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Effect of An Atmospheric Plasma Jet on the Differentiation of Melanoblast Progenitor

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[Abstract] Objective: Melanoblasts are the cell source of regeneration for pigment restoration. The ability to differentiate into mature melanocytes is the essential feature of melanoblasts in depigmentation diseases. Cold atmospheric plasma is an ionized gas with near-room temperature and highly reactive species that has been shown to induce stem cell differentiation. The aim of the study was to explore the effect of cold atmospheric plasma on the differentiation of melanoblast progenitor cells. **Methods:** In this study, melanoblasts were exposed to the plasma jet and the cell morphology was observed. The cell cycle and cell proliferation were detected. Furthermore, the cell immunofluorescence and the detection of melanin particle and nitric oxide were carried out to investigate the differentiation of melanoblast progenitor cells. **Results:** Cells that were treated with the plasma had longer and more synaptic structures, and the G1 phase of cell cycle was prolonged in the treated group. More melanin synthesis-related proteins and melanin particles were produced after plasma treatment. Nitric oxide was one of the active components generated by the plasma jet, and the nitric oxide content in the cell culture medium of the treated group increased. **Conclusion:** These results indicate that an increase in nitric oxide production caused by a plasma jet can promote cell differentiation. The application of plasma provides an innovative strategy for the treatment of depigmentation diseases.
Key words: atmospheric plasma jet; melanoblasts; differentiation; nitric oxide, depigmentation disease

Melanoblasts are skin stem cells with differentiation potential and self-renewal ability. Research on melanoblast differentiation is of great significance for the prevention and treatment of hypopigmented skin diseases, such as vitiligo[1, 2]. Melanoblasts differentiate into mature melanocytes, which play important roles in maintaining the periodic coloration of hair[3, 4]. Research has shown that melanocyte stem cells (MSCs) derived from hair follicles could be used in the treatment of clinical depigmentation diseases[1].

Cold atmospheric plasma (CAP) is an ionized gas with temperatures close to room temperature. CAP has been reported to be effective in many biomedical applications including cancer treatment, stem cell differentiation, and skin wound treatment[5–9]. Previous studies have shown that the components produced by a plasma jet include reactive oxygen species, reactive nitrogen, ultraviolet rays, charged ions, and electrons[10]. Nitric oxide (NO) is a highly reactive gas that participates in a variety of physiological processes in biological systems[11–14]. In addition, NO has been reported to play an important role in regulating the proliferation, migration, and differentiation of progenitor and stem cells[9, 15, 16]. Therefore, we explored the effect of plasma on melanoblast function. Treatment with plasma promoted the biological characteristics and differentiation potential of cultured melanoblasts. Thus, an increase in NO production could promote cell differentiation.

1 MATERIALS AND METHODS

1.1 Cell Culture

iMC23 cells were provided by Professor Ke YANG from the Stem Cell Therapy Center of Children’s Hospital of Chongqing Medical University. iMC23 cells are derived from the skin of newborn C57BL/6 mice, and these murine melanoblast lines do not produce melanin particles[17]. Cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM)

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(Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA). The cells were incubated in 5% CO_2 at 37°C.

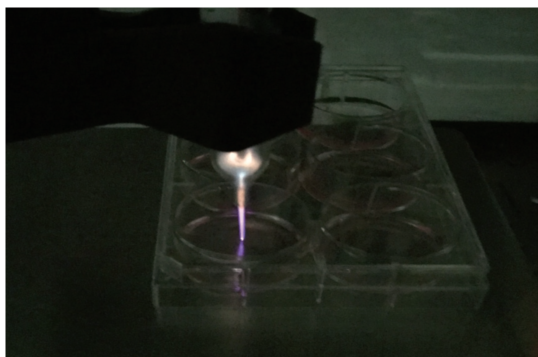
1.2 Plasma Jet Device and Cell Treatment

The single-electrode cold atmospheric plasma jet device was used to treat the iMC23 cells as shown in fig. 1A. The plasma jet device was provided by Professor Zi-lan XIONG from the State Key Laboratory of Advanced Electromagnetic Engineering and Technology, Huazhong University of Science and Technology. The working gas was a mixture of helium (He) and O_2 (0.5%). The device was driven by a pulsed-DC power supply, and the amplitude of the applied voltage, the pulse frequency, and the pulse width were fixed at 6 kV, 8 kHz, and 2 μs, respectively. The cells were divided into three groups: control group, gas flow group, and 60 s plasma treatment group. The distance between the cell culture medium and the syringe nozzle was 15 mm.

1.3 Cell Proliferation Assay

The CCK-8 kit (Yeasen, China) was used to measure cell viability. A total of 100 μL of cell suspension per well was inoculated in the 96-well plates. After the corresponding experimental treatment was applied, the plates were placed in the incubator (37°C, 5% CO_2). Next, 10 μL of CCK solution was added to each well, the culture plates were incubated for 1.5 h, and the absorbance (A) was measured at 450 nm. Each treatment factor was repeated three times.

A



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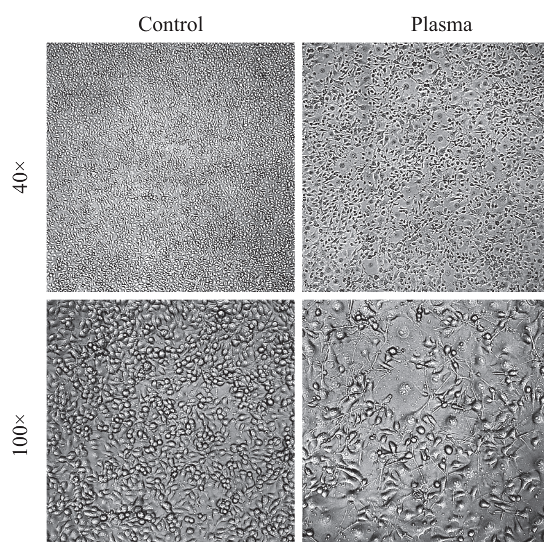


Fig. 1 Plasma treatment changed melanoblast morphology

A: the single-electrode atmospheric pressure room-temperature plasma jet device used to treat iMC23 cells. The working gas was a mixture of helium (He) and O_2 (0.5%). B: comparison of cell morphology changes between the control group and the plasma treatment group. There were more synaptic structures in the cells 48 h after the plasma treatment.

1.4 Cell Cycle Analysis

The cell cycle detection kit was provided by Wuhan Servicebio Biotechnology Company (China), and the experimental procedures were performed in accordance with the requirements of the kit. The cells were washed with the buffer solution and centrifuged after digestion. Then, 1 mL of pre-cooled 70% ethanol was added to fix and resuspend the cells for more than 12 h. On the next day, the fixed cells were centrifuged, resuspended in the buffer solution, and centrifuged again. Next, 0.5 mL of staining buffer, 25 μL of propidium iodide staining solution (20×), and 10 μL of RNase A (50×) were added to each sample in order. Finally, the cells were resuspended and bathed in a 37°C water bath for 30 min. Cell cycle analysis was performed using a flow cytometer at 488 nm wavelength. Each treatment factor was repeated three times.

1.5 Immunofluorescence

The iMC23 cells were fixed in a 4% paraformaldehyde solution for 20 min. After fixation, the cells were treated with 0.1% Triton-100 solution for 10 min. Next, the cells were washed with the buffer solution and blocked with 5% BSA solution for 30 min. Subsequently, the cells were incubated with a freshly prepared primary antibody tyrosinase related protein 2 (TRP-2) (1:100; Abcam, England) working solution for 12 h. On the next day, the cells were washed with the buffer solution and incubated with a freshly prepared fluorescent secondary antibody for 1 h under dark conditions. Finally, DAPI was added to stain the cells at room temperature for 10 min. After the cells were washed, they were observed under a fluorescence microscope and the images were acquired for analysis.

1.6 Melanin Particle Detection

Cells in the logarithmic growth phase were digested and dispersed into a single cell suspension. Totally, 1×10^6 cells were centrifuged at 1000 r/min for 5 min, and then the pellet was washed twice with the buffer solution. After centrifugation, 20 μL of a 1 mol/L NaOH lysate (containing 10% DMSO) was added. Next, the mixture was incubated in the water bath at 80°C for 2 h, and then the A was immediately measured at 405 nm. Each treatment factor was repeated three times.

1.7 Nitric Oxide Detection

To detect the extracellular aqueous NO, conditioned medium from the iMC23 cells was collected as the test sample immediately after the plasma treatment.

We used an NO detection kit (Jiancheng, China) to determine the concentration of extracellular NO according to the manufacturer's instructions.

1.8 Statistical Analysis

GraphPad Prism 6.0 software was used for statistical data analysis. The results are expressed as mean±standard error of the mean (SEM). The Student's *t*-test was used to analyze the quantitative data. $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 Plasma Treatment Changed Melanoblast Morphology

The iMC23 cell culture was exposed to CAP jet as shown in fig. 1A. The mixture of He and O_2 (0.5%) was served as the working gas. The amplitude of the applied voltage, the pulse frequency, and the pulse width were fixed at 6 kV, 8 kHz, and 2 μs, respectively. Cell morphology analysis showed that more synaptic structures were induced 48 h after the plasma treatment (fig. 1B). Changes in cell morphology indicated melanoblast differentiation into mature melanocytes.

2.2 Effect of Plasma Treatment on Cell Cycle and Cell Proliferation

The number of cells in the G0/G1 phase increased after the plasma treatment, which indicated that the cells were blocked in the prolonged G0/G1 phase (fig. 2A). There was a statistically significant difference between the groups (fig. 2B). To detect whether the cells were damaged under the influence of plasma treatment, cell proliferation was continuously monitored. As shown in fig. 2, there was no statistical difference in the cell *A* between the control group and the plasma-treated group (fig. 2C). In addition, the *A* of cells increased with time in the cell proliferation curve, indicating that the number of cells increased with time (fig. 2C). Taken together, these results further suggested that the plasma treatment promoted melanoblast differentiation without inducing cell damage.

2.3 Effect of Plasma Treatment on Melanin Particles and Melanin Synthesis-related Proteins

Melanin particles cannot be generated by melanoblasts since melanoblasts lack functional tyrosinase, but the particles can be produced by mature melanocytes. Therefore, the expression of

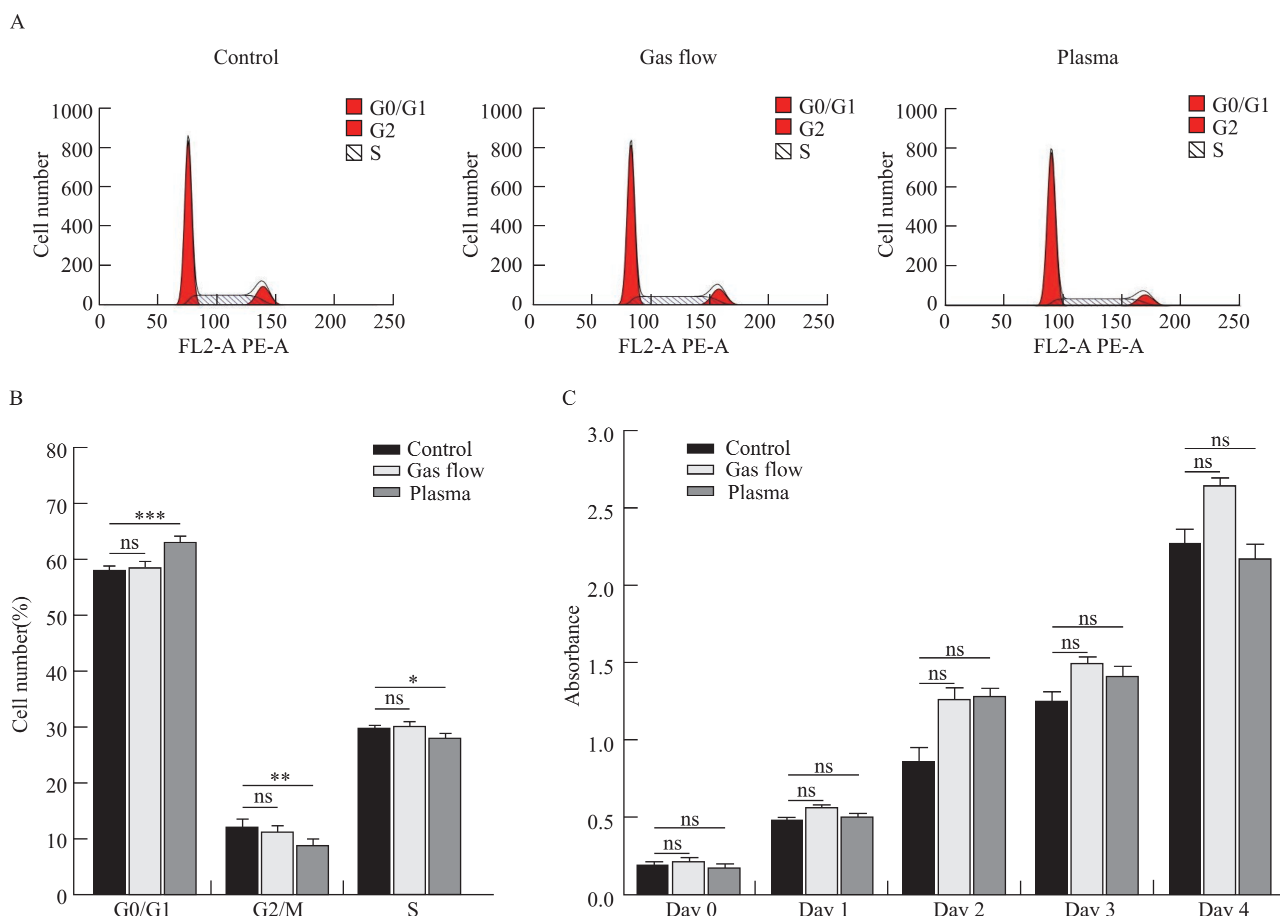


Fig. 2 Effect of plasma treatment on cell cycle and cell proliferation

A: cell cycle in each group. Compared with the control group, the number of cells in the G0/G1 phase in the plasma treatment group increased. B: the difference of cells in the G0/G1 phase between the plasma treatment group and the control group was statistically significant. C: cell proliferation in the groups. There was no statistically significant difference in cell proliferation among the three groups, indicating that plasma treatment did not cause cell damage. ns: not significant. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

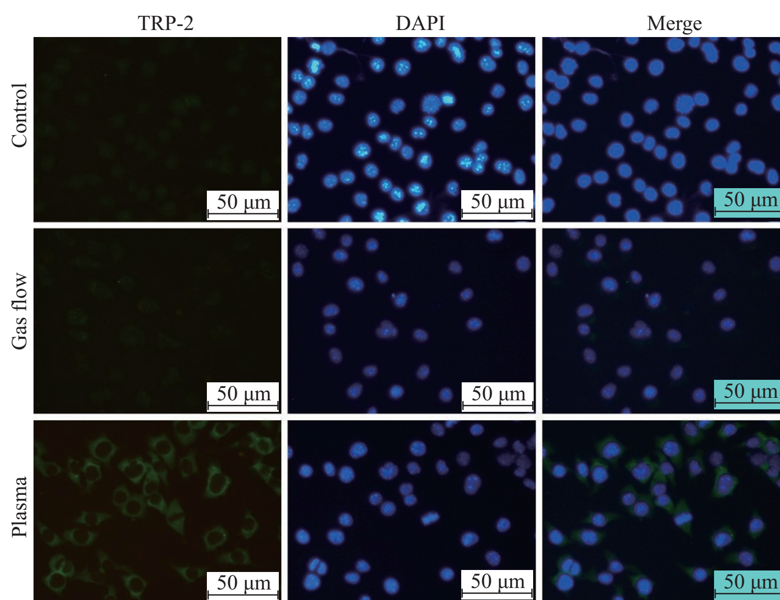
melanin synthesis-related proteins was measured. We found an increase in the expression of TRP-2 after plasma treatment (fig. 3A). Next, the content of melanin particles in each group was measured, and the results indicated that the content of melanin particles significantly increased in the plasma-treated group as compared with that in the control group (fig. 3B). Accordingly, the plasma treatment induced cell differentiation of melanoblasts at the protein level and the cell product level.

2.4 Plasma Treatment Increased Extracellular Nitric Oxide

In our study, the optical emission spectroscopy of the plasma jet was detected, and NO was one of the active species that was generated (fig. 4A). Subsequently, the cell culture media were collected as the test samples. There was a significant increase in the concentration of extracellular NO in the plasma-treated group compared to the control group (fig. 4B). Therefore, these findings indicated that the NO generated by the plasma promoted the differentiation of iMC23 cells.

3 DISCUSSION

In the late embryonic stage, neural crest cells differentiate into melanoblasts, which migrate through the dermis to the epidermis and enter the developing hair follicle[18, 19]. When the melanoblasts enter the hair follicle, some of the cells migrate to the hair matrix area and differentiate into mature melanocytes, while other cells settle in the hair follicle bulge and become MSCs[20, 21]. Mature melanocytes produce pigment and pass it to the keratinocytes, while MSCs are responsible for the



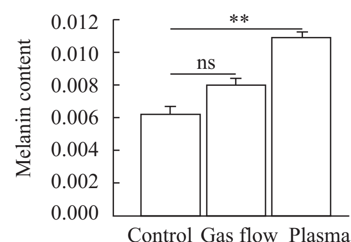
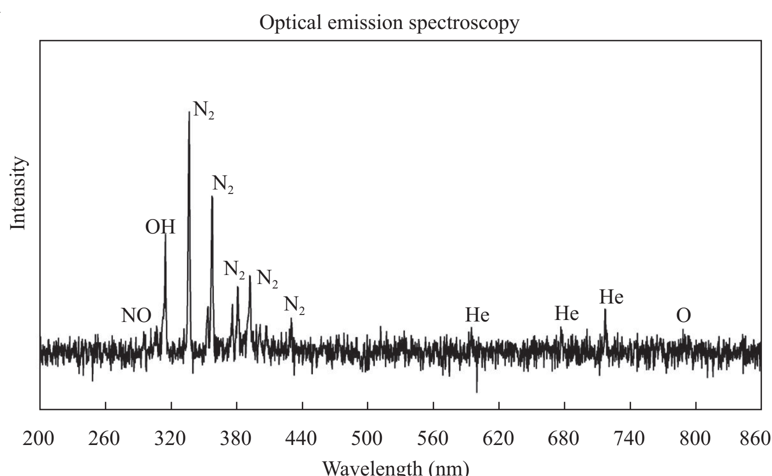


Fig. 3 Effect of plasma treatment on melanin particles and melanin synthesis-related proteins

A: the immunofluorescence results of TRP-2 protein showed that plasma treatment up-regulated the TRP-2 expression. ×200. B: melanin particles in each group. The content of melanin particles in the plasma treatment group increased and the difference was statistically different as compared with the control group. ns: not significant. $^{**}P<0.01$



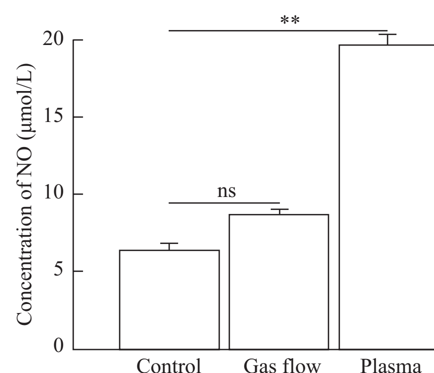


Fig. 4 Plasma treatment increased the extracellular NO

A: optical emission spectroscopy of the plasma jet showed that NO was one of the active species that was generated. B: NO concentration in the cell culture medium of each group. The extracellular NO concentration in the plasma treatment group increased significantly as compared with the control group. ns: not significant. $^{**}P<0.01$

subsequent regeneration of the melanocyte lineage[21, 22]. A lack of melanocytes in the human epidermis is closely related to pigment-deficient diseases such as vitiligo, while the MSC system in hair follicles can be reconstructed by undifferentiated melanoblasts[22–24]. Previous studies suggested that MSCs could be induced to participate in the repigmentation of the epidermis after ultraviolet irradiation[25, 26]. However, the effect of CAP on the differentiation of melanoblasts has not been studied. In this study, plasma treatment increased the dendritic structure of melanoblasts, which indicated morphological differentiation of these stem cells. At the same time, the cells were blocked in the G0/G1 phase, and cell proliferation was not affected in the plasma-treated group. The prolongation of the G1 phase in the stem cells might lead to the accumulation of the key differentiation factors[27]. Therefore, these results indicated that CAP treatment promoted the differentiation of melanoblasts without causing significant damage to the cells.

The application of CAP in biomedicine has been studied for many years, but research on the effect of plasma on stem cell differentiation is an innovative direction[28–30]. Researchers found that the level of differentiation of murine neural stem cells was increased by plasma treatment, and the murine neuroblastoma stem cell showed higher proliferation after CAP treatment[16, 31]. Furthermore, the active effect of CAP on osteoprogenitor cells and hematopoietic stem cells has also been studied[15, 32]. In the current research on the effect of CAP on stem cells, there is no reported relationship between CAP treatment and melanoblasts. Therefore, we considered the effect of plasma treatment on the differentiation of melanoblasts. Melanin can be produced by mature melanocytes, while melanoblasts cannot produce melanin since they lack functional tyrosinase. Therefore, the related proteins of melanin synthesis were detected, and the results showed that plasma treatment resulted in an increase in TRP-2 expression. TRP-2 can form a complex with tyrosinase and play a catalytic role in the process of melanin synthesis[33]. In this study, the melanin particle content increased after plasma treatment. These findings further demonstrated that melanoblasts could differentiate into melanocytes in response to plasma exposure.

It is well known that NO can be induced in the process of plasma discharge, and in recent years plasma-related studies have shown that exogenous NO in plasma was a key factor in directional neuron differentiation[9, 34]. In addition, in a study on the osteoprogenitor cells, CAP increased extracellular NO[35]. In another study, CAP increased NO, which induced an inhibitory effect on cell oxidase in neuroblastoma stem cells[16]. Reports also have shown that exogenous NO might be a potent activator of melanin synthesis[36, 37]. Therefore, in this study, we preliminarily considered whether CAP treatment induced NO production and determined the subsequent effects on melanoblasts. NO production of the plasma jet was detected using optical emission spectrum and we observed an increase in the extracellular NO concentration after the plasma treatment of the cultured cells. This indicated that NO was induced by the plasma treatment and that melanoblast differentiation might be promoted by NO.

In conclusion, plasma-treated melanoblasts underwent changes in morphology and cell cycle. Furthermore, more melanin synthesis-related proteins and melanin particles were produced after CAP exposure. The plasma treatment increased the active species of NO, which further promoted the differentiation of melanoblasts. However, the effect of NO on cell differentiation needs to be further clarified. By studying the differentiation-promoting effect of plasma treatment on stem cells, we showed that plasma treatment might be an innovative therapeutic strategy for promoting melanoblast differentiation to treat depigmentation diseases such as vitiligo.

Conflict of Interest Statement

The authors declare no potential conflicts of interest.

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