ORIGINAL CONTRIBUTION

The effect of hypoxia on the proliferation capacity of dermal papilla cell by regulating lactate dehydrogenase

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Abstract

Hypoxia is of great significance for stem cells to maintain the proliferation and differentiation capacity. As a specialized mesenchymal component of the hair follicle (HF), the dermal papilla cell (DPC) not only regulates HF cycle, but also plays a pivotal role in differentiating hair follicle stem cell(HFSC) into HF. However, whether hypoxia could affect DPCs on proliferation or metabolism remains unclear. In our study, DPCs were cultured in normoxia (20%O_2) or hypoxia (5%O_2). Cell viability assays were performed, and lactate dehydrogenase (LDH) activity and lactate level in DPCs were detected. After that, LDH was overexpressed or knocked down in DPCs; then, the expression of protein markers (ALP, Ki-67) was assessed by Western blotting, and cell proliferation was also detected after overexpression or knockdown of LDH. Hypoxia did show positive effect on proliferation of DPCs. The LDH activity of DPCs cultured under hypoxic condition was significantly higher than that of cultured under normoxic condition. Overexpression of LDH significantly up-regulates the expression of ALP and Ki-67 compared with knockdown and negative control. Cell proliferation was also promoted in DPCs with elevated LDH. Our findings showed that the proliferation activity of DPCs could be stimulated under hypoxia. Meanwhile, LDH plays an important role in maintaining the activity of DPCs in hypoxic condition.

KEYWORDS
cell proliferation, cell regeneration, Dermal papilla cell, Hypoxia, lactate dehydrogenase

1 | INTRODUCTION

Dermal papilla cells (DPSs) play a key role in hair follicles (HF) cycling as its capacity to differentiate into many cell types under different microenvironment stimulations, providing structural or functional repairs for hair formation or growth.[1-5] Due to its hair inductivity, DPSs are considered a therapeutic target for treatment of alopecia.

As a specialized mesenchymal stem cell, despite DPCs have the potential of regeneration and differentiation, they undergo replicative senescence in the natural process, resulting in cellular phenotype changes and lower the potential of hair regeneration. Recent studies have focused on DPCs as a therapeutic target of hair loss; thus, maintaining the hair inductivity of DPCs becomes critical for hair loss treatment.

Previous studies have shown that stem cells are found in the hypoxic microenvironments,[6,7] suggesting that hypoxia plays an important role for stem cell stimulation. Hypoxia has been shown to regulate various cell transformation processes and signal transductions by regulating the expression of hypoxia-inducible factor 1 (HIF-1), which plays a key role in several transactive genes.[8,9] Existed studies on neural and hematopoietic stem cells have shown that hypoxic condition in cell culture in vitro is beneficial for survival and self-renewal of stem cells.[10,11] Hypoxic microenvironment assists in maintaining the multipotent differentiation of embryonic stem cells (ESC).[12,13] In addition, research has explored the effects of glucose metabolism on hair follicle stem cell from the perspective of cell metabolism and confirmed that lactate dehydrogenase (LDH), a key enzyme of glucose metabolism, can affect the viability of hair follicle stem cell by activating the initiation of a new hair cycle under certain conditions.[14,15]

Many recent studies have focused on DPCs and hair loss treatment; yet, the stimulation effect of hypoxic on DPCs is not well illustrated. As a population of mesenchyme in the skin, regulated by multiple factors, DPCs have the ability to induce proliferation of HFs resulting in hair regeneration.[1] Whether LDH took part in the process of proliferation and regeneration of DPCs under hypoxia remained undefined. In the presented study, we analyzed the cell vitality and proliferation capacity of DPCs under both normoxic and hypoxic conditions, and evaluated the expression level of LDH and lactate of DPCs from two different conditions. Then, we overexpressed and knocked down LDH in DPCs under hypoxia conditions to see if changes could be reversed. We found that hypoxia could promote the proliferation of DPCs via up-regulation of LDH.

2 | MATERIALS AND METHODS

2.1 | Animals

4-6-week-old C57BL/6J mice were purchased from the Experimental Animal Center of Southern Medical University (Guangzhou, China). All animal experiments were carried out with the approval of the Institutional Animal Care and Use Committee.

2.2 | Isolation and culture of DPCs

For the culture of DPCs, anagen vibrissae follicles of 4-6-week-old C57BL/6J mice were surgically dissected with fine forceps; meanwhile, the subcutaneous fat tissue and excess hair shafts outside the skin were removed using microscissors. After rinsing with PBS containing 1% penicillin-streptomycin for 3 times, the surrounding collagen capsules in the follicles were digested with 2.5 mg/ml collagenase IV at 37°C for 2h. Most DPCs were completely or partially exposed at the bulbs of vibrissae follicle, and then, DPCs were dissected microscopically. Isolated DPCs were planted in the plastic dishes and supplemented with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C

in a humidified atmosphere with 5% CO^2. The cell culture medium was changed every 3 days in further experiments. After reaching 80% confluence, DPCs were harvested with 0.25% trypsin-EDTA and transferred to new culture dishes with a split ratio of 1:2. Cell characterization for DPCs has been described in previous study.[16,17] For hypoxia exposure, cells were cultured in a hypoxia chamber flushed with $5\%O_2$ and $5\%CO_2$, with balance of $90\%N_2$ at 37°C.

2.3 | Evaluation of DPCs proliferation

Dermal papilla cells were seeded and cultured in 96-well plates either in normoxia (20% O_2) or hypoxia (5% O_2) at an initial density of 4×103 cells per well. During the different time points at 6, 12, 24, 48 and 72 hours, 10 μL CCK-8 reagent was added to each well and cells were incubated for 1.5 hours at 37°C. The absorbance was measured at a wavelength of 450 nm using a microplate reader (SpectraMax, Plus 384 Molecular Devices, Inc). For clone formation, transfected DPCs were counted and seeded into 6-well plates (1000 cells per wall). Cells culture medium was replaced every 3 days. Cells colonies were counted after 14 days after fixation and dyeing with crystal violet.

2.4 | LDH activity and lactate level assay in DPCs

Dermal papilla cells were cultured on 20% or 5% oxygen condition for different time before assay, then were collected with 0.25% trypsin-EDTA, and washed twice with phosphate-buffered saline (PBS). Generally, 5×106 DPCs were transferred to a 2-ml EP tube, and the medium was removed. According to the manufacturer's instructions of LDH activity and lactate level Assay Kits (Solarbio), 1 mL extraction fluid was added and the tube was gently shaken. Next, the cells were broken using ultrasonic cell disruptor (JY92-IIN, scientz), and the mixture was centrifuged (8000 g for LDH and 12 000 g for Lactate) at 4°C for 10 minutes. The supernatants were then collected at 4°C for the following steps. After that, the samples were mixed with different reagents from LDH activity Assay Kit and lactate level Assay Kit. Finally, equal sample was prepared in triplicate across a 96-well plate. A microplate reader(mentioned before) held at 37°C measured 450 nm absorbance for LDH activity and 530nm absorbance for lactate level.

2.5 | Cell transfection

For knockdown of LDHA, lentivirus vector purchased from GenePharma(Shanghai, China) was used. DPCs seeded in a 24-well plate with 1×105 cells per well were cultured and stabilized. We used shRNAs targeting LDHA and related NC to lower the expression of LDHA in DPC. The shRNAs were purchases from GenePharma (Shanghai, China), and the following sequences were applied:5'-ttgttgatgtcatcgaag-3'(sense),5'-gggtccttggggaacatg-3'(antisense);NC 5'-gtacatagggacgtaacg-3'. We used Lipofectamine® 2000 (Invitrogen) to transfect DPCs under the manufacturer's protocol.
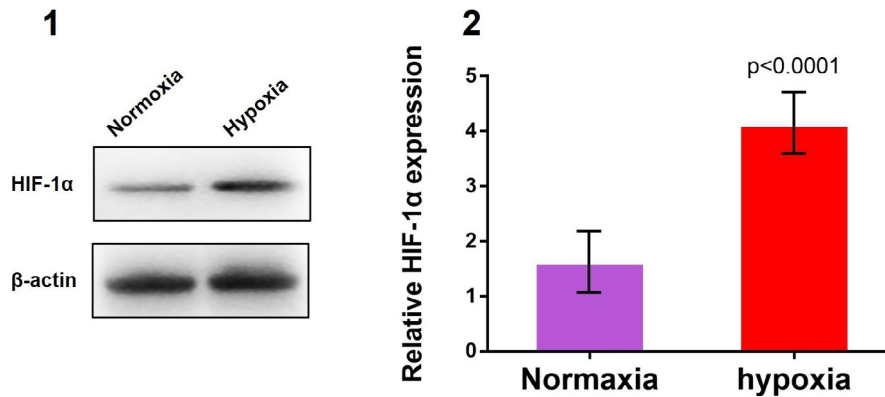
FIGURE 1 Confirmation of DPC hypoxia. DPCs were microdissected from anagen vibrissae follicles and incubated in 5% or 20% oxygen for 24 hours. Western blotting analysis revealed the protein expression levels of HIF-1α in DPCs cultured in normoxia and hypoxic samples. β-actin was used as a loading control. B, Quantification of the Western blots. Dates for WB were the mean values ± SEM of three separate experiments. (*P < .05). HIF-1α, hypoxia-inducible factor 1α

To higher expression of LDHA in DPCs, lentiviral vectors (GenePharma) were also used, and the following sequence was used: 5'-tcgagctcgagtccaatatggcaactctaaagg −3' and 5'-cccctctagaaaattgcagctgctcctttttggat −3'. After incubation of 24h, RT-qPCR and Western blot were used to access the efficiency of transfection.

RNA extraction, reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR).

Total RNA was extracted from appropriately cultured cells by Fast Pure Cell/Tissue Total RNA Isolation Kit (Vazyme, Nanjing, China) under the manufacturer's instructions. The reverse transcription was carried out using Hiscript High Fidelity One Step RT-PCR Kit (Vazyme, Nanjing, China). The primers for LDHA were designed as follows: 5′-tatggagtggaatgaatgttgc-3′; 5′-cccttaatcatggtggaaactc-3′, primers for β–actin:5′-aaagacctgtacgccaacac-3′;5′-gtcatactcct-gcttgctgat-3′. Relative RNA expression between different groups was calculated using the △△Ct method. The quantitative real-time PCR system we used was ABI Step One Plus system with SYBR Green Master Mix (Vazyme, Nanjing, China) for RNA detection.

2.6 | Western blotting

Total protein from DPCs was extracted using 100 μL RIPA lysis buffer (20-188; Millipore) according to the manufacturer's instructions. Equal amount of protein extracts (50 μg) were separated using 8% SDS-PAGE and transferred to a polyvinylidene fluoride membrane using a wet transfer system. After blocked with 5% bovine serum albumin (BSA) powder in Tris-Buffered Saline and Tween 20 (TBST) for 1 hour, the blotted membranes were first incubated with the following primary antibodies at 4°C overnight: rabbit anti-LDHA (ab226016, Abcam); rabbit anti-HIF-1 alpha (HIF-1α)(ab179483; Abcam), rabbit anti-alkaline phosphatase (ALP)(ab108337; Abcam), rabbit anti-Ki 67 (27309-1-AP; Proteintech), rabbit anti-β-actin (ab8227; Abcam). Then, the blots were washed three times in TBST buffer for 5 minutes and incubated with corresponding

secondary antibodies (CST7074; Cell signaling) at room temperature for 2 hours. The immune complexes were detected using a Western blotting enhanced chemiluminescence (ECL) kit (64-202; Millipore), using the ALLIANCE 4.7 gel imaging system for photography. Band intensities of HIF-1α were quantified by using the Image-Pro Plus 6.0 software (Media Cybernetics).

2.7 | Statistical analysis

One-way analysis of variance was performed (ANOVA) for comparison of different groups using SPSS17.0 software. Date from three repeated experiments was analyzed with the unpaired Student's t test (two-tailed). All of the experimental data were expressed as mean ± SEM. A P-value < 0.05 was considered to denote statistical significance. All the results of these tests were visualized using GraphPad Prism 6 software.

3 | RESULTS

3.1 | Confirmation of DPCs viability under hypoxia

To confirm that DPCs metabolically respond under hypoxic condition, we assessed the expression level of hypoxia-inducible factor-1α (HIF-1α) in the DPCs exposed to 5% O_2 by conducting Western blot assay (Figure 1A), and HIF-1α was detected in DPCs after hypoxia-preconditioned for 24 hours. At the same time, compared with normoxia (20% O_2), the protein expression levels of HIF-1α in DPCs were remarkably increased under hypoxic condition (Figure 1B).

3.2 | Effect of hypoxia on the morphology of DPCs

There was no obvious difference in the morphology and growth pattern of DPCs cultured under normoxic and hypoxic conditions.

FIGURE 2 Cell proliferation and morphology of DPCs. A, No significant difference was observed in the morphology and growth pattern of DPCs cultured in normoxia and hypoxia. B, CCK-8 curve analysis revealed the proliferation rate of DPCs cultured in normoxia and hypoxia. C, The proliferation rate of DPCs significantly increased after exposure to hypoxia for 24 h. Scale bars: 200 μm. (*P < .05; #P > .05)
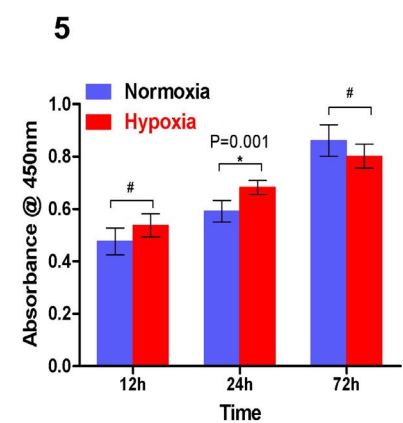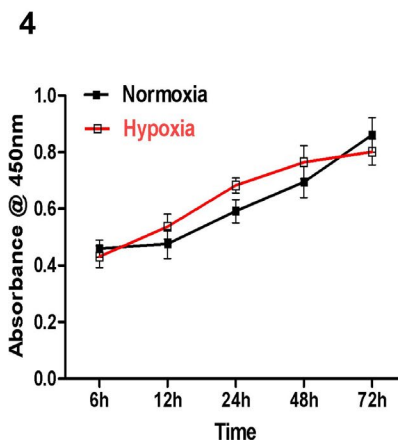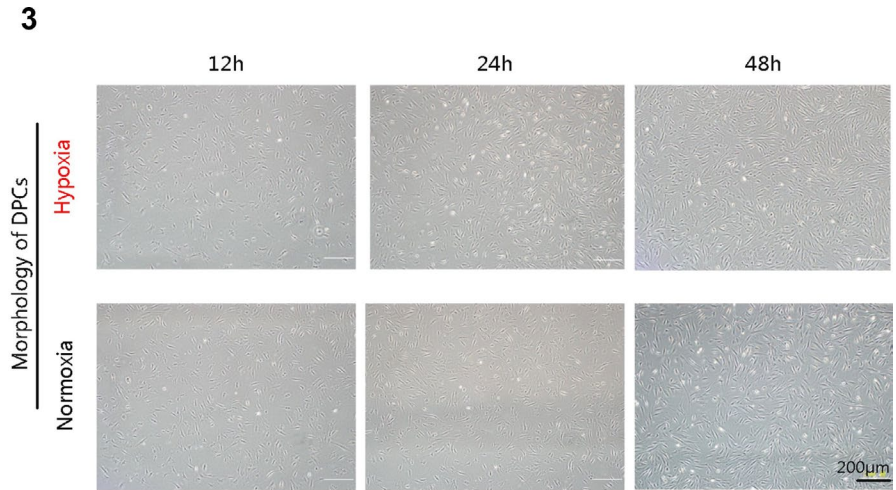
However, most of DPCs had earlier adhesion to the wall and stronger proliferation ability in hypoxia (Figure 2A).

3.3 | Effects of hypoxia on DPCs viability

To evaluate the proliferation capacity of DPCs, the cells were incubated in 5% or 20% oxygen for 3 days and then subjected to CCK-8 analysis (Figure 2B). The proliferation rate of DPCs cultured in hypoxia was a gradually upward trend during the first 12 hours and began to surpass the rate of DPCs cultured in normoxia. After incubated for 24 hours, significantly increase was observed in the proliferation rate of DPCs under hypoxic condition as compared to that of those under normoxic condition (Figure 2C), which was consistent with our previous morphological observation of DPCs.
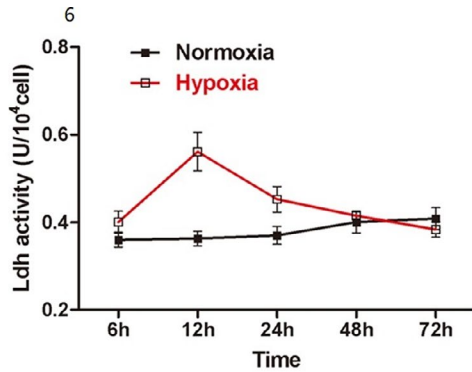
3.4 | Effects of hypoxia on LDH and lactate levels in DPCs metabolism

Dermal papilla cells cultured in both normoxic and hypoxic conditions for 72 hours. At the same time, LDH activity and lactate levels

in the cells were detected at different time. Hypoxia led to an increased level of LDH activity in DPCs within 24 hours (Figure 3A), and the LDH activity of DPCs cultured under hypoxic condition was significantly higher than that of cultured under normoxic condition (Figure 3B). With the prolongation of the culture, we found that there was no significant different in the LDH activity of DPCs under the normoxic and hypoxic conditions (Figure 3A). The lactate levels of DPCs showed a similar trend of up-regulation in hypoxia; however, the change was not statistically significant compared to that cultured in normoxia (Figure 3C).

3.5 | LDH enhanced the proliferation of DPCs

To further determine whether LDH could affect the proliferation of DPCs, overexpression and knockdown of LDH in DPCs were applied in further experiments. Firstly, we conducted RT-qPCR and Western blot to confirm the overexpression and knockdown of LDH in DPCs (Figure 4A-C). Then, the protein expression of cell proliferative markers (ALP, Ki-67)[18-20] in DPCs was detected via Western blot. The results showed that the intrinsic protein expression of ALP and Ki-67 was markedly increased when LDH was overexpressed in

FIGURE 3 Effects of hypoxia on LDH and lactate levels in DPCs. A, Curve-reader assay for LDH activity in DPCs cultured in the normoxic and hypoxic conditions for 72 h. B, Plate-reader analysis of LDH activity in DPCs after incubated 24 h. C, Plate-reader analysis of lactate levels in DPCs after incubated 24 h. All data were mean ± SEM of three separate experiments. (*$P < .05$; #$P > .05$)
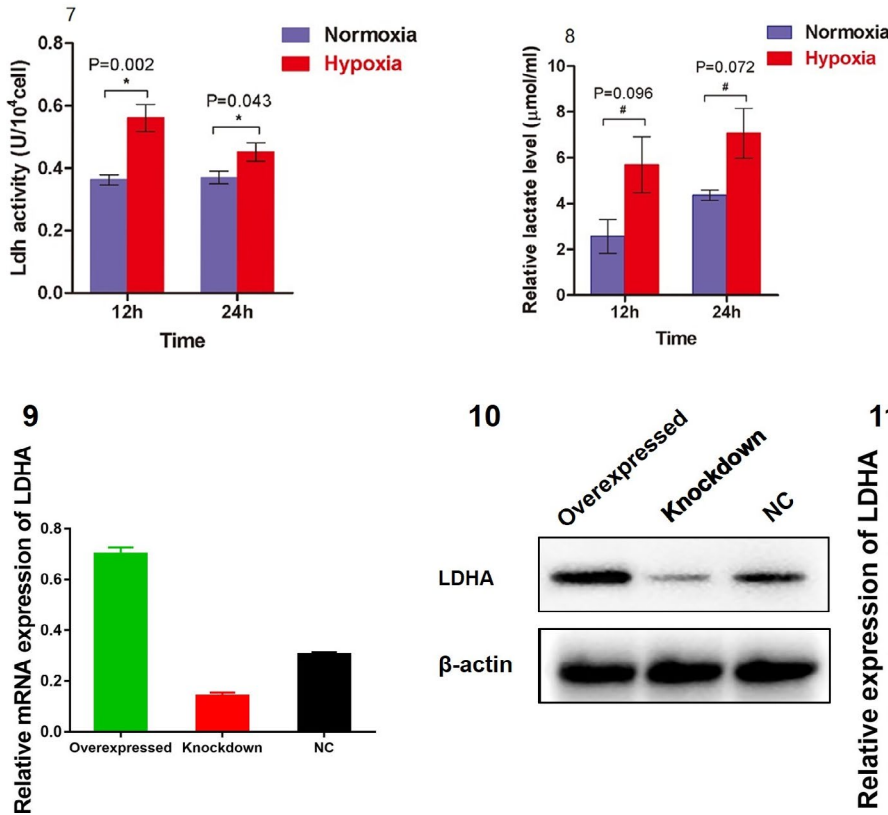
FIGURE 4 Protein expression analysis of DPCs transfected with lentivirus. A, RT-qPCR of DPCs transfected with overexpression, knockdown, and negative control of LDHA. B, Western blot of DPCs transfected with overexpression, knockdown, and negative control of LDHA. C, Quantitative comparison of LDHA in DPCs transfected with overexpression, knockdown, and negative control of LDHA

DPCs (Figure 5A,B). CCK-8 and clone formation were also used to detect the proliferation of DPCs. Compared with knockdown group, overexpression of LDH significantly up-regulate the proliferation of DPCs (Figure 5C,D). These results suggest that LDH can indeed affect the proliferation capacity of DPCs by influencing the relevant characteristics of the cell proliferation and metabolism.

4 | DISCUSSION

Hypoxia regulates diverse physiological processes, especially in energy metabolism and cell proliferation through hypoxia induction factor (HIF-1), which mainly consisted of two subtypes: HIF-1α and HIF-1β.[21-23] HIF-1α is regulated by intracellular oxygen concentration and determines the activity of HIF-1.[24] Therefore, we detected HIF-1α in our experiments as the index of cell respondence under hypoxia. Lactate dehydrogenase (LDH) is an important part in the process of glycolytic metabolism in most cells. A recent study confirmed that LDH activity could increase hair follicle stem cell activation.[14] Meanwhile, some studies had also revealed that LDH was significant in maintaining proliferative and differentiative potential of intestinal stem cells.[25] Taken together, we hypothesized that hypoxia could induce proliferation of DPCs by increasing the activity of LDH in cellular glycolytic metabolism.

In our study, DPCs were cultured under the hypoxic condition (5%O_2) as well as normoxic condition (20%O_2). The results of CCK-8
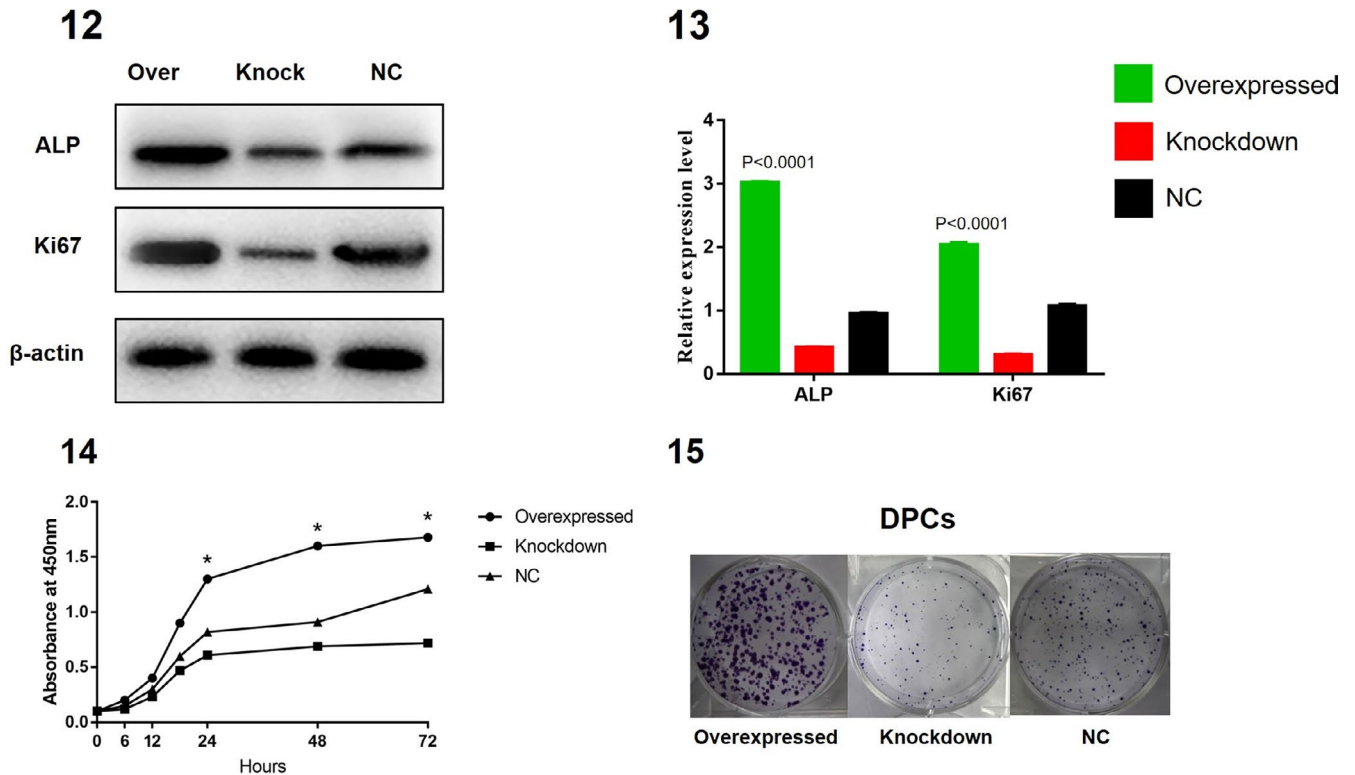
FIGURE 5 Effect of LDH on key protein markers and cellular proliferation in DPCs. A, Western blotting (WB) was showed the protein expressions of ALP Ki-67 in DPCs significantly increased in overexpression group. β-actin was used as an internal control. B, Quantification of the Western blots further confirmed the result. Relative protein expression of ALP and Ki-67 in DPCs transfected with overexpression was up-regulated. A P value less than 0.05 was considered statistically significant. Data for WB were the mean values ± SEM of three independent experiments. C and D, Cellular proliferation of DPCs transfected with overexpression, knockdown, and negative control of LDHA, CCK-8 in C and clone formation in D

cell proliferation assay indicated that the proliferation of DPCs could be stimulated in the hypoxic environment, especially in the early stage of conditioned culture. Glycolysis is the main way of cell metabolism under hypoxia, so the changes of LDH activity and lactate production level in DPCs were detected simultaneously to evaluate the level of cellular metabolism. Our results showed that the LDH activity and lactate level of DPCs cultured in hypoxic state was higher than normoxia, especially in the early time of proliferation. By comparing the CCK-8 curve and LDH activity curve, it was not difficult to find that the change of LDH activity was earlier than the proliferation of DPCs. We figured that DPCs entered into glycolytic metabolism in hypoxia environment, which stimulated the enhancement of LDH activity and thus promoted cell proliferation. Similar conclusion reported in the activity of LDH in hair follicle stem cells supports our current finding.[14,15] Meanwhile, we found that LDH activity in DPCs obviously increased at the stage of 12-24h in vitro hypoxic culture. This result suggested that the suitable time for LDH to induce the proliferation of DPCs might be 24 hours in vitro.

Furthermore, to confirm the proliferative potential of DPCs was affected by LDH, We overexpressed and knock down the LDH in DPCs by transfecting lentivirus. The efficacy of overexpression and knockdown was confirmed by RT-qPCR and Western blot. Then, we found that overexpression of LDH could increase the expression level of ALP and Ki-67 in DPCs comparing with knockdown, and

negative control group. The high expression of ALP had been shown to enhance hair follicle induction and the proliferation of DPCs.[20] Ki-67 is a classic marker of cellular proliferation.[26,27] In further experiments, compared with knockdown and NC, overexpression of LDH could increase proliferation of DPCs, as the results of CCK-8 and clone formation assays indicated.

There are some limitations in the present study. Firstly, we only used one cell line in our experiments instead of two or more cell lines. Secondly, we did not analyze the cellular proliferative activity of transfected DPCs under hypoxia and normoxia to confirm the effects of LDH.

In conclusion, our experiments suggested that LDH was highly activated in DPCs under hypoxia, resulting in the increase of the several key proteins expression, which led to the improvement of the proliferation capacity of DPCs. As a result, LDH in DPCs could be a novel therapeutic target for treatment hair loss.

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