

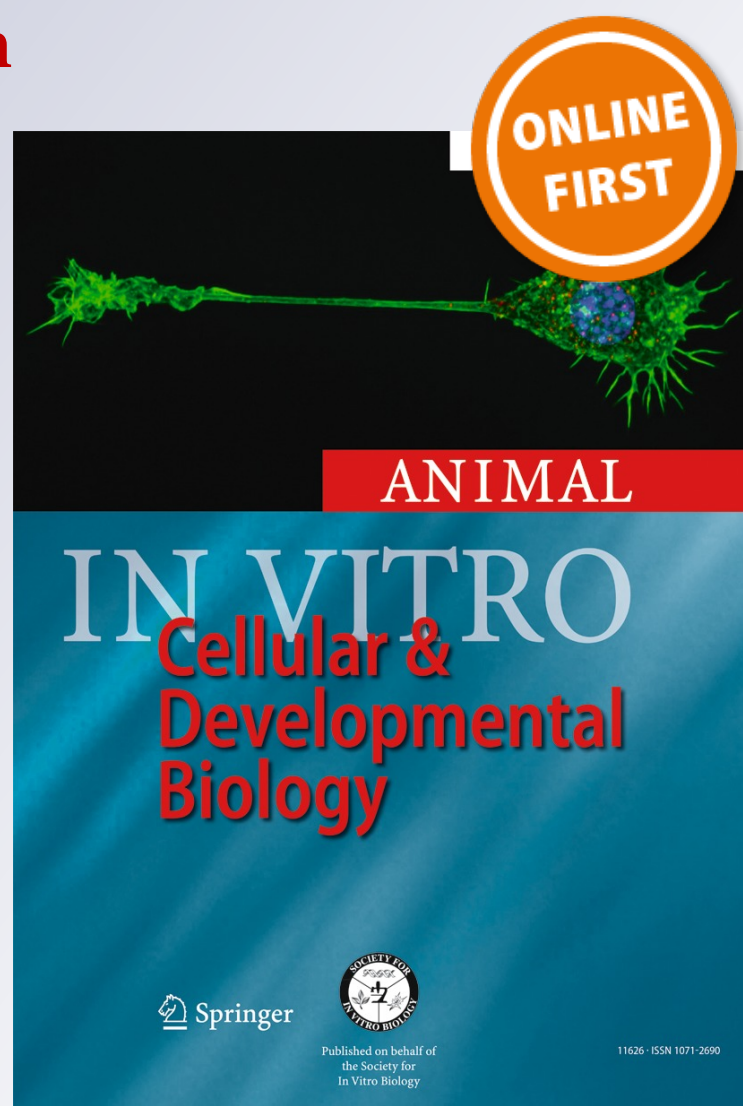
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Effect of EGF and FGF on the expansion properties of human umbilical cord mesenchymal cells

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Abstract Mesenchymal stem cells have been increasingly introduced to have great potential in regenerative medicine, immunotherapy, and gene therapy due to their unique properties of self-renewal and differentiation into multiple cell lineages. Studies have shown that these properties may be limited and changed by senescence-associated growth arrest under different culture conditions. This study aimed to present the ability of some growth factors on human umbilical cord mesenchymal (hUCM) cells expansion and telomerase activity. To optimize hUCM cell growth, epidermal growth factor (EGF) and fibroblast growth factor (FGF) were utilized in culture media, and the ability of these growth factors on the expression of the telomerase reverse transcriptase (TERT) gene and cell cycle phases was investigated. TERT mRNA expression increased in the hUCM cells treated by EGF and FGF. So, the

untreated hUCM cells expressed $30.49 \pm 7.15\%$ of TERT, while EGF-treated cells expressed $51.82 \pm 12.96\%$ and FGF-treated cells expressed $33.77 \pm 11.55\%$ of TERT. Exposure of hUCM cells to EGF or FGF also promoted the progression of cells from G1 to S phase of the cell cycle and induced them to decrease the number of cells entering the G2/M phase. Our study showed that EGF and, to a lesser extent, FGF amplify the proliferation and expansion of hUCM cells.

Keywords Cell cycle · Growth factors · TERT · Umbilical cord matrix-derived cells

Introduction

The primitive mesenchymal-like cells of Wharton's jelly are trapped at a very early embryological age and retain the properties of primitive stem cells. They can be more easily obtained and processed than embryonic and adult mesenchymal stem cells (MSC) such as bone marrow-derived and hematopoietic stem cells (Tamura et al. 2011). These cells have the ability to self-renew and to differentiate into multiple cell lineages (Jeon et al. 2011). A necessary factor for the self-renewal, proliferation, and expansion of these cells is the maintenance of telomere length (Bayne and Liu 2005; Jeon et al. 2011). Short telomere length induces the limitation of the cells' proliferative life span (Chomal 2003). Shortening of the telomeres can lead to threefold greater mortality in heart disease, eightfold greater mortality in infectious disease, and some other problems like coronary atherosclerosis, vascular dementia, Alzheimer's disease, and several cancers (Bayne and Liu 2005). Telomerase, the enzyme of telomere synthesis (Masutomi et al. 2003), is a ribonucleoprotein enzyme that synthesizes the telomeric DNA sequence onto the 3' ends of chromosomes (Miura et al. 2001). Telomerase has three subunits, one of them named telomerase reverse transcriptase (TERT) has an important

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role in the replication of telomere repeat sequences and the extension of telomere sequence (Wojtyla et al. 2011; Liang et al. 2012). Cells with higher proliferation capacity such as cells in Wharton's jelly can express a high telomerase activity (Tamura et al. 2011). Considerable evidence suggests that growth factors such as fibroblast growth factor (FGF), IGF-1, and epidermal growth factor (EGF) (Maida et al. 2002) are crucially involved in regulating telomerase activity and gene expression of TERT (Bayne and Liu 2005).

The regulation of telomere length and telomerase activity is a complex and potent process which leads to cell cycle regulation in human stem cells (Hulleman and Boonstra 2001). MSCs, after a limited number of cell division in culture, enter a senescence situation which is considered as the permanent state of cell cycle arrest (Liang et al. 2012). Growth factors or signals escape the cells during cell cycle arrest, so the resting G0 cells prepare themselves for the process of multiplication in the G1 phase. During passing of the cells into the S phase, new DNA synthesis takes place and the cells progress into the G2 phase, which is the correction process where any aberrations in DNA synthesis are corrected. Cell proliferation rate is characterized by greater numbers of cells in the S phase and fewer numbers of cells in the G0/G1 phase, with G2/M phase cells remaining constant (Dhulipala et al. 2006). However, in the absence of growth factors, cells will stop dividing and enter the quiescent state (G0) (Hulleman and Boonstra 2001).

Investigation about the normal tissue-specific modulators of the growth factors in regulating telomerase activity and cell cycle may introduce more specific tools for controlling the cells' proliferation and expansion. Therefore, in this study, the effects of EGF and FGF on TERT expression and cell cycle stages were investigated on MSCs isolated from the human umbilical cord.

Materials and Methods

Cell isolation and culture. Mesenchymal cells were isolated from human umbilical cords using an explant culture approach as described earlier (Salehinejad et al. 2012a, b). Briefly, Wharton's jelly fragments were seeded onto the surface of a culture dish with Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (2.5 µg/ml) for 2 wk. Within this period, every 5 d, half of the medium was refreshed until the cells migrated from the fragment borders and reached approximately 80% confluence. From passage 2, human umbilical cord mesenchymal (hUCM) cells were either used for the experiments or cryopreserved by a conventional freezing protocol using DMSO as cryoprotectant.

Detection of hUCM cells phenotype. Cells were prepared at a concentration of 1×10^5 cells/ml in DMEM/F12 with 10% FBS. The harvested cells were washed, fixed, and incubated for 15 min at 4°C with a 1:9 dilution of normal goat serum in PBS to block nonspecific binding of the primary antibody. The cells were then labeled with the following antibodies: FITC-conjugated anti-CD34, FITC-conjugated anti-CD44 (Chemicon, Temecula, CA), FITC-conjugated anti-CD45 (eBioscience, San Diego, CA), PE-conjugated anti-CD90 (Dako, Glostrup, Denmark), and PE-conjugated anti-CD105 (R&D, Minneapolis, MN) for 1 h and analyzed using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) machine. The control population was stained with matched isotype antibodies (FITC-conjugated and PE-conjugated mouse IgG monoclonal isotype standards), which were confirmed by positive fluorescence of the limbal samples. At least 10,000 events were recorded for each sample and data were analyzed using WinMDI software (West Lafayette, IN).

Differential capability of hUCM cells. Osteogenic and adipogenic differentiation of the hUCM cells was carried out as described elsewhere (Salehinejad et al. 2012a, b). The cells were cultured in DMEM/F12 medium with either osteogenic (10 nM dexamethasone, 50 µg/ml ascorbate-phosphate, and 10 mM β-glycerophosphate) or adipogenic (100 nM dexamethasone, 50 µg/ml ascorbate-phosphate, and 50 µg/ml indomethacin) materials for 21 and 14 d, respectively. Adipogenic differentiation was detected by Oil Red O staining, while osteogenic differentiation was detected by Alizarin Red S staining.

Alkaline phosphatase staining. The hUCM cells from the fourth passage were grown on clean, sterile glass slides for 2–3 wk. The medium was refreshed every 5 d. Alkaline phosphatase (ALP) activity was detected using an ALP Kit (R-87 Sigma, St. Louis, MO) according to the manufacturer's instructions. A dark red product after 20 min exposure to the substrate confirmed ALP activity. The cells were then counterstained with hematoxylin and mounted and photographed using an Olympus DP71 digital camera attached to an IX71 inverted microscope (Tokyo, Japan).

Treatment of the cells with growth factors. To determine the effects of EGF and FGF on telomerase activity and cell cycle phases, the hUCM cells from the fourth passage were treated with 20 ng/ml EGF or 20 ng/ml FGF for 48 h, while the control group was incubated only with DMEM/F12 supplemented with 10% FBS. The breast cancer MCF-7 cells, as a positive control, were incubated in DMEM/F12 containing 10% FBS. After 48 h, the cells from each group were trypsinized and divided into two parts for reverse transcription polymerase chain reaction (RT-PCR) and cell cycle analysis.

RNA isolation and cDNA synthesis. To determine the expression of TERT gene and housekeeping gene β -actin, the total RNA was extracted using Tripure Isolation Reagent (Roche, Mannheim, Germany). For this purpose, $1 \text{ ml}/10^6$ cells of Tripure was used for each of the groups. Subsequently, $0.5\text{--}1 \text{ }\mu\text{g}$ of DNase-digested total RNA was used for oligo(dT) reverse transcription to generate single-stranded cDNA. cDNA synthesis was completed by using the M-MuLV reverse transcriptase enzyme. The concentration of cDNA was determined by measuring the absorbance at 260 nm by using a spectrophotometric measurement of ultraviolet (UV) light absorbance.

Semiquantitative reverse transcription polymerase chain reaction. Semiquantitative RT-PCR assays were performed with $25 \text{ }\mu\text{l}$ reaction mixture for each sample containing $12.5 \text{ }\mu\text{l}$ master mix (CinnaGen, Tehran, Iran) including 0.1 U Taq DNA polymerase, $200 \text{ }\mu\text{M}$ dNTP, $1.5 \text{ }\mu\text{M}$ MgCl_2 , $1\times$ PCR buffer, 100 ng MCF-7, hUCMs/EGF, and hUCMs/FGF cDNA and 200 ng hUCMs cDNA, $2.5 \text{ }\mu\text{M}$ TERT primer (Metabion, Martinsried, Germany) and $0.5 \text{ }\mu\text{M}$ β -actin primer (CinnaGen, Tehran, Iran), and RNase-free water. The conditions used for amplification were an initial activation step at 94°C for 5 min followed by 10 cycles of denaturation at 94°C for 30 s , annealing at 65°C for 30 s , and extension at 72°C for 30 s . The first cycle was followed by 30 cycles of denaturation at 94°C for 30 s , annealing at 55°C for 30 s , and extension at 72°C for 30 s . The reaction was completed with a final extension step at 72°C for 10 min . The gene-specific primer sequences, annealing temperatures, and size of the PCR products are listed in the Table 1. The PCR products were analyzed using an ethidium bromide-stained 3% (w/v) agarose gel. Electrophoresis was then carried out at a voltage of 110 V for approximately 35 min . When electrophoresis was complete, the gels were visualized on a UV transilluminator and were photographed. The photographs of the gels were then scanned using the National Institutes of Health (NIH) image analysis software (Scion) to determine the density of individual bands on the gels. The volume density of the amplified products was calculated and normalized against the β -actin housekeeping gene control values.

Cell cycle analysis. Cell cycle was analyzed through propidium iodide staining. Cultured hUCM cells at three

different conditions and MCF-7 cells were used for cell cycle analysis. One million cells were incubated with a solution containing 0.25 g sodium citrate, 0.005 g ribonuclease A, 0.025 g propidium iodide, and 0.75 ml Triton X-100 in 250 ml distilled water at 4°C for 30 min . Cells were analyzed by FACS, and the proportion of cells in the G0/G1, S, and G2/M phases was estimated using the FlowJo software (version 7.5). The proliferative index (PI) was calculated according to the following formula, published by Wang et al. (2007):

$$\text{PI} = \left[\left(\text{S} + \text{G2/M} \right) / \left(\text{G0/G1} + \text{S} + \text{G2/M} \right) \right] \times 100\%$$

Statistical analysis. Statistical analyses were performed using the SPSS software (version 12.0), and data were presented as the mean \pm standard deviation (SD). One-way ANOVA followed by Tukey's post hoc test was performed to compare the data among different groups. All p values < 0.05 were considered significant.

Results

hUCM cells phenotypic. The isolated cells were negative for the hematopoietic cell surface markers CD34 and CD45; the expression of CD34 was 13% , whereas the expression of CD45 was 9% . These cells were positive for the MSC markers CD44, CD90, and CD105 (99.5% , 57% , and 84% , respectively) (Fig. 1).

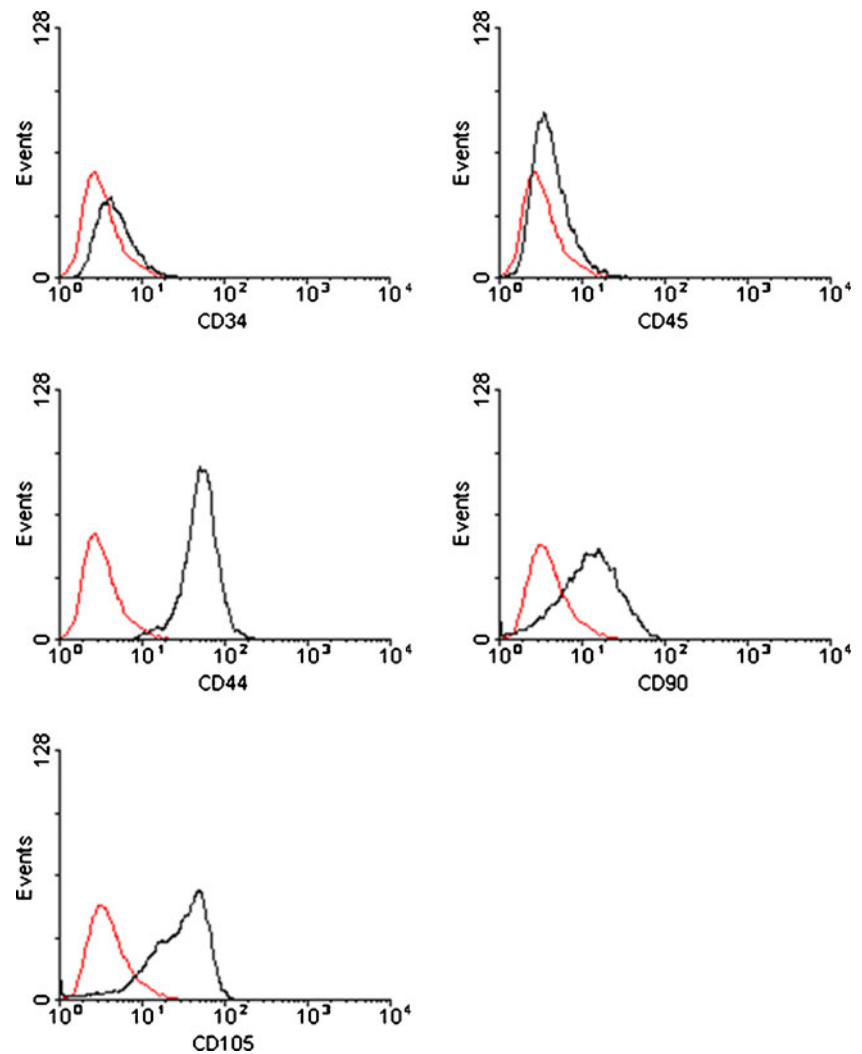
Differentiation into adipogenic and osteogenic lineages. On day 14 of adipogenic treatment, an accumulation of lipid-containing vacuoles was detected by Oil Red O staining (Fig. 2a). At day 21, the MSCs became Alizarin Red S-positive (Fig. 2c). Control cells, maintained in regular medium, showed no Oil Red O-positive or Alizarin Red S-positive cells (Fig. 2b, d).

Alkaline phosphatase activity. Approximately 14 d after the onset of culture, the hUCM cells formed colonies which exhibited ALP activity. The ALP reaction was most intense within the compactly cell colonies (Fig. 3).

Table 1. Primer sequences, primer annealing temperatures, and sizes of PCR products

Lineage	Gene/gene accession number	Direction	Sequence (5'–3')	Annealing temperature ($^\circ\text{C}$)	Amplicon size (bp)
hUCMs	TERT	Forward	CGGAAGAGTGTCTGGAGCAA	59.1	145
		Reverse	GGATGAAGCGGAGTCTGGA		
MCF-7	β -Actin	Forward	GATGATGATATCGCCGCGCT	55.1	351
		Reverse	CTTCTCGCGGTTGGCCTTGG		

Figure 1. Representative of flow cytometry results. The *red histograms* show the isotype control-stained cells and the *black histograms* show the antibody stained cells.



TERT expression. The 145-bp TERT fragment of various cDNA was detected in the hUCMs, hUCMs/EGF, hUCMs/FGF, and MCF-7 groups (Fig. 4A). The 351-bp internal PCR control was observed in all the groups, which indicates that none of the samples contained an unusual amount of Taq polymerase inhibitor (Fig. 4B). MCF-7, which has been confirmed to be hTERT-positive, expressed $37.33 \pm 11.19\%$ of TERT. The untreated hUCM cells expressed $30.49 \pm 7.15\%$ of TERT, while the EGF-treated cells expressed $51.82 \pm 12.96\%$ and FGF-treated cells expressed $33.77 \pm 11.55\%$ of TERT (Fig. 5A). The ratio of TERT banding to the internal control was also measured. This ratio was greater in hUCMs/EGF followed by MCF-7 cells, hUCMs, and hUCMs/FGF, respectively (Fig. 5B).

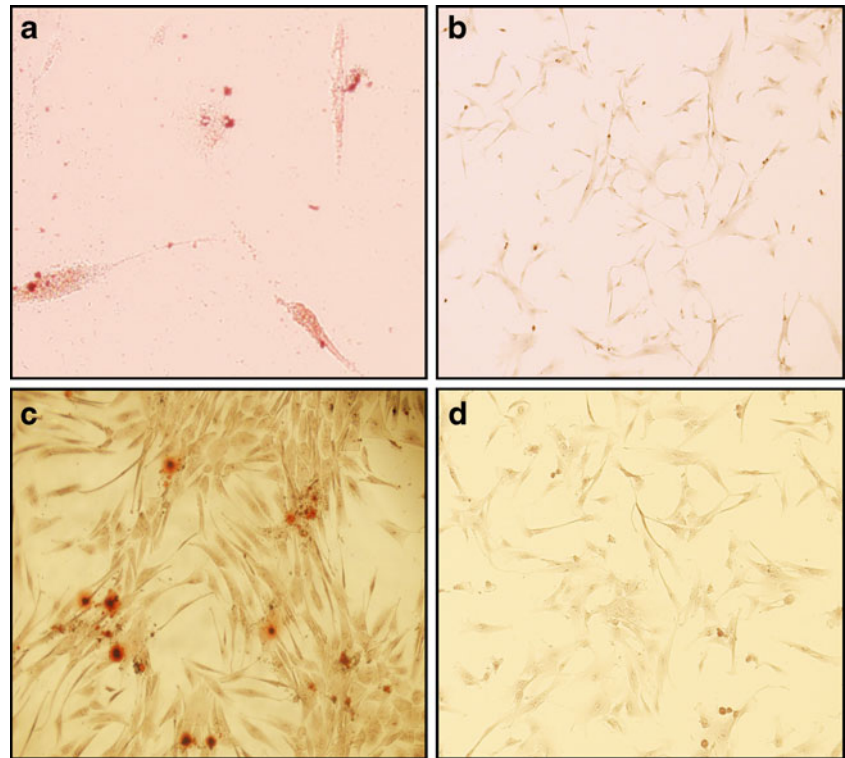
Cell cycle. DNA content analysis revealed that most of the cells were in the G0/G1 phase in all the growth conditions (Fig. 6a). Forty-eight hours after stimulation with EGF, $37.34 \pm 9.8\%$ of the hUCM cells had entered the S phase, whereas $26.3 \pm 2.55\%$ of the cells treated with FGF and $27.88 \pm 8.84\%$ of the untreated cells had done so. Also,

$7.71 \pm 0.53\%$ of the cells treated with EGF progressed to the G2/M phase, while $9.58 \pm 0.24\%$ of the cells treated with FGF and $11.31 \pm 0.79\%$ of the untreated cells had entered the G2/M phase. A similar slowing of cell cycle progression was observed in MCF-7 cells. So, $18.83 \pm 6.18\%$ and $1.51 \pm 1.11\%$ of the mentioned cells had entered the S and G2/M phases, respectively (Fig. 6b). The PI, calculated according to the formula, was 41.13%, 49.18%, 41.62%, and 22.51% for hUCMs, hUCMs/EGF, hUCMs/FGF, and MCF-7, respectively.

Discussion

The extension of telomerase in adult stem cells is still unclear, and since these are the founder cells of all the tissues in the body, understanding the expression of telomerase in adult stem cells is very important (Serakinci et al. 2008). However, it is certain that, in stem cells, the level of telomerase activity is not sufficient to maintain telomere

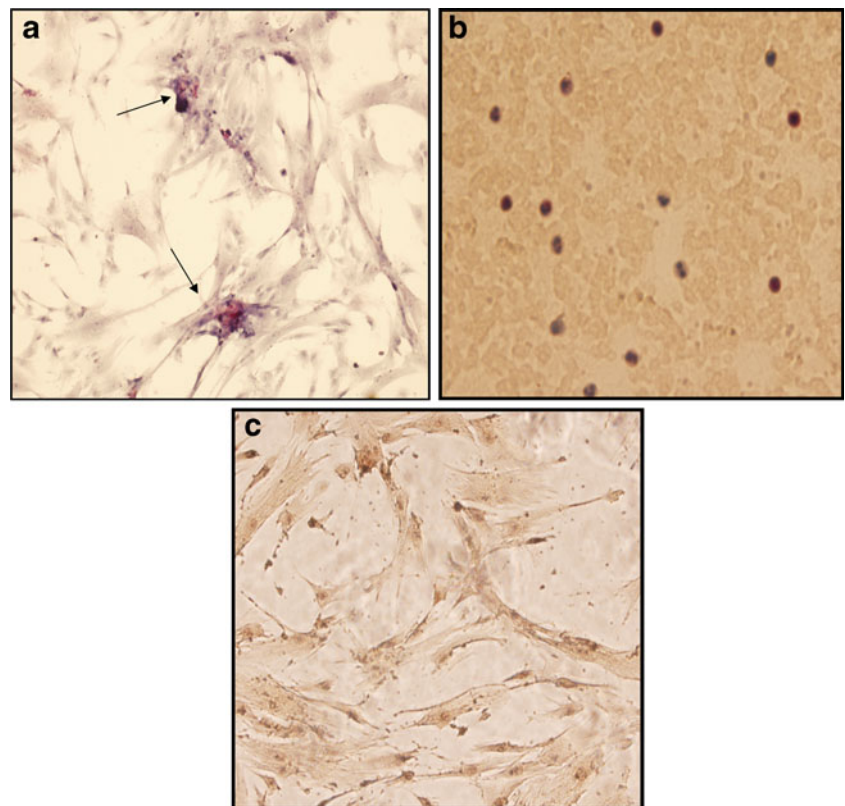
Figure 2. (a) Adipogenic differentiation in the hUCMCs. Oil Red O-positive adipocytes stained as *bright orange*. (c) Osteogenic differentiation by Alizarin Red S-positive calcium deposition appeared as a *red* precipitate. (b, d) No Oil Red O-positive or Alizarin Red S-positive cells were detected in the control samples. Original magnification, $\times 100$.



length during aging (Alenzi et al. 2009). Telomerase decreases with the reduction of cell renewal and expansion potential (Murthy 2002). It was shown that the lack of telomerase in mouse mesenchymal cells lose multipotency

and the differentiation capacity. These results suggest that, in addition to its known role in cell replication, telomerase is required for the differentiation of mMSCs in vitro (Liu et al. 2004). Moreover, studies have shown that, when telomerase

Figure 3. The cell colonies positively stained with ALP (a, *dark red color*). Leukocytes of blood smear as positive control stained with ALP (b). No ALP-positive cells were detectable in hUCMs treated with levamisole (c). Original magnification, $\times 200$.



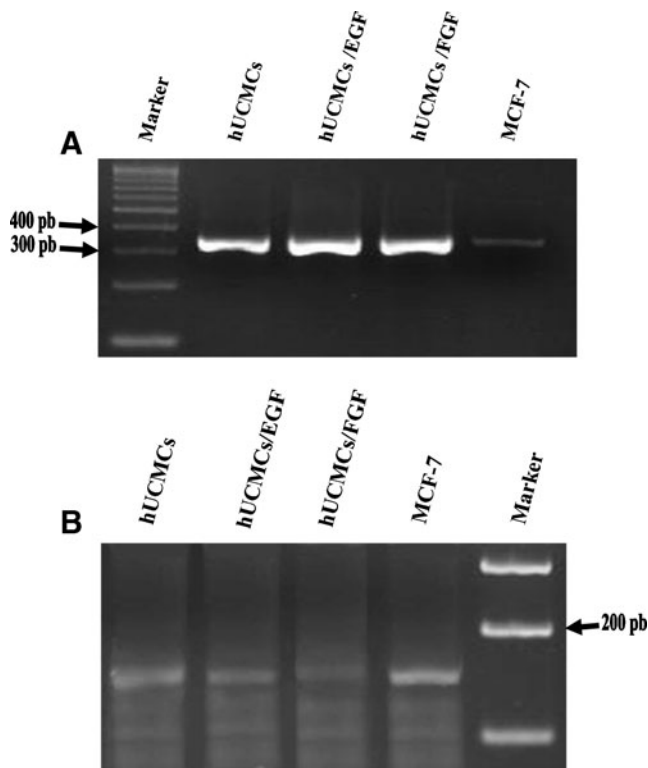


Figure 4. RT-PCR assays for β -actin and TERT mRNA expression. (A) β -Actin as the internal standard expressed in 351 bp and (B) TERT mRNA expressed in 145 bp. The MCF-7 was used as a positive control. Each image is the representative of at least three independent experiments.

is upregulated at cell cycle entry, somehow the cells progress through the S phase and they are repressed in quiescent G0 cells (Murthy 2002). The mechanisms of this association are unclear, but specific growth factors may be effective on it. It appears that these growth factors are necessary at the early stage of the cell cycle at the G1 phase to support the subsequent DNA replication (Hulleman and Boonstra 2001). So, the present study examined the effect of EGF and FGF on the telomerase activity and cell cycle of hUCMs. Our findings showed that EGF caused an increase in the relative TERT expression and PI.

Unlike most normal somatic cells, which are telomerase-negative, low to moderate levels of the enzyme have been described in adult stem cells. But it should be noted that the absence of telomerase activity in hMSCs, obtained from the bone marrow, was observed by several investigators (Stenderup et al. 2003; Zimmermann et al. 2003). These findings suggest that these cells have a different telomere biology compared to other adult stem cells. Alternatively, true MSC might have a very rare subpopulation that has a detection level below the sensitivity of the telomerase assay (Zimmermann et al. 2003). On the other hand, the isolated cells are not homogeneous and a pure cell population; in reverse, they are a mixture of stem and precursor cells

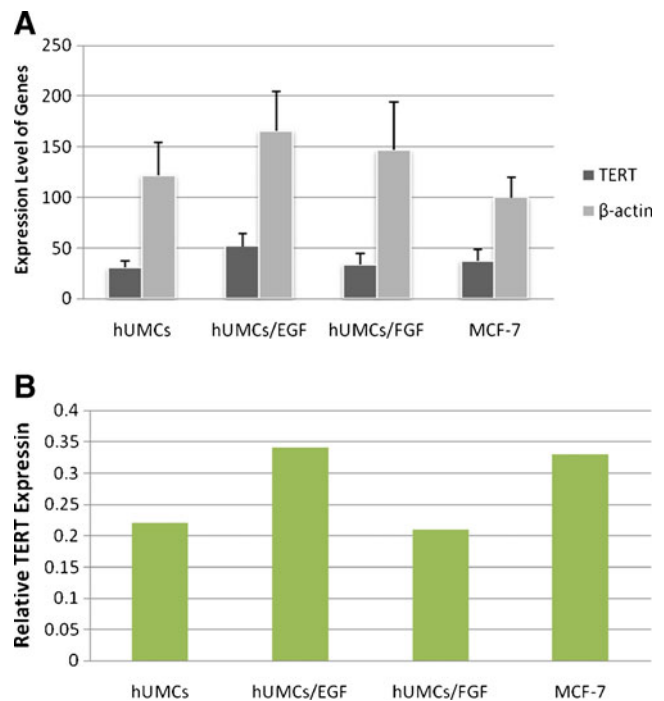


Figure 5. hTERT and β -actin expression. (A) hTERT mRNA expression was detected by RT-PCR compared with β -actin in hUCMs, hUCMs/EGF, hUCMs/FGF, and MCF-7 (as positive control). There was no statistically significant difference among the groups ($p > 0.05$). All data are expressed as the mean \pm SD, $n = 3$. (B) Relative TERT expression was assessed by determining the ratio of the TERT band to the β -actin using the NIH image analysis software (Scion).

(Stenderup et al. 2003). Considering these interpretations, in this study, the explant method was applied for the isolation of mesenchymal cells from Wharton's jelly since the cells obtained by the explant method have homogeneous morphology and the highest cell proliferation compared to the other methods (Salehinejad et al. 2012a, b).

Besides, the culture method affects the expression of genes. Gene expression may be more in vivo-like in three-dimensional culture than in two-dimensional monolayer cultures. For example, tumor antigens may be expressed in three-dimensional culture and not in monolayer culture (Hoffman 1993). Meanwhile, utilization of Gelfoam as three-dimensional culture is more current. But, at present, utilization of Gelfoam is restricted to the investigation of tumors in vitro, drug response assay, stem cell differentiation (Hoffman 2010; Duong et al. 2012), or histoculture that can be transplanted to injured tissue in cell therapy (Hoffman 2010; Liu et al. 2011). While in our work on hUCMs, we did not need to maintain the cells in Gelfoam for forming the actual position of Wharton's jelly. At present, more studies use the monolayer culture for the investigation of these cells. But, future studies utilizing three-dimensional cultures may significantly enhance our understanding of gene expression (Hoffman 1993).

In any case, it seems that TERT can express in different cell conditions with different levels. Comparison of our

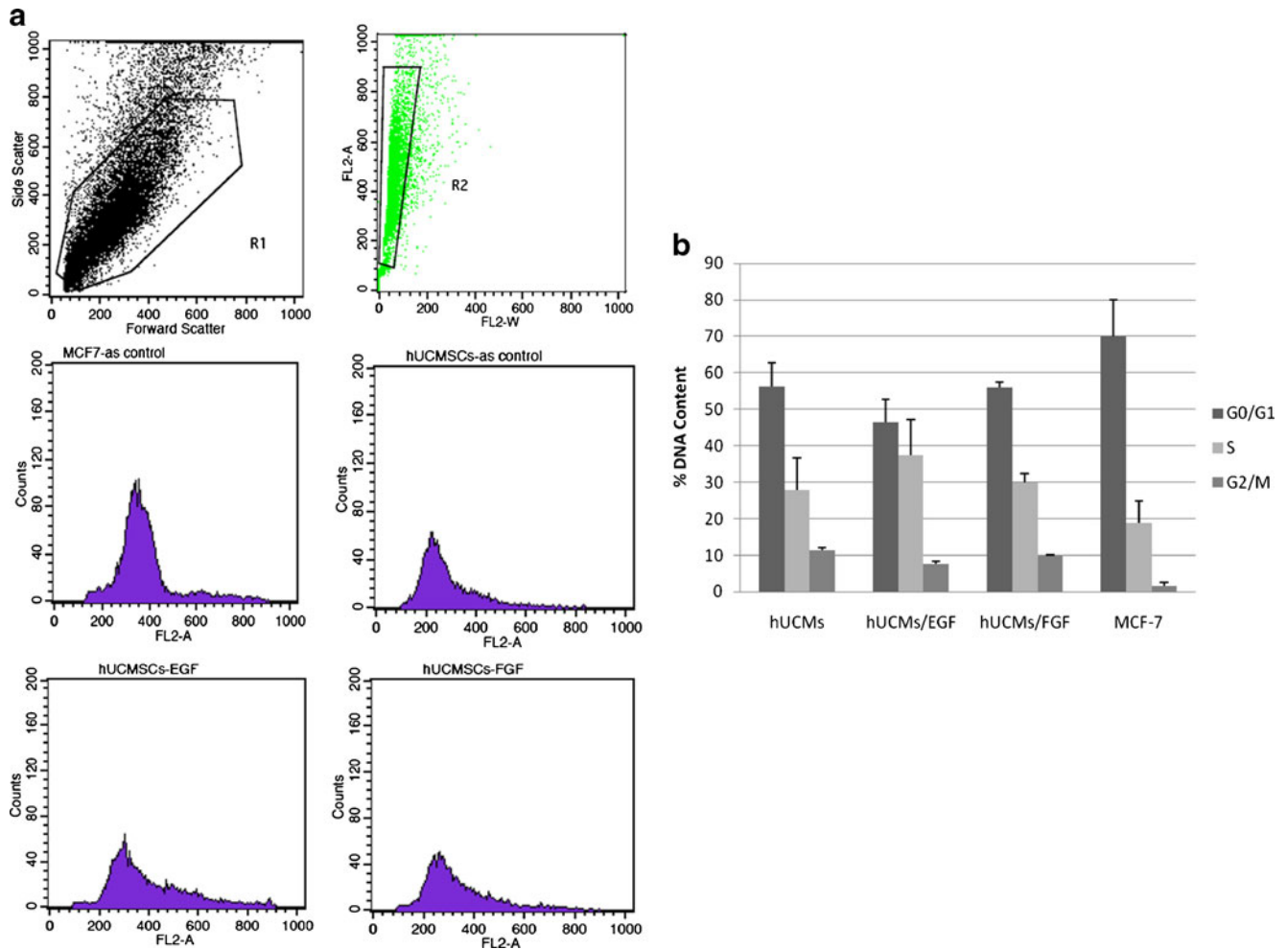


Figure 6. (a) Representative of the flow cytometry results of the cell cycle and (b) distribution of the cells in the different phases of the cell cycle. In all of the groups, a higher percentage of the cells are in the

G0/G1 phase. The cell percentage in the S/G2/M phase was higher in hUCMs/EGF, hUCMs/FGF, hUCMs, and MCF-7 respectively. All data are expressed as the means \pm SEM, $n=3$.

results with Liang et al. (2012) show that TERT was expressed in hUCM cells at a level of 0.50 ± 0.02 (Liang et al. 2012), while in our results, hUCMs expressed the TERT gene at a level of only 0.26 ± 0.06 . An interpretation of this diversity may be that they used whole umbilical cord mesenchymal cells, while we used only Wharton's jelly mesenchymal cells.

Certainly, in pluripotent stem cells, which are telomerase-positive (Young and Black 2004), the high concentrations of trophic growth factors, such as EGF, aFGF, and bFGF (Majore et al. 2011), cause high expansion of the cells. Growth factors such as EGF directly regulate telomerase via specific signal transduction pathways (Maida et al. 2002). Budiyo et al. (2003) have demonstrated that EGF activates TERT in NIH3T3 and A431 cells, whereas EGF cannot increase TERT in HSC-1. However, when hTERT-negative normal cells (normal foreskin fibroblasts or endometrial stromal cells) were treated with EGF, no induction of hTERT mRNA expression was observed.

Thus, the effect of EGF is limited to telomerase-positive cells which constitutively express hTERT and does not apply to hTERT-negative cells (Maida et al. 2002).

Our results showed that the presence of EGF considerably promoted the expression level of TERT (from $30.49\pm7.15\%$ to $51.82\pm12.96\%$), so that TERT was expressed more than our positive control cells (MCF-7) ($37.33\pm11.19\%$). ES cells treated with FGF-2 expressed TERT for a long term (Schwob et al. 2008). The findings show that the presence of FGF in culture may lead to different expression levels of TERT. In this research, it was found that, in the presence of FGF, TERT is expressed only slightly higher than the absence of FGF (from $30.49\pm7.15\%$ to $33.77\pm11.55\%$). Considering that the usage of FGF-2 with a lower dose causes stimulation of cell proliferation and a higher dose leads to cell differentiation (Garcia et al. 2005), it seems that the FGF dosage used in this study was inadequate for cell proliferation, which needs further research.

Investigation of cell cycle phases is another aspect of the cell expansion survey. Both intracellular and extracellular

control mechanisms are used for the regulation of cell cycle progression, but extracellular factors may determine cell fate such as differentiation, proliferation, or apoptosis. Cell cycle progression depends on the presence of growth factors. As long as growth factors are present, most adherent cells will continue their proliferation (Hulleman and Boonstra 2001).

Cell cycle analysis has revealed that the DNA content of most of the cells is higher in the G1 phase (Seshareddy 2008). However, some of the determining properties of ES cells are that they can be induced to continue proliferating or differentiating, they lack the G1 checkpoint in the cell cycle, and they spend most of their time in the S phase of the cell cycle, whereas cell cycle analysis have demonstrated that more than 80% of umbilical cord-derived cells (P2 to P6) are in the G0/G1 phase ($90.0 \pm 0.4\%$) and only a small proportion of cells are engaged in proliferation (G2/M phase, $6.0 \pm 0.3\%$; S phase, $4.0 \pm 0.7\%$) (Cao and Feng 2009). Liang et al. (2012) have shown that hUCMs are $84.0 \pm 2.4\%$ in the G0/G1 phase, $7.7 \pm 0.9\%$ in the S phase, and $8.3 \pm 1.5\%$ in the G2/M phase. Also, Zeng et al. (2011) got similar results as the hUCMs they used were $80.03 \pm 4.28\%$ in the G0/G1 phase, $18.45 \pm 4.10\%$ in the S phase, and $1.52 \pm 0.57\%$ in the G2/M phase. Another report has indicated that $60 \pm 2.9\%$ of hUCM cells were located in the G0/G1 phase, $8.9 \pm 1.6\%$ in the S phase, and $31.1 \pm 1.3\%$ in the G2/M phase of the cell cycle at the same time (Majore et al. 2011). Our results were closer to the results of Majore et al. (2011) that $56.09 \pm 6.56\%$ of the hUCMs located in the G0/G1 phase, while the percentage of cells located in the S and G2/M phases were slightly different from the others ($27.88 \pm 8.84\%$ and $11.31 \pm 0.79\%$, respectively). The PI for hUCMs calculated by Liang et al. (2012) was 16 ± 2.4 , while in our study, the PI was calculated as 41.13% for hUCMs and 22.51% for MCF-7 cells, as positive control, which was a considerable finding.

The end of the G1 phase or restriction point (R) has been defined as the point in G1 and the time that the cells no longer respond to the withdrawal of growth factors because, when the cells pass the restriction point, they will continue cell cycle progression independent of the presence of growth factors and enter the S phase. So, it seems that limited exposure of the cells to growth factors is enough for cell proliferation and when growth factors are removed before the restriction point. In our experiments, we kept the cells for 48 h in the presence of EGF and FGF (Hulleman and Boonstra 2001).

On the bases of the cell type, EGF causes diverse effects on cell growth (Maida et al. 2002). When Liang et al. (2010) exposed the hUCMs to EGF, after 24 h, 68.66 ± 3.43 of cells were in the G0/G1 phase, while 11.0 ± 0.55 and 20.34 ± 1.02 were in the S and G2/M phases, respectively (Liang et al. 2010). It was shown that, via using EGF, after 21 h of treatment with 5 ng/ml EGF, the G1/G0 phase ratio of

MCF10A cells moved from 78% to 42% and the S phase increased from 1% to 20% and the G2/M phase changed from 21% to 38% (LeVeal et al. 2004). On the other hand, the PI in these cells progressed from 31% to 58%. Cell cycle analysis on our cells after exposure to EGF for 48 h showed that $46.55 \pm 6.25\%$ of cells remained in the G0/G1 phase, while $37.34 \pm 9.8\%$ and $7.71 \pm 0.53\%$ of cells moved to the S and G2/M phases, respectively. So, it can be concluded that EGF has the ability to increase the PI from 41.13% to 49.18%; besides this, it can facilitate the progression of cells from G1 to S phase.

FGFs are usually used at lower doses (Garcia et al. 2005) in cell cultures for the propagation of hUCMs (Gauthaman et al. 2010). Upadhyay et al. (2007) showed that FGF-10 protects the cells against oxidant-induced DNA damage and apoptosis, so they focused on examining the effect of FGF-10 on A549 cells in the G1 phase. The rate of cell proliferation is normally controlled by the duration of the G1 phase of the cell cycle (Zhu et al. 1996). Cell cycle analysis of AEC revealed that 92.2% of the examined cells were in the G0/G1 phase, with 5.43% in the S phase and 2.37% in the G2/M phase. Following exposure to 10 ng FGF-10, $44.1 \pm 2.9\%$ of the treated cells moved to the S/G2/M phase, while $56.9 \pm 2.1\%$ of the cells remained in the G0/G1 phase (Upadhyay et al. 2007). We found similar results in this study, so that 41.62% of hUCM cells exposed to FGF were in the S/G2/M phase.

Conclusion

We may conclude that growth factors amplify the proliferation and expansion of hUCM cells. So that EGF caused the increase in the expression levels of TERT in these cells. We also conclude that the ratio of hUCMs in the G0/G1 phase decreased, while those in the S/G2/M phase increased after EGF and FGF treatments.

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