Cell Tissue Bank (2015) 16:487–495
DOI 10.1007/s10561-015-9494-9

Comparison of human dermal fibroblasts (HDFs) growth rate in culture media supplemented with or without basic fibroblast growth factor (bFGF)

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Received: 21 July 2014 / Accepted: 8 January 2015 / Published online: 21 January 2015

Abstract Basic fibroblast growth factor (bFGF or FGF-2) is a member of the FGF family secreted by different kinds of cells like HDFs and it is an important nutritional factor for cell growth and differentiation. The HDFs release bFGF in culture media at very low. The present study aims to investigate the HDFs growth rate in culture media supplemented either with or without bFGF. In brief, HDFs were isolated from human foreskin sample and were cultured in vitro in media containing bFGF and lack of this factor. The cells growth rate was calculated by trypan blue. The karyotyping was performed using G-banding to investigate the chromosomal abnormality of HDFs in both groups. Total RNA of each groups were extracted and cDNA samples were synthesized then, real-time Q-PCR was used to measure the expression level of *p27kip1* and *cyclin D1* genes normalized to internal control gene (*GAPDH*). The karyotype analysis showed that HDFs cultured in media or without bFGF had normal karyotype (46 chromosomes, XY) and chromosomal abnormalities were not observed. The cell growth rates in both groups were normal with proliferated exponentially but the slope of growth curve in HDFs cultured in media containing bFGF was increased. Karyotyp test showed that bFGF does not affect on cytogenetic stability of cells. The survey of *p27kip1* and *cyclin D1* genes by real-time Q-PCR showed that the expression level of these genes were up-regulated when adding bFGF in culture media ($p < 0.05$). The findings of the present study demonstrate that appropriate supplementation of culture media with growth factor like bFGF could enhance the proliferation and differentiation capacity of cells and improve cells growth rate. Similarly, fibroblast growth factors did not induce any chromosomal abnormality in cells. Furthermore, in HDFs cultured in bFGF supplemented media, the *p27kip1* and *cyclin D1* genes were up-regulated and suggesting an important role for bFGF in cell-cycle regulation and progression and fibroblast division stimulation. It also suggests that the effects of bFGF on different cell types with/or without production of bFGF or other regulation factors be investigated in future.

Keywords HDF · bFGF · Cell growth rate · Karyotyping · Real-time PCR

Abbreviations

HDFs	Human dermal fibroblasts
bFGF	Basic fibroblast growth factor

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Introduction

Human dermal fibroblasts (HDFs) are belonged to a dermis cell type with mesenchymal origin and found in all connective tissues. These cells are achievable with good kinetic of growth and can be easily cultured in vitro. HDFs could be obtained from adult normal dermis or neonatal foreskin (Chang et al. 2002; French et al. 2004; Wong et al. 2007). These cells play a critical role in epithelial-mesenchymal interactions and wound healing, as well as synthesis and secretion of extracellular matrix proteins (including laminin and fibronectin) and collagen under cell culture conditions (Mizuno and Glowacki 1996; Wong et al. 2007). Fibroblasts play a potential function in proliferation and migration in response to chemotactic, mitogenic and modulatory cytokines, and also autocrine and paracrine interactions (Jongkind and Verkerk 1984; Mastromonaco et al. 2006). These cells have many applications in tissue engineering, genetics and aging research, diagnosis of peroxisomal disorders, cell nuclear transfer, and cell reprogramming (Jongkind and Verkerk 1984; Mastromonaco et al. 2006; Wong et al. 2007).

HDFs secrete many factors in their culture media such as acidic and basic fibroblast growth factor (FGF-1 and -2 or bFGF, respectively). Human *FGF-2* gene is 70,990 bp in length, composed of a 5′UTR, 3 exons, 2 introns and the protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. This growth factor is a small polypeptide heparin-binding proteins which can interact with cell-surface-associated heparan sulfate proteoglycans and in many biological processes, such as brain development, can control the secretion of the ovarian protein hormone relaxin (RLX), limb blood pressure regulation, nervous system development, wound healing, and tumor growth (Taylor and Clark 1992; Coumoul and Deng 2003; Schalper et al. 2008). FGFs are members of a large family and have been identified in several cell types, including fibroblasts, endothelial cells, epithelial cells, macrophages, and neurons. They could affect the cell proliferation, growth, differentiation, chemotaxis, cell migration, and survival of several cell types in their culture (Jih et al. 2001; Böttcher and Niehrs 2005; Schalper et al. 2008).

The expression of *cyclin D1* (*CCND1*) and cyclin-dependent kinase inhibitor *p27kip1* (*CDKN1B*) genes (as a cell-cycle and transcription control) is induced by adding bFGF in culture media or by secretion of this factor in both human tumors and normal cells such as HDFs (Fredersdorf et al. 1997; Chassot et al. 2007). Moreover, bFGF is important for embryonic development and maintaining of stem cell cultures in an undifferentiated state (Roletto et al. 1996; Liang et al. 2012; Lotz et al. 2013). In the present study the cell culture growth rate of human dermal fibroblasts (HDFs) were compared with and without bFGF.

Materials and methods

HDFs isolation and cell culture

The foreskin specimens of healthy male newborns were obtained from Kashani Hospital (Shahrekord city, Iran) and transferred to the Cellular and Molecular Research Center of Shahrekord University of Medical Sciences in transfer media (Dulbecco's modified Eagle's medium (DMEM) contain 1 % penicillin/streptomycin). Foreskin tissue was dissected and washed with phosphate-buffered saline (PBS) and centrifuged in 1,200 rpm for 5 min. After discarding the supernatant, HDFs were isolated from human foreskin tissue by a combination of mechanical disaggregation and enzymatic digestion (0.25 % Trypsin–EDTA solution, 100 U/mL Collagenase type IV, and 100 μg/mL DNase). Single cells were cultured at 37 °C in a 5 % CO_2 atmosphere in two separate groups of flasks [techno plastic products (TPP)] containing DMEM plus 10 % fetal bovine serum (FBS), and 1 % penicillin/streptomycin antibiotics (all reagents purchased from Gibco, NY, USA). After 2 days the media together with floating cells and red blood cells (RBC) were discarded and replaced by fresh media (with and without bFGF).

Cells count

In this study HDFs cultured in two groups (with and without bFGF) were seeded in a 6-well plate (TPP) with a concentration of 4×10^4 per well. The culture media of each plate were changed again every 3 days through the 6 days and cells viability and growth curves of HDFs were determined using trypan blue under microscope by counting unstained cells every

24 h. The experiment was performed in three biological replicates.

HDFs preparation and karyotyping

The HDFs (cultured in media with and without bFGF) were washed twice with phosphate-buffered saline (PBS) and dissociated with 0.25 % Trypsin–EDTA solution at 37 °C for 5 min. The enzyme activity was neutralized by DMEM contain 15 % FBS. HDFs were counted by Neubauer Lam under microscope and cells were seeded into 6-well plates at a concentration of 150×10^3 in 2 mL of DMEM with 15 % FBS per well, and incubated in a CO_2 incubator at 37 °C. After cells confluence reach to 80–90 % in each well, the culture medium was replaced with media containing 0.1 μg/mL Karyomax colcemid solution (Cat. No. 15212-012. Invitrogen, Carlsbad, CA, USA) and returned into the CO_2 incubator. After 20 min the cells of each group were trypsinized (0.25 % Trypsin–EDTA solution) and cells sediment were collected and suspended in 5 mL of 0.075 M KCl solution and the suspension was incubated in 37 °C for 20 min. Then, 1 mL cold Carnoy's fixative (methanol/acetic acid, 3:1) was added and mixed with cells and were centrifuged at 900 rpm for 10 min at room temperature (RT) and cells pellet were collected. After two rounds of fixation (add 5 mL fixative and centrifuge at 900 rpm for 10 min), the pellet were fixed via suspending in 200 μL of cold fixative and cells from each suspension were dispensed onto glass slides and baked at 75 °C for 3 h and after that routine chromosome G-banding analysis was carried out on HDFs cultured in normal media and supplemented with bFGF. Twenty karyotypes per slide were examined.

Reverse transcriptase PCR

Total RNA was isolated from HDFs using BIOZOL Kit (BSC51M1) according to the manufacturer's instructions. The extracted RNA was measured by NanoDrop ND-1000 (Peqlab, Erlangen, Germany) according to the method described by Sambrook and Russell (2001). One μg of each RNA samples was subjected to synthesize of cDNA using the PrimeScript™ RT Reagent Kit (Takara Bio Inc, RR037A) according to manufacturer's instruction. The specific oligonucleotide primers for amplification of *cyclin D1* (*CCND1*) and *p27kip1* (*CDKN1B*) genes as well as *GAPDH* (internal control gene) were designed using Gene Runner software version 3.05 (Hastings Software, Inc.) and sequences were analyzed by basic local alignment search tool (BLAST) in GenBank data (Table 1). The PCR reaction performed in a total volume of 25 μL containing 2 mM $MgCl_2$, 200 μM dNTP mix, 2.5 μL of 10X PCR buffer (all Fermentas, Germany), 1 μg of template cDNA, 0.25 μM of each primer, and one unit of *Taq* DNA polymerase (Roche Applied Science, Germany). The negative control was sample without DNA. The PCR temperature conditions in a Gradient Palm Cycler (Corbett Research, Australia) involved an initial denaturation at 94 °C for 5 min; followed by 35 cycles at 94 °C for 30 s, annealing at 63 °C (*p27kip1* gene) and 66 °C (*cyclin D1* gene) for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min was done at the end of the amplification.

A total amount of 10 μL of amplified cDNA were applied to the 3 % agarose gel electrophoresis and constant voltage of 80 V for 30 min was used for products separation. The 50 bp DNA ladder (Fermentas, Germany) was used as a molecular weight marker to determine the length of the amplified fragments. After electrophoresis, the gel was stained with ethidium bromide and examined under UV light and photograph was obtained in UVIdoc gel documentation system (Uvitec, UK).

Real-time PCR assay

The expression levels of *p27kip1* and *cyclin D1* genes in HDFs that cultured in media supplemented with bFGF and without this factor were measured by quantitative real-time PCR (real-time Q-PCR) using a SYBR Green master mix (Roche Applied Science, Indianapolis, IN, USA). Furthermore, the GAPDH primers were used as internal control of the reaction for comparing the cell-cycle gene expression. The real-time PCR reaction was performed in 10 μL reaction containing 5 μL of SYBR Green master mix, 2.5 nM concentrations of each forward and reverse primers, and 60 ng/μL of cDNA sample. The micro-tubes were placed into Rotor-Gene 3000 (Corbett, Australia) for gene amplification. The reaction program consist of an initial denaturation step at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 10 s, annealing at 63, 66 and 61 °C for *p27kip1*, *cyclin D1* and *GAPDH* genes, respectively for 20 s, and

Table 1 The sequence of primers used for gene amplification

Gene	Primers name	Sequence	Product length (bp)	GenBank accession number
cyclin D1	CD1-F	5′-AAGTTGCAAAGTCCTGGAGCC-3′	125	NM_053056
	CD1-R	5′-TCGGCTCTCGCTTCTGCTG-3′		
p27kip1	p27-F	5′-TCGCCGTGTCAATCATTTTC-3′	91	NM_004064
	p27-R	5′-AACACCCCGAAAAGACGAG-3′		
GAPDH	GAPDH-F	5′-TCATTTCCTGGTATGACAACG-3′	121	NM_001256799
	GAPDH-R	5′-TTCCTCTTGTGCTCTTGCTG-3′		

extension at 72 °C for 20 s. The experiments were carried out in triplicate for each data point. The fold changes average of *p27kip1* and *cyclin D1* genes was analyzed based on threshold cycles (Ct) and expression level of each target gene was normalized to the corresponding internal control gene (*GAPDH*). The relative quantification of gene expression in two groups was determined using the $2^{-\Delta Ct}$ method. Finally the melting curve was generated immediately after amplification by holding the reaction at 95 °C for 60 s, and then lowered the temperature to 45 °C with transition rate of 0.1 °C and maintained for 120 s, which was then followed by heating slowly at transition rate 0.05 to 80 °C with continuous collection of fluorescence at 640 nm.

Statistical analysis

The data were collected in statistics programs for the Social Sciences software, version 20 (SPSS, Inc., Chicago, IL, USA) and differences between genes expression of cells that cultured in media supplemented with and without bFGF were considered significant at $p < 0.05$. Graphs were prepared using Microsoft excel and the Graph Pad Prism version 5.01 (2003, San Diego, CA) software.

Ethical approval

In present study informed consent forms were approved by the Regional Research Ethical Committee of Shahrekord University of Medical Sciences. The foreskin samples of healthy male newborns were obtained and consent forms were filled by parents of each infant. Male newborns with genetic abnormalities were excluded from the study.

Results

HDFs preparation

HDFs cultured in media supplemented with bFGF and in normal condition were used for karyotyping, real-time PCR, and cell growth analysis. Figure 1 shows the HDFs isolated from human foreskin samples that cultured in media containing bFGF and without bFGF.

Cell growth rate

After cultured HDFs confluence in different conditions (with and without bFGF) reached to 80–90 %, each cell groups were seeded into 6 wells plate and the cell growth rate were calculated using trypan blue by counting unstained cells for each triplicate wells every 24 h. The growth curves showed that the number of HDFs in both groups was not increased in the first 48 h after passage. From 24 to 120 h, HDFs that cultured in media with and without bFGF had normal growth with proliferated exponentially but the slope of growth curve in cultured cells in presence of bFGF was significantly higher and numbers of cells were increased. In 120–144 h after passage HDFs were entered in stationary and then death phase (Fig. 2).

Karyotype test

Karyotyping by G-banding was conducted to investigate the chromosomal stability of HDFs cultured in media supplemented either with bFGF or without this factor. A number of 20 karyotypes per each slide were analyzed and showed that HDFs in both group had normal karyotype (20/20, 46 chromosomes, XY). The karyotype analysis did not show any chromosomal

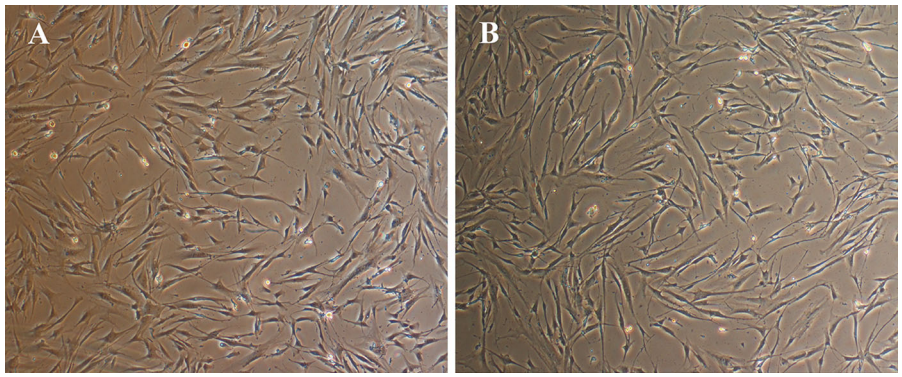

Fig. 1 **a** HDFs are cultured in media contain bFGF and **b** without this growth factor

abnormalities and bFGF did not affect the cytogenetic stability of these cells (Figs. 3 and 4).

Conventional reverse transcriptase PCR analysis

The amplified cDNA samples using designed specific oligonucleotide primers on 3 % agarose gel electrophoresis revealed a 125, 121, and 91 bp fragments for *cyclin D1*, *GAPDH* (as a internal control), and *p27kip1* genes, respectively (Fig. 5).

Real-time PCR assay

The expression level of *p27kip1* (0.008745897 vs. 0.000226589, $p < 0.05$) and *cyclin D1* (0.007526952 vs. 0.000221258, $p < 0.05$) genes in HDFs cultured in media with bFGF was compared to corresponding levels in cells cultured without this factor after normalization to *GAPDH* expression level (0.002185814) (Figs. 6 and 7). This assay is representative of three times for comparison of groups. According to these results both genes were up regulated in HDFs after adding bFGF in culture media.

Discussion

Growth factors are important agents secreted by various cells in tissues. They can affect and control proliferation, signaling processes, healing, regulating a variety of cellular processes, cellular differentiation and cellular growth (Schuldiner et al. 2000). In the present study we used isolated HDFs from foreskin specimens because these cells are easily accessible and can be used for generation of induced pluripotent stem cells (iPSCs) as well as supporting cell line (as a feeder layer) and cell nuclear transfer in many researches. In addition these cells are cytogenetically stable and can be maintained in vitro for long-term

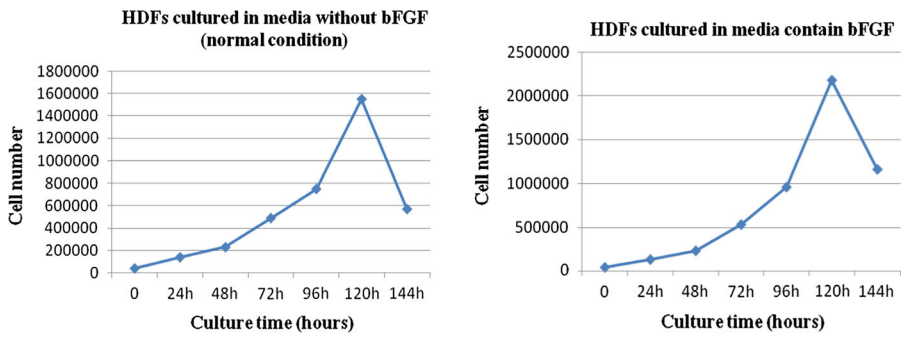

Fig. 2 Growth *curves* of HDFs cultured in media without bFGF (*left*) and supplemented with this growth factor (*right*) through 6 days. The growth *curves* shows normal exponential growth in both groups but in first 48 h the *numbers* of cells was not increased and in cultured cells in media contain bFGF the slope of growth curve was high, and after 120–140 h the cells were entered to stationary and death phase finally

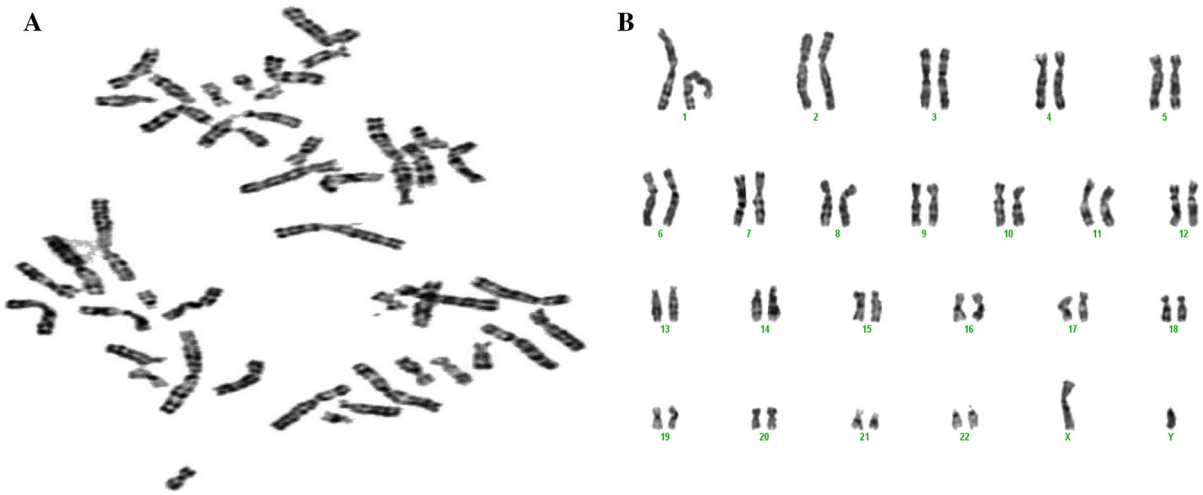

Fig. 3 **a** The metaphase and **b** karyotype of HDFs cultured in media without bFGF

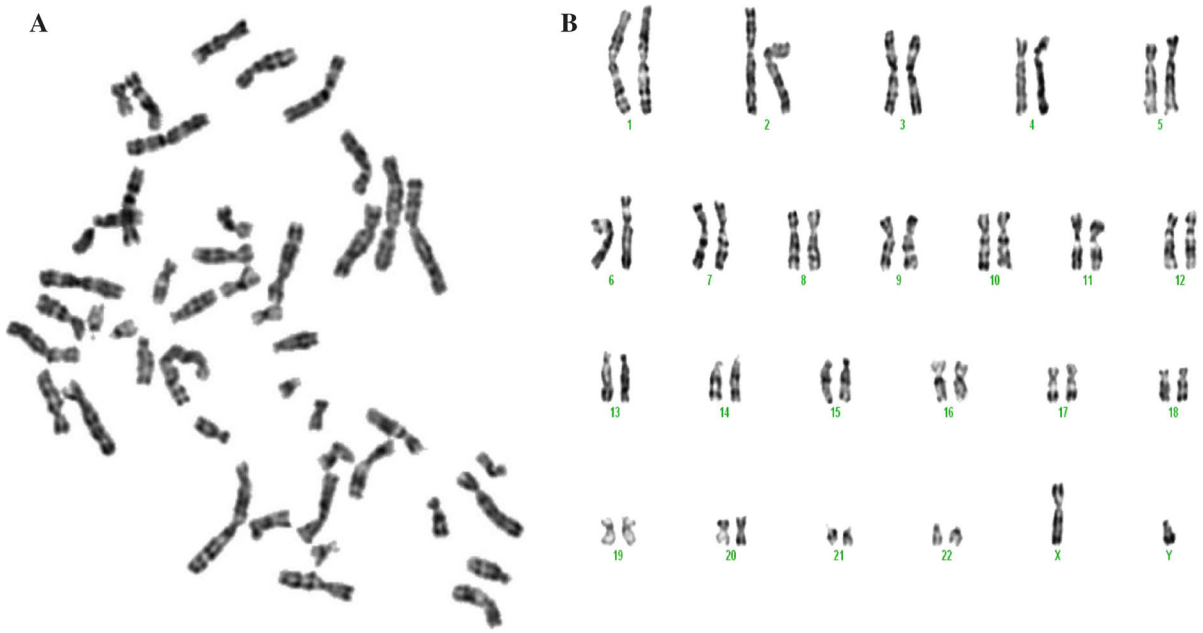

Fig. 4 **a** The metaphase and **b** karyotype of cultured HDFs in media supplemented with bFGF

culture (Bisson et al. 2013; Ghasemi-Dehkordi et al. 2014). Although, HDFs are able to secrete bFGF in their culture media but the secretion level is very low and its effect on these cells has not been clarified yet. In this study the growth rate of HDFs cultured in media supplemented with and without bFGF was compared. Cell growth rate was calculated using trypan blue and karyotype test using G-banding was conducted to investigate the chromosomal stability of HDFs in both groups. Also, total RNA were extracted from each group and cDNA samples were synthesized. Then, expression level of *p27kip1* and *cyclin D1* genes was studied by real-time Q-PCR and compared between the groups. The results showed that HDFs cultured in media with and without bFGF had normal karyotype and cell growth rate but the slope of growth curve in cultured cells in presence of bFGF was increased. The karyotyping showed no chromosomal abnormalities

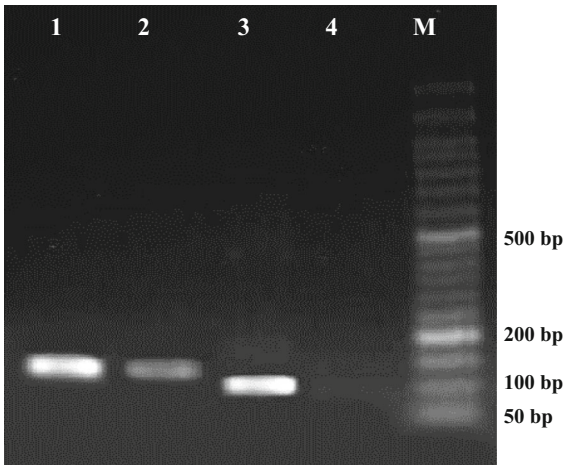

Fig. 5 The amplified cDNA samples of cell-cycle genes (*p27kip1* and *cyclin D1*) and *GAPDH* gene on agarose gel electrophoresis (*Lane* M is 50 bp molecular weight marker (Fermentas, Germany), *lanes 1* and *3* are the results for *cyclin D1* and *p27kip1* genes respectively, *lane 2* is *GAPDH* (internal control gene), and *lane 4* is negative control (no DNA)

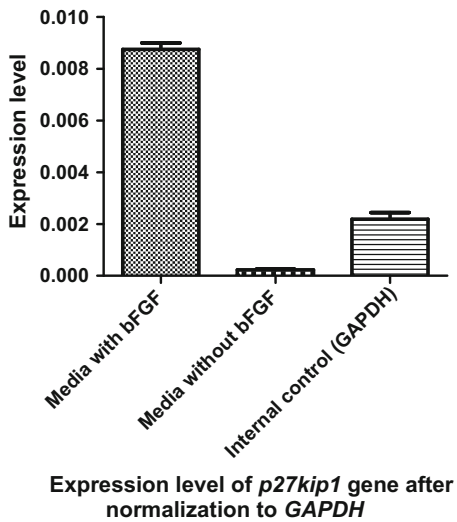

Fig. 6 Comparison of the expression level of *p27kip1* gene in HDFs cultured in media containing bFGF to the cells grown in media without this factor

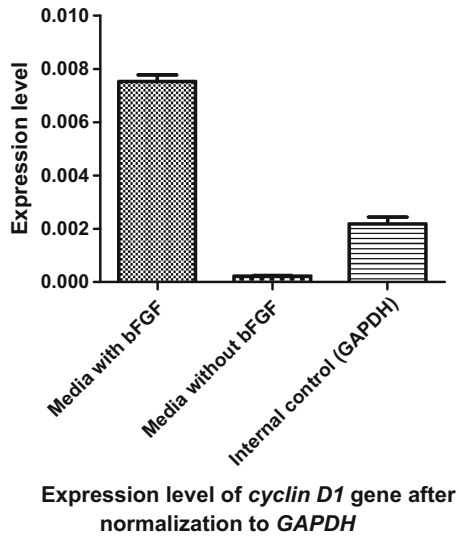

Fig. 7 Comparison of the expression level of *cyclin D1* gene in HDFs cultured in media containing bFGF to the cells grown in media without this factor

and bFGF did not cause cytogenetic instability in these cells. The real-time Q-PCR analysis showed that *p27kip1* and *cyclin D1* genes were up-regulated after adding bFGF in culture media.

The effects of bFGF in different cell lines were investigated. Powers and colleagues mentioned that fibroblast growth factor play a role in tumor growth and angiogenesis and Vander Heiden et al. indicated that growth factors can influence cell growth (Powers et al. 2000; Vander Heiden et al. 2001). So, their findings confirmed the increase of HDFs cell number cultured in bFGF supplemented media. Roletto et al. observed the secretion of hepatocyte growth factor/scatter factor (HGF/SF) by MRC-5 cells and by other fibroblast-derived cell cultures in conditioned media are enhanced by exposure to bFGF. Their findings articulated interaction can be speculated for bFGF, HGF/SF, and IL-1, e.g., in tissue regeneration during inflammatory processes or in wound healing (Roletto et al. 1996). It has been reported that there is a correlation between the high level expression of *p27kip1* and *cyclin D1* genes and human breast cancer cells. Also an inverse correlation between the expression of *p27kip1* gene and the degree of tumor malignancy in human breast and colorectal cancers has been observed (Fredersdorf et al. 1997). So, up-regulation of these genes after adding bFGF in present study is not surprising. Schuldiner and co-workers evaluated the potential of eight growth factors including bFGF, transforming growth factor b1 (TGF-β1), activin-A, bone morphogenic protein 4 (BMP-4), HGF, epidermal growth factor (EGF), β nerve growth factor (bNGF), and retinoic acid to direct the differentiation of human ES-derived cells in vitro. They showed that each growth factor has a unique effect that

may result from directed differentiation and/or cell selection and indicated that bFGF together with retinoic acid, EGF, and BMP-4 activate the ectodermal and mesodermal markers (Schuldiner et al. 2000). Jeffery et al. indicated that fibroblast growth factor-2 enhances expansion of human bone marrow-derived mesenchymal stromal cells without diminishing their immunosuppressive potential (Auletta et al. 2011). In another study the precise effects of bFGF on fibroblast proliferation and the signaling pathways responsible for bFGF-induced proliferation in cultured HDFs was investigated and the results showed that bFGF increased the number of HDFs in a dose- and time-dependent manner (Makino et al. 2010).

Conclusion

Although HDFs can secrete low level of bFGF, direct supplementation of this factor in culture media could increase the cells rate growth and improve the proliferative capacity and decrease doubling time of these cells. Furthermore, the findings of present study showed that supplementation of growth factor like bFGF does not affect the chromosomal stability of cells. Also, *p27kip1* and *cyclin D1* genes are up-regulated in HDFs upon supplementation of culture with bFGF. This finding suggests that this growth factor affect the regulation and progression of cell cycle genes and leads to increase the cells number. It is recommended in future studies the effects of bFGF or other regulation factors with or lack of ability to secretion factors on different cell types examined.

Acknowledgments The authors would like to express sincere thanks to the staffs of Cellular and Molecular Research Center of Shahrekord University of Medical Sciences for their cooperation.

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