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Original Paper

Inhibition of Cardiomyogenesis in Embryocarcinoma Cells Induced by Long-Term High Level of Glucose

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Key Words
Differentiation • Embryocarcinoma Cells • Cardiomyocytes • PDGM • Hyperglycemia • GATA4 • Nkx2.5 • PGC-1α

Abstract
Background/Aims: Cardiac myocytes constitute the first differentiated cell type during mammalian heart formation with the ability to beat spontaneously and rhythmically. Hyperglycemia is a primary risk factor for cardiovascular disease in pre-gestational diabetes mellitus (PGDM). However, the impact that hyperglycemia has on cardiac progenitors or on precursors differentiation remains poorly understood. The aim of the present study is to investigate whether hyperglycemia affects cardiomyogenesis of embryocarcinoma cells. ***Methods:*** P19CL6 cells differentiation induced by 1% DMSO was evaluated under either normal glucose (5.6 mmol/L) or high level of glucose concentrations (20 mmol/L or 40 mmol/L). To investigate the effect of long-term high level of glucose on cardiomyocytes differentiation, sarcomeric α-actinin, peroxisome proliferator–activated receptor coactivator-1 (PGC-1α), transcription factor GATA4 and Nkx2.5 were assessed by qRT-PCR analysis, western blot and immunofluorescence. ***Results:*** We observed that long-term high level of glucose markedly reduced P19CL6 cells differentiation into cardiomyocytes. The change in PGC-1α expression was consistent with changes in cardiac muscle myosin expression after exposure to 20 mmol/L or 40 mmol/L of glucose. On the other hand, the high level of glucose concentration profoundly decreased both GATA4 and Nkx2-5 expressions from day 6 to day 12 after differentiation, which was induced by 1% DMSO. ***Conclusion:*** Our results elucidate that the effect resulting from the long-term exposure of cardiac progenitors to high level of glucose is associated with decreased expression of GATA4 and Nkx2.5, providing a novel mechanism by which high glucose is able to affect cell differentiation.

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Introduction

Diabetes mellitus is a common complication in pregnancy, and pre-gestational diabetes mellitus (PGDM) is one of the leading risk factors for birth defects. PGDM is associated with a wide spectrum of anomalies affecting many organs during development. In an *in vivo* rat embryo model, approximately 50% of the rat embryos which were exposed to 600 mg/dl of glucose post-implantation for 2 hours or longer, during early stage of organogenesis, were malformed [1]. Among the numerous congenital malformations with PGDM, cardiovascular defects are the most frequent [2, 3]. Studies performed in animal models demonstrated that offspring of diabetic mice show increased myocardial collagen deposition and myocardial hyperplasia in late gestation [4, 5]. To date, hyperglycemia is considered to be a primary teratogen affecting organ development. Some retrospective reviews of diabetic pregnancies in human reported that poor glycemic control in early pregnancy is associated with increased risk of heart defects in offspring; the incidence of CHD in PGDM patients with a level of hemoglobin A1c (HbA1c) above 8.5% was almost three times higher than the incidence observed in patient with HbA1c levels lower than 8.5% [6, 7]. Furthermore, fetal glucose concentration depends on fetal glucose uptake. This uptake relates to the maternal-fetal glucose concentration gradient and glucose-stimulated insulin secretion. Constant hyperglycemia is usually responsible for downregulating insulin secretion whereas pulsatile hyperglycemia in fetus enhances it [8]. Thus, hyperglycemia has more impact on the fetus than on its mother. In fact, many pregnant women with PGDM are unaware of the increased risk of cardiovascular defects that hyperglycemia has on their unborn child, especially during early gestational phase; The reason behind that relate to the late diagnoses of gestational diabetes, which usually occurs after heart formation is completed. Although high maternal glucose concentration in late gestation is found to be associated with heart defects, the impact that long-term hyperglycemia has on cardiac progenitors or on precursor differentiation still needs to be elucidated.

During cardiomyogenesis, a transition from glycolytic metabolism to mitochondrial oxidative phosphorylation is required in order to meet the high energy levels required by the cardiomyocytes. Fatty acid oxidation is considered the major source of mitochondrial energy generation in mature cardiomyocytes [9, 10]. Over the last few years, Peroxisome proliferator–activated receptor g coactivator-1 (PGC-1α) was found to be highly expressed in tremendous energy consumption organs such as the brain, heart and skeletal muscles. It has been also shown to play critical regulatory roles in stimulating mitochondrial biogenesis and modulating mitochondrial functions in many cell types. Overexpression of PGC-1α in rat neonatal cardiomyocytes was reported to induce an increase in oxygen consumption. On the other hand, a decrease in the expression of Fatty acid oxidation (FAO) gene and ATP synthesis genes was observed in mice deficient in PGC-1α [11, 12]. GATA4, a member of the GATA family of transcription factors (TF), is a key cardiogenic regulator of heart development. GATA4 is found to be expressed in cardiac progenitors and its expression usually persists till later stages of embryogenesis. GATA-4 has been shown to regulate the expression of many other cardiac genes during cardiomyogenesis including A- and B-type natriuretic peptides as well as alpha myosin heavy chain (aMHC). Inhibition of GATA-4 expression has been shown to block *in vitro* cardiac muscle differentiation [13]. Nkx-2.5 homeobox gene, another TF expressed in cardiac progenitors, plays an important role in the differentiation of the myocardial lineage [14, 15]. In mice, loss of Nkx2.5 revealed an essential role in the establishment of the ventricular gene expression program [16]. Unlike GATA-4, Nkx2.5 does not appear to be directly required for myogenic genes regulation but is able to exert its role in cardiomyogenesis via combinatorial interaction with other cardiac TF [17, 18].

Embryonic stem cells (ES) or induced pluripotent stem cells (iPS) are considered as useful *in vitro* cardiomyogenesis models for their ability to self-renew and remain pluripotent [19-21]. However, the effects of different glucose concentration on embryoid body (EB) formation, derived from mouse ES cells or iPS cells, differed from human ES cells. Glucose concentration is found to affect the propensity for cell differentiation after

formation of the EB [22, 23]. P19CL6 cells were established from colonel derivatives of P19 cells by Habara-Ohkubo [24]. It has been reported that P19CL6 cells differ from P19 cells in their pluripotency state and have properties similar to cardiac precursors [25, 26]. On the other hand, P19CL6 cells remain important for investigating the molecular mechanism of cardiomyogenesis as authentic cardiomyocytes precursors; these cells can be successfully and effectively differentiated into beating cardiomyocytes using 1% DMSO over a period of 10 days, without EB formation. Expression of many cardiac differentiation markers as well as cardiac myosin occurs during the differentiation period of these cells, whereas no expression of skeletal muscle-specific markers, such as MyoD and myogenin is present [27, 28]. Therefore, P19CL6 cells have been used as an *in vitro* embryocarcinoma cells model to examine the differentiation mechanisms of cardiac precursors.

In this paper, we investigated whether P19CL6 cells can successfully differentiate into contractile cardiomyocytes under long-term high level of glucose concentration. We report here that high levels of glucose are able to inhibit P19CL6 cells differentiation into cardiomyocytes by downregulation of GATA4 and Nkx2-5 expressions and disturbing the switch to mitochondrial oxidative metabolism during differentiation. The result has provided important clues regarding the effect that hyperglycemia has on cardiac progenitors or precursors.

Materials and Methods

Cell culture and differentiation

P19CL6 cells were kindly provided by Prof.Yunzeng Zou (Institutes of Biomedical Sciences, Fudan University, Shanghai, China). Cells were cultured in α-MEM medium containing 5.6 mmol/L glucose which is normally maintained and differentiated into cardiomyocytes of P19CL6 cells; the medium also supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 10% fetal bovine serum, and were maintained in a 5% CO_2 atmosphere at 37 degree. The cells were reseeded every 3 days. Cells were used for experimental differentiation between 5-10 passages. To induce differentiation, P19CL6 cells were seeded at 1.8 x 10^5 cells in 6-well plates or 1.0 x 10^4 cells in 4 well glass slide, followed by incubation with 1% DMSO added in growth medium over 12 days, Cells cultures incubated in high glucose were also incubated with 20 mmol/L or 40 mmol/L D-glucose, respectively. The medium was changed every day.

Immunofluorescence

For sarcomeric α-actinin staining, cells were harvested at day 12 after induction, while for GATA4 and Nkx2-5 staining, cells were harvested at day 9 after induction. After fixation with 4% paraformaldehyde at room temperature for 15 min, followed by permeatigation with 0.3% TritonX-100 for 15 min and blocked with 5% BSA for 60 min, respectively, cells were then incubated with primary antibody(1:800 monoclonal anti- sarcomeric α-actinin (SAB3300072, Sigma-Aldrich), 1:500 polyclonal anti-GATA4 (sc-1237, Sant Cruz) or 1:800 polyclonal anti-NKX2-5(ab35842, Abcam)) at 4 degree overnight, and followed by incubation with Alexa Fluor® 594 or 488 conjugate secondary antibody(Life technologies) at room temperature in dark for 45 min. DAPI (ab104139, Abcam) was used for nuclear stain. The images were acquired using fluorescence microscopy at 200X or 400X magnification using Image pro Plus software.

Quantitative real-time PCR

Total RNA was extracted from cells at indicated time pints by TRIzol. RT-PCR was performed as previously described. cDNAs were generated from 1 ug of total RNA using the Omniscript RT Kit (QIAGEN, Inc.) QPCR was carried out with the Quantitect SYBR Green PCR kit (QIAGEN) in a MX4000 real-time PCR machine. Relative gene expression levels were calculated using the DDCt method, with the TBP as normalizing gene. The primers used were as followed: GATA4: 5'- CAC AAG ATG AAC GGC ATC AAC -3' and 5'- AGG CAT TAC ATA CAG GCT CAC-3'; Nkx2-5: 5'- GTG GGT CTC AAT GCC TATGG-3' and 5'- TCA TCG CCC TTC TCC TAAAG-3'; BNP: 5'- CAG CTC TTG AAG GAC CAAGG-3' and 5'- AGA GAC CCA GGC AGA GTCAG-3'; α-MHC: 5'- TCA AGG AGC TCA CCT ACCAG-3' and 5'- GTT GCA AAG GCT CCA GGTC-3'; β-MHC: 5'- AAG CTG CAG TTG AAG GTG AAGG-3' and 5'- AGC TTG TTG ACC TGG GACTC-3'; PGC-1α: 5'- AAG AGC GCC GTG TGA

TTTAC-3' and 5'- AGC AGG GTC AAA ATC GTCTG-3'; MCAD: 5'- ACC CTC GTG TAA CTA AGCTC-3' and 5'- AAT GCT GCT ATG TCA CAGTC-3'; MyoD; 5'- CCC CGG CGG CAG AAT GGC TACG-3' and 5'- GGT CTG GGT TCC CTG TTC TGTGT-3'.

Western Blot analysis

Cells were harvested in presence or absence of high glucose at different time points. The protein was extracted as previously described. A total of 20ug of extracts was subjected to immunoblotted. The antibodies were used as followed: Rabbit polyclonal anti-GATA4 1:1000 (sc-1237, Sant Cruz), Rabbit polyclonal anti-Nkx2-5 1:1000 (ab35842, Abcam)), Rabbit polyclonal anti- PGC-1α 1:1000, mouse monoclonal sarcomeric α-actinin 1:1500 (SAB3300072, Sigma-Aldrich) and peroxidase conjugated secondary antibody (Jackon).

Statistical analysis

Data are showed as means ± SEM. Two-way analysis of variance was used to compare between groups. P- Value < 0.05 was considered as an index of statistical significance.

Results

DMSO-induced P19CL6 cells differentiation into cardiomyocytes

To investigate the effect that long-term high levels of glucose concentration has on cardiomyocytes differentiation; a stable *in vitro* model of induction of P19CL6 cells differentiation into cardiomyocytes was used. Following exposure of the cells to 1% DMSO, differentiation of these cells was induced: the cells were forming patches and contracting over a period of 12 days as presented by spontaneous and rhythmic beating, comparable to the state of differentiated cardiomyocytes. To further verify whether these differentiated cells were actually cardiomyocytes, immunofluorescence at day 12 after induction was conducted to check the expression of sarcomeric α-actinin protein (differentiation marker, Green) (Fig. 1A-F). In the presence of 1% DMSO, sarcomeric α-actinin was highly expressed in these cells indicative of a cardiac differentiated phenotype. 5-6 random fields per coverslip were photographed and counted out autofluorescence regions, in the presence of 1% DMSO, the area of sarcomeric α-actinin staining could reached to (15.44% ± 4.19%). The expression of cardiac specific TF and genes was also examined by qRT-PCR analysis and western blot during P19CL6 differentiation. Increased expression of GATA4 was detected at day 3 which increased exponentially till day 9 after induction (Fig. 1G), whereas Nkx2-5 and BNP expressions were detected from day 9 to day 12 after induction (Fig. 1H, I). Both cardiac myosinα-MHC and β-MHC expressions peaked at day 9 (Fig. 1J-K). These results were recapitulated in Western blot analysis where increasing protein expressions of α-actinin and NKX2.5 were shown, although slight difference of GATA4 expression pattern was observed (Fig. 1L, M). however, we did not detect skeleton muscle markers expression increased during differentiation (Fig. 1N). In contrast to cardiomyocytes precursors, establishment of a mitochondrial oxidative metabolism is required to meet the energy demands in functional cardiomyocytes. The expression of FAO genes, including medium-chain acyl CoA dehydrogenases (MCAD) and PGC-1α are considered as markers of functional cardiomycocytes. The expression of these genes was detected by qRT-PCR at late stages after differentiation induction and results showed a high expression of MCAD and PGC-1α mRNA at day 9 and day12 (Fig. 1 O,P). Together, these data prove that P19CL6 cells successfully differentiated into cardiomyocytes after exposure to 1% DMSO.

Long-term high glucose inhibits P19CL6 cells differentiation into cardiomyocytes

To elucidate the effect of long-term high concentration of glucose on P19CL6 cells differentiation, immunofluorescence of sarcomeric α-actinin was carried out on P19CL6 differentiated cells treated with 5.6mmol/L (normal concentration), 20 mmol/L and 40 mmol/L D-glucose at day 12 after DMSO-induction (Fig. 2A). We measured ratios of cardiac α-actin-positive cell areas to total areas of microscopic fields, the positive cell areas in high

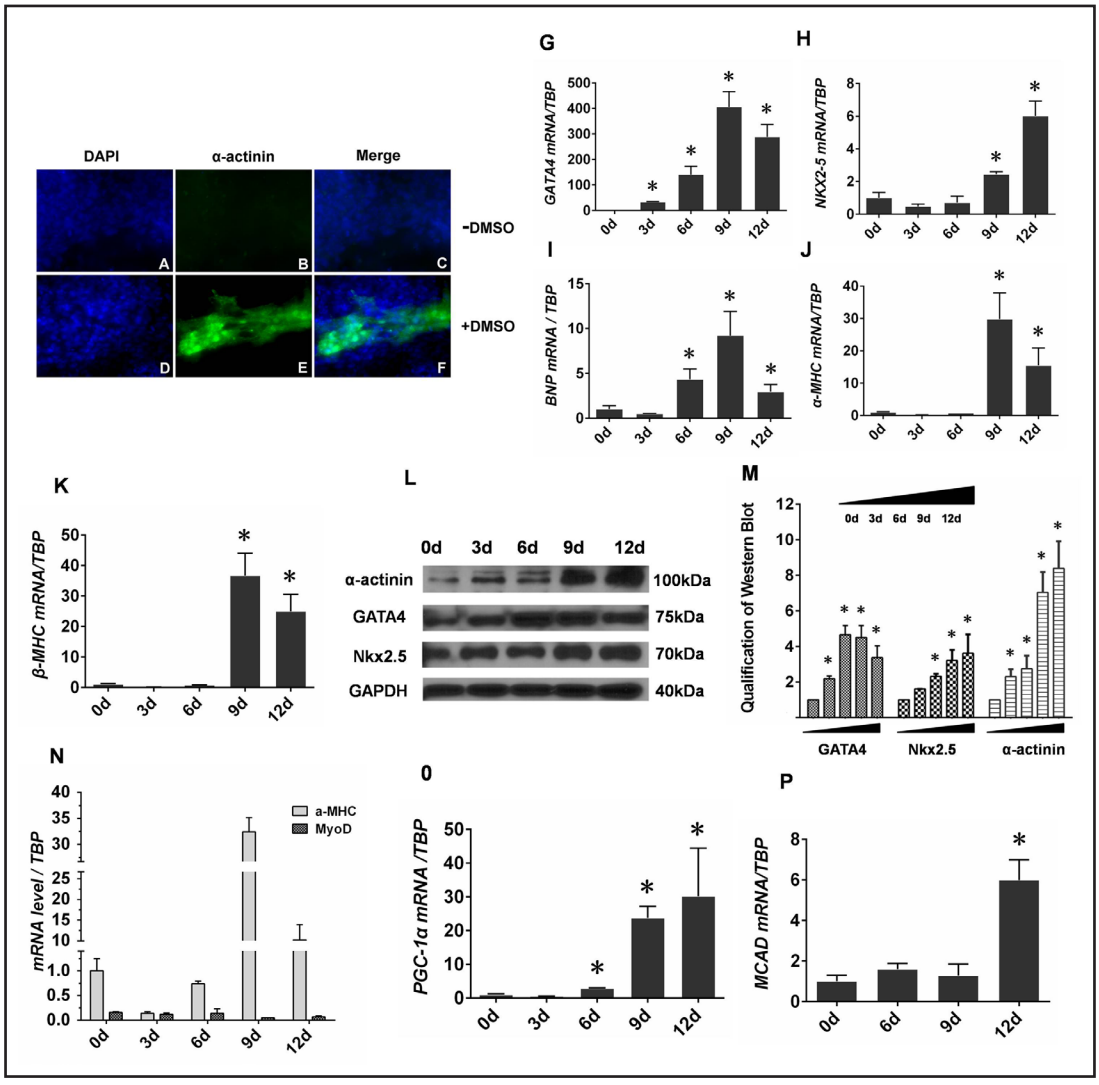

Fig. 1. P19CL6 cells differentiation into cardiomyocytes induced by 1% DMSO. (A-C) Immunofluorescence of α-cardiac actinin on P19CL6 cells treated without 1% DMSO at day 12; (D-F) Immunofluorescence of sarcomeric α-actinin on P19CL6 cells treated with 1% DMSO at day 12, note the positive cells (green). (G-K) Gene expression profile by qRT-PCR analysis using RNA extracted from P19CL6 cells at indicated times during P19CL6 cells differentiation, showed increased expression of TF GATA4 detected at day 3, which increased sharply after that, while Nkx2-5 and BNP increased 6 days after the induction. Expressions of cardiac myosin α-MHC and β-MHC were detected at day 9. (L-M) Western blot demonstrating the expression of GATA4, Nkx2-5, sarcomeric α-actinin, note the increase in the protein levels of GATA4, Nkx2-5 and sarcomeric α-actinin which were consistent with the qRT-PCR analysis. (N) Expression of skeleton muscle markers during P19CL6 cells differentiation induced by 1% DMSO as compared to α-MHC. (O-P) increased expression of fatty acid oxidation (FAO) genes, medium-chain acyl CoA dehydrogenases (MCAD) and peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α), during induction of P19CL6 differentiation (*$p < 0.05$, Statistical significant vs day 0).

level of glucose treatment group were reduced to (7.10% ± 2.57%) and (3.65% ± 0.83%), respectively. Results showed a reduction in the expression of sarcomeric α-actinin after increasing the doses of glucose in the medium. 20 mmol/L D-glucose treatment resulted in downregulation of the expression of cardiac myosin genes (α-MHC and β-MHC) during differentiation process (Fig. 2B-C), whereas exposure to 40 mmol/L D-glucose almost diminished its expression as shown by immunofluorescence and qRT-PCR. These data

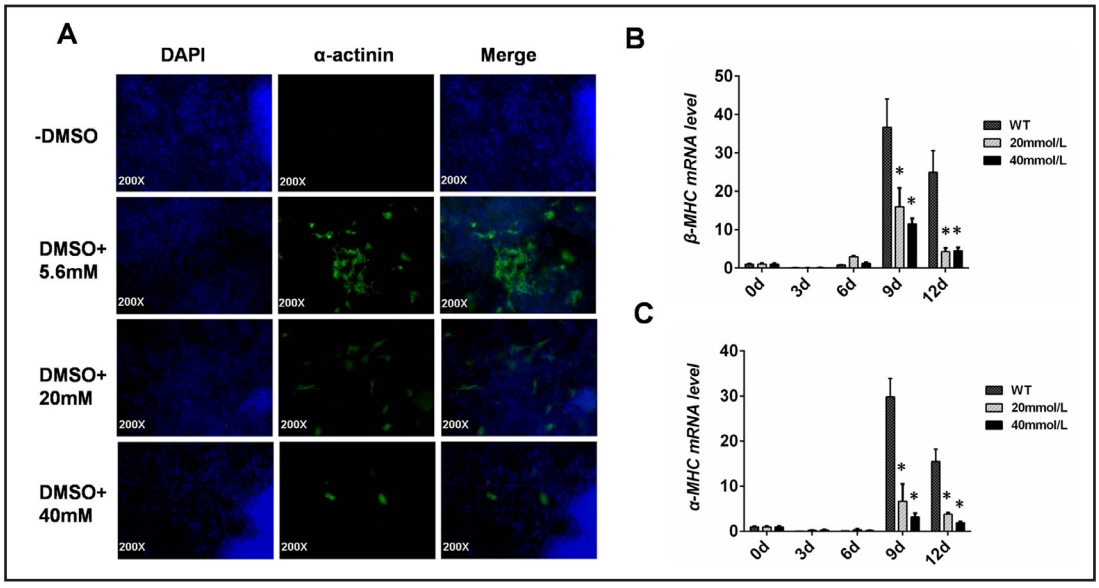

Fig. 2. High glucose inhibited P19CL6 cells differentiation into cardiomyocytes. (A) Immunofluorescence of sarcomeric α-actinin (green) on differentiated P19CL6 cells with different concentration of D-glucose at day 12 after induction, less positive cells were observed in 20 mmol/L or 40 mmol/L glucose treated cells in comparison to normal concentration (5.6 mmol/L). (B-C) qRT-PCR analysis using RNA from differentiated P19CL6 cells demonstrating significant changes in the m RNA levels of α-MHC and β-MHC when P19CL6 cells were treated with 20mmol/L or 40mmol/L glucose. (*$p < 0.05$, Statistical significant vs the WT of the same day).

indicated that long-term exposure to high level of glucose has a drastic effect on P19CL6 cells by inhibiting their differentiation.

Long-term high glucose is associated with decreased PGC-1α expression during P19CL6 differentiation

To further investigate the mechanisms underlying the effect of long-term high glucose concentration on P19CL6 differentiation, we examined the levels of mitochondrial oxidative metabolism protein PGC-1α. Compared to controls, PGC-1α protein level was profoundly reduced in differentiated P19CL6 cells treated with 20 mmol/L or 40 mmol/L D-glucose at day 12 after induction (Fig. 3A-B). Unexpectedly, exposure to 20 mmol/L D-glucose resulted in a temporary increase in mRNA level of PGC-1α at day 9 followed by a decrease in its expression at day 12 after induction. However, exposure to 40 mmol/L D-glucose induced a lower expression of PGC-1α at day 6, day 9 and day 12 after induction (Fig. 3C). Moreover, mRNA level of FAO genes such as MCAD also decreased in differentiated cells treated with 20 mmol/L D-glucose or 40 mmol/L D-glucose at day 12 after induction (Fig. 3D). These results suggest that long-term exposure to high glucose decreases expression of genes involved in the regulation of mitochondrial biogenesis and function during P19CL6 cells differentiation, which might underlie the defects associated with the switch to mitochondrial oxidative metabolism in differentiated cells.

Long-term high glucose decreased GATA4 and Nkx2.5 expression during P19CL6 differentiation

To further investigate cardiac gene expression profile following high glucose concentration exposure, qRT-PCR and Western blot analysis were performed to detect the expression of GATA4 and Nkx2-5 transcription factors. The data revealed that treatment with 20mmol/L D-glucose decreases the expression of both GATA4 and Nkx2-5 throughout day 6, 9 and 12 after induction when compared to cells treated with 5.6 mmol/L of glucose.

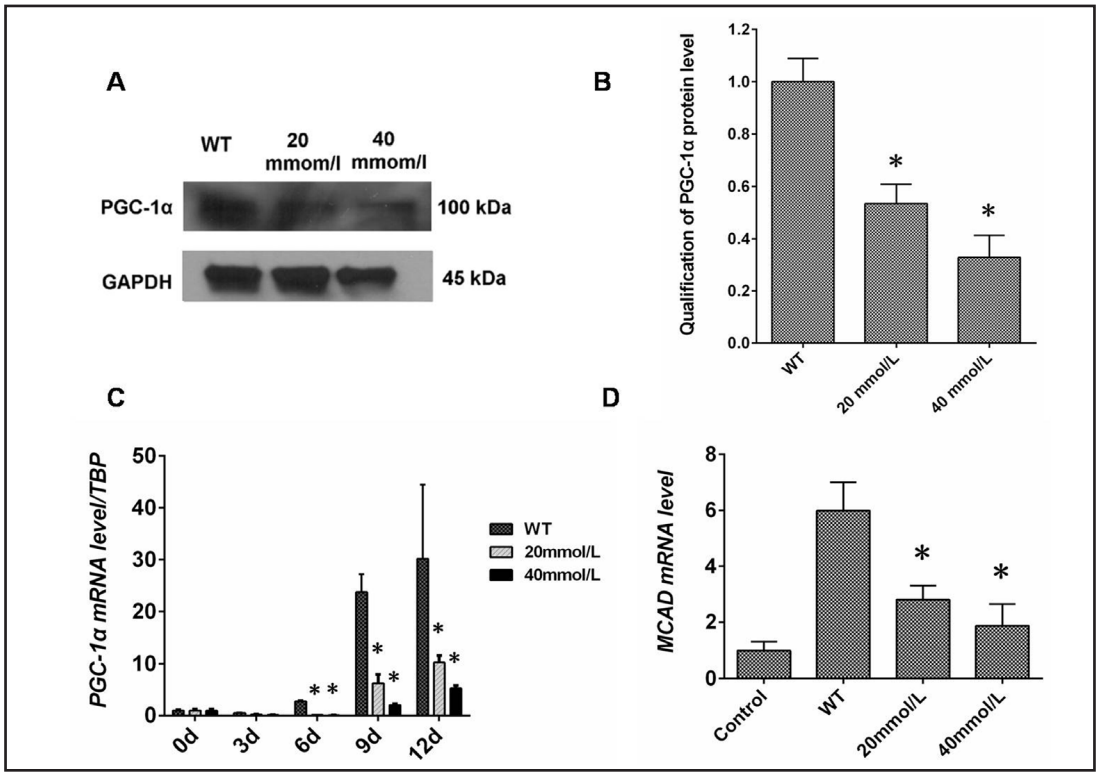

Fig. 3. High glucose decreased PGC-1α and MCAD genes expression during P19CL6 cells differentiation. (A-B) Western blot showing significant decrease in PGC-1α expression at day 12 after treatment with high D-glucose concentration following induction of differnetiation. (C) qRT-PCR analysis demonstrated significant changes in the m RNA levels of PGC-1α during P19CL6 cells differentiation. (D) qRT-PCR analysis at day 12 demonstrated significant changes in MCAD after cells where treated with high D-glucose concentration after induction. (*p < 0.05, Statistical significant vs the WT of the same day).

Exposure of the cells to 40 mmol/L D-glucose further reduces their expression (Fig. 4A-D). As show in Fig. 4E, immunofluorescence staining of GATA4 and Nkx2-5 performed at day 9 after induction also indicated lower detection of GATA4 and Nkx2-5 positive cells in P19CL6 cells treated with 20 mmol/L or 40 mmol/L D-glucose, respectively, when compared to cells treated with 5.6 mmol/L of glucose. The findings indicate that exposure to high level of glucose for a long term inhibits P19CL6 cells differentiation into cardiomyocytes by decreasing GATA4 and Nkx2-5 expressions.

Discussion

PGDM is considered as a rising problem with increasing impact on embryonic development especially that defects in the cardiovascular system development are the main defects associated with PGDM. Spontaneously beating cardiomyocyte are the first type of differentiated cells to appear during mammalian heart formation [29]. Throughout heart formation, cardiomyocytes proliferate and organize themselves to give rise to the mature four-chambered heart. Up until recently, it has been known that hyperglycemia is a primary factor affecting heart development. However, little is known about the impact of hyperglycemia on cardiac progenitors or precursors. Here we show that long-term high glucose levels inhibit cardiac differentiation of P19CL6 cells. These results, revealing that cardiac differentiation is altered in response to high glucose, provide important clues regarding the effect of hyperglycemia on cardiac progenitors or precursors.

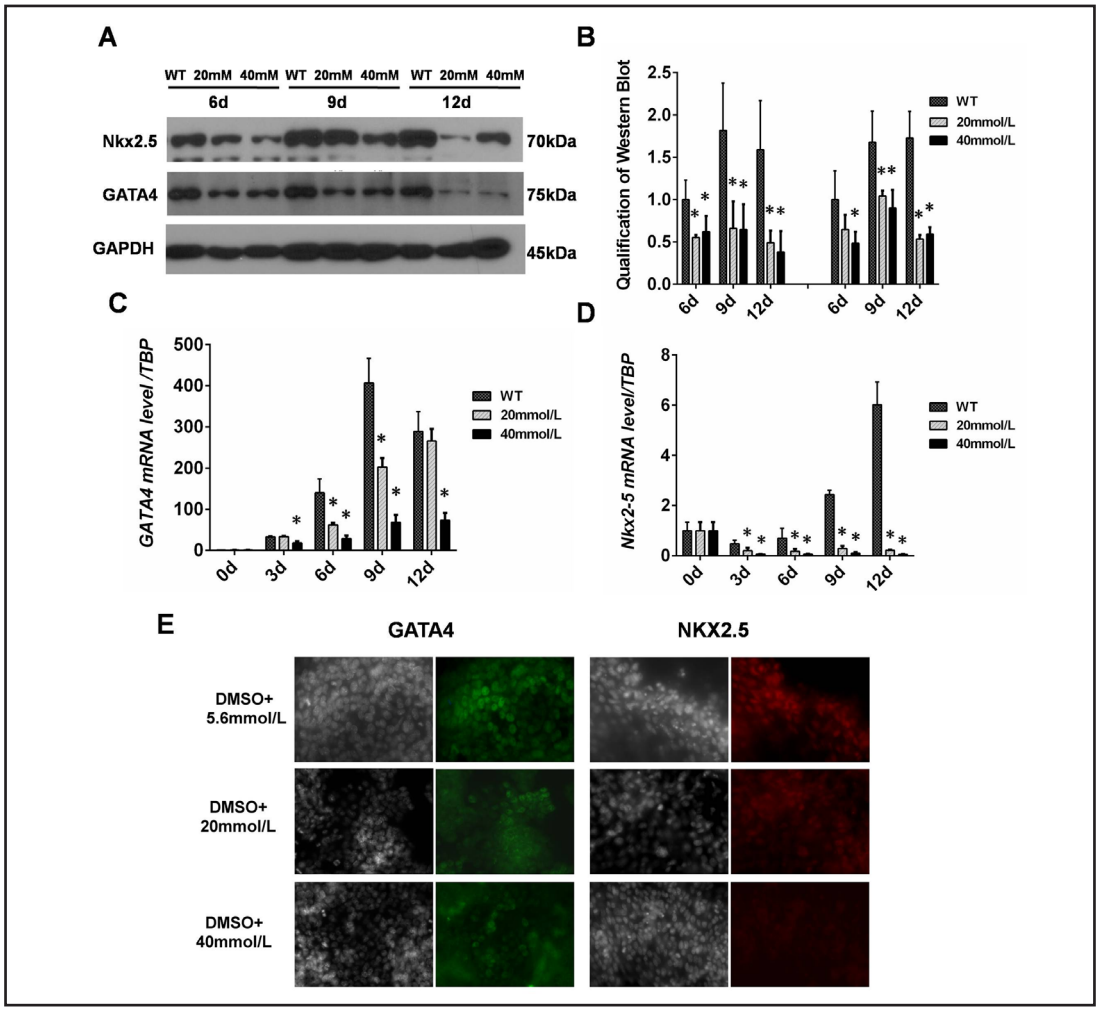

Fig. 4. High glucose decreased expression of GATA4 and Nkx2-5 expression during P19CL6 cells differentiation. (A-B) Western blot showing small protein amounts of GATA4 and Nkx2-5 in cells treated with high glucose at day 6, day 9 and day 12. (C-D) qRT-PCR analysis demonstrating a significant decrease in mRNA level of GATA4 and Nkx2-5, which were consistence with imunofluorescence and western blot findings. (E) Immunofluorescence of GATA4 and Nkx2-5 on differentiated P19CL6 cells with different concentration of D-glucose at day 9, showing less GATA4 (green) and Nkx2-5 (red) positive cells detected in cells treated with 20 mmol/L and 40 mmol/L glucose in comparison to the normal concentration (5.6 mmol/L) (*p < 0.05, Statistical significant vs the WT of the same day).

During four-chamber formation, the number of mitochondria increases along with important mitochondrial proteins in order to meet the increases in ATP consumption due to the elevation of embryonic heart output [9, 10]. Several lines of evidence indicated that PGC-1α is a key regulator of mitochondrial functional capacity in the heart, and is involved in cardiomyocyte differentiation of murine embryonic stem cells *in vitro*[12, 30-32]. Vega-Naredo et al. reported that P19 cells were able to increase the intrinsic mitochondrial membrane potential (Δψm) and respiration, reduce their proliferation and differentiation, when mitochondrial metabolism was forced using OXPHOS medium [33]. In this study, and under physiological glucose concentration, the trend of PGC-1α expression changed in accordance with changes in cardiac muscle myosin expression, suggesting that successful transition of embryonic stem cells to functional cardiomyocytes comes with an increase in mitochondrial function in differentiated cells. Gao et al. using differentiated 3T3-L1

adipocytes model showed that high level of glucose are able to induce mitochondrial dysfunction by inducing downregulation of PGC-1α [34]; On the other hand, our study showed that PGC-1α expression was sensitive to high glucose concentrations during P19CL6 cell differentiation, with alteration of genes involved in mediating fatty acid oxidation. Thus deficiency in the switch to mitochondrial oxidative metabolism is maybe associated with decreased expression of PGC-1α that occurs under high level of glucose during P19CL6 cells differentiation. Recently, several signaling pathways have been implicated in the regulation of PGC-1α promoter activation and expression in response to various stimuli [35, 36]. Irrcher et al identified three GATA sequence in PGC-1α promoter by cloning the proximal 2-kb human PGC-1α promoter, suggesting that GATA family of TF could regulate the activation of this promoter. In fact, GATA4 overexpression alone increased the activation of hPGC-1α promoter in C2C12 cells [37].

Transcription factor GATA4, expressed in early cardiac progenitor cells prior to cardiomyogenesis and then throughout adulthood, has a key role in cardiomyogenesis. The impact that hyperglycemia has on GATA4 expression was previously reported: the study elucidated that high glucose is able to induce GATA4 depletion in cardiomyocytes by increasing the degradation of GATA4 protein or by affecting the phosphorylation of GATA4, which is induced by MEK/ERK signaling [38, 39]. Interestingly, we found in our study using qRT-PCR analysis, Western blot and immunofluorescence assays that the decrease in GATA4 expression happens under high level of glucose concentration, suggesting that GATA4 is sensitive to glucose concentration stimulation both in cardiac progenitors and mature cardiomyocytes. Furthermore, exposure to high levels of glucose concentration showed trend changes in GATA4 expression similar to what was seen with cardiac myosin expression, suggesting that GATA4 is the first cardiac TF to be induced during P19CL6 cells differentiation; our findings are consistent with previously reported studies which show that absence of GATA4 in P19 cells or ES cells render them unable to form beating cardiomyocytes [40, 41].

Nkx2-5 is another important TF expressed throughout cardiac development, with high levels in embryonic differentiated cardiomyocytes [15]. *In vitro* Nkx2.5 gain and loss-of-function studies indicated that Nkx2.5 function appears to be essential for cardiomyogenesis in P19 cells by activation of myocyte enhancer factor 2C (MEF2C) [18, 42]. These results are in accordance with our findings showing a decrease in the expression of Nkx2.5 and cardiac myosin gene during the process of differentiation, after treatment with high levels of glucose. However, ectopic expression of Nkx2.5 in frog and zebrafish led to the recruitment of additional myocytes into the heart, but was not sufficient to initiate cardiac cell differentiation into functional cardiomyocytes [14, 43], suggesting that Nkx2.5 is able to determine the cardiac cell fate via acting in cooperation with other TF. Since the expression pattern of GATA4 and Nkx2.5 factors is largely overlapping in cardiomyogenesis, it is possible that these two factors might be regulating each other's expression or are physically interacting. In fact, GATA4 binding sites are indeed present in the mouse Nkx2.5 promoter and inactivation of Nkx2.5 did not affect expression of GATA-4 [44-47]. These results suggest that GATA4 may function upstream of Nkx2.5, confirming the results reported in this study: expression of GATA4 precedes Nkx2.5 expression. Furthermore, it is well-known that Nkx2.5 and GATA4 are capable of co-activating early cardiac gene promoters including cardiac actin and ANF, through binding to their adjacent DNA binding sites [48-50] .These findings could potentially explain why a decrease in expression of cardiac myosin was detected along with the decrease in GATA4 expression after exposure to high glucose concentration. Thereby, inhibition of cardiogenesis in P19CL6 cells, exposed to high levels of glucose, is associated with decreases in GATA4 and Nkx2.5 expression, which eventually results in the down-regulation of cardiac myosin and other TF such as PGC-1α.

In conclusion, high level of glucose is able to inhibit P19CL6 cell differentiation via targeting the expression of genes involved in mitochondrial oxidative metabolism; the mechanism is mediated via the regulation of GATA4 and Nkx2.5 expressions. These findings represent a novel mechanism by which glucose concentration affects cell differentiation and shed more light on the effect of hyperglycemia on heart development.

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Disclosure Statement

The authors declare that they have no competing interests.

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