Metabolic shift and the effect of mitochondrial respiration in osteogenic differentiation of dental pulp stem cells (#79009)

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Metabolic shift and the effect of mitochondrial respiration in osteogenic differentiation of dental pulp stem cells

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Background.

Metabolism shifts from glycolysis to mitochondrial oxidative phosphorylation is important during differentiation of stem cells. Mitochondria function as directors in cell differentiation rather than bystanders. However, metabolic shift and the effect of mitochondria regulating differentiation of human dental pulp stem cells (hDPSCs) remain unclear.

Methods.

Human dental pulp stem cells were collected from 5 healthy donors. Cell differentiation was induced by osteogenic mineralized medium. The activities of alkaline phosphatase, hexokinase, pyruvate kinase, and lactate dehydrogenase were measured by enzyme activity kits. The extracellular acidification rate and the mitochondrial oxygen consumption rate were detected. The mRNA level of *COL-1, ALP, TFAM* and *NRF1* was analyzed. The protein level of p-AMPK and AMPK were measured by western blot.

Results.

Glycolysis decreased after a slight increase, while mitochondrial oxidative phosphorylation kept on rising when cells growing in osteogenic mineralized medium. Thereby, the metabolism of differentiating cells switched to mitochondrial respiration. Next, inhibition of mitochondrial respiration with carbonyl cyanidem-chlorophenylhydrazone, a mitochondrial uncoupler, depressed hDPSCs differentiation with less ALP activity and decreased *ALP* and *COL-1* mRNA expression. Furthermore, mitochondrial uncoupler leaded to AMPK activation.5-aminoimidazole-4-carboxamideribonucleotide, an AMPK activator simulated the effect of mitochondrial uncoupler by inhibiting cell differentiation, mitochondrial biogenesis and mitochondrial morphology. Mitochondrial uncoupler and activation of AMPK depressed mitochondrial oxidative phosphorylation and inhibited differentiation, suggesting that they may serve as a regulator to quit osteogenic differentiation from impaired mitochondrial oxidative phosphorylation.

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Abstract

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Keywords: dental pulp stem cells; osteogenic differentiation; glycolysis; mitochondrial oxidative phosphorylation;

Introduction

Human dental pulp stem cells (hDPSCs), as a source of adult multipotent stem cells, present the ability to differentiate into multiple types of cell types, including but not limited to odontoblast/osteoblast, chondrocyte, adipocytes, myogenic and neural cells(Sui et al., 2020; Tsutsui, 2020). Due to their easily isolation and great potential in tissue engineering and regenerative medicine, DPSCs are widely used in various fields. Numerous studies are dedicating to uncover the detailed mechanisms involved in their self-renewal ability and multi-lineage differentiation potential. The capacity of dental pulp stem cells to differentiate is important for dental pulp repair and dentin rebuilding(Sui et al., 2020).

Energy metabolism plays the critical role in guiding the proliferation, differentiation and many life processes of stem cells. Energy production by aerobic oxidative phosphorylation is the main function of mitochondria(Rossmann et al., 2021). In stem cells, mitochondria produce the energy not only for the maintenance of homeostasis, but also for differentiation(Tsutsui, 2020).The metabolism predilection shifts between mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis change along cell status and different levels of mitochondrial maturation, which depend on the pluripotency stage.(Khacho & Slack, 2018; Wanet et al., 2015). For instance, fully differentiated cells rely more on OXPHOS, while undifferentiated stem cells rely more on glycolysis(Ly ,Lynch & Ryall, 2020). Some evidence also shows a metabolic switch from anaerobic glycolysis toward OXPHOS upon differentiation of hematopoietic stem cells (HSCs). Quiescent HSCs rely on glycolysis, while the active HSCs change to OXPHOS metabolism when cells undergo differentiation(Morganti ,Cabezas-Wallscheid & Ito, 2022).

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Moreover, the dynamic distribution, subcellular content and structural of mitochondria, have been shown to exhibit peculiar functions in the processes of cellular differentiation and reprogramming(Khacho & Slack, 2017). Mitochondrial-mediated shift accompanied by mitochondrial remodeling and dynamics are vital to the differentiation and the fate of neural stem cells(Coelho et al., 2022). Our previous study demonstrated that at the initial stage of hDPSCs differentiation, both the OXPHOS and glycolysis were upregulated(Wang et al., 2016). It should be emphasized whether the mitochondrial and glycolysis change exist during hDPSCs continuous differentiation. Other questions that need to be addressed are the role of mitochondria upon differentiation of hDPSCs.

Some studies are underway to unveil the bidirectional crosstalk between mitochondria and differentiation, such as reactive oxygen species production, energy-sensing pathways, hypoxia-inducible factor pathway. Adenosine monophosphate (AMP)–activated protein kinase (AMPK), as a central metabolic sensor sensitive to the AMP/ATP ratio, can be activated by various types of cellular stress(Aslam & Ladilov, 2022; Li et al., 2013). AMPK is involved in the regulation the fate of stem cell, and serves as a key regulator of cell differentiation(Liu et al., 2021; Sun et al., 2017; Yang et al., 2016)Previous reports showed that AMPK was required for immune cell differentiation and adipogenic differentiation(Rambold & Pearce, 2018; Son et al., 2019; Yang et al., 2016). AMPK activation established a metabolic barrier to impede the cellular reprogramming into iPSCs(Vazquez-Martin et al., 2013; Vazquez-Martin et al., 2012). AMPK influenced osteogenic differentiation of human dental pulp mesenchymal stem cells (*Pantovic et al., 2013*). The study also detected the change of AMPK when OXPHOS was intervened.

Metabolism is an important regulator for stem cells and fate decision. Anaerobic glycolysis may be an adaption to the low oxygen niche for stem cells and maintain their stemness. The shift from glycolysis to OXPHOS is required for stem cells to differentiate. The reverse transition from OXPHOS to glycolysis is required for the induction of pluripotency from somatic cells(Zhang ,Menzies & Auwerx, 2018). Metabolism regulating of stem cells would be a potential target for tissue engineer or regeneration medicine.

In all, the aim of the study is to study the change of mitochondrial OXPHOS and glycolysis during hDPSCs differentiation and its possible regulating factors.

Materials & Methods

Reagents and antibodies

Hexokinase (HK), pyruvate kinase (PK), lactate dehydrogenase (LDH), and alkaline phosphatase(ALP) enzyme activity kits were obtained from Jiancheng (Nanjing, China). MitoTracker Red CMXRos (M7512) and Alexa Fluor 488 Phalloidin (A12379) were obtained from Invitrogen (Carlsbad, CA, USA). XF Cell Energy Phenotype Test Kit, XFp extracellular flux analyzer, XFp culture microplates and bicarbonate-free DMEM were obtained from Seahorse Bioscience Agilent Technologies (North Billerica, MA, USA). Hoechst 33342 (B2261), Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, C2759) was purchased from Sigma-Aldrich (St Louis, MO, USA). The following antibodies were used: AMPK (1:1000, Abcam, ab80039), p-AMPK (1:1000, Abcam, ab133448), anti-GAPDH (1:1000, Abcam, ab9485), and HRP-conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA, USA),

Alexa Fluor 594-conjugated secondary antibody (Invitrogen).

Cell culture and treatment

After receiving the informed consents from patients and their parents, human dental pulp tissues were collected from caries- and periodontitis-free third molars extracted for orthodontic purposes from healthy donors (n=5). The experimental procedures were conducted according to the Declaration of Helsinki. The protocol was approved by the Institutional Ethics Committee of West China Hospital of Stomatology (WCHSIRB-D-2017-052; WCHSIRB-D-2020-075-R1). Briefly, after washing with sterile PBS, dental pulp tissues were cut into fragments, digested with 3 mg/ml type I collagenase at 37℃ for 30 min, placed on 25 cm^2 culture dishes, and maintained in standard medium which was low glucose Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), penicillin (100 units/mL), and streptomycin (100 mg/mL) at 37°C in 5% CO_2. Cells were sub-cultured when cells reached 80% confluence, and used at passage 3 in this study. Differentiation was induced by osteogenic mineralized medium (MM), which was the standard medium supplemented with10 mM b-glycerophosphate, 50 mg/mL ascorbic acid and 100 nM dexamethasone (Sigma–Aldrich, St. Louis, MO, USA). All media were replaced every 2 days. Flow cytometry was used to analyze cell surface marker expressions and the cells were also incubated in MM for further alizarin red staining (Supplementary Fig. S1).

To inhibit OXPHOS activity, cells were cultured in a mineralized medium with a mitochondrial uncoupler, carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 2μM). In some experiments, cells were treated with 5-aminoimidazole-4-carboxamideribonucleotide (AICAR,

500μM)

Enzyme activities assay

Cells were collected and ultrasonically decomposed on ice. After centrifugation, the supernatant was to measure the activities of ALP, HK, PK and LDH with corresponding enzyme activity kits. Enzyme activities were calculated from optical density value and total protein.

Cell energy phenotype analysis

Our previous results showed that hDPSCs initiated to differentiate on day 3 induced in MM. At this critical point, cells possessed increased glycolytic and mitochondrial function. With the further differentiation, extracellular flux rates were measured in this study. Cells (4×10^3 cells/well) were plated on XFp cell culture microplates and divided into experimental group and control group. The experimental group was cultured in MM, while the control was grown in standard medium at 37 °C and 5% CO_2. On day 1, 3, 5 and 7, the extracellular acidification rate (ECAR) as an indicator of the glycolysis potential, and the mitochondrial oxygen consumption rate (OCR) as parameter of mitochondrial respiration were analyzed on a Seahorse XFp Bioanalyzer, using the Seahorse XF Cell Energy Phenotype Test Kit according to the manufacturer's instructions. One hour before beginning the measurement, cells were switched to Seahorse bicarbonate-free DMEM, adjusted to pH 7.4 with NaOH, were subsequently incubated in a non-CO_2 incubator at 37°C for 1 h. Then, OCR and ECAR tests were performed under baseline and stressed conditions, after Oligomycin (1 μM), an ATP synthase inhibitor, and CCCP, 2μM, a mitochondrial uncoupling agent, were injected into the microplates. Extracellular flux rates were analyzed by using Seahorse XF software after normalized to the total protein units,

and the ratio of OCR/ECAR were calculated.

Real-time quantitative PCR (qPCR)

Total RNA was extracted from hDPSCs treated in different conditions using TRIzol (Invitrogen) and cDNA was reverse transcribed with PrimeScript RT reagent Kit (TakaKa, Osaka, Japan). A negative control without the reverse transcriptase was also implemented. Real-time PCR was performed using a SYBR Green PCR kit (Takara) and the Roche LightCycler480 Real-Time PCR System (Roche, Basel, Switzerland). GAPDH was used as the internal reference gene, and levels were relativized to the control group. The sequences of specific primers were: 5'-AAGGAGAAAGAGGAGCCAAAGG-3'(forward) and 5'-AGCACCAGGGAAACCAGTCATAC-3'(reverse) for *COL-1*; 5'-CAGATGAAGTGGGAGTGCTTGT-3'(forward) and 5'-CTGATGTGGAGTATGAGAGTGACG-3'(reverse) for *ALP*; 5'-ATGGCGTTTCTCCGAAGCAT-3'(forward) and 5'-TCCGCCCTATAAGCATCTTGA-3'(reverse) for *TFAM*; 5'-AGGAACACGGAGTGACCCAA-3'(forward) and 5'-TATGCTCGGTGTAAGTAGCCA-3'(reverse) for *NRF1*; 5'-CTTTGGTATCGTGGAAGGACTC-3'(forward) and5'-GTAGAGGCAGGGATGATGTTCT-3'(reverse) for *GAPDH*.

Fluorescence microscopy

Cells were seeded on the coverslips at 1×10^4 cells/well, and incubated with MitoTracker Red CMXRos (MRC) in the FBS-free DMEM at a concentration of 100 nM at 37℃ for 30 min. After washing, cells were fixed in 4% paraformaldehyde for 10 min, and permeabilized with

0.05% Triton X-100 in PBS for 10 min at room temperature. Then, cells were incubated with Alexa Fluor 488 Phalloidin to show the cytoskeleton for 1 hour at room temperature. Nuclei were stained with Hoechst 33342 for 5 min.

For the immunofluorescence experiment, cells were first fixed in 4%paraformaldehyde for 10 min, and were permeabilized with 0.05% Triton X-100 in PBS for 10 min. After washing, cells were blocked with 5% BSA in PBS for 30 min and incubated with primary antibody against p-AMPK overnight at 4℃. Following further washing, cells were incubated with Alexa Fluor 594-conjugated secondary antibody. Nuclei were also visualized by Hoechst 33342 for 5 min. Fluorescence signals were obtained using an Olympus microscope (Olympus, Munster, Germany).

Immunoblotting

Total proteins from hDPSCs were extracted using total protein extraction kit (KeyGEN BioTECh, Nanjing, China) according to the manufacturer's recommendation. SDS/PAGE was performed on Bio-Tris 5-10% gradient polyacrylamide gels acrylamide. Proteins were transferred to PVDF membranes (Bio-Rad), and membranes were blocked with 5% BSA in TRIS-buffered saline, 0.1% Tween (TBS-T) for 60 min at room temperature. The membranes were incubated overnight with appropriate primary antibodies. Detection was subsequently performed with HRP-conjugated secondary antibody for 1 hour at room temperature, and the membranes were scanned using the GelDoc XR+ System (Bio-Rad Laboratories, Hercules, CA, USA). The density of Western blot band signals was monitored using Quantity One software.

Statistical analysis

Every experiment was independently replicated a minimum of three times, ANOVA and Student's t-test were performed to determine statistical significance between control group and experimental group using SPSS version 17.0 software. *P*-values were considered to be significant when $p < 0.05$ ($*p< 0.05$).

Results

During osteogenic differentiation, glycolysis function declined after an initial slight increase

In order to assess the status of osteogenic differentiation, the mRNA expression of cell differentiation makers (*ALP* and *COL-1*) and ALP activity were detected on day 0, 1, 3, 5, and 7 cultured in control and mineralized medium (MM). By q-PCR, the expression of *ALP* mRNA increased at 3 days after differentiation induction (p=0.005), and *COL-1* started to upregulate 5 days after treatment (p=0.044), compared with the control (Fig. 1A and B). To further understand the hDPSC differentiation, ALP activity was also monitored. As is reported in Fig. 1C, the increase of ALP activity occurred from day 3, and kept on rising during the process of differentiation (p=0.000).

The activity of several important enzymes in glycolysis was measured. The activities of HK (p=0.023), PK (p=0.013) and LDH (p=0.005) had a slight increase at day 3 when hDPSC started to differentiate, then gradually decreased to a much lower level than that of control at day 7 during differentiation of hDPSC (Fig. 1D, E, and F).

Mitochondrial OXPHOS predominated in energy production of the differentiating hDPSCs.

The data showed that mitochondrial transcription factor A (*TFAM*, controlling mtDNA

Makers– markers

expression and mitochondrial biogenesis) expressions were elevated at day 3，5 and 7 compared to the control (Fig. 2A, p=0.016, p=0.002, p=0.001). The expressions of *TFAM* in MM were also gradually upregulated since day 3 (Fig. 2A, p=0.000). However, the expression level of Nuclear Respiratory Factors 1 (*NRF1*, controlling nuclear encoded respiratory chain components expression) was unchanged compared to control cells (Fig. 2B).

Later, the mitochondrial OCR(mitochondrial respiration indicator) and ECAR(glycolysis indicator) during hDPSCs differentiation in baseline and stressed condition were analyzed by Seahorse XF Cell Energy Phenotype Test Kit. Two stressor compounds, oligomycin that causes an increase of glycolysis and carbonyl cyanide p-trifluoro methoxyphenylhydrazone that drives OCR rates higher, were used as to induce a stressed condition. Under the basal condition, consistent with upregulated *TFAM* mRNA expression, OCR of differentiating cell was greater than that of control at day 3 (Fig. 2C), but the ratio of OCR/ECAR had no difference (Fig. 2D). At the days 5 and 7, ECAR was down-regulated, and OCR was still rising. Moreover, the cells in MM exhibited higher OCR/ECAR compared to the control at day 5 and 7(Fig. 2D, p=0.004, p=0.001). On the metabolic stressed condition, a similar pattern of specific dynamic changes in mitochondrial OXPHOS and glycolysis could be observed (Fig. 2E).

Mitochondrial uncoupling interfered with hDPSCs' differentiation

A mitochondrial uncoupler, CCCP, was used to attenuate mitochondrial respiration(Kane et al., 2018). The cells were cultured in osteogenic mineralized medium with or without CCCP. Upon differentiation, both the basal and stressed mitochondrial OCR depressed after CCCP treatment, compared to cells cultured in MM (Fig. 3A). The ECAR of cells growing in presence

spaces should be left

237 of CCCP showed a decrease compared to the control at day 3 in baseline conditon (p=0.002),
238 while there was no difference in other days (Fig. 3B). With the MitoTracker Red CMXRos
239 staining, the mitochondrial morphology showed to be punctate, and failed to form functional
240 network at the initial stage of differentiation (yellow arrow, Fig. 3C). Importantly, this
241 suppression of mitochondrial respiration attenuated the differentiation of hDPSCs, manifested as
242 the inhibited ALP activity and the restrained *ALP* and *COL-1* mRNA expression, compared to
243 cells growing in absence of CCCP (Fig. 3D).

244 **The activation of AMPK impaired OXPHOS-driven differentiation**

245 The activation of AMPK was detected in cells cultured in MM with or without CCCP (2μM)
246 for 1-7 days. The ratio of AMPK phosphorylation/AMPK protein were increased at day 3,5,7 by
247 using CCCP (Fig. 4A). Would AMPK influence hDPSCs differentiation? As shown on Fig. 4B,
248 the activation of AMPK was induced significantly by AICAR treatment. Crucially, consistent
249 with CCCP treatment, AICAR suppressed hDPSCs differentiation (Fig. 4C). Furthermore, the
250 mRNA expression of *TFAM* was suppressed, and the mitochondrial fragmentation was observed,
251 compared to the control since day 3 after treatment of AICAR (Fig. 4D and 4E). Collectively,
252 these results indicated that the activation of AMPK contributed to hDPSCs differential inhibition
253 and impaired OXPHOS.

254

255 **Discussion**

256 In this study, it was demonstrated that the dynamic shift of glycolysis and mitochondrial
257 OXPHOS took place during differentiation of hDPSCs. Attenuating mitochondrial respiration

Conditon– condition

with uncoupler CCCP suppressed hDPSCs differentiation and activated the central metabolic sensor, AMPK.

Metabolic shift accompanied hDPSCs' osteogenic differentiation

Recent studies have displayed that stem cells depend mostly on anaerobic glycolysis for ATP supply, while the differentiating cells depend on mitochondrial aerobic respiration. The metabolic shift and mitochondrial resetting into mature bioenergetic states are considered a hallmark of stem cell differentiation(Wanet et al., 2015). When hDPSCs differentiated, cells presented distinct energy phenotypes. At day 3, the level of ECAR showed that glycolysis increased rather than decreased. Studies have shown that glycolysis supplies rapid energy generation and substrates for the biosynthesis. Increased glycolysis can provide a quick production of ATP and sufficient substrates for the biosynthesis, which meet the anabolic demands of initial differentiation(Palmer et al., 2015). This implies that differentiation requires the supply of enough energy and substrates. The joint effects of glycolysis and mitochondrial OXPHOS may support the tremendous energy demand on day 3 of differention. Simutanously, higher level of OCR and increased ratio of OCR/ECAR herald a shift toward a characteristic of mitochondrial OXPHOS. The results showed that mitochondrial OXPHOS predominated in energy production of the differentiating hDPSCs at day 5 and day 7, when metabolic shift of hDPSCs were observed. The finding was similar to other reported stem cells(Coelho et al., 2022; Folmes et al., 2011; Prigione et al., 2010).

Some studies suggested that mitochondrial content, such as mitochondrial DNA (mtDNA) copy number, mitochondrial mass and biogenesis, tightly adapted to the change of cell

metabolism during pathological or physical activity(Clark & Parikh, 2020; Procaccio et al., 2014). In order to assay the mitochondrial content-related modulating factors during hDPSCs differentiation, the mRNA expression of *TFAM* and *NRF1* were detected in the study(Kang ,Chu & Kaufman, 2018; Quan et al., 2020).As hDPSCs differentiation went onto day 5 and 7, up-regulated *TFAM* expression were observed, which might induced increased mtDNA content and increased mitochondrial biogenesis(Tsutsui, 2020; Vega-Naredo et al., 2014). Nevertheless, it seemed that *NRF1* was not involved in the process of hDPSCs differentiation. The reason might be related to the difference of cell types.

AMPK participated in differential inhibition when mitochondrial activity attenuated

Metabolic shift plays an essential role in regulating cellular function, particularly in stem cell self-renewal, pluripotency, and plasticity(Andre et al., 2019). Mitochondrial biogenesis and metabolic shift toward OXPHOS are deemed as early events in differentiation process of multiple stem cells. Furthermore, Inoue S et al. suggested that mitochondrial respiration was more important in mouse hematopoietic stem cell commitment and differentiation than in reaching the final phase of cell differentiation(Inoue et al., 2010). Consistent with previous reports, our data showed that inhibiting mitochondrial function by CCCP from the beginning prevented differentiation of hDPSCs. It turned out that mitochondrial OXPHOS was required for differentiation. CCCP-treated hDPSCs exhibited an apparent fragmentation of mitochondrial tubules, likely due to a block in mitochondrial fusion.

The energy sensor AMPK was activated by the decreased intracellular ATP due to impaired OXPHOS. It was speculated that AMPK may be the direct regulator of the mitochondrial fission

and fusion machinery to mediate subsequent events. As an intracellular energy sensor, the vital role of AMPK was to restore the energy balance. Once activated, AMPK induces catabolic pathways to produce energy and prevents anabolic pathways, such as lipid and protein synthesis, to save energy(Wanet et al., 2015). On the other hand, considerable evidence shows that AMPK is involved in regulating cell fate, including controlling stem cell pluripotency and differentiation(Afinanisa ,Cho & Seong, 2021; Fernandez-Veledo et al., 2013; Liu et al., 2021).In the study, CCCP-treated hDPSCs exhibited an elevated level of p-AMPK, showing that the energy sensor AMPK was involved in the process of metabolism that influenced differentiation. AMPK activation was a potential modulator when the differentiation of hDPSCs had switched on.

To further verify the effect of AMPK activation. AICAR, a direct AMPK activator was applied. AICAR resulted in mitochondrial fragmentation and differential inhibition, which were similar to that induced by CCCP. For cell differentiation, numerous anabolic pathways, including the biosynthesis of proteins and lipids, are required to support specific function of differentiated cells. The mitochondrial biogenesis and stimulation of metabolism are also needed to produce adequate energy for the processes. CCCP-induced or AICAR-induced AMPK activation may serve as a regulator for the cells to quit differentiation from impaired OXPHOS, which might be useful for keeping stemness or to maintain homeostasis. Mitochondrial dysfunction as a regulator may be a viable therapeutic target for stem-cell-based therapies and interventions for cognitive defects(Khacho ,Harris & Slack, 2019).

However, the detailed mechanism between mitochondria and cell differentiation requires

321 further investigation. Whether metabolic shift is prerequisite for cell differentiation and whether
322 mitochondrial OPHOXS can regulate cell differentiation *in vivo* remains to be further studied.
323
324 **Conclusion**
325 It was demonstrated that increased mitochondrial function was indispensable for hDPSCs
326 differentiation, while the glycolysis gradually decreased in the stage of energy supply. It was also
327 unveiled that mitochondrial uncoupler CCCP depressed mitochondrial OXPHOS and inhibited
328 hDPSCs differentiation. Activation of AMPK also interfere with the mitochondrial morphology,
329 mitochondrial OXPHOS and cell differentiation. The findings helped to reveal the relationship
330 among glycolysis, mitochondrial OXPHOS and cell differentiation. CCCP or AMPK activation
331 may be a potential regulator to quit differentiation from impaired OXPHOS or to maintain
332 homeostasis.
333
334 **Acknowledgments**
335 Not applicable.
336

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Figure 1

Glycolysis increased first and then decreased during hDPSC differentiation.

Cells were cultured in control and mineralized medium for 0, 1, 3, 5, and 7 days, respectively. (A) mRNA expression of ALP was upregulated from day 3, (B) COL-1 was upregulated from day 5. (C) HK, a key glycolytic enzyme, increased at day 3 when cells initiated to differentiate, and then decreased from day 5. (D, E) The same trend was detected in PK and LDH. Data were mean ±SD, *p<0.05, **p<0.01, ***p<0.001.

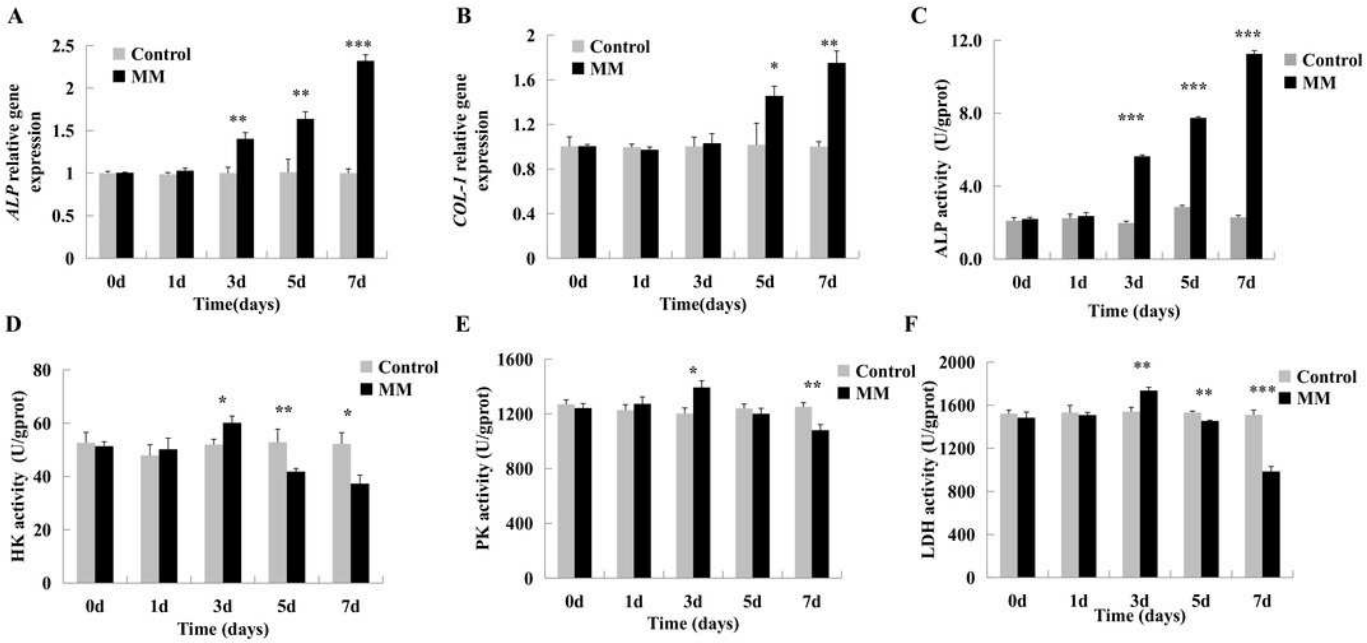

Figure 2

Cell energy phenotype shifted to mitochondrial OXPHOS as hDPSCs differentiated.

(A) TFAM expression, which is involved in mitochondrial biogenesis, upregulated from the initial stage of hDPSCs differentiation, (B) while NRF1 was not greatly changed upon differentiation. (C) On day 1, 3, 5, and 7, the OCR and ECAR of cells growing in basal condition were firstly measured, and then those of stressed condition in the absence of Oligomycin and FCCP were assayed to show the max metabolic potential. The ECAR was on the horizontal axis, and the OCR was on the vertical axis. The hollow rhombus was for basal condition, while the solid was for stressed condition. (D) For the differentiating cells, the ratio of basal OCR/ECAR, which was calculated to show the major energy phenotype, increased from day 5. (E) The stressed OCR/ECAR ratio had the similar tendency. Data were mean±SD, *p<0.05, **p<0.01, ***p<0.001.

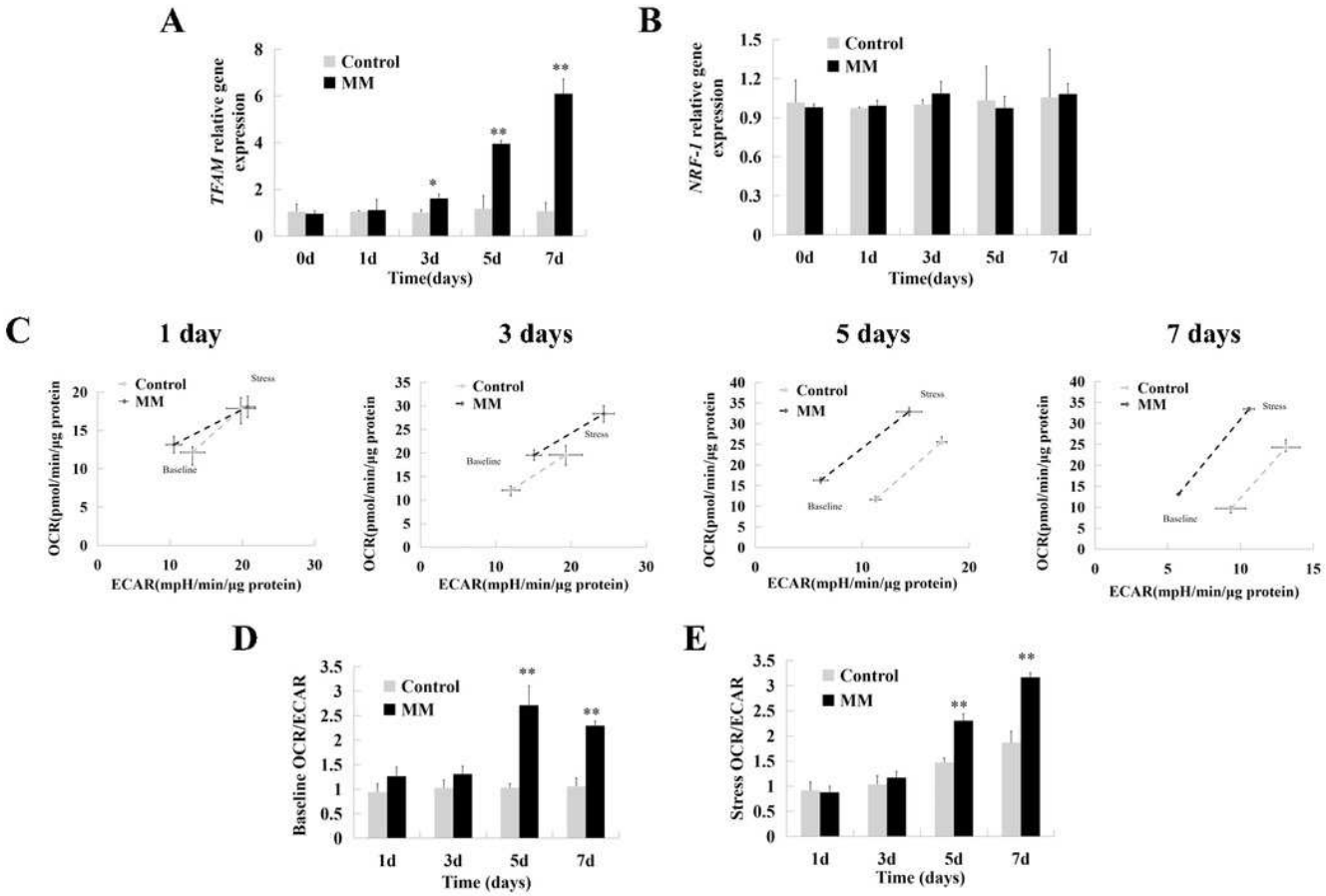
A
TFAM relative gene expression
Control
MM
0d
1d
3d
5d
7d
Time(days)
B
NRF-1 relative gene expression
Control
MM
Time(days)
C
1 day
3 days
5 days
7 days
Control
MM
Stress
Baseline
OCR(pmol/min/μg protein
ECAR(mpH/min/μg protein)
D
Baseline OCR/ECAR
Control
MM
Time (days)
E
Stress OCR/ECAR
Control
MM
Time (days)

Figure 3

As hDPSC differentiation, cell energy phenotype shifted to mitochondrial OXPHOS.

Cells were cultured in the MM with or without CCCP (2 μM) for 1-7 days. (A) The change of OCR was monitored over time. With the CCCP treatment, cells9 OCR was depressed, compared to the control. (B) Except for the day 3, the ECAR of CCCP group had no significant change. (C) At day 3, fluorescence was used to detect the morphology of mitochondria: mitochondria - MitoTracker Red CMXRos (MRC), red; F-actin-Phalloidine, green; nucleus - Hoechst 33342, blue. The local magnification showed CCCP inhibited the form of mitochondria functional network. (D) The osteogenic differential makers, the mRNA expression of ALP and COL-1 and the activity of ALP were suppressed by CCCP. Scale bars:20 μm. Data were mean ±SD, *p<0.05, **p<0.01, ***p<0.001.

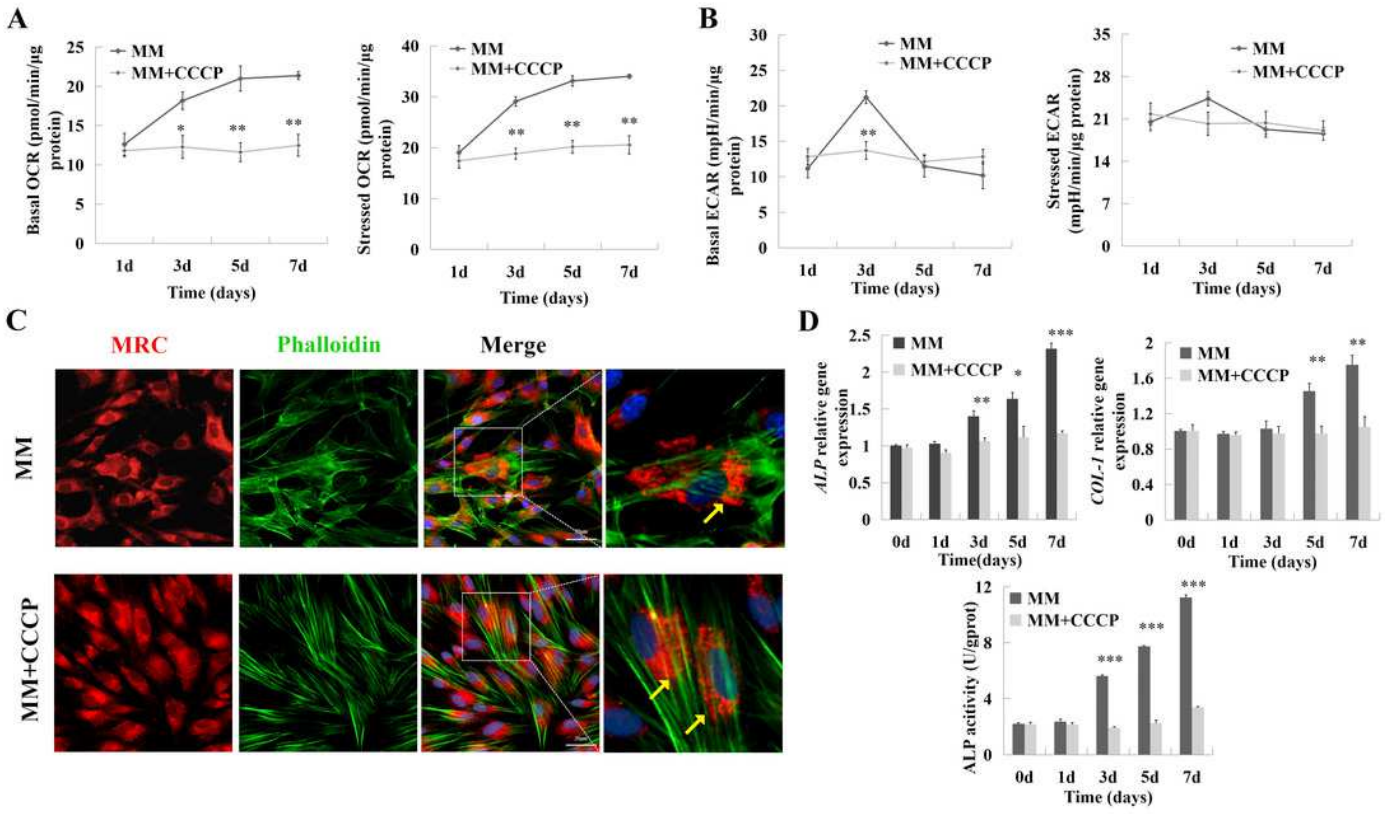

Figure 4

The activation of AMPK contributed to differential inhibition.

(A) Immunoblot analysis for AMPK and phosphorylated AMPK in cell lysates from hDPSCs treated with CCCP (2 μM) or MM only for 1, 3, 5, and 7 days. GAPDH served as the standard. (B) Cells were treated with AMPK agonist AICAR (500 μM) for 3 days, the p-AMPK was observed using immunofluorescence: p-AMPK, red; nucleus, blue. Scale bar: 50 μM. (C) The ALP, COL-1 expression and ALP activity were detected to unveil the effect of AICAR in cell differentiation. (D) qPCR analysis of mitochondrial factor TFAM in AICAR-treated (500 μM) cells. (E) Mitochondrial morphology in AICAR-treated and control cells were evaluated by fluorescence staining. Mitochondria-MitoTracker Red CMXRos (MRC), red; F-actin -Phalloidine, green; nucleus - Hoechst 33342, blue. Scale bar: 20μM. Data were mean±SD, * p <0.05, ** p <0.01, *** p <0.001.

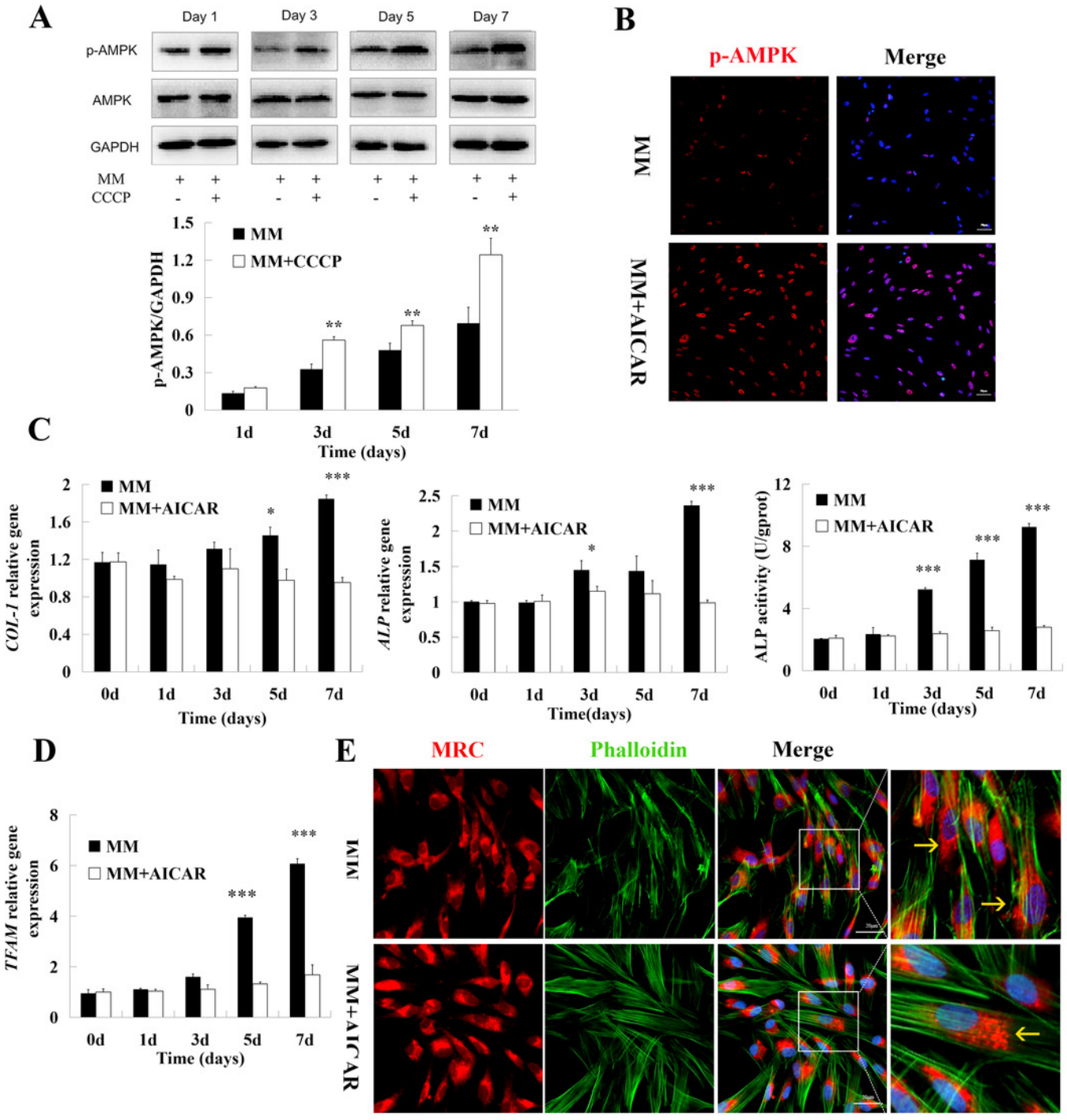

Figure 4–E X column text should be made clearly visible