy inhibitory function (Figure 1E). miR-22 has been reported to be highly enriched in cardiomyocytes compared with other cardiac cell types (14); thus, we focused on this specific miRNA in the following analyses.

**CARDIAC AUTOPHAGY, CARDIAC REGULATION, AND miR-22.** Autophagy is a complex, dynamic, multistep process; therefore, potential autophagic regulators should be validated by different methods (15). The functional miRNA precursor screen and validation experiments on the basis of LC3 degradation confirmed that miR-22 functions as an autophagic inhibitor. We next studied p62 in miR-22-modified cardiomyocytes; p62 is an autophagic substrate that shows a diffuse pattern of distribution at the basal level, whereas cellular aggregation is observed during autophagic inhibition (15). Indeed, overexpression of miR-22 in HL-1 cells increased aggregated p62 (Figures 2A to 2C), whereas miR-22 inhibition reversed this effect (Online Figures 2A to 2C). As proof of concept, another strong autophagic inhibitor, miR-9 derived from the screen, also led to accumulated punctated p62 (Figures 2A to 2C), again reversed by miR-9 inhibition (Online Figures 2A to 2C).

We next confirmed our findings from the HL-1 cell line in primary cardiomyocytes; here, we counted the autophagic puncta (GFP-LC3) after miR-22 overexpression in neonatal rat cardiomyocytes. Cardiomyocytes with miR-22 overexpression showed fewer autophagic puncta, confirming miR-22’s inhibitory effects (Figures 2D to 2F). Also, accumulation of p62 protein levels was shown after pre-miR-22 transfection in neonatal rat cardiomyocytes (Figure 2G). Thus, using different methods, we demonstrated that miR-22 is a strong autophagic inhibitor in cardiomyocytes.

miR-22 has been reported as a pro-hypertrophic miRNA (14,16,17). Its inhibition abolished cardiac hypertrophy, whereas its overexpression resulted in massive hypertrophy (14,16). Previously, our group reported miR-22 to be induced during aging in murine hearts (18). Cardiac miR-22 expression levels were confirmed in mice of different ages, from 4 days to 1.7 years. We found a 3-fold up-regulation at 3 months of age compared with younger control subjects followed by a further gradual increase with age (Figure 3A). Furthermore, miR-22 expression increased in human myocardium with age (Figure 3B), with human fetal hearts showing significantly lower levels of miR-22 compared with adults (Figure 3C). Apart from real-time measurements, RNA sequencing of miR-22 showed a progressive increase from 12 to 52 to 104 weeks during natural aging in murine hearts (Figure 3D). Similar to natural aging, we used an accelerated aging model (Lamin A/C heterozygous mice), where the hearts also had higher sequencing reads for miR-22 already at mid-age (Figure 3D). We then checked whether the aging-mediated miR-22 induction was restricted to cardiomyocytes. Interestingly, miR-22 was specifically increased in cardiomyocytes from older hearts, whereas no change was evident in other cardiac cell population (Figure 3E). Collectively, these results confirmed induction of miR-22 during cardiac aging.

A decline in cardiac autophagic activity with age was previously reported (4), leading to our hypothesis that miR-22 regulated the decrease in autophagy during aging. To study the effects of miR-22 on cardiac aging and autophagy, we used an established in vitro cardiomyocyte aging model (19) where we cultivated neonatal rat cardiomyocytes for 2 weeks and compared older to early (day 3) cultured cardiomyocytes. miR-22 was significantly up-regulated in old compared to young cardiomyocytes (Figure 3F). Interestingly, the autophagic substrate p62 was also up-regulated in old (vs. young) cardiomyocytes, confirming cardiac autophagic inhibition with age (Figures 3G and 3H). Next, we inhibited miR-22 by a locked nucleic acid (LNA)-based anti-miR approach (Online Figure 3A). Inhibition of miR-22 in aging cardiomyocytes led to partial restoration of myocardial autophagy, demonstrated by lower levels of p62 (Figures 3G and 3H). A culture of cardiomyocytes for 2 weeks resulted in cellular hypertrophy, which was partially inhibited by miR-22 silencing, suggesting that miR-22 inhibition can reverse some of the aging effects in cultured cardiomyocytes (Online Figures 3B to 3D). Another miRNA, miR-9, identified from the screen as an autophagic inhibitor, also exerted pro-hypertrophic effects in neonatal rat cardiomyocytes, showing an opposite relation of autophagy and hypertrophy (Online Figure 4).

**UPSTREAM REGULATORS OF miR-22.** We searched for potential upstream regulators of miR-22 and identified p53 as a transcriptional activator of miR-22 (20). To determine whether p53 is a direct inducer of miR-22 during aging, we treated neonatal rat cardiomyocytes with the p53 inhibitor pifithrin-alpha, which prevented the miR-22 induction during aging of cardiomyocytes (Figure 3I). Furthermore, small
**FIGURE 3**

miR-22 Up-Regulation During Cardiac Aging

**A**

miR-22/5sno202

- P4
- P7
- P10
- 3 weeks
- 3 months
- 6 months
- 1 year
- 1.7 years

**B**

miR-22/U6 RNA

- Below 40 Years
- Above 40 Years

**C**

- Fetal
- Adult

**D**

- 52 weeks
- 12 weeks
- 52 weeks
- 104 weeks

**E**

- Cardio myocyte
- Fibroblast
- Endothelial cells

**F**

miR-22/5sno202

- Day 3
- Day 14

**G**

p23/GAPDH

- Day 3 LNA Scr LNA 22
- Day 14

**H**

- p62
- GAPDH

**I**

miR-22/5sno202

- Day 3
- Day 14
- Day 14 Pre-miR-Neg
- Day 14 Pre-miR-22
- Day 14 Pre-miR-Neg+Pifi-α

**J**

Green Fluorescence Log

- Count

**K**

- GFP MFI
interfering RNA-mediated inhibition of p53 in aging cardiomyocytes partially decreased miR-22 expression levels (Online Figures 5A and 5B), confirming a direct role of p53 in miR-22 induction during myocardial aging. For confirmation, we analyzed the effect of pifithrin-alpha on cardiac autophagy. Indeed, pifithrin-alpha induced autophagy at basal levels, and overexpression of miR-22 prevented pifithrin-alpha activated autophagy (Figures 3J and 3K). Furthermore, pifithrin-alpha completely prevented the development of cellular hypertrophy in aging cardiomyocytes, which was only partially rescued by miR-22 overexpression (Online Figures 5C to 5E). Thus, p53 appears to be an upstream inducer of miR-22 during aging.

**THERAPEUTIC POTENTIAL OF miR-22 INHIBITION.** On the basis of these in vitro results, we speculated that inhibition of miR-22 in vivo would be beneficial for the treatment of age-related cardiac diseases (a specific situation where both miR-22 expression is increased and cardiac autophagy impaired). Thus, we inhibited miR-22 in both older (age 10 to 11 months) and juvenile (age 9 weeks) mice post-MI. miR-22 was successfully down-regulated in both border and remote myocardium of older infarcted mice after LNA targeting miR-22 treatment compared with LNA-scramble control treated subjects (Online Figures 6A and 6B). Echocardiographic assessment of cardiac function showed a reduced ejection fraction in mice with MI compared to sham, which was restored in the LNA-anti-miR-22-treated older group, depicting a beneficial effect of miR-22 inhibition post-MI (Figure 4A). Additionally, the Tei index and cardiac output were impaired after MI but restored in older animals with MI treated with the miR-22 inhibitor (Figures 4B and 4C). Systolic volume was increased post-MI, which again was attenuated by LNA-anti-miR-22 treatment (Figure 4D).

We next performed speckle tracking strain imaging to evaluate myocardial wall motion post-MI. Regional evaluation of peak longitudinal strain rate showed a significant decrease in the posterior apex of hearts post-infarction that was significantly improved with miR-22 inhibition; other cardiac regions showed similar trends (Figure 4E). Average peak longitudinal strain rate of the complete myocardium was significantly recovered in mice with miR-22 inhibition compared with scrambled control subjects (Online Figure 6C). Global longitudinal strain was also lower in infarct animals compared with sham control animals but was significantly improved in older animals treated with LNA-anti-miR-22 (Figure 4F). At the histological level, a significant increase in cardiomyocyte size was evident in remote regions of infarcted hearts treated with LNA-scramble compared with sham, whereas LNA-anti-miR-22-treated mice were protected from development of cellular cardiomyocyte hypertrophy, suggesting improved cardiac remodeling (Figures 4E to 4G). Taken together, these results showed that miR-22 inhibition is beneficial for the restoration of cardiac function post-MI in older mice.

As miR-22 is increased in old myocardium and significantly lower in younger mice (Figure 5A), we also tested potential beneficial effects of miR-22 inhibition in young mice post-MI. For this, we performed MIs in juvenile mice and inhibited miR-22 similar to the older mouse study. Real-time polymerase chain reaction analysis showed inhibition of miR-22 after LNA-anti-miR-22 treatment in myocardium compared to mice receiving the scrambled control (Figure 5B). Echocardiographic assessment indicated lesser improvement in ejection fraction and cardiac output in the LNA-anti-miR-22-treated group compared with scrambled-control-treated mice (Figures 5C and 5D). Other parameters such as systolic volume and Tei index were not significantly altered by miR-22 inhibition in young mice post-MI (Figures 5E and 5F). Similar to the standard echocardiography data, strain imaging also showed no improvements with miR-22 blockade in young MI mice. Global longitudinal strain (Figure 5G) and global

**FIGURE 3 Continued**

(A) miR-22 gradually increased during aging in murine hearts (n = 4 to 8). (B) Higher levels of miR-22 expression were seen in human hearts >40 years of age compared with those <40 years of age and adult compared with fetal myocardium (C). (D) Sequencing of miR-22 showed a gradual increase during natural cardiac aging and higher expression levels in accelerated aging model LMNA−/− hearts. (E) In fractionated young and old hearts, cardiomyocyte restricted induction of miR-22 during aging. (F) Increased miR-22 levels in aging cardiomyocytes in vitro. p62 levels in younger and older cardiomyocytes treated with either locked nucleic acid (LNA)-scramble (Scr) control or LNA 22 are (G) significantly different in the complete dataset and (H) visibly different in the representative western blot. (I) Pifithrin-α (Pif-α) repressed miR-22 in aging cardiomyocytes. Pif-α activated autophagy in HL-1 cells as seen by lower GFP intensity via (J) a representative FACS plot and (K) GFP MFI; GFP intensity was restored by overexpression of miR-22. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. RNA = ribonucleic acid; SnoRNA = small nucleolar ribonucleic acid; other abbreviations as in Figures 1 and 2.
FIGURE 4 Pharmacological Inhibition of miR-22 in Older Mice Post-MI

A

% Ejection Fraction

Sham LNA Scr LNA 22

B

Tel index

Sham LNA Scr LNA 22

C

Cardiac Output

Sham LNA Scr LNA 22

D

Vol %

Sham LNA Scr LNA 22

E

PSSR

0.14 0.08 0.09 0.10

F

GLS

Sham LNA Scr LNA 22

G

Cross-Sectional Area

Sham LNA Scr LNA 22

H

% of Cells

Cross-Sectional Area

Sham LNA Scr LNA 22

I

Sham LNA Scr LNA 22

Continued on the next page
longitudinal peak strain rate (Figure 5I) were significantly lower in hearts with MI versus sham, but were only slightly improved by LNA-anti-miR-22 treatment. Analysis of regional wall motion measured by peak strain rate showed no significant improvement in any myocardial region in hearts with miR-22 inhibition compared with scramble control subjects (Figure 5H).

Evaluation of cardiomyocyte size found increased cell size in the infarcted heart compared with sham, but no significant effect on cardiomyocyte cell size in LNA-anti-miR-22-treated animals (Figures 5J to 5L). After picrosirius red staining, we found fibrosis was higher in older sham compared with younger sham hearts (Online Figures 6D and 6E), but no increased fibrosis was evident 2 weeks post-MI either in young or old hearts treated with LNA scramble or with LNA-anti-miR-22 (Online Figures 6F and 6G). Per these results, young mice had a significantly weaker response to miR-22 therapy, whereas older mice, which show higher cardiac miR-22 expression levels, had a very strong beneficial response, demonstrating significant age-dependent effects of a cardiac miRNA therapy on the basis of age-dependent expression of miR-22 levels.

**DOWNSTREAM TARGETS OF miR-22.** To identify downstream targets responsible for beneficial effects of miR-22 inhibition, we listed all validated targets of downstream targets responsible for benefit of miR-22 inhibition, specifically in older mice (Figure 6C). The peroxisome proliferator-activated receptor-alpha (PPAR-α) agonist GW7647 was reported to activate autophagy in the liver (21), but the effects of PPAR-α activity on cardiac autophagy have not yet been studied. We treated HL-1 cells with GW7647; they responded with a moderate activation of autophagy as depicted by increased GFP degradation. This was prevented after overexpression of miR-22, suggesting a direct involvement of PPAR-α in cardiac autophagy regulation (Figures 6D and 6E). Furthermore, in vivo myocardial levels of the autophagic substrate p62 were lower after miR-22 inhibition, revealing activation of autophagy (Figure 6F). Although miR-22 inhibition prevented cardiac hypertrophy and activated autophagy, a direct correlation of both hypertrophy and autophagy remained inconclusive. To determine whether hypertrophic inducers may affect autophagy, we implanted phenylephrine (PE) and isoproterenol (ISO)-containing minipumps in adult mice for 1 week to induce cardiac hypertrophy in vivo. In that scenario, p62 accumulated in PE/ISO-treated hearts compared with control hearts (Figure 6G). Similarly, neonatal rat cardiomyocytes treated with PE/ISO showed an accumulation of p62, confirming inhibition of autophagy with hypertrophic agonists (Online Figures 7B and 7C). miR-22 deletion was reported to impede cardiac hypertrophy in calcineurin transgenic hearts, another model of massive hypertrophy (14). We thus determined the levels of p62 in calcineurin transgenic and wild-type hearts, identifying an accumulation of p62, which confirmed reduced autophagic activity and provided a potential link between hypertrophy and impaired cardiac autophagic activity (Figure 6H).

**CIRCULATING miR-22 AS A PROGNOSTIC BIOMARKER.** Our studies of intracellular miR-22 levels in vitro and in vivo prompted us to also assess levels of circulating miR-22 in patients with systolic HF from ischemic and nonischemic origin. Indeed, the existence of

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**FIGURE 4 Continued**

Decline in cardiac function after myocardial infarction (MI) assessed by echocardiography, as measured by (A) ejection fraction, (B) Tei index, (C) cardiac output, and (D) systolic volume, was restored in LNA-anti-miR-22-treated mice. Strain imaging of hearts by Vevo strain (Fujifilm VisualSonics, Toronto, Ontario, Canada) showed improved regional (E) peak longitudinal strain rate (PLSR) and (F) global longitudinal strain (GLS) with miR-22 inhibition post-MI. (G) Increased cardiomyocyte cross-sectional area in infarcted hearts compared with sham was absent in LNA-anti-miR-22-treated hearts, which was reflected in (H) the frequency distribution of area measurements and (I) stained segments. n = 5 sham; n = 8 to 10 LNA-scramble; and n = 10 LNA-anti-miR-22. Scale bar = 100 μm.

*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. AA = anterior apex; AB = anterior base; AM = anterior mid; PA = posterior apex; PB = posterior base; PM = posterior mid; other abbreviations as in Figures 1 and 3.
FIGURE 5  Inhibition of miR-22 in Young Mice Post-MI

A

B

C

D

E

F

G

H

I

J

K

L

Continued on the next page
tissue-free, circulating miRNAs and other RNAs in body fluids have suggested the possibility of developing noncoding RNA biomarkers for heart diseases (22,23). miR-22 had been reported as cardiomyocyte-enriched miRNA, playing a very important role in cardiovascular pathophysiology (14,16). We studied the association of circulating miR-22 levels with the prognosis of HF patients in 154 patients in a case/control setting (patient characteristics are summarized in Online Table 2). The patients who died during the 3-year follow-up had significantly higher New York Heart Association functional class, brain natriuretic protein, and creatinine levels, and lower peak VO2.

The mean normalized delta cycle threshold (dCT) value for miR-22 was \(-4.11 \pm 0.91\) (range: \(-6.57\) to \(-1.94\)). There was no association between age and circulating miR-22 levels (\(r = 0.0128; p = 0.162\)). The levels of miR-22 were similar in ischemic HF (\(-4.19 \pm 0.87\)) and nonischemic HF (\(-3.98 \pm 0.94; p = 0.159\)). miR-22 levels at the time of prognostic evaluation were higher in case patients than control subjects (\(-3.80 \pm 0.94\) vs. \(-4.42 \pm 0.74; 1.54\)-fold change; \(p < 0.0001\) (Figure 7A)). In a model adjusting for age, sex, New York Heart Association functional class, HF etiology, serum creatinine, left ventricular ejection fraction, brain natriuretic protein, and peak VO2, miR-22 levels remain independently associated with cardiovascular mortality. Compared with patients in the first quartile of miR-22 levels, patients in the third and fourth quartiles had significantly increased cardiovascular mortality (Figure 7B). The prognostic association of miR-22 levels was observed in ischemic HF (cases: \(-3.89 \pm 0.92\); control subjects: \(-4.48 \pm 0.72; p = 0.0009\)) and nonischemic HF (cases: \(-3.68 \pm 0.97\); control subjects: \(-4.33 \pm 0.78; p = 0.0054\)). Similarly, the association was observed in patients >60 years of age (cases: \(-3.89 \pm 0.94\); control subjects: \(-4.44 \pm 0.66; p = 0.0036\)) and in patients ≤60 years of age (cases: \(-3.71 \pm 0.94\); control subjects: \(-4.40 \pm 0.83; p = 0.0012\)). To identify the probable source of circulating miR-22, we assessed the expression of miR-22 in different mouse organs and found miR-22 to be highly expressed in muscle tissue followed by aorta, lung, and liver (Figure 7C). Thus, the source of higher circulating miR-22 likely is the heart together with other organs, such as the liver.

**DISCUSSION**

We demonstrated that miR-22 is a key regulator of cardiac autophagy and shows therapeutic potential as a novel target to treat post-infarct remodeling, especially in older mice. Balanced autophagic activity is necessary for the healthy functioning of the heart. Autophagy is reported to be regulated by several Atg genes, whereas only some miRNAs are known to control myocardial autophagy (10). We performed a large functional screening approach for miRNAs to identify new regulators of myocardial autophagy. In our phenotypic functional miRNA screening, we found miR-22 to be a strong inhibitor of cardiac autophagy. miR-22 was increased during aging in murine and human myocardium. Our results showed that miR-22 inhibition post-infarction led to improved cardiac function and inhibited cardiac remodeling in older mice (Central Illustration) but not young mice, correlating with the observation that miR-22 expression levels are high in older but not young myocardium. Thus, our results demonstrated that pharmacological inhibition of miR-22 is of therapeutic relevance especially in older myocardium.

Several studies report miR-22 as a prohypertrophic miRNA, and permanent deletion of miR-22 blunted hypertrophy induced by various cardiac stress but led to cardiac dilation (14,16,17). Previous studies have investigated miR-22 knockout mice submitted to various forms of cardiac stress, such as pressure overload, isoproterenol infusion, or calcineurin overexpression (14,24). Our study was the first to evaluate the effect of pharmacological inhibition of miR-22 in an MI model. Moreover, the MI performed in older animals in our study is more

**FIGURE 5 Continued**

(A) Myocardial expression levels of miR-22 were higher in old mice (age 10 to 11 months) relative to young mice (age 9 weeks). (B) LNA-anti-miR-22 significantly reduced miR-22 compared with LNA-scramble (sc). Echocardiographic analysis of (C) ejection fraction, (D) cardiac output, (E) systolic volume, and (F) Tei index showed deteriorated cardiac function with infarction and minor improvements with miR-22 inhibition. (G) Global and (H) regional PLSR showed decrease in wall motion of infarcted hearts compared with sham. (I) Global longitudinal strain (GLS) also decreased upon myocardial infarction with no improvement in the LNA-anti-miR-22 group. (J) Cardiac cross-sectional measurements with wheat germ agglutinin staining showing increased area in infarcted hearts compared with sham; this was also reflected in (K) the frequency distribution of area measurements and (L) stained segments. n = 5 to 6 sham; n = 11 to 12 LNA-scramble; and n = 11 LNA-anti-miR-22. Scale bar = 100 μm. *p ≤ 0.05; **p ≤ 0.001. Abbreviations as in Figures 1, 3, and 4.
**FIGURE 6** Downstream Effectors of miR-22

(A) A total of 20 cardiac (orange) and autophagy (blue) relevant targets were identified from the list of all confirmed targets (gray) of miR-22. (B) Ppara varied significantly in older sham hearts and post-MI hearts with either LNA-scramble or LNA-anti-miR-22 treatment, but no significant differences were seen in Ppara levels in young hearts (C). Fluorescence-activated cell sorting plot (D) and GFP mean intensity (E) of HL-1 cells treated with GW7647 or control after transfection with either pre-miR-neg or pre-miR-22. Myocardial p62 levels are compared in infarcted hearts with LNA-scramble or LNA-anti-miR-22 (F), in hearts with phenylephrine/isoprenaline (PE/ISO) minipumps or control (G), and in calcineurin transgenic (CnA TG) hearts compared with wild-type (WT) hearts (H). *p ≤ 0.05, **p ≤ 0.01. Abbreviations in Figures 1 to 4.
relevant to clinical settings, as hospitalization rates with cardiovascular abnormalities are significantly higher in older people (25).

We showed that miR-22 inhibition activated cardiac autophagy, leading to improved function post-MI. Similarly, Ruozi et al. (26) discovered that ghrelin improves cardiac function after MI by activating myocardial autophagy. Another study also demonstrated the beneficial effect of autophagic activation by rapamycin post-MI (27). Taken together, these studies illustrated the benefits of autophagic activation post-infarction.

Among several targets of miR-22, we provided new evidence for de-repression of PPAR-\(\alpha\) by miR-22 inhibition, specifically in older hearts. A moderate activation of autophagy has been seen with PPAR-\(\alpha\) agonist, indicating that PPAR-\(\alpha\) is at least partially responsible for the autophagic inhibitory function of miR-22. Apart from the intracellular function of miR-22, we found that circulating levels of miR-22 can predict mortality in HF patients. Higher levels of miR-22 showed a significant association with cardiovascular mortality, reflecting its prognostic potential.

**STUDY LIMITATIONS.** A multifactorial process, cardiac aging includes several cell types. Our study cannot rule out the possible effect of LNA-mediated miR-22 inhibition on other cell types; thus, the beneficial effects we identified may be due to a

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**FIGURE 7** Prognostic Effect of miR-22 Levels in Chronic Heart Failure

(A) Comparative scatterplots showing normalized miR-22 levels at inclusion according to outcome (no cardiovascular death vs. cardiovascular death) during 3-year follow-up. (Mean delta cycle threshold \([\text{dCt}}\) values calculated as mean Ct value of normalizer average – Ct value of miR-22; \(p\) value calculated by unpaired Student \(t\) test.) Orange lines = respective medians; blue circles = individual patients. (B) Adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for cardiovascular death during 3-year follow-up according to miR-22 levels at inclusion (quartile 1: -6.57 to -4.69; quartile 2: -4.68 to -4.03; quartile 3: -4.02 to -3.39; quartile 4: -3.38 to -1.94). (C) MiR-22 expression levels in different organs of the adult mice.
cumulative effect rather than cardiomyocyte specific. Another limitation of our study was the in vitro aging model, which is not completely similar to the in vivo model. However, similar results from both models confirmed miR-22 to be a pro-aging candidate.

**CONCLUSIONS**

We have reported a new functional role of miR-22 in older myocardium, putting forward a new prognostic tool for patients with MI and a treatment strategy on the basis of miR-22 inhibition for elderly individuals with myocardial infarction.

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COMPETENCY IN MEDICAL KNOWLEDGE: Inhibition of a noncoding older-cardiomyocyte-enriched microRNA, miR-22, attenuated adverse remodeling and improved cardiac function in an animal model of MI in older but not younger mice, and circulating miR-22 levels predicted death in patients with systolic HF.

TRANSLATIONAL OUTLOOK: Future studies should explore the potential roles of miR-22 as both a prognostic marker in patients with HF and a treatment target for elderly patients with MI.

REFERENCES


KEY WORDS aging, autophagy, circulating miRNA, MiR-22, p62

APPENDIX For an expanded Methods section as well as supplemental figures and tables, please see the online version of this article.