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Glutamine regulates cardiac progenitor cell metabolism and proliferation

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Abstract

Autologous transplantation of cardiac progenitor cells (CPCs) alleviates myocardial dysfunction in the damaged heart; however, the mechanisms that contribute to their reparative qualities remain poorly understood. In this study, we examined CPC metabolism to elucidate the metabolic pathways that regulate their proliferative capacity. In complete growth medium, undifferentiated CPCs isolated from adult mouse heart proliferated rapidly ($T_d = 13.8$ h). CPCs expressed the Glut1 transporter and their glycolytic rate was increased by high extracellular glucose concentration, in the absence of insulin. Although high glucose concentrations did not stimulate proliferation, glutamine increased CPC doubling time and promoted survival under conditions of oxidative stress. In comparison with glucose, pyruvate or BSA-palmitate, glutamine, when provided as the sole metabolic substrate, increased ATP-linked and uncoupled respiration. Although fatty acids were not used as respiratory substrates when present as a sole carbon source, glutamine-induced respiration was doubled in the presence of BSA-palmitate, suggesting that glutamine stimulates fatty acid oxidation. Additionally, glutamine promoted rapid phosphorylation of the mTORC1 substrate, p70S6k, as well as retinoblastoma protein, followed by induction of cyclin D1 and cdk4. Inhibition of either mTORC1 or glutaminolysis was sufficient to diminish CPC proliferation, and provision of cell permeable α -ketoglutarate in the absence of glutamine increased both respiration and cell proliferation, indicating a key role of glutamine anaplerosis in cell growth. These findings suggest that glutamine, by enhancing mitochondrial function and

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stimulating mTORC1, increases CPC proliferation, and that interventions to increase glutamine uptake or oxidation may improve CPC therapy.

Keywords

stem cells; heart failure; cell therapy; bioenergetics; mitochondria; proliferation

INTRODUCTION

The adult mammalian heart contains a distinct population of self-renewing, multipotent, cardiac progenitor cells (CPCs) that can partially differentiate into cells with endothelial, cardiac, and smooth muscle cells characteristics [1–4]. While it is unclear whether [5] or not [6] these cells are sufficient or necessary for spontaneous regeneration of the heart after injury, several laboratories have shown that transplantation of CPCs improves myocardial recovery and function after infarction [7–17]. Cell therapy with CPCs has also been found to improve cardiac function in heart failure patients [18]. Nevertheless, in both animal models and humans, the improvements are marginal; therefore, it is unclear whether the benefits of CPC therapy have been maximized or if CPC therapy is equally efficacious for all heart failure patients.

Individuals in need of cell therapy often have metabolic defects that could affect stem cell function and stem cell-mediated tissue repair. For example, heart failure patients are often insulin resistant, diabetic [19–21] or cachexic [22, 23]. These conditions profoundly affect both systemic metabolism and tissue nutrient availability, which could negatively impact cell therapy. Because of their unique metabolic requirements [24], stem cells may be particularly affected by metabolic conditions of the host. Previous work has shown that metabolism influences the proliferation, stemness, and differentiation of embryonic and mesenchymal stem cells [25]; however, nothing is currently known regarding the metabolic properties of CPCs or how metabolism affects CPC function. This is important not only because perturbations in systemic metabolism impair CPC-mediated myocardial repair [26, 27], but also because understanding how metabolism regulates CPC function could enable the development of interventions to increase the clinical efficacy of CPC therapy.

In the current study, we sought to identify the metabolic programs that support CPC function and regulate their proliferation. Our studies show that CPCs utilize glucose in an insulin-independent manner and that the preferred substrate for mitochondrial oxidation is glutamine, which is important for coordinating intermediary metabolism and stimulating cell signaling pathways that regulate cell division.

MATERIALS AND METHODS

Materials

The following antibodies were used for flow cytometry: anti-Sca-1, anti-c-kit (2B8 clone), and anti-lineage antibodies were purchased from eBioscience. Anti- α -smooth muscle actin, anti-cardiac troponin T2, and anti-sarcomeric- α -actinin were purchased from Abcam (Cambridge, MA, USA). Anti-Flk1 antibody was purchased from eBioscience (San Diego,

CA, USA). The following antibodies were used for Western blotting: anti- α -smooth muscle actin and anti- α -tubulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against GAPDH, IR- β , IRS-1, p-p70S6K, p70S6K, VEGFR2, ASCT2, p-Akt, Akt, and p-Rb were purchased from Cell Signaling (Danvers, MA, USA). Antibodies against Rb, Cdk4, and cyclin D1 were from Santa Cruz Biotechnology, Inc. Antibodies against GLUT1 and GLUT4 were purchased from Millipore (Darmstadt, Germany). Dexamethasone, succinic acid, pyruvic acid, L-glutamic acid, saponin, oligomycin, antimycin A, rotenone, L-carnitine, sodium palmitate, FCCP, bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES), dimethyl 2-oxoglutarate (DMK), aminooxyacetic acid (AOA), pyruvic acid, NADH, and D-glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rapamycin was purchased from Cell Signaling Technology. Koningic acid (KA) was purchased from TMS Co., LTD (Japan). L-Glutamine powder was purchased from Cellgro. Fatty-Acid Free BSA was purchased from Roche Diagnostics (Indianapolis, IN, USA). The following DMEM/F-12 powders were purchased from US Biological (Swampscott, MA, USA): DMEM/F12 containing L-glutamine, D-glucose, and pyruvic acid; DMEM/F12 containing L-glutamine and pyruvic acid but no D-glucose; and DMEM/F12 lacking L-glutamine, D-glucose, and pyruvic acid. EmbryoMax[®] ES Cell Qualified Fetal Bovine Serum, EmbryoMax[®] ES Cell Qualified Penicillin-Streptomycin Solution, ESGRO[®] (LIF) were purchased from Millipore (Darmstadt, Germany). Recombinant Human FGF-basic was purchased from Peprotech (Rocky Hill, NJ, USA). Recombinant epidermal growth factor was purchased from Sigma. Insulin, Transferrin and Selenium (500 $\times$) was purchased from Lonza (Walkersville, MD, USA).

Cell isolation and culture

All animal procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Louisville Institutional Animal Care and Use Committee. Murine cardiac progenitor cells (mCPCs) were isolated from adult, male C57BL/6J mouse heart cell outgrowth cultures. After outgrowth, the mCPCs were subjected to sequential sorting for c-kit⁺/lin⁻ markers using magnetic immunobeads CPC [14, 28]. As described previously [28], the mCPCs were cultured in growth medium (DMEM/F12 medium) containing the following constituents: glutamine (2.5 mM), glucose (17.5 mM), pyruvate (0.5 mM), leukemia inhibitory factor (1000 U/mL), basic fibroblast growth factor (20 ng/mL), epidermal growth factor (20 ng/mL), 10% embryonic stem cell fetal bovine serum, penicillin/streptomycin (1 $\times$), and insulin-transferrin-selenium (1 $\times$). Expression of cell surface markers was assessed by flow cytometry. Harvested mCPCs (passage 3–6) were blocked with FcBlock (0.005 mg/mL in PBS + 1% BSA) for 10 minutes at 4°C. The mCPCs were stained with a cocktail of anti-mouse c-kit (eBioscience, 2B8 clone), Ly6AE (Sca-1), and hematopoietic lineage antibodies (eBioscience). P815 mastocytoma cells were used as a positive control for c-kit expression. Data were acquired on an LSRII flow cytometer (BD BioSciences) using BD FACSDiva software and analyzed using FlowJo software. Unstained samples were used for setting discrimination gates. Human CPCs (hCPCs) were isolated from discarded atrial appendages and cultured as described previously [29]. Written consent for collection of CPCs was obtained in accordance with a protocol approved by the Institutional Review Boards of the University of Louisville.

Differentiation, proliferation, and viability measurements

Differentiation of mCPCs was assessed by incubating cells in DMEM/F-12 medium containing 2% FBS, 1% penicillin/streptomycin, and 10 nM dexamethasone, essentially as described [4, 30, 31]. This differentiation medium was changed every two days throughout the differentiation protocol (up to 10 d).

Cell proliferation was assessed by counting cells using a BD Accuri C6 flow cytometer. Briefly, mCPCs were seeded in complete growth medium in 6-, 12-, or 24-well plates. Then, 16 h later, a group of cells was counted and recorded as time = 0 h. The medium in the remaining plates was then replaced with medium containing the desired substrates, and the cells were cultured for the desired duration. The mCPCs were harvested by trypsinization and suspended in medium. A small volume of suspended cells was used for recording cell events using the Accuri flow cytometer, with proper gating to exclude cell debris. To calculate the cell doubling time, the following formula was used; $T_d = T \ln 2 / \ln(X_e/X_b)$, where T is the incubation time, X_b is the cell number at the beginning of the incubation time (i.e., t = 0 h), and X_e is the cell number at the end of the incubation time; X_e and X_b were recorded in the log phase of cell growth. Cell viability was assessed by measure lactate dehydrogenase (LDH) activity in the cell medium and lysates, as described previously [32].

Gene expression and protein abundance analyses

Commitment to cardiovascular lineages was assessed by measuring cell type-specific gene expression by qRT-PCR and by measuring abundances of proteins specific to particular cardiovascular phenotypes. For qRT-PCR, mRNA was isolated from mCPCs using TRIZOL reagent (Invitrogen) and RNA quantity and purity were estimated by measuring absorbances at 260 and 280 nm using a Nanodrop spectrophotometer (Thermo scientific). Two µl of cDNA was used in a reaction mixture containing SYBR green (VWR, Radnor, PA, USA) and oligo primers (Integrated DNA Technologies, Inc; Coralville, IA, USA). The following primers were used for qRT-PCR: SM-α-actin, 5'-cccagacatcagggagtaatg-3' (sense), 5'-ctttccatgtcgtccagt-3' (anti-sense); Tnnt2, 5'-tgatgacatccacaggaagc-3' (sense), 5'-gacgcttttcgacgtctct-3' (anti-sense); and Flk1, 5'-atgttcgagattacagatcac-3' (sense), 5'-cgcaaagagacacattgagg-3' (anti-sense). For Western blotting, 0.5–25 µg protein from crude CPC lysates were applied to each lane of a 10.5–14% Bis-Tris-HCl gel and electrophoresed. The separated proteins were then electroblotted onto a PVDF membrane. The membrane was incubated overnight at 4°C using appropriate dilutions of primary antibodies. The following day, PVDF membranes were incubated at room temperature with HRP-conjugated secondary antibodies. Immunoreactive bands were detected using a Typhoon variable mode imager after exposure to ECL detection reagent (GE Healthcare). Band intensities were quantified using Total Lab TL120 software.

Extracellular flux analysis

Oxygen consumption and proton production rates (OCR and PPR, respectively) in intact, adherent mouse and human CPCs were measured using a Seahorse XF24 analyzer, as described [33–35]. Before provision of the indicated substrates, CPCs were cultured in unbuffered DMEM (pH 7.4) containing no substrates for 1 h at 37°C in a non-CO₂ incubator.

Measurement of glutamine in mouse plasma

Glutamine in naïve mouse plasma was measured by GC/MS using the EZ:Faast GCMS Physiological amino acid analysis kit (Phenomenex KG0-7166), as described [36].

Determination of glycolytic flux

Glycolytic flux was determined by analyzing the conversion of [5-³H]-glucose to [³H]₂O [37–39]. Briefly, mCPCs were grown in 6-well plates to ~80% confluency. Reaction medium ([DMEM/F-12 medium containing 1 mM L-glutamine, 5.5, 25 mM D-glucose or 5 mM glucose with 20 mM D-mannitol (osmotic control) and 2 µCi/ml [5-³H]-glucose (Moravek Biochemicals)] was added and incubated at 37°C for 3 h. In some reactions, koniginic acid (10 µg/ml), an inhibitor of glyceraldehyde-3-phosphate dehydrogenase [40, 41], was included. Then, 50 µl of the reaction medium was pipetted into 1.5 ml microcentrifuge Eppendorf tubes containing 50 µl 0.2 N HCl. The microcentrifuge tubes, with tube tops removed, were placed in 20 ml scintillation vials containing 0.5 ml of distilled water. The vials were sealed and incubated for 48 h at room temperature to allow for evaporation diffusion of the [³H]₂O in the microfuge tubes into the scintillation vials. To account for incomplete equilibration of [³H]₂O and background, in parallel vials, known amounts (µCi) of [5-³H]-glucose and [³H]₂O (Moravek Biochemicals) were placed in microcentrifuge tubes, which were also placed into separate scintillation vials containing 0.5 ml distilled water. Following the incubation, the microcentrifuge tubes were removed and 10 ml of scintillation fluid was added to each vial. Radioactivity was then measured by scintillation counting. Glucose utilization was calculated using the formula reported by Ashcroft et al. [37], taking into account the specific activity of the [5-³H]-glucose, incomplete equilibration and background, the ratio of unlabeled and labeled glucose, and scintillation counter efficiency.

Statistical analysis

Data are mean ± SEM. Multiple groups were compared using one-way or two-way ANOVA, followed by Tukey post-tests. Unpaired Student's *t* test was used for two-group comparisons. A *p* value < 0.05 was considered significant.

RESULTS

Characterization of CPCs from adult murine heart

After isolation based on c-kit positivity and lineage negativity, mCPCs were grown in culture for up to 8 passages (Fig. 1A). In accordance with previous findings [28], mCPCs examined at passage 3 were negative for hematopoietic lineage markers and showed a high level of Sca-1 positivity (~90%), but only retained ~10% positivity for c-kit (Fig. 1B,C).

To determine their differentiation capacity, mCPCs were incubated in differentiation medium [42] lacking LIF and growth factors for 2–10 days, after which markers of cardiovascular cell lineages were examined. Two days of exposure to the differentiation medium led to a 60-fold induction of cardiac troponin T (*Tnnt*) and ~5-fold increases in the messages of both vascular endothelial growth factor receptor 2 (*Flk1*) and smooth muscle α-actin (*Acta2*), suggesting a capacity to express proteins indicative of cardiac myocyte,

endothelial, and smooth muscle lineages (Fig. 1D). Although protein abundance by flow cytometry showed relatively large, statistically significant increases in fluorescence intensity of smooth muscle α -actin (SM α -actin), the median fluorescence intensities of Flk1 and cardiac sarcomeric α -actin (SA α -actin) did not reach statistical significance (Fig. 1E,F), suggesting that the cells primarily express markers indicative of smooth muscle cells upon differentiation. Indeed, Western blot analysis of lysates from mCPCs treated with differentiation medium showed remarkable increases in the abundance of SM α -actin (Fig. 1G); however, there was no increase in the markers of either cardiomyocytes or endothelial cells. These data suggest that, under the conditions tested, mCPCs can partially differentiate into cardiovascular cells of the smooth muscle cell lineage.

We next examined the proliferative capacity of mCPCs in culture. As shown in Fig. 1H, undifferentiated CPCs expanded in complete growth medium proliferated rapidly ($T_d = 13.8$ h).

Glucose metabolism in CPCs

Doubling total biomass rapidly requires extensive integration of cellular biosynthesis and energetic pathways. Because glucose metabolism regulates both ATP production and is a precursor for the biosynthesis of metabolites such as nucleotides and phospholipids, we next examined glucose metabolism in mCPCs. Since nothing is known about the basic metabolic characteristics of these cells, we first examined the expression of glucose transporters, measured glycolytic flux, and determined how the cells respond to insulin. As shown in Fig. 2A, mCPCs expressed insulin receptor- β and insulin receptor substrate-1, and upon stimulation with insulin, demonstrated increased Akt phosphorylation. Relative to whole heart, mCPCs expressed high levels of glucose transporter 1 (Glut1) and low levels of Glut 4, indicating that glucose uptake in these cells is likely to be independent of insulin action. Indeed, exposure of cells to 25 mM glucose resulted in a 40% increase in glycolytic flux compared with cells in 5 mM glucose ($p < 0.01$), and this was inhibited by the glyceraldehyde-3-phosphate dehydrogenase inhibitor, koniginic acid (KA) (Fig. 2B). Assessment of the glycolytic activity by extracellular flux analysis yielded similar results, with a larger increase in the proton production rate (PPR) in mCPCs treated with 25 mM glucose compared with those treated with 5 mM glucose (Fig. 2C). Similar to results from radiometric assays, the increase in glycolysis due to glucose treatment was almost completely inhibited by KA (Fig. 2D). The glycolytic rate of mCPCs was not significantly affected by insulin treatment (data not shown). ATP levels in cells cultured in growth medium were increased in a concentration-dependent manner by glucose (Fig. 2E). Collectively, these observations suggest that, unlike differentiated cardiac myocytes, glucose transport in mCPCs is not regulated by insulin and is likely to be regulated by levels of extracellular glucose.

To determine whether extracellular glucose concentration regulates the proliferative capacity of mCPCs, we cultured these cells in growth media containing 0–25 mM glucose and measured their rate of proliferation. Over the first 48 h, when mCPCs transition into the log phase of expansion (see Fig. 1H), glucose had little effect on proliferative capacity; however, after 48 h, there was a large decrease in the number of cells grown in growth

medium lacking glucose, and LDH assays indicated significant cell death (Fig. 2F,G), which could be due to decreased availability of glucose-derived macromolecules for biosynthesis. Compared with cells grown in euglycemic culture conditions, there were fewer mCPCs present under conditions of high glucose treatment at 96 h. This effect, however, appears to be due to osmotic changes, because increasing the osmotic strength of the medium by adding 20 mM mannitol had a similar effect (Fig. 2F). No loss in cell viability occurred in cells incubated in high glucose or in the corresponding osmotic control (Fig. 2G). Collectively, these results indicate that, in mCPCs, the absence of glucose promotes cell death and that high extracellular glucose concentrations regulate both glucose utilization and proliferation.

Glutamine is the preferred substrate of CPCs for mitochondrial oxidation

Because glucose withdrawal did not affect mCPC proliferation for the initial 48 h, we examined whether there were other energy sources that could sustain CPC proliferation. To test the relative capacity of mCPCs to oxidize glucose, pyruvate, fatty acids, and glutamine, we used high throughput respirometry. After equilibration of mCPCs in DMEM containing no substrates for 1 h, baseline oxygen consumption rates (OCRs) were recorded, which was followed by treatment of CPCs with glutamine, glucose, pyruvate, palmitate conjugated to BSA, or BSA control. As shown in Fig. 3, exposure of mCPCs to glutamine resulted in stimulation of basal oxygen consumption, whereas treatment with palmitate (and its corresponding BSA control) or pyruvate did not affect basal OCR (Fig. 3A). Glucose, however, appeared to decrease OCR in a time-dependent manner (Fig. 3A). Concordant with glutamine-stimulated respiration, we found that mCPCs expressed the major glutamine transporter, ASCT2 (Fig. 3A, inset).

Exposure of CPCs sequentially to oligomycin, FCCP, and antimycin A/rotenone allowed for interrogation of specific components of mitochondrial metabolism [33, 35, 43]. The opposite effects of glutamine and glucose on mitochondrial OCR manifested primarily as changes in ATP-linked OCR (ATP demand) and maximal mitochondrial activity, i.e., glutamine increased ATP demand and supported uncoupled respiration, whereas glucose diminished ATP demand and decreased maximal mitochondrial respiratory activity (Fig. 3A,B). Similar to control mCPCs not exposed to substrate (BSA control), palmitate had no effect on mitochondrial metabolism. Proton leak and non-mitochondrial OCR were not affected by any substrate (Fig. 3B). Based on these data, it appears that the substrate preference for mitochondrial oxidation in mCPCs is as follows: glutamine > pyruvate > fatty acids > glucose.

To determine whether glutamine-induced stimulation of respiration in CPCs might be unique, or if it could be typical of all proliferating and/or differentiated cells, we compared the effects of glutamine on mitochondrial activity in mCPCs with that of differentiated cardiovascular cells (cardiomyocytes, smooth muscle cells, and endothelial cells) and highly proliferative cells with a doubling time similar to that of CPCs (i.e., 3T3L1 cells [44, 45]). While mCPCs showed a stimulation of respiration upon addition of glutamine, none of the other cell types demonstrated this response (Fig. 3C). Human CPCs showed a bioenergetic profile similar to mCPCs: although overall OCR was lower in hCPCs, they demonstrated an

increase in respiratory activity upon glutamine treatment as well as inhibition of mitochondrial activity upon exposure to glucose (Fig. 3D). Taken together, these results reveal a potential bioenergetic signature of CPCs that is conserved from rodents to humans.

Glutamine coordinates CPC metabolism and regulates their proliferative capacity

To understand substrate-regulated mitochondrial metabolism in CPCs more fully, we further examined the effects of each substrate on oxygen consumption rate or proliferation. Glucose, provided as the sole carbon substrate, decreased OCR by up to 50%, with maximal responses near the normal plasma levels of glucose (5 mM, Fig. 4A). Such glucose-induced decreases in respiration are suggestive of the Crabtree effect [46, 47]. To determine whether the suppression of mitochondrial metabolism by glucose is affected by glutamine, we examined OCR in mCPCs when glutamine was provided along with glucose and pyruvate. As shown in Fig. 4B, glutamine rescued the suppression of mitochondrial activity by glucose, suggesting that glutamine could be a primary coordinator of intermediary metabolism (Fig. 4B). To test this idea further, we assessed mitochondrial activity in mCPCs provided with BSA-palmitate in the absence or presence of glutamine. Although BSA-palmitate, by itself, did not increase mitochondrial OCR, the presence of glutamine increased OCR by 66% when fatty acids were present, and this stimulation of respiration significantly exceeded that when glutamine was provided as the sole substrate (Fig. 4C). These findings indicate that glutamine is sufficient to increase mitochondrial metabolism and suggest that it plays a permissive role in mitochondrial fat oxidation by CPCs.

Because glutamine stimulates mitochondrial activity and appears to coordinate major pathways of intermediary metabolism, we hypothesized that glutamine may be a primary regulator of CPC growth, differentiation and/or survival. Indeed, the absence of glutamine in the culture medium prevented mCPC proliferation, whereas the addition of glutamine increased proliferation in a concentration-dependent manner (Fig. 4D). To determine whether glutamine regulates CPC differentiation, we assessed differentiation of vascular and cardiac markers by qPCR and assessed the protein expression of α -smooth muscle actin by Western blotting. Under the conditions tested, glutamine did not affect differentiation into cardiac- or smooth muscle-like lineages (Supplementary Fig. 1).

To determine whether glutamine regulates CPC survival, the cells were incubated in the absence or presence of glutamine, without or with the redox stressor hydrogen peroxide (500 μ M). As shown in Fig. 4E and F, absence of glutamine had no effect on cell viability in unstressed cells. However, oxidative stress induced by incubation with 500 μ M H₂O₂ caused >50% cell death in mCPCs cultured in the absence of glutamine, and the presence of glutamine prevented oxidative stress-induced cell death. Collectively, these data suggest that glutamine regulates CPC proliferative capacity and is important for maintaining cell viability under conditions of oxidative stress.

Glutamine functions as a growth factor in CPCs by stimulating mTOR

Simultaneous permissive effects of glutamine on both metabolism and proliferation suggest that glutamine likely influences signaling mediators that control cell growth. Hence, we tested whether glutamine regulates the activation of mTORC1—a central signaling hub that

controls cellular metabolism and energy homeostasis in highly proliferative cells [48]—by assessing the phosphorylation of its downstream substrate, P70S6K. Stimulation of mCPCs with glutamine resulted in a time-dependent increase in p-P70S6K, which was followed by the phosphorylation of Rb and increases in the abundances of cyclin D1 and cdk4 (Fig. 5A,B). The stimulation of phosphorylation of P70S6K was completely inhibited by rapamycin (Fig. 5C,D), and rapamycin inhibited glutamine-induced cell proliferation by ~80% (Fig. 5E). These results indicate that glutamine coordinates the cell cycle in CPCs by activating mTORC1.

Glutaminolysis regulates CPC proliferation

Because rapamycin did not fully inhibit proliferation, we hypothesized that glutaminolysis may play a significant role in glutamine-induced stimulation of CPC proliferative capacity. The stimulatory effect of glutamine is likely due in part to glutamine anaplerosis, where glutamine is converted to glutamate by glutaminase (GA), and glutamate is further oxidized to α -ketoglutarate (α -KG) by glutamate dehydrogenase (GDH) or aspartate aminotransferase (AAT). The α -KG entering the tricarboxylic acid (TCA) cycle can be used to generate reducing equivalents (NADH) or can be used for cataplerotic reactions (e.g., lipid and nucleotide biosynthesis) in the cell (Fig. 5F). Thus, we reasoned that if glutamine-derived α -KG is important to CPC metabolism and proliferation, inhibiting its formation would affect proliferation and respiration. Indeed, inhibitors of glutaminolysis, BPTES and AOA, decreased CPC proliferation by ~45 and 100%, respectively (Fig. 5G), suggesting that proliferation is supported by the conversion of glutamine to α -KG.

Regulation of CPC proliferation via α -ketoglutarate

Because glutaminolysis has been shown to activate mTORC1 signaling via increasing intracellular α -KG levels [49], we next examined whether glutamine-derived α -KG regulates mTORC1 activation in CPCs. For this, AOA was incubated with mCPCs prior to treatment with glutamine. As shown in Fig. 6A, AOA inhibited glutamine-induced phosphorylation of P70S6K, indicating a requirement of glutaminolysis for mTORC1 activation. To determine the relative importance of glutaminolysis versus mTOR activation, we assessed whether providing glutamate or a cell-permeable form of α -KG (dimethyl α -KG; DMK) would be sufficient to increase respiratory activity and CPC proliferation. As shown in Fig. 6B, both glutamate and DMK increased OCR independent of the presence of glutamine, and DMK increased the FCCP-stimulated rate of respiration to the same level as glutamine. Furthermore, DMK increased cell proliferation even in the absence of glutamine (Fig. 6C); although DMK-induced proliferative activity appeared to plateau after 48 h. This relatively late loss of proliferative activity is likely due to lack of sufficiency of DMK, by itself, to stimulate mTORC1 activity (Fig. 6D). Although DMK was not sufficient to activate mTORC1, loss of glutamine-induced mTORC1 activation by AOA was restored by provision of DMK, indicating that α -KG is necessary but not sufficient for glutamine-induced mTOR signaling (Fig. 6E). Consistent with this finding, DMK was sufficient to restore the loss of proliferative capacity observed in AOA-treated cells (Fig. 6F). Collectively, these data demonstrate that bioenergetic changes and mTORC1 signaling triggered by glutamine-derived α -KG are major determinants of the proliferative capacity of CPCs.

DISCUSSION

Several studies have shown that CPC transplantation can improve myocardial function after injury [7–14]. Nevertheless, the mechanisms underlying the reparative actions of CPCs are not well understood. Recent work suggests that it is unlikely that a significant number of transplanted CPCs differentiate into mature cardiac myocytes [6, 50], and few CPCs (~1%) are retained in the myocardium after transplantation [51]. Hence, it has been suggested that transplanted CPCs secrete trophic factors that recruit endogenous stem cells or stimulate myocyte proliferation [52]. Regardless of the specific mechanism by which they promote cardiac recovery, it is expected that an increase in CPC survival and proliferation would improve therapeutic outcomes after their transplantation. Therefore, we focused on understanding the metabolic features of CPCs and how they might regulate CPC proliferation.

We found that glucose transport in mCPCs is independent of insulin and that increasing extracellular glucose concentration elevates glycolytic rate and augments ATP levels. Nonetheless, low or high glucose levels had only marginal effects on early stages of mCPC proliferation, indicating that there might be other substrates that support CPC metabolism, survival and proliferation. High throughput respirometric analyses revealed that, of the major substrates tested, glutamine was most efficacious in supporting mitochondrial respiration and CPC proliferation and viability. Glutamine not only supported mitochondrial respiration in CPCs, but also further increased mitochondrial activity when fatty acids were provided. Importantly, these studies show that the permissive effect of glutamine on cell proliferation appears to be due its stimulatory effect on mitochondrial metabolism as well as its ability to activate the mTOR signaling pathway. The salubrious effects of glutamine on CPCs appear to be due to generation of products of glutamine oxidation, as provision of cell permeable α -KG was sufficient to stimulate both mitochondrial activity and cell proliferation (Fig. 7). These data reveal, for the first time, a unique metabolic feature of CPCs, showing how metabolic activity regulates CPC proliferation. Taken together, these findings suggest that interventions to increase glutamine uptake or consumption could increase *in vitro*, and potentially, *in vivo*, the proliferative capacity of CPCs.

Our studies reveal a unique metabolic phenotype of CPCs that distinguishes them from other stem cells as well as differentiated cells in the myocardium. We found that unlike hematopoietic progenitors [53], mCPCs express high levels of the Glut1 glucose transporter in the undifferentiated state, a property they share with embryonic stem cells [54]. Hematopoietic stem cells such as the CD34⁺/CD38⁻ or more differentiated CD34⁺/CD38⁺ cells do not express Glut1, although the levels of Glut1 are increased 40-fold upon erythropoietin-induced erythropoiesis [55]. However, like HSCs [55], CPCs express the ASCT2 glutamine transporter, suggesting that while sharing metabolic characteristics with both embryonic stem cells and hematopoietic progenitors, CPCs retain a unique phenotype that distinguishes them from other stem/progenitor cells. The metabolic characteristics of CPCs also distinguish them from differentiated cells in the myocardium as suggested by our finding that, unlike differentiated cardiac myocytes, smooth muscle cells, endothelial cells and fibroblasts, mitochondrial activity of CPCs is stimulated by glutamine. These observations suggest that CPCs have a distinct metabolic identity, which, in addition to

surface markers, could be used for their identification. These metabolic characteristics could also be exploited to enhance their therapeutic efficacy.

The high levels of Glut1 and the dependency of glycolytic rate on extracellular glucose concentration suggest that CPCs could be prone to hyperglycemic injury. Indeed, it was recently reported that culturing CPCs in high glucose reduces CPC yield from cardiac biopsies, impairs their angiogenic and chemotactic ability, and reduces their ability to repair the heart [27]. Similarly, hyperglycemia associated with insulin deficiency results in increased reactive oxygen species generation and CPC aging [26]. While the effects of hyperglycemia on CPC function remain unclear, our observations that CPCs express Glut1 and that their glycolytic activity increases concomitantly with extracellular glucose concentration suggest that an increase in glycolysis may be an important factor in inducing diabetic dysfunction in CPCs. Like CPCs, other stem cells such as ESCs and HSCs, rely on glycolysis for stem cell maintenance and stemness [24, 25]. In HSCs, an increase in glycolysis leads to an increase in cell number and inhibition of hematopoietic differentiation, whereas attenuation of glycolysis prevents HSC reconstitution capacity. In contrast, we found that high levels of glucose did not increase CPC proliferation and that extracellular glucose inhibited mitochondrial activity. These observations suggest that, in CPCs, glucose inhibits mitochondrial respiration via a Crabtree-like effect and that high rates of glycolysis may be required for maintaining quiescence, whereas for cell proliferation and growth, mitochondrial activity—supported by glutamine—is required.

In different types of stem cells, glutamine has been shown to be important for differentiation, survival and proliferation [55–57]. In agreement with these observations we found that glutamine had a strong stimulatory effect on CPC proliferation and promoted survival under conditions of oxidative stress. Because exogenous glutamine was found to stimulate proliferation over the basal rate of cell growth, it appears that CPCs have an inadequate ability to synthesize a glutamine pool large enough to provide α -KG, which is required for CPC proliferation. Our data also show that, although CPCs do not require glutamine for survival under unstressed conditions, their survival under conditions of oxidative stress is highly dependent on glutamine availability. This is consistent with the fact that glutamine is important for maintaining glutathione levels [58], which would be required to combat oxidative stress-mediated tissue damage in the failing heart [59, 60].

The reliance of CPCs on glutamine for cell division appears to derive from their unique metabolism. Data from proliferation assays and respirometry experiments support the view that CPCs may have a “truncated” Krebs cycle, such as that described in some cancer cells [48], that is enabled by the provision of glutamine or cell permeable α -KG. Interestingly, α -KG alone may not be adequate to maintain high rates of cell proliferation for >48 h, likely because it cannot substitute for the nitrogen donation role of glutamine [61] and was insufficient to stimulate mTORC1, which was found to increase phosphorylation of retinoblastoma protein and is required to maintain cell cycle progression by promoting translation of key mediators such as Cyclin D1 and Cdk4 [62, 63].

Although the metabolic signature of CPCs is somewhat similar to that found in cancer cells [48], which utilize large amounts of glutamine and demonstrate a Warburg phenotype, there

are notable differences. For example, α -KG is sufficient to activate mTORC1 in osteosarcoma cells [49], which was not observed in CPCs. Moreover, c-Myc, which is commonly amplified in tumor cells and regulates glutaminolysis [64], was not detectable in CPCs (data not shown). Nevertheless, the finding that α -KG is necessary for activation of mTORC1 by glutamine is consistent with a previously proposed model, where glutaminolysis is necessary for GTP loading of RagB and activation of mTORC1 signaling [49]. These properties of CPCs distinguish them from cancer cells and reveal a potentially unique stem cell metabolic signature not shared by other fast-dividing cells reliant on glucose and glutamine.

While the precise molecular mechanisms supporting high rates of glutaminolysis in CPCs are currently unknown, the results of our study have important clinical implications for CPC therapy. Importantly, we found that the concentrations of glutamine that affected cell division were in the physiological range. This is based on our measurement of glutamine in the plasma of naïve mice, which we found to be $443 \pm 30 \mu\text{M}$ ($n = 4$). Thus, it appears likely that fluctuations in glutamine could be a major regulator of CPC proliferative capacity *in vivo*. In the context of inactive or catabolic states, plasma concentrations of free glutamine in humans decrease remarkably [65–67], and several studies have shown that critically ill patients have low plasma levels of glutamine (e.g., $< 400 \mu\text{M}$) [68–70]. Similarly, patients with ischemic cardiomyopathy have plasma levels of glutamine approximating $400 \mu\text{M}$ [71]. Hence, the results of our study suggest that the efficacy of CPC therapy may be improved by increasing plasma levels of glutamine in patients undergoing CPC transplant, expectedly by increasing the survival and proliferation of the transplanted cells.

Current pre-clinical and clinical regimens for autologous cell therapy involve isolating and culturing CPCs for several passages prior to myocardial implantation. Clinical scenarios require at least 1 million cells per patient, while preclinical studies in mice require $\sim 100,000$ – $500,000$ cells, depending on the route of delivery; hence, augmenting or improving CPC metabolism to decrease the time between cell isolation and transplant could be useful in expanding the number of CPCs isolated from patients. In addition, the results of our study may also help optimize cell delivery protocols. Most stem cell delivery procedures suspend the cells in Plasma-lyte or a similar saline solution, neither of which contain substrates that maintain cellular bioenergetic health. A nutrient delivery solution containing glutamine or cell permeable α -KG could improve the health of CPCs after harvest and might improve their proliferative capacity, survival, and retention upon transplantation.

In summary, our results indicate that glutamine is a central regulator of CPC metabolism, survival, and proliferation. Although mitochondrial metabolism of glutamine for energy provision (as well as nucleotide and lipid biosynthesis) appears to be a major mechanism underlying the stimulatory effects of glutamine on CPC proliferation, other mechanisms remain to be tested. For instance, in addition to stimulating mitochondrial respiration, glutamine could also modulate the levels of citric cycle intermediates, which can activate G protein coupled receptors [72] and thereby regulate cell growth [73]. Additional studies are required to identify the molecular regulators that support high rates of glutaminolysis and glucose metabolism in CPCs and to determine the effects of glutamine on their paracrine properties. Nevertheless, the present *in vitro* study suggests that modulating glutamine

uptake, metabolism, or biological targets might increase CPC proliferation, survival and efficacy upon transplantation. Further studies are required to determine whether glutamine or its downstream effectors can be exploited to optimize CPC therapy *in vivo*.

Supplementary Material

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References

1. Smith AJ, Lewis FC, Aquila I, et al. Isolation and characterization of resident endogenous c-Kit(+) cardiac stem cells from the adult mouse and rat heart. *Nature protocols*. 2014; 9:1662–1681. [PubMed: 24945383]
2. Toko H, Hariharan N, Konstandin MH, et al. Differential regulation of cellular senescence and differentiation by prolyl isomerase Pin1 in cardiac progenitor cells. *J Biol Chem*. 2014; 289:5348–5356. [PubMed: 24375406]
3. Matuszczak S, Czaplá J, Jarosz-Biej M, et al. Characteristic of c-Kit progenitor cells in explanted human hearts. *Clinical research in cardiology: official journal of the German Cardiac Society*. 2014
4. Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell*. 2003; 114:763–776. [PubMed: 14505575]
5. Ellison GM, Vicinanza C, Smith AJ, et al. Adult c-kit(pos) cardiac stem cells are necessary and sufficient for functional cardiac regeneration and repair. *Cell*. 2013; 154:827–842. [PubMed: 23953114]
6. van Berlo JH, Kanisicak O, Maillet M, et al. c-kit+ cells minimally contribute cardiomyocytes to the heart. *Nature*. 2014; 509:337–341. [PubMed: 24805242]
7. Fischer KM, Cottage CT, Wu W, et al. Enhancement of myocardial regeneration through genetic engineering of cardiac progenitor cells expressing Pim-1 kinase. *Circulation*. 2009; 120:2077–2087. [PubMed: 19901187]
8. Hong KU, Guo Y, Li QH, et al. c-kit+ Cardiac Stem Cells Alleviate Post-Myocardial Infarction Left Ventricular Dysfunction Despite Poor Engraftment and Negligible Retention in the Recipient Heart. *PLoS One*. 2014; 9:e96725. [PubMed: 24806457]
9. Zhang LX, DeNicola M, Qin X, et al. Specific Inhibition of HDAC4 in Cardiac Progenitor Cells Enhances Myocardial Repairs. *American journal of physiology Cell physiology*. 2014
10. Zakharova L, Nural-Guvener H, Feehery L, et al. Retrograde coronary vein infusion of cardiac explant-derived c-Kit+ cells improves function in ischemic heart failure. *The Journal of heart and lung transplantation: the official publication of the International Society for Heart Transplantation*. 2014; 33:644–653.
11. Bolli R, Tang XL, Sanganalmath SK, et al. Intracoronary delivery of autologous cardiac stem cells improves cardiac function in a porcine model of chronic ischemic cardiomyopathy. *Circulation*. 2013; 128:122–131. [PubMed: 23757309]
12. Loffredo FS, Steinhauser ML, Gannon J, et al. Bone marrow-derived cell therapy stimulates endogenous cardiomyocyte progenitors and promotes cardiac repair. *Cell stem cell*. 2011; 8:389–398. [PubMed: 21474103]
13. Dawn B, Stein AB, Urbanek K, et al. Cardiac stem cells delivered intravascularly traverse the vessel barrier, regenerate infarcted myocardium, and improve cardiac function. *Proc Natl Acad Sci U S A*. 2005; 102:3766–3771. [PubMed: 15734798]

14. Li Q, Guo Y, Ou Q, et al. Intracoronary administration of cardiac stem cells in mice: a new, improved technique for cell therapy in murine models. *Basic research in cardiology*. 2011; 106:849–864. [PubMed: 21516491]
15. Williams AR, Hatzistergos KE, Addicott B, et al. Enhanced effect of combining human cardiac stem cells and bone marrow mesenchymal stem cells to reduce infarct size and to restore cardiac function after myocardial infarction. *Circulation*. 2013; 127:213–223. [PubMed: 23224061]
16. Welt FG, Gallegos R, Connell J, et al. Effect of cardiac stem cells on left-ventricular remodeling in a canine model of chronic myocardial infarction. *Circulation Heart failure*. 2013; 6:99–106. [PubMed: 23212553]
17. Takamiya M, Haider KH, Ashraf M. Identification and characterization of a novel multipotent sub-population of Sca-1(+) cardiac progenitor cells for myocardial regeneration. *PLoS One*. 2011; 6:e25265. [PubMed: 21980409]
18. Bolli R, Chugh AR, D'Amario D, et al. Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. *Lancet*. 2011; 378:1847–1857. [PubMed: 22088800]
19. Agoston I, Cameron CS, Yao D, et al. Comparison of outcomes of white versus black patients hospitalized with heart failure and preserved ejection fraction. *Am J Cardiol*. 2004; 94:1003–1007. [PubMed: 15476612]
20. Rathore SS, Foody JM, Wang Y, et al. Race, quality of care, and outcomes of elderly patients hospitalized with heart failure. *JAMA*. 2003; 289:2517–2524. [PubMed: 12759323]
21. Singh H, Gordon HS, Deswal A. Variation by race in factors contributing to heart failure hospitalizations. *J Card Fail*. 2005; 11:23–29. [PubMed: 15704060]
22. von Haehling S, Doehner W, Anker SD. Nutrition, metabolism, and the complex pathophysiology of cachexia in chronic heart failure. *Cardiovascular research*. 2007; 73:298–309. [PubMed: 17034772]
23. Anker SD, Chua TP, Ponikowski P, et al. Hormonal changes and catabolic/anabolic imbalance in chronic heart failure and their importance for cardiac cachexia. *Circulation*. 1997; 96:526–534. [PubMed: 9244221]
24. Ito K, Suda T. Metabolic requirements for the maintenance of self-renewing stem cells. *Nature reviews Molecular cell biology*. 2014; 15:243–256. [PubMed: 24651542]
25. Gaspar JA, Doss MX, Hengstler JG, et al. Unique metabolic features of stem cells, cardiomyocytes, and their progenitors. *Circ Res*. 2014; 114:1346–1360. [PubMed: 24723659]
26. Rota M, LeCapitaine N, Hosoda T, et al. Diabetes promotes cardiac stem cell aging and heart failure, which are prevented by deletion of the p66shc gene. *Circ Res*. 2006; 99:42–52. [PubMed: 16763167]
27. Molgat AS, Tilokee EL, Rafatian G, et al. Hyperglycemia inhibits cardiac stem cell-mediated cardiac repair and angiogenic capacity. *Circulation*. 2014; 130:S70–76. [PubMed: 25200058]
28. Zafir A, Readnower R, Long BW, et al. Protein O-GlcNAcylation is a novel cytoprotective signal in cardiac stem cells. *Stem Cells*. 2013; 31:765–775. [PubMed: 23335157]
29. He JQ, Vu DM, Hunt G, et al. Human cardiac stem cells isolated from atrial appendages stably express c-kit. *PLoS One*. 2011; 6:e27719. [PubMed: 22140461]
30. Fransioli J, Bailey B, Gude NA, et al. Evolution of the c-kit-positive cell response to pathological challenge in the myocardium. *Stem Cells*. 2008; 26:1315–1324. [PubMed: 18308948]
31. Mohsin S, Khan M, Toko H, et al. Human cardiac progenitor cells engineered with Pim-I kinase enhance myocardial repair. *Journal of the American College of Cardiology*. 2012; 60:1278–1287. [PubMed: 22841153]
32. Perez J, Hill BG, Benavides GA, et al. Role of cellular bioenergetics in smooth muscle cell proliferation induced by platelet-derived growth factor. *Biochem J*. 2010; 428:255–267. [PubMed: 20331438]
33. Hill BG, Dranka BP, Zou L, et al. Importance of the bioenergetic reserve capacity in response to cardiomyocyte stress induced by 4-hydroxynonenal. *Biochem J*. 2009; 424:99–107. [PubMed: 19740075]

34. Dranka BP, Hill BG, Darley-USmar VM. Mitochondrial reserve capacity in endothelial cells: The impact of nitric oxide and reactive oxygen species. *Free Radic Biol Med*. 2010; 48:905–914. [PubMed: 20093177]
35. Dranka BP, Benavides GA, Diers AR, et al. Assessing bioenergetic function in response to oxidative stress by metabolic profiling. *Free Radic Biol Med*. 2011; 51:1621–1635. [PubMed: 21872656]
36. Sansbury BE, DeMartino AM, Xie Z, et al. Metabolomic analysis of pressure-overloaded and infarcted mouse hearts. *Circulation Heart failure*. 2014; 7:634–642. [PubMed: 24762972]
37. Ashcroft SJ, Weerasinghe LC, Bassett JM, et al. The pentose cycle and insulin release in mouse pancreatic islets. *Biochem J*. 1972; 126:525–532. [PubMed: 4561619]
38. Elstrom RL, Bauer DE, Buzzai M, et al. Akt stimulates aerobic glycolysis in cancer cells. *Cancer research*. 2004; 64:3892–3899. [PubMed: 15172999]
39. Vander Heiden MG, Plas DR, Rathmell JC, et al. Growth factors can influence cell growth and survival through effects on glucose metabolism. *Mol Cell Biol*. 2001; 21:5899–5912. [PubMed: 11486029]
40. Kato M, Sakai K, Endo A. Koningic acid (heptelidic acid) inhibition of glyceraldehyde-3-phosphate dehydrogenases from various sources. *Biochim Biophys Acta*. 1992; 1120:113–116. [PubMed: 1554737]
41. Sansbury BE, Riggs DW, Brainard RE, et al. Responses of hypertrophied myocytes to reactive species: implications for glycolysis and electrophile metabolism. *Biochem J*. 2011; 435:519–528. [PubMed: 21275902]
42. Bailey B, Izarra A, Alvarez R, et al. Cardiac stem cell genetic engineering using the alphaMHC promoter. *Regenerative medicine*. 2009; 4:823–833. [PubMed: 19903002]
43. Hill BG, Benavides GA, Lancaster JR Jr, et al. Integration of cellular bioenergetics with mitochondrial quality control and autophagy. *Biol Chem*. 2012; 393:1485–1512. [PubMed: 23092819]
44. Phillips M, Enan E, Liu PC, et al. Inhibition of 3T3-L1 adipose differentiation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Cell Sci*. 1995; 108 (Pt 1):395–402. [PubMed: 7537747]
45. Li R, Blanchette-Mackie EJ, Ladisch S. Induction of endocytic vesicles by exogenous C(6)-ceramide. *J Biol Chem*. 1999; 274:21121–21127. [PubMed: 10409665]
46. Crabtree HG. Observations on the carbohydrate metabolism of tumours. *Biochem J*. 1929; 23:536–545. [PubMed: 16744238]
47. Diaz-Ruiz R, Rigoulet M, Devin A. The Warburg and Crabtree effects: On the origin of cancer cell energy metabolism and of yeast glucose repression. *Biochim Biophys Acta*. 2011; 1807:568–576. [PubMed: 20804724]
48. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, et al. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab*. 2008; 7:11–20. [PubMed: 18177721]
49. Duran RV, Oppliger W, Robitaille AM, et al. Glutaminolysis activates Rag-mTORC1 signaling. *Molecular cell*. 2012; 47:349–358. [PubMed: 22749528]
50. Tang XL, Rokosh G, Sanganalmath SK, et al. Intracoronary administration of cardiac progenitor cells alleviates left ventricular dysfunction in rats with a 30-day-old infarction. *Circulation*. 2010; 121:293–305. [PubMed: 20048209]
51. Hong KU, Li QH, Guo Y, et al. A highly sensitive and accurate method to quantify absolute numbers of c-kit+ cardiac stem cells following transplantation in mice. *Basic research in cardiology*. 2013; 108:346. [PubMed: 23549981]
52. Hong KU, Bolli R. Cardiac stem cell therapy for cardiac repair. *Current treatment options in cardiovascular medicine*. 2014; 16:324. [PubMed: 24903489]
53. Montel-Hagen A, Blanc L, Boyer-Clavel M, et al. The Glut1 and Glut4 glucose transporters are differentially expressed during perinatal and postnatal erythropoiesis. *Blood*. 2008; 112:4729–4738. [PubMed: 18796630]
54. Tonack S, Rolletschek A, Wobus AM, et al. Differential expression of glucose transporter isoforms during embryonic stem cell differentiation. *Differentiation; research in biological diversity*. 2006; 74:499–509.

55. Oburoglu L, Tardito S, Fritz V, et al. Glucose and glutamine metabolism regulate human hematopoietic stem cell lineage specification. *Cell stem cell*. 2014; 15:169–184. [PubMed: 24953180]
56. Wang Y, Huang Y, Zhao L, et al. Glutaminase 1 Is Essential for the Differentiation, Proliferation, and Survival of Human Neural Progenitor Cells. *Stem cells and development*. 2014
57. Vega-Naredo I, Loureiro R, Mesquita KA, et al. Mitochondrial metabolism directs stemness and differentiation in P19 embryonal carcinoma stem cells. *Cell death and differentiation*. 2014; 21:1560–1574. [PubMed: 24832466]
58. Amores-Sanchez MI, Medina MA. Glutamine, as a precursor of glutathione, and oxidative stress. *Molecular genetics and metabolism*. 1999; 67:100–105. [PubMed: 10356308]
59. Ide T, Tsutsui H, Kinugawa S, et al. Direct evidence for increased hydroxyl radicals originating from superoxide in the failing myocardium. *Circ Res*. 2000; 86:152–157. [PubMed: 10666410]
60. Giordano FJ. Oxygen, oxidative stress, hypoxia, and heart failure. *J Clin Invest*. 2005; 115:500–508. [PubMed: 15765131]
61. Voet, D.; Voet, JG. *Biochemistry*. Vol. xvii. New York: J. Wiley & Sons; 1995. p. 1361
62. Gingras AC, Raught B, Sonenberg N. Regulation of translation initiation by FRAP/mTOR. *Genes Dev*. 2001; 15:807–826. [PubMed: 11297505]
63. Gao N, Flynn DC, Zhang Z, et al. G1 cell cycle progression and the expression of G1 cyclins are regulated by PI3K/AKT/mTOR/p70S6K1 signaling in human ovarian cancer cells. *American journal of physiology Cell physiology*. 2004; 287:C281–291. [PubMed: 15028555]
64. Wise DR, DeBerardinis RJ, Mancuso A, et al. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc Natl Acad Sci U S A*. 2008; 105:18782–18787. [PubMed: 19033189]
65. Askanazi J, Elwyn DH, Kinney JM, et al. Muscle and plasma amino acids after injury: the role of inactivity. *Annals of surgery*. 1978; 188:797–803. [PubMed: 736657]
66. Smith RJ, Wilmore DW. Glutamine nutrition and requirements. *JPEN Journal of parenteral and enteral nutrition*. 1990; 14:94S–99S. [PubMed: 2119461]
67. Rogeri PS, Costa Rosa LF. Plasma glutamine concentration in spinal cord injured patients. *Life sciences*. 2005; 77:2351–2360. [PubMed: 16024049]
68. Umpleby AM, Carroll PV, Russell-Jones DL, et al. Glutamine supplementation and GH/IGF-I treatment in critically ill patients: effects on glutamine metabolism and protein balance. *Nutrition*. 2002; 18:127–129. [PubMed: 11844642]
69. Askanazi J, Carpentier YA, Michelsen CB, et al. Muscle and plasma amino acids following injury. Influence of intercurrent infection. *Annals of surgery*. 1980; 192:78–85. [PubMed: 7406567]
70. Gottschalk A, Wempe C, Goeters C. Glutamine in the ICU: who needs supply? *Clinical nutrition*. 2013; 32:668–669. [PubMed: 23290873]
71. Khogali SE, Pringle SD, Weryk BV, et al. Is glutamine beneficial in ischemic heart disease? *Nutrition*. 2002; 18:123–126. [PubMed: 11844641]
72. He W, Miao FJ, Lin DC, et al. Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors. *Nature*. 2004; 429:188–193. [PubMed: 15141213]
73. Luttrell DK, Luttrell LM. Not so strange bedfellows: G-protein-coupled receptors and Src family kinases. *Oncogene*. 2004; 23:7969–7978. [PubMed: 15489914]

Figure 1. Characterization of murine CPCs

Mouse CPCs were isolated from mouse hearts based on c-kit positivity and lineage (Lin) negativity and then examined for their differentiation and proliferative capacity. (A) Photomicrograph of mCPCs in culture under normal growth conditions. Scale bar = 400 μ m; (B and C) Flow cytometry plots and relative quantification of Sca-1, c-kit and Lin after four passages in culture; (D) Relative transcript levels of markers of cardiomyocytes (*Tnnt2*), endothelial cells (*Flk1*) and smooth muscle cells (*Acta2*) after 2 d in differentiation medium (DIFF); (E) Representative images of flow cytometry plots showing the relative expression of sarcomeric actin (SA α -actin), vascular endothelial growth factor receptor-2 (Flk1), and smooth muscle actin (SM α -actin) after differentiation for 5 d; (F) Median fluorescence intensity of groups from panel E, n = 3 per group, *p<0.05, **p<0.01 vs. undifferentiated group (UNDIFF); (G) Expression of cardiovascular lineage markers by Western blotting; lysates from the following cells or tissues were used as positive controls: for SM- α -actin, rat aortic smooth muscle cell lysate; for cardiac troponin T (Tnnt2) and SA- α -actin, whole mouse heart homogenate; for VEGFR2, human umbilical vein endothelial cell lysate; and (H) proliferation rate of mCPCs in growth medium; plot is derived from three independent experiments.

Figure 2. CPCs utilize glucose in a non-insulin-dependent manner

(A) Western blots showing the expression of the insulin receptor (IR), insulin receptor substrate (IRS) and glucose transporter Glut1 and Glut4 as well as the increase in Akt phosphorylation upon stimulation with insulin (10 nM); (B) Radiometric measurement of glycolytic flux in mCPCs in the presence of 5 mM glucose and 25 mM glucose. Medium containing 5 mM glucose and 20 mM D-mannitol was used as an osmotic control. Koningic acid (KA, 5 μM), an inhibitor of glyceraldehyde-3-phosphate dehydrogenase, was used as an additional control and to ensure specificity of the assay. Graph is representative of 3 independent experiments. **p<0.01 vs. 5 mM Glc, #p<0.001 vs. non-KA groups; (C) Extracellular flux measurements of the proton production rate (PPR) of mCPCs incubated with no glucose (0 mM Glc), low glucose (5 mM Glc), or high glucose (25 mM Glc); (D) PPR of mCPCs in the presence of high glucose and treated with KA; (E) ATP levels measured after 3 h in 0–25 mM glucose; (F) Cell proliferation assay of mCPCs in normal growth medium without glucose (0 mM Glc) or with 5 or 25 mM Glc; 20 mM mannitol (Mann) was used as an osmotic control. n = 3 per group, *p<0.05 vs. 5 mM Glc at 96 h; and (G) LDH assay of mCPCs grown for 96 h in 0, 5, or 25 mM glucose or in 5 mM glucose + 20 mM mannitol (Osm. CTRL). Graph represents 3 independent experiments. ***p<0.001 vs. all other groups.

Figure 3. Glutamine is the major substrate utilized by proliferating CPCs in culture

Respiratory activity of mCPCs when provided with different carbon substrates: **(A)** Oxygen consumption rates (OCR) of mCPCs incubated with no substrate (BSA control) or glutamine (Gln, 4 mM), glucose (Glc, 5 mM), pyruvate (Pyr, 1 mM) or BSA-conjugated to palmitate (Palm, 100 μM). After addition of each substrate, two OCR measurements were recorded, after which oligomycin (Oligo), FCCP, and antimycin A and rotenone (AA/Rot) were injected sequentially. *Inset*: Representative Western blot of the glutamine transporter ASCT2 (apparent M_r, 49 kDa); **(B)** The OCRs after each compound was injected were used to calculate the basal level of mitochondrial OCR (Mito_{Basal}), ATP-linked OCR, proton leak, maximal mitochondrial OCR (Mito_{Max}), and the non-mitochondrial OCR (N.M.). The bar graph represents the combined values from four independent experiments. *p<0.05 vs.

BSA, # $p < 0.05$ vs. Glc, @ $p < 0.05$ vs. BSA-palm; (C) Baseline OCR of mCPCs and differentiated cells upon glutamine injection: NRCMs, neonatal rat cardiomyocytes; RASMCs, rat aortic smooth muscle cells; HUVECs, human umbilical vein endothelial cells; 3T3L1, mouse fibroblast cell line; and (D) Responses of mouse and human CPCs to glucose and glutamine: CPCs were provided glucose (Glc, 5 mM) or glutamine (Gln, 4 mM) at the indicated arrow (substrate), and OCRs were measured. The mitochondrial function assay using oligomycin, FCCP, and AA/Rot was then performed as in panel A.

Figure 4. Glutamine stimulates oxygen consumption and proliferation in CPCs

(A) Effect of glucose (Glc) on the oxygen consumption rate (OCR) of mCPCs. The CPCs were incubated in DMEM devoid of all substrates prior to addition of 0–25 mM glucose; (B) Effects of glucose, glutamine, and pyruvate provided alone or in combination on mitochondrial OCR; (C) Effect of glutamine (Gln, 4 mM) on basal mitochondrial OCR in the absence (BSA) or presence of palmitate (BSA-Palm, 100 μM). Results represent a mean of three independent experiments, ** $p < 0.01$ vs. indicated groups; (D) Proliferation assay of mCPCs in growth medium containing 0–2.5 mM Gln; (E) Representative photomicrographs of mCPCs cultured without (0 mM Gln) or with (2.5 mM Gln) glutamine in the presence or absence of H₂O₂ (500 μM) for 24 h; and (F) LDH assay from panel E. Data represent 3 independent experiments; # $p < 0.005$ vs. 0, 0.1, 0.5, and 2.5 mM Gln in the absence of H₂O₂, * $p < 0.05$ vs. 0 mM Gln + H₂O₂ group.

Figure 5. Glutamine-induced proliferation of CPCs is dependent on mTOR and glutaminolysis
 Effects of glutamine on cell cycle signaling: (A) Mouse CPCs were deprived of glutamine for 24 h and then treated with glutamine (Gln; 2.5 mM) for 0.5–24 h and the activation of the mTOR substrate P70S6K was assessed by immunoblotting. Cell signaling downstream of mTOR, i.e., p-Rb, cyclin D1 and cdk4, were also examined; (B) Group data for panel A. (C) mCPCs were deprived of glutamine for 24 h followed by pre-incubation with rapamycin (10 nM) or vehicle (DMSO) for 1 h and then treated without or with Gln for 3 h; (D) Group data for panel C. For panels A and C, representative blots of three independent experiments are shown; *p<0.05, **p<0.01, ***p<0.001 vs. non-glutamine treated cells; (E) Proliferation of mCPCs in absence or presence of Gln and/or Rap. n = 3 per group, ***p<0.001 vs. Gln + Rap; (F) Diagram of typical glutamine anaplerosis reactions in mitochondria: Glutamine is hydrolyzed by glutaminase (GA) to yield glutamate and ammonium (not shown). Glutamate can be further metabolized to α-ketoglutarate (α-KG) via glutamate dehydrogenase (GDH) or aspartate aminotransferase (AAT). The α-KG can then be used in Krebs cycle reactions to fuel mitochondrial activity and ATP production. Inhibitors of GA and GDH/AAT, i.e., BPTES and AOA, respectively, as well as cell permeable α-KG (dimethyl α-KG; DMK) can be used to assess the relative importance of this glutaminolysis pathway; and (G) Cell proliferation assay in the absence or presence of Gln (2.5 mM) without or with AOA (0.5 mM) or BPTES (50 μM). Results are representative of three independent experiments. **p<0.01 vs. Gln alone, ***p<0.001 vs. Gln alone.

Figure 6. α-ketoglutarate sustains CPC metabolism and is sufficient to increase CPC proliferation

(A) Western blot of p-P70S6K in the absence or presence of Gln (2.5 mM) and AOA (0.5 mM): mCPCs were deprived of glutamine for 24 h followed by pre-incubation with AOA for 1 h. The cells were then treated with Gln for 3 h. The CPCs were then lysed and phosphorylation of P70S6K was assessed by immunoblot densitometry. Results are representative of three independent experiments. *** $p < 0.001$ vs. all other groups; (B) Oxygen consumption rate (OCR) trace of CPCs treated with no substrate (CTRL) or DMK (10 mM), Glu (10 mM), or Gln (4 mM). After substrate addition, two OCR measurements were recorded followed by the mitochondrial function assay. Results are representative of three independent experiments; (C) Cell proliferation assays in the absence of Gln and presence of 0–10 mM DMK. Results are representative of three independent experiments. * $p < 0.05$, *** $p < 0.001$ vs. 0 mM DMK; (D) Representative Western blots of p-P70S6K and Cyclin D1 in mCPCs deprived of glutamine but provided with DMK (5 mM) for 0–24 h; (E) mTORC1 activation, assessed by Western blotting for p-P70S6K, in mCPCs treated without or with Gln (2.5 mM), AOA (0.5 mM), and/or DMK (5 mM); and (F) Proliferation of mCPCs incubated in the absence or presence of glutamine (2.5 mM), AOA (0.5 mM), and/or DMK (5 mM). Cell number was counted after 48 h. *** $p < 0.001$ and **** $p < 0.0001$ vs. cells not treated with glutamine, AOA, or DMK; ### $p < 0.001$ and #### $p < 0.0001$ vs. cells treated

with glutamine alone; @p<0.05 and @@@p<0.001 vs. DMK alone; \$\$\$p<0.001 vs. cells treated with glutamine and AOA; &p<0.05 vs. cells treated with glutamine, AOA and DMK.

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Fig. 7. Working model of metabolic regulation of cardiac progenitor cell proliferation

Cardiac progenitor cells utilize glucose in an insulin-independent manner and take up glutamine to maintain their bioenergetic demands. The CPCs demonstrate an α -KG-enabled Krebs cycle, characterized by requirement of glutamine-derived α -KG to fuel mitochondrial respiration and to allow for oxidation of other substrates such as fat and glucose. Glutamine is necessary for mTORC1 activation, which is important for cell cycle regulation and optimal cell proliferation, and appears to require glutaminolysis-derived α -KG.