Editorial

Induction of cardiomyocyte proliferation, a new way forward for true myocardial regeneration?

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Heart failure and current therapies

Ischemic heart disease (IHD) and heart failure (HF) are major causes of morbidity and mortality worldwide and although acute treatment during myocardial infarction has improved significantly, chronic burden of the disease gained much attention recent years (1,2). The major cause of the failing hearts, however, the loss of contractile cardiomyocytes is currently still a problem to overcome. The only treatment modalities that are nowadays available for contractile dysfunction includes left ventricle assist devices (LVAD) and heart transplantation. Both are well accepted therapies but with different draw-backs for large patients groups, thereby pointing to the lack of enough donor hearts and the need for endured medication use, respectively (3).

Regenerative medicine is a direction in which the loss of contractile tissue is aimed to be replaced by the exogenous addition of progenitor or stem cells. Although originally having high expectations, which was implicated by scientific progress in preclinical models, and long-term, randomized clinical trials with good safety profiles for delivery of cells to the myocardium, regenerative therapy for cardiovascular disease had been inconsistent and shown modest efficacy thus far. Recent reviews have nicely illustrated current drawback and the need for improvements for these strategies, using cells or cell-derived products (4-6). Due to the lack of major steps forward other directions have been explored, boosted by the observations of Bergmann and his colleagues that cardiomyocytes from the human heart have a certain degree of renewal capacity (7). This is combination with the work of Poss, which demonstrated the potential of cardiomyocytes to divide, has pushed new regenerative strategies forward (8).

Cardiomyocytes renewal and the cell cycle

The cardiac regenerative capacity mainly depends on the ability of cardiomyocytes to proliferate after injury (9,10). Adult amphibian hearts can completely regenerate after apex resection through cell cycle re-entry of pre-existing cardiomyocytes in the infarct borderzone (9,11). In the mammalian heart, cardiomyocytes proliferate prenatally and withdraw from the cell cycle 7 days after birth (12). At 3 days after birth, cardiomyocytes retain a constant cell volume and are primarily mononucleated (13). This is in contrast to the abundance of multinucleated cardiomyocytes through DNA replication in the absence of cytokinesis 1–2 weeks after birth (13,14). Under basal conditions in the adult mammalian heart, proliferation of cardiomyocyte replaces the cardiomyocytes that die due to apoptosis at a rate of 0.5–1% a year (7). Although after cardiac injury the cardiomyocyte proliferation rate increases, it remains insufficient to regenerate the heart (15). The endogenous repair mechanisms that occur after injury in the neonatal and the amphibian heart do lead to complete restoration of the myocardium. Stimulating the same endogenous repair

mechanism in the adult mammalian heart, through the proliferation of pre-existing cardiomyocytes, can currently be considered the most feasible method to regenerate the heart in a clinical setting. The program that regulates cardiomyocyte cell cycle withdrawal remains largely unknown but is hypothesized to result from the relative hypoxia and the postnatal metabolic shift from anaerobic glycolysis to oxidative phosphorylation (16). This is in line with studies demonstrating that in amphibian hearts, cardiac regeneration depends on hypoxia signaling (17). Furthermore, the rare population of cycling cardiomyocytes in the adult mammalian heart were found to be located in hypoxic niches (18). Even though it might seem contradictory to the role of hypoxia in regeneration, the infarcted myocardium requires sufficient revascularization for functional regeneration to occur. The neonatal and zebrafish heart are heavily vascularized after cardiac injury and without vascularisation of the infarct area, regeneration ends prematurely (12,19).

To investigate the regulators of cardiac regeneration studies have analyzed the changes in expression of coding and the non-coding genes at p7 when the mammalian heart loses is capacity to regenerate. Analysis of the non-coding transcriptome in cardiac disease indicated the essential role of non-coding RNAs in cardiac biology and in epigenetic control of gene expression in the diseased heart (20,21). miRNAs regulate the expression of a target mRNA by binding to the 3'UTR and hereby either preventing the translation of the mRNA or aiding in its degradation. LncRNAs (>200 nucleotides) can regulate epigenetic gene silencing, bind to miRNAs, and function as protein scaffolds (22). Exploring the expression at P1, P3, P5, P7, and P10 showed differential expression of the 413 miRNAs and 545 lncRNAs in both endothelial cells and cardiomyocytes (23). A marked transition between P3 and P5 was found of 240 miRNAs including the increase in expression of miR-451, miR-195, and miR-22 and a decrease in expression of miR-6240. miR-451 was previously reported to protect erythrocytes against oxidative stress which could also be a potential explanation of its upregulation in cardiac development towards relative hyperoxia (24). miR-195 is member of the miR-15 family which has been described to be upregulated in the postnatal mammalian heart and contribute to postnatal cardiomyocyte cell cycle withdrawal (25). miR-22 induces cardiac senescence by preventing cardiomyocyte proliferation and by activating cardiac fibroblasts (26). Other miRNAs that have been associated with the induction of cardiomyocyte proliferation include miR-590, miR199a (27), miR-210 (28), and miR-133 (29). Recent evidence identified 96 miRNAs to be able to induce cardiomyocyte proliferation in human induced pluripotent stem cell derived cardiomyocytes (30). Sixty-seven of these miRNAs targeted the Hippo pathway through the repression of Hippo and the activation of Yap, suggesting this pathway might be a promising target for cardiac regenerative therapy.

miRNA-128, a new player that promotes cardiomyocyte proliferation

Recently in *Nature Communications*, Huang and colleagues reported a new player, microRNA-128 (miRNA-128), which is upregulated in cardiomyocytes during the postnatal switch from proliferation to terminal differentiation (31). miR-128 is an intronic miRNA, encoded by two isoforms; miR-128-1 and miR-128-2 (32). The pri-miR-128-1 gene resides within the R3H domain containing protein 1 gene (R3HDM1) and pri-miR-128-2 lies within the cAMP-regulated phosphoprotein, 21 kDa gene (ARPP21, also known as regulator of calmodulin signaling, RCS). This organization is conserved in human, rat, and mouse genomes (32). Early studies pointed to a role of miRNA-128 as a tumor suppressor, but others found a role in neural and brain development and in behavior disorders (33-35). In the myocardium, however, the expression of miR-128 is low during early developmental stages but is increased during postnatal heart growth.

The cardiac-specific overexpression of miR-128, via a tet-off transgenic mouse system, impaired cardiac homeostasis by inducing a progressive heart mass and thereby to increased heart-to-body weight ratios. Detailed analyses suggested that cardiomyocytes increased in size, leading to a hypertrophic response and thereby to a reduced fractional shortening and ejection fraction. More detailed analyses displayed that cardiomyocyte proliferation was reduced upon overexpression of miR-128, with no effect on apoptosis rates, thereby suggesting an early cell cycle exit for cardiomyocytes. Interestingly, the cardiac-specific deletion of miR-128 in both *in vitro* cultured neonatal cardiomyocytes as *in vivo* mouse hearts resulted in an increased dedifferentiation of cardiomyocytes and increased proliferation rates. Interestingly, although the number of cardiomyocytes was larger, being smaller in size, the mice developed normally and exhibited no cardiac dysfunctions. Confirmatory, the overexpression of miR-128 in neonatal mice at P1 inhibited cardiac regeneration upon myocardial

apex resection, whereas deletion of miR-128 in adult mice promoted cardiac regeneration upon ischemic injury. The authors further explored putative targets and suggest a role for Suz12, thereby affecting histone modifications and p27, and promoting cardiomyocytes into the S-phase to divide.

Potential impact and future directions

miR-128 was previous being reported as a neuronal-enriched miRNA, but also involved in cardiac repair of lower vertebrates such as the newt (36). In these species, however, the effects observed where mainly attributed to proliferation in the non-cardiomyocyte populations and on extracellular matrix deposition in which inhibition of miR-128 led to these effects. Although the authors suggest this might be due to differences in complex of the heart cell types between the species, it also points towards some concerns for translation of these findings regarding miRNA therapeutics for patient care if we cannot control potential off-target cell effects (31).

In the past few years, several types of miRNA inhibitors have been developed such as microRNA antagomiRs, antimiRs and LNAs which have been shown to be very effective in inhibiting their specific targets. We and many others labs have shown that these microRNA inhibitors demonstrate much longer effects than conventional pharmaceutics (37). This long-lasting effect will definitely increase the possibility of side-effect, especially in this case of miR-128, which is a potent cell cycle regulator with known effects in other cell types and organs. For these reasons, there is a strong need to develop new methods to increase miRNA therapeutic delivery, prevent off-target cellular uptake and, in the case of miR-128, increase cardiac-specific uptake. Our lab has further developed a local delivery technology called ultrasound-triggered microbubble destruction (UTMD) for microRNA delivery. In this technology, positive charged gas-filled microbubbles can be loaded with negative charged microRNA inhibitors, antimiR or antagomirs. By locally applying ultrasound, gas-filled microbubbles can be destructed and thereby deliver their cargo to the targeted area (38). Using this technology, we have been able to significantly increase local delivery of antagomir to the healthy heart with minimum side-effects. Interestingly, similar results were also observed in a myocardial infarction and reperfusion model, even without ultrasound treatment. This is probably caused by the fact that the inhibitors can be enriched in the infarcted region through local injury-induced vascular leakage, which is a possible mechanism how ultrasound triggered microbubble destruction delivers its cargo too (39). Another possible strategy to achieve targeted delivery will be nanovectors which are armed with cardiac homing peptide. Inspired by the observation that post myocardial infarction AT1 levels are increased, Xue *et al.* have developed a nanovector with AT1 targeting peptides on the surface. Armed with miR-1 inhibitors, this nanovector can significantly inhibit miR-1 induced apoptosis in the infarcted region (40). Although only a few cases have been successful to specific delivery miRNAs into the myocardium, we believe with the continuous efforts to develop new delivery vehicles such as polymers, lipid nanoparticles, and extracellular vesicles with difference formulations in combination with difference targeting strategies, it is possible to take full advantage of microRNA therapeutics while minimize their potential side-effect.

Conclusions

The growing burden of chronically diseased cardiovascular patients that develop HF needs new treatment modalities to restore contractile properties of the myocardium. Although attempts to minimize the damage to the cardiac tissue in acute settings is successful, still new approaches are needed. Cardiac regenerative medicine offers potential inspiring strategies, including the use of endogenous or exogenous injection of progenitor cell population. Since the simple injection of cell types is hampered by retention of high cell numbers, other options are explored as well, including the stimulation of post-natal cardiomyocyte proliferation. These novel approaches are extremely exciting, especially by the use of miRNA therapeutics, which can lead to long-lasting and potent effects. The future directions of these approaches are aiming to further explore the potential of the identified molecules and improve local targeting and timed exposure of the therapeutics to reduce costs and toxic effects and, maybe of most importance for the stimulation of cell division, prevent off-target effects in other cell types.

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