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Epimorphin-induced differentiation of human umbilical cord mesenchymal stem cells into sweat gland cells

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Abstract. – OBJECTIVES: Mesenchymal stem cells (MSCs) have the potential for multi-directional differentiation and can be induced to differentiate into sweat gland cells under certain conditions. Epimorphin (EPM) plays an important role in the promotion of epithelial cell morphogenesis; however, its effect on sweat gland-cell differentiation of MSCs remains unknown. The purpose of this study was to investigate how EPM regulates sweat gland cell differentiation of human umbilical cord mesenchymal stem cells (hUCMSCs).

MATERIALS AND METHODS: hUCMSCs were labeled with 5-bromo-2-deoxyuridine (BrdU) before differentiation induction; were cultured in common culture medium, conditioned medium, or EPM-conditioned medium; and then induced to differentiate into sweat gland cells. Five days after induction, the expression rates of the sweat gland-cell antigens cytokeratin 14 (CK14), cytokeratin 19 (CK19), and carcinoembryonic antigen (CEA) in hUCMSCs were detected by flow cytometry, and the messenger ribonucleic acid (mRNA) and protein levels of CK14, CK19, and CEA were determined by reverse transcription polymerase chain reaction (RT-PCR) and western blot, respectively.

RESULTS: hUCMSCs can be induced to differentiate into sweat gland cells in conditioned medium, and expression of CEA was detected by immunofluorescence assay. Flow cytometry results showed that the expression rate of the sweat gland-cell antigens CK14, CK19, and CEA in the conditioned medium were significantly lower than that in the EPM conditioned medium ($p < 0.05$). RT-PCR and western blot results showed that the mRNA and protein levels of CK14, CK19, and CEA in the conditioned medium were all significantly lower than that in the EPM-conditioned medium ($p < 0.01$).

CONCLUSIONS: These results suggest that EPM can effectively induce the differentiation of hUCMSCs into sweat gland cells.

Key Words:
Sweat glands, Umbilical cord, Mesenchymal stem cells, Epimorphin, Cell differentiation.

Introduction

Wound healing has been studied in depth in the field of surgery. Skin defects are frequently caused by burns, chronic ulcers, and trauma, and autologous skin grafting has been commonly used for clinically repairing wound surfaces. However, autologous skin grafting is not beneficial for patients with large areas of severe burns. Due to sweat gland defects or scar hyperplasia, normal skin functions such as the ability to secrete sweat and regulate temperature are severely affected, which in turn affects the quality of patients' lives[1].

Previous investigations have found that bone marrow mesenchymal stem cells (BMSC) can be differentiated into sweat gland cells *in vitro* under certain conditions. Stem cell-derived gland-cell transplantation in skin defect wounds could help regenerate sweat gland tissues and enable sweat secretion[2]. However, some reports have shown that the differentiation rate of BMSC-derived sweat gland cells is very low, and it is difficult to meet the needs of large-area transplantations in case of wounds.

In 1992, Hirai et al[3], for the first time, identified epimorphin (EPM) from embryo stromal-cell surfaces in mice and found that EPM plays an important role in embryonic lung and skin morphogenesis in mice. Current research suggests that EPM can induce liver stem cells to dif-

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ferentiate into liver epithelial cells[4]. In this study, we used EPM to regulate the differentiation of human umbilical cord mesenchymal stem cells (hUCMSCs) into sweat gland epithelial cells *in vitro* under certain conditions. In addition, we observed the role of EPM in differentiation induction. We hope that these findings pave the way for the transplantation of stem cell-derived sweat gland cells in case of wounds.

Methods

Cell Culture

hUCMSC cells and human sweat gland cells were obtained from the Cell Bank of PLA General Hospital, isolated and cultured as previously reported[5,6], and maintained in stem cell medium (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) medium containing 10% fetal bovine serum, 100 units mL^{-1} penicillin, and 100 μg/mL streptomycin sulfate). Sweat gland cells were cultured in a sweat gland cell medium (serum-free keratinocyte media (FSKM; Gibco, Carlsbad, CA, USA), 100 units mL^{-1} penicillin, and 100 μg/mL streptomycin sulfate).

BrdU Labeling

After 3 passages, hUCMSCs were digested and plated onto seeding-treated 6-well tissue culture plates at a concentration of 1×10^5 cells per plate, and 10 μmol/L 5-bromo-2-deoxyuridine (Brdu) was added when the cells reached 40% confluence. Coverslips were removed after incubation for 72 h, washed with phosphate buffered saline (PBS), and fixed by 40 g/L paraformaldehyde for 60 min. The cells were identified with fluorescence staining and flow cytometry (FCM) analysis. hUCMSCs without BrdU labeling were selected as the negative control, and PBS was used instead of the primary antibody in the blank control group.

Induction of Differentiation

To induce hUCMSC differentiation into sweat gland cells, hUCMSCs cultured in the stem cell medium were selected as the normal medium group, and 30% volume of sweat gland-cell supernatant and 50 μg/L epidermal growth factor (EGF) was added to the culture medium of the conditioned medium group. The morphological changes in each group were observed daily. On the 5th day, carcinoembryonic antigen (CEA) antigens in hUCMSCs were detected by immunofluorescence staining, and 100 μmol/L EPM was added to the conditioned medium to make EPM-conditioned medium.

Flow Cytometry Detection

On the 5th day of cultivation, the cells were resuspended with PBS at a concentration of 1×10^6 cells/mL, and 2×10^5 cells were harvested from every tube. Antibodies were added and cultivated, and expression rates for cytokeratin 14 (CK14), cytokeratin 19 (CK19), and CEA in the conditioned medium and the EPM-conditioned medium groups were detected by FCM after washing.

Western Blot

The cells exposed to the various treatments were harvested and lysed using the radio-immunoprecipitation assay (RIPA) methods (with 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1% protease inhibitors). The protein concentration of the supernatants was determined by bicinchoninic acid (BCA) assay, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to methanol-activated polyvinylidene difluoride membranes, which were then sealed at room temperature for 0.5 h. The membrane was incubated at 4°C overnight with antibodies against CK14, CK19, and CEA, washed with Tris-buffered saline and 0.1% Tween (TBST), and developed with an alkaline phosphatase color development kit. The ECL electrochemiluminescence (ECL) reagent kit was used for the visual detection of RIPA. The proteins of interest were visualized using an enhanced chemiluminescence system (Millipore, Billerica, MA, USA). β-actin expression was used as internal control.

RT-PCR

Total ribonucleic acid (RNA) was isolated from the cells in each group using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed in a 20 mL reaction volume with 5 μL total RNA. Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed in a 50 μL reaction volume with upstream and downstream primers for CK14, CK19, and CEA messenger RNA (mRNA). Appropriate amounts of polymerase chain reaction (PCR) amplification products were analyzed by performing 1.5% agarose gel

electrophoresis and the levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. The results were analyzed using a gel imaging analysis system.

Statistical Analysis

The data were analyzed using the SPSS 17.0 software package (SPSS Inc. Chicago, IL, USA). Data are expressed as mean ± standard deviation ($x \pm s$), and a *t*-test for independent samples was used to estimate the difference among the groups. A *p*-value of < 0.05 was considered statistically significant.

Results

Cell Culture and BrdU Labeling

The results for the morphological analysis and identification for hUCMSC and human sweat gland cell were shown in our previous report. After labeling with BrdU for 72 h, hUCMSCs showed substantial growth, as observed under an inverted microscope, and the fluorescence microscopy revealed stained nuclei (Figure 1).

Morphological Observation and Phenotypic Characterization of the Differentiation of hUCMSCs to Sweat Gland Cells

hUCMSC cells in the control group were grown in the common culture medium for five days. After five days, long, spindle-shaped cells without morphological change were observed, and some cells in the conditioned medium group showed morphological characteristics of sweat gland epithelial cells. After BrdU and CEA double-labeling, the control group only showed BrdU signal, but some cells in the conditioned medium group showed double signals (Figure 2).

Effect of EPM on the Differentiation of hUCMSCs to Sweat Gland Cells

As shown in Figure 3, FCM detection results showed that the expression rates of cell antigens CK19, CK14, and CEA in the EPM group (26.58% ± 4.75%, 29.92% ± 2.15%, and 29.68% ± 1.83%, respectively) were significantly higher than that in the conditioned medium (13.65% ± 1.08%, 15.38% ± 1.08%, and 14.65% ± 1.96%, respectively) ($p < 0.05$) (Figure 3).

Effect of EPM on the Gene Expression of Cell Differentiation in Cell-Specific Antigens

EPM and the conditioned medium synergistically induced hUCMSC differentiation into sweat gland cells. mRNAs of the sweat gland cell-specific antigens CK14, CEA, and CK19 were analyzed by fluorescence quantitative PCR. The relative expression levels of the CEA gene in the control, conditioned medium, and EPM-conditioned medium groups were 1.00 ± 0.09, 25.40 ± 6.19, and 66.61 ± 12.30, CK19 being 1.00 ± 0.13, 33.12 ± 7.43, and 88.44 ± 16.49, CK14 being 1.00 ± 0.15, 27.86 ± 6.43, and 69.35 ± 14.26, respectively. These results show that the expression levels were significantly higher in the condi-

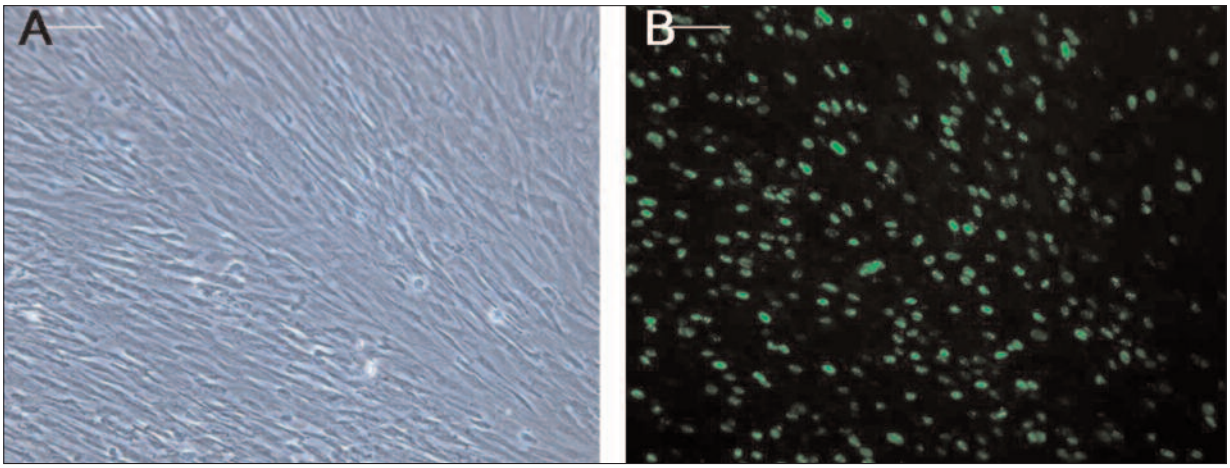

Figure 1. Characteristics of HUCMSCs. ***A,*** The growth of HUCMSCs after labeling with BrdU for 72h under the bright field. ***B,*** Immunofluorescence staining of HUCMSCs after labeling with BrdU for 72h under fluorescence microscope. Abbreviation: hUCMSCs, Human umbilical cord-derived mesenchymal stem cells. BrdU, 5-bromo-2-deoxyuridine.

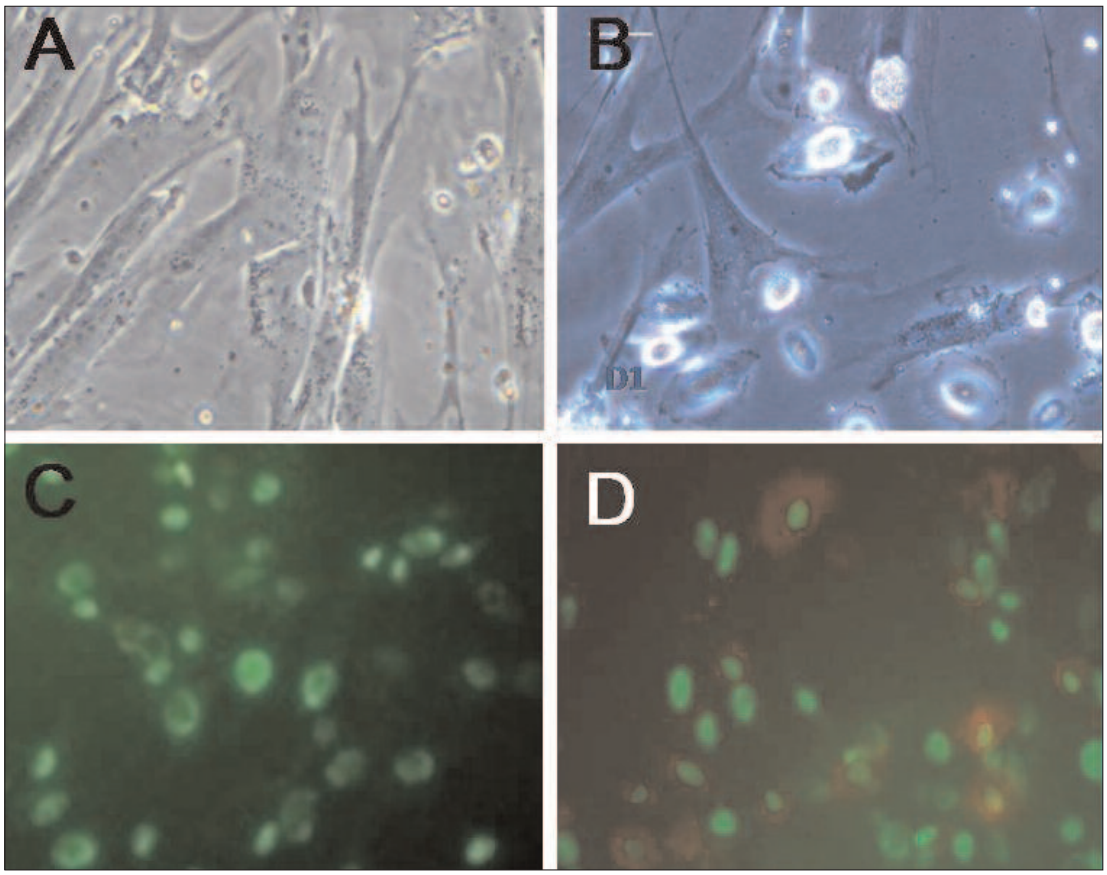

Figure 2. Morphological observation and phenotypic characterization of the differentiation of hUCMSCs to sweat gland cells. ***A,*** Morphological characterization of hUCMSC culture in the common medium for five days. ***B,*** Morphological characterization of hUCMSC culture in the conditioned medium for five days. ***C,*** BrdU and CEA double-labeling of hUCMSC culture in the common medium for five days. ***D,*** BrdU and CEA double-labeling of hUCMSC culture in the conditioned medium for five days. Abbreviation: hUCMSCs, Human umbilical cord-derived mesenchymal stem cells. BrdU, 5-bromo-2-deoxyuridine. CEA, carcinoembryonic antigen.

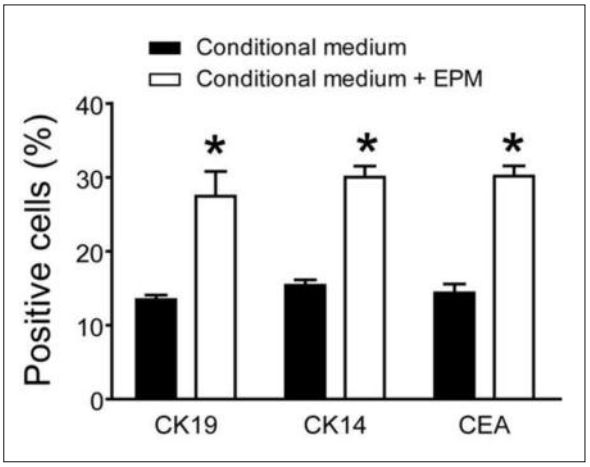

Figure 3. Comparison of the differentiation rate of hUCMSCs to sweat gland cells between EPM group and the conditioned medium group. *Abbreviation:* hUCMSCs, Human umbilical cord-derived mesenchymal stem cells. EPM, epimorphin.

tioned medium group than in the control group ($p < 0.01$), and the expression was significantly higher in the EPM-conditioned medium group than in the conditioned medium group ($p < 0.01$) (Figure 4).

Effect of EPM on the Protein Expression of Cell Differentiation in Cell-Specific Antigens

EPM and sweat gland-cells synergistically induced hUCMSC differentiation into sweat gland cells in the conditioned medium. The relative expression levels of the CEA in the control, conditioned medium, and EPM-conditioned medium groups were 0.08 ± 0.01, 1.00 ± 0.28, and 4.02 ± 0.44, CK19 being 0.09 ± 0.00, 1.00 ± 0.06, and 2.19 ± 0.10, CK14 being 0.08 ± 0.02, 1.00 ± 0.16, and 3.14 ± 0.48, respectively. The sweat

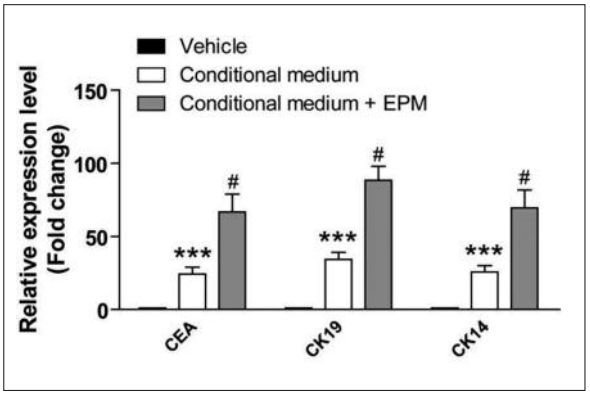

Figure 4. The mRNA expression of CEA, CK19 and CK14 in hUCMSCs. Abbreviation: hUCMSCs, Human umbilical cord-derived mesenchymal stem cells. mRNA, messenger ribonucleic acid. CEA, carcinoembryonic antigen. CK19, cytokeratin 19. CK14, cytokeratin 14.

gland cell-specific antigens CK14, CEA, CK19 were analyzed by western blotting. The expression levels in the conditioned medium group were significantly higher than that in the control group ($p < 0.01$), and the EPM-conditioned medium group showed significantly higher expression than the conditioned medium group did ($p < 0.01$) (Figure 5).

Discussion

Sweat gland defects after severe large-area burns lead to the loss of normal abilities of skin, such as the ability to secrete sweat and regulate temperature, and can severely affect the quality of patients' lives[7]. Therefore, methods to repair defective sweat glands in patients with severe large-area burns have considerable clinical significance. It has been found that BMSCs can be induced through subjecting to heat-shock to produce phenotypic structures and characteristics of sweat glands cells, and these cells have been used for the repair and regeneration of damaged sweat gland. However, due to the low differentiation rate, this technology cannot meet the needs of sweat gland repair and regeneration for patients with large-area skin defects.

In order to improve stem cell-derived sweat-gland regeneration therapy, in the current study, we used EPM to regulate hUCMSC differentiation into sweat gland cells and increase the differentiation rate by using umbilical cord Wharton's jelly-derived mesenchymal stem cells (MSCs), which have low immunogenicity and strong proliferative abilities. hUCMSCs have characteristics similar to those of BMSCs, and they express MSC surface antigens, cluster of

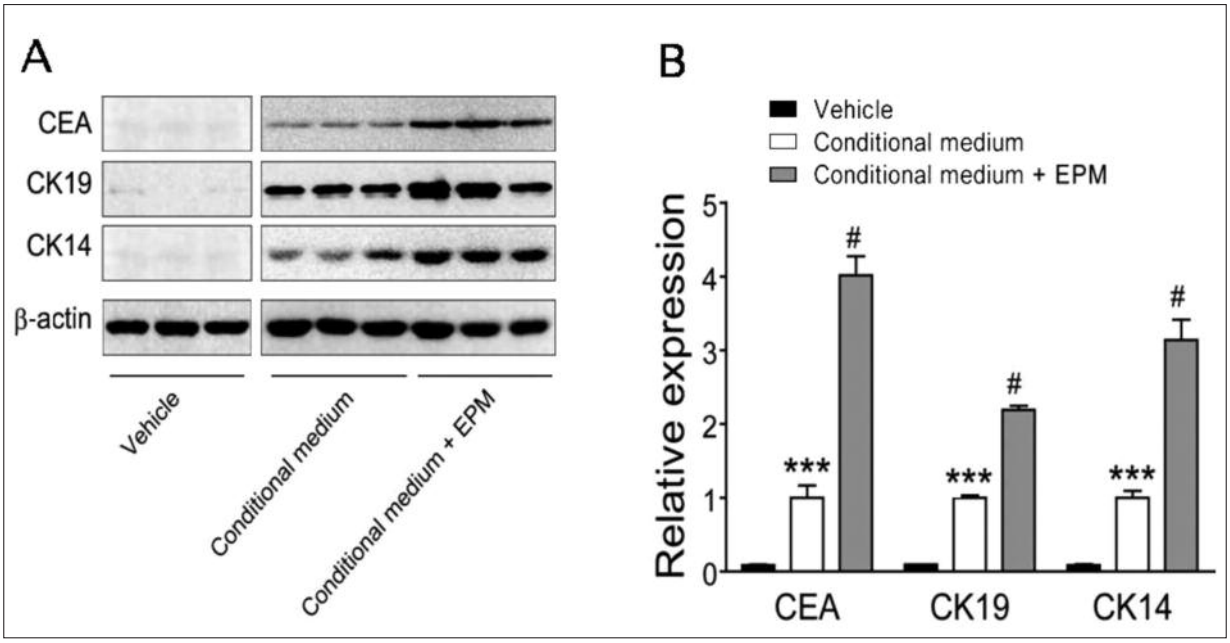

Figure 5. Comparison expression of CEA, CK19 and CK14 antigen among three groups. ***A,*** Western blot showing the protein band of CEA, CK19 and CK14 antigen, respectively. ***B,*** Histogram comparison; The expression of CEA, CK19 and CK14 antigen in EPM group increased significantly compared with the other two control groups. *Abbreviations:* CEA, carcinoembryonic antigen. CK19, cytokeratin 19. CK14, cytokeratin 14. EPM, epimorphin.

differentiation 44 (CD44) and cluster of differentiation 105 (CD105), but not with the hematopoietic cell marker cluster of differentiation 34 (CD34) and the sweat gland cell-specific marker CEA[8]. CK14, CK19, and CEA can be used as sweat gland epithelial cell antigen markers; in particular, CEA is expressed in adult-skin sweat glands with high specificity[9].

In this study, under conditioned medium induction, some hUCMSCs stained positive for CEA in the immunofluorescence analysis. Flow cytometry showed that the expression rates of CK19, CK14, and CEA in the EPM-conditioned medium group were significantly higher than that in the conditioned medium group ($p < 0.05$). RT-PCR results showed that the expression levels of CK19, CK14, and CEA genes in the conditioned medium group were significantly higher than that in the control group ($p < 0.01$), and the EPM-conditioned medium group showed significantly higher expression than the conditioned medium group did ($p < 0.01$). Western blot results revealed that the expression levels of CK19, CK14, and CEA proteins in the conditioned medium group were significantly higher than that in the control group ($p < 0.01$), and it was significantly higher in the EPM-conditioned medium group than in the conditioned medium group ($p < 0.01$). Therefore, EPM significantly increased the differentiation rate of hUMSCs into sweat gland cells.

Previous research has shown that EPM plays an important role in epithelial morphogenesis through epidermal-mesenchymal interactions that mediate typical morphogenesis, such as cutaneous branches and cavity formation in the lungs[3], pancreas[10], breasts[11], gallbladder epidermis cavity[12], endothelial tubular structure formations[13], etc. Its underlying mechanism may be that EPM through activation of mitogen-activated protein kinase (MAPK) signaling pathways (MAPK/ERK kinase (MEK) and extracellular signal-regulated kinase (ERK)) and epidermal growth factor receptor (EGFR) phosphorylation[14].

In the present study we used EPM to promote the differentiation of hUCMSCs into sweat gland epithelial cells, and our results are consistent with those of previous reports, showing that EPM can induce liver stem cells to differentiate into epithelial cells. Therefore, EPM can improve the rate of differentiation of MSCs into sweat gland cells. We hope that our findings pave the way for the transplantation of stem cell-derived sweat gland cells in case of wounds.

Conclusions

hUMSCs can differentiate into sweat gland cells under certain conditions at a low differentiation rate. The differentiation rate can be increased by adding 100 μmol/L EPM into the induction solution, and our results suggest that EPM can promote MSCs to differentiate into sweat gland cells. The present study can help improve the low differentiate rate of stem cells, and these finding provide useful information for the future clinical applications of stem cell-directed differentiation and transplantation.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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