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Wnt3a promotes human umbilical cord mesenchymal stem cells to differentiate into epidermal-like cells

T.-J. SUN[1,2], R. TAO[3], Y.-Q. HAN[4], G. XU[5], J. LIU[5], Y.-F. HAN[5]

[1]Department of Burn and Plastic Surgery, Hainan Branch of People's Liberation Army General Hospital, Sanya, People's Republic of China
[2]Department of Burn and Plastic Surgery, Burns Institute, The First Affiliated Hospital of PLA General Hospital, Beijing, People's Republic of China
[3]Department of Plastic Surgery, PLA General Hospital, Beijing, People's Republic of China
[4]School of Electrical and Information Engineering, Wuhan Institute of Technology, Wuhan, People's Republic of China
[5]Department of Plastic Surgery, Affiliated Beijing Shijitan Hospital, Capital Medical University, Beijing, People's Republic of China

Tian-Jun Sun and *Ran Tao* should be regarded as co-first authors

Abstract. – OBJECTIVE: Mesenchymal stem cells are a population of pluripotent cells that can differentiate into epidermal-like cells under certain conditions. Wnt3a can promote the proliferation and differentiation of stem cells. However, the role of Wnt3a in the differentiation of human umbilical cord mesenchymal stem cells (hUCMSCs) into epidermal-like cells is unknown.

MATERIALS AND METHODS: Third-generation hUCMSCs were cultured in normal medium, epidermal stem cell-conditioned medium, and conditioned medium with added Wnt3a. After culturing for 5 days, the expression of cytokeratin 19 (CK19), an antigen specific for epidermal-like cells, was assessed by immunofluorescence and flow cytometry. The expression of CK19 mRNA was confirmed by reverse transcription-polymerase chain reaction(RT-PCR), and β-catenin expression was detected by western blot.

RESULTS: hUCMSCs differentiated into epidermal-like cells when cultured in conditioned medium as shown by positive immunofluorescence staining for CK19. Flow cytometry showed that the number of cells positive for CK19 in the epidermal stem cell-conditioned medium group was significantly higher than that of control group, but lower than that of the Wnt3a-conditioned group ($p < 0.05$). RT-PCR showed that the expression level of CK19 mRNA in the conditioned medium group was significantly lower than that of the Wnt3a group ($p < 0.01$). Westernblots showed that the expression of β-catenin in the conditioned medium group was significantly lower than that of the Wnt3a group ($p < 0.01$).

CONCLUSIONS: These results suggest that Wnt3a can effectively promote the differentiation of hUCMSCs into epidermal-like cells.

Key Words:
Epidermal-like cells, Umbilical cord, Mesenchymal stem cells, Wnt3a, Cell differentiation.

Introduction

Wound repair has been increasingly studied in the field of general surgery. Skin defects caused by burns, chronic ulcers, trauma, and other types of damage are quite common in surgery clinics. Autologous skin transplantation is commonly used in wound repair. However, patients with large areas of damaged skin, such as from burns or skin defects, lack sufficient skin for such transplants. The recent development and application of skin tissue engineering technology has provided new insights to improve the quality of wound repair and to solve the problem of sufficient autologous skin[1].

The application of skin tissue for the timely covering of a wound can reduce wound shrinkage and minimize scar hyperplasia, thereby improving the ultimate appearance of the skin. This is the most basic approach to treat skin defects, including large burn areas and chronic ulcers. Stem cells have been the most active area of tissue engineering research, and can contribute the healing of injured skin appendages[2].

Corresponding Author: Yan-Fu Han, MD; e-mail: dochyf@163.com

Theoretically, human umbilical cord mesenchymal stem cells (hUCMSCs) can differentiate into epidermal cells, fibroblasts, and sweat gland epithelial cells[3], and are regarded as an ideal source of seed cells. However, the *in vivo* and *in vitro* differentiation rates of hUCMSCs into epidermal-like cells is very low and cannot meet the clinical needs for the repair of major skin defects. Finding ways of increasing the differentiation rate is of great clinical interest.

Previous research indicated that Wnt3a can promote the proliferation and differentiation of mesenchymal stem cells[4]. However, little is known about whether Wnt3a can promote the differentiation of hUCMSCs into epidermal-like cells. In this study, the ability of Wnt3a to stimulate the differentiation of hUCMSCs into epidermal-like cells was determined.

Materials and Methods

Cell Culture

hUCMSCs were obtained from the Cell Bank of the Chinese PLA General Hospital. These cells were cultivated as described previously[5,6] in stem cell growth medium (SCGM), containing Dulbecco's modified Eagle medium/F12 (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 1 × 10 U/L penicillin, and 0.1 g/L streptomycin. hUCMSCs were passaged to the third generation, and then used in differentiation experiments.

Induction of Differentiation

hUCMSCs cultivated in SCGM were used as the normal culture medium group, in Epidermal stem cell-conditioned medium (normal culture medium with added epidermal growth factor, basic fibroblast growth factor, insulin, retinoic acid, and $CaCl_2$) used as the conditioned medium group, in conditioned medium supplemented with 100 ng/mL Wnt3a used as the Wnt3a group. Cell morphological changes were observed daily for 5 days. Expression of the CK19 cell antigen biomarker was detected by immunofluorescence staining.

Flow Cytometry

Cells were cultivated for 5 days and then washed with phosphate-buffered saline (PBS) and suspended at 10^6 cells/mL. Cells (2×10^5) were aliquoted into separate tubes and mixed with antibodies. After incubation for 24 hours at room temperature, cells were washed with PBS and expression of the cell antigen biomarker CK19 was detected by flow cytometry.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

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. RT-PCR was performed in a 50 μL reaction volume with upstream and downstream primers for CK19 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.

Western Blot Analysis

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 (SDSPAGE) 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 β-catenin, 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.

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

Morphological Observations in Cell Culture

hUCMSCs were isolated using an improved enzyme digestion method[4]. After 3 days, hUCMSCs exhibited rod-like and irregular shapes, and grew in a dispersed pattern. After the third generation, fibroblast-like hUCMSCs could be found with a parallel arrangement or growing in a spiral pattern (Figure 1).

Morphological Observations and Phenotypic Characterization of Epidermal-like Cells Derived from hUCMSCs

hUCMSCs in the control group, grown in normal culture medium, exhibited a long spindle-like shape that was unchanged during the 5-day culture period. Some cells in the conditioned medium group exhibited morphological characteristics of epidermal-like cells and were positive for CK19, as determined by immunofluorescence staining (Figure 2).

Wnt3a Enhances the Differentiation of hUCMSCs Into Epidermal-like Cells

Flow cytometry results showed that the percentage of CK19-positive cells in the Wnt3a-conditioned group (46.53 ± 6.85%) was significantly higher than that of the single-conditioned group (24.68 ± 4.08%) ($p < 0.05$), and the control group (1.68 ± 0.75%) ($p < 0.01$). The percentage of CK19-positive cells in the conditioned medium group was also significantly higher than that in the control group ($p < 0.01$) (Figure 3).

Wnt3a Regulates the Expression of a Characteristic Antigen for Differentiated Cells

Both Wnt3a and epidermal stem cell-conditioned medium induced hUCMSCs to differentiate into epidermal-like cells. The expression of the CK19 antigen was analyzed by quantitative PCR. Relative levels of *CK19* mRNA in the control group, conditioned medium group, and Wnt3a group were 1.00 ± 0.05, 26.8 ± 7.2, and 65.6 ± 10.3, respectively. These results showed that the *CK19* expression level in the conditioned medium group was significantly higher than that in the control group ($p < 0.01$), and that *CK19* expression was significantly higher in the Wnt3a group than in the conditioned medium group ($p < 0.01$) (Figure 4).

Wnt3a Regulates the Expression Level of β-catenin in Differentiated Cells

Both Wnt3a and conditioned medium could induce hUCMSCs to differentiate into epidermal-like cells. Western blots showed that the relative expression levels of theWnt3a downstream protein, β-catenin, were 0.92 ± 0.12, 3.75 ± 0.26, and 7.84 ± 1.45, in control, conditioned, and Wnt3a group, respectively. The β-catenin level in the conditioned medium group was significantly higher than that of the control group ($p < 0.01$), and the level in the Wnt3a group was significantly higher than that in the conditioned medium group ($p < 0.01$) (Figure 5).

Discussion

Wound repair has been a subject of high interest in the fields of general surgery, as skin de-

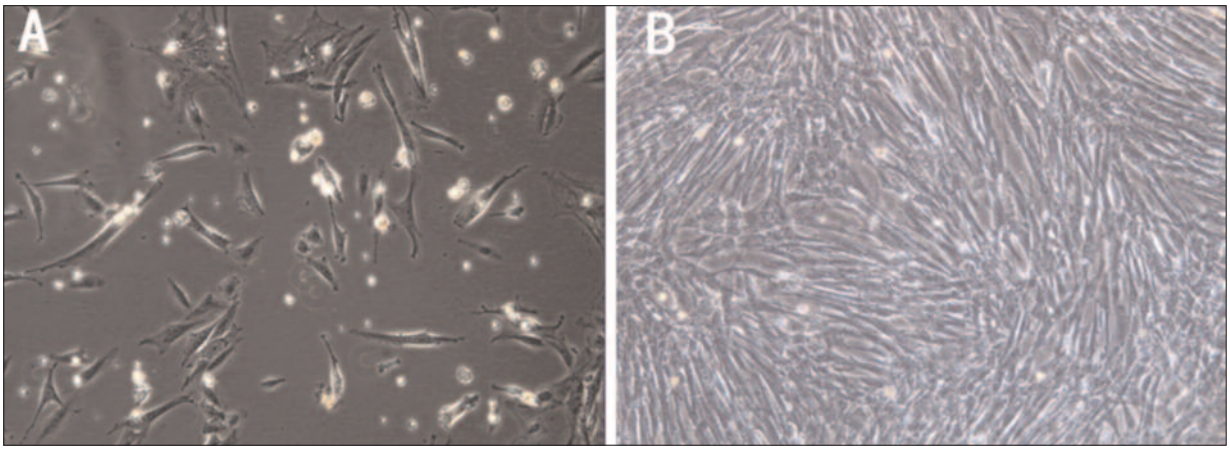

Figure 1. Characteristics of HUCMSCs. ***A,*** The growth of HUCMSCs after primary culture for 3 days. ***B,*** The fussion growth of HUCMSCs at passage 3. Abbreviation: hUCMSCs, Human umbilical cord-derived mesenchymal stem cells.

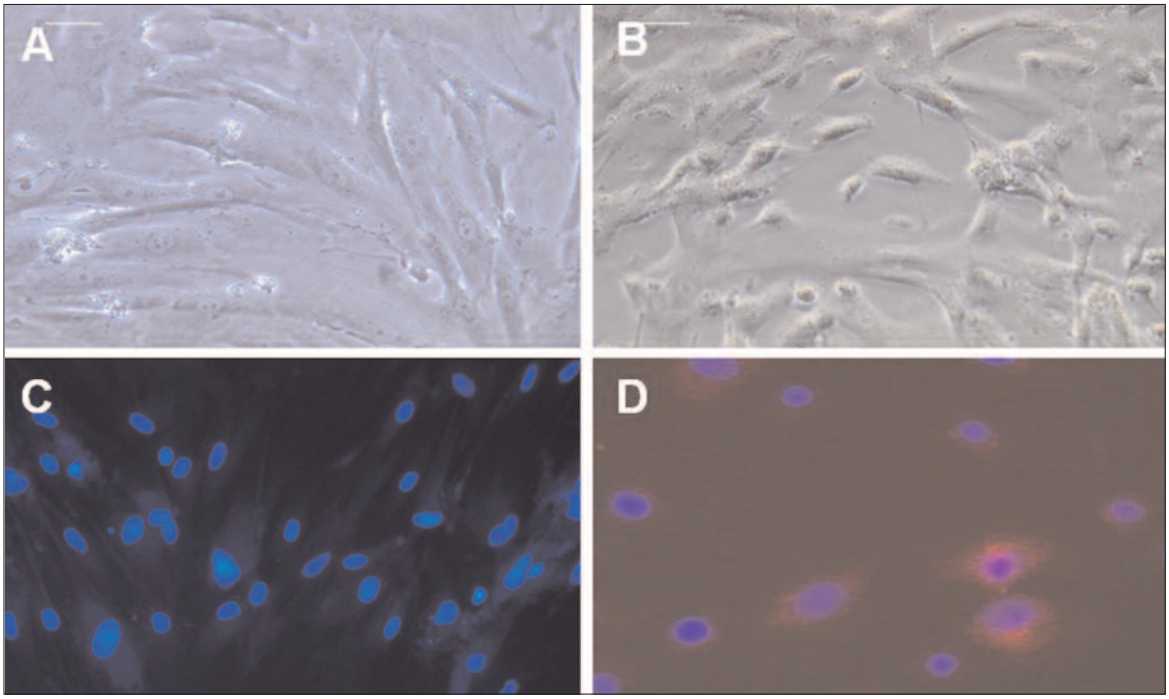

Figure 2. Morphological observation and phenotypic characterization of the differentiation of hUCMSCs to epithelial-like cells. ***A,*** Morphological characterization of hUCMSC culture in the common medium for five days. ***B,*** Morphological characterization of hUCMSC culture in the conditioned medium for five days. ***C,*** DAPI and CK19 double-labeling of hUCMSC culture in the common medium for five days. ***D,*** DAPI and CK19 double-labeling of hUCMSC culture in the conditioned medium for five days. Abbreviation: hUCMSCs, Human umbilical cord-derived mesenchymal stem cells. DAPI, 4',6-diamidino-2-phenylindole. CK19, cytokeratin 19.

fects caused by burns, chronic ulcers, trauma, etc. are quite common in clinical settings, and autologous skin transplantation is commonly used in wound repair. However, patients with large area severe burns and skin defects are severely lack of autologous skin for such transplantation. Therefore, development of improved methods to repair large areas of damaged skin has important clinical significance. Previous research has shown that bone marrow mesenchy-

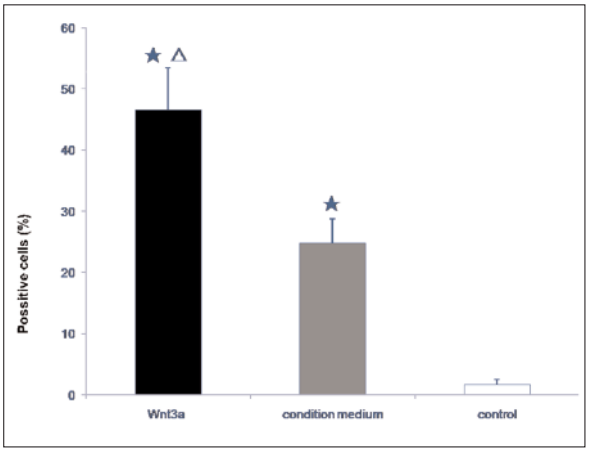

Figure 3. Comparison of the differentiation rate of hUCMSCs to epithelial-like cells among control group, the conditioned medium group and Wnt3a group. Abbreviation: hUCMSCs, Human umbilical cord-derived mesenchymal stem cells.

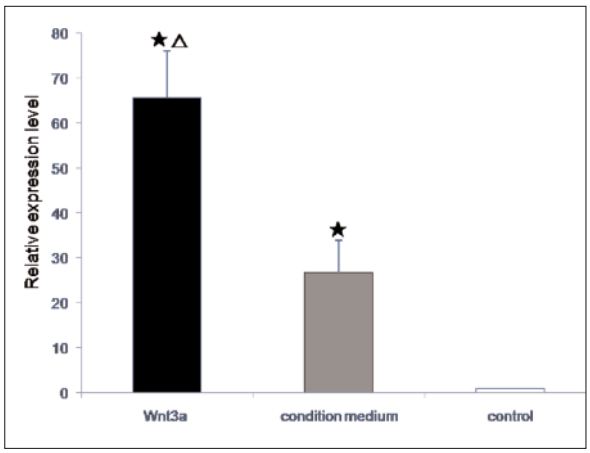

Figure 4. The mRNA expression of CK19 in hUCMSCs. Abbreviation: hUCMSCs, Human umbilical cord-derived mesenchymal stem cells. mRNA, messenger ribonucleic acid. CK19, cytokeratin 19.

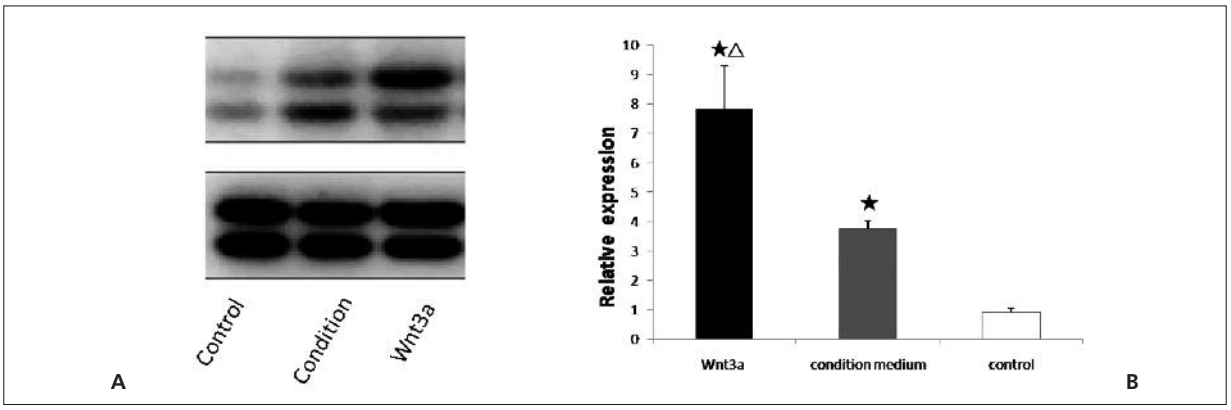

Figure 5. Comparison expression of β-catenin protein among three groups. ***A,*** Western blot showing the protein band of β-catenin, respectively. ***B,*** Histogram comparison; the expression of β-catenin in Wnt3a group increased significantly compared with the other two control groups.

mal stem cells (BMSCs) can be induced to present the phenotypic structure and properties of epidermal-like cells[7]; accordingly, BMSCs have been used for the repair and regeneration of skin damage[8]. However, because of their low differentiation rate, this approach is unable to meet the skin repair and regeneration needs of patients with large areas of damaged skin.

To improve skin regeneration through the use of stem cell therapy technology, in the present study we examined the ability of Wnt3a to differentiate hUCMSCs into epidermal-like cells. Umbilical cord Wharton's jelly-derived MSCs (with low immunogenicity and strong proliferative ability) were selected as the source of hUCMSCs. These hUCMSCs have similar characteristics to BMSCs, expressing the MSC surface antigens CD44 and CD105, but not the hematopoietic cell biomarker CD34 or the epidermal-like cell biomarker CK19[9]. CK19, which is expressed with high specificity in epidermal cells, can thus be used as a specific antigen marker of these cells[10]. Our results showed that some hUCMSCs were positive for CK19 immunofluorescence staining when cultured in conditioned medium. However, flow cytometry showed that Wnt3a-conditioned medium resulted in significantly more CK19-positive cells than the single-conditioned medium group ($p < 0.05$). RT-PCR confirmed that the expression level of *CK19* mRNA in the conditioned medium group was significantly higher than that of the control group ($p < 0.01$), and that in the Wnt3a group was significantly higher than both the control and the conditioned medium groups ($p < 0.01$). The western blot results showed that the level of β-catenin in the conditioned medium group was significantly higher than that of the control group ($p < 0.01$). Furthermore, in the Wnt3a group, β-catenin expression was significantly higher than in both the control and the conditioned medium groups ($p < 0.01$). Overall, our results showed that Wnt3a significantly increased the differentiation of hUMSCs into epidermal-like cells.

Wnts are powerful proteins in the regulation of stem cell differentiation and proliferation and in skin self-stability and regeneration[11]. Wnt3a has been confirmed as a typical member of the classical Wnt signaling protein family. Exogenous Wnt3a can activate the Wnt/β-catenin signaling pathway both *in vivo* and *in vitro*, and plays an important role in regulating the proliferation and differentiation of various stem cells[4]. β-catenin is the key signal transduction molecule of the classic Wnt signaling pathway[12-14]. The activity of downstream GSK3β is inhibited when Wnt ligands combine with their receptors, further hindering the phosphorylation and degradation of β-catenin, and upregulating the expression of intracellular β-catenin, which translocates into the nucleus where it regulates gene expression. Our results showed that Wnt3a can promote the differentiation of hUMSCs into epidermal-like cells in the presence of conditioned medium, and can upregulate the expression of β-catenin, for example, c-Myc[15]. Together, these data indicate that exogenous Wnt3a promotes the differentiation of hUMSCs into epidermal-like cells in *vitro* through the activation of Wnt/β-catenin signaling pathways. However, the regulatory role of Wnt/β-catenin signaling in cell fate determination remains controversial, the activated Wnt/β-

catenin signaling being able to enhance cell proliferation but also induce apoptosis in a variety of cells[16]. The apoptosis effect of Wnt on hUMSCs remains to be further studied.

The ability of Wnt3a to regulate the differentiation of hUMSCs is consistent with a previous study of its effects in promoting BMSC alveolar epithelial cell differentiation[17]. This provides an experimental basis for the application of stem cell-derived epidermal-like cells in skin transplantation research.

Conclusions

hUMSCs can be induced to differentiate into epidermal-like cells under certain conditions. The differentiation rate is normally very low but can be increased *in vitro* by adding Wnt3a to a conditioned culture medium. The current study suggests that Wnt3a can promote the differentiation of hUMSCs into epidermal-like cells through activation of the classic Wnt signaling pathway. This effect has the potential to improve the low differentiation rate of stem cells, and may be beneficial to the clinical application of stem cells in transplantation in the future.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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