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Targeting the miRNA-106b-25 cluster as a potential regenerative therapeutic approach for myocardial injury

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Background: Myocardial infarction (MI) causes cell death and triggers hypertrophic remodeling of surviving cardiomyocytes, resulting in heart failure. A therapeutic approach aiming at inducing cardiomyocyte proliferation and inhibiting hypertrophy.

Hypothesis: Overexpression of the miR-106b-25 cluster to potentially target a network of negative cell cycle regulators, as well as series of pro-hypertrophic factors, indicating this microRNA cluster to possess a central role in recovering the heart muscle after myocardial injury.

Methods and Results: The miR-106b-25 cluster contains 3 co-expressed miRNAs: miR-106b, miR-93 and miR-25. Experiments in primary cardiomyocytes demonstrated that inhibition of miR-106b, miR-93 and miR-25 increases cell size, while overexpression of the miR-106b-25 cluster inhibited phenylephrine (PE)-induced hypertrophy. By performing fluorescent EdU labeling, we demonstrate that the miR-106b-25 cluster stimulates cardiomyocyte proliferation. Also in vivo overexpression of miR-106b, miR-93, and miR-25 using adenovirus-associated virus 9 (AAV9) in neonatal mice induce an increased incorporation of EdU into cardiomyocytes, combined with increased levels of the proliferative markers Aurora-B and phospho-histone 3 (pH3), while cardiomyocyte size was not affected. In line, in adult mice, AAV9-miR106b-25 treatment increases heart mass without inducing hypertrophy, as indicated by both WGA staining and RT-qPCR experiments measuring transcript abundance of the hypertrophy genes Nppa, Nppb and Myh7, showing no difference between the experimental groups. To test the regenerative potential of myocyte proliferation secondary to AAV9-miR106b-25 gene transfer, mice were randomized to receive a control AAV9-MCS (empty vector) or AAV9-miR106b-25 and sham surgery or myocardial infarction (MI). Echocardiographic measurements demonstrate improvement of cardiac function after MI upon AAV9-miR106b-25 treatment versus AAV9-MCS, which is associated with a reduced infarction size and a clear decrease in fibrotic damage, as shown by Sirius-Red staining. Finally, to unravel the underlying mechanism by which the miR-106b-25 cluster stimulates cardiomyocyte proliferation, we performed luciferase-reporter assays, which identified negative cell cycle inhibitors Cdk1a, Cdk1c, and E2F5 as direct downstream targets.

Conclusion: Our data demonstrate that AAV9-miR106b-25 treatment reduces scar formation and improves cardiac function after MI by stimulating cardiomyocyte proliferation and inhibiting hypertrophic remodeling. These findings might give rise to a new regenerative approach aiming at healing the cardiac muscle after myocardial injury.

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An allogeneic bioengineered myocardial graft limits infarct size and improves cardiac function: pre-clinical study in the porcine myocardial infarction model

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Purpose: There are currently no efficient strategies to prevent chemotherapy-induced heart disease because of limited understanding of the underlying molecular mechanisms. We demonstrated previously that phosphoinositide 3-kinase gamma inhibition prevents pressure overload-induced heart failure. Therefore, in this study, the cardioprotective effects of PI3K-gamma inhibition were tested in a murine model of cardiomyopathy induced by the chemotherapeutic agent doxorubicin (DOX).

Methods: Mice expressing a kinase inactive PI3K-gamma selective inhibitor (PI3K-gamma kinase-dead; KD) and their wild-type counterparts (WT) were exposed to DOX administration (a cumulative dose of 12 mg/kg, 4 mg/kg i.p. at 0, 7 and 14 days). Another group of WT mice was pre-treated with a PI3K-gamma selective inhibitor (AS-605240; 10 mg/kg) before each DOX injection. Echocardiography was used to evaluate heart function 6 weeks after the first DOX injection. Cardiomyocyte apoptosis, collagen deposition, cardiac surface area of allogeneic swine after MI induction. Cardiac function was assessed using magnetic resonance imaging. Infarct size, scar healing, and vascularization were evaluated using quantitative morphometry and histopathology. Animals were sacrificed one month post-MI.

Results: Compared to control-MI animals, EMG-treated animals exhibited significantly higher left ventricular ejection fraction relative to baseline (3.26 ± 2.85 vs. 7.97 ± 4.29, P=0.047) and post-MI (61.30 ± 3.07% vs. 50.76 ± 2.62%, P=0.001). Relative to control-MI animals, EMG-treated animals had a 68% smaller infarct size (2.46 ± 0.60% vs. 7.63 ± 2.26%, P=0.048) and significantly lower type IIII collagen ratio (2.26 ± 0.72 vs. 13.01 ± 6.85, P=0.032). GFP + pATDPCs migrated to underlying ischemic myocardium and expressed cardiac lineage markers (GATA4, MEF2, SERCA2, connexin43, and cTnI) and endothelial makers (IsOB8 and CD31). Newly-formed blood vessels were connected to host myocardium. EMG promoted increased infarct myocardium vascularization, with blood vessel densities of 3.64 ± 0.50% for EMG-treated animals and 1.72 ± 0.12% for control-MI animals (P=0.01).

Conclusions: In the pre-clinical swine MI model, the newly developed EMG improved cardiac function and reduced myocardial neovascularization, reduced infarct size, and limited fibrosis progression. This scalable allogeneic EMG is ready for clinical translation.

Figure 1. Improvements promoted by EMG
morphological alterations and signaling transduction were studied by TUNEL assay, Electron microscopy and Western blot assay.

**Results:** Survival in the KD mice was significantly improved compared to the WT counterparts (% mortality: WT DOX: 50%; KD DOX: 20%; *p < 0.05), within 6 weeks from the first DOX administration. This was paralleled by preserved left ventricular systolic function in KD animals while WT mice suffered severe systolic impairment (% FS WT DOX: 20.5 ± 1.9; KD DOX: 36.6 ± 2.7, ***p < 0.001). In line with these findings, cardiac atrophy, cardiomyocyte apoptosis and collagen deposition were significantly lower in KD than in WT hearts. Importantly, pharmacological inhibition of PI3Kγ by AS-605240 improved systolic function and survival in DOX-treated WT mice (% FS WT DOX: 23.4 ± 3.7; WT + AS DOX: 38.9 ± 1.9, ***p < 0.001). Mechanistically, PI3Kγ was found to serve as a negative regulator of cardiomyocyte autophagy, through the phosphoAkt/mTOR signaling axis. Autophagy was more pronounced in DOX-treated KD hearts than in WT counterparts, as evidenced by increased expression of the autophagy marker LC3II. The enhanced autophagy was correlated with ultrastructural preservation of KD cardiomyocytes, while WT hearts displayed marked mitochondrial damage and vacuolization. Intriguingly, PI3Kγ inactivation fully prevented DOX-associated systolic failure and synergistically enhanced the anti-tumor activity of the drug, in both 4T1 and Hei2/TuNeT transgenic breast cancer models. This is beneficial from the role of PI3Kγ in regulating inflammation, as tumor-associated macrophage polarization from M1 (tumor suppression) to M2 (tumor promotion) was dampened by PI3Kγ inhibition.

**Conclusions:** Thus, targeting PI3Kγ may serve as a potential strategy to reduce the cardiotoxicity and enhance the anti-cancer effect of anthracyclines.

43 Functional screening of microRNAs identifies miR-22 as a regulator of cardiac autophagy and aging

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**Purpose:** Aging individuals are more prone to incidences of myocardial infarction and heart failure. Cardiac remodelling post myocardial infarction leads to progressive impaired cardiac function and is caused by a variety of several processes including deranged autophagy. MicroRNAs are known to be a key player in cardiovascular disease but their involvement in cardiac autophagy and ageing is less understood.

**Method and Results:** We exploited a high-throughput FACS-based LC3-GFP detection method to measure the autophagic flux in cardiomyocytes after transfection of precursor microRNA library consisting of 380 miRNAs. Together with additional expression screenings, we identified miR-22 to be an abundant and strong inhibitor of the cardiac autophagy process. Cardiac MI-22 expression levels increased during ageing of mice as well as in ageing neonatal cardiomyocytes in vitro by a PS3-dependent mechanism. This was paralleled by accumulation of p62, a marker for lower autophagic activity. Inhibition of miR-22 in ageing cardiomyocytes in vitro activated autophagy and inhibited cellular hypertrophy. PS3 inhibition activated autophagy and led to atrophy in cardiomyocytes, but this was reversed by miR-22 overexpression. Pharmacological inhibition of miR-22 post myocardial infarction in aged mice, activated cardiac autophagy, prohibited post infarction remodelling and led to improved cardiac function compared to controls. Interestingly, similar effects were significantly less pronounced when studies were done in younger mice with significantly lower cardiac miR-22 expression levels. PPARα with a more pronounced disorder when induced at earlier stages.

**Conclusion:** Based on our data, we conclude miR-22 to be an important regulator of cardiac autophagy and a potential therapeutic target to treat cardiac remodelling post infarction especially in the aged myocardium. Finally, circulating miR-22 provides prognostic information for patients post myocardial infarction highlighting miR-22 as a promising therapeutic and biomarker candidate for cardiovascular disorders.

44 Functional defects and molecular mechanisms of left ventricular non-compaction in nko2.5 mutant mice

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Left Ventricular Non-Compaction (LVNC) is a rare cardiomyopathy, characterized by hypertrodevalution and deep trabecular recesses in the left ventricle. This genetic disorder, associated with several mutations, exhibits clinical, morphological and functional heterogeneity. The main complications are heart failure, systemic embolic events and malignant arrhythmias. It is still unclear whether LVNC results from a defect occurring during cardiac development. One hypothesis to consider is that the severity of LVNC depends on which embryonic stage the arrest of myocardial compaction occurs. Our aim was to study the pathological evolution of LVNC by characterizing functional defects and identifying molecular mechanisms in mouse models with abnormal ventricular trabecular development.

To establish a LVNC mouse model, we performed conditional inactivation of Nkx2.5 in ventricular trabeculae using tamoxifen-inducible Cre+O-CreERT2 mice. We used deletion at embryonic day E10.5/E11.5 when ventricular trabeculae form or at E13.5/E14.5 when the myocardium condenses. To quantify the degree of non-compaction, we carried out immunofluorescence analyses on left ventricular sections. We also performed a thymine Masson coloration to quantify the subendocardial and interstitial fibrosis present in Nkx2-5 mutant hearts. Functional analyses show defects in ECG recordings with increased PR and QRS durations. This phenotype worsens with age with bundle branch blocks and a diminution in the ejection fraction at 6 months following deletion at E10.5/E11.5 but not E13.5/E14.5, consistent with a more pronounced phenotype after deletion at the earlier developmental stage. Microarray measurement of the transcriptomes of 6-month-old mice with different LVNC levels induced by Nkx2-5 deletion at the two timepoints, revealed differences in ventricular gene expression between the two groups, predominantly in pathways involved in calcium signaling, blood vessel development and immune processes. Deregulated expression of numerous genes playing a crucial role in cardiac cell activity (action potential, contraction) or cardiomyocyte differentiation correlates with the different phenotypes observed in these mutant mice. This study demonstrates that deletion of Nkx2-5 during trabecular development represents a good mouse model for studying the cellular and molecular mechanisms associated with LVNC. Furthermore, our results show a differential phenotype associated with the timing of deletion of Nkx2-5 with a more pronounced disorder when induced at earlier stages.

45 PITX2 modulates atrial membrane potential, potentiating the antiarrhythmic effects of sodium channel blockers

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**Background:** Antiarrhythmic drugs are variably effective in patients. The mechanisms modifying their effectiveness are unknown. Recent data suggest that PITX2 expression may have an important role in regulating gene expression and electrical function of the adult left atrium.

**Purpose:** To determine whether overexpression of PITX2 expression modulate the effects of anti-arrhythmic drugs on left atrial electrophysiology.

**Methods and Results:** Atrial PITX2 mRNA expression varied markedly in 95 AF patients receiving rhythm control therapy. In murine hearts with reduced Pitx2c expression (Pitx2c+/− mice), flecainide (1µmol/L) was remarkably more effective in suppressing atrial arrhythmias (6/18 base vs 0/15 Flec, p < 0.05) and induced marked post-repolarisation refractoriness (PPI, Pitx2c+/− 21 ± 3 ms, n=6 vs WT 6 ± 4 ms, n=6, p < 0.01). Resting membrane potential was more depolarized in Pitx2c+/− atria (WT -70 ± 0.7 mV, n=30 cells vs Pitx2c+/− -48 ± 0.7 mV, n=32 cells, p < 0.05), and TASK-2 gene and protein expression were decreased. This resulted in more effective sodium channel inhibition in isolated atrial cardiomyocytes. Equalizing holding potentials replicated the increased effectiveness of flecainide in blocking human Nav1.5 channels in HEK293 cells. Computer modelling showed a similar effect when resting membrane potential was slightly increased in the Courtemanche-Nattel model.

**Conclusions:** PITX2 can modulate atrial resting membrane potential with important effects on sodium channel inhibition by flecainide. PITX2 and ion channels regulating the resting membrane potential may provide novel targets for antiarrhythmic drug development and companion therapies in AF.