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Cardiac mesenchymal cells from diabetic mice are ineffective for cell therapy-mediated myocardial repair

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Abstract

Although cell therapy improves cardiac function after myocardial infarction, highly variable results and limited understanding of the underlying mechanisms preclude its clinical translation. Because many heart failure patients are diabetic, we examined how diabetic conditions affect the characteristics of cardiac mesenchymal cells (CMC) and their ability to promote myocardial repair in mice. To examine how diabetes affects CMC function, we isolated CMCs from non-diabetic C57BL/6J (CMC^{WT}) or diabetic B6.BKS(D)-*Leprdb*/J (CMC^{db/db}) mice. When CMCs were grown in 17.5 mM glucose, CMC^{db/db} cells showed > twofold higher glycolytic activity and a threefold higher expression of *Pfkfb3* compared with CMC^{WT} cells; however, culture of CMC^{db/db} cells in 5.5 mM glucose led to metabolic remodeling characterized by normalization of metabolism, a higher NAD⁺/NADH ratio, and a sixfold upregulation of Sirt1. These changes were associated with altered extracellular vesicle miRNA content as well as proliferation and cytotoxicity parameters comparable to CMC^{WT} cells. To test whether this metabolic improvement of CMC^{db/db} cells renders them suitable for cell therapy, we cultured CMC^{WT} or CMC^{db/db} cells in 5.5 mM glucose and then injected them into infarcted hearts of non-diabetic mice (CMC^{WT}, *n* = 17; CMC^{db/db}, *n* = 13; Veh, *n* = 14). Hemodynamic measurements performed 35 days after transplantation showed that, despite normalization of their properties in vitro, and unlike CMC^{WT} cells, CMC^{db/db} cells did not improve load-dependent and -independent parameters of left ventricular function. These results suggest that diabetes adversely affects the reparative capacity of

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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CMCs and that modulating CMC characteristics via culture in lower glucose does not render them efficacious for cell therapy.

Keywords

Cell therapy; Heart failure; Metabolism; Glycolysis; Mitochondria; Extracellular vesicle

Introduction

Cell therapy is a potential treatment for restoring cardiac function in heart failure patients. Promising results with animal models and in humans suggest that cell transplantation after myocardial infarction (MI) improves cardiac function, which is associated with decreased scar size, angiogenesis, or increased viable tissue [4, 5, 12, 24, 25, 41, 49, 50, 59, 60, 69, 71, 73]. Nevertheless, the ability of cells isolated from diabetic hearts to promote cardiac recovery remains unclear. Previous work has shown that diabetes inhibits the ability of numerous types of cells to promote tissue repair. For example, diabetes diminishes the ability of cardiac progenitor cells (CPCs) [44, 54, 57], endothelial progenitor cells, mesenchymal stem cells and hematopoietic stem cells [16, 17, 30, 31, 36, 55, 64] to migrate, proliferate, secrete paracrine factors, differentiate or engraft [2, 3, 7, 15, 18, 29, 33, 38, 39, 42, 63, 68, 77, 80]. This is significant because, in the US, ~ 25% of heart failure patients are diabetic, and up to 65% of hospitalized HF patients have type 2 diabetes (T2D) [14, 58, 61]. Therefore, understanding the mechanisms by which T2D affects cell therapy could provide a conceptual framework for improving the reparative properties of multiple cell types for patients with or without metabolic disease.

In diabetes, systemic insulin resistance—leading to poor glucose control and hyperglycemia—could negatively affect several cell types, particularly cells with mesenchymal characteristics. Cells of mesenchymal origin typically do not require insulin for glucose uptake (e.g., CPCs [56, 57]), leaving them exceptionally vulnerable to hyperglycemic injury. Accordingly, the purpose of this study was to examine whether diabetes damages the mesenchymal cell pool, which represents a common source of cells used for transplantation, and whether modifying culture conditions might render them efficacious for therapy. We found that cardiac mesenchymal cells (CMCs) [21, 72, 73] from the *db/db* diabetic mouse model (CMC^{*db/db*}) show marked metabolic derangements that mirror those found in diabetic CPCs [57]. Passaging of CMC^{*db/db*} cells in low glucose conditions normalizes their metabolic profile and robustly augments the expression of Sirt1. Despite this, correction of CMC^{*db/db*} properties via culture in low glucose was not sufficient to render them suitable for cardiac repair.

Materials and methods

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. The CMCs were isolated from 3-

month-old male B6.BKS(D)-*Lepr^{db}/J* (*db/db*) and C57BL/6 J wild-type (WT) mice (Jackson Laboratories, Bar Harbor, ME) without sorting for surface markers such as c-kit [73]. This procedure for cell isolation has been described before [72]. Briefly, excised hearts were washed in PBS and enzymatically dissociated with Collagenase II (5 mg/mL in PBS; Worthington) by gentle agitation for 45 min at 37 °C. After Collagenase II inactivation with DMEM/F12 medium containing 10% FBS, the cells were centrifuged at 600 *g* for 10 min. The collected cell pellet was suspended in growth medium consisting of DMEM/F12 (Invitrogen) containing 17.5 mM glucose, 10% FBS (Seradigm, VWR), bFGF (10 ng/ml), EGF (10 ng/ml), ITS (insulin/transferrin/selenium), glutamine, and penicillin–streptomycin. The CMCs were cultured and passaged twice in this growth medium. The cells were then frozen in liquid nitrogen. Upon thawing, the cells were propagated in the same growth medium containing either 5.5 mM or 17.5 mM glucose for 2–5 passages prior to in vitro or in vivo experiments.

Flow cytometry

Expression of cell surface markers was assessed by flow cytometry. Harvested CMCs (passage 3–6) were blocked with FcBlock (0.005 mg/ml in PBS containing 11% BSA) for 10 min at 4 °C. The CMCs were stained with the antibodies (eBioscience) listed in Supplementary Table 1. 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.

Proliferation and cytotoxicity measurements

Cell proliferation was assessed by counting cells using a hemocytometer, as described previously [56]. Cell viability and cytotoxicity was assessed by lactate dehydrogenase assay, as described previously [56].

Gene expression and protein abundance analyses

Gene expression was assessed by measuring cell type-specific gene expression by qRT-PCR. For qRT-PCR, mRNA was isolated from CMCs 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) and oligo primers (Integrated DNA Technologies, Inc). The *Pfkfb3* primers used for qRT-PCR are forward primer, GTG ACA GGG ACT TGT CAC TC; reverse primer, GCC ATG CCG ACA CAG GTA.

For Western blotting, protein from CMC lysates was applied to each lane of a 12.5% or 10.5–14% Bis–Tris–HCl gel and electrophoresed. The separated proteins were then electroblotted onto a PVDF membrane and immunoblotted as described [56, 57]. The following antibodies were used for these studies: anti-Sirt1 antibody (Cell Signaling Technology; 2496S), anti-Sirt3 antibody (Cell Signaling Technology; 5490S), anti-Sirt6 antibody (Cell Signaling Technology; 12486S) and anti-β-actin antibody (Cell Signaling Technology; 12262S). Immunoreactive bands were detected using a Fuji LAS-3000 Bio Imaging analyzer after exposure to ECL detection reagent. Band intensities were quantified using TotalLab TL120 or ImageQuantTL software.

Extracellular vesicle (EV) isolation

CMC-derived EVs were isolated using previously published protocols [10, 34, 65]. Briefly, CMCs were cultured in growth medium. At 80% confluency, the cells were washed in PBS and suspended in basal DMEM/F12 media supplemented with 0.5% BSA. After 24 h, conditioned media were collected and centrifuged (3000g, 15 min, 4 °C) to pellet cells and large debris. The EVs in the supernatant were precipitated with one-third volume of polyethylene glycol (PEG) buffer (33.4% w/v PEG 4000, 50 mM HEPES [pH 7.4], 1 mM NaCl) overnight at 4 °C, followed by centrifugation at 3000g for 30 min. The EV pellets were resuspended in PBS and then centrifuged at 100,000g (Beckman Coulter Optima L-90 K ultracentrifuge) for 2 h at 4 °C.

miRNA array

Total RNA was isolated from EVs using the mirVana™ miRNA Isolation Kit (Ambion). A rodent miRNA microarray kit (Applied Biosystems) was used to assess the miRNA profile according to the manufacturer's protocol. Briefly, 350 ng of total RNA (combined RNA from three independent isolations) was reverse-transcribed with Megaplex RT primers (Megaplex RT Rodent Pool A) and pre-amplified using Megaplex PreAmp primers followed by a real-time PCR with a TaqMan Rodent MicroRNA Array (panel A) performed on an Applied Biosystems ViiA™ 7 Real-Time PCR System. SDS software version 2.3 (Applied Biosystems) was used to obtain the comparative threshold cycle (Ct) value. U6 small nuclear RNA included in the TaqMan Rodent MicroRNA Array was used as an endogenous control and the relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

Extracellular flux analysis

Mitochondrial activity and surrogate measures of glycolytic flux were measured in WT and *db/db* CMCs, using procedures described previously [27, 56, 57].

Radiometric determination of glycolytic flux

Glycolytic flux was determined by analyzing the conversion of [5-³H]-glucose to [³H]₂O, as described previously [19, 56, 57].

Stable isotope tracing

Isolated WT and *db/db* CMCs were incubated with 5.5 mM ¹³C₆-glucose in 6-well plates for 12 h, quenched in cold acetonitrile, and extracted in acetonitrile:water:chloroform (v/v 2:1.5:1), similar to that described previously [19, 57]. Lipid and nucleotide spectra were acquired using a hybrid linear ion trap–FT-ICR mass spectrometer (Finnigan LTQ FT, Thermo Electron), as described previously [19, 57]. The FT-ICR MS spectra were exported as exact mass lists into a spreadsheet file using QualBrowser 2.0 (Thermo Electron). Assignments of all isotopologue peaks of metabolites were performed based on their accurate mass matching (m/z window $\pm$ 3 ppm), and natural isotope abundance contributions from each of the isotopologues in the MS data were stripped using MetSign [19, 57, 67]. To calculate relative abundances of NAD⁺, NADH, and adenine nucleotides, the intensities of each spectral peak for each individual metabolite were summed. These total abundances were then used to calculate NAD⁺/NADH ratios and relative adenylate abundances.

Murine model of myocardial infarction

C57BL/6/J female mice (12 weeks of age), purchased from The Jackson Laboratory (Bar Harbor, ME, USA) [74], were maintained under specific pathogen-free conditions and a 12-h light–dark cycle. Food and water were provided ad libitum. For ischemia–reperfusion, mice were anesthetized with sodium pentobarbital (60 mg/kg i.p.). The chest was opened through a midline sternotomy, and a nontraumatic balloon occluder was implanted around the mid-left anterior descending coronary artery using an 8–0 nylon suture. Rectal temperature was carefully monitored and maintained between 36.7 and 37.3 °C throughout the experiment. In all groups, MI was produced by a 60-min coronary occlusion followed by reperfusion. Successful performance of coronary occlusion and reperfusion was verified by visual inspection and by observing S-T segment elevation and widening of the QRS on the electrocardiogram during ischemia and their resolution after reperfusion.

Preparation of cells for injection

The CMCs were expanded for 3–5 passages in growth medium containing 5.5 mM glucose. On the day of injection, cells were 60–80% confluent. The cell monolayer was washed two times with room temperature PBS, and the cells were harvested by enzymatic digestion with 0.25% trypsin–EDTA (Invitrogen). The resulting cell suspension was trypsin-inactivated with DMEM/F12 supplemented with 10% FBS. After centrifugation at 600g for 10 min at room temperature (RT), the cells were suspended in PBS and counted using a hemocytometer. Next, the cells were filtered through a 40-µm filter and centrifuged for 10 min at 600g at RT. An aliquot of 2.5×10^6 cells was suspended in 1000 µL of sterile PBS (pH 7.4) at room temperature and transported to the mouse surgery laboratory for injection.

Intramyocardial cell delivery

At 48 h after I/R, the mice were anesthetized and the chest reopened through a central thoracotomy. The CMCs (10^5 cells in 40 µL) or, an equivalent volume of vehicle, were injected intramyocardially using a 30-gauge needle. A total of four injections (10 µL each) were made in the peri-infarct region in a circular pattern, at the border between infarcted and noninfarcted myocardium, as described [73].

Invasive hemodynamic measurements

Left ventricular (LV) mechanical performance was assessed using a Millar pressure–conductance catheter 35 days after cell transplantation, as described [73]. The hemodynamic studies were performed just before euthanasia using a MPVS Ultra pressure–volume (PV) loop system (Millar Instruments) and 1.0 French PVR-1035 microtip ultra-miniature PV catheters (Millar Instruments). Mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and rectal temperature kept between 36.7 and 37.3 °C. A 1.0 French Millar catheter was inserted into the LV via the right carotid artery. After 30 min stabilization, the PV signals were recorded continuously. Inferior vena cava occlusion was performed with external compression to produce variably loaded beats for determination of LV performance. The LabChart 7 Pro data acquisition and analysis software (with built-in PV loop module; AD Instruments) was used to calculate cardiovascular parameters.

Statistical analysis

All values are mean $\pm$ SEM. All comparisons were made to the relevant control group, and statistical tests were performed using either *t* test or one-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons [35]. The null hypothesis was rejected when $p > 0.05$.

Results

Diabetic CMCs demonstrate a higher reliance on glycolysis

We first examined the cell surface marker expression profile of CMC^{WT} and CMC^{db/db} cells. Both cell types expressed similar profiles of the mesenchymal markers CD90.2, CD29, and CD105. Both preparations also showed ~ 80% positivity for Sca-1 and ~ 15% positivity for CD34. Similar to diabetic CPCs [57], CD73 expression appeared lower in CMC^{db/db} cells; however, further analysis in four independent isolations showed no statistical significance for CD73 abundance (CMC^{WT}: $60.2 \pm 6.6\%$; CMC^{db/db}: $45.6 \pm 6.1\%$, $p > 0.05$). The cells were mostly negative for CD31, c-kit, and CD45 (Fig. 1).

To determine whether diabetes affects the metabolic characteristics of CMCs (similar to that of CPCs [57]), we measured mitochondrial activity and extracellular acidification rates (ECAR) in CMC^{WT} and CMC^{db/db} cells (Fig. 2a). The CMC^{db/db} cells showed lower basal oxygen consumption rates (OCR; Fig. 2b) and a > twofold increase in basal ECAR (Fig. 2c). The lower OCR/ECAR ratio in CMC^{db/db} cells suggests lower reliance on oxidative phosphorylation for energy (Fig. 2d). Consistent with findings in diabetic CPCs [57], CMC^{db/db} cells had threefold higher expression of *Pfkfb3* (Fig. 2e), which encodes an enzyme known to activate glycolysis [45, 57, 75]. Collectively, these findings indicate that diabetes promotes a glycolytic phenotype in slowly adherent CMCs, which is similar to its effects on CPCs.

Culture of CMC^{db/db} cells in low glucose normalizes their metabolic profile

Typical growth medium for mouse CMC and CPC culture contains 17.5 mM glucose, which is hyperglycemic (315 mg/dl) in mammals. Therefore, we examined whether culturing the CMC cells in low glucose conditions (i.e., 5.5 mM, simulating 99 mg/dl) might improve the metabolic profile of CMCs. For this, CMC^{WT} and CMC^{db/db} cells that were isolated in 17.5 mM glucose-containing medium were cultured in low (5.5 mM) glucose-containing medium and passaged twice over the course of 2 weeks (Fig. 3a). We then examined the metabolism of the CMCs to determine if metabolic changes in CMC^{db/db} cells persist upon switching from 17.5 to 5.5 mM glucose. Surprisingly, we found no differences in OCR or ECAR between CMC^{WT} and CMC^{db/db} cells (Fig. 3b–d). Furthermore, glycolytic flux measured via the tritiated glucose assay was not different between the groups (Fig. 3e). Moreover, *Pfkfb3* expression levels were similar in both cell preparations (Fig. 3f).

To assess metabolism in the cells further, we cultured both cell preparations in medium containing 5.5 mM ¹³C₆-glucose for 12 h and performed stable isotope resolved metabolomics. As shown in Suppl. Figure 1, end products of ancillary biosynthetic pathways of glucose metabolism, such as those from the pentose phosphate pathway (e.g., ATP), the

hexosamine biosynthetic pathway (i.e., UDP-Glc(Gal)NAc), and the glycerophospholipid pathway (e.g., phosphatidylinositol), showed no differences in ^{13}C labeling. Similarly, we found no differences in labeling of other phospholipids and nucleotides (data not shown). Because cells of mesenchymal origin may also use glutamine as a fuel source [56], we also incubated cells in $^{15}\text{N}^{13}\text{C}_5$ -glutamine for 12 h and assessed labeling. We found no differences in labeling of the detected metabolites (data not shown). Taken together, these data indicate that the diabetes-induced changes in metabolism in $\text{CMC}^{db/db}$ cells are normalized upon culture in medium containing 5.5 mM glucose.

Culture of $\text{CMC}^{db/db}$ cells in low glucose medium upregulates Sirt1

Although culture conditions mimicking hyperglycemia appear to have little effect on the metabolism of CMC^{WT} cells, in $\text{CMC}^{db/db}$ cells, hyperglycemic conditions maintain metabolic abnormalities. We reasoned that $\text{CMC}^{db/db}$ cells might perceive the 5.5 mM glucose culture condition as a substrate restriction, which is similar to calorie restriction. This could elevate the NAD^+/NADH ratio. Indeed, mass spectrometry-based assay of the relative levels of NAD^+ and NADH suggested that the NAD^+/NADH ratio in $\text{CMC}^{db/db}$ cells cultured in 5.5 mM glucose was ~ 9 , while CMC^{WT} cells demonstrated a ratio of 2. Adenylate levels ($\text{ATP}/\text{ADP}/\text{AMP}$) were not different between the cell preparations (data not shown).

Because NAD^+ levels regulate the epigenetic landscape and may be involved in diabetic memory, we examined the sirtuin family of proteins, which are known to use NAD^+ as a cofactor in deacetylation reactions. Although $\text{CMC}^{db/db}$ cells cultured in high glucose showed no difference in sirtuin expression compared with CMC^{WT} cells, $\text{CMC}^{db/db}$ cells cultured in 5.5 mM glucose showed sixfold higher levels of Sirt1 and lower Sirt6 compared with CMC^{WT} cells (Fig. 4). Sirt3 expression was unchanged in both conditions (data not shown).

Given that Sirt1 regulates nutrient signaling, promotes mitochondrial biogenesis, prevents senescence, improves cell survival, combats oxidative stress, and diminishes genomic instability [9, 40, 43, 62, 70], we next assessed proliferation of the CMC^{WT} and $\text{CMC}^{db/db}$ cells cultured in low glucose and examined their viability after a 16 h challenge with hydrogen peroxide. As shown in Fig. 5a and 5b, doubling time and cell viability were not statistically different between CMC^{WT} and $\text{CMC}^{db/db}$ cells. However, extra-cellular vesicles (EVs) isolated from the culture medium of CMC^{WT} and $\text{CMC}^{db/db}$ cells showed heightened levels of numerous miRNAs (Fig. 5c), including miR-182, which has been shown to both upregulate Sirt1 and be a downstream effector of Sirt1, regulating nerve regeneration [66].

Low glucose culture of $\text{CMC}^{db/db}$ cells does not restore their therapeutic efficacy

That low glucose culture of $\text{CMC}^{db/db}$ cells rendered them metabolically and functionally similar to CMC^{WT} cells in vitro as well as upregulated Sirt1 and influenced EV cargo suggested to us that their reparative properties in vivo may be similarly influenced. To test this hypothesis, we injected CMC^{WT} and $\text{CMC}^{db/db}$ that were acclimated to low glucose culture conditions into the border zone of the infarcted murine heart. The cells (or vehicle) were injected 48 h after I/R injury, and cardiac function was assessed after 35 days (Fig. 6a).

Just prior to euthanasia, we performed hemodynamic assessment of LV function using a pressure–volume catheter. Similar to our previous reports [73], CMC^{WT} cells improved the load-dependent parameters of LV ejection fraction and dP/dt as well as the load independent parameter, end-systolic elastance; however, CMC^{db/db} cells failed to improve LV function (Fig. 6b–e). Collectively, these data demonstrate that the apparent improvement of CMC^{db/db} cell competence caused by 5.5 mM glucose culture in vitro does not extend to reparative efficacy in vivo.

Discussion

Our previous studies suggest that CMCs isolated on the basis of their adherent properties exhibit robust reparative properties [73]. In this study, we examined whether diabetes affects this population of cells in a manner similar to that of CPCs, which are sensitive to hyperglycemic damage [44, 54, 56, 57]. We found that, like CPCs isolated from diabetic mice, CMC^{db/db} cells show higher levels of glycolysis, which may be related in part to upregulation of *Pfkfb3*. Because murine CPCs and CMCs are typically cultured in hyperglycemic (17.5 mM glucose) medium, we tested whether switching cells a lower glucose (5.5 mM) medium would restore metabolic defects and render them efficacious for myocardial repair. We found that culturing the cells in 5.5 mM glucose fully normalized catabolic and anabolic CMC metabolism, robustly upregulated Sirt1, and changed the miRNA profile in EVs. Despite this apparent improvement in metabolic profile, the CMCs were ineffective for cardiac repair, which suggests that the impact of diabetes on the reparative qualities of CMCs extends beyond short-term changes in intermediary metabolism.

Our findings that reparative defects persist in CMCs despite normalization of glucose metabolism is, in a general sense, consistent with results of the ADVANCE and ACCORD trials, which show that strict glucose control does not diminish the macrovascular and cardiac complications of diabetes [1, 20]. Lack of efficacy of CMC^{db/db} cells could be explained via persistence of hyperglycemia-induced defects. This phenomenon is termed metabolic memory—a hypothesis that cells and tissues remember their glycemic environment [8]. The mechanisms underlying metabolic memory in diabetes are thought to involve mitochondrial dysfunction [48] and flooding of ancillary biosynthetic pathways [6]. Our data show that culture of CMC^{db/db} cells in medium containing 5.5 mM glucose normalized glycolysis, mitochondrial activity, and biosynthetic pathway activity, which indicates that acute reversal of metabolic abnormalities caused by diabetes does not render CMCs effective in myocardial repair.

A remarkable consequence of switching CMC^{db/db} cells to low glucose medium was stark upregulation of Sirt1, which was accompanied by a modest downregulation of Sirt6. This is interesting because sirtuins regulate metabolism: both Sirt1 and Sirt6 affect glycolytic and mitochondrial activity [23, 52, 81]. We speculate that culturing CMC^{db/db} cells in medium containing 5.5 mM glucose leads to a form of energy restriction, which promotes upregulation of Sirt1 and normalizes metabolism. This notion would be consistent with previous studies showing that glucose restriction (or calorie restriction) upregulates Sirt1 and promotes metabolic remodeling in cultured cells as well as in whole organisms (e.g., [22, 26,

32, 78]). Our initial view that the SIRT1-over-expressing, metabolism-corrected CMC^{db/db} cells might be able to promote cardiac repair was strengthened by studies showing that SIRT1 is a cardioprotective enzyme [28, 46, 47, 76] and that it can improve cardiac repair after injury [13, 79]. In addition, it is also worth noting that mesenchymal stem cells cultured for up to 12 h without glucose showed higher cardiac repair capacity than cells cultured with glucose [11], which further strengthened our rationale for testing the 5.5 mM glucose culture strategy.

Despite these expectations, our in vivo studies showed that CMC^{db/db} cells cultured in low glucose failed to improve cardiac function, even though they showed improvement in not only metabolic parameters, but also indices commonly related to cell competence. The CMC^{db/db} cells cultured in 5.5 mM glucose showed similar, if not slightly improved, proliferation and viability in vitro compared with CMC^{WT} cells cultured under the same conditions. Furthermore, analysis of nucleic acid cargo in EVs isolated from CMC^{db/db} cells showed numerous upregulated miRNAs including miR-182, which has been shown to be upregulated in a SIRT1-dependent manner and to promote corneal nerve regeneration [66]. Moreover, several other miRNAs that were in higher abundance in CMC^{db/db}-derived EVs have been shown to have beneficial actions. For example, the levels of miR-15a, mi339–5p, and mi-370 were higher in EVs derived from CMC^{db/db} cells, and these are thought to be involved in tissue regeneration or fibrosis [37, 51, 53]. That these in vitro measures of cell competence do not equate to in vivo efficacy is an important outcome of this study that merits further investigation.

In summary, our study shows that even though CMCs are effective in promoting cardiac repair, their reparative capacity is severely compromised by diabetes. We found that culturing diabetic cells (CMC^{db/db} cells) in what is typically considered normal glucose (5.5 mM) restores their metabolic activity to levels similar to that of CMC^{WT} cells and augments Sirt1 levels. Despite these changes, CMC^{db/db} cells were ineffective in promoting cardiac repair. These observations suggest that diabetes induces deleterious changes to CMCs that are difficult to reverse. Future studies are required to determine whether diabetes-induced attenuation of CMC-mediated repair is due to other factors, such as an inability to promote an adequate immune response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Surface marker expression profile of CMC^{WT} and CMC^{db/db} cells. Slowly adherent CMCs were isolated from WT and *db/db* mouse hearts and stained for CD90.2, CD29, CD105, CD73, CD31, CD34, c-kit, Sca-1, and CD45. Unstained samples were used for setting discrimination gates. Representative flow cytometry plots are shown

Fig. 2.

Diabetic CMCs show higher rates of glycolysis than nondiabetic CMCs. Extracellular flux analysis of mesenchymal cells from WT and *db/db* mice isolated and cultured continuously in medium containing 17.5 mM glucose: **a** Simplified schematic of isolation and culture procedures; **b** Mitochondrial bioenergetic profile of CMC^{WT} and CMC^{*db/db*} cells (OCR, oxygen consumption rate); **c** Extracellular acidification rate (ECAR), indicative of aerobic glycolytic activity; **d** OCR/ECAR ratio, which delineates the relative energetic reliance of the cells on mitochondrial activity versus glycolysis. Data are mean $\pm$ SEM, $n = 5$ replicates per group; and **e** *Pfkfb3* abundance in CMCs. Data represent four independent experiments. * $p < 0.05$, *** $p < 0.001$ vs. CMC^{WT}

Fig. 3.

Growth of diabetic CMCs in medium containing low glucose normalizes their metabolic phenotype. Metabolic flux analysis of CMCs derived from WT and *db/db* mice grown in medium containing 5.5 mM glucose: **a** Schematic illustrating protocol for isolation and switching to low glucose medium; **b** Mitochondrial bioenergetic profile of CMC^{WT} and CMC^{*db/db*} cells (OCR, oxygen consumption rate); **c** Extracellular acidification rate (ECAR), indicative of aerobic glycolytic activity; and **d** OCR/ECAR ratio, which delineates the relative energetic reliance of the cells on mitochondrial activity versus glycolysis; **e** Glycolytic flux measured via the [5-³H]-glucose method; and **f** Relative expression of *Pfkfb3*. *n* = 3 biological replicates per group

Fig. 4.

Growth of diabetic CMCs in low glucose medium increases Sirt1 abundance. CMCs isolated from WT and *db/db* mice were grown in culture medium containing 17.5 mM glucose (HG). At passage 2, medium was changed to medium containing 5.5 mM glucose (LG) or the cells were kept in HG medium. After 5 days, the cells were harvested, and protein abundance was measured. **a** Sirt1 and Sirt6 abundance in CMCs cultured in HG; **b** Sirt1 and Sirt6 expression in CMCs cultured in LG; and **c** Quantification of immunoblots. Data represent three biological replicates per group. * $p < 0.05$

Fig. 5.

In vitro competency factors of CMC^{WT} and CMC^{db/db} cells grown in low glucose medium. CMCs isolated from WT and *db/db* mice were expanded in low glucose medium for two passages and the following competency factors were examined: **a** Cell proliferation; **b** cytotoxicity caused by incubation with 500 μ M hydrogen peroxide. *n* = 3 biological replicates per group; and **c** miRNA (miR) analysis in extracellular vesicles isolated from the culture medium. Data are derived from pooled samples of 3 biological replicates per group

Fig. 6.

CMCs from diabetic mice fail to promote myocardial repair. **a** Adult mice were subjected to a 60-min coronary occlusion followed by reperfusion. Two days later, CMCs isolated from 12-week-old wild-type (WT) or *db/db* mice and cultured in low glucose were transplanted into the infarcted hearts. Infarcted mice receiving phosphate-buffered saline (vehicle) served as controls. After 35 days, hemodynamic measurements were performed using a Millar catheter. Shown are: **a** LV ejection fraction, **b** end-systolic elastance, **c** dP/dt max, and **d** dP/dt min. $n = 13$ – 17 mice per group; ** $p < 0.01$ vs. vehicle, ## $p < 0.01$ vs. CMC^{WT}, *** $p < 0.001$ vs. vehicle, ### $p < 0.001$, #### $p < 0.0001$ vs. CMC^{WT}