Cell Proliferation

Cell Prolif., 2012, **45**, 132–139 doi: 10.1111/j.1365-2184.2012.00808.x

Basic fibroblast growth factor modulates cell cycle of human umbilical cord-derived mesenchymal stem cells

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Received 24 September 2011; revision accepted 17 December 2011

Abstract

Background: Mesenchymal stem cells (MSC) have great potential in regenerative medicine, immunotherapy and gene therapy due to their unique properties of self-renewal, high plasticity, immune modulation and ease for genetic modification. However, production of MSC at sufficient clinical scale remains an issue as *in vitro* generation of MSC inadequately fulfils the demand with respect to patients.

Objectives: This study has aimed to establish optimum conditions to generate and characterize MSC from human umbilical cord (UC-MSC).

Materials and methods: To optimize MSC population growth, basic fibroblast growth factor (bFGF) was utilized in culture media. Effects of bFGF on expansion kinetics, cell cycle, survival of UC-MSC, cytokine secretion, expression of early stem-cell markers and immunomodulation were investigated.

Results: bFGF supplementation profoundly enhanced UC-MSC proliferation by reducing population doubling time without altering immunophenotype and immunomodulatory function of UC-MSC. However, cell cycle studies revealed that bFGF drove the cells into the cell cycle, as a higher proportion of cells resided in S phase and progressed into M phase. Consistent with this, bFGF was shown to promote expression of cyclin D proteins and their relevant kinases to drive UC-MSC to transverse cell cycle check points, thus, committing the cells to DNA synthesis. Furthermore, supplementation with bFGF changed the cytokine profiles of the cells and reduced their apoptotic level.

Conclusion: Our study showed that bFGF supplementation of UC-MSC culture enhanced the cells' growth kinetics without compromising their nature.

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Introduction

During the last decade, mesenchymal stem cells (MSC) have become an important component of stem cell-based neo-therapies for tissue regeneration and transplantation. MSC constitute a rare non-haematopoietic population of the adult bone marrow (BM), which can be defined according to its ability to self-renew and differentiate into tissues of mesodermal origin (osteoblasts, adipocytes, chondrocytes) (1). MSC have been isolated from various tissues, and are constantly present, or their pool is replenished, due to migration there from the bone marrow (2). Furthermore, it has recently been demonstrated that MSC are also present in umbilical cord blood (CB) and other foetal tissues (3,4). Physiologically, MSC serve as progenitors of bone marrow stroma and thus play a crucial role in supporting haematopoiesis by providing haematopoietic progenitors with their necessary cytokines and cell contact-mediated signals (5). However, additional importance has been realized as MSC can suppress immune responses and have been exploited in treating autoimmune diseases and graft-versus-host disease after allogenic transplantation (6–8). Animal studies of cerebral injury, myocardial infarction, muscular dystrophy and bone fractures appear to confirm their potential clinical usage (9). Furthermore, phase I clinical studies employing MSC demonstrate that they are non-toxic and non-immunogenic to recipients. Consistent with phase I studies, administration of autologous and third party MSC into osteogenesis imperfecta patients corrects the bone disorder, thus leading to successful phase II clinical trial (10).

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Increasing demands of tissue regeneration and transplantation cases necessitates a readily available source of MSC as an 'off-the-shelf' product for quick and effective treatment. Consistent with this, several attempts have been made to generate MSC from various tissues, especially tissue origin from human delivery wastes, such as CB (11,12), umbilical cord (UC) tissue (4,13) and placenta (14,15). Although CB is a reliable source for haematopoietic stem cells (16), presence of MSC in CB is controversial and remains to be confirmed (17). Alternatively, accumulating data of Wharton Jelly's of UC has shed further light on generating MSC. Various approaches have been applied to generate MSC from UC (18,19), nevertheless, reproducibility is a foremost issue and needs to be resolved.

Basic fibroblast growth factor (bFGF) is a commonly used population growth supplement in MSC culture (20–23); it has been reported that bFGF increases MSC proliferation capacity and telomere length, while retaining the cells' multipotential differentiation ability (23–25). However, controversial data suggest that bFGF favours osteogenic (26) and chondrogenic (27) differentiation of MSC. To complement the current data, we have attempted to investigate effects of bFGF on UC-MSC morphology, population growth kinetics and cell functions, such as proliferative capacity, immunosuppressive activity and survival rate.

Materials and methods

Generation of human UC-MSC

Human UC samples were obtained at delivery, from full-term pregnancies after obtaining written informed consent of volunteers, in accordance with consent of the Ethics Committee of the Faculty of Medicine and Health Sciences, Universiti Putra,Malaysia. UC samples were dissociated into single cell suspensions using a combination of enzymatic digestion and mechanical dissociation (4). Cells were cultured in MSC complete media made up of Dulbecco's modified Eagle's medium with nutrient mixture F-12 (HAM)[1:1] (DMEM/F12) with GLUTAMAX-I (Gibco-Invitrogen, Grand Island, NY, USA), supplemented with 10% pre-selected foetal bovine serum (Stem Cell Technology Inc., London, UK), 1% penicillin and streptomycin (Gibco, Invitrogen), 0.5% fungizone (Gibco, Invitrogen), 0.1% gentamicin (Gibco, Invitrogen), with/without 40 ng/ml bFGF (Peprotech, Rocky Hill, NJ, USA). Cells were incubated for 5–10 days to allow their adherence to plastic dishes, and non-adherent cells were removed by replacement with fresh media. At least 20 UCs were processed to generate UC-MSC and a minimum of six UC-MSC were further utilized for subsequent experiments. All UC-MSC met minimal criteria set by the International Society for Cellular Therapy (ISCT) to define human MSC (data not shown).

Growth kinetic analysis and proliferation assay

Population growth kinetics of live UC-MSC was performed using the trypan blue exclusion cell count method. Briefly, UC-MSC were cultured in presence or absence of bFGF in six-well plates at 4×10^3 cells/well and were harvested every 2 days over a period of 12 days, for haemocytometer cell counting. Dose-dependent mitogenic effect of bFGF was assessed using tritiated thymidine (^{3}H-TdR) incorporation assays. Cells were plated in 96 wells at 5×10^3 cells/well and treated with a variety of concentrations of bFGF (0, 10, 20 and 40 ng/ml) for 72 h, and 0.5 μCi/well ^{3}H-TdR (Pelkin Elmer Inc, Wellesley, MA, USA) was added for the final 18 h incubation. Cells were harvested on to glass fibre filter mats A (Perkin Elmer) using a 96-well plate manual cell harvester (MACH IIIM-FM) (Tomtec Inc., Hamden, CT, USA) and scintillation cocktail was added to amplify the signal. Liquid scintillation spectroscopy was used for counting in a Microbeta Trilux beta counter (Pelkin Elmer).

Cell cycle analysis

UC-MSC were seeded at 0.2×10^6 cells/per 25 cm^2 flask and cultured in presence or absence of bFGF supplementation. At 80–90% confluence, cells were harvested for cell cycle analysis. Briefly, cells were washed and fixed overnight in 70% ethanol at −20 °C. Fixed cells were washed and incubated in 100 μg/ml propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO, USA) and 20 ng/ml RNAase (Sigma-Aldrich) in PBS for 30 min. Cell cycles were assessed by flow cytometry and analysis was performed using FCS Express V3 software (BD Biosciences, San Jose, CA, USA).

Caspase 3/7 activity assay

Cells were seeded into 96 wells at 5×10^3 cells/well and were incubated for 24 h. Caspase 3/7 activity assay was performed by using Caspase-Glo® 3/7 kit (Promega, Madison, WI, USA) and measured using GlowMAX Bioluminometer (Promega). Results were expressed in relative light units.

Human cytokine antibody array

Conditioned media were harvested from UC-MSC grown in MSC complete media with or without bFGF

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supplementation, for 48 h. Human cytokine antibody array (Panomics, Fremont, CA, USA) was incubated in 2 ml of conditioned media and processed according to the manufacturer's instructions.

Western blot analysis

UC-MSC were seeded at 0.5×10^6 cells/60 mm petri dish and cultured in presence or absence of bFGF supplementation for 72 h. At the end of each experiment, cells were harvested for western blot analysis as described previously (28) Primary antibodies and respective dilutions used are listed in Table 1.

Isolation of T cells

Fresh heparinized peripheral blood samples from the healthy donors were collected and diluted in 1× PBS at 1: ratio and were layered on Ficoll Paque for density gradient separation. The peripheral blood mononuclear cells (PBMC) were washed in 1× PBS and trypan blue exclusion cell counting was performed. PBMC were cultured in complete T-cell medium containing RPMI 1640 (Gibco-Invitrogen) supplemented with 10% FBS serum (Gibco-Invitrogen) and 1% penicillin/streptomycin (Gibco-Invitrogen).

T-cell proliferation assay

Ability of UC-MSC to inhibit T-cell proliferation was tested using the tritiated thymidine [^{3}H]dT incorporation assay. MSC were co-cultured with fixed numbers of T cells at 1:5, 1:10, 1:50 and 1:100 ratios, in 96-well plates. T cells were stimulated with PHA-L (Roche, Pitsacaway, NJ). Cultures were incubated for 72 h and were pulsed with tritiated thymidine [^{3}H]dT (0.037 MBq/well) [0.5 μCi/well] (Perkin Elmer) during the final 18 h incubation. Cell proliferation was measured by [^{3}H]dT incorporation, which reflects percentage of cells in the S phase of the cell cycle. At 72 h, cells were harvested on to glass fibre filter mats A (Perkin Elmer) using a 96-well plate, manual cell harvester (MACH IIIM-FM; Tomtec Inc., Hamden, CT, USA). Scintillation cocktail was added and thymidine incorporation was measured by liquid scintillation spectroscopy using a Microbeta Trilux beta counter (Pelkin Elmer).

RT-PCR

Total RNA was extracted from early passage of MSC (P3-P10) using TRIzol® Reagent (Invitrogen). Reverse transcription reactions were carried out using the ImPromII™ Reverse Transcription System (Promega) kit, and cDNA strands were generated. PCR was performed with the Taq DNA Polymerase kit (Qiagen, Hilden, Germany). Genes of interest were obtained using primers (EUROGENTEC AIT) for Nanog, Oct3/4, Sox2, Rex1 and GAPDH.

Statistical analysis

Values for all measurements are presented as mean ± SD. Comparisons for all pairs were performed with Student's *t*-test. Significance levels were set at P-value <0.05.

Results

bFGF promoted population growth of UC-MSC

bFGF profoundly increased proliferation rate of UC-MSC in a dose-dependent manner (Fig. 1a). Cell population growth is revealed by a typical logarithmic curve, with initial lag or stationary period followed by rapid logarithmic expansion until cells reached a plateau at the end of culture period. Non-supplemented UC-MSC had an initial lag phase of 4 days, followed by exponential

Table 1. Primary antibodies used for western blot analysis

Protein	Clone	Company	Catalogue no.	Antibody dilution/incubation time
p-Rb (S807/811)	Polyclonal	Cell Signaling Technology (Danvers, MA, USA)	9308	1:1000/o.n
Rb	4H1	Cell Signaling Technology	9309	1:1000/o.n
p-CDC2 (Y15)	Polyclonal	Cell Signaling Technology	9111	1:1000/o.n
CDC2/CDK1	A17.1.1	Thermo Fisher Scientific (Lab Vision), Fremont, CA, USA	MS-110-P0	1:3000/o.n
CDK4	DCS156	Cell Signaling Technology	2906	1:1000/o.n
CDK6	DCS83	Cell Signaling Technology	3136	1:1000/o.n
Cyclin D1	DCS6	Cell Signaling Technology	2926	1:1000/o.n
Cyclin D3	DCS22	Cell Signaling Technology	2936	1:1000/o.n
PCNA	PC10	Dako, Carpinteria, CA, USA	M0879	1:3000/o.n
Cyclin E	HE12	Thermo Fisher Scientific (Lab Vision)	MS-870-P0	1:1000/o.n
α-Tubulin	DM1A	Thermo Fisher Scientific (Lab Vision)	MS-581-P	1:5000/1 h

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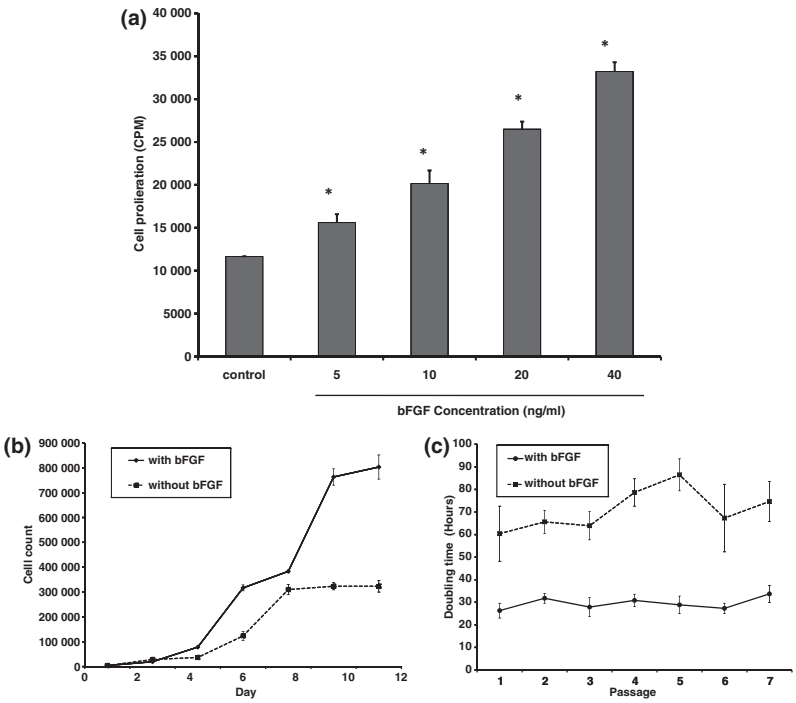

Figure 1. Supplementation of bFGF promotes population growth kinetics of UC-MSC by increasing cell proliferation. (a) UC-MSC plated at 5000 cells/well in 96-well plate were treated with bFGF in a variety of concentrations for 72 h. Cells were pulsed with [^{3}H]-thymidine over the final 18 h and incorporated [^{3}H]-thymidine was measured using a Microbeta Scintillation Counter. Results are expressed as mean CPM ± SD. (b) At passage 3, cells were plated in six-well plates at 4000 cells/well, and medium was changed twice weekly. Triplicate cultures were harvested for trypan blue exclusion cell counting every 2 days. Results represent mean cell number ± SD. (c) Comparison of doubling times of UC-MSC with and without bFGF supplementation. 0.2×10^6 cells were seeded into T25 culture flasks and cultured with or without bFGF. Cells were cultured to confluence and subjected to trypan blue exclusion cell counting. Doubling time was determined using the Patterson Formula* and expressed as mean doubling time ± SD. *P*-values <0.05 were compared with and without bFGF. *$T_d = T\lg 2/\lg(N_t/N_0)$ where T_d is doubling time (h), T is time cells proliferated from N_0 to N_t (h), and N is cell count.

log phase of 3–4 days before their plateau. bFGF supplemented UC-MSC on the other hand, progressed aggressively with initial lag phase of only1–2 days and a longer exponential logarithmic phase of 6–7 days before reaching their plateau (Fig. 1b). Moreover, cell yield of bFGF-supplemented UC-MSC was approximately 2.5-fold higher than non-supplemented UC-MSC. Consistent with this, doubling times of bFGF-supplemented UC-MSC were approximately 3- to 4-fold shorter than those of non-supplemented UC-MSC in reflection of their rapid cell proliferation (Fig. 1c).

bFGF induces cell proliferation and cell cycle protein expression

Cell cycle status of UC-MSC was determined by assessing their DNA content after PI staining. Non-supplemented UC-MSC were in a quiescent state with 92.7% cells in G_0/G_1, 3.6% in S phase and 3.7% in G_2/M (Fig. 2a). bFGF-supplemented UC-MSC were actively in the cell cycle with 66.27% in G_0/G_1, 24.23% in S and 9.5% in G_2/M (Fig. 2b). Western blotting results revealed that bFGF augmented expression of cyclin D proteins (Fig. 2c) followed by increase in Cdk4, PCNA, Rb, pRb-s807 and pCDC2 proteins.

bFGF supplementation reduced secretion of VEGF and MMP3

In response to bFGF supplementation, level of vascular endothelial growth factor (VEGF) and matrix metalloprotease 3 (MMP3) was significantly reduced in UC-MSC conditioned media (Fig. 3). However, production of interleukin-6 (IL-6), interleukin-8 (IL-8), tumour necrosis factor receptor-I (TNFRI) and interleukin-1α (IL-1α) remained unaffected.

bFGF maintained stem-cell characteristics of UC-MSC by reducing apoptotic activity and increasing expression of stem-cell markers

The effect of bFGF on UC-MSC cell death by apoptosis was measured by evaluation of caspase 3/7 activity. UC-MSC supplemented with 40 ng/ml bFGF for 24 h

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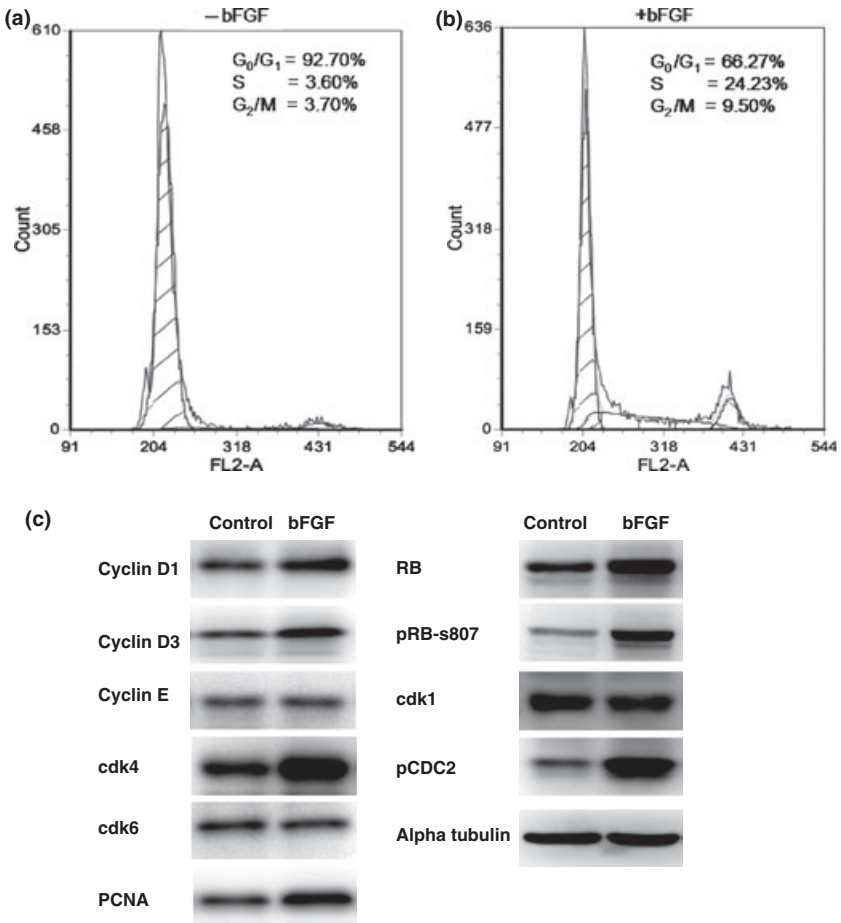

Figure 2. Activation of cell cycle machinery of UC-MSC by bFGF supplementation. (a) DNA content as quantified by PI staining. More than 90% of non-supplemented UC-MSC were in G_0 and G_1 phases, but only a small population was actively proliferating ($S+G_2/M$ = 7.30%). bFGF-supplemented UC-MSC were actively engaged in mitosis (G_0/G_1 = 66.27; $S+G_2/M$ = 33.73%). (b) Protein analysis by western blotting indicated that bFGF supplementation increased cyclins and Cdks that actively participate in G_1 and S phases of the cell cycle. Data are representative of six experiments.

had significantly less activity and production of caspases 3/7. Furthermore, bFGF supplementation increased expression of typical early embryonic transcription factors, namely, Nanog, Sox2 and Rex1; however, expression of Oct4 remained unaffected (Fig. 4b).

Immunomodulatory activity of UC-MSC was unaffected by bFGF

When UC-MSC were co-cultured with human T cells with PHA stimulation, both bFGF-supplemented and non-supplemented UC-MSC had similar patterns of T-cell anti-proliferation in a dose-dependent manner (Fig. 5).

Discussion

Robust progress in regenerative medicine has created a huge demand for clinical scale production of MSC. This has necessitated maximal stem-cell yield without compromising their multi-differentiation potential while preserving the cells' stemness during *in vitro* expansion. This includes identification of various stem cell sources that promise more primitive and higher stem cell yield and culture protocols for stem cell expansion to more specific and complex stem cell sorting methods. Although BM serves as a reliable source of MSC, many constraints such as invasive procedures and availability of donors, often limit their accessibility. This paradigm has initiated exploration of alternative sources of stem cells, such as newborn-derived tissues and other postnatal tissues. Among these, UC and CB are of great potential for being utilized in generating MSC.

In this study, we have generated UC-MSC and evaluated the effect of bFGF some aspects of their cell physiology. Although stem cells from earlier ontogeny have higher proliferative indices, yet requirement of vast cell

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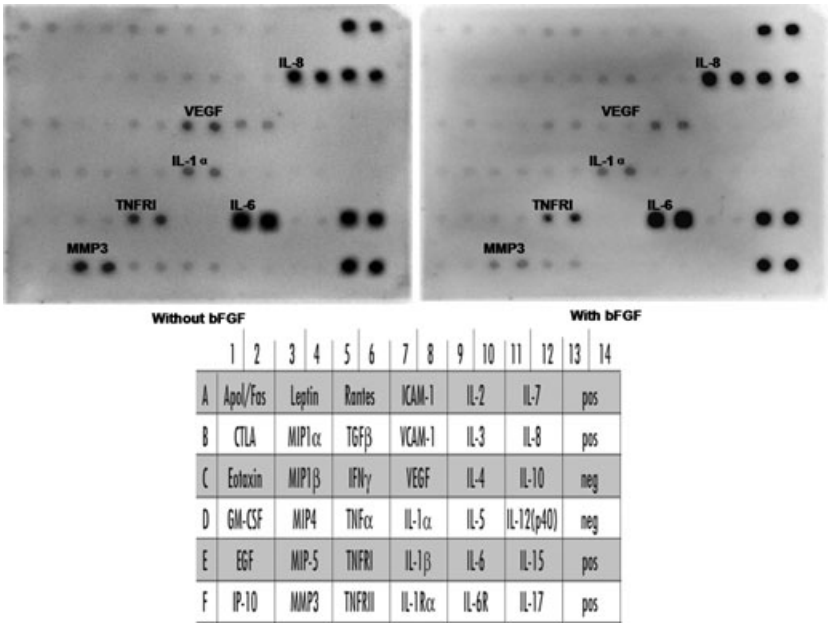

Figure 3. bFGF supplementation did not alter cytokine secretion profile of UC-MSC. Supernatants from bFGF supplemented and non-supplemented UC-MSC cultures were harvested and analysed for 36 human cytokines. Black points indicate presence of cytokine in supernatant from UC-MSC cultures. In presence of bFGF, VEGF and MMP3 were down-regulated compared to common cytokines produced by MSC.

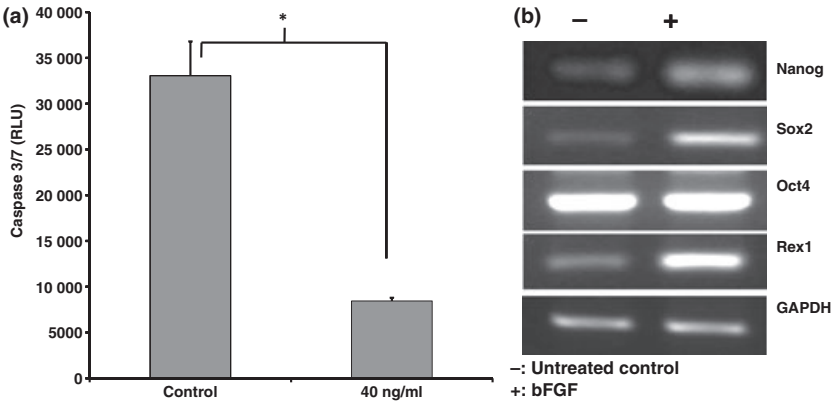

Figure 4. Early apoptosis prevention and enhanced stemness of UC-MSC by bFGF. (a) Caspase 3/7 activity in UC-MSC treated for 24 h with 40 ng/ml bFGF, as measured with Caspase-Glo® 3/7 Kit. Results are expressed as mean relative light unit ± SD. *P*-values <0.05 were compared to controls. Data shown are representative of five experiments. (b) Expression of early embryonic transcriptional markers Nanog, Sox2, Rex1 and Oct4 were measured by PCR. GAPDH served as housekeeping gene.

numbers for regenerative and immunotherapeutic approaches is a prerequisite and thus, extensive *in vitro* expansion of stem cells is necessary. Mitogens are frequently used in mesenchymal stem cell culture to enhance their propagation, however, the mechanism contributing to activation of cell proliferation is still unclear.

Here, we have documented that bFGF profoundly increases UC-MSC proliferation by driving them actively into the cell cycle. Similar to their bone marrow counterpart, our study reveals that bFGF supplementation enhances population growth kinetics of UC-MSC in a dose-dependent manner (23–25,27) (Fig. 1a). Although various cell cycle proteins contribute to MSC proliferation by regulation of cyclins, bFGF particularly increases synthesis of cyclin D *via* the ERK signalling pathway, thus enabling traverse of the cell cycle check point at G_0/G_1 (29) (Fig. 2c). Transitions between phases of the cell cycle are controlled by sequential activation of series of cyclins, cyclin-dependent protein kinases (CDK) and cyclin-dependent kinase inhibitors. Upon stimulation, cells undergo transition from G_0 to G_1 phase and both cyclin D and cyclin E are induced (30). bFGF drives UC-MSC into S phase, as indicated by elevated levels of cyclin D1 and D3 proteins and cyclin-dependent kinase,

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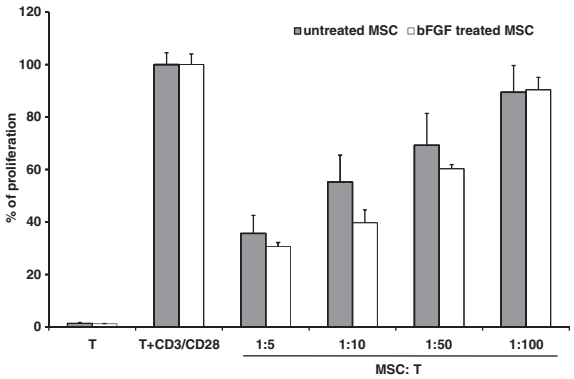

Figure 5. bFGF supplementation did not affect immunomodulatory activity of UC-MSC. One hundred thousand human T cells were co-cultured with various ratios of UC-MSC in presence or absence of PHA, for 3 days. T-cell proliferation rate was assessed using tritiated thymidine incorporation, pulsed for final 18 h incubation. Data are representative of three experiments.

Cdk4. Raised levels of cyclin D/Cdk complexes directly increased expression of phosphorylated retinoblastoma protein (pRb), which would release E2F to trigger transcription of genes encoding proteins, such as cyclin E, cyclin A, DNA polymerase and further proteins that drive cells through S phase (31). Meanwhile, bFGF supplementation also promoted progression of the cell cycle through M phase by augmentation of PCNA expression. Our results clearly reflect advocacy of bFGF in progress of UC-MSC cycle phases, specially G_1 and M, by facilitating cell cycle check points.

Our data show that bFGF did not alter osteogenic nor adipogenic differentiation potential; immunophenotype (data not shown) and immunosuppressive activity of UC-MSC. However, other investigations into animal and human bone marrow MSC have reported that bFGF supplementation encourages MSC differentiation to osteogenic (23) and adipogenic (32) phenotypes and enhances immunosuppressive activity, even with up-regulation of HLA class I and low levels of HLA-DR (23). It is worth mentioning that we have only tested immunosuppressive activity of bFGF-treated MSC in presence of PHA, a polyclonal stimulator of T cells. Nevertheless, it is still unclear whether similar immunosuppressive activity would be preserved if other stimulators such as allogenic and antigen-specific peptides were used to activate T cells.

Despite increases in cell proliferation, bFGF also preserved primitive status of UC-MSC by increasing expression of Nanog, Sox2 and Rex1 transcription factors (Fig. 4b). These are early transcription markers that are expressed in stem cells to direct them to self-renewal and cell proliferation. It has been reported that co-localization of Nanog, Oct4, Sox2 and Rex1 plays important roles in self-renewal and differentiation potentials of MSC (33). No increase in osteogenic and adipogenic differentiation potential of UC-MSC was observed after bFGF supplementation, even though there was enhanced expression of Nanog, Sox2 and Rex1.

Elevated expression of stem cell-associated transcription factors was further supported by reduction in cell death (apoptosis) as bFGF-supplemented UC-MSC resulted in significantly reduced caspase activity. In addition, bFGF supplemented UC-MSC caused reduced secretion of VEGF and MMP3. Other cytokines, such as TGF-β, IL-6 and IL-8, which previously have been postulated to be mediators of MSC-exerted immunosuppression, remained unchanged. This could contribute to maintenance of a similar magnitude of immunosuppression in both bFGF-treated and non-treated UC-MSC (34). Secretion of VEGF and MMP3 are closely associated with tumour progression and development of metastases. It has been shown that solid tumour cells can exploit MSC as tumour-supportive stromal cells, to produce VEGF (35). Although this would be beneficial to limit tumour progression and metastasis, yet their repair capability in organ injury that requires reperfusion and angiogenesis, such as myocardial regeneration, might be affected.

In conclusion, we have demonstrated that bFGF serves as a potent mitogen to modulate MSC population growth kinetics in terms of cell proliferation capacity, cell cycle status, stemness, apoptosis and cytokine secretion profile. Thus, such effects of bFGF could be exploited towards generating umbilical cord-derived MSC for future clinical use.

Acknowledgements

This work was supported by Research University Grant Scheme (04-01-09-0781RU & 04-01-07-0113RU) from Universiti Putra Malaysia and e-Science Fund (02-01-04-SF1022) from Ministry of Science, Technology and Innovation (MOSTI), Malaysia. Chih Kong Tong and Shalini Vellasamy are supported by the Graduate Research Fellowship Scheme at Universiti Putra Malaysia. Wai Kien Yip is a recipient of the Universiti Putra Malaysia Postdoctoral Fellowship.

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