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In Vitro Culture and Multipotency Evaluation of Broiler Umbilical Cord Mesenchymal Stem Cells

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HIGHLIGHTS

- Broiler-derived UMSCs line was successfully isolated for the first time.
- UMSCs possessed stemness characteristics, such as hereditary stability, self-renewal ability, and expression of specific markers.
- UMSCs possessed multilineage potential and could be differentiated into cells of three layers.

Abstract: Umbilical cord mesenchymal stem cells (UMSCs) from fetal tissues have become a valuable source of cell regeneration therapy due to their low immunogenicity, high plasticity and antimicrobial peptides. Most researches about UMSCs mainly focused on humans, mice, rabbits and other mammals, but it is still unknown in broiler. To the best of our knowledge, we first established a broiler-derived UMSCs line from noncontroversial Wharton's jelly of umbilical cord. The detection of biological characteristics showed UMSCs showed the morphological characteristics of fibroblasts and could be cultured to 26 generations in vitro, and maintained diploid chromosomes (2n=78) by G-banding analysis. Cell cycle of UMSCs showed the rate of G0/G1 was about 76.7-80.9%. Moreover, UMSCs could express mesenchymal markers (CD29, CD90, CD71, and CD44), but not hematopoietic marker (CD34) and endothelial marker (CD31). Additionally, UMSCs could be efficiently induced to transdifferentiate into three germ layer cells, such as adipocytes, osteoblasts, chondrocytes, hepatocytes, epithelia, and neurogenic cells. These results demonstrated UMSCs have the essential characteristics of MSCs, and can be used as an attractive candidate for cell regenerative therapy and the study of regulatory mechanisms associated with selective differentiation.

Keywords: UMSCs; broiler; biological characteristics; multilineage potential; regenerative therapies.

INTRODUCTION

MSCs, origined from mesodermal, also known as mesenchymal stromal cells, which retained self-renewal and multipotential differentiation capability in vitro [1]. Unlike the totipotent embryonic stem cells, MSCs are characterized by easy acquisition, rapid proliferation and low tumorigenicity. More importantly, they are not involved in ethical issues [2, 3]. In view of these practical advantages, MSCs have attracted great interest as an ideal seed cells in tissue reconstruction. Bone marrow MSCs (BMSCs), as the first type of MSCs, have been regarded as the golden standard for research in regenerative medicine and tissue engineering [4]. However, BMSC is not always available for many reasons, such as the incidence of a high degree of microbial exposures, the low yield of cell numbers, and the decrease in proliferation and differentiation capacity as the donor ages [5]. Therefore, it is urgent to find an alternative stem cell type.

Various tissues considered as medical waste, such as birth-related placenta, umbilical cord (UC), umbilical cord blood (UCB), and amnion and amniotic fluid are also the abundant source of MSCs [6-12]. Moreover, these MSCs proliferate faster than BMSCs, which is attributed to their earlier stages of development. Furthermore, due to their minimal costs and little ethical issues, UMSCs have attracted increasing attention for research in recent years and can be treated as an appealing candidate cell.

Recently, many studies associated with UMSCs had been carried out, but few research has been conducted on broiler [13-15]. To date, owing to the stable genetic performance and excellent productivity, broiler have been a vital chicken breed for development of the global economy. The present study aimed to isolate the UMSCs from the Wharton's Jelly of 14-day-old broiler embryos, and detect their biological characteristics, such as phenotype, proliferation, and multipotency.

MATERIAL AND METHODS

UMSCs isolation and in vitro culture

The UC were obtained from broiler embryos (14-day-old, 12 samples) and cleaned six times with PBS under sterile conditions. Then, after removed amnion layer and vascular tissue, the cords were chopped into about 1 mm^3 pieces and digested with 0.1% type IV collagenase (Invitrogen, Carslbad, CA, USA) for 25 min at 37°C, followed by termination of the enzymatic activity with fetal bovine serum (FBS; Biochrom, Germany).Then, the cell suspension was plated into 6-well microplates at 1×10^5 cells/mL, and incubated at 37 °C with 5% CO_2 atmosphere. At about 80%–90% confluence, the primary cells were digested with 0.125% trypsin-EDTA regeant for passaging at a ratio of 1:1. Then, programmed freezing broiler-derived UMSCs suspension (2×10^6 cells/mL) were achieved using a freezing container (Nalgene), subsequently transferred to a liquid nitrogen storage system for long-term preservation.

Culture systems optimization and G-banding analysis

Three culture systems were tested as shown below using the UMSCs from passage 5 (P5), system I (DMEM/F-12, 10% FBS, 10 ng/mL bFGF), system II (DMEM/F-12, 15% FBS, 10 ng/mL bFGF), system III (DMEM/F-12, 15% FBS, 10 ng/mL bFGF, 10 ng/mL LIF, 10 ng/mL SCF). Cells were reseeded in 96-well plates at a density of 1×10^4 cells/well. Proliferation abilities in three different culture systems were produced by judging the average count data. After prepared and fixed, metaphase chromosomes spreads of UMSCs were stained by Giemsa according to previous studies [16].

RT-PCR analysis

The total RNAs were extracted from UMSCs and induced cells using Trizol reagent (Invitrogen, USA). RT-PCR analysis of cultured UMSCs group and differentiation-induced UMSCs group was performed according to our previous studies [16]. For PCR reactions, the primers designed are presented in Table 1.

Table 1. Primer sequences of specific genes used in this study.

Gene	Primer sequences (5'-3')
GAPDH	F: TGAAAGTCGGAGTCAACGGA
	R: GCACGATGCATTGCTGACAA
CD29	F: AATGCCAAGTGGGATACGGG
	R: TCAGACCCCTGGCACTGATT
CD44	F: TCCTGCAAGTACTAGGTCTACG
	R: TGACTAGCTTTTTCTTCTGCCCA
CD71	F: GTTATCGTGGACGAATCGAGC
	R: TCATCGTTCCACACTTTATCCA
PPAR-γ	F: TAAAGTCCTTCCCGCTGACC
	R: TAATCTCCTGCACTGCCTCC
LPL	F: CGATCCCGAAGCTGAGATGAA
	R: CCAATCAATGAACATGGCAACA
SPP1	F: ATTGAGGATGACGCCACCG
	R: CGCTCTCTAGCGTCTGGTTG
COL1A1	F: AGGGAAACAACGGTGCTCAAG
	R: AGATGGACCACGGCTTCCAAT
CK19	F: GGAGTGTCAGTCTCTTCCGC
	R: GGCGGTCATTCAGGTTTTGC
CK18	F: ACAAAGGAGGTGGACACAGC
	R: GACACCACTTTGCCATCCAC
COL2A1	F: ATTAAAGATGTTGTAGGACCCCGAG
	R: CTCCACCCGCCTTCTCATCG
SOX9	F: ACCAGAACTCCGGCTCCTAC
	R: CTTTAAGGCCGGGTGAGCTG
AFP	F: TCGGGCACGCTTGATCTTTA
	R: GACTTCTTGGCAGAGCCAATC
ALB	F: AGGCAGTTGCCATGATCACA
	R: CAGGGGCATACAAGAAGGGG
MAP2	F: ATCAATGGAGAGCTGTCGGC
	R: CGGAATTCCATCTTCGTGGC

[1] F: forward; R, reverse.

Immunofluorescence staining and flow cytometry analysis

Upon proliferating to about 60-70% confluency, UMSCs of P5 were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.25% Triton X-100 for 15 min, and then blocking with 2% BSA and 10% normal goat serum for 30 min. After that, anti-CD29, anti-CD71, anti-CD44, anti-CD90, anti-CD34, anti-CD31, anti-CK19, anti-MAP2 and anti-NESTIN (1:300, Bioss, China) were incubated with the cells overnight at 4 °C. Subsequently, cells were incubated in the diluted secondary antibody for 1 h at 37 °C in the dark according to instructions. DAPI was used to counterstain the nuclear. Fluorescence signals were detected using a confocal microscopy (Mshot, Guang zhou) and quantified by video densitometric analysis.

After stained with Propidium Iodide (PI), the distributions of cell cycle at different passages were analyzed by FACS (BD, FACSVerse, USA). Expression of specific cell markers were further identified by FACS with primary antibodies (anti-CD44, anti-CD29, anti-CD71 and anti-CD90) according to previous studies [17].

Adipogenic, osteogenic, and chondrogenic differentiation

UMSCs were incubated in adipogenic induction media (AID; DMEM/F-12, 10% FBS, 1 mmol/L dexamethasone, 8 μg/mL insulin, 200 μM indomethacin and 0.5 mM IBMX) for 14 days to assess the potential of differentiation into adipogenic lineages [18, 19]. Adipogenesis was showed by the experiments of Oil Red staining and adipocytes specific genes (LPL and PPAR-γ) detection. For osteogenic differentiation, the cells in a 6-well plate were incubated by osteogenic induction media (OID), which consisted of DMEM/F-12 and supplemented with 0.1 mM dexamethasone, 10 mM β-glycerophosphate and 50 μM vitamin C. The differentiation potential of UMSCs to osteoblast was verified by analyzing the calcium accumulation stained by Alizarin Red test and osteogenic markers (SPP1 and COL1A1) expression. For chondrogenic

differentiation, UMSCs were cultured in chondrogenic induction medium (CID) supplemented with 1% ITS, 0.1 μm dexamethasone, 50 μg/mL L-proline, 50 μg/mL L-ascorbic acid, 0.9 mM sodium pyruvate, and 10 ng/mL TGF-β3. Chondrogenesis was processed by the experiment of Alcian Blue positive staining and detection of chondrogenic markers (SOX9 and COL2A1) expression.

Epithelial, hepatogenic and neural differentiation

Epithelial-like cell differentiation were exposed to epithelia inducing medium (EID) supplemented with 10% FBS, 20 ng/mL EGF, 10 ng/mL KGF, 60 ng/mL IGF-2 and 10 ng/mL HGF for 14 days [20]. After reaching 60-70% confluence, the UMSCs at P5 were cultured by hepatocyte inducing medium (HID) containing DMEM/F-12, 5% FBS, 20 ng/mL HGF, 10 ng/mL EGF, 20 ng/mL FGF-4, and 1% ITS [21-22]. Hepatic glycogen deposits were detected by Periodic Acid Schiff (PAS) staining. To identify the neuronal differentiation potential, UMSCs were treated with neurocyte inducing medium (NID) for 14 days according to our previous studies [16]. And, the multipotency of UMSCs to neurocytes were assayed by the expression of neurocyte-related genes (NESTIN, and MAP2) using immunofluorescence detection and RT-PCR analysis.

Statistical analysis

Experimental data are expressed as the means ± standard deviation (SD) from at least three independent experiments. The Student's t-test was used to determine statistical significance ($P < 0.05$). The software of GraphPad Prism 7.0 (San Diego, CA, USA) was used for statistical analysis and the generation of graphs.

RESULTS

Culture, morphology, and karyotype of UMSCs

Primary UMSCs consisted of heterogeneous population with round, flattened and spindle shape under the inverted microscope. After 3-4 passages, cultured cells possess homogeneity traits and exhibited a fibroblast-like morphology in a swirl-like pattern. Moreover, the UMSCs were passaged every 2 days and highest number of passages was P26. Shortly afterwards, the majority of UMSCs exhibited extensive vacuolization or karyopyknosis (Figure 1A). According to the results of tests on culture system, system III differs from other two systems for generation time of about 2.3 days ($P < 0.01$). Chemistry reagents b-FGF, SCF, LIF and FBS concentrations (15%) could be conducive to UMSCs proliferation. And, culture system III is believed to be a better alternative to other culture systems and used to culture UMSCs in further trials (Figure 1B).

Chromosome G-Banding analysis verified the frequencies of UMSCs cell line with 2n=78 were 96.4% (P5), 94.2% (P10) and 92.2% (P15), respectively, which indicated that there was no cross-contamination, and supported the that the UMSCs cell line was reproducibly diploid (Figure 1C). Flow cytometric experiments confirmed that most of UMSCs entered a phase of G0/G1 (76.7-80.9%), and a few proliferating cells population entered the S/M phase (Figure 1D). This phenomenon was closely related to specific cell cycle stages of standard MSCs. Moreover, the proportion of UMSCs in different cell phases is not markedly different among P5, P10 and P15.

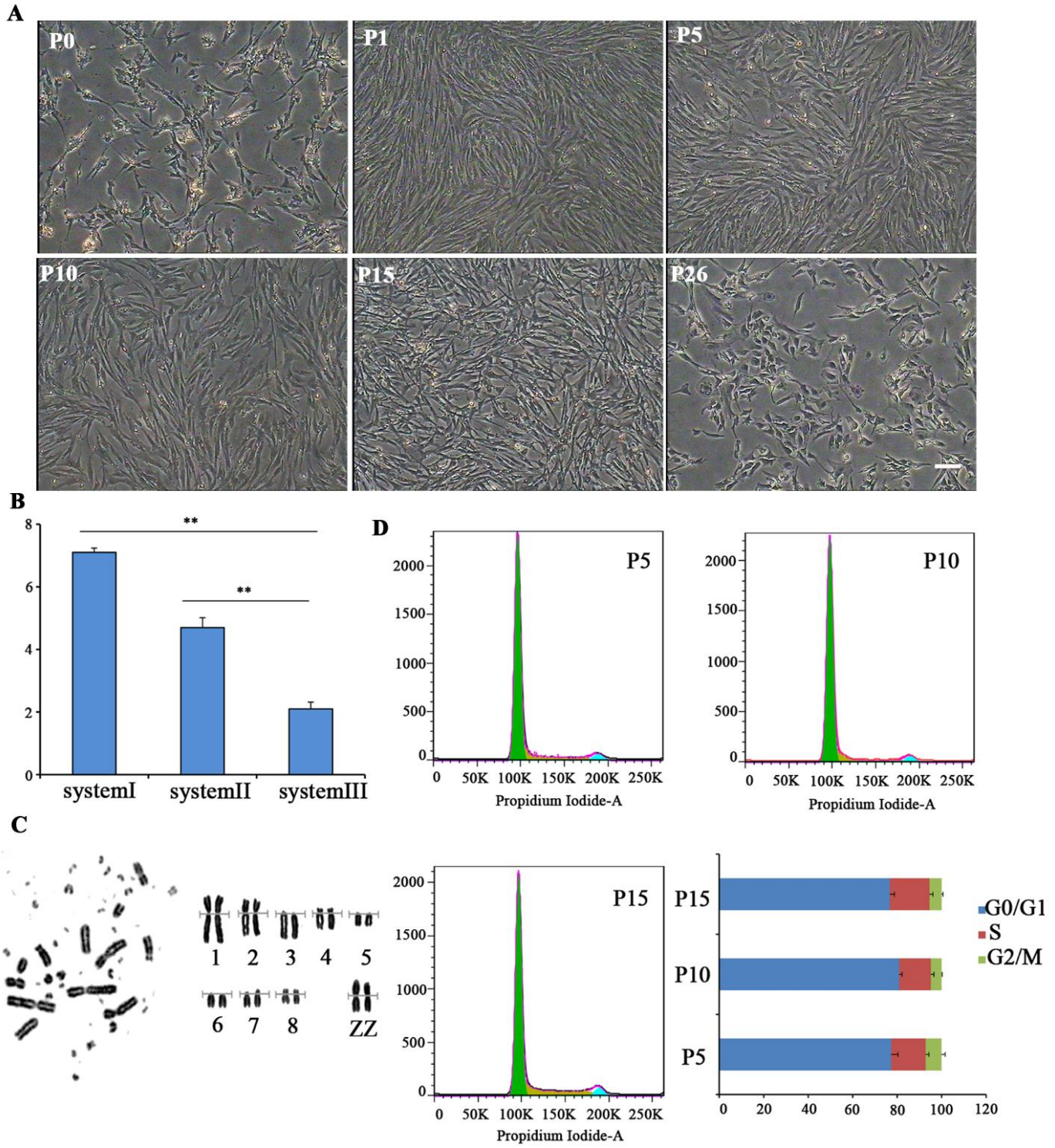

Figure 1. Morphology, G-banding and cell cycle analysis. (A) Cell morphology observation of UMSCs. (B) Assessment of UMSCs proliferation ability in three different culture systems. **P<0.01. (C) Chromosome G-banding of UMSCs. The chromosomes number was 2n = 78. (D) The proportion of UMSCs (P5, P10 and P15) entered G0/G1 phase by cell cycle analysis. Scale bars, 50 μm.

Characterization of stem cell markers in UMSCs

The result of immunofluorescence showed that UMSCs could clearly express the positive surface markers of MSCs (CD29, CD44, CD71 and CD90), but not CD34 and CD31 (Figure 2A). Moreover, FACS analysis demonstrated that the viable cells which express above-mentioned molecular surface markers of MSCs occupied more than 90% (Figure 2C). In accord with the results of immunofluorescence, RT-PCR further demonstrated fundamental characteristics of UMSCs, which could express mesenchymal marker genes CD29, CD44, and CD71 (Figure 2B).

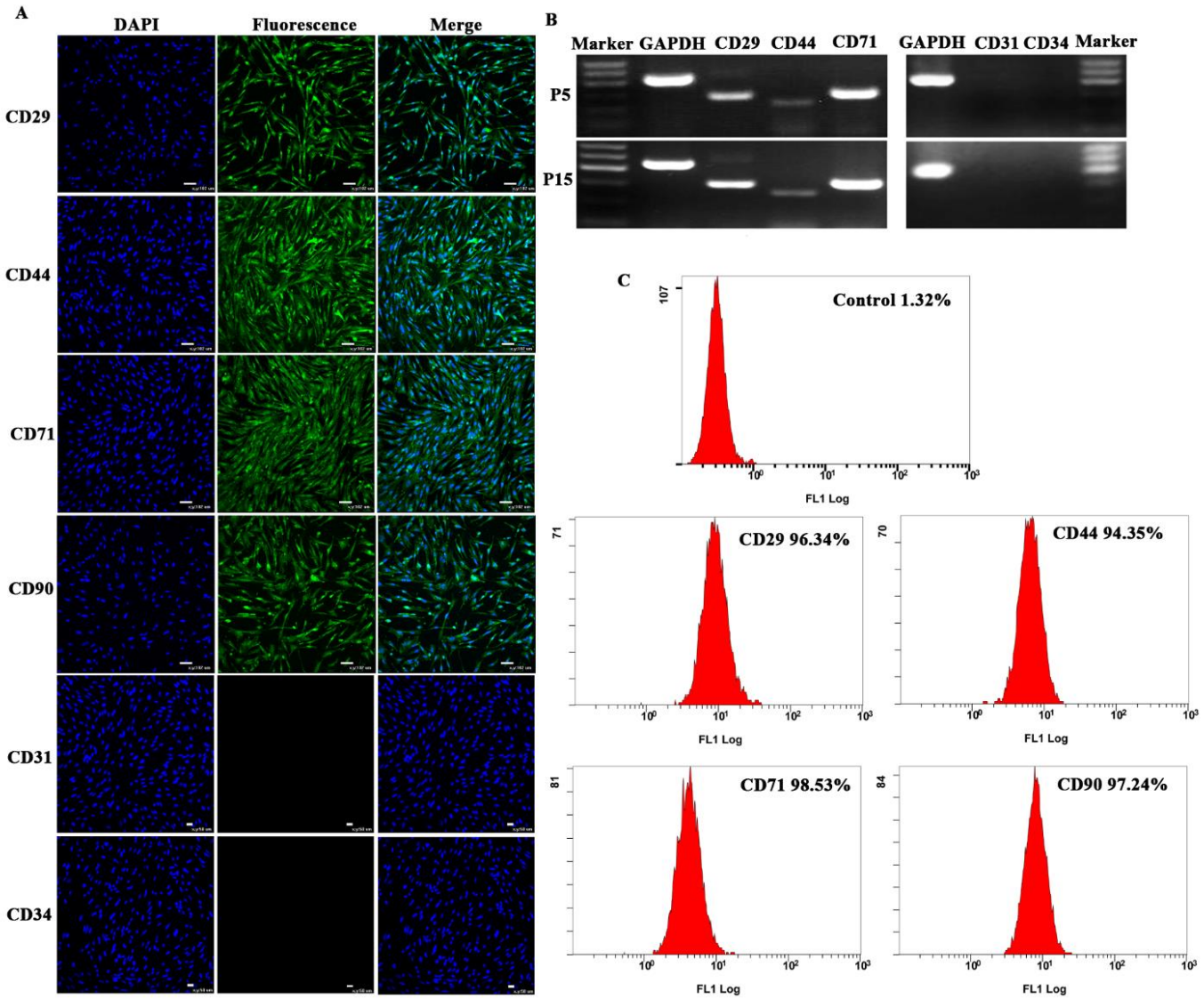

Figure 2. Cell surface markers expression. (A) Immunofluorescence identification of surface markers in UMSCs. (B) The expression of surface marker genes in UMSCs by RT-PCR. (C) Flow cytometric analysis of surface antigens in UMSCs.

Adipogenic, osteogenic, chondrogenic differentiation of UMSCs

When cultured in AID for 3 days, the UMSCs grew slowly, and the morphology of cells became flat with shiny intracellular lipid droplets. Over time, the number and size of oil droplets gradually increased. Large fat droplets were positive for oil red O solution staining by day 14 (Figure 3A). Moreover, RT-PCR analysis identified the expression of specific marker genes of adipogenesis, including PPAR-γ and LPL (Figure 3B). After induction with OID for 14 days, the appearance of UMSCs was transformed from spindle to polygonal shape, and many mineralized nodules became evident. The numbers of the larger calcium deposits nodules increased and differentiated osteocytes were positive by Alizarin Red S staining (Figure 3C). Next, the expression of osteoblasts specific genes SPP1 and COL1A1 was observed in the experimental group treated with OID by RT-PCR analysis (Figure 3D). Under CID for 14 days, the UMSCs tended to proliferated and formed the cluster-like aggregation gradually. Chondrogenic differentiation of UMSCs was further showed using Alcian Blue staining experiment (Figure 3E). Besides that, RT-PCR analysis further demonstrated the differentiation through the expression of COL2A1 and SOX9 (Figure 3F).

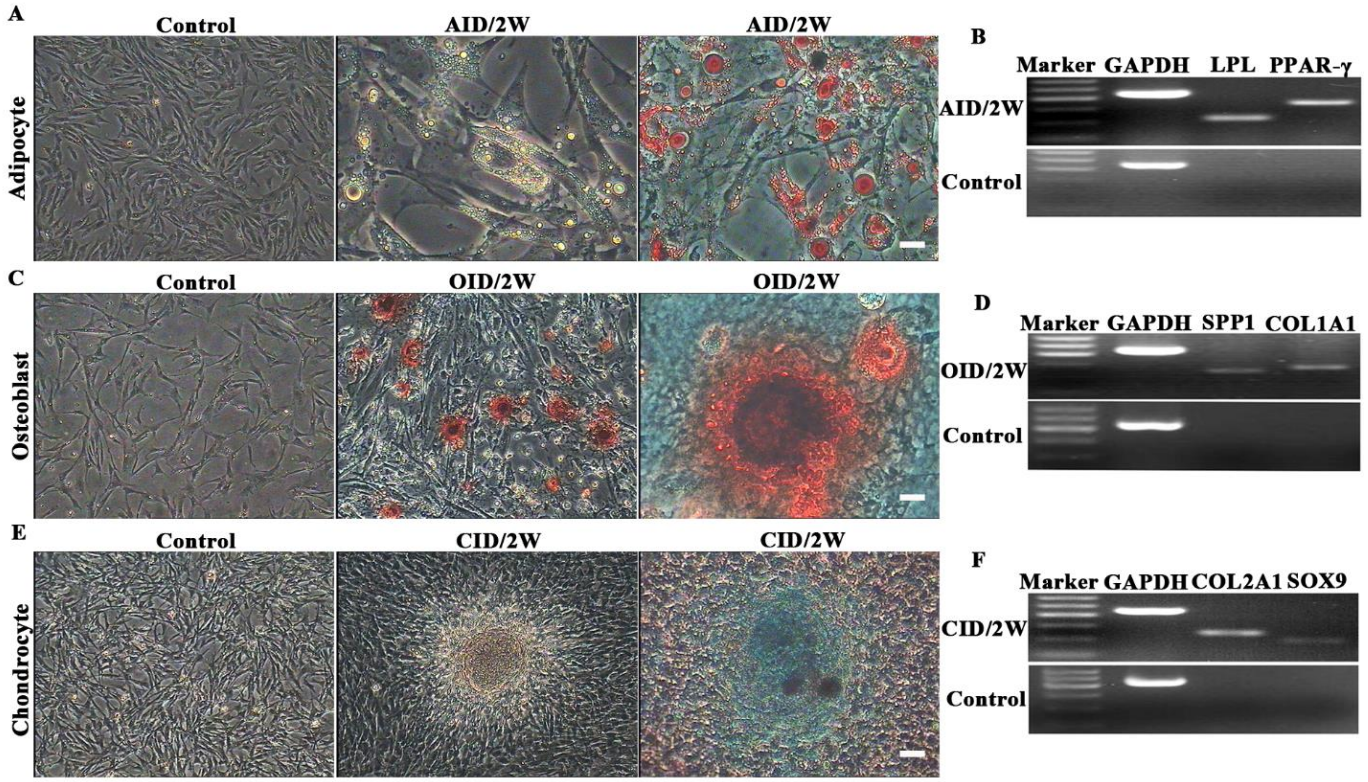

Figure 3. Adipogenic, osteogenic, and chondrogenic differentiation. (A) Lipid droplets were stained using Oil Red O. (B) RT-PCR analysis of PPAR-γ, and LPL expression in differentiated cells. (C) Calcium nodules were indicated by Alizarin Red S staining. (D) RT-PCR analysis of COL1A1 and SPP1 expression in differentiated cells. (E) Chondrogenic differentiation was confirmed by Alcian Blue staining. (F) RT-PCR analysis of COL2A1 and SOX9 expression in differentiated cells. Scale bars, 20 μm.

Epithelial, hepatogenic, and neural differentiation of UMSCs

When cultured in EID, the morphology of UMSCs gradually transformed from fibroblastic into flatter over time. After 14 days of culture, UMSCs acquired a cobblestone shape and formed single-layer (Figure 4A). And, the expression of epithelial cells marker genes CK18 and CK19 were significant increased by RT-PCR analysis (Figure 4B). In addition, CK19 was also validated in differentiated cells by immunofluorescence detection (Figure 4A).

Hepatic differentiation was evaluated by the morphological changes and the ability of glycogen-storage of UMSCs at P5 during the induction. The fibroblast-like morphology of broiler UMSCs were transformed into distinctive polygonal shape of mature hepatocyte-like cells under HID conditions for 21 days (Figure 4C). Moreover, the ability of glycogen synthesis of the induced cells was confirmed by Periodic Acid-Schiff (PAS) staining (Figure 4C). The expression of the hepatocytes specific markers genes AFP and ALB were further validated for differentiated cells (Figure 4D).Under NID induction for 14 days, immunofluorescence evidenced the expression of MAP2 and NESTIN in the induced cells (Figure 4E). Furthermore, the expression of neural marker MAP2 gene was also confirmed by RT-PCR (Figure 4F).

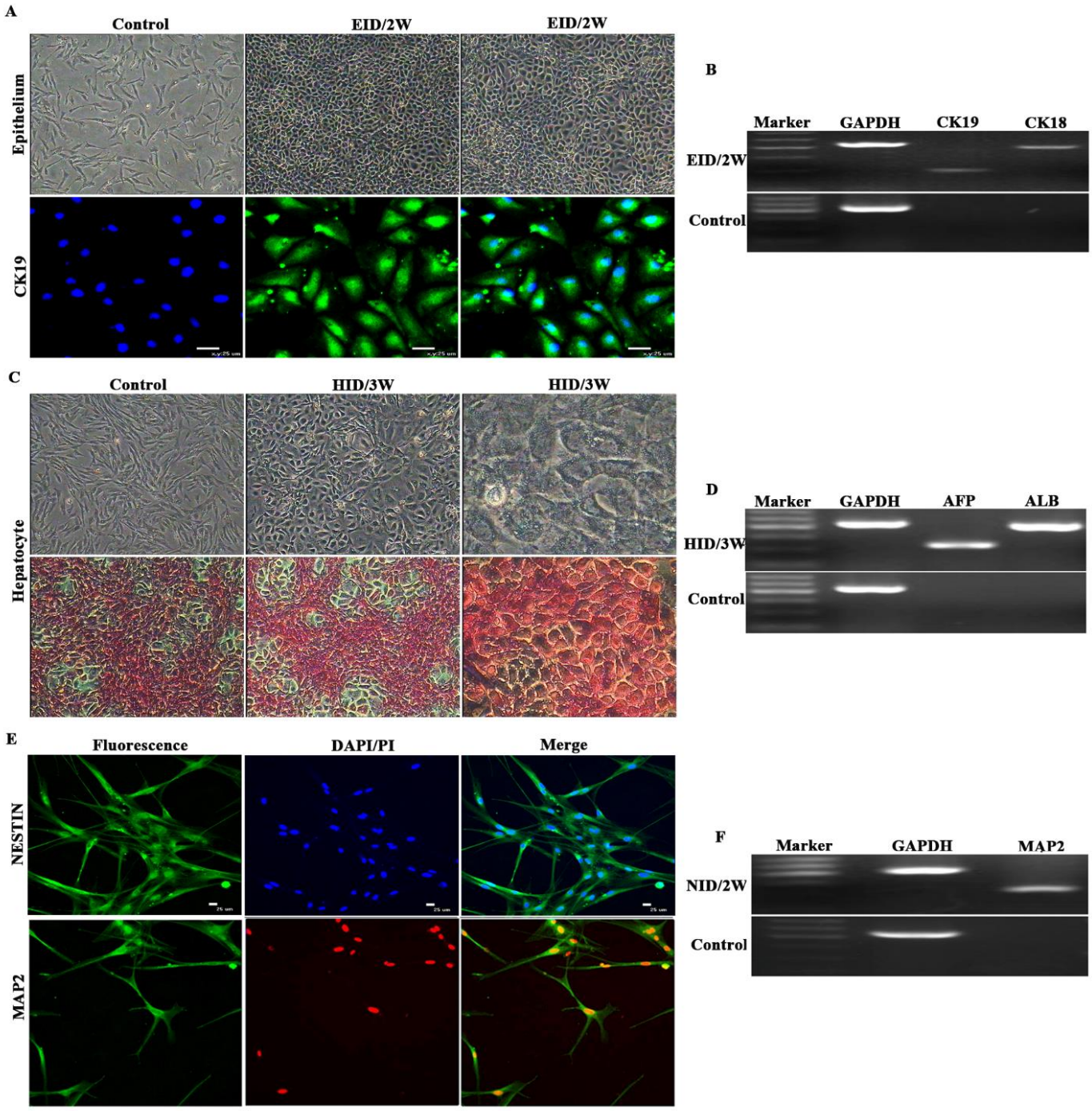

Figure 4. Epithelial, hepatogenic and neural differentiation. (A) The expression of CK19 in induced cells of UMSCs using immunofluorescence. Scale bars, 50 μm. (B) RT-PCR analysis of CK18, and CK19 expression in differentiated broiler UMSCs. (C) Hepatic glycogen deposits were detected by PAS staining. Scale bars, 20 μm. (D) RT-PCR analysis of AFP and ALB expression in differentiated broiler UMSCs. (E) Immunofluorescence analysis of MAP2 and NESTIN expression in differentiated broiler UMSCs. (F) RT-PCR analysis of MAP2 expression in differentiated broiler UMSCs.

DISCUSSION

Stem cell-based therapy is a fascinating new therapeutic approach for clinical treatment of a variety of diseases, especially advanced diseases [23]. MSCs, appealing multipotent candidate cells using for cell-based therapies, could be isolated from almost all tissues and organs compared with other stem cells. Among these potential sources, UMSCs is considerably populated with MSCs and serves as a better alternative [24, 25]. Whereas, many factors could do a great influence on UMSCs generation and culture such as period of pregnancy, anatomical region of cord, process of transportation and conservation [5].

In this study, we successfully isolated the UMSCs from noncontroversial Wharton's jelly of umbilical cord of broiler embryos, and also identified their self-renewing ability, multi-differentiating potential in vitro. In comparison to conventional explant method, enzymatic digestion method was well employed to yield UMSCs and enhanced the rate of successive expansion of MSCs. Therefore, we consider that collagenase digestion is a better alternative way to purify the explant-derived endothelial cells from Wharton's jelly. The culture medium was DMEM/F-12 supplemented with 15% FBS, 10 ng/mL bFGF, 10 ng/mL LIF, 10 ng/mL SCF. These bioactive factors in serum can promote the proliferation of UMSCs in a robust and three-dimensional state of growth [7]. LIF are directly related to the maintenance of pluripotency [26]. To sustain proliferation, the addition of 10 ng/mL SCF was required to provide nutrients [27]. Besides that, bFGF has the effects of promoting mitosis and stimulating anabolism [28]. This culture system was potential contributors to promote the better growth of UMSCs. No dramatic morphological changes were identified during the following serial passage from P1 to P25, and the undifferentiated cell state were stable. However, UMSCs gradually entered into the aging period by P26, probably due to enzyme digestion in subculture, growth characteristics or culture conditions. However, the reasons for the aging of UMSCs cells may need further research. The multipotency of stem cells is the most important prerequisites for autologous cell therapy. UMSCs were effectively induced to differentiate into mesoblastic origin cells (adipocytes, osteoblasts and chondrocytes) and non-mesenchymal origin cells (hepatocytes, epithelial and nerve cells). Broiler UMSCs showed a promising cell source for cell transplantation treatment and tissue engineering applications accordingly. Nevertheless, the mechanisms of UMSCs differentiation and the function of induced cells are still not fully understood. Therefore, further research on the differentiation mechanisms of UMSCs will promote its effective application in transplantation therapy. LPL and PPAR-γ are two key marker genes for adipocyte differentiation [29]. In this study, the addition of IBMX can enhance the expression of PPAR-γ at mRNA level and further facilitate adipocyte formation. Dexamethasone could promote adipocytes and osteoblasts differentiation, and its concentration was critical for regulating the direction of differentiation of UMSCs [30]. The concentration of dexamethasone between 10^{-8}-10^{-10} mol/L could stimulate osteoblasts differentiation. Whereas, at a higher concentration of dexamethasone, UMSCs would differentiate into adipocytes instead of osteoblasts [31]. Among chondrogenic bioactive factors, L-ascorbic acid and TGF-β3 appeared to play most effective roles in successful chondrogenic differentiation [7]. Additionally, serum-free media were used during chondrogenic differentiation processes, because the lack of antigrowth factors in serum can reduce cellular apoptosis [7]. In hepatocyte-promoting medium, HGF stimulated mitosis and enhanced glycogen formation. The addition of FGF-4 had been found to promote the growth of hepatocytes by underlying mechanisms, probably due to the increased expression of AFP and ALB [32]. Furthermore, EGF would stimulate epithelial cell regeneration, and IGF-2 could inhibit fibroblast growth and promoted epithelial cell proliferation [33]. Collectively, various key inducers were identified to determine the differentiation direction of UMSCs in this study.

Although the multiple differentiations of UMSCs were successfully confirmed in vitro, the differentiation mechanisms remain to be elucidated. The main limitation is the lack of effective application of these cells in regenerative therapies in vivo, such as very limited levels of engraftment and unstable phenotype. Further in vivo studies are warranted to fully understand its regulatory mechanisms in future research.

CONCLUSION

In this study, UMSCs were isolated from 14-day-old broiler embryos. The in vitro culture systems for UMSCs were further optimized. Moreover, the self-renewal ability and differentiation potential of the isolated UMSCs was elucidated. These results demonstrated that UMSCs have the essential characteristics of MSCs, and may contribute to future applications in regenerative medicine.

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Conflicts of Interest: No conflict of interest.

Ethical approval: Experimental procedures in our present study were approved by the Institutional Animal Care and Use Committee (IACUC) for Ethics of Bengbu Medical College.

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