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Original Article

Enhancement of early cardiac differentiation of dedifferentiated fat cells by dimethyloxalylglycine via notch signaling pathway

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Abstract: *Background:* Hypoxia has been reported to possess the ability to induce mature lipid-filled adipocytes to differentiate into fibroblast-like multipotent dedifferentiated fat (DFAT) cells and stem cells such as iPSCs (interstitial pluripotent stem cells) and ESCs (embryonic stem cells) and then to differentiate into cardiomyocytes. However, the effect of hypoxia on cardiac differentiation of DFAT cells and its underlying molecular mechanism remains to be investigated. *Objective:* To investigate the role of hypoxia in early cardiac differentiation of DFAT cells and the underlying molecular mechanism. *Methods:* DFAT cells were prepared from 4 to 6 week-age mice and cultured under hypoxic conditions by adding Prolyl hydroxylase inhibitor and dimethyloxalylglycine (DMOG) into the culture media. To inhibit or block Notch signaling, γ-secretase inhibitor-II (GSI-II) and Notch1 siRNA (si-Notch1) were used. DFAT cell viability was detected using MTT assay. qRT-PCR, immunofluorescence microscopy and western blotting were used to evaluate the cardiac differentiation of DFAT cells and co-immunoprecipitation was used to study the interaction between HIF-1α and Notch signaling. *Results:* 0.6-mM DMOG failed to affect the viability of DFAT cells, but stimulated the cells to express early cardiac transcription factors including Islet1, Nkx2.5 and Gata4 in a time-dependent manner and increase the number of $cTnT^+$ cardiomyocytes (detected at the 28^{th} day after stimulation). It was also demonstrated that DMOG was involved in HIF-1α and Notch signaling as well as HIF-1α-NICD complex formation. *Conclusion:* Hypoxia enhanced early cardiac differentiation of DFAT cells through HIF-1α and Notch signaling pathway.

Keywords: Dedifferentiated fat cell, hypoxia, notch signaling pathway

Introduction

Although therapies including both invasive (eg. primary percutaneous coronary intervention) and noninvasive (eg. new antithrombotic medicines) techniques have been applied to patients with ischemic heart disease, a substantial number of them have little benefit from these approaches. Recent therapeutic strategies for chronic ischemic cardiac disease and heart failure have been partially focused on myocardial regeneration therapy [1] and a variety of stem and progenitor cells have been employed in cardiac regeneration [2]. However, these cells have failed to show great promise in cardiac regeneration therapy.

Recent studies have demonstrated that dedifferentiated fat (DFAT) cells are a homogeneous group of multipotent cells similar to mesenchymal stem cells [3]. It has been reported that DFAT cells could be dedifferentiated into cardiomyocyte-like cells [4]. DFAT cells could be produced from lipid-filled mature adipocytes through traditional ceiling culture method, a hypoxic condition [5, 6]. Since DFAT cells are easily isolated and induced, have rich source and homogeneity, they show great promise in being differentiated into cardiac regeneration cells, although the molecular mechanisms underlying that are yet to be investigated. The ceiling culture technique induced mature adipocyte dedifferentiation that are involved in lipid

metabolism [7]. Therefore, we hypothesized that the mature adipocyte dedifferentiation may be initiated by hypoxia-induced lipid metabolism.

Hypoxia plays an important role in fetal heart development and *in-vitro* cardiac differentiation of pluripotent stem cells. Hypoxia was recognized as a cause of cardiac malformations, including myocardial and valvular hypoplasia [8, 9]. Moreover, hypoxia was also reported to promote the differentiation of MSCs into CMCs in myocardial medium or in a CMC and MSC coculture system [10, 11]. Exogenous expression of HIF-1α promoted cardiac differentiation of ESCs [12] and MSCs [13], whereas cultured ESCs lost the ability to express HIF-1α and failed to form embryoid bodies (EBs) [14]. It has also been reported that hypoxia impaired cardiomyocyte differentiation of iPSCs [15]. However, the relationship between HIF-1α and cardiac differentiation is not yet fully understood. The hypoxia-inducible factor (HIF) prolyl hydroxylase enzymes, termed as prolyl hydroxylase domain (PHD), play important roles in oxygen regulation [16]. The prolyl hydroxylase inhibitor, dimethyloxalylglycine (DMOG) has been used to produce hypoxic conditions through inhibiting the activity or expression of PHD and stabilizing HIF enzyme family [17].

The mutations of several members of the Notch signaling pathway have been implicated in the pathogenesis of various human cardiovascular diseases. It has been suggested that Notch signaling pathway played important roles in cardiac morphogenesis [18, 19]. Although Notch signaling pathway was reported to play multiple roles in diverse stages of heart development [20-22], its role in cardiac differentiation remained controversial. Previous studies have demonstrated that Notch signaling activation could promote cardiac differentiation [23-25]. Based on a previous report that HIF-1α could directly interact with the Notch signaling intracellular domain (NICD) [26], we hypothesized that Notch signaling pathway was involved in modulating cardiac differentiation in response to hypoxia.

In this study, we investigated the effect of hypoxia on early cardiac differentiation of dedifferentiated fat (DFAT) cells and its underlying molecular mechanism.

Methods

Preparation and treatment of DFAT cells

The preparation of DFAT cells from adipose tissue was performed as described previously by Sugihara *et al.* [5]. Briefly, lipid-filled mature adipocytes were obtained from the inguinal fat pads of 4-6-week-old female Sprague-Dawley rats (Guiyang Medical University, China). The inguinal fat pads were completely digested and thoroughly pipetted to avoid contamination with preadipocytes, fibroblasts and/or stromal-vascular cells in order to obtain pure unilocular adipocytes [27, 28]. Briefly, approximately 2.0 g of fat tissue was washed repeatedly with phosphate-buffered saline (PBS) until the wash solution was clear, followed by mincing and digesting using 0.1% (w/v) collagenase type I (1 mg/ml, Invitrogen, Carlsbad, CA, USA) at 37°C for 45 min with gentle agitation. The cell suspension was filtered through 250-μm nylon meshes to remove the debris but allow cells to pass through. After centrifugation at 220 g for 5 min, the floating unilocular mature adipocytes were collected. Subsequently, the mature adipocytes were induced into DFAT cells using ceiling culture technique reported by Sugihara [5]. Briefly, the cells (5×10^4 cells) were transferred into 25 cm^2 culture flasks filled with fresh complete Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (HyClone, USA) and 0.5% antibiotic-antimycotic solution (Sigma, USA). The cells floated up and adhered to the ceiling of the flask. After culturing for 7 to 10 days, the cells could be transformed into fibroblast-like DFAT cells. Subsequently, the medium was removed and the flasks inverted so the remaining cells adhered to the bottom. The medium was changed every 3 to 4 days. The cells were subcultured at a ratio of 1:2 or 1:3 when they reached 80% confluence. DFAT cells from P2 or P3 were used in this study.

To induce cardiac differentiation, DFAT cells (1×10^4 cells/well) were seeded in 24-well plates and cultured with fresh complete medium supplemented with DMOG (Frontier Scientific, Logan, UT). DMOG concentrations and treatment time are shown in **Figures 2A** and **3A**. γ-secretase inhibitor-II (GSI-II) (catalog no. 565755; Calbiochem, San Diego) (20 μM) was added to the medium to inhibit Notch signaling

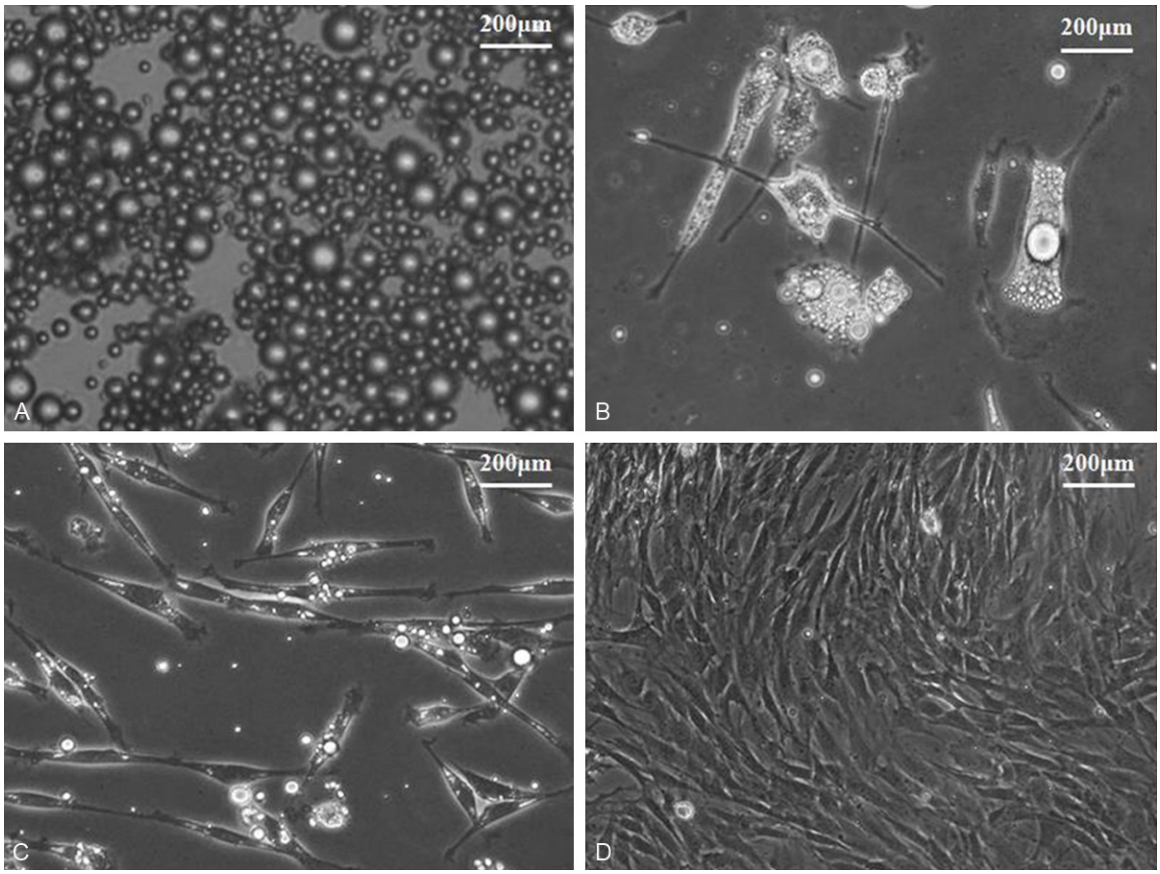

Figure 1. Morphology of mature adipocytes and DFAT cells in vitro. The floating top layer containing unilocular adipocytes was collected and incubated in culture flasks that were completely filled with medium. The flasks were then turned upside down. A. Representative image of mature adipocytes after cell separation. Mature adipocytes were filled with single large lipid droplets and floated on the medium. (Scale bar: 200 μm). B, C. Representative images of mature adipocyte transformation. Mature adipocytes adhered to the flask ceiling 4 days after culture and single large lipid droplet formed in some. Then they gradually poured out the intracellular small lipid droplets and transformed into fibroblast-like morphology. D. Representative image of DFAT cells. Most of the adipocytes became fibroblast-like and began to proliferate after 10 days of ceiling culture.

pathways for 24 h. GSI-II concentration was selected based on a previous study by Jiho Jang et al., [23]. Notch1 siRNA (si-Notch1: sense: GGACCUCAUCAACUCA-CAUTT; antisense: AUGUGAGUUGAUGAGGUCCTT) duplexes were synthesized by Shanghai Sangon Biotech Co., Ltd. Transfection of siRNA was performed using the lipofection method as recommended (Promega). After transfection for 24 h, the cells were cultured for 3 or 28 days until they were harvested to be used for further assays.

MTT reduction assay

To perform MTT assay, exponentially growing DFAT cells were plated onto 96-well plates (5000 cells in 100 ml medium containing 20% dialyzed fetal bovine serum per well). The next day, fresh medium containing different concentrations (0.2 to 2.0 mM) of DMOG (**Figure 1B**) was added, followed by culture for 48 h. Subsequently, 10 ml MTT solution (5 mg/ml) was added into each well and the cells were incubated for an additional 4 h. Thereafter, 100 ml solution containing 20% SDS and 50% dimethyl formamide (pH 4.8) was added into each well followed by incubation overnight and then the absorption was read at 570 nm.

Immunofluorescence assay

The cells cultured on coverslips were fixed in 4% paraformaldehyde for 10 min at room temperature. After rinsing with PBS, the cells were permeabilized with PBS containing 0.3% Triton-X 100 (Sigma, USA) for 15 min, then blocked with PBS containing 10% goat serum and 1% BSA for 30 min. Next the cells were

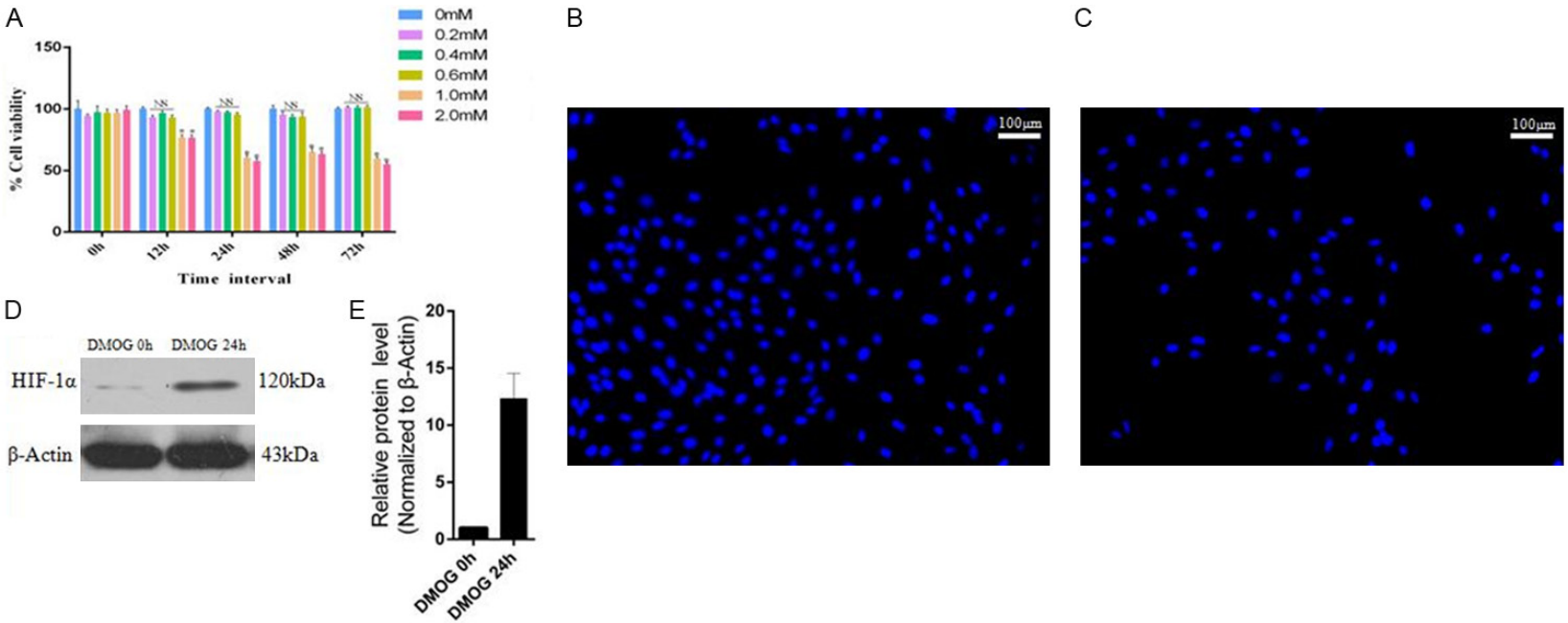

Figure 2. The effect of DMOG on cell viability of DFAT cells. (A) Representative MTT assay to measure cell viability in DFAT cells following treatment with different concentrations of DMOG (0 to 2.0 mM) at 0, 12, 24, 48 and 72 hrs; 0 mM: control. The results indicated that there was no statistically significant effect on cell viability following treatment with DMOG at 0.2 to 0.6 mM concentrations, whereas DMOG at 1 to 2 mM concentration reduced cell viability by approximately 25% compared to control. The OD 570 values are represented as % cell viability and the OD of the untreated cells (0 mM) is taken as 100% viability for each time point. Values marked with asterisk are statistically significant (*P<0.05) compared to control; NS means no significant difference (P>0.05) compared to control. Data are mean ± SD of three independent experiments. (B, C) Representative graph showing cell viability as measured using 4',6-diamidino-2-phenylindole (DAPI) staining following treatment with DMOG at 0.6 mM (B) and 1 mM (C) for 24 hrs. No condensed and fragmented nuclei in DFAT cells was observed after treatment with 1 mM DMOG for 24 hrs, wheresas the cell number was significantly reduced compared to treatment with 0.6 mM DMOG. Figures were selected as representative data from three independent experiments. Scale bar: 100 μm. (D, E) Representative Western blots of HIF-1α protein in DFAT cells incubated with or without DMOG (0.6 mM) for 24 hrs. DMOG markedly increased HIF-1α protein levels. β-Actin was used as a loading control (D). Fold difference in HIF-1α protein expression by densitometry analysis of HIF-1α in DFAT cells lysates normalized to β-actin content. HIF-1a protein expression was significantly up-regulated compared with those incubated without DMOG. Densitometry analysis is representative of three Western blot experiments. *P<0.05, as compared to control (E).

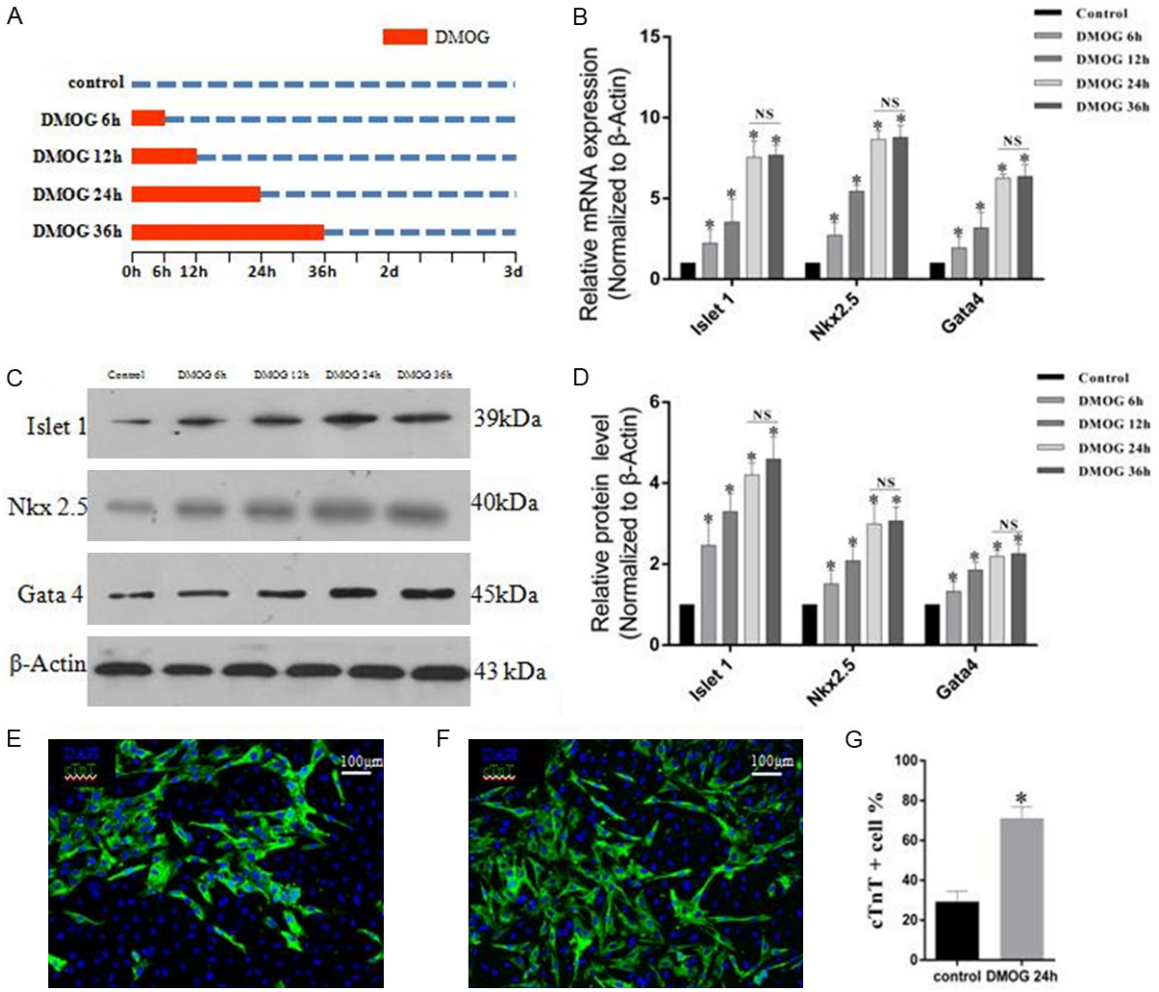

Figure 3. Effect of DMOG on cardiomyocyte differentiation of DFAT cells. (A) The effects of different durations of stimulation of DFAT cells by DMOG. DFAT cells were treated with 0.6 mM DMOG for different time periods as shown in the schematic diagram. The early cardiac transcription factors expression was detected on day 3. (B) Real-time RT-PCR was performed to evaluate the changes in genes expression of early cardiac transcription factors Islet1, Nkx2.5 and Gata4 following treatment with 0.6 mM DMOG for 6, 12, 24, or 36 hrs. Total RNA was isolated on day 3 and β-Actin was used as an internal control. DMOG-treatment increased the genes expression levels of early cardiac transcription factors Islet1, Nkx2.5 and Gata4 in a time-dependent manner and reached a peak at 24 hrs. The data is representative of three independent experiments. Each bar represents mean ± SD from three experiments (*P<0.05, vs. the control group). (C, D) Representative Western blots demonstrating the expression profile of early cardiac transcription factors Islet1, Nkx2.5 and Gata4 following treatment with 0.6 mM DMOG for 6, 12, 24, or 36 hrs and total protein was extracted from DFAT cells on day 3 (C). Fold difference in early cardiac transcription factors (Islet1, Nkx2.5 and Gata4) expression by densitometry analysis in DFAT cells lysates normalized to β-actin content (D). Relative protein expression levels were up-regulated in a time-dependent manner and reached a peak around 24 hrs (protein relative to β-actin and normalized to the control group). The data is representative of three independent experiments. Each bar represents mean ± SD from three samples (*P<0.05, vs. the control group). (E, F) Immunofluorescence staining showing the cTnT$^+$ cardiomyocytes (green) with (F) or without (E) DMOG for 24 hrs on day 28. Scale bars = 100 μm. Nuclei were counterstained with DAPI (blue). (G) Quantification of the cTnT$^+$ cardiomyocytes. Data were quantified from five random fields in two assays. Bar represents mean±SD (*P<0.05, vs. incubated without DMOG group). DMOG significantly increased the number of cTnT$^+$ cardiomyocytes.

incubated with the appropriate primary antibodies or nonspecific IgG control antibodies overnight at 4°C. On the next day, the cells were rinsed with PBS and incubated with secondary FITC-conjugated goat anti-mouse or anti-rabbit IgG (1:200) for 30 min at room temperature. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma, USA). To quantitatively evaluate the differentiation efficiency, the fluorescence was visualized by fluorescence

Table 1. Primers used in real-time PCR

Primers	Primer sequences (5'-3')	Product size (bp)
Nkx2.5	F: CACCCACGCCTTTCTCAGTC	361
	R: CCATCCGTCTCGGCTTTGT	
Gata4	F: AAGGTACCTGGACTTTGCCTGTTGGGG	230
	R: CTAAGCTTACTGCGGCTGAGCCTCGG	
β-actin	F: TTTAATGTCACGCACGATTC	184
	R: ACTCCTATGTAGGTGACGAGGC	
Islet1	F: CTGCTTTTCAGCAACTGGTCA	123
	R: TAGGACTGGCTACCATGCTGT	

microscopy and the positive-stained cells were counted. No less than five random image fields of each coverslip were used to count the positively-stained cells. The percentage of positively-stained cells represented the differentiation efficiency.

Western blotting and co-immunoprecipitation assays

The cells were harvested and washed twice with ice-cold PBS and then incubated in lysis buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% [vol/vol] Nonidet P-40 and protease inhibitor mixture) for 10 min on ice. The lysates were then centrifuged to remove the debris at 14,000 g for 30 min at 4°C. The total protein concentration in the supernatant was determined using the BCA protein assay kit (Pierce). 30-μg total protein was loaded per well in 10%-12% SDS-PAGE gel, followed by electrophoresis. The protein bands were blotted onto polyvinylidene difluoride (PVDF) membranes using a transfer unit (Bio-Rad Inc., USA). After washing with 0.1% TBS-T, the membranes were incubated in blocking buffer (5% skimmed milk in TBS-T) for 1 h at room temperature and then incubated with appropriate antibodies (GATA-4:sc-25310, Santa Cruz, CA; Islet-1:sc-390793, Santa Cruz, CA; NICD-1:ab8925, Abcam, Hong Kong, China; Nkx-2.5:sc-8697, SantaCruz, CA; HIF-1α:NB100-105, Novus, USA; Lamin B1:ab-8982, Abcam, Hong Kong, China; β-tubulin: ab7792, Abcam, Hong Kong, China; β-Actin: sc-130656, Santa Cruz, CA.) overnight at 4°C. After washing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz, CA) for 2 h at room temperature. After further washing with TBS-T, the membranes were incubated in ECL plus reagent for 5 min and the signals were visualized using Hyper Performance Chemiluminescence film.

To perform co-immunoprecipitation experiments, total cell lysates and nuclear lysates from DFAT cells following treatment with 0.6 mM DMOG, 0.6 mM DMOG+20 μM GSI or siRNA transfection for 24 h, were incubated with anti-HIF-1α antibody (NB100-105, Novus USA) or normal IgG (sc-2762, Santa Cruz, CA) for 2 h with gentle shaking, and then with 50 ml of protein G-sepharose slurry (Santa Cruz, CA) at 48°C for 1 h. Immunoprecipitates were washed three times with wash buffer and subjected to SDS-PAGE electrophoresis and then detected with anti-NICD-1 antibody (ab8925, Abcam, Hong Kong, China).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen, USA) and isolated according to the manufacturer's protocol. The RNA pellets were dissolved in diethylpyrocarbonate (DEPC)-treated H_2O. The purity of the RNA was confirmed by the 260/280 nm absorbance ratio (>1.8). 3 μg total RNA per sample was transcribed into first-strand cDNA employing an appropriate kit (Promega, USA) and oligo-d (T) 18 primers, in accordance with the protocol recommended by the manufacturer. The primers were designed on the basis of the complete cDNA sequences deposited in the GenBank (see **Table 1**). qRT-PCR was carried out using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, USA) in accordance with the manufacture's protocol and analyzed using GeneAmp 7300 SDS software. In brief, 20-μl solution containing 2-μl first-strand cDNA, 2 X QPCR SYBR® Green Mix and 1 mM each of forward and reverse primers was subjected to thermal cycling as follows: pre-incubation at 95°C for 10 min, then 40 cycles of denaturation at 95°C for 30 seconds; primer annealing at 52-60°C for 30 seconds and extension step at 72°C for 1 min. Finally, a post extension step was performed at 72°C for 7 min. Both RT-PCR and melting curve analysis were routinely performed to check the specificity of this PCR procedure. The C*t* value of a target mRNA was normalized against the C*t* value of GAPDH ($\Delta Ct = Ct_{target}-Ct_{GAPDH}$). C*t* was the threshold cycle of quantitative PCR (qPCR) defined by ABI PRISM

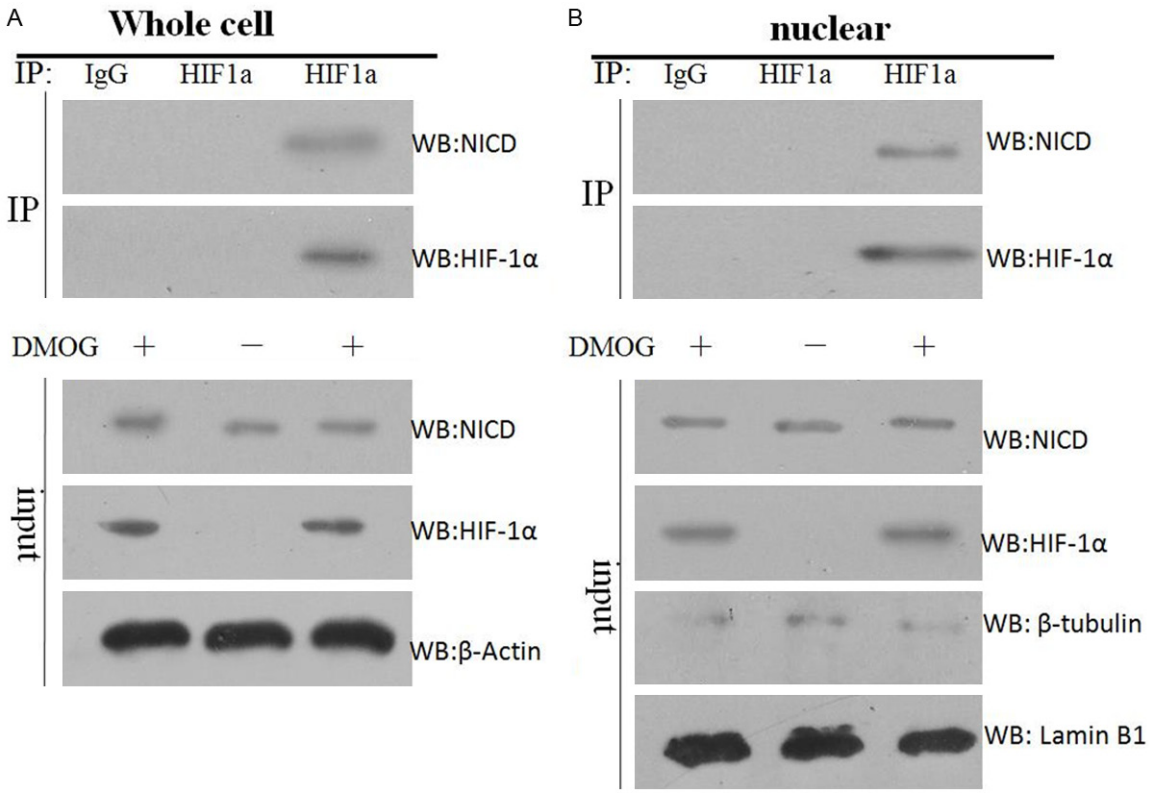

Figure 4. DMOG induced HIF-1α-NICD complex formation and translation into nucleus. Co-immunoprecipitation was performed with total cell lysates (A) and nuclear lysates (B) from DFAT cells following incubation with or without 0.6 mM DMOG for 24 hrs, precipitated with anti-HIF-1α antibody (IP) or normal IgG as a control antibody and then detected by anti-NICD-1. Anti-β-actin monoclonal antibody was used as loading control. Laminin B1 and β-tubulin were used to assess unclear integrity and purity, respectively. NICD-1 protein was co-precipitated with HIF-1 ± in total cell lysates and nuclear lysates. The result is representative of three independent experiments.

7300 software. The relative mRNA level of a target gene was obtained by $2^{-\Delta\Delta Ct}$; $\Delta\Delta Ct = \Delta Ct_{\text{treated value}} - Ct_{\text{untreated value}}$.

Statistical analysis

The data are expressed as mean ± standard deviation (SD), with all experiments being performed in triplicate. Data were analyzed by analysis of variance (ANOVA), followed by post-tests using the Bonferroni method, with $P<0.05$ being considered significant. Statistical analysis was performed using SPSS10.0 software.

Results

Induction of mature adipose cells into DFAT cells by ceiling culture

During the first 2 days, the lipid-filled unilocular mature adipocytes (**Figure 1A**) with strong light refraction floated to the top of the flask and gradually adhered to the top inner surface. Approximately on the 3rd day, the cytoplasmic rims of some mature adipocytes began to stretch out protuberances. Thereafter, the protuberances became more significant and a single large lipid droplet divided into some small ones, the cells became flat and multilocular (**Figure 1B**). In the next few days, the multilocular cells expand to the outside of the lipid droplets and gradually become fibroblast-like cells (**Figure 1C**). Approximately after 10 days, the cells discharged all lipid droplets and became fibroblast-like cells without lipid droplets, namely DFAT cells. Thereafter, the DFAT cells possessed proliferative phenotype and entered a logarithmic proliferative phase and formed a clone (**Figure 1D**).

Effect of different concentrations of DMOG on cell viability and HIF-1α expression in DFAT cells

To determine the possible cytotoxic effects of DMOG, MTT assay was performed to measure

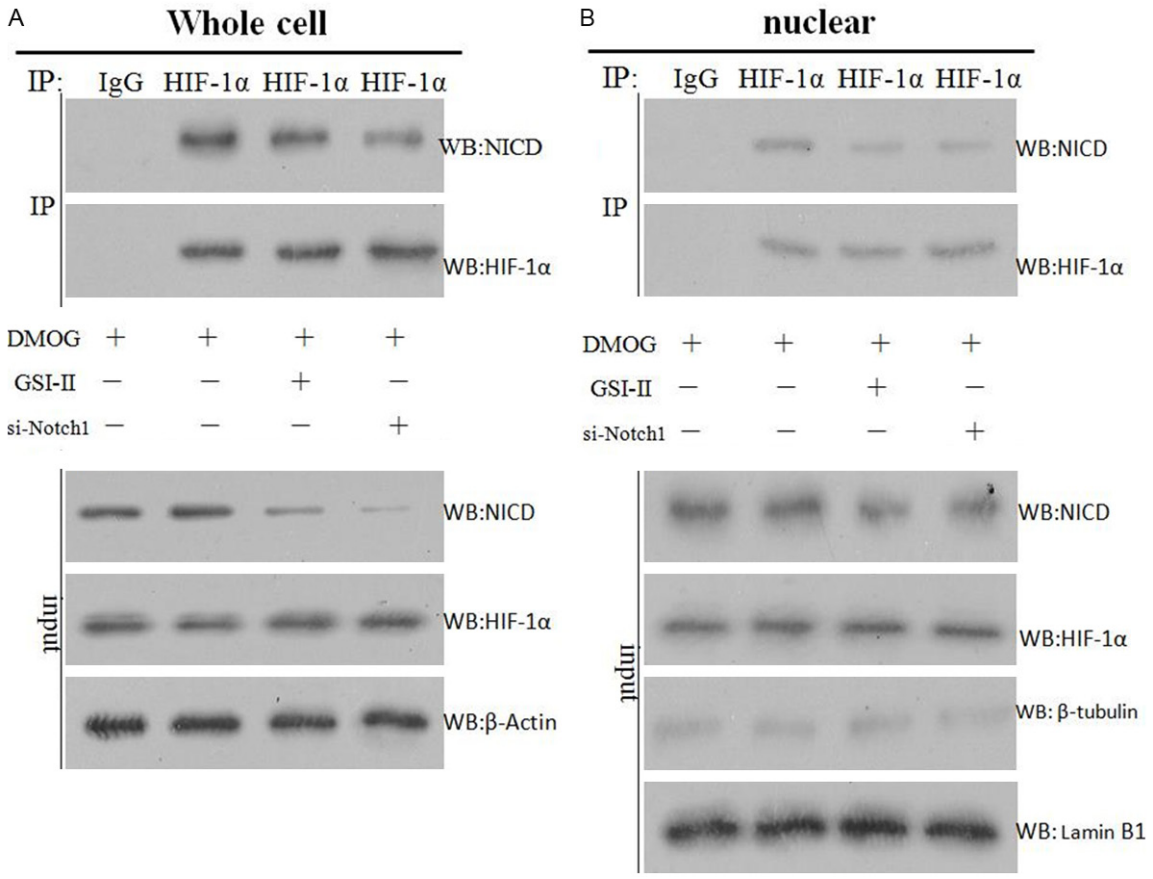

Figure 5. Blocking Notch signaling pathways inhibits DMOG induced formation of HIF-1α-NICD complex. Co-immunoprecipitation demonstrated the changes of HIF-1α-NICD complex formation in response to treatment with γ-secretase inhibitor-II (GSI-II) and transient transfection with Notch1 siRNA (si-Notch1) in DMOG treated DFAT cells. Total cell lysates (A) and nuclear lysates (B) from DFAT cells after treatment with DMOG or DMOG+GSI-II or DMOG+si-Notch1 were prepared for co-immunoprecipitation. The formation of HIF-1α-NICD complex could be inhibited by GSI-II and si-Notch1. The result is representative of three independent experiments.

the viability of DFAT cells treated with different concentrations (from 0 to 2.0 mM) of DMOG at 0, 12, 24, 48 and 72 h. The results indicated that 1 to 2 mM DMOG reduced cell viability by approximately 25% compared to control, whereas no statistically significant difference was demonstrated between 0.2 to 0.6 mM doses (**Figure 2A**). Subsequently, DAPI assay was also performed to confirm if 1 mM or higher concentration of DMOG reduced the viability of DFAT cells by apoptosis, as shown in **Figure 2B** and **2C**. No condensed, fragmented or crescent-shaped nucleus was observed in the cells treated with 1 mM DMOG for 24 hrs, whereas the cell number was significantly reduced compared to the cells treated with 0.6 mM DMOG. These results suggest that 1 mM or higher concentration of DMOG showed no toxic effect on DFAT cells, but inhibited their proliferation. As expected, the protein expression of HIF-1α was increased in DFAT cells treated with 0.6 mM 2-oxoglutarate analog DMOG for 24 h (**Figure 2D** and **2E**). This result demonstrated that HIF-1α protein was highly stabilized by DMOG.

DMOG promoted cardiac differentiation of DFAT cells

To elucidate the effect of DMOG on cardiomyocyte differentiation of DFAT cells, the cells were treated with 0.6 mM DMOG for 6, 12, 24 or 36 h (**Figure 3A**). Early cardiac transcription factors were detected using Western blotting and qRT-PCR on the 3rd day. The results indicated that the protein and mRNA expression levels of early cardiac transcription factors Islet1, Nkx2.5 and Gata4 were increased by 0.6 mM DMOG in a time-dependent manner and reached a peak at 24 hours (**Figure 3B-D**). On the 28th day, cTnT^{+} cardiomyocyte-like cells could be detected by immunofluorescence

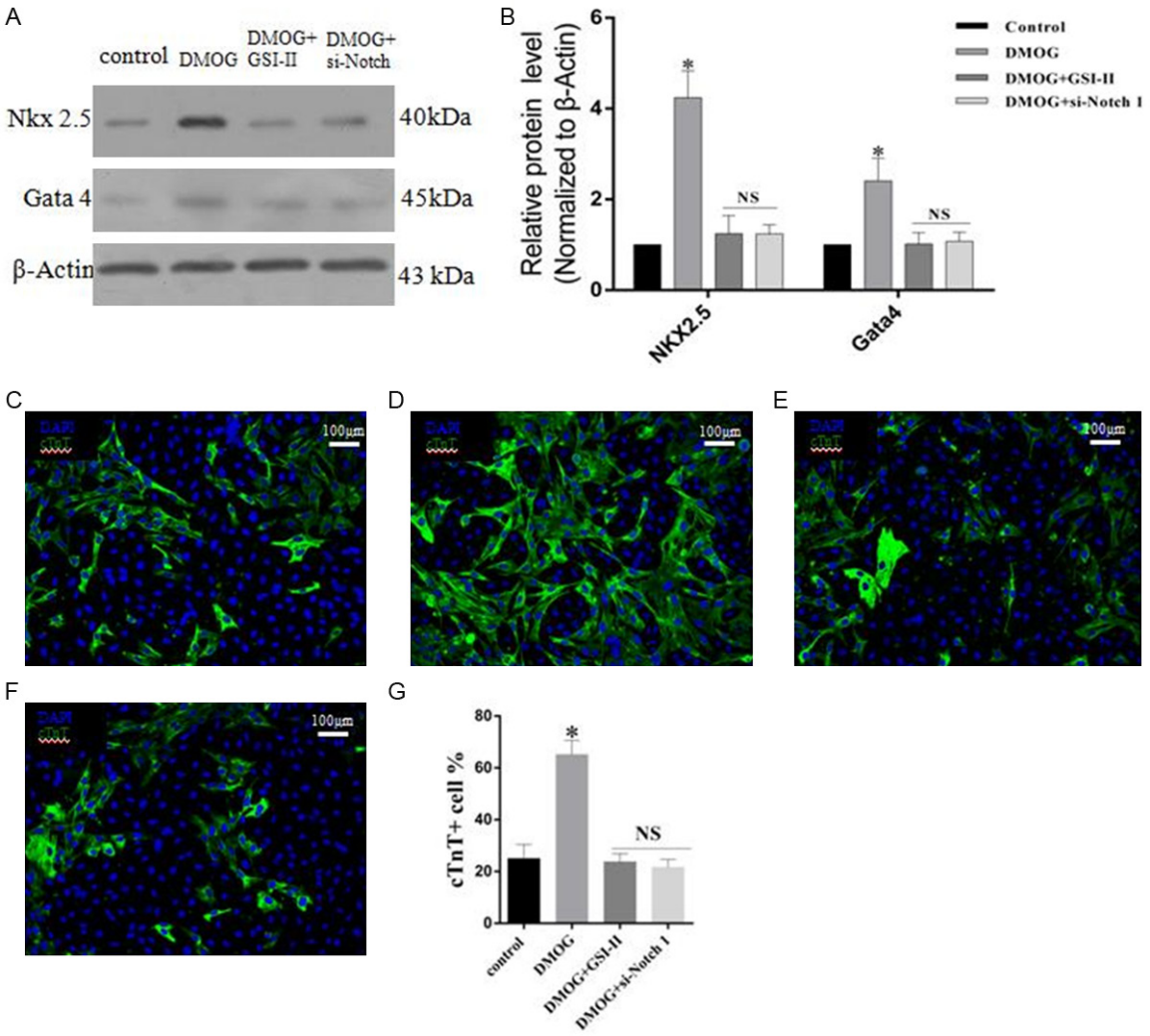

Figure 6. Inhibition of HIF-1α-NICD complex formation abolished DMOG induced cardiac differentiation of DFAT cells. A. The protein expression changes of early cardiac transcription factors Nkx2.5 and Gata4 were analyzed by western blotting in response to treatment with DMOG or DMOG+GSI-II or DMOG si-Notch1 on day 3 with untreated DFAT cells as control and β-Actin was used as an internal control. The densitometry analysis was performed with images of three independent experiments. B. GSI-II and si-Notch1 abolished DMOG induced cardiac differentiation of DFAT cells. Each bar represents mean ± SD from three samples (*P<0.05, vs. control group). C-F. Immunofluorescence staining was performed to demonstrated the cTnT$^+$ cardiomyocytes (green) on day 28 following treatment with DMOG or DMOG+GSI-II or DMOG+si-Notch1 with untreated DFAT cells as control. Scale bars = 100 μm. Nuclei were counterstained with DAPI (blue). G. Quantification of the cTnT$^+$ cardiomyocytes. Data were quantified from five random fields in two assays. Bar represents mean ± SD (*P<0.05, vs. control group). GSI-II and si-Notch1 reduced the number of cTnT$^+$ cardiomyocytes which were increased by DMOG.

staining. The number of cTnT$^+$ cardiomyocyte-like cells were significantly increased by a 0.6 mM dose of DMOG for 24 h but not by 20% FBS (**Figure 3E-G**).

DMOG stimulated cardiac differentiation of DFAT cells through the formation of HIF-1α-NICD-1 complex

To understand the molecular mechanisms underlying the cardiac differentiation induced by DMOG, confocal immunofluorescence microscopy was used to examine the interaction of HIF-1α with NICD-1. HIF-1α protein in total cell lysates and nuclear lysates of DFAT cells treated with or without 0.6 mM DMOG for 24 h were precipitated with anti-HIF-1α antibody and then immunoblotted with anti-NICD-1 antibody. It was found that NICD-1 protein was co-precipitated with HIF-1α protein in both total cell lysates and nuclear lysates (**Figure 4A** and **4B**). To further clarify this phenomenon, we used

γ-secretase inhibitor-II (GSI-II) and transient transfection with Notch1 siRNA (si-Notch1) to block the Notch signaling pathways. Co-immunoprecipitation was performed after DFAT cells were treated with 0.6 mM DMOG with or without 20 μM GSI-II or si-Notch1 for 24h. Our results revealed that the formation of HIF-1α-NICD complex was inhibited by GSI-II and si-Notch1 (**Figure 5A** and **5B**). We also examined the early cardiac transcription factors Nkx2.5 and Gata4 on day 3 and the number of $cTnT^+$ cardiomyocyte-like cells on the 28^{th} day following treatment with GSI-II and si-Notch1. The results indicated that Notch signaling pathways inhibitor quelled the upregulation of the early cardiac transcription factors and decreased the number of $cTnT^+$ cardiomyocyte-like cells induced by DMOG (**Figure 6A-G**). Taken together, our results demonstrate that DMOG promotes early cardiac differentiation through the formation of HIF-1α-NICD-1 complex in DFAT cells.

Discussion

Ceiling culture stimulated mature adipocytes to differentiate into DFAT cell by altering lipid metabolism, cell proliferation and differentiation-related genes, via changing morphological and biological properties [7, 29]. Hypoxia has been found to play a significant role in this pathway. DFAT cells have similar gene expression, DNA methylation and cell-surface antigen levels as BM-derived MSCs [references]. When cultured to 60 passages, DFAT cells retained high viability and the ability to differentiate into different cell types [3, 29]. Thus, DFAT cells were considered to be an attractive source for cell-based cardiac regeneration.

Hypoxia and HIF1α play a vital role in mammalian cardiac development by regulating the morphogenesis of the developing heart as well as the development of myocardium [30]. However, the role of hypoxia and HIF1α in stem cell cardiac differentiation has been controversial and thus poorly understood. Hypoxia has been reported to either promote [11-15] or inhibit [16] stem cell cardiac differentiation. In this study, we activated the HIF-1 pathway using prolyl-hydroxylase inhibitor Dimethyloxalylglycine (DMOG) and the results showed that both early cardiac transcription factors (Islet1, Nkx2.5 and Gata4) and cardiac specific structural protein $cTnT^+$ were enhanced. Our study also indicated that hypoxia promoted cardiac differentiation of DFAT cells.

Notch signaling pathway participated in the regulation of almost every aspect of cardiomyocyte differentiation and heart development. The effect of Notch signaling pathway on stem cell cardiac differentiation has been also controversial [24-26] and it may play a stage-specific biphasic role in cardiac differentiation [21, 31]. Notch signaling pathway was reported to interact with hypoxia to retain the myogenic cell line C2C12, satellite cells and neural stem cells in an undifferentiated state [26]. The present study found that hypoxia promoted the formation of NICD-1-HIF-1α complex, induced by the HIF-1α overexpression, in early cardiac differentiation but this process could be blocked by a Notch signaling pathway inhibitor.

Taken together, our study implicated that both HIF-1 α and Notch signaling played potential roles in hypoxia-induced DFAT cell cardiac differentiation. However, the exact molecular mechanism underlying the hypoxia-induced DFAT cell cardiac differentiation has yet to be fully investigation.

Disclosure of conflict of interest

None.

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