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Inhibiting mitochondrial fission with Mdivi-1 directs cardiac differentiation of human induced pluripotent stem cells via protein kinase CK2
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Introduction: Mitochondria are morphologically dynamic organelles which are constantly undergoing fusion and fission, processes essential to maintain the organelle fidelity and these also determine cell fate. Human induced pluripotent stem cells (iPSCs) showed fragmented mitochondria, and we set out to explore whether inhibiting mitochondrial fission affects differentiation of iPSCs, using Mdivi-1, a known inhibitor of mitochondrial fission protein DRP1.

Aims: To examine the potential of Mdivi-1 to promote cardiac differentiation of human iPSCs.

Methods: Cardiac differentiation of human iPSCs (iPSc(foreskin)-2 and CERA007c6 cell lines) was induced by forming embryoid bodies (EB) for 6 days, which were then transferred to gelatin-coated plates. iPSCs were treated with either DMSO or 5 µM Mdivi-1 with or without 5 µM TBCA (a selective inhibitor of protein kinase CK2) during EB formation. End point analysis includes mitochondrial morphology using MitoTracker red staining, the percentage of beating EBs, expression of cardiac markers by RT-qPCR, calcium imaging by fluorescence Flu-4 dye, electrophysiological behaviour by micro-electrode arrays and protein kinase profiling by KINOMEscan assays.

Results: Mitochondria in undifferentiated iPSCs were mainly fragmented with perinuclear localisation, while mitochondria in differentiated cardiomyocytes appeared elongated and distributed throughout the cytoplasm. Treatment of iPSCs with Mdivi-1 during EB formation resulted in a 3-fold increase in beating EBs. The beating cardiomyocytes expressed several key cardiac markers, cyclic calcium ion and were responsive to isoprenaline and carbachol. When screened against a panel of 468 protein kinases, which covers over 80% of the human kinome, Mdivi-1 showed only one candidate kinase target – CSN1S2A (protein kinase CK2α). Western blotting analysis showed a significant increase in OXG6 protein expression in EBs treated with Mdivi-1, and treatment with TBCA attenuated the cardiogenic effect of Mdivi-1.

Conclusion & significance: Mdivi-1 can significantly enhance differentiation of human iPSCs into functional cardiomyocytes. These cardiomyocytes can be used for drug screening, disease modelling or for treatment of heart disease. Mdivi-1 is a highly selective inhibitor that shows a clinically safe profile. This inhibitor allows timely and short-term manipulation of mitochondrial morphology for benefit in cell differentiation.

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A novel role of tristetraprolin in preventing mitochondrial dysfunction in the heart against iron deficiency by optimizing expression of Rieske iron-sulfur protein
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Background: Patients with severe iron deficiency generally have normal cardiac function. Considering that iron is a vital molecule for normal cellular function, a mechanism to protect the heart against iron deficiency must exist. We recently discovered that an mRNA binding protein tristetraprolin (TTP) is activated in iron-deprived cells independently of the canonical iron regulatory proteins. The yeast homolog of TTP has been shown to regulate proteins associated with mitochondrial respiratory chain under iron deficiency in yeast; however, the role of TTP in mammalian mitochondrial regulation is not known. Here, we hypothesized that TTP protects the heart against iron deficiency through optimizing iron requiring mitochondrial proteins, and that disruption of this pathway will make the heart vulnerable to iron deficiency.

Results: Consistent with the above hypothesis, iron deficient TTP-KO mice induced by low iron diet for 6 weeks showed significant reduction in cardiac function compared with iron deficient TTP-WT mice in spite of similar reduction in mitochondrial iron levels, while TTP-KO mice at baseline have normal cardiac function. Since TTP is known to reduce protein expression via mRNA degradation, we hypothesized that it halts some iron-requiring proteins and optimizes their expressions in parallel to iron deficiency. We identified Rieske iron-sulfur proteins encoded by UQCRFS1, which has TTP-binding AUUUA motif in 3' untranslated region of its mRNA, to be negatively regulated by TTP. Specifically, we showed that TTP binds to and degrades its mRNA through three different findings: 1) decreased steady-state mRNA level of UQCRFS1 with iron chelation, but no reduction with TTP deletion, 2) increased mRNA stability of UQCRFS1 assessed by actinomycin-D treatment with TTP deletion, 3) more TTP-binding with mRNA of UQCRFS1 assessed by RNA co-immunoprecipitation. We also confirmed that Rieske protein level under iron deficiency was preserved in TTP-deleted cells. Additionally, in TTP-knockdown HR2 cells with iron chelation, the production of superoxide via complex III captured by amplex red was increased, and oxygen consumption rate was decreased with increased lactate production, suggesting disruption of electron flow in mitochondrial electron transport chain. Interestingly, enzyme activity of mitochondrial complex III was decreased in TTP-depleted cells with iron chelation in spite of intact whole complex III assembly assessed by non-denatured gel.

Conclusions: Our results provide a novel role for TTP in mitochondrial adaptation to iron deficiency by optimizing the expression of Rieske protein. Mitochondrial complex III which contains an appropriately expressed Rieske protein lacking iron-sulfur cluster under iron deficiency may underlie mitochondrial dysfunction.
In vitro and in vivo genome engineering of Dilated Cardiomyopathy caused by phospholamban R14 deletion.

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Introduction: familial Dilated cardiomyopathy (DCM) is a genetic form of heart disease characterized by biventricular dilatation and progressive cardiac dysfunction and caused by mutations in several cardiomyopathy genes, many of which play important roles in the contraction of the cardiac muscle. Recent studies have identified a number of pathogenic DCM-causing mutations in the phospholamban (PLN) gene which encodes a critical regulatory protein that controls calcium cycling and contractility in cardiomyocytes. The most commonly identified PLN mutation in DCM patients is a deletion of amino acid arginine 14 in the coding region of the PLN gene (R14del). It was first discovered in a large Greek family and recently found to have a very high prevalence in the Netherlands. Heterozygous carriers develop left ventricular dilatation, contractile dysfunction and episodic ventricular arrhythmias, with overt heart failure by middle age in some cases.

Purpose: In this study we aimed to (i) establish PLN-R14del-induced DCM disease models in vitro and in vivo and (ii) to correct R14del mutation by using genome engineering.

Methods: We generated induced pluripotent stem cells (iPSCs) from a patient harboring the PLN-R14del mutation and differentiated them into cardiomyocytes (iPSC-CMs). We performed transient Ca2+ analysis and PLN immunocytochemistry to characterize PLN-R14del-induced DCM in vitro. We generated both a TALEN engineered gene editing and an adenovirus-assisted vector (AAV)-mediated gene therapy approaches to correct R14del mutation. For the in vivo study, we have generated a mouse model expressing the “human” sequence of PLN, carrying the R14del mutation.

Results: We found that the PLN-R14del mutation induces Ca2+- handling abnormalities, abnormal cytoplasmic distribution of PLN protein and increases expression of molecular markers of cardiac hypertrophy (bNP, ANP, cTnI). Using genome engineering, we corrected the genetic mutation and rescued the disease phenotype.

Conclusions: Our findings suggest that (i) iPSC-CMs can be used as a platform to model PLN-R14del-induced DCM in vitro and (ii) targeted gene correction of R14del PLN mutation is sufficient to restore a normal cardiac phenotype. We will use our transgenic mouse model of human PLN-R14del-induced DCM in vitro and (ii) targeted gene correction of R14del PLN mutation is sufficient to restore a normal cardiac phenotype. We will use our transgenic mouse model of human PLN-R14del-induced DCM in vitro.

Cardiac specific genes, many of which play important roles in the contraction of the cardiac muscle.

Cigarette smoking increases expression of the G protein-coupled receptor 15 mRNA by change in Cpg methylation.

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Background: cigarette smoking increases the risk for cardiovascular disease. Recent studies investigated the effect of smoking habit on DNA methylation. Within the GRPR gene, encoding the G protein-coupled receptor 15, the Cpg locus cg19895270 has been identified to be hypomethylated in smokers, which correlated with the GRPR expression in white blood cells. Other molecular mechanisms by which smoking might influence GRPR expression have not been analysed so far.

Purpose: The aims of this study were: (i) to analyse the longitudinal effect of smoking behaviour on GRPR expression in monocytes and peripheral blood mononuclear cells (PBMCs); (ii) to investigate if this change is mediated by a change in cg19895270 methylation or GRPR promoter activity.

Methods: To determine the effect of changes in smoking behaviour over time, monocyte GRPR mRNA expression was measured by qPCR in smokers (n = 179), ex-smokers (n = 369) and never-smokers (n = 480) from the population-based Gutenberg Health Study at baseline and five-year follow-up visit.

The DNA methylation status of the GRPR cg19895270 locus was determined in PBMCs from 262 subjects by bisulfite conversion and high resolution melting and mRNA expression was measured by microarray. Regression analysis was performed to estimate the association between smoking, gene expression and GRPR Cpg methylation.

To investigate the effect of smoking on GRPR promoter activity, the putative GRPR promoter was cloned into the pGL4.10 luciferase reporter gene vector and transfected into HEK293A cells. The transfection was stimulated with 20% smoker’s or non-smoker’s plasma and 0, 10 or 40 μg/mL liquid smoke extract for 6 h and luciferase activity was measured.

Results: Monocyte GRPR expression was significantly higher in smokers compared to ex-smokers (p < 0.001) and never-smokers (p < 0.001). Ex-smokers had significantly higher GRPR expression levels than never-smokers up to ten years after quitting (p < 0.001). Smoking initiation between baseline and follow-up visit (n = 17) led to an increase in GRPR expression, whereas smoking cessation resulted in decreased GRPR expression (n = 42, p < 0.001).

In PBMCs, the correlation between smoking and GRPR expression (p = 0.001) was associated with cg19895270 hypomethylation (p = 0.05). Likewise, smoking cessation was linked to cg19895270 methylation as well as reduced GRPR expression (p < 0.05). Furthermore, higher cumulative smoking exposure correlated with lower GRPR Cpg site methylation and as well as higher GRPR mRNA expression (p = 0.0001).

In the luciferase reporter gene assay, neither smoker’s extract nor liquid smoke extract changed GRPR promoter activity.

Conclusion: This was the first longitudinal study showing that changing smoking behaviour changed GRPR expression. This time- and dose-dependent effect was mediated by changes in DNA methylation. Hence, GRPR is an interesting new candidate gene for the link between smoking and cardiovascular disease.

Cardiogenic potential of iPSC from cardiac progenitor cells

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Introduction: the reprogramming of adult somatic cells (ASC) to an embryonic stem cell-like state (induced Pluripotent Stem Cells, iPSCs) has paved the way for patient-specific personalized medicine. Founder cell type could influence iPSC molecular and differentiation properties after establishing their pluripotent state.

We evaluated the presence of a phenotypic memory related to the tissue of origin in iPSC from cardiac progenitor cells (CPC).

Methods: CPC were derived from right atrial appendage explants from patients who underwent heart valve surgery. Cells were reprogrammed using Sendai virus carrying four transcription factors (Sox2, Oct4, Cdx4, Myf5 and Klf4) in order to activate the stemness pathways. iPSC were amplified and the colonies were selected and split, in order to limit cellular degeneration and increase cell number.

CPC-derived iPSC were differentiated toward human cardiomyocytes (hCM) by the addition of defined growth factor in order to modulate the Wnt signalling pathways (upregulation in the early stage for 24h and subsequently re-inhibited for 24h).

The expression of endogenous pluripotency markers including Nanog, SSEA-4, Lefty) at significant levels. As shown by ICC each line expressed SSEA-4 and Tra-1-60 at the protein level and beating rate of 63 bpm as measured in control condition. Application of epinephrine (0.1 mM) for 24h and subsequently re-inhibited for 24h). The expression of endogenous pluripotency markers including Nanog, SSEA-4, Lefty, TrA-1-60 was quantified in embryonic developmental stage by RT-qPCR and Immunocytochemistry (ICC). Cardiogenic differentiation was evaluated by the expression of cardiac specific markers such as α-Actin, α-myosin and γ-actin by electrophysiology recordings performed on dissected spontaneously beating areas using MEA4 multisite arrays system (MEA).

Results: CPC-derived iPSC clones showed human embryonic stem cell-like morphology. RT-qPCR analysis revealed that all iPSC clones expressed endogenous pluripotency genes (Nanog, SSEA-4 and Lefty) at significant levels. As shown by ICC each line expressed SSEA-4 and Tra-1-60 as the protein level. Beating areas appear in culture at day 11-16 (starting from the day 0 of differentiating protocol). Beating cells showed typical striped pattern when stained for α-Actin and α-myosin. The gap junction proteinCx43 was wildly expressed in differentiated cells. Spontaneous field potential (FP) waveforms were recorded from MEA electrodes with RR intervals (430 ms), QTcB (227 ms, Bazett’s correction) and beating rate of 63 bpm as measured in control condition. Application of ephrinephrine (0.1 mM) revealed an increase of 57% in beating rate and 30% in QTcB band of 52% in RR interval.

Conclusions: CPC were successfully reprogrammed into pluripotent embryonic stem cells. CPC-derived iPSC exhibit cardiogenic properties when induced to differentiate trough cardiomyocytes.