EFFECTS OF METABOLIC MODULATION ON CARDIOMYOCYTE PHENOTYPE

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Tiivistelmä:

Sepelvaltimotauti ja sen aiheuttama sydämen vajaatoiminta ovat seurausta sydämen hapenpuutteen aiheuttamasta palautumattomasta sydänlihassolujen menetyksestä. Sepelvaltimotauti on maailmanlaajuisesti merkittävin kuolinsyy, joten sydänlihassolukatoa ennaltaehkäiseville tai kumoaville regeneratiivisille hoidoille on valtava tarve. Ennen syntymää sikiökaudella sydän kasvaa sydänlihassolujen jakautumisen kautta, ja sydänlihassolujen käyttämä energia tuotetaan pääsääntöisesti anaerobisen glykolyysin avulla. Syntymän jälkeen veren happi- ja rasvahappopitoisuuden kasvaessa sydänlihassolut siirtyvät rasvahappojen hapettamiseen, ja samanaikaisesti solusykli ja jakautuminen pysähtyvät. Tämän jälkeen sydän ei pysty regeneroitumaan vaurion jälkeen, mikä johtaa sydämen vajaatoiminnan kehittymiseen.

Tämän työn tavoitteena oli määrittää sydänlihassolujen aineenvaihdunnan sekä hapenpuutteen (hypoksian) vaikutuksia solusyklin aktivaatioon tutkimalla aineenvaihdunnan modulaation ja hapenpuutteen vaikutuksia sydänlihassolujen fenotyyppiin ja hypoksiavasteeseen. Tavoitteenamme oli lisäksi selvittää hapenpuutteen vaikutuksia aineenvaihduntaan ja hypoksiaan liittyvien geenien ilmentymiseen, sekä sitä, kuinka sydänlihassolujen kypsyminen aineenvaihdunnan muutoksen seurauksena vaikuttaa yllä mainittuihin. Hypoksian ja kypsymisen vaikutuksia ihmisen monikykyisistä kantasoluista erilaistettujen sydänlihassolujen (hiPSC-sydänlihassolu) geenien ilmentymiseen tutkittiin qRT-PCR:llä. Viiden aineenvaihduntaa säätelevän yhdisteen vaikutuksia solusykliin ja hiPSC-sydänlihassolujen fenotyyppiin tutkittiin automatisoidun kuva-analyysin avulla. Hypoksia havaittiin hiPSC-sydänlihassoluissa lisääntyneenä *VEGFA*-geenin ilmentymisenä kolmen tunnin hypoksia-altistuksen seurauksena. HiPSC-sydänlihassolujen kypsyminen ja siihen liittyvä oksidatiivinen aiheenvaihdunta havaittiin lisääntyneenä *SDHA*:n ilmentymisenä, joka väheni hypoksia-altistuksen seurauksena. Solujen kypsyminen aiheutti jakautumisen lisääntymistä ja stressisignaloinnin vähentymistä. Krebsin syklin metaboliitti sukkinaatti vähensi kypsytettyjen hiPSC-sydänlihassolujen jakautumista hypoksiassa 8,2 prosentilla, viitaten oksidatiivisen aineenvaihdunnan solujakautumista estävään vaikutukseen. Mevalonaattireitin ja ketogeneesin inhibitio ei vaikuttanut solusykliin tai fenotyyppiin, mutta β-hydroksibutyraatti hieman lisäsi solujakautumista, vahvistaen nykyistä käsitystä siitä, että ketogeneesillä on rooli sydänlihassolujen solusyklin säätelyssä. Havaintomme perusteella hiPSC-sydänlihassolut voivat olla hyödyllisiä fenotyypin ja geenien ekspression analyyseissä. Kuitenkin toisenlaisia tutkimusmenetelmiä tarvitaan aineenvaihdunnan modulaation ja sen vaikutusten yksityiskohtaiseksi määrittämiseksi.

Abstract

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Abstract:

Ischemic heart disease (IHD) and subsequent heart failure are caused by irreversible loss of contractile cardiomyocytes due to low oxygen supply to the heart. As the leading cause of death worldwide, IHD raises an urgent need for regenerative therapies that prevent or reverse loss of cardiomyocytes. The fetal mammalian heart grows by cardiomyocyte proliferation and utilizes glycolysis as main energy metabolism pathway, until it is introduced to increased oxygen and fatty acid supply at birth. Subsequently, cardiac energy metabolism shifts from glycolysis to β-oxidation of fatty acids and cardiomyocytes exit the mitotic cell cycle. Due to cessation of proliferation the heart can no longer regenerate after ischemic injury and responds to it by introduction of maladaptive pathological processes leading to heart failure.

To gain deeper insight on the roles of cardiac metabolism pathways and hypoxia in cell cycle activation, we evaluated the effects of pharmacological metabolic modulation and oxygen supply on cardiomyocyte phenotype and hypoxia response. Furthermore, we studied the changes in the metabolic genotype of cardiomyocytes under alterations of oxygen supply. We utilized quantitative reverse transcription PCR (qRT-PCR) to evaluate the effects of hypoxia and metabolic maturation on the expression of genes involved in hypoxia signaling and metabolism of human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs). Additionally, we investigated the effects of five metabolism-modulating compounds on cell cycle and phenotype of both metabolically matured and unmatured hiPSC-CMs, by utilizing high content analysis. We observed presence of hypoxia signaling as an increase in vascular endothelial growth factor A (*VEGFA*) expression following 3-hour hypoxic exposure. High expression of succinate dehydrogenase complex flavoprotein subunit A (*SDHA*) in hiPSC-CMs, which was downregulated at hypoxia, confirmed occurrence of oxidative metabolism induced by metabolic maturation. Surprisingly, metabolic maturation tended to increase proliferation and decrease stress response signaling of hiPSC-CMs. Introduction of the TCA cycle intermediate succinate decreased proliferation of metabolically unmatured hypoxic hiPSC-CMs by 8.2 %. Finally, inhibition of the mevalonate pathway and ketogenesis caused no alterations in hiPSC-CM phenotype or cell cycle, but introduction of the ketone body β-hydroxybutyrate tended to increase proliferation, supporting current evidence that ketogenesis plays a role in cardiomyocyte cell cycle regulation. Our observations suggest that hypoxic hiPSC-CMs can be useful in investigating gene expression and phenotype. Even so, additional methodologies are needed for in-depth evaluation of metabolic reprogramming and its effects on cardiomyocyte phenotype.

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1 INTRODUCTION

Ischemic heart disease (IHD), or coronary artery disease, affects over 120 million people worldwide, and is the leading cause of death, with over 9 million yearly deaths (WHO 2020; Khan et al. 2020). It can lead to myocardial infarction, which is a major cause of heart failure (Townsend et al. 2016). Low oxygen supply to the heart during myocardial infarction (MI) leads to irreversible loss of cardiomyocytes and fibrotic scarring of the cardiac tissue (Braunwald 2013). The adult mammalian heart possesses poor regenerative capacity because proliferation of cardiomyocytes terminates shortly after birth as they exit the mitotic cell cycle (Li et al. 1996; Soonpaa et al. 1996). Consequently, the heart responds to injury through maladaptive pathological hypertrophy, often leading to contractile dysfunction (Nakamura & Sadoshima 2018). Primary prevention and increase in awareness and healthcare quality have reduced mortality, prevalence, and incidence of IHD regionally over the decades (Khan et al. 2020). Nonetheless, mortality remains alarmingly high in numerous countries, as prevalence of IHD risk factors continues to increase (Nowbar et al. 2019; Khan et al. 2020). Mortality from myocardial infarction and subsequent heart failure combined with a scarcity of regenerative therapies raises an urgent need for the discovery of novel strategies to induce regeneration in human hearts.

Heart regeneration occurs in certain cold-blooded vertebrates and neonatal rodents (Becker et al. 1974; Jopling et al. 2010, Porrello et al. 2011; González-Rosa & Mercader 2012). It has been demonstrated that heart regeneration takes place through dedifferentiation and proliferation of surviving cardiomyocytes in the injury site, accompanied by coronary revascularization, activation of the epicardium and immune system activation (Godwin & Brockers 2006; Jopling et al. 2010; Porrello et al. 2013; Xin et al. 2013). Some post-mortem case studies and reports of corrective heart surgery and recovery from myocardial infarction at birth suggest that cardiac regeneration might occur during early development in humans as well (Haubner et al. 2015). The current evidence on post-injury regenerative capacity has given reason for extensive research aiming to a deeper comprehension of the pathways underlying cardiomyocyte proliferation. Despite numerous approaches to induce cardiac regeneration through increasing the number of cardiomyocytes in the injury site, advances have not yet

achieved sufficient regenerative responses (Fernandes et al. 2010; Ieda et al. 2010; Jiang et al. 2010; Zakharova et al. 2010; Makkar et al. 2012; Cahill et al. 2017).

Glycolysis as the main energy production source is associated with proliferative capacity of cells (Ito & Suda 2014; Gu et al. 2017). Indeed, energy for the proliferating cardiomyocytes in the fetal heart is obtained mostly from anaerobic glycolysis (Asciutto & Ross-Asciutto 1996, Lopachuck et al. 1991; Nakano et al. 2017). At birth the heart is introduced to an increase in cardiac workload and oxygen concentration, together with an alteration in energy substrate supply in the circulation. Consequently, transcriptional regulation of signaling pathways related to energy metabolism guide a transition of the metabolic phenotype of cardiomyocytes from glycolysis to β-oxidation of fatty acids (Lopachuck et al. 1992; Lehman et al. 2000; Chung et al. 2007; Puente et al. 2014). The cessation of proliferation during terminal differentiation of cardiomyocytes and onset of cell growth by adaptive physiological hypertrophy within the first week after birth is concomitant to the transition of energy metabolism. Correspondingly, *in vitro* studies have shown that transition of cardiomyocyte metabolism from glycolysis to oxidative phosphorylation is enough to induce cell cycle exit of immature cardiomyocytes (Mills et al. 2017).

Unlike other organs, the adult heart is characterized by a remarkable metabolic flexibility as it can utilize multiple energy substrates in order to maintain homeostasis and repetitive contractile function (Lopachuck et al. 2010; Wentz et al. 2010; Murashige et al. 2020). Based on current knowledge, the aim of inducing cell cycle re-entry and cardiomyocyte proliferation through modulation of metabolic components is based on promoting a shift of the metabolic phenotype of cardiomyocytes towards that of the regenerative neonatal heart (Fillmore et al. 2014; Nakada et al. 2017; Cardoso et al. 2020; Bae et al. 2021). Hypoxic conditions and induction of glycolysis through metabolic reprogramming in adult cardiomyocytes promote cell cycle re-entry and post-infarction regeneration *in vivo* (Nakada et al. 2017). Additionally, the TCA cycle intermediate succinate makes an interesting target for cardiac metabolism modulation, as its accumulation in the heart has been shown to reduce cardiomyocyte proliferation and cardiac regeneration *in vivo* (Bae et al. 2021). Reversal of succinate accumulation through inhibition of the succinate

dehydrogenase (SDH) with the small molecule compound , in turn, promotes cardiomyocyte proliferation and cardiac regeneration *in vivo* by inhibition of oxidative metabolism.

On top of glycolysis and oxidation of fatty acids, alterations in other metabolic pathways of the heart seem to serve a role in the cell cycle exit as well. Cardiac ketogenesis and the mevalonate pathway seem to be involved in the cell cycle exit and regulation of proliferation during in the postnatal regenerative phase, making these pathways an interesting research topic (Talman et al. 2018). Proliferation of neonatal rat cardiomyocytes *in vitro* is attenuated by inhibition of both the mevalonate pathway and ketogenesis, but not by inhibition of the mevalonate pathway alone, suggesting that ketogenesis might have a role in the regulation of cardiomyocyte cell cycle.

The introduction of human induced pluripotent stem cells (hiPSC) has allowed advances in cardiovascular research and development of regenerative therapies. Cardiomyocytes derived from hiPSC (hiPSC-CMs) exhibit somewhat similar properties to cardiomyocytes in the developing human heart. However, hiPSC-CMs possess a morphological, functional and physiological phenotype of immature cells, which differ in many ways from postnatal cardiomyocytes that undergo extensive developmental maturation (Zhang et al. 2009; Burridge et al. 2014; Karbassi et al. 2020). Metabolic maturation is one approach to maturation of hiPSC-CMs, and it can be achieved by supplying the cells with energy substrates that induce oxidative metabolism (Mills et al. 2017).

To gain better understanding of the dynamic role of metabolic pathways in cell cycle activity, this study examines the effects of pharmacological modulation of cardiomyocyte metabolism and oxygen supply on cardiomyocyte phenotype and hypoxia response. Additionally, we evaluated the changes in the metabolic genotype of cardiomyocytes under alterations of oxygen supply.

2 MATERIALS AND METHODS

2.1 Compounds and reagents

Cell culture media, reagents and supplements used in cell culture were purchased from Gibco (Thermo Fisher Scientific, Paisley, UK). Dimethyl sulfoxide (DMSO), 4′,6-diamidino-2-phenylindole (DAPI), Triton™-X-100, sodium tetraborate, β-mercaptoethanol and hydrochloric acid were purchased from Sigma-Aldrich (Darmstadt, Germany). Small-molecule inhibitors CHIR99021, Wnt-C59 and ROCK-inhibitor (ROCKi) (Y-27632) were bought from Tocris Bioscience (Bristol, UK). Small molecule compounds Simvastatin (sc-200829), Hymeglusin (sc-203077A) and Sodium (S)-3 hydroxybutyrate (sc-236887) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany), while MitoKit-CII/Succinate-nv (NV118) and MitoKit-CII/Malonate-nv (NV161) (60200-01) were purchased from Oroboros Instruments GmbH (Innsbruck, Austria).

The primary antibodies used in immunofluorescence staining were purchased from Abcam (Cambridge, UK): polyclonal rabbit anti-cardiac troponin T (anti-cTnT) (#ab45932), monoclonal rat anti-BrdU (#ab6326) and monoclonal mouse anti-proBNP (#ab13115). The secondary antibodies used were from Life Technologies (Eugene, Oregon, USA): Alexa Fluor 488 goat anti-rabbit lgG (Invitrogen #A11034), Alexa Fluor 647 goat anti-rat IgG (#A21247) and Alexa Fluor 594 donkey anti-mouse lgG (#A21203). TaqMan® Gene Expression Assays for Nppa (Rn00664637_g1; probe NPPA HS00383230_g1), Nppb (Rn00580641_m1; probe NPPB HS01057466_g1), FOS (Hs04194186_s1), VEGFA (Hs00900055_m1), SDHA (Hs07291714_mH), HMGCR (Hs00168352_m1), Actb (Mm00607939_s1; probe ACTB Cat# 4333762T) and eukaryotic 18S rRNA (Hs99999901_s1; probe 18S Cat# 4352930E) were purchased from Thermo Fisher Scientific.

2.2 Cell culture and metabolic maturation

iPS(IMR90)-4 cells (Yu et al. 2007), purchased from WiCell (Madison, Wisconsin, USA), were cultured on Matrigel®-coated (1:50; Corning, Bedform, MA, USA) 6-well plates in Essential eight™ medium (E8) and passaged 1:15 approximately every four days. For passaging, the cells were dissociated using Versene and resuspended in E8 containing 10 μM ROCK-inhibitor. Differentiation protocol for iPS(IMR90) cells was conducted as described earlier by Burridge et al. (2014). Briefly, cardiomyocyte differentiation was started when the culture was approximately 80–95 % confluent (Day 0) by induction of Wnt signaling pathway with the glycogen synthase kinase 3 (GSK3) inhibitor CHIR99021 at a concentration of 6.5–7 μM in RPMI 1640 medium supplemented with B-27 without insulin (RB-). On the next day, the medium was changed to RB- without CHIR99021. On day 3 the medium was changed to RB- containing Wnt signaling pathway inhibitor C59 at a concentration of 2.5 μM. On days 5, 7 and 9 of differentiation, cells were fed with RB-. Beating of cardiomyocytes was observed from days 7–9 onwards. After 48 hours, metabolic selection of cardiomyocytes was initiated by changing RB- to RPMI 1640 without glucose supplemented with B-27 (with insulin). On day 13, the cells were fed with fresh metabolic selection medium. From day 15 onwards, the cardiomyocytes were cultured in RPMI 1640 supplemented with B-27 (RB+). The differentiated cardiomyocytes (hiPSC-CMs) were dissociated on day 15 or 16 using TrypLE™ select 1.5× solution in PBS and 1 mM EDTA for 7–8 minutes at 37 °C. Cells were centrifuged and resuspended in RB+ containing 10 % fetal bovine serum (FBS) and ROCKi at 10 μM and replated on Matrigel®-coated (1:200 or 1:50) 12-well plates at 500 000 cells/well for quantitative real-time polymerase chain reaction (qRT-PCR), or on black CellCarrier™ 96-well plates at 10 000 cells/well for immunofluorescence staining. After allowing the cells to attach for 48 hours, the medium was changed to serum-free and ROCKi-free RB+ or maturation medium (MM) (Mills et al. 2017) containing 4×B27 supplement, 1 × GlutaMAX, L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate at 200 μM, glucose at 1 mM and BSA-conjugated palmitic acid (Cayman Chemical Company) at 100 μM, diluted in DMEM without D-glucose, L-glutamine, phenol red and sodium pyruvate. To allow metabolic maturation to take place,

hiPSC-CMs were incubated in MM for six days. Cell cultures were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 at all times.

2.3 Hypoxia exposure and treatment with small-molecule compounds

hiPSC-CMs on 12-well plates were exposed to hypoxic conditions for three hours. Hypoxic conditions were created using ProOx C21 Oxygen and Carbon Dioxide Subchamber Controller (BioSpherix, Ltd., RRID: SCR_021131) installed in a cell culture incubator. Next, hiPSC-CMs were incubated in normoxia at 37 °C (5 % CO_2) for 0, 1, 3 or 21 hours, after which they were washed twice with PBS and lysed on ice with 350 µl cell lysis buffer from MN NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany) with 1 % β-mercaptoethanol. Cell lysates were stored at –80°C until RNA extraction.

hiPSC-CMs on 96-well plates were treated with five compounds at three different concentrations for 24 hours. Treatments with succinate, malonate and β-hydroxybutyrate were performed at concentrations of 10 µM, 30 µM and 100 µM. Treatments with hymeglusin and simvastatin were performed at concentrations of 1 µM, 3 µM and 10 µM. Serial dilutions of compounds were made in RB+ medium and MM. All dilutions contained 5′-bromo-2′-deoxyuridine (BrdU) at 10 µM. Two controls were used: one control consisted of RB+ medium with BrdU at 10 µM and 1 % DMSO, and the other of MM with BrdU at 10 µM and 1 % DMSO. The experiment was carried out at 37 °C in a humidified atmosphere of 5 % CO_2 both in normoxic and hypoxic conditions (0.0–0.5 % O_2). Exposure time to hypoxia was 3 hours, after which cells were incubated in normoxia for 21 hours at 37 °C in a humidified atmosphere of 5 % CO_2.

2.4 RNA isolation and qRT-PCR

For gene expression analysis, RNA extraction was performed according to manufacturer's instructions and RNA concentration and quality were measured with the spectrophotometry device NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, United States). Complementary deoxyribonucleic acid (cDNA) synthesis from RNA

samples was performed from 31– 324 ng of total RNA in 10 µl reactions using protocol of Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) and MJ Mini Personal Thermal Cycler (Bio-Rad). The cDNA was diluted to 1:10 in PCR-grade H_2O and stored at –20°C. TaqMan® Gene Expression Assays were used for qRT-PCR analysis for genes of interest (*NPPA*, *NPPB*, *VEGFA, SHDA, HMGCR*) and reference genes (*Actb* and *18S rRNA*). Assays were conducted with LightCycler® 480 probes Master reagent (Roche Life Science, Mannheim, Germany)) according to manufacturer's instructions. qRT-PCR was run with 4.5 µl of diluted cDNA in reaction volumes of 10 µl on a white LightCycler® 480 Multiwell Plate 384 (Roche) with LightCycler® 480 Real-Time PCR System (Roche). Each reaction was run 2–3 times and analyses were conducted with average values of 2–3 technical replicates obtained from each reaction. The ΔΔCt method was used for analysis: quantification cycle (Cq) values of the genes of interest were normalized to the average of the Cq of the reference genes Actb/ACTB and 18S rRNA. The obtained values (ΔCq) were normalized to those of the control sample. No-template controls were utilized to verify absence of PCR contamination.

2.5 Immunofluorescence staining and high content analysis

When compound exposure time reached 24 h, the experiment was terminated, and the hiPSC-CMs were washed twice with PBS and fixed using 4 % paraformaldehyde (PFA) at room temperature (RT) for 15 minutes. The cells were then washed 3x5 minutes with PBS, permeabilized with 0.1 % Triton-X-100 in PBS for 10 minutes and washed with PBS 2x5 minutes. As the hiPSC-CMs had been previously labeled with BrdU, DNA was hydrolyzed by incubating cells with 2 M hydrochloric acid (HCl) at RT for 30 minutes. HCl was replaced with 0.1 M sodium borate buffer (pH 8.5) at RT for 30 minutes, and then washed three times with PBS. Prior to immunofluorescence staining, non-specific antibody binding sites were blocked with 4 % FBS in PBS for 45 minutes. Primary antibody solutions were prepared into 4 % FBS in PBS: anti-cTnT-rabbit at 1:400, anti-BrdU-rat at 1:350 and pro-BNP-mouse at 1:350. Cells were incubated with primary antibodies at RT for 1 hour, protected from light, and washed 3x5 minutes with PBS. Next, hiPSC-CMs were incubated at RT for 45 minutes in secondary antibody solution containing DAPI at 1:100 and AlexaFluor-conjugated secondary antibodies at 1:200. The

cells were then washed with PBS for 3x5 minutes and stored in PBS at 4 °C protected from light until imaged. High content imaging was performed with the ImageXpress® Micro Confocal High-Content Imaging System (Molecular Devices) using a 20X Plan Apo Lambda ImageXpress Micro Objective with four sites per well, and analysis was carried out with MetaXpress® High-Content Image Acquisition and Analysis Software (Molecular Devices). Identification of hiPSC-CMs was based on DAPI and cTnT stainings: nuclei of all cells were identified with DAPI and hiPSC-CMs were identified with cTnT. The data were considered only from cTnT positive cells. Mononucleated and binucleated hiPSC-CMs were included in the analysis. For identification of BrdU+ cells, the roporte intensity of BrdU staining was measured. To classify hiPSC-CMs as BrdU+ or BrdU-, the reference level for BrdU intensity was set individually for each experiment. For confirmation of cell proliferation shown by BrdU staining and more accurate assessment of cell cycle activity, we performed cell cycle analysis based on measurement of total intensity of nuclear DAPI staining in individual hiPSC-CMs and generation of histograms of DNA content. Visually defined cut-offs were set manually on histograms to identify cardiomyocytes in phases G_0/G_1, S and G_2 of mitotic cell cycle. For identification of proBNP+ cardiomyocytes, the perinuclear area of the cells was determined as a 10-pixel ring around the nucleus and the average intensity of perinuclear proBNP staining was measured. The intensity threshold for proBNP+ cells was set manually in each experiment. Each experiment included 2–3 technical replicates of each treatment group and the average of technical replicates was used in the statistical analysis as n=1.

2.6 Statistical analysis

Single outliers of technical replicates were identified from raw data utilizing Grubbs' test and removed. Data were analyzed using IBM SPSS Statistics 28 software. Levene's test was used to analyze the equality of variances, and due to unequal variances Welch's ANOVA was carried out and followed by Games-Howell post-hoc test, and differences at the level of $P < 0.05$ were considered statistically significant. For the analysis of gene expression, non-normalized ΔCt values were used in statistical analysis.

3 RESULTS

3.1 The effects of hypoxia on cardiomyocyte gene expression

To evaluate the effects of cardiomyocyte exposure to hypoxic conditions followed by reoxygenation in normoxic conditions, we studied the expression of genes related to cardiac hypertrophy, hypoxic signaling and metabolism. We measured mRNA levels with qRT-PCR at four different time points after a three-hour exposure to hypoxia (Fig 1A). None of the genes of interest showed statistically significant changes in mRNA expression. Hypoxia did not induce significant changes in the expression of genes encoding natriuretic peptides A (*NPPA*) and B (*NPPB*), which are markers for cellular stress under hypoxia and pressure overload (Lang et al. 1985; Chen et al. 1997; Clerico et al. 1998). However, in metabolically matured hiPSC-CMs (maintained in MM) baseline *NPPA* expression was 2.0-fold higher in normoxia in comparison with unmatured hiPSC-CMs (maintained in RB+) (Fig 1B). *NPPA* expression was slightly downregulated during three-hour hypoxia and gradually restored during the reoxygenation period. *NPPB* expression, in turn, tended to increase slightly after three-hour hypoxia and returned to baseline in a similar pattern in both metabolically matured and unmatured hiPSC-CMs (Fig. 1C). Hypoxia induced the expression of vascular endothelial growth factor A (*VEGFA*), regulator of angiogenesis, in both metabolically unmatured and matured hiPSC-CMs: expression was 3.7-fold higher in unmatured cells and 2.7-fold higher in matured cells after exposure to hypoxia (Fig 1D). During reoxygenation, *VEGFA* expression was lowest at three hours post-hypoxia, yet higher than relative controls (1.4-fold for unmatured and 1.4-fold for matured hiPSC-CMs). At baseline, succinate dehydrogenase complex flavoprotein subunit A (*SDHA*) expression was 2.1-fold higher in metabolically matured than in unmatured hiPSC-CMs (Fig. 1E). In these cells *SDHA* expression was downregulated at hypoxia and upregulated again during reoxygenation. In hiPSC-CMs maintained in RB+ there was no detectable change in SDHA expression, indicating that oxidative metabolism does not have a significant role in energy metabolism of unmatured hiPSC-CMs. Finally, at baseline *HMGCR* expression was 2.9-fold higher in metabolically matured cells than in unmatured cells, and it decreased 1.8-fold following hypoxia exposure. Reoxygenation had no marked

effect on decreased *HMGCR* expression. Hypoxia and reoxygenation had no effect on *HMGCR* expression in unmatured hiPSC-CMs.

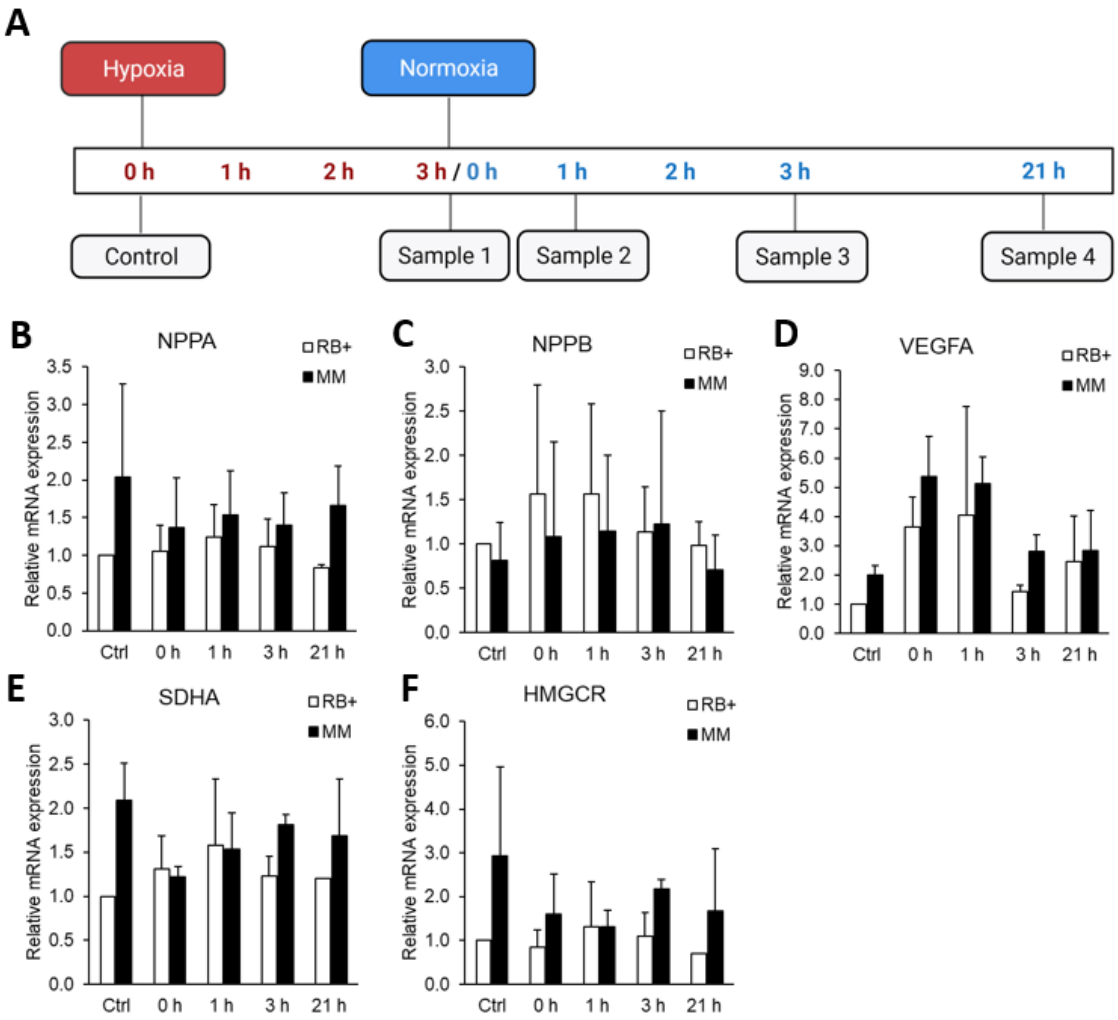

Fig. 1. Effects of exposure to three-hour hypoxia and subsequent reoxygenation on cardiomyocyte gene expression. A) Timeline of exposures to hypoxic and normoxic conditions. hiPSC-CMs were exposed to hypoxia (red color) for three hours, after which they were collected or incubated in normoxia for one hour, three hours or 21 hours (blue color). B) Relative expression of natriuretic peptide A (*NPPA*). C) Relative expression of natriuretic peptide B (*NPPB*). D) Relative expression of vascular endothelial growth factor A (*VEGFA*). E) Relative expression of succinate dehydrogenase complex flavoprotein subunit A (*SDHA*). F) Relative expression of HMG-CoA reductase (*HMGCR*). Results are presented as mean + SD of independent experiments with hiPSC-CMs from individual differentiations (n=3 for *NPPA*, *NPPB* and *VEGFA*; n=2 for *SDHA* and *HMGCR*). Relative gene expression was normalized to the respective controls from cells kept in normoxic conditions in RB+ medium.

3.2 The effects of metabolic maturation and hypoxia on cardiomyocyte phenotype

To investigate the effects of hypoxia and metabolic maturation on cell cycle activity and cellular stress response we first incubated metabolically matured and unmatured hiPSC-CMs in hypoxic conditions for three hours, and in normoxic conditions for 21 hours, with concomitant BrdU-loading (24 hours). Next, we utilized HCA of DAPI, cTnT, BrdU and proBNP (Fig. 2A–B). BrdU is a thymidine analog that is incorporated in the newly synthetized DNA of dividing cells during S phase of cell cycle. 24-hour BrdU-loading allowed detection of cells in S phase and cells transitioned to G_2 phase after completion of S phase. Hypoxia had no effect on percentage of BrdU+ hiPSC-CMs in either metabolically matured or unmatured cells (Fig 2C). However, in both normoxic and hypoxic conditions metabolically matured hiPSC-CMs tended to have a higher percentage of BrdU+ cells in comparison with unmatured hiPSC-CMs. Cell cycle analysis showed that most hiPSC-CMs (80.8 % of unmatured normoxic cells, 73.2% of matured normoxic cells, 84.6 % of unmatured hypoxic cells and 74.6% of matured hypoxic cells) were in G_0/G_1 phase of cell cycle (Fig. 2D). Percentage of hiPSC-CMs in S phase of the cell cycle tended to increase and percentage of cells in G_2 phase to decrease following hypoxia in both unmatured and matured hiPSC-CMs. This result suggests that hypoxia induced transition of cells from G_0/G_1 phase to S phase, where DNA replication for cell division initiates. Interestingly, hypoxia did not induce changes in proBNP expression (Fig. 2E), which is normally induced in cardiomyocytes by stress signaling related to hypoxia and mechanical stress and used as a biomarker in the diagnostics of heart failure (Goetze et al. 2003; Casals et al. 2009). Nonetheless, proportion of proBNP+ hiPSC-CMs was markedly higher in metabolically unmatured cells, yet not statistically significantly.

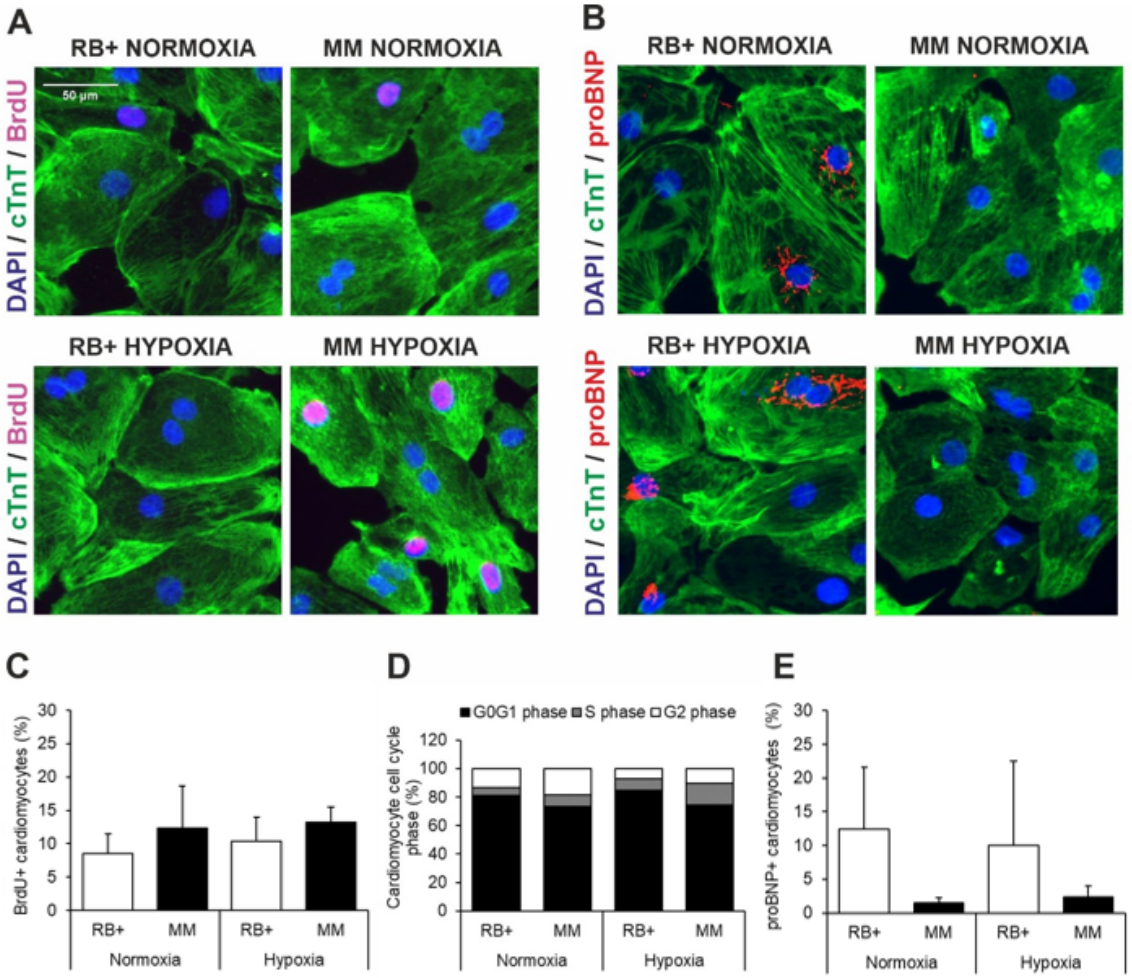

Fig 2. Effects of six-day metabolic maturation on cardiomyocyte phenotype and cell cycle in normoxic conditions and after three-hour hypoxia followed by 21-hour reoxygenation, compared to corresponding conditions in RB+ medium with no metabolic maturation. A) Representative images of metabolically unmatured and matured hiPSC-CMs stained for DNA (DAPI), cTnT and BrdU. B) Representative images of metabolically unmatured and matured hiPSC-CMs stained for DNA (DAPI), cTnT and proBNP. C) Percentage of BrdU+ hiPSC-CMs D) Percentage of hiPSC-CMs in G_0/G_1 phase, S phase and G_2 phase of cell cycle. E) Percentage of proBNP+ hiPSC-CMs. Results are presented as mean + SD of independent experiments with hiPSC-CMs from individual differentiations (n=3)

3.3 The effects of succinate and malonate on cardiomyocyte phenotype

To investigate the role oxidative metabolism in hiPSC-CM phenotype and cell cycle activity in normoxic and hypoxic conditions, we treated hiPSC-CMs with succinate and malonate for 24 hours and performed HCA. Inhibition of glucose metabolism with succinate at concentrations of 10 µM and 30 µM had no significant effect on percentage

of BrdU+ hiPSC-CMs in any of the treatment groups (Fig 3A). At the highest concentration (100 μM) succinate reduced the percentage of BrdU+ hiPSC-CMs in all treatment conditions, although significantly (8.2 %, $p = 0.03$) only in unmatured cells exposed to hypoxia. The decrease in the proportion of BrdU+ hiPSC-CMs was slightest in metabolically matured hiPSC-CMs, which do not prioritize glycolysis in energy production. Cell cycle analysis showed no statistically significant differences in percentage of hiPSC-CMs in any phase of cell cycle (Fig. 3B). Yet, the proportion of hiPSC-CMs in G_2 phase tended to increase in all treatment groups, the most with the concentration of 30 μM. Surprisingly, at the concentration of 100 μM, succinate did not reduce the total percentage of hiPSC-CMs in phases S and G_2, like it did to the percentage of BrdU+ cells. In all treatment groups except in matured hiPSC-CMs in normoxia the percentage of cells in S phase followed an increasing pattern with increasing succinate concentrations. Only in metabolically matured hiPSC-CMs maintained in normoxia the percentage decreased as succinate concentrations increased. Malonate treatment, in turn, had no significant effect on the percentage of BrdU+ hiPSC-CMs or percentage of hiPSC-CMs in phases of cell cycle (Fig. 3C–D). In all treatment groups the proportion of BrdU+ hiPSC-CMs was highest at the concentration of 30 μM, and the highest concentration 100 μM tended to decrease BrdU+ cells at hypoxic conditions. Cell cycle analysis of unmatured hiPSC-CMs showed a similar trend in percentages of hiPSC-CMs in phases S and G_2 of cell cycle as the percentage of BrdU+ positive cells did: the percentages were highest with malonate at 30 μM. In matured hiPSC-CMs malonate □roporti increased the □roportion of cells in S and G_2 phases, but there were no differences between concentrations.

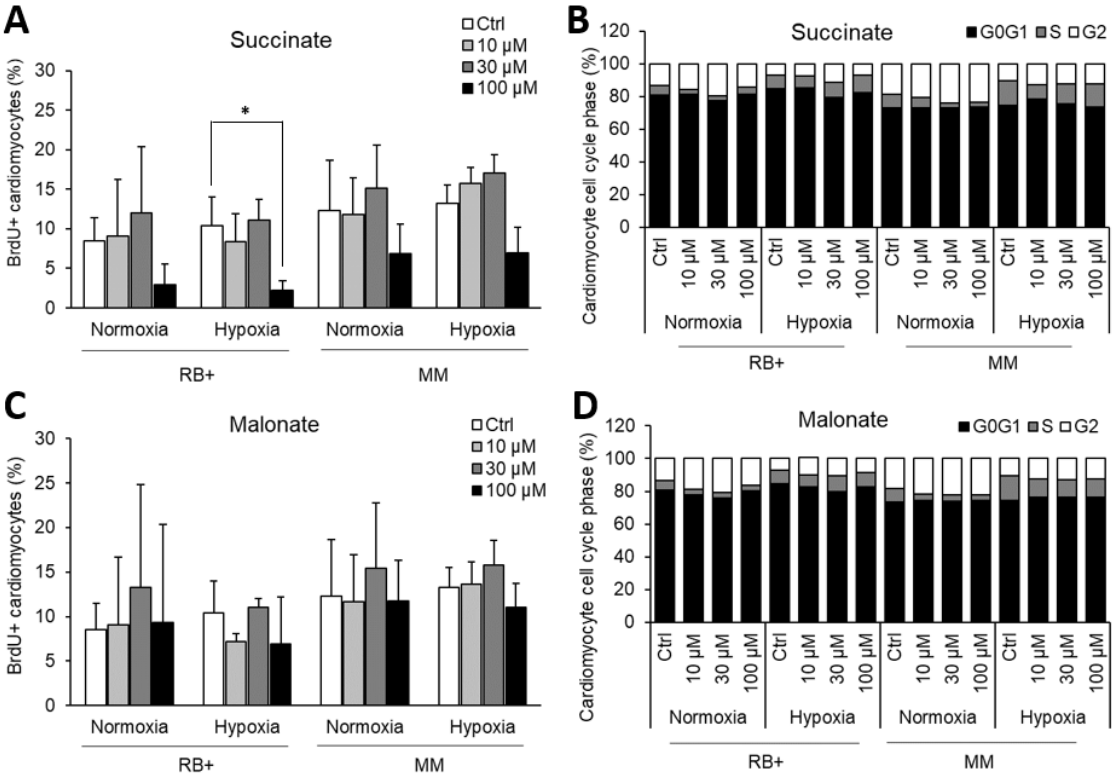

Fig. 3. Effects of 24-hour exposure to succinate and malonate on the percentage of BrdU+ hiPSC-CMs and cell cycle phase of metabolically unmatured and matured hiPSC-CMs. 24-hour compound exposure took place either in fully normoxic conditions or hypoxic conditions (three hours) followed by reoxygenation (21 hours) in normoxic conditions. A) Percentage of BrdU+ hiPSC-CMs after succinate treatment B) Percentage of hiPSC-CMs in G_0/G_1 phase, S phase and G_2 phase of cell cycle after succinate treatment. C) Percentage of BrdU+ hiPSC-CMs after malonate treatment D) Percentage of hiPSC-CMs in G_0/G_1 phase, S phase and G_2 phase of cell cycle after malonate treatment. Results are presented as mean (A–D) + SD (A; C) of independent experiments with hiPSC-CMs from individual differentiations (n=3). *$p < 0.05$ (Welch's ANOVA followed by Games-Howell).

Neither succinate or malonate induced statistically significant alterations in expression of proBNP in hiPSC-CMs. Expression of proBNP tended to increase with the lowest concentration (10 µM) of succinate at all experimental conditions (Fig. 4A). At other concentrations there were no changes in proBNP expression of hiPSC-CMs with succinate, but expression tended to decrease with increasing concentrations. Similarly to succinate, malonate also tended to increase proportion of proBNP+ hiPSC-CMs at lowest concentration (Fig. 4B). Malonate also decreased proBNP expression at the concentration of 100 µM, suggesting attenuating effects on cardiomyocyte stress response.

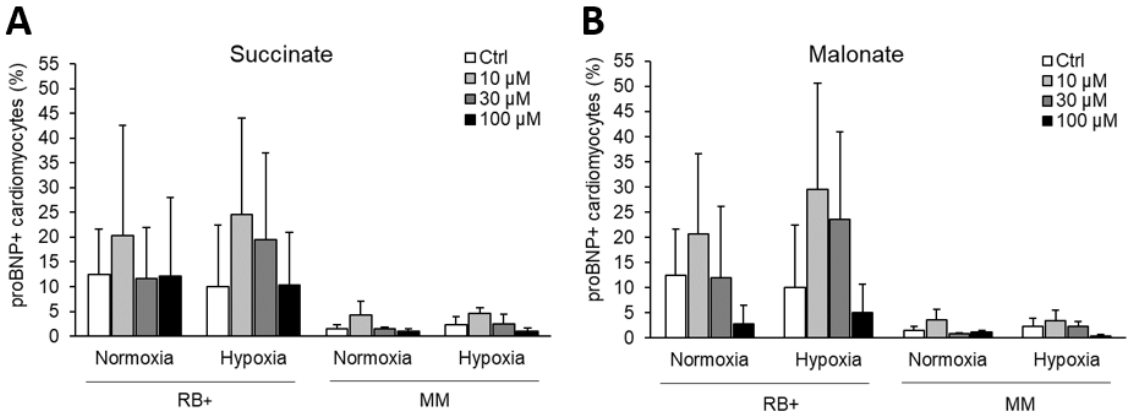

Fig. 4. Effects of 24-hour exposure to succinate and malonate on proBNP expression of metabolically unmatured and matured hiPSC-CMs. The 24-hour compound exposure took place either in fully normoxic conditions or hypoxic conditions (three hours) followed by reoxygenation (21 hours) in normoxic conditions. A) Percentage of proBNP+ hiPSC-CMs after succinate treatment B) Percentage of proBNP+ hiPSC-CMs after malonate treatment. Results are presented as mean + SD of independent experiments with hiPSC-CMs from individual differentiations (n=3).

3.4 The effect of modulation of the mevalonate pathway and ketogenesis on cardiomyocyte phenotype

We evaluated the roles of the mevalonate pathway and ketogenesis on cardiomyocyte phenotype and cell cycle activity by treating metabolically matured and unmatured hiPSC-CMs with hymeglusin (inhibitor of mevalonate pathway and ketogenesis) and simvastatin (inhibitor of the mevalonate pathway). Hymeglusin had no significant effect on the percentage of BrdU+ hiPSC-CMs in any experimental condition, although at concentrations of 1 µM and 10 µM it tended to decrease BrdU+ cells in metabolically unmatured hiPSC-CMs (Fig. 5A). Cell cycle analysis showed a similar trend: percentages of hiPSC-CMs in S phase of cell cycle were lower in all groups treated with hymeglusin (Fig. 5B). Correspondingly, proportion of hiPSC-CMs in G_2 phase tended to increase with hymeglusin treatment. The effects of simvastatin on the percentage of BrdU+ hiPSC-CMs were similarly modest. The proportion of BrdU+ cells was slightly reduced in unmatured hiPSC-CMs. In matured hiPSC-CMs simvastatin had no effect on the percentage of BrdU+ hiPSC-CMs in normoxic conditions, but slightly increased it in a concentration-dependent manner in cells treated in hypoxic conditions (Fig. 5C). There were no significant changes in proportions of hiPSC-CMs in each cell cycle phase after

simvastatin treatment (Fig. 5D). To further determine the role of ketogenesis we investigated the effects of β-hydroxybutyrate exposure following the same experimental conditions as described above. Effects of β-hydroxybutyrate showed as slight increase in the proportion of BrdU+ cells in a concentration-dependent manner in normoxia both in unmatured and matured hiPSC-CMs (Fig. 5E). In hiPSC-CMs exposed to hypoxia, the percentage of BrdU+ cells increased with concentrations of 30 µM and 100 µM, but also not significantly. β-hydroxybutyrate-induced alterations in percentages of hiPSC-CMs in cell cycle phases S and G_2 followed a similar pattern than the percentage of BrdU+ cells (Fig. 5F).

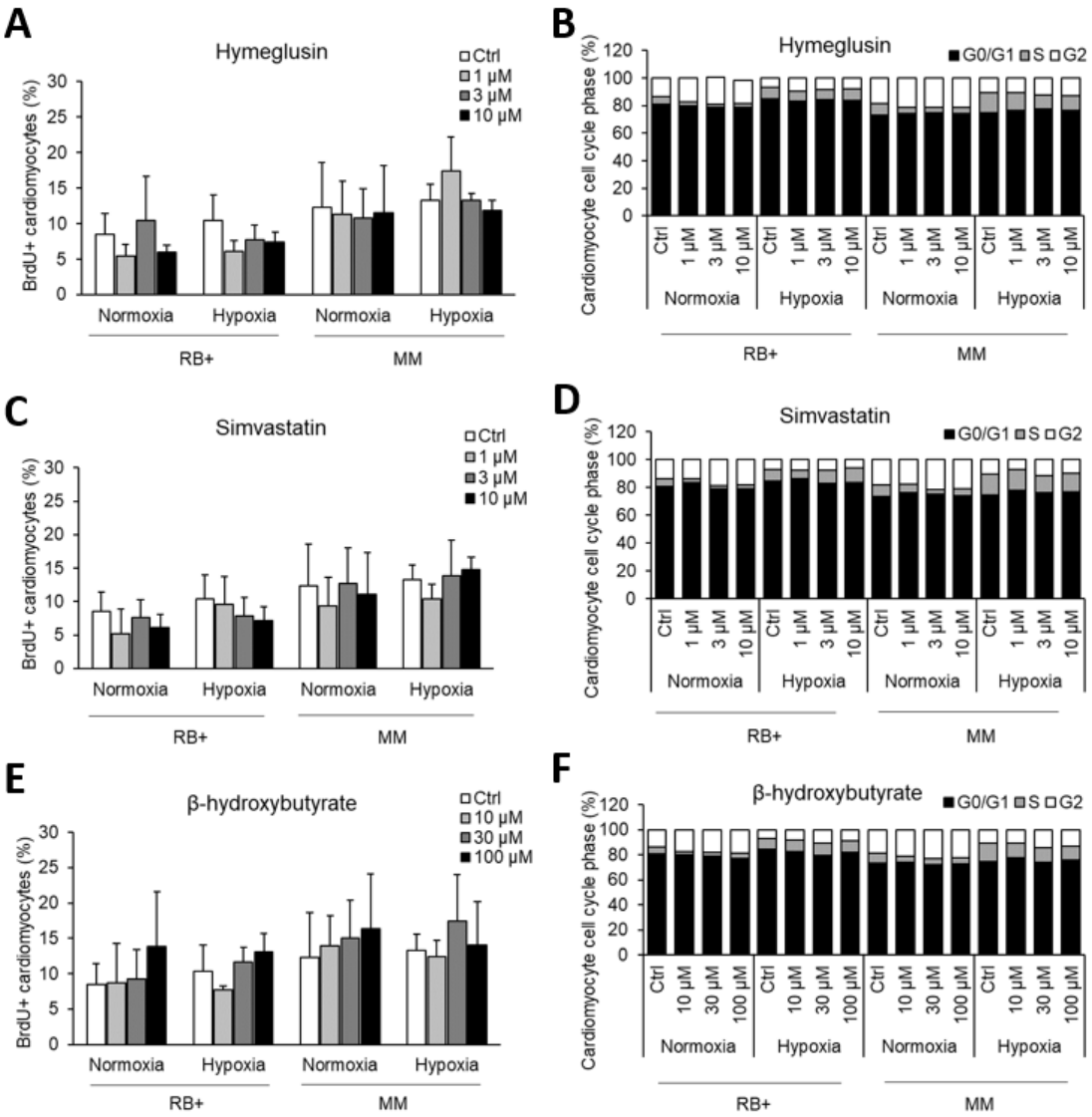

Fig. 5. Effects of 24-hour exposure to hymeglusin, simvastatin and β-hydroxybutyrate on percentage of BrdU+ hiPSC-CMs and cell cycle phase of metabolically unmatured and matured

hiPSC-CMs. The 24-hour compound exposure took place in either in fully normoxic conditions or hypoxic conditions (three hours) followed by reoxygenation (21 hours) in normoxic conditions. A) Percentage of BrdU+ cardiomyocytes after hymeglusin treatment B) Percentage of hiPSC-CMs in G_0/G_1 phase, S phase and G_2 phase of cell cycle after hymeglusin treatment C) Percentage of BrdU+ hiPSC-CMs after simvastatin treatment. D) Percentage of hiPSC-CMs in G_0/G_1 phase, S phase and G_2 phase of cell cycle after simvastatin treatment E) Percentage of BrdU+ hiPSC-CMs after β-hydroxybutyrate treatment. F) Percentage of hiPSC-CMs in G_0/G_1 phase, S phase and G_2 phase of cell cycle after β-hydroxybutyrate treatment. Results are presented as mean (A–F) + SD (A; C; E) of independent experiments with hiPSC-CMs from individual differentiations (n=3).

ProBNP expression was increased by hymeglusin, simvastatin and β-hydroxybutyrate at lowest concentrations, yet not statistically significantly (Fig. 6). Hymeglusin increased proBNP expression at all concentrations in all conditions except for unmatured hiPSC-CMs in normoxic conditions. The increase was strongest in unmatured hiPSC-CMs under hypoxia exposure (21.6 %) (Fig. 6A). Simvastatin increased proBNP expression at all concentrations in all conditions, with most marked increase of 18.8 % in unmatured hiPSC-CMs maintained in normoxic conditions. β-hydroxybutyrate induced a decrease in proBNP expression at 100 µM in all conditions, with a 9 % decrease in unmatured hiPSC-CMs maintained in normoxic conditions.

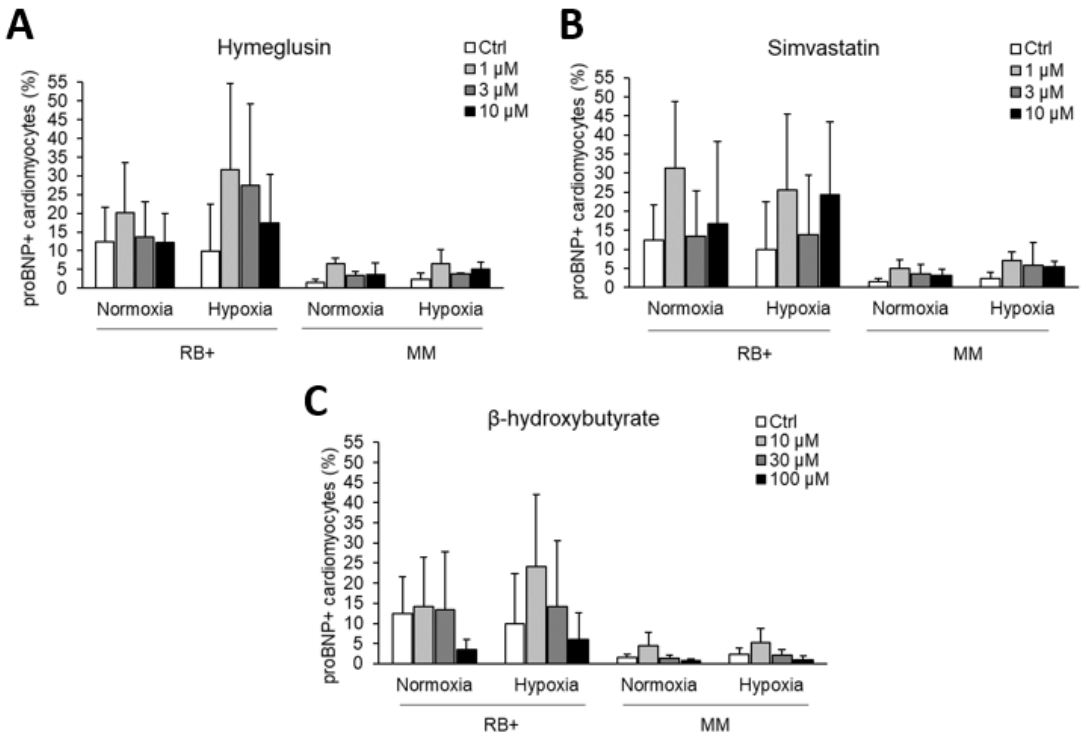

Fig. 6. Effects of 24-hour exposure to hymeglusin, simvastatin and β-hydroxybutyrate on proBNP expression of metabolically unmatured and matured hiPSC-CMs. The 24-hour compound exposure took place in either in fully normoxic conditions, or hypoxic conditions (three hours) followed by reoxygenation (21 hours) in normoxic conditions. A) Percentage of proBNP+ hiPSC-CMs after hymeglusin treatment. B) Percentage of proBNP+ hiPSC-CMs after simvastatin treatment. C) Percentage of proBNP+ hiPSC-CMs after β-hydroxybutyrate treatment. Results are presented as mean + SD of independent experiments with hiPSC-CMs from individual differentiations (n=3).

4 DISCUSSION

In search of therapeutic strategies for cardiac regeneration, induction of cell cycle re-entry of existing cardiomyocytes is an ever-lasting goal. Evidence on the dynamic nature of cardiac energy metabolism throughout cardiac development, disease and regeneration gives reason to evaluate extensively the role of cellular processes related to cardiomyocyte metabolism and the contribution of different metabolic pathways to cardiomyocyte cell cycle activity.

Metabolic maturation of hiPSC-CMs occurs through introduction of high concentration of palmitic acid combined with limited glucose and no insulin supply (Mills et al. 2017). The metabolic shift induces cardiomyocyte cell cycle exit and decreases proliferation rate of hiPSC-CMs, together with increased production of reactive oxygen species (ROS) due to onset of mitochondrial oxidative metabolism (Puente et al. 2014; Mills. et al. 2017). In this study, *SDHA* expression was more pronounced in matured hiPSC-CMs compared to unmatured cells, indicating onset of oxidative metabolism due to metabolic maturation. This SDHA expression was reduced in hypoxia, further implying the occurrence of metabolic maturation. Interestingly, metabolic maturation of hiPSC-CMs increased proliferation rate and decreased hypertrophic stress response in comparison with unmatured hiPSC-CMs. This discrepancy raises a suspicion about the success and extent of metabolic maturation of hiPSC-CMs. On the other hand, one approach to hiPSC-CM maturation is most likely not enough to promote sufficient maturation, as cardiomyocyte maturation is regulated by multiple complex and overlapping signaling networks. In addition to metabolic maturation, mammalian cardiomyocyte maturation consists of diverse overlapping developmental changes in morphology and functions of cells (Chung et al. 2007; Mills et al. 2017; Karbassi et al. 2020). Functionally differentiated and

metabolically matured hiPSC-CMs therefore do not entirely represent the properties of terminally differentiated and fully matured adult human cardiomyocytes. Additionally, to properly characterize metabolic phenotype of hiPSC-CMs, more accurate analyses of metabolism, such as oxygen consumption rate measurement and assay of metabolites, are needed. Mills et al. (2017) showed that in hiPSC-CMs cultured in MM for 9 days insulin treatment and introduction of the potent pro-proliferative agent CHIR99021 could not restore proliferation lost during metabolic maturation. Considering this, the lack of marked pro-proliferative effects by compounds experimented in this study is not surprising.

In regard to hypoxia exposure, we expected to see pro-proliferative effects especially in metabolically matured hiPSC-CMs, as hypoxia has been found to induce cardiomyocyte proliferation after myocardial infarction (Nakada et al. 2017). Also, chronic hypoxic exposure of adult mice reduces mitochondrial oxidative metabolism and oxidative DNA damage, resulting in cell cycle re-entry of cardiomyocytes, attenuated by introduction of a ROS generator (Nakada et al. 2017). Oxygen promotes ROS production, leading to oxidative DNA damage and intracellular stress response signaling, which contribute to cell cycle exit of cardiomyocytes (Puente et al. 2014; Nakada et al. 2017). In our study, however, pro-proliferative effects of hypoxia, indicated by BrdU+ hiPSC-CMs, were modest, even if cell cycle analysis showed an increase in proportion of cells in S phase of cell cycle, which suggests induction of DNA replication prior to cell division.

Based on current knowledge, hypoxia signaling plays a crucial role in cell cycle control of cardiomyocytes. Hypoxia signaling by Hypoxia Inducible Factor 1α (HIF-1α) regulates angiogenesis and hypoxic stress response through modulation of VEGF. HIF-1α is an important factor participating in fetal cardiomyocyte development as it directly induces glycolysis and regulates cell cycle and stress response (Guimarães-Camboa et al. 2015). Additionally, HIF-1α activation is associated with cell cycle re-entry during cardiac regeneration. Here, gene expression analysis of *VEGFA* showed activation of hypoxic stress signaling in both metabolically matured and unmatured hiPSC-CMs after hypoxia exposure, confirming presence of hypoxia signaling. The observed upregulation of *VEGFA* under hypoxia is in line with a previous study demonstrating upregulation of

HIF-1α and VEGFA expression in cultured primary neonatal rat cardiomyocytes at hypoxia exposure (Sano et al. 2007). Surprisingly, we did not detect significant changes in proliferation and hypertrophy of metabolically matured cardiomyocytes under hypoxia.

In hypoxia or ischemia, cessation of oxidative phosphorylation causes SDH reversal and accumulation of succinate in the mitochondria (Chouchani et al. 2014). When oxygen supply is restored, re-oxidation of succinate causes formation of ROS, further promoting cell-cycle exit and cellular stress responses. In this study, introduction of succinate decreased hiPSC-CM proliferation in hypoxic unmatured cardiomyocytes. In line with this, we detected increased expression of the hypertrophy and stress biomarker proBNP in these conditions. This experimental condition mimics hypoxic fetal/neonatal cardiomyocytes. A recent study by Bae et al. (2021) emphasized that succinate causes cardiomyocyte cell cycle exit and inhibits heart regeneration in post-infarcted neonatal mice, where MI was generated one day after birth. Unexpectedly, our results show that promotion of glycolysis through inhibition of SDH with the small-molecule compound malonate could not induce proliferation in hiPSC-CMs regardless of the maturation state. After all, malonate decreased hypertrophy and stress signaling, suggesting reduction in DNA damage by ROS.

A previous study reported marked reduction in cardiomyocyte proliferation after three-day exposure to simvastatin at 10 μM in proliferative hiPSC-CMs (Mills et al. 2019). The study also demonstrated *in vivo* that the mevalonate pathway is required for postnatal heart growth by cardiomyocyte proliferation. On the other hand, another study on cholesterol biosynthetic processes with metabolomics and proteomics found that cardiomyocyte cell cycle might actually be regulated rather by ketogenesis than the mevalonate pathway, observed as hymeglusin-induced decrease of proliferation of neonatal rat ventricular cardiomyocytes, while simvastatin had no effect (Talman et al. 2018). In our study, there was no difference between effects of mevalonate pathway inhibition with simvastatin or inhibition of both the mevalonate pathway and ketogenesis with hymeglusin on hiPSC-CM proliferation and stress response. This finding together with our data on the pro-proliferative tendency of β-hydroxybutyrate also supports the current assumption that the contribution of ketogenesis to cell-cycle entry is more

significant than that of the mevalonate pathway. Mevalonate pathway activity was however more pronounced in metabolically matured cells, seen as higher *HMGCR* expression, and hypoxia seems to have downregulating effects on pathway activities. We detected no correlation between *HMGCR* expression and proportion of BrdU+ cells in metabolically matured hiPSC-CMs.

Metabolic phenotype and proliferation of unmatured cardiomyocytes utilizing glycolysis and metabolically matured hiPSC-CMs utilizing fatty acid oxidation were not altered by hypoxia and did not differ significantly from each other. Consequently, with methodology used in this study, comparison of effects of metabolic modulation at the four experimental conditions could not provide in-depth information on alterations in metabolic phenotype and cell cycle regulation. Due to the low number of biological replicates, the statistical significance of results was very limited. We plated hiPSC-CMs on each well of the 96-well plates and located control wells at the edges of the plates. Higher evaporation of the wells close to the edge may have contributed to the high variation and reverse concentrations-responses observed with compound treatments. Also, proBNP results need to be interpreted cautiously, because prior to staining for proBNP, exocytosis of intracellular vesicles was not prevented with Brefeldin A, which caused low signal-to-noise ratio in HCA. To allow better interpretation of proBNP results, a positive control could have been added in the experimental design. This would have allowed to compare effects hypertrophic effects of hypoxia, metabolic maturation and metabolic modulators to a known pro-hypertrophic treatment, such as endothelin-1. Correspondingly, pro-proliferative effects of hypoxia, β-hydroxybutyrate and malonate, and anti-proliferative effects of succinate, simvastatin and hymeglusin could have been better evaluated if a known potent pro-proliferating compound, such as a p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, was used as positive control for cardiomyocyte proliferation (Uosaki et al. 2013).

In conclusion, we observed occurrence of metabolic maturation and hypoxia signaling at gene expression level, but effects on hiPSC-CMs phenotype were modest. Promotion of mitochondrial oxidative metabolism and ROS production with succinate attenuated proliferation of metabolically unmatured hypoxic hiPSC-CMs, which supports current

evidence of anti-proliferative effects of oxidative metabolism. Results obtained in this study reinforce current knowledge and hypotheses about the involvement of oxidative metabolism, glycolysis and ketogenesis in the regulation of cardiomyocyte cell cycle. Based on our results, hypoxic hiPSC-CMs can be a useful tool to study outcomes of different experimental conditions on gene expression and phenotype. However, to verify the accuracy and extent of metabolic maturation, modulation of metabolism and the relation between hypoxia signaling and different metabolism pathways, additional methodologies are required.

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8 ANNEXES

8.1 Literature review

Literature review:

THE ROLE OF ENERGY METABOLISM IN CARDIOMYOCYTE PROLIFERATION

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1 INTRODUCTION

The mammalian heart contracts repetitively and continuously, which means its energy demand is constant. Most of the energy requirements of the adult heart are met by mitochondrial oxidative metabolism, of which fatty acids are the main source (Murashige et al. 2020). Electric function and contractility of the myocardium together with mitochondrial adenosine-5-triphosphate (ATP) production and availability of metabolic substrates are the basic requirements for normal cardiac function. The heart has a unique ability to utilize multiple energy substrates in order to maintain contractile function and homeostasis of the heart, which is vital for the whole organism (Murashige et al. 2020).

In the fetal heart anaerobic glycolysis is the main source for ATP production (Piquereau & Ventura-Clapier 2018). As cardiomyocytes differentiate during maturation in the perinatal period, the energy metabolism shifts to β-oxidation of fatty acids as a result of several dynamic changes in the energy demand, substrate availability and regulation of signaling pathways (Lopaschuk et al. 2010). This transition correlates with mitotic cell cycle exit of cardiomyocytes, resulting in cessation of growth by cell division (proliferation) and onset of cell growth by adaptive physiological hypertrophy (Puente et al. 2014; Piquereau & Ventura-Clapier 2018). Hypertrophy of the cells is characterized by an increase in length and width as a response to hemodynamic resistance, and it allows an increase in the contractility of the heart and reduction of ventricular wall stress (Kolwicz et al. 2013). Onset of hypertrophy involves activation of signaling pathways involved in cell growth, proliferation, survival and angiogenesis (Nakamura & Sadoshima 2018).

Myocardial injury associated to ischaemic heart disease is the major cause of heart failure, characterized by death of cardiomyocytes and fibrotic scarring of the heart tissue due to low oxygen supply to the heart (Nakamura & Safoshima 2018). The adult mammalian heart has very limited regenerative capacity, as most of differentiated cardiomyocytes permanently exit the cell cycle shortly after birth and lose proliferation capacity. Instead, cardiomyocytes respond to injury by induction of maladaptive pathological hypertrophy, often leading to

contractile dysfunction of the heart accompanied by several intracellular and extracellular changes that negatively affect the homeostasis of the heart. During heart failure the energy metabolism of cardiomyocytes also transitions to a state resembling the metabolic profile of the fetal heart: preference for glycolysis increases and oxidation of fatty acids decreases.

Impressively, hearts of certain cold-blooded vertebrates, such as zebrafish and some amphibians have the ability to regenerate through the adult life of the animal (Becker et al. 1974; Jopling et al. 2010). Neonatal rodents can also regenerate their hearts after injury in the first postnatal week before cell cycle exit (Porrello et al. 2011). Regeneration has been shown to occur through dedifferentiation and proliferation of surviving cardiomyocytes in the injury site (Porrello et al. 2013). This post-injury regenerative capacity has given reason for extensive research with the aim of understanding the pathways underlying proliferation of cardiomyocytes in order to find strategies to induce regeneration in human hearts. Indeed, several approaches have been taken to enhance regeneration through increasing the amount of cardiomyocytes in the injury site, but none of them have shown optimal properties for a wide and cost-effective use, which is required in the treatment of heart diseases. Therefore, in depth understanding of the factors underlying proliferation of cardiomyocytes, including the role of cardiac metabolism, is needed in to develop better tools to fight heart diseases.

In this literature review, I will summarize changes in energy metabolism of maturating cardiomyocytes along with the current knowledge on mechanisms of endogenous post-injury cardiomyocyte renewal. In addition, I will discuss previous attempts of energy metabolism related regeneration-inducing therapies, as well as ongoing research and future directions.

2 ENERGY METABOLISM OF MAMMALIAN CELLS

Cell energy metabolism is the prerequisite of all activities of the cell, including cell growth and proliferation. Proliferation of mammalian cells is related to various cell activities, such as embryogenesis, development of immune cells and growth of tumors (Rafalski et al. 2012).

Differentiation of stem cells into mature proliferating cells is associated to a shift in the energy metabolic phenotype of the cells. Also, reprogramming of glucose metabolism is considered typical in certain tumor cells (Hanahan & Weinberg 2011). When differentiated cells proliferate and grow, high amounts of macromolecules are required to support the needs of cell replication, cell division and energetic requirements of different anabolic processes (Rafalski et al. 2012).

Energy-releasing catabolic processes in mammalian cells are fueled mostly by glucose, fatty acids and amino acids. Energy production from these molecules normally occurs through mitochondrial oxidative phosphorylation, leading to generation of energy stored in the form of ATP molecules.

2.1 Glycolysis, TCA cycle and oxidative phosphorylation

Energy from glucose is produced by glycolysis. A glucose molecule is catabolized by multiple enzymes into two pyruvate molecules and NADH in the cytosol, producing two ATP molecules. In aerobic conditions the pyruvate enters the mitochondria and is decarboxylated to acetyl-coenzyme A (Acetyl-CoA), which next enters the tricarboxylic acid (TCA) cycle and energy from its chemical bonds is gradually stored by reducing the energy shuttles NAD^+ and FAD molecules to three NAHD and one $FADH_2$ molecules as illustrated in Figure 1 (Martínez-Reyes & Chandel 2020). Succinate dehydrogenase (SDH) is considered a particular enzyme, as it is involved in both the TCA cycle and the electron transport chain. It is responsible for the oxidation of succinate into fumarate and transfers two hydrogen atoms to FAD, generating two $FADH_2$ molecules. NAHD and $FADH_2$ in turn undergo an electron transfer process and oxidative phosphorylation to generate ATP molecules. The energy shuttles oxidize by transferring high-energy molecules to protein complexes that finally link electrons and protons to oxygen, forming H_20. An electrochemical gradient is formed between the two sides of the inner mitochondrial membrane, and as protons travel across it, ADP is converted to ATP. As a result of oxidative phosphorylation 36 ATP molecules are formed.

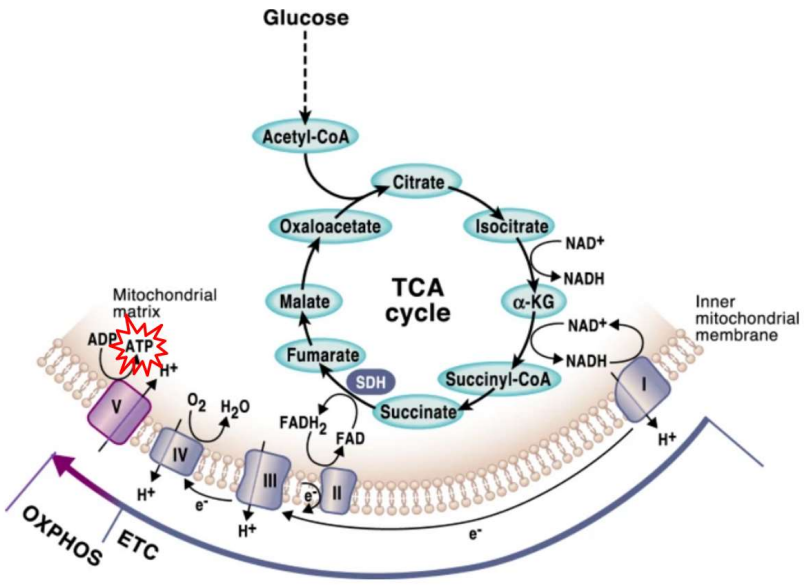

Figure 1. Overview of the TCA cycle, electron transport chain and oxidative phosphorylation in ATP production. OXPHOS = oxidative phosphorylation; ETC = electric transport chain; SDH = succinate dehydrogenase. Picture modified from: Martínez-Reyes & Chandel 2020.

In the absence of oxygen after glycolysis the pyruvate is further processed to lactate, as it cannot be oxidized in anaerobic conditions. Anaerobic glycolysis is markedly less efficient than oxidative phosphorylation, as it produces only two ATP molecules. Exogenous lactate can also be taken up by cells and be converted to pyruvate via lactate dehydrogenase (LDH), as described in Figure 2. (Lopachuck et al. 1992; Makinde et al. 1998) Pyruvate is converted to Acetyl-CoA by pyryvate dehydrogenase (PHD) and processed in the TCA cycle. The uptake of glucose is regulated by growth factor-mediated signaling pathways. The intermediates of glycolysis and TCA cycle can be also used for the biosynthesis of nucleotides, non-necessary amino acics and fatty acids.

2.2 β-oxidation of fatty acids

ATP can be generated through degradation of triglycerides and phospholipids into fatty acids. Fatty acids are taken up to cells by lipoprotein lipase and fatty acid translocase (Mashima et al. 2009). Also, they can be synthetized *de novo* by retracting citrate removed from TCA

cycle of pyruvates. Processing of citrate releases acetyl-CoA, in turn converted to malonyl-CoA and further utilized in fatty acid synthesis.

Fatty acids are esterified to fatty acyl-CoA by fatty acyl-CoA synthase (FACS) (Lopaschuk et al. 2010). Next the molecules can be modified to more complex lipids such as triacylglycerol, or the acyl group is transferred to carnitine via carnitine palmitoyltransferase 1 (CPT 1). Next, acylcarnitine is converted back to fatty acyl-CoA by CPT 2 in the mitochondria, and it enters the fatty acid β-oxidation cycle. End-products of the cycle are Acetyl-CoA, NADH, and $FADH_2$. Acetyl-CoA is processed in the TCA cycle, followed by oxidative phosphorylation as described above. This pathway is described in Figure 2.

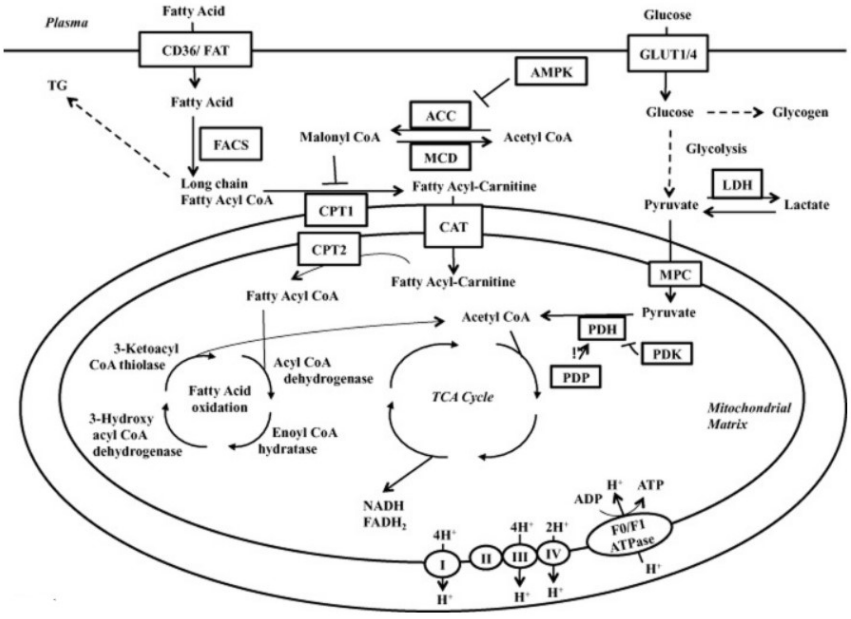

Figure 2. Fatty acid and glucose oxidation. ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; CPT, carnitine palmitoyl transferase; CAT, carnitine translocase; FACS, fatty acyl CoA synthetase; FAT, fatty acid transporter; GLUT, glucose transporter; LDH, lactate dehydrogenase; MCD, malonyl CoA decarboxylase; MPC, mitochondrial pyruvate carrier; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PDP, pyruvate dehydrogenase phosphatase; TCA, tricarboxylic acid; TG, triacylglycerol. Source: Fillmore et al. 2014

2.3 Other energy metabolism pathways

Glucose oxidation can also occur through the pentose phosphate pathway (PPP) parallel to glycolysis (Alfarouk et al. 2020). It uses glucose 6-phosphate (G6P) as a starting product and consists of an oxidative phase and non-oxidative phase, ending up in the production of 5-carbon sugars (further used for nucleotide and nucleic acid synthesis) and NADPH. The PPP pathway is considered anabolic rather than catabolic, as its end products contribute more to biological functions than energy production.

Ketone body oxidation becomes an important energy metabolism pathway when low amounts of dietary carbohydrates are available (Cotter et al. 2013). Ketone bodies are derived from the TCA cycle of oxidized fatty acids (see 3.4.1 for more detailed pathway). Oxidation of ketone bodies (mostly beta-hydroxybutyrate, βOHB) is composed of a series of reactions resulting in formation of Acetyl-CoA that next enters the TCA cycle for ATP production. The most important enzyme of the reaction is succinyl-CoA:3-oxoacid-CoA transferase (SCOT).

3 CARDIOMYOCYTE ENERGY METABOLISM

Cardiomyocytes are responsible for the generation of contraction of the heart. Maturation of cardiomyocytes from the fetal state to adult state consists of a sequence of dynamic changes in the structure, metabolism and transcriptional regulation of the cells (Chung et al. 2007). The metabolic phenotype of the cells at different stages is affected and determined by cell signaling pathways, the demands of energy, and the availability of metabolic substrates. Additionally, the type of energy source affects the differentiation and self-renewal of cells, as well as the susceptibility of the heart to hypoxia or ischaemia (Kolwicz et al. 2013; Nakano et al. 2017).

3.1 Energy metabolism in cardiogenesis and cell differentiation

During maturation of the embryo cardiogenesis is driven by cardiac progenitors that derive from the mesodermal germ layer of the early embryo (Olson & Srivastava 1996). Two types of progenitor cells are responsible for the formation of the heart tube and ultimately the 4 chambers of the heart. Transition from noncontractile pluripotent stem cells to beating cardiomyocytes raises a need for an organized and efficient energetic network.

Differentiation of progenitor cells to first immature and then mature proliferative cardiomyocytes is driven by changes in the expression of pro-proliferative transcription factors. Glycolysis is considered a determining factor for pluripotency, as its reduction is associated to decreased capacity of proliferation (Gu et al. 2017). As the environment of the embryo and fetus has low oxygen concentrations, the energy supply of the cells is depending mostly on anaerobic glycolysis, regulated by hypoxia-inducible factor 1α (HIF-1α) (Guimarães-Camboa et al. 2015). Anaerobic glycolysis is characterized by high lactate production and low oxygen consumption (Chung et al. 2010; Gu et al. 2017). Low oxygen consumption also correlates to the poorly developed and sparse mitochondria and enzymes involved in oxidative phosphorylation in the differentiating pluripotent embryonic stem cells (Mackler et al. 1971; Chung et al. 2007). The preference of the heart to glycolysis as energy source can be in part explained by the high concentration of lactate in the environment of the embryo. Lactate is an important source of energy in the fetal heart, and most of the oxygen consumption of the heart is used for lactate oxidation (Comline & Silver 1976; Werner & Sicard 1987). Physiological concentrations of lactate inhibit fatty acid oxidation and consequently concentrations of fatty acids are low in the environment of the fetus in comparison with newborns (Warshaw 1972; Lopachuck et al. 2017).

Differentiation of pluripotent stem cells towards cardiomyocytes is driven by transcriptional regulation of Myc, Glycogen synthase kinase 3β (GSK3β), insulin and Wnt/β-catenin signaling pathways (Cliff et al. 2017; Quaife-Ryan et al. 2020). These pathways determine the expression of mesodermal and cardiogenesis modulators, rate of glycogen synthesis and

factors in cell fate and proliferation. Differentiation is associated to a decrease in anaerobic glycolysis and increase in oxidative phosphorylation of fatty acids as source of ATP production of the cells (Chung et al. 2010). Transcriptional regulation leading to introduction of fatty acid oxidation is mediated by a circuit of peroxisome proliferator-activated receptor (PPAR), estrogen-related receptor (ERR) and PPARγ coactivator-1 α (PGC1α) (Lai et al. 2008; Lehman et al. 2000). The metabolic shift is precisely timed and balanced as at early phases of differentiation the need for glucose is still high, whilst inactivation of glycolysis is essential for proper maturation of cardiomyocytes (Chung et al. 2007; Nakano et al. 2017).

3.2 Energy metabolism of fetal and neonatal heart

The final phase of heart development is the maturation of the cells, preparing the heart for life-long repetitive beating. Maturation includes changes in the transcriptional expression, metabolism and structural features of cardiomyocytes (Nakano et al. 2017). As cells differentiate into mature cardiomyocytes, their mitochondria undergo structural reorganization and increase in number (Piquereau & Ventura-Clapier 2018). They develop to reach a tubular structure and occupy larger volumes of the cells. Mature cardiomyocytes are characterized by mitochondria with densely packed cristae (Chung et al. 2007). Energetic and metabolic cellular networks develop and couple mitochondria with myofibrillar proteins and cytoplamic complexes to provide efficient energy substrate metabolism. These processes are associated to the initiation of electric conduction and contractile function, approximately at third week of gestation in humans, increasing the necessity for efficient energy substrate metabolism. Higher energetic needs are met by the onset of oxidative phosphorylation, observed as a higher mitochondrial membrane potential, increase in consumption of oxygen and expression of enzymes involved in the electron transport chain. Although the onset of contraction of cardiomyocytes happens in early gestation period, terminal differentiation of the cells takes place in the postnatal period (Li et al. 1996).

At birth the heart is facing major changes in the energy substrate supply and hemodynamic resistance, and it is forced to quickly adapt to a new environment and increase cardiac output

to sustain the systemic circulation. The postnatal heart seems to become dependent on oxidative metabolism when proliferative capacity decreases, probably due to oxygen-rich environment (Puente et al. 2014). Increased oxygen and fatty acid supply in the circulation induce the metabolic shift to fatty acid β-oxidation. By 7 days after birth the contribution of glycolysis and lactate oxidation to ATP production is markedly decreased (Piquereau & Ventura-Clapier 2018).

3.2.1 Factors contributing to energy metabolism shift

Onset of fatty acid β-oxidation is affected by multiple regulators of substrate metabolism. Factors involved in regulation are the supply of fatty acids and alternative energy substrates, oxygen supply, regulation of fatty acid uptake and transport and regulation of specific phases of oxidation in mitochondria (Lopaschuk et al. 2010). Kinases, proteins and hormones involved in the allosteric and transcriptional regulation of signaling in energy metabolism pathways are presented in Table 1. Carnitine palmitoyltransferase (CPT) 1 is essential in regulating the rate of mitochondrial metabolism of fatty acids, as it catalyzes the conversion of long-chain Acyl-CoA to acylcarnitine, which then enters the mitochondria for ATP production (Fillmore et al. 2014). CPT 1 is regulated by malonyl-CoA levels. The steps of CTP 1 regulation initiated by a change in insulin and glucagon levels are shown in Figure 3.

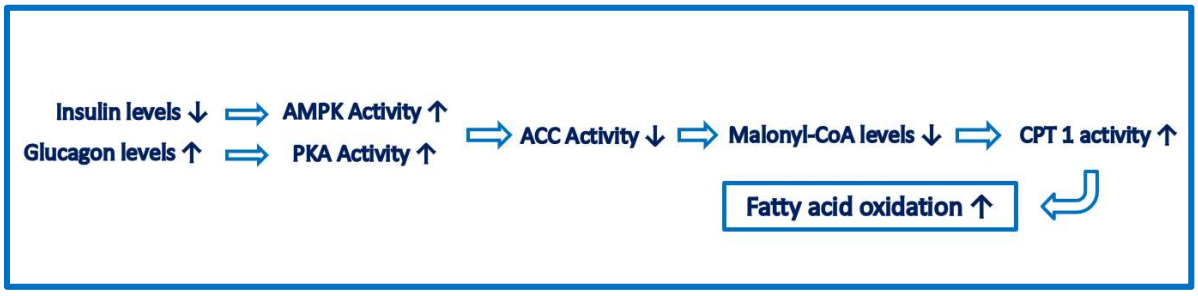

Figure 3. The effect of changes in insulin and glucagen levels on fatty acid oxidation in cardiomyocytes after birth. AMPK = AMP-activated protein kinase; ACC = Acetyl-CoA carboxylase; CPT 1 = carnitine palmitoyltransferase 1.

The availability of fatty acids suppresses numerous steps of the glycolytic pathway, mostly through inhibition of pyruvate oxidation. Uptake of glucose in cardiomyocytes is reduced by

lowering of insulin levels and elevation of glucagon levels in comparison with the fetal period. Fatty acid oxidation increases the amounts of NADH and acetyl-CoA, which inhibit PDH and the oxidation of pyruvate in the mitochondria via the activation of pyruvate dehydrogenase kinase (PDK) (Kerbey et al. 1976; Lopaschuk et al. 2010). Glycolysis is also decreased by acetyl-CoA through the inhibitory effects of citrate on phosphofructokinase-1 (PFK-1) (Lopaschuk et al. 2010). These and other changes in the transcriptional regulation of the metabolic network contributing to shift in energy metabolism are shown in Table 1.

Table 1. Transcriptional regulation and signaling pathway components contributing to shift of cardiomocyte energy metabolism after birth.

Change in metabolic pathway	**Change in signaling pathway**		**References**
	Increase in activity / upregulation	**Decrease in activity / downregulation**	
Glycolysis ↓	ATP, NADH, G6P, citrate, PDH	HIF1α, PPAR/PGC-1α/ERR, insulin, adrenaline, AMP, AMPK, ADP, NAD+	Semenza et al. 2014, Alaynick et al. 2007, Collins-Nakai et al. 1994, Kerbey et al. 1976, Lopaschuk et al. 2010
Fatty acid β-oxidation ↑	PPAR/PGC-1α/ERR, FOXO, AMPK, CoA decaboxylase, fatty acids, glucagen	ACC, Malonyl-CoA, glucose, lactate, ketone bodies, insulin	Alaynick et al. 2007, Lopaschuk et al. 2010, Lai et al. 2008; Lehman et al. 2000, Makinde et al. 1998
Glucose oxidation ↓	PPAR, FOXO, PDK4 Fatty Acids, Acetyl CoA, NADH, ATP	Insulin, adrenaline	Collins-Nakai et al. 1994, Kerbey et al. 1976, Lopaschuk et al. 2010, Makinde et al. 1998, Li et al.
Ketone body oxidation ↓		3-hydroxybutyrate dehydrogenase	Talman et al. 2018
BCAA catabolism ↑		BCKDK, NADH, CoA esters	Talman et al. 2018
Mevalonate ↓		HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase	Talman et al. 2018

3.2.2 Cell cycle arrest

As the metabolic phenotype of terminally differentiating cardiomyocytes transitions, proliferation of the cardiomyocytes decreases, and the cells exit the mitotic cell cycle. Exit from cell cycle is concomitant to the completion of final round of DNA replication without cytokinesis, resulting in binucleated myocytes (Alkass et al. 2015). Proliferation is replaced with cellular growth by hypertrophy, consistent through the perinatal period (Li et al. 1996; Alkass et al. 2015). The drastic increase of oxygen might contribute to the cell cycle arrest of cardiomyocytes. Puente et al. (2014) hypothesized that aerial oxygen may induce upstream signaling leading to production of reactive oxygen species (ROS), which causes oxidative DNA damage and intracellular stress response pathways. These changes seem to contribute to the cessation of proliferative capacity of the cells and induce cell cycle arrest by the end of the first postnatal week.

3.3 Energy metabolism of the adult heart

As the neonatal heart grows, fatty acids remain the main source of ATP production under normal aerobic conditions (Lopaschuk et al. 2010). Fifty to seventy percent of of the acetyl-CoA is derived from β-oxidation of fatty acids and 30–50% from the oxidation of pyruvate, which is obtained in equal amounts from glycolysis and lactate oxidation. The adult heart is characterized by remarkable metabolic flexibility, and the energy metabolism pathway is determined by concentrations of different energy metabolism substrates in the circulation. The cardiac metabolic network is highly adaptive in using substrates other than fatty acids, such as glucose, lactate, ketone bodies and amino acids, when they become abundantly available (Wentz et al. 2010)

Reprogramming of metabolism in response to pathological hypertrophy is characterized by remodeling of the ATP production system by reduction of oxidation of fatty acids and glucose with an increase of glycolysis (Nakamura & Sadoshima 2018). Also, the use of ketone bodies and lactate as energy substrate increases (Murashige et al. 2020). These

changes are driven by the downregulation of transcriptional regulators of fatty acid oxidation via PPARα in response to pressure overload (Young et al. 2001). The reduction of fatty acid oxidation is not fully compensated by glycolysis and the introduction of other substrates, leading to a decrease in overall energy supply and development of heart failure (Nakamura & Sadoshima 2018). These changes lead the metabolism of hypertrophic cardiomyocytes to a state that resembles the metabolic profile of the fetal heart.

3.4 Other metabolic pathways involved in cardiomyocyte metabolism

As seen in Table 1, alteration of other metabolic pathways than glycolysis and β-oxidation of fatty acids is involved in the terminal differentiation and cessation of proliferation of cardiomyocytes after birth. Alterations in these pathways after birth and during myocardial injury make them an interesting subject for research in finding strategies for regenerative therapies.

3.4.1 Mevalonate pathway and ketogenesis

The mevalonate pathway (Figure 4) is a metabolic pathway that produces sterol isoprenoids, such as cholesterol, and nonsterol isoprenoids, essential for multiple cellular processes, such as post-translational modification of proteins involved in intracellular signaling of cell growth and differentiation (Buhaescu & Izzedine 2007). HMG-CoA serves as a substrate for the ketogenesis route producing 3-hydroxybutyrate.

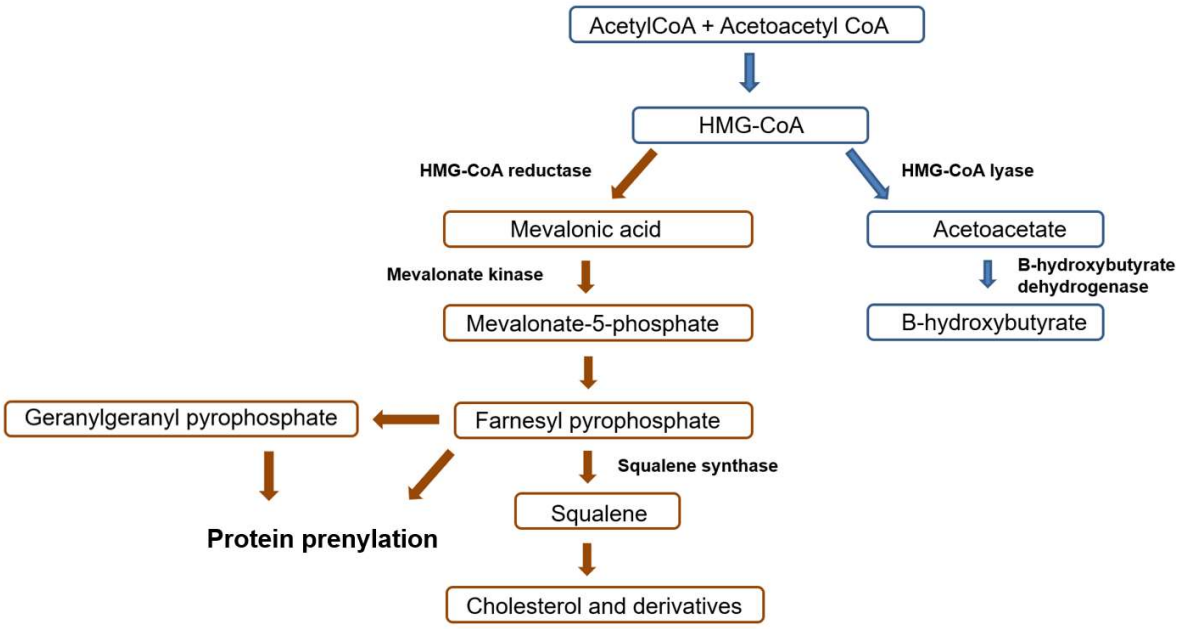

Figure 4. The simplified mevalonate pathway. The pathway starts in the cytosol with the synthesis of 3-hydroxy-3-metylglutaryl-CoA (HMG-CoA) from Acetyl-CoA. Next HMG-CoA is converted to mevalonic acid by HMG-CoA reductase (HMGR), which is the first rate-limiting enzyme of the pathway. Next, the mevalonate kinase catalyzes the phosphorylation of mevalonic acid to phosphomevalonate. Multiple steps lead to the formation of the intermediates farnesyl-pyrophosphate and geranylgeranyl pyrophosphate, which are involved in the prenylation of proteins (essential in intracellular protein-protein interactions) in cells. In ketogenesis HMG-CoA is converted to Acetoacetate by HMG-CoA lyase. The end product of ketogenesis, β-hydroxybutyrate, is oxidized in the heart for ATP production.

The intermediates of the mevalonate pathway have been found to regulate the proliferation of cells both *in vivo* and *in vitro* (Mills et al. 2019). Talman et al. (2018) demonstrated a marked increase in the mevalonate pathway after birth in mice. The increase was followed by downregulation of multiple enzymes and kinases involved in the pathway with increasing postnatal age. Accordingly, it has been shown that inhibition of mevalonate pathway at post-natal days 1–15 decreases cardiomyocyte proliferation and reduces heart size in mice (Mills et al. 2019). These findings suggest that the mevalonate pathway is involved in the transition of cardiomyocytes from proliferative to non-proliferative state during the postnatal period. Moreover, Mills et al. (2019) demonstrated that introduction of mevalonate and geranylgeranyl pyrophosphate initiate proliferation of adult cardiomyocytes *in vivo* when mevalonate pathway had been previously suppressed. These results indicate that intermediates of the mevalonate pathway are required for proliferation of cardiomyocytes.

Ketogenesis takes place predominantly in the liver and produces ketone bodies (acetone, acetoacetate, and β-hydroxybutyrate) that supply energy to the heart when low concentrations of carbohydrates are available (Cotter et al. 2013). Ketone bodies are synthetized from acetyl-CoA derived from the TCA cycle of oxidized fatty acids (Robinson & Williamson 1980). The contribution of ketone body oxidation to the overall cardiac energy metabolism is increased in the neonatal period and at physiological states related to low glucose supply. The pathway seems to take place and be regulated locally in the heart as well, shown as upregulation of the rate-limiting enzyme HMG-CoA lyase after birth (Talman et al. 2018). Similarly to the mevalonate pathway, transient activation of ketogenesis at birth followed by decrease in the postnatal days in the mouse heart has been reported (Talman et al. 2018). Proliferation of cardiomyocytes *in vitro* is attenuated by the inhibition of both the mevalonate pathway and ketogenesis, but not the mevalonate pathway alone, suggesting that ketogenesis might have a role in the regulation of cardiomyocyte cell cycle.

3.4.2 Branched-chain amino acids

Branched-chain amino acids (BCAAs), including leucine, isoleucine and valine, are essential amino acids for mammals (Huang et al. 2011). Additional to serving as substrate for peptide synthesis, they are sources of sterol, ketone bodies and glucose synthesis. In the BCAA catabolic pathway, BCAAs are converted into branched-chain α-ketoacids (BCKAs), decarboxylated and metabolized to acetyl-CoA or succinyl-CoA prior to oxidation in the TCA cycle. BCAAs are essential in cellular function and growth, as they regulate signaling pathways promoting protein synthesis, cellular metabolism and cell growth. Importantly, they activate mTOR (mammalian target of rapamycin) signaling involved in regulation of cardiac homeostasis and cardiac hypertrophy (Sciarretta et al. 2018). Analysis of BCAA concentrations in the postnatal mice heart has shown an increase of levels in the first 9 days, after which concentrations start to decrease (Talman et al. 2018).

Elevated levels of BCAAs are associated with suppression of glucose metabolism by inhibiting the PDH activity and thus interfering mitochondrial pyruvate utilization *in vivo* (Li

et al. 2017). Also, glucose oxidation is reduced by BCAAs through downregulation of the hexosamine pathway. Observational studies indicate that high plasma concentrations of BCAAs align with obesity, insulin resistance and cardiovascular diseases (Newgard 2012; Tobias et al. 2019).

4 ENDOGENOUS CARDIOMYOCYTE RENEWAL

Several vertebrates, such as newts and zebrafish are capable of regenerating body parts after amputation (Becker et al. 1974; Jopling et al. 2010). The regeneration process is characterized by dedifferentiation of cells in the area surrounding the injury in order to generate a mass of proliferating undifferentiated cells (Bryant et al. 2002). Zebrafish are capable of fully regenerating their hearts within 30 days post-injury, when up to 20 % of the ventricle has been removed (Jopling et al. 2010). Labelling of cardiomyocytes showed that the regenerated cardiomyocytes had origin from dedifferentiated pre-existing cardiomyocytes. Regenerating cardiomyocytes undergo metabolic reprogramming to glycolysis from oxidative phosphorylation (Honkoop et al. 2019). Correspondingly, inhibition of glycolysis attenuates cardiomyocyte proliferation.

Mammalian hearts have been shown to have regenerative capacity as well (Porrello et al. 2011). Porrello et al. showed that partial surgical resection performed on hearts of 1-day-old mice stimulates a regenerative response, restoring the heart to normal function and structure with minimal fibrosis and hypertrophy. The regenerative capacity decreases after birth and ends by 7 days of age. This period is consistent with the postnatal period of proliferation prior to cell cycle arrest. A multicellular transcriptional analysis of mouse heart regeneration showed that as a consequence of myocardial infarction adult fibroblasts and leukocytes undergo a transition to a transcriptional state similar to that in the neonatal period, but cardiomyocytes and endothelial cells do not (Quaife-Ryan et al. 2017).

According to the prevalent hypothesis proliferation capacity of most human cardiomyocytes ceases after the first year of life. Mollova et al. (2013) detected evidence of cardiomyocyte division that decreases throughout childhood and adolescence to reach a non-detectable level in adult life. Also, cardiomyocyte growth and proliferation seem to contribute to myocardial growth for up to the first 20 years of life. These low levels of proliferation are, however, far from sufficient for cardiac regeneration after injury. Nonetheless, post-mortem case studies and reports of corrective heart surgery and recovery from severe myocardial infarction at birth suggest the possibility of cardiac regeneration at early development in humans (Vivien et al. 2016).

Differences between the physiological and structural properties of cold-blood vertebrates and mammalians partly explain the dissimilarities in their heart regeneration capacities. Lower vertebrates are facing less hemodynamic resistance and functional requirements than mammals. Cardiomyocytes of highly regenerative vertebrates and neonatal mice are mostly mononucleated and diploid and rely predominantly on glycolysis (Oberpriller et al. 1988; Vivien et al. 2016). Adult mammalian cardiomyocytes, in turn, are polyploid and mononucleated (human) or binucleated (mouse) (Soonpaa et al. 1996; Bergmann et al. 2015). Mononucleated mammalian cardiomyocytes have more proliferative potential than binucleated cardiomyocytes, and diploid zebrafish cardiomyocytes seem to proliferate more effortlessly than polyploid cardiomyocytes. This information indicates that the structure of mammalian cardiomyocytes is not ideal for maintenance of cell cycle. Also, the complexity of the sarcomere structure is substantially different between the species. In the postnatal period, sarcomere structure develops to meet the increasing requirements for contractile function (Guo & Pu 2020). To proliferate, myocytes must rapidly disassemble and reassemble structural and cytoskeletal complexes (Vivien et al. 2016). Lower vertebrates have less complex intracellular structures and therefore the functions related to proliferation can take place more effortlessly.

5 APPROACHES TO METABOLISM-MEDIATED CARDIAC REGENERATION

The high incidence of myocardial infarction and subsequent severe damage by it maintains the urge to find effective regenerative therapies. Indeed, in the course of decades, various approaches have been taken to increase the number of cardiomyocytes after injury (Vagnozzi et al. 2018). These approaches comprise reprogramming of fibroblasts into cardiomyocytes, transplantation of cardiomyocytes differentiated *in vitro* from embryonic stem cells or induced pluripotent stem cells (iPSC), genetic and pharmacological modulation of cell cycle genes, and modulation of growth and proliferation signaling of endogenous proliferation. Overall, advances in these fields have not yet yielded proliferation rates high enough to drive a sufficient regenerative response.

The aim of promoting cell cycle re-entry and cardiomyocyte proliferation through manipulation of metabolic components is based on promoting the shift of the metabolic state of cardiomyocytes towards that of the regenerative neonatal heart. This comprises induction of glucose metabolism and reduction of fatty acid oxidation (Table 2). Justification for the favoring of these metabolic settings lies in multiple studies indicating regenerative effects of increased glucose metabolism and reduced fatty acid oxidation (Kolwicz et al. 2013; Fillmore et al. 2014; Bae et al. 2020a). The mitochondrial content of cardiomyocytes rises after birth due to shift to oxygen-dependent mitochondrial metabolism and the related increase in ROS induces DNA damage and cell cycle exit of cardiomyocytes (Puente et al. 2014). Thus, the decrease of mitochondrial ROS is one approach to cell cycle re-entry as well.

The accumulation of succinate via SDH in heart failure due to low oxygen supply is an interesting target for metabolism modulation as well. Succinate accumulation results from the reversal of SDH due to fumarate overflow from purine nucleotide breakdown, causing reduction of fumarate to succinate (see Figure 2). At reperfusion SDH rapidly re-oxidizes succinate and generates high amounts of ROS through reverse electron transport at mitochondrial complex I (Chouchani et al. 2014). Bae et al. (2021) recently showed that succinate reduces cardiomyocyte proliferation and inhibits heart regeneration after

myocardial injury in neonatal mice. Also, inhibition of SHD with malonate promotes cardiomyocyte proliferation and regeneration in mice with myocardial infarction generated 7 days after birth. Malonate increases proliferation of adult cardiomyocytes and reduces DNA damage caused by myocardial injury. Since malonate inhibits oxidation of succinate and $FADH_2$ production for the electron transport chain, it inhibits oxidative phosphorylation and promotes a shift to glucose metabolism. This data suggests that malonate might promote the reprogramming of metabolism to a regenerative state and cell cycle activity.

Table 2. Approaches to pharmacological regulation of cardiomyocyte metabolism to promote a metabolic state ancillary to cardiac regeneration.

TARGET	MOLECULE	MODE OF ACTION	REFERENCES
FAO inhibition			
CPT1 inhibition	Perhexiline, etomoxir, oxfenicine	Mitochondrial long-chain fatty acid uptake ↓	Fillmore et al. 2013, Cao et al. 2019
MCD inhibition	CBM-301106	Malonyl CoA ↑ and mitochondrial long-chain fatty acid uptake ↓	Fillmore et al. 2013, Dyck et al. 2004
PPAR agonism	Fibrates and thiazolidinediones	Circulating fatty acids ↓ Glucose uptake and oxidation ↑	Fillmore et al. 2013, Magadum et al. 2017
Glucose oxidation → FAO inhibition			
PDK inhibition	Dichloroacetate	PDH activity ↑	Heggermont et al. 2016
SDH inhibition	Malonate	(Accumulation of succinate and ROS production ↓) Glucose metabolism ↑	Bae et al. (2021
Insulin sensitivity	Glucagon-like peptide 1 agonists	Insulin sensitivity ↑ Glucose uptake ↑	Nikolaidis et al. 2005

On top of fatty acid oxidation and glycolysis, alterations in other metabolic pathways of the heart also need to be taken into consideration in metabolic reprogramming aiming for regeneration. As stated before, in heart failure myocardial utilization of ketone bodies increases. β-hydroxybutyrate has beneficial hemodynamic effects both *in vivo* and in patients with heart failure (Horton et al. 2019; Nielsen et al. 2019). β-hydroxybutyrate has been

observed to be an endogenous inhibitor of histone deacetylases, which are involved in cell signaling promoting oxidative stress resistance (Shimazu et al. 2013). As cardiac ketogenesis and the mevalonate pathway (described earlier in this review) seem to be involved in the cell cycle exit and regulation of proliferation during the postnatal regenerative phase, these pathways are of great interest for further research in understanding the dynamic role of metabolism in cell cycle activity.

6 SUMMARY AND CONCLUSIONS

The heart has remarkable metabolic flexibility that allows it to maintain the vital contractile function that supplies blood to the whole body at different physiological states. The function of the heart is compromised in myocardial infarction, where the damaged cardiac tissue is repaired by fibrotic scarring, leading to heart failure due to impaired contractile function of cardiomyocytes. The adult heart has poor regenerative capacity due to lack of proliferation of cardiomyocytes once they exit the mitotic cell cycle shortly after birth. However, the cardiac regenerative capacity of some cold-blood vertebrates, neonatal mammal hearts together with case reports of cardiac regeneration at early development in humans has created faith within the field of regenerative research.

Multiple studies have demonstrated that cardiac metabolism is highly dynamic and switches its preference to different energy metabolism substrates during development, disease and regeneration. Linkage between metabolic profile and cardiomyocyte cell cycle activity is an intriguing topic of research, making targeting of cardiac metabolism a potential option for heart regeneration therapy.

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