Research Article

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Umbilical Cord Tissue Derived Mesenchymal Stem Cells Can Differentiate into Skin Cells

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Abstract: Autologous skin grafts are used to treat severe burn wounds, however, the availability of adequate donor sites makes this option less practical. Recently, stem cells have been used successfully in tissue engineering and in regenerative medicine. The current study aims to differentiate umbilical cord tissue derived mesenchymal stem cells (CT-MSCs) into skin cells (fibroblasts and keratinocytes) for use to treat severe burn wounds. After isolation, MSCs were characterized and their growth characteristics were determined. The cells were induced to differentiate into fibroblasts and keratinocytes using respective induction medium. Results indicated that CT-MSCs were spindle shaped, plastic adherent and positive for CD29, CD44, CD73, CD90 markers. CT-MSCs also showed high proliferative potential as indicated by cumulative population doubling, doubling time and plating efficiency. The MSCs were successfully differentiated into fibroblast and keratinocytes as indicated by morphological changes and expression of lineage specific genes. We propose that these differentiated skin cells which are derived from CT-MSCs can thus be used for the development of bioengineered skin; however, further studies are required to evaluate the utility of these substitutes.

Keywords: mesenchymal stem cells, cord tissue, bioengineered skin, skin cells, keratinocytes, fibroblasts

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Abbreviations

MSCs: Mesenchymal Stem Cells
CT-MSCs: Cord tissue derived Mesenchymal Stem Cells
KGF: Keratinocyte growth factor
FGF: Fibroblast growth factor
PBS: Phosphate buffer saline
DMEM: Dulbecco's modified eagle medium
PE: Plating efficiency
cPDs: Cumulative population doublings
DT: Doubling time

1 Introduction

Skin wound healing requires restoration of both the dermal and epidermal layers of skin. Normally re-epithelization occurs by proliferation of keratinocytes that migrate from the wound edges. Similarly, fibroblast proliferation and release of growth factors restore dermis. In severe burn injuries, the normal repair process becomes defective. Autologous skin grafts is a treatment for severe burn wounds. However, problems such as the availability of adequate donor sites (for use as a graft) and chances of infection (due to additional injury) make this option less practical [1]. Recently, the use of adult stem cells has become a promising approach for the treatment of several diseases and disorders [2]. These cells are found in different adult and neonatal tissues. Stem cells possess two important characteristics, this is the ability of self-renewal and the ability to differentiate into specific cell types under appropriate culture conditions. Mesenchymal stem cells (MSCs) are a type of adult stem cells that have remarkable differentiation potential and could be utilized for repair and regeneration of lost tissues [3].

The high proliferation, multi-lineage differentiation capacity, immunomodulatory and immunosuppressive properties have made MSCs promising therapeutic candidates [4-6]. The MSCs can be isolated from adult (such as bone marrow, adipose tissue, synovial membrane, teeth etc.) and neonatal sources (such as cord blood, cord tissue) [7-10]. The current study has focused on umbilical

cord tissue derived MSCs (CT-MSCs) as human cord tissue is readily available. There are certain advantages in using cord tissue, firstly, a large number of MSCs can be harvested and secondly, its isolation poses no risk to donors. Finally, CT-MSCs are less immunogenic and can be expanded extensively *in vitro* [11-12].

In the current study, MSCs were isolated from human umbilical cord tissue by an explant culture technique. These cells were characterized and their growth characteristics were determined. CT-MSCs were differentiated into fibroblasts and keratinocytes by culturing in the respective induction medium. Results show that CT-MSCs exhibited spindle shape morphology, plastic adherent growth and expression of CD29, CD44, CD73, CD90 markers. The CT-MSCs exhibited high proliferative potential as indicated by cumulative population doubling and plating efficiency. On the other hand, the differentiated fibroblast and keratinocyte exhibited a changed morphology and had expression of lineage specific genes. We show that CT-MSCs can be differentiated into both types of skin cells (i.e. keratinocytes and fibroblasts) that could be used in the future development of bioengineered skin. However, further studies will be required to evaluate the utility of such substitutes.

2 Material And Methods

2.1 Collection and isolation of cord tissues

Human umbilical cord tissues were collected following full-term cesarean births. Around 3-5 inches of cord tissue was transferred from the hospital to the cell processing facility under sterilized conditions. All samples were obtained after written consent from the donors. All procedures were performed according to the protocol approved by local Institution Review Board at the King Edward Medical University.

CT-MSCs were obtained using the explant culture technique as previously described [13]. Briefly, after washing with PBS (phosphate buffered saline), tissue pieces were minced into small pieces. Complete culture medium (Dulbecco's Modified Eagle Medium (DMEM) + 1% non-essential amino acids + 1% penicillin/streptomycin solution) supplemented with 10% fetal bovine serum (FBS) was added and minced tissue pieces were incubated in culture flasks at 37°C with 5% CO_2 in humid conditions.

Informed consent: Informed consent has been obtained from all individuals included in this study

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by Institution Review Board at the King Edward Medical University.

2.1.1 Plating efficiency (PE)

At passage 1, 40 cells/cm^2 were plated into a 25cm^2 culture flask containing complete medium and incubated at standard culture conditions for 2 weeks. After 2 weeks of culture, the medium was removed and cell colonies were fixed with absolute methanol and stained with 0.1% crystal violet dye. Cell colonies (with more than 30 cells) were counted under phase contrast microscope and PE was measured using the following formula [13]

PE: [Total number of colonies/ number of cells initially plated] x 100

2.1.2 Number and time of population doublings

To determine population doublings, initial cell number and number of cells harvested at each passage was recorded. The following formulae were used to calculate the cumulative population doublings and population doubling time [13]

$$cPDs = \text{Log } N/\ No \times 3.33$$

$$DT = CT/cPDs$$

Where, 'cPDs' represent cumulative population doublings, 'No' is the number of cells plated, 'N' is the cells number harvested, 'CT' is the time in culture and 'DT' is the doubling time.

2.2 Differentiation of CT-MSCs into skin cells

For differentiation, CT-MSCs were cultured in keratinocyte or fibroblast induction medium for 2 weeks. The fibroblast differentiation medium contained DMEM (Gibco, Cat#11995-065), 10% FBS (Merck, Cat# 2020-07-31), 1% penicillin/streptomycin solution (Capricon, Cat# CP13-1019), 5 ug/ml insulin (Sigma, Cat# 19278-5ml) and 1 ng/ml basic fibroblast growth factor (Sigma, Cat# F02901) while keratinocyte differentiation medium consisted of DMEM, 10% FBS, 1% penicillin/streptomycin solution, 0.5 mg/ml hydrocortisone (Sigma, Cat# H-4001), 1% insulin transferrin (Roche, Cat# 13532600) and 15 ng/ml keratinocytes growth factor (Sigma, Cat# H6666). Differentiation of CT-MSCs into fibroblasts and

keratinocytes was confirmed by morphological changes and through polymerase chain reaction (PCR) using marker genes for fibroblast (desmin, collagen 3, vimentin, FGF7) and keratinocytes (CK1, CK10, CK14).

2.3 Reverse transcription polymerase chain reaction (RT-PCR)

Expression of lineage specific genes was carried out by RT-PCR. Briefly, cells were cultured for 15 days in the respective differentiation medium and total RNA was extracted using Trizol. Following extraction, the RNA was quantified using NanoDrop. For cDNA synthesis, 1.5ug of RNA sample was used using Wizscript cDNA synthesis kit. The WizPure™ PCR master mix was used for quantitating the expression level of genes. Sequences for primer pairs and their product lengths (bp) are shown in Table 1. Gel bands were measured with image J software.

2.4 Data Analysis Procedure

Statistical analysis of data was performed using GraphPad Prism version 6. The data was expressed as mean ± standard deviation. All experiments were performed on at least three samples in triplicate.

3 Results

3.1 Isolation and characterization of MSCs

We used an explant culture technique to obtain pure MSC population (Figure 1A). Within 7 days, MSCs appeared around the small pieces of cord tissue (Figure 1B). Cells grew rapidly around the tissue pieces (Figure 1C) and become confluent within two weeks (Figure 1D). CT-MSCs exhibited plastic adherent growth and spindle shaped morphology (Figure 1E). This spindle shaped morphology

Table 1: List of used primers and their sequences.

Genetic Markers	5'-3'sequence	Product size
Beta Actin	CGCATGGGTCAGAAGGATTC (F) TAGAAGGTGTGGTGCCAGATTT (R)	137
CD29	GCAGTTGGTTTTGCGATTAAG (F) AAGGCATCACAGTCTTTTCCA (R)	233
CD44	AGAAAAATGGTCGCTACAGCA (F) CTGAAGTGCTGCTCCTTTCAC (R)	571
CD45	CACTGCAGGGATGGATCTCA (F) ACTCGTGGGTTCAGAACCTTCA (R)	312
CD73	ACAACAGCCAACTGCTTTCAT (F) TTCTCAGCATTCCCGAAAT (R)	154
CD90	ATGAACCTGGCCATCAGCATGC (F) CACGAGGTGTTCTGAGCCAGCA (R)	344
CK1	GGAGGAGGAGGTGGTAGATTTT (F) GAGGTTGCTGATGTATGACTCG (R)	388
CK10	GAGCAAGGAACTGACTACAG (F) CTCGGTTTCAGCTGCAATCT (R)	249
CK14	TGCTATTGGTGTCAGGGAAG (F) GTGGCAAGGTTCTTTTCTCC (R)	277
Collagen-3	GTTGACCCTAACCAAGGATGCA (F) GGAAGTTCAGGATTGCCGTAG (R)	203
Vimentin	CTGCGGGAGTAGTTGGAAAGT (F) GGAAATGGGACAAAACATCCT (R)	241
FGF 7	TGGTGAAGTTCATGGATGTCTATC (F) CACAGGATGGCTTGAAGATGTA (R)	212
Desmin	CATCCTCAAGAAGGTGTTGGAG (F) CAAAGAGACGTGGGACGAGT (R)	112
4-Oct	GGCGTTCTCTTTGGAAAGGTGTTC (F) CTCGAACCACATCCTTCTCT (R)	145
SSEA4	CCGCGTCAAGAGGCCCATGAA (F) CCCGCTTCTCGGTCTCGGACAA (R)	148

was maintained even in late passages (Figure 1F). In addition, CT-MSCs were positive for the expression of mesenchymal lineage markers CD29, CD44, CD73, CD90 while being negative for CD45 as determined by RT-PCR (Figure 1G).

3.2 Growth Characteristics

The growth characteristics of CT-MSCs were assessed by measuring plating efficiency, cumulative population doublings, and doubling time. Plating efficiency of

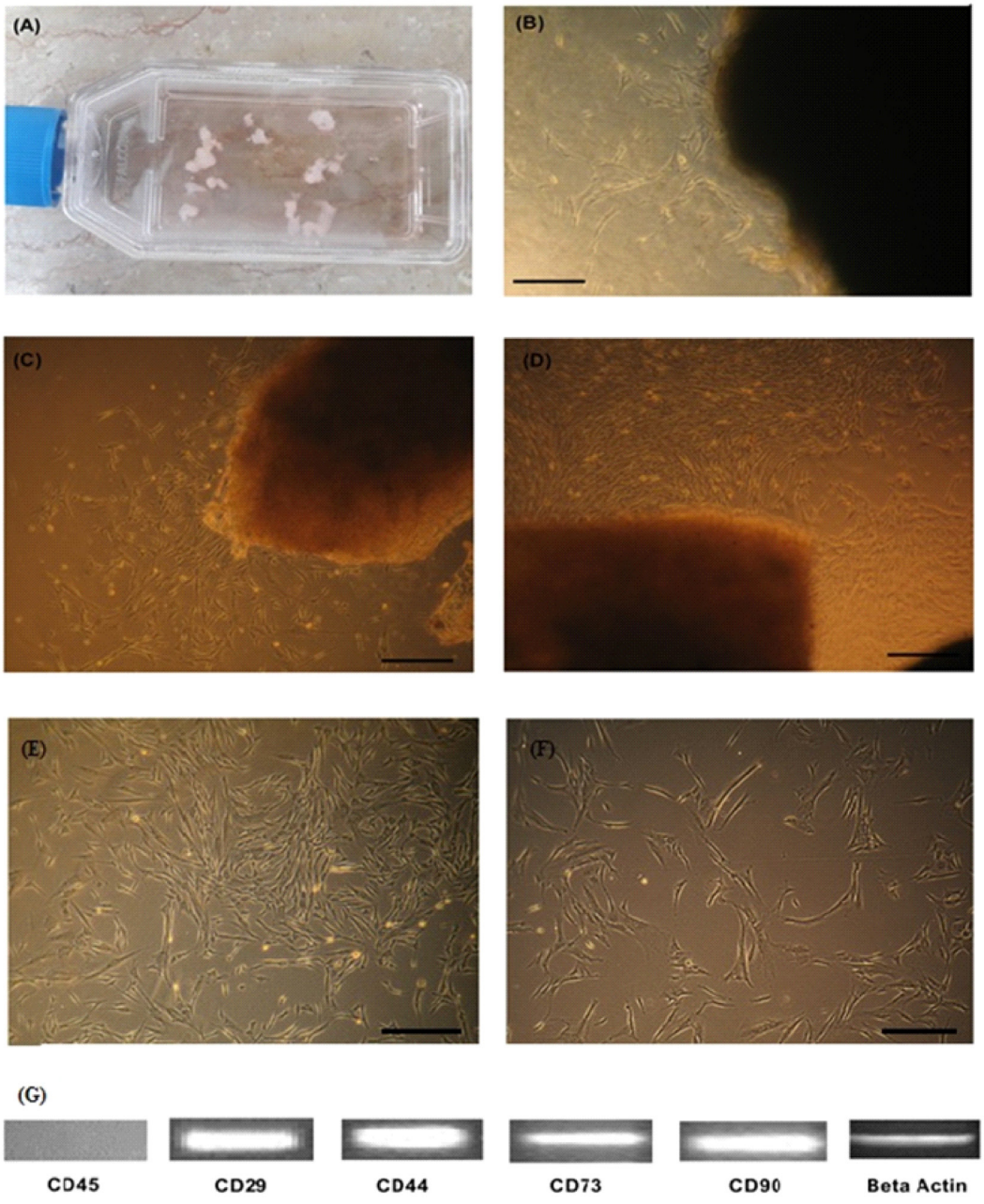

Figure 1. Isolation and characterization of CT-MSCs: MSCs from cord tissue pieces were isolated using explant tissue culture (A). MSCs started to grow out of small cord tissue pieces within a week (B). Cells around cord tissue pieces after 10 days of culturing (C). Cells after 2 weeks of culture (D). CT-MSCs showed homogeneous spindle shaped morphology (E).CT-MSCs maintained their spindle shped morphology during passaging. The cells shown here are at passage 7 (F). PCR analysis showed positive expression of CD29, CD 44, CD73 and CD90, and negative expression of CD45 in CT-MSCs (G).

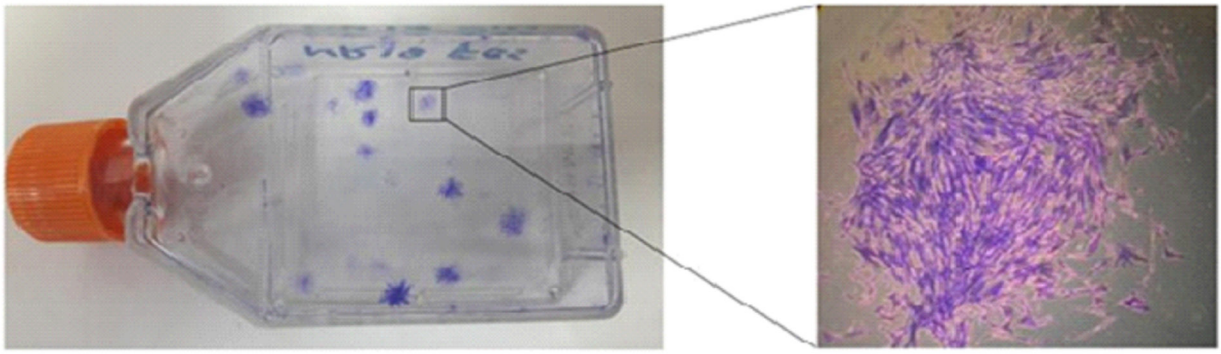

Figure 2. Plating efficiency of CT-MSCs. Inset shows a single colony of MSCs at high power (10X).

CT-MSCs was evaluated by culturing the cells at low numbers for the eventual growth into colonies. The number of colonies were counted after 2 weeks and this number was used to determine plating efficiency which was 4.225±1.7 (Figure 2).

To determine the number and time of population doublings at each passage, cells were counted with a hemocytometer and 10% cells were seeded into new culture flasks. The number of cumulative population doublings was 24.67±0.445 for CT-MSCs. Similarly, the average population doubling time for CT-MSCs was 51±3 hours.

3.3 CT-MSCs can differentiate into keratinocytes and fibroblasts

CT-MSCs at passage 2 (n=5) were differentiated into skin cells, either fibroblasts or keratinocytes. For differentiation into fibroblasts and keratinocytes, respective differentiation medium was used for 2 weeks. To serve as a control, cells were cultured in parallel in regular expansion medium.

Compared to the spindle shaped morphology of control group (Figure 3A), MSCs in the keratinocyte differentiated medium showed polygonal shape (Figure 3B). Similarly, RT-PCR results indicated that differentiated MSCs were positive for keratinocyte lineage markers (CK1, CK10, CK14) at day 7 and day 14 (Figure 3C, D). In contrast, the expression of OCT4 and SSEA4 stem cell markers was downregulated (Figure 3E, F).

Differentiation of CT-MSCs into fibroblasts was terminated after 14 days. Untreated CT-MSCs at 7 and 14 days were used as controls and showed no morphological changes (Figure 4A). This is compared to the prominent morphological changes when cultured in fibroblast induction medium (Figure 4B). The morphology of CT-MSCs cultured in fibroblast induction medium changed considerably from spindle shape to a more elongated shape (Figure 4B). Furthermore, the expression of collagen-3, desmin, FGF-7, and vimentin was up-regulated after 14 days (Figure 4C, D) while the expression of SSEA4 and OCT4 was down-regulated (Figure 4E, F).

4 Discussion

Rapid repair and management of large skin ruptures is often necessary for the survival of patients. Minor wounds may heal without intervention, however, severe skin injuries require additional care. For severe burn injuries, autologous skin grafting is the best option available. However, there are certain limitations that make this treatment option less practical [14, 15]. In the current study, CT-MSCs were differentiated into keratinocytes and fibroblasts with the future goal of using these to construct skin constructs. In the previous studies the MSCs isolated from different parts of placenta were either differentiated into fibroblasts or keratinocytes [16, 18]. However, the current study is novel in that MSCs are isolated from umbilical cord tissue and we demonstrate successful differentiation into both types of skin cells (fibroblasts and keratinocytes). The isolation and subsequent differentiation of same cells into both types of cells is more convenient for the reconstruction of artificial skin substitutes. A caveat of our study is that we have not evaluated the utility of these differentiated skin cells *in vitro* or *in vivo*.

MSCs from the umbilical cord tissue were isolated by the explant culture method. This method is inexpensive and provided pure MSC population [13, 16]. We observed cell outgrowth within a week after the initial culture. Following 2-3 weeks of culture, a sufficient number of cells was obtained, which allowed for further experiments. The MSCs from tissue pieces exhibited plastic adherent growth,

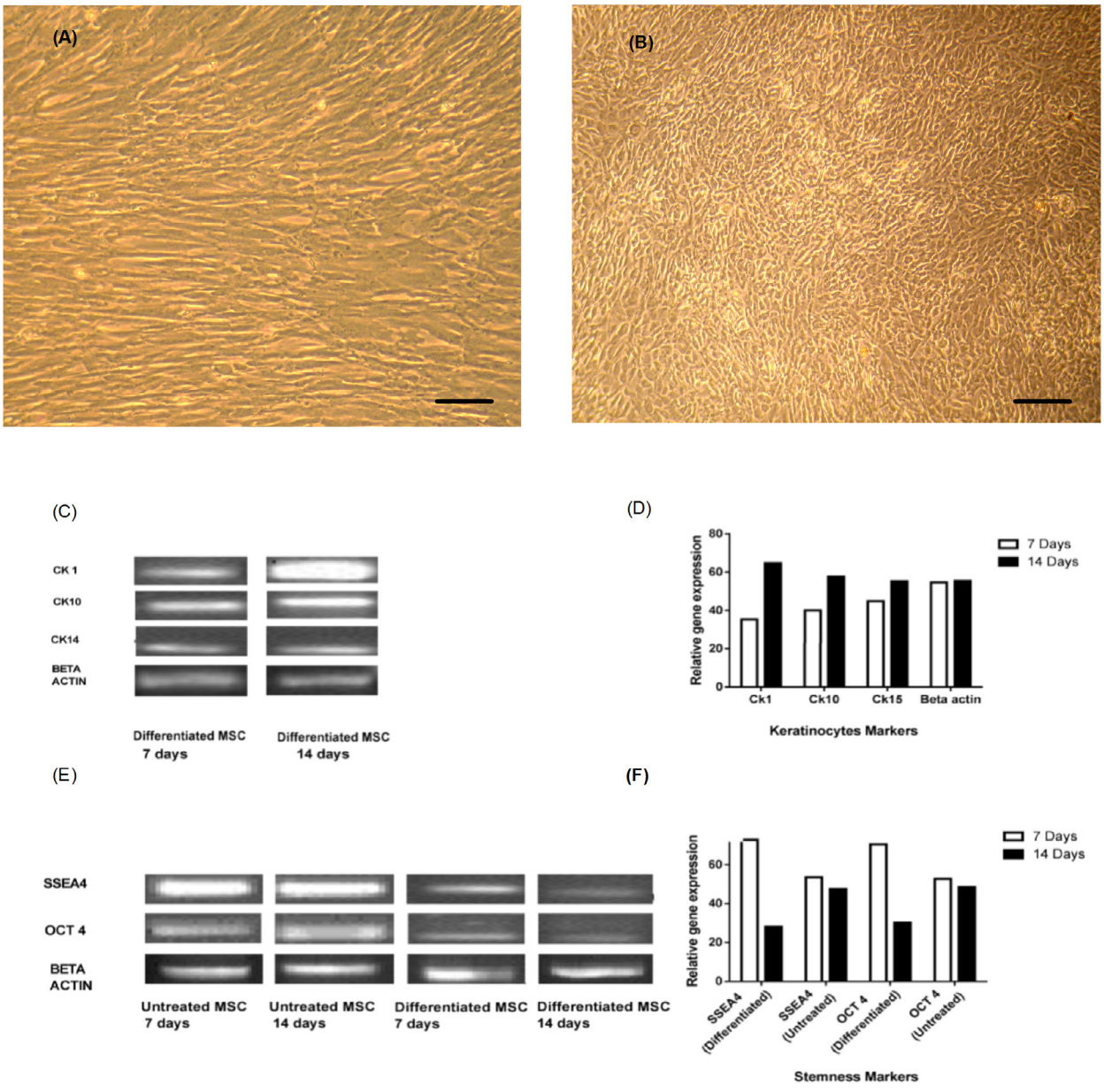

Figure 3. In vitro differentiation of CT-MSCs into keratinocytes: Untreated CT-MSCs exhibit spindle shape morphology (A). MSCs induced into keratinocytes exhibits a polygonal shape (B). Results of RT-PCR show that differentiated cells were positive for keratinocyte specific genes CK1, CK10 and CK14 (C). Quantification of gel bands using ImageJ software (D). Expression of SSEA4 and OCT4 decreased during differentiation into keratinocytes (E, F).

spindle shaped morphology and positive expression of MSCs genes (CD29, CD44, CD73 CD90) and no expression of CD45 (a hematopoietic marker). These results were similar to already published reports [4, 10, 19-23]. CT-MSCs showed high proliferative potential as determined by plating efficiency and number of population doublings. The clonogenic potential of CT-MSCs was determined by plating efficiency amd was 4.225±1.7. This was also similar to previous studies in the literature [24, 27]. The number population doublings of CT-MSCs was 24.67±0.445 and their doubling time was 51±3 hours. Similar results were reported by Choudhery and colleagues [13], and Mahmoud *et al.* [16].

To differentiate CT-MSCs into skin cells (fibroblasts and keratinocytes), they were cultured in respective differentiation medium for 2 weeks. In keratinocyte induction medium, differentiated CT-MSCs exhibited

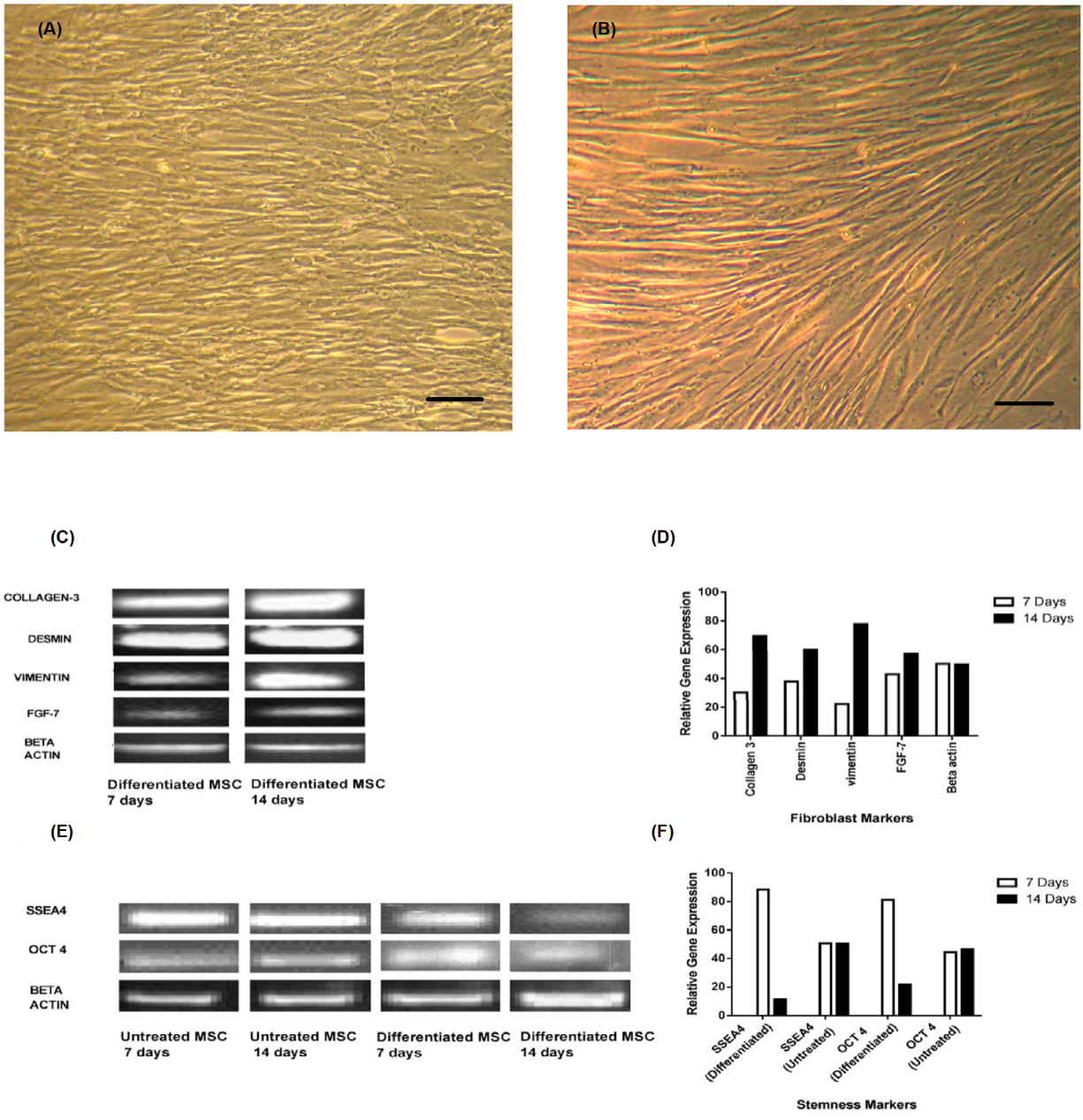

Figure 4. In vitro differentiation of CT-MSCs into Fibroblasts. Untreated CT-MSC exhibit spindle shape morphology (A). Treated CT- MSCs exhibited more elongated spindles morphology after culturing in fibroblast differentiation medium (B). Results of RT-PCR indicate that differentiated cells were positive for fibroblast specific genes (collagen-3, vimentin, FGF-7, desmin). RT-PCR analysis also showed upregulated expression of fibroblast specific markers, collagen-3, vimentin, FGF-7, and desmin after 14 days of differentiation versus 7 days (C). Quantification of gel bands with ImageJ software (D). Expression of stem cell markers was downregulated at 14 days of culture in fibroblast differentiation media versus 7 days culture

polygonal morphology which was similar to keratinocytes [28]. The differentiated cells were positive for the expression of CK1, CK10 and CK14 markers. Previous studies have demonstrated that keratinocytes express these markers [29- 30]. The CT-MSCs which were differentiated into fibroblasts with fibroblast differentiation medium, became more elongated when compared to the control. Furthermore, these induced cells were positive for collagen-3, desmin, FSP-1 7 and vimentin which are fibroblast specific markers [31-33]. We also showed that during differentiation into skin cells (fibroblasts and keratinocytes), MSCs exhibited a progressive down regulation of pluripotency markers SSEA4 and OCT4 [34-35]. In this study we demonstrate that MSCs with standard

characteristics can be isolated from human placenta using the explant culture technique. These CT-MSCs can then be induced *in vitro* to differentiate into skin cells such as fibroblasts and keratinocytes. This successful differentiation was characterized by morphological changes and changes in gene expression profile. We propose that these cells may be used in the future to prepare an artificial skin substitute for use as a treatment for severe burn injuries.

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Conflict of interest: Authors state no conflict of interest

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