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The involvement of TWEAK and FN14 in murine autoimmune myocarditis
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Background: Myocarditis, defined as an inflammation of the myocardium, can be caused by autoim-mune and infectious mechanisms. To this end, we have generated a mouse model of experimental autoimmune myocarditis (EAM). Little is known about the role of the innate immune system in the pathogenesis of this autoimmune disorder. However, it was already observed that the cytokine tumor necrosis factor-like weak inducer of apoptosis (TWEAK) and its receptor fibroblast growth factor-inducible 14 (FN14) play a pivotal role in different inflammatory diseases. Thus, we studied the involvement of TWEAK and FN14 in the development of inflammatory cardiomyopathy.

Methods and Results: Aj mice were immunized with cardiac troponin I (cTnI) to induce an EAM followed bycardiomyopathy, fibrosis and reduced ejection fraction (EF). To determine the severity of myocardial damage, hs-TnT levels were measured. Here, mice lacking FN14 (FN14-/-) displayed a significantly lower hs-TnT level compared to wild type (wt) littermates (FN14-/-: 625 ± 193 pg/mL vs. wt: 3119 ± 1047 pg/mL). Furthermore, transcardiac echocardiography showed an improved EF (FN14-/-: 79 ± 3% vs. wt: 75 ± 3%). Histological examination of heart sections demonstrated less inflammation and fibrosis of the myocardium in FN14-/- mice (inflammation score: FN14-/-: 2.64 ± 0.39 vs. wt: 3.56 ± 0.53; fibrosis score: FN14-/-: 2.46 ± 0.55 vs. wt: 3.22 ± 0.66). Moreover, lower cTnI antibody titers were detectable in ELISA.

In contrast, TWEAK-/- mice displayed a higher hs-TnT level (TWEAK-/-: 3749 ± 442 pg/mL vs. wt: 1171 ± 499 pg/mL), a decreased EF (TWEAK-/-: 79 ± 2% vs. wt: 84 ± 2%), severe inflammation and fibrosis (inflammation score: TWEAK-/-: 3.4 ± 0.6 vs. wt: 2.57 ± 0.53; fibrosis score: TWEAK-/-: 3.29 ± 0.75 vs. wt: 2.43 ± 0.57) and higher cTnI antibody titers compared to wt littermates.

Conclusion: TWEAK and FN14 may play an important role in the pathogenesis of autoimmune myocarditis. While the absence of FN14 led to a better disease outcome, mice lacking TWEAK are heavily affected by cTnI immunisation and showed an increased impairment of cardiac function. This result refers to a TWEAK-independent FN14 signalling cascade, which seems to be involved in disease development. Thus, inhibition of FN14 might represent a novel therapeutic strategy in the treatment of inflammatory cardiomyopathy.

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Sympathetic neurons that innervate the heart locally modulate cardiomyocyte trophic and electrophysiological properties
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Purpose: The myocardium is highly innervated by Sympathetic Neurons (SNs), that distribute within the tissue with a well-defined pattern. We have previously shown that neuronal input to cardiomyocytes (CMs) represses proteolysis and activates protein synthesis, through the β2-AR dependent signaling. We here tested the hypothesis that regional differences in SN distribution reflect on heterogeneity in CM protein turnover.

Methods: In situ CM immunochemistry was performed in situ on cryosections from wild type and SN ablated hearts. CM size, CM protein turnover and CM proteolysis were assessed by labelling with antibodies specific for the CM component and by immunoblotting, respectively. CMs were classified into three groups based on the innervation pattern as in the heart of other mammals, including humans, characterized by different SN distribution, CM size follows neuronal density. Moreover, using cardiac optogenetics in denervated hearts, we observed changes in the transmural electrophysiology and increased arrhythmia vulnerability, suggesting that the control of structural CM properties, the physiologic innervation pattern is fundamental to allow normal cardiac electrophysiology.

Conclusion: This is the first evidence, to the best of our knowledge, of an otherwise homogeneous tissue, shaped, after development, by a superimposed innervation network, through the modulation of cellular protein turnover.

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W4R variant of CSR3P leads to the expression of a novel alternate reading frame protein due to alternative splicing
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Background: CSR3P or MLP protein is expressed in striated muscle and localizes, among others, to the sarcomere Z-disc and nucleus. CSR3P interacts with telethonin and α-actinin, acting as cardiomyocyte mechanical stretch sensor, and plays important roles in the regulation of sarcomere architecture. Several cardiomyopathies have been associated with various CSR3P mutations. Among those, we previously demonstrated that W4R missense mutation (10TtoC in exon 2) in the CSR3P gene leads to dilated and hypertrophic cardiomyopathies in patients and experimental animal models.

Aim: We investigated the molecular interplay between the W4R mutation and development of heart failure. Particularly, we focused on CSR3P transcriptional regulation, showing that W4R mutation promoted CSR3P alternative splicing and the translation of a novel protein: ARF-CSR3P.

Results: The CSR3P gene consists of 6 exons which encode a 21 kDa protein. RT-PCR analysis performed on Csrp3W4R/- and Csrp3W4R/W4R mice revealed the presence of different CSR3P splice variants. Notably, a CSR3P mRNA missing exon 2 (2ΔmRNA) is highly prevalent. Minge experiments conducted in various different cell lines, including neonatal rat cardiomyocytes, confirmed alternative splicing and skipping of exon 2 in the presence of 10TtoC substitution. Interestingly, alternative splicing also impairs translation of the mature CSR3P protein. Indeed, splicing affects wild type CSR3P, leading to its degradation through the ubiquitine proteasome system, which is rescued after treatment with MG132 (10 μM). Finally, generation of antibodies against ARF-CSR3P, which has been deduced from the 2ΔmRNA, led to the identification of this protein. Animals over-expressing ARF-CSR3P develop heart failure reminiscent of what can be observed in human mutation carriers.

Conclusions and future perspectives: CSR3P or MLP protein is implicated in dilated and hypertrophic cardiomyopathies and as- treated heart failure via multiple molecular mechanisms, including a splicing defect leading to the expression of a novel ARF-CSR3P mRNA and protein, loss of CSR3P protein, defects in protein /protein interaction and mislocalization of the mature protein. Therefore, a single mutation may cause disease via multiple mechanisms and hence explain different phenotypes in different individuals.

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Glucocorticoid intervention prenataly: effects on fetal heart maturation
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Glucocorticoids are routinely administered to pregnant women at risk of pre-term delivery to mature fetal organs and improve neonatal survival. We have shown that glucocorticoid action is essential to
mature the fetal heart. Here, we tested the hypotheses that (i) antenatal glucocorticoid exposure, prior to the normal increase in glucocorticoid levels, will advance fetal heart maturation and (ii) this will depend on cardiovascular glucocorticoid receptor (GR).

Male SMGRKO mice, which have Sm22α-Cre mediated deletion of GR in cardiomyocytes and vascular smooth muscle cells (VSMC), were crossed with female control (Cre-) mice to generate SMGRKO and control fetuses within the same pregnancy. Dexamethasone (Dex, 10μg/kg/d) or Vehicle (Veh) was administered in the drinking water of pregnant dams from E11.5 (n=3-4 dams/group), 3d prior to the initiation of fetal glucocorticoid synthesis. In utero high frequency ultrasound was performed at E15.5.

Initial results show that dexamethasone increased isovolumetric contraction time (indicating impaired systolic function) in control fetuses (Dex, 36.3±6.1ms vs Veh,28.1±4.6ms, p<0.01). Heart rate was also increased by Dex treatment in control (Dex, 191±20 bpm vs Veh, 172±17 bpm, p<0.05), but not in SMGRKO mice (Dex214±0.7 bpm vs Veh,221±72 bpm, p=0.5).

Comparison of Veh treated SMGRKO and control mice showed no differences in E/A wave ratio, a marker of cardiac maturity (SMGRKO +Veh, 0.3±0.03 vs Con +Veh, 0.4±0.06, p=0.49). However, in Dex treated mice, E/A wave ratio was lower in SMGRKO mice than in controls (SMGRKO +Dex,0.3±0.01 vs Con +Dex,0.4±0.01, p<0.01), suggesting greater cardiac immaturity in SMGRKO mice than controls following exogenous glucocorticoid treatment. This finding is consistent with previous findings that glucocorticoids effects on E/A wave ratio are independent of cardiovascular GR.

Precocious glucocorticoid exposure may impair fetal cardiac systolic function via activation of cardiovascular GR. It may also impair cardiac maturation via actions that are independent of cardiovascular GR. These could be mediated via GR elsewhere, via MR activation by endogenous corticosterone (in face of removal of competing GR) or via maternal or blood pressure effects. However, this requires further investigation and experiments are ongoing. Nevertheless, these data suggest that early exposure to potent synthetic glucocorticoids antenatally, prior to the normal increase in endogenous glucocorticoids, may be detrimental to cardiovascular health.

249 Uncoupling of myofilament Ca2+ sensitivity from troponin I phosphorylation by hypertrophic and dilated cardiomyopathy mutations can be reversed by EGGC and related Hsp90 inhibitors.

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Introduction: Heart muscle contraction is regulated by Ca2+- and modulated via the β-adrenergic response that leads to phosphorylation of Troponin I (TnI) at Ser22/23, which changes the Ca2+-sensitivity of the cardiac myofilament. Previously, it has been shown that mutations in sarcomeric proteins that cause Dilated Cardiomyopathy (DCM) or Hypertrophic Cardiomyopathy (HCM) abolish the relationship between TnI phosphorylation and Ca2+-sensitivity. We have termed this “uncoupling”.

Ca2+-desensitisers (such as Epigallocatechin-3-gallate (EGGC) and Silybin) act upon troponin and alter the Ca2+-sensitivity of the myofilament but their relationship with TnI phosphorylation has never been studied before. Using the in vitro motility assay (IVMA) we have found that Ca2+-desensitisers can reverse or “recouple” this relationship.

Purpose: To test the ability of EGGC and related compounds to reverse the uncoupling effect of DCM and HCM mutations.

Methods: The in vitro motility assay was mainly used in this study.

Results: EGGC decreased Ca2+-sensitivity of phosphorylated and unphosphorylated wild-type thin filaments equally retaining the coupling. In contrast, in thin filaments with the DCM-causing TPM1 E40K mutation, Ca2+-sensitivity was uncoupled. EGGC reduced Ca2+-sensitivity of phosphorylated but not unphosphorylated thin filaments containing DCM or HCM-causing mutations, reversing the uncoupling effect of the mutation. Thus, in the presence of EGGC, Ca2+-sensitivity dependence on phosphorylation is restored (re-coupling). The same pattern was observed with four other DCM mutations (TPM1 E54K, TNNC1 G159D, TNNI3 K364Q, ACTC E361G).

For the HCM-related mutation TNN2T K280N mutation, troponin from a patient with this mutation showed no difference in Ca2+-sensitivity when compared with donor heart troponin and the Ca2+-sensitivity was also uncoupled. EGGC was able to restore coupling to this mutation and to other mutations in TNN2T (114, 728±7, R92Q, 'E160, S179F and K273E), TPM1 (E180G) and ACTC (E99K).

However, EGGC is promiscuous and has many off-target effects and so an alternative compound is needed. 30 EGGC and Silybin related drug compounds were studied using IVMA for their ability to recouple. Some of these analogues indicated potent recoupling properties and their effects were preserved across three mutations causing DCM or HCM. As both EGGC and Silybin inhibit heat shock protein 90 (Hsp90), preliminary investigations were made into 4 other Hsp90 inhibitors (Ravatrin, Geldanamycin, Novobinin and Pterostilbene), they were all found to be recouplers. The Ca2+-desensitisising and recoupling effects of EGGC were also seen in contracting myofibrils.

Conclusion: The effect of EGGC and its analogues demonstrates that it is possible to reverse the pathological defects in troponin caused by DCM and HCM mutations pharmacologically and has the potential for treatment.

250 Investigating inherited HCM caused by SC02 and PRKAG2 mutations using the patients’ induced pluripotent stem cell-derived cardiomyocytes.

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Mutations in PRKAG2 gene encoding the γ subunit of AMPK cause hypertrophic cardiomyopathy (HCM) and familial Wolf-Parkinson-White syndrome (WPW). Patients with R302Q mutation in PRKAG2 suffer from sinus bradycardia, escape rhythms, atrial fibrillation and supraventricular tachycardia. This mutation affects AMPK activity and causes elevated glycogen storage in cardiomyocytes. The link between glycogen storage and WPW syndrome, HCM and arrhythmia remains unknown. A mutation in SC02 gene encoding for mammalian cytochrome-c oxidase, a crucial part of the mitochondrial electron transport-chain, causes HCM and infants death. To investigate the pathological mechanisms underlying these HCM-causing mutations and search for novel pharmaceutical and genetic therapeutic modalities, we generated induced Pluripotent Stem Cells-derived cardiomyocytes (iPSC-CMs) from patients’ somatic cells, attempting to recapitulate the disease phenotype in vitro. The diseases we explored are: (1) HCM with familial WPW by caused by R302Q mutation in PRKAG2 gene. (2) Concentric HCM caused by G1925 mutation in SC02 gene. Successful reprogramming of respective patients’ skin-derived fibroblasts resulted in iPSC-CMs expressing R302Q or G1925 mutations. Action potentials were recorded from cardiomyocytes and extracellular electrograms from beating cardiomyocytes clusters using patch clamp and Micro Electrode Array (MEA) techniques, respectively. [Ca2+]i transients and contractions were recorded by means of fura-2 and video edge detector, respectively. The major findings were: (1) PRKAG2 mutated iPSC-CMs exhibited spontaneous delayed-afterdepolarizations (DADs), slow firing rates and irregular rhythms (the latter two at the single cell and network level). Further, these phenomena were intensified with culture age, suggesting inter-relations between glycogen storage and electrophysiological abnormalities. (2) SC02- mutated iPSC-CMs exhibited attenuated inotropic response to isoproterenol as well as DADs and irregular rhythms. Importantly, transmission electron microscopy analysis of SC02-mutated iPSC-CMs displayed abnormal mitochondria size and morphology. Conclusions: PRKAG2 and SC02 mutated iPSC-CMs displayed abnormal functional features resembling the clinical phenotype expressed in patients carrying the mutations. In these cases of life threatening arrhythmias the cause is neither mutations in structural proteins nor ion channels; the cause for arrhythmias involved with hypertrophic cardiomyopathy, here, lies within mutated metabolism regulators.