Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family

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We recently identified a brief time period during postnatal development when the mammalian heart retains significant regenerative potential after amputation of the ventricular apex. However, one major unresolved question is whether the neonatal mouse heart can also regenerate in response to myocardial ischemia, the most common antecedent of heart failure in humans. Here, we induced ischemic myocardial infarction (MI) in 1-d-old mice and found that this results in extensive myocardial necrosis and systolic dysfunction. Remarkably, the neonatal heart mounted a robust regenerative response, through proliferation of preexisting cardiomyocytes, resulting in full functional recovery within 21 d. Moreover, we show that the miR-15 family of microRNAs modulates neonatal heart regeneration through inhibition of postnatal cardiomyocyte proliferation. Finally, we demonstrate that inhibition of the miR-15 family from an early postnatal age until adulthood increases myocyte proliferation in the adult heart and improves left ventricular systolic function after adult MI. We conclude that the neonatal mammalian heart can regenerate after myocardial infarction through proliferation of preexisting cardiomyocytes and that the miR-15 family contributes to postnatal loss of cardiac regenerative capacity.

Results

Neonatal Heart Regenerates After MI. To establish how the neonatal mouse heart responds to ischemic injury, we permanently ligated the left anterior descending (LAD) coronary artery of 1-d-old mice (Fig. 1A). Evidence of myocardial necrosis was noted at day 3 after LAD ligation, where staining with the viability indicator triphenyltetrazolium chloride (TTC) revealed that the majority of the myocardium below the ligature (∼75%) was nonviable (Fig. 1 B and C). LAD ligation was accompanied by a marked decline in left ventricular systolic function at 4 d after injury (Fig. 1D). Histological analysis confirmed that extensive cardiomyocyte loss and intramyocardial hemorrhaging had occurred after LAD ligation (Fig. 1E).

To assess the regenerative capacity of the neonatal mouse heart after ischemic injury, histological and functional analyses were performed at multiple time points after LAD ligation. Serial

Conflict of interest statement: E.N.O. is a cofounder of miRagen Therapeutics, a company focused on developing miRNA-based therapies for cardiovascular disease.

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Neonatal MI Induces Cardiomyocyte Proliferation. Heart regeneration in neonatal mice after ischemic injury was associated with robust and widespread induction of cardiomyocyte proliferation and sarcomere disassembly (Fig. 2). Induction of cardiomyocyte mitosis and cytokinesis were assessed by colocalization of phospho-histone H3 (pH3) and aurora B kinase, respectively, with cardiac troponin T. A significantly higher number of cardiomyocytes with disassembled sarcomeres (Fig. 2 D and H–I), and a significantly increased number of pH3-positive (Fig. 2 C and G) and aurora B-positive (Fig. 2 E and F) cardiomyocytes were identified in mice at day 7 after MI compared with sham-operated controls. Approximately 3–5% of cardiomyocytes were undergoing mitosis at day 7 after MI. Notably, proliferating cardiomyocytes were not restricted to the ischemic and border zones of the infarct but were also identified in the remote zone (i.e., posterior and lateral walls) (Fig. 2 B–D and F). Furthermore, pulse-chase labeling with BrdU (administered at days 1, 7, and 14) identified many BrdU-positive cardiomyocytes in the newly formed myocardium at day 21 after MI (Fig. 3 A–C). The number of BrdU-positive cardiomyocyte nuclei was augmented in the ischemic, border, and remote zones of the myocardium at day 21 after MI, indicating that many cardiomyocytes within the newly formed myocardium had undergone at least one round of DNA replication during the course of post-MI regeneration.

Newly Formed Cardiomyocytes Are Derived from Preexisting Cardiomyocytes. To establish the lineage of origin of regenerated cardiomyocytes, Rosa26-lacZ reporter mice were crossed with Myh6-MerCreMer mice, which harbor a tamoxifen-inducible Cre recombinase transgene under control of Myh6 cardiomyocyte-specific promoter. Neorenes were then given a single s.c. dose of tamoxifen at birth to label the preexisting cardiomyocyte population before LAD ligation (Fig. 3D), as described (8). Administration of a single dose of tamoxifen at birth resulted in ~70% of the myocardium being labeled with lacZ in the sham-operated group (Fig. 3E). Histological sections were acquired from sham and infarcted hearts at day 21 after MI. The majority of the newly formed myocardium stained positive for lacZ, with no differences in the percentage of lacZ-positive myocardium between sham and infarcted hearts in the ischemic, border, and remote zones (Fig. 3E). These findings provide genetic evidence that the majority of newly formed cardiomyocytes after MI in 1-d-old mice are derived from preexisting cardiomyocytes, rather than from a stem cell population.

Lack of Regeneration of 7- and 14-d-Old Infarcted Hearts. To determine whether the cardiac regenerative response to ischemic injury is lost when neonatal mouse cardiomyocytes withdraw from the cell cycle, we performed LAD ligation on 7- and 14-d-old mice. In contrast to 1-d-old mice, 7- and 14-d-old mice failed to regenerate after LAD ligation (Fig. 4 A and B). Whereas 1-d-old mice were capable of clearing fibrotic tissue and replenishing cardiomyocytes (Fig. 1E), 7-d-old mice retained significant scar tissue at day 21 after MI (Fig. 4A) and the cardiac remodeling response of 14-d-old mice after MI was similar to that of adult mice and was characterized by extensive fibrosis, wall thinning, and ventricular dilatation (Fig. 4B). Fewer proliferating cardiomyocytes with disassembled sarcomeres were identified after MI in 7-d-old mice (Fig. 4 C and D). In contrast to the regenerative response of the 1-d-old mouse heart, which occurred in the absence of any appreciable hypertrophic remodeling, cardiac repair in 14-d-old mice occurred coincident with cardiac and cardiomyocyte hypertrophy (Fig. 4 E–G),
which is a hallmark feature of pathological remodeling in the adult heart. These results indicate that cardiac regenerative capacity in mice is lost by 1 wk of postnatal life.

**miR-195 Transgenic Hearts Fail To Regenerate After MI.** The molecular mechanisms regulating cardiac regenerative capacity after birth in mammals are unknown. We recently discovered that multiple members of the miR-15 family of microRNAs are up-regulated in the mouse heart shortly after birth and contribute to cardiomyocyte mitotic arrest (12). Consistent with their inhibitory effect on myocyte proliferation, we found that several members of the miR-15 family were down-regulated in infarcted myocardium (below the LAD ligature), and in noninfarcted myocardium (above the ligature), suggesting that these differences were not a consequence of myocyte loss in the infarct zone. To test whether the miR-15 family regulates cardiac regenerative capacity in neonatal mice, miR-195 (a member of the miR-15 family) was overexpressed in the developing heart (12). MYH7-miR-195 transgenic (TG) mice underwent LAD ligation at P1. We reported that a small subset (~22%) of these TG mice have preexisting ventricular septal defects at birth (12). These mice were identified after MI by histological analysis and by dilation of the right ventricle and cardiac wall motion abnormalities upon echocardiography. TG mice that did not have preexisting VSDs had normal baseline cardiac function at 3 wk of age (Fig. 5C). At day 7 after MI, MYH7-miR-195 TG mouse hearts showed fewer proliferating cardiomyocytes (Fig. 5 A and B and Fig. S3 B), more proliferating nonmyocytes (Fig. S3 C), and hypertrophic cardiomyocytes (Fig. 5 E and F). Echocardiography showed that MYH7-miR-195 TG mice had significantly depressed contractile function at day 21 after MI compared with wild-type mice (Fig. 5C). Moreover, histological assessment of the MYH7-miR-195 TG hearts 21 d after MI showed extensive anterior wall fibrosis extending to the apex and defective regeneration (Fig. 5D). Microarray analyses on injured WT and miR-195 TG mouse hearts indicated that miR-195 overexpression in cardiomyocytes was associated with the modest repression of a number of cell cycle and mitochondrial genes, and the induction of many inflammatory genes after MI (Fig. S4). These findings demonstrate that miR-195 overexpression in cardiomyocytes is sufficient to impair the regenerative response of the 1-d-old mouse heart resulting in adult-like remodeling after MI.
time (Fig. 6 E and F and Fig. S5 B and C), indicating that postnatal inhibition of the miR-15 family promoted myocardial regeneration. Because of the lack of cardiomyocyte specificity of the anti-miR administration protocol, inhibition of the miR-15 family was also associated with the robust induction of proliferation in the nonmyocyte compartment after MI (Fig. SSD), but the significance and therapeutic relevance of these non-cardiomyocyte effects are unclear. These findings suggest that postnatal inhibition of the miR-15 family prevents cardiomyocyte cell cycle arrest in a subset of cardiomyocytes and improves cardiac function after MI.

**Inhibition of the miR-15 Family Increases Adult Myocyte Proliferation and Improves Cardiac Function After Adult MI.** To determine whether postnatal inhibition of the miR-15 family can induce adult myocyte proliferation, we administered locked nucleic acid (LNA)-modified anti-miRs to neonatal mice by s.c. injection at postnatal days (P)1, 7, and 14 to inhibit the expression of the miR-15 family until adulthood (Fig. 6A and Fig. S5A). At P21, MI was induced by using an LAD ligation/reperfusion model. Given that the effects of inhibition of the miR-15 family on the coronary vasculature are uncharacterized, we used an ischemia-reperfusion model rather than permanent LAD ligation to better control the effects of the anti-miR treatment on the myocyte compartment. We found that inhibition of the miR-15 family was associated with similar infarct size between the two groups (Fig. 6 B–D). Moreover, inhibition of the miR-15 family from birth until adulthood resulted in induction of myocyte proliferation in the infarcted adult heart and a gradual improvement in left ventricular systolic function after MI over time (Fig. 6 E and F and Fig. S5 B and C), indicating that postnatal inhibition of the miR-15 family promoted myocardial regeneration. Because of the lack of cardiomyocyte specificity of the anti-miR administration protocol, inhibition of the miR-15 family was also associated with the robust induction of proliferation in the nonmyocyte compartment after MI (Fig. SSD), but the significance and therapeutic relevance of these non-cardiomyocyte effects are unclear. These findings suggest that postnatal inhibition of the miR-15 family prevents cardiomyocyte cell cycle arrest in a subset of cardiomyocytes and improves cardiac function after MI.

**Fig. 3.** Determining the lineage origin of newly formed cardiomyocytes. (A) Schematic of BrdU administration protocol. (B) Immunostaining showing colocalization of BrdU and Nkx2.5 at day 21 after MI. (Scale bar: 20 μm.) (C) Quantification of the number of BrdU+/Nkx2.5+ nuclei at day 21 after MI. Quantitative analysis represents counting of multiple fields from three independent samples per group (~9 fields per region). (D) Schematic of cardiomyocyte lineage tracing study design. (E Upper) β-galactosidase enzymatic staining of MYH6-MerCreMer; Rosa26-lacZ reporter mouse heart showing similar staining in sham and MI hearts at day 21 after surgery. Basal and apical sections (below ligature) are shown for each heart. (E Lower) Quantification of the percentage of lacZ+ myocardium in sham and MI hearts showing no difference across regions of the heart. Quantitative analysis represents counting of multiple fields from three independent samples per group (~9 fields per region). Values presented as mean ± SEM; *P < 0.05.

**Fig. 4.** Lack of regeneration of 7- and 14-d-old hearts after MI. (A) Masson’s trichrome staining of hearts infarcted at P7 showing fibrosis and lack of regeneration at 7, 14, and 21 d after MI. (B) Masson’s trichrome staining of hearts infarcted at P14 showing a lack of regeneration and significant pathological remodeling at 7, 14, and 21 d after MI. (C and D) Immunostaining and quantification of pH3+ cardiomyocytes and cardiomyocytes with disassembled sarcomeres at day 7 after MI showing lack of myocyte proliferation after MI at P7. Quantitative analyses represent counting of multiple fields from three independent samples per group (~9 fields per region). Values presented as mean ± SEM. (E) Ventricles from the body weight ratios for sham and MI groups at 21 d after surgery at either P1 or P14. Values presented as mean ± SEM; n = 5 per group; *P ≤ 0.005. (F) Cell size quantification showing no change in cell size at day 21 after MI at P1 and significant myocyte hypertrophy in the ischemic, border, and remote zones at day 21 after MI at P14. (G) Wheat germ agglutinin staining for sham and MI groups at 21 d after surgery at either P1 or P14. Quantitative analyses represent counting of multiple fields from three independent samples per group (~90 cells assessed per heart). Values presented as mean ± SEM; *P < 0.05. (Scale bars: A and B, 1 mm; C, 20 μm; G, 50 μm.)
All protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. Timed-pregnant ICR/CD-1 mice (Charles River Laboratories) were used to deliver pups for neonatal surgical procedures. MYH7-miR-195 transgenic mice were maintained on a mixed genetic background (12) and were bred to female ICR/CD-1 mice to deliver pups for neonatal surgeries.

Neonatal MI. MI surgeries were performed on neonatal mice (ICR/CD-1 strain; Charles River Laboratories) at P1 and P7. Neonates were anesthetized by cooling on an ice bed, as described previously (8). Lateral thoracotomy was performed, and the intercostal space was performed by blunt dissection of the intercostal muscles after skin incision. A tapered needle (C-1) attached to a 6-0 prolene suture (Ethicon) was passed through the midventricle below the origin of the LAD coronary artery and tied to induce infarction. The pericardial membrane remained intact after LAD ligation. Myocardial ischemia was indicated by the light pallor of the myocardium below the ligature after suturing. After LAD ligation, neonates were removed from the ice bed, thoracic wall incisions were sutured with a 6-0 nonaxorbable prolene suture, and the skin wound was closed by using skin adhesive. Sham-operated mice underwent the same procedure involving hypothermic anesthesia and thoracotomy without LAD ligation. No differences in the myocyte proliferative response were observed.

Conclusions
Although the majority of mammalian cardiomyocytes undergo cell cycle arrest shortly after birth, modest, but measurable, cardiomyocyte turnover occurs in adult mouse and human hearts (2, 3, 13–17). However, this myocyte turnover is insufficient for restoration of cardiac contractile function after injury. In contrast to the adult mammalian heart, the neonatal mouse heart has significant regenerative potential for the first few days of life. Our findings indicate that the neonatal mouse heart is capable of mounting a robust regenerative response after ischemic myocardial necrosis, which is mediated primarily through proliferation of preexisting cardiomyocytes.

In our genetic labeling studies of cardiomyocytes, only 70% of cardiomyocytes were labeled by lacZ expression, raising the possibility that 30% of unlabeled cardiomyocytes are derived from an unidentified stem or progenitor population. However, no fate mapping studies have been performed to date to examine this possibility. Although a recent report showed activation of a c-kit reporter after myocardial cryoinjury in the neonate (18), it is difficult to reach a conclusion about some minor role of progenitor cells in neonatal heart regeneration without carefully designed fate mapping studies. Although c-kit is expressed in a subset of cardiac progenitor cells, it is known that c-kit is also expressed during cardiomyocyte dedifferentiation (19, 20), and proliferation (21), which are known features of the neonatal cardiac regenerative response. Therefore, future studies using detailed analysis of the extent and mechanism of regeneration in the neonatal cryoinjury model are warranted.

Although a full understanding of the mechanism of neonatal heart regeneration remains elusive, the current report highlights a role of the miR-15 family in this process. Using both gain- and loss-of-function genetic and pharmacological approaches in the neonatal cardiac injury model, we have found that the miR-15 family, which inhibits cardiomyocyte proliferation and represses a number of cell cycle genes in the heart, modulates neonatal heart regeneration. These results suggest that postnatal up-regulation of the miR-15 family may be an important regulatory component of a molecular pathway that arrests cardiomyocyte proliferation and cardiac regenerative capacity after birth. However, there are several limitations to the transgenic and anti-miR experiments reported in our study that warrant further discussion and that will require future follow-up studies. For instance, a subset (22%) of MYH7-miR-195 transgenic mice are born with preexisting congenital heart abnormalities (12). These mice were excluded from our functional analyses and endpoints. The MYH7 transgene is activated during early embryonic cardiac development, and although cardiac function is normal in the majority of MYH7-miR-195 transgenic mice prior to injury, we cannot completely exclude a potential contribution of an underlying subtle pathology in these mice to the impaired regenerative phenotype after MI. Future studies using inducible transgenic mice or other technologies that allow for more precise temporal control of microRNA expression in vivo may help to resolve this question.

Members of the miR-15 family, like other microRNAs, engage a broad collection of mRNA targets including numerous cell cycle regulatory proteins and survival factors to exert their functions (22). Thus, the potential suppression of the regenerative response by the miR-15 family likely involves actions of numerous protein targets. Although the current study design points to a developmental contribution of the miR-15 family to regenerative arrest in the neonatal period, acute inhibition of this microRNA family after MI is obviously more therapeutically relevant experimental paradigm. In this regard, acute inhibition of the miR-15 family in adult mice is associated with improved contractile function after ischemia-reperfusion injury (22).

The current findings in neonatal mice suggest that therapeutic strategies aimed at restoring the proliferative potential of adult mammalian cardiomyocytes will be an important component of attempts to reactivate the dormant regenerative capacity of the adult mammalian heart after MI. The neonatal mouse model represents a useful tool for modulation of mammalian cardiac regeneration and for understanding the complex cellular and molecular mechanisms that govern cardiomyocyte proliferative capacity.

Methods
Experimental Animals. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. Timed-pregnant ICR/CD-1 mice (Charles River Laboratories) were used to deliver pups for neonatal surgical procedures. MYH7-miR-195 transgenic mice were maintained on a mixed genetic background (12) and were bred to female ICR/CD-1 mice to deliver pups for neonatal surgeries.
between sham-operated mice (hypothermic anesthesia and thoracotomy) and unoperated control mice. After surgery, neonates were warmed for several minutes under a heating lamp until recovery. The entire procedure lasted ~10 min. Ninety percent of sham-operated and LAD-ligated P1 and P7 neonates survived the surgical procedure, with all deaths occurring during or on the day of surgery. However, maternal cannibalization reduced survival rates in the MI group the following day to ~70%. For MI surgeries at P14, mice were anesthetized with isoflurane, followed by endotracheal intubation for ventilation by using a small animal ventilator (Harvard Apparatus).

**Adult MI.** Ischemia/reperfusion (I/R) surgeries were performed on 3-wk-old mice. After isoflurane anesthesia, the mice underwent endotracheal intubation and mechanical ventilation, followed by thoracotomy and I/R (45 min of ischemia induced by LAD ligation). The chest was closed, and the animals were extubated after recovery.

**Genetic Fate Mapping Study.** We crossed cardiomyocyte-specific MerCreMer mice [Tg(Myh6-cre/Esr*)1Jmk/J; The Jackson Laboratory] with Rosa26-lacz-flox-targeted mice (Gtrosa26tm1Sor; The Jackson Laboratory) to generate a tamoxifen-inducible cardiomyocyte reporter strain for genetic fate mapping (23). Induction of Cre recombinase activity and laZ reporter expression was achieved by administering a single 2-mg dose of tamoxifen (Sigma), dissolved in sesame oil (Sigma), s.c. to neonatal mice at birth, as described (8). The following day, neonates underwent LAD ligation. At 21 d after MI, hearts were harvested, embedded in tissue freezing medium and flash frozen in 2-methylbutane cooled on liquid nitrogen (Sigma). Cryosections (8 μm) of the myocardium below the ligature were stained with X-gal staining solution for 48 h at 37 °C to detect laZ activity. Sections were counterstained with nuclear fast red, and the percentage of myocardial tissue stained positive for laZ was quantified by using Image J software.

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