

Original Article

Basic Fibroblast Growth Factor Enhances the Expansion and Secretory Profile of Human Placenta-Derived Mesenchymal Stem Cells

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ABSTRACT

Introduction: Mesenchymal stem cells (MSCs) hold a great therapeutic potential for regenerative medicine and tissue engineering due to inherent immunomodulatory and reparative properties. Hence, it necessitates a readily available supply of MSCs to meet the clinical demands adequately. Although, a human placenta can produce MSCs, the *in vitro* culture-mediated cellular senescence often affect the quality of cell product. Thus, the current study has explored the feasibility of basic fibroblast growth factor (bFGF) to enhance the growth of placenta-derived MSCs (PLC-MSCs). **Methods:** The basic fibroblast growth factor (bFGF) was supplemented to optimise the growth of MSCs. The effects of bFGF on morphology, growth kinetics and cytokine secretion of PLC-MSCs were assessed. **Results:** The bFGF supplementation increased the proliferation of PLC-MSCs in a dose-dependent manner and 40 ng/ml showed a high trophism effect on PLC-MSC's growth. In the presence of bFGF, PLC-MSCs acquired a small and well-defined morphology that reflect an active proliferative status. bFGF has induced PLC-MSCs to achieve a shorter doubling time (45 hrs) as compared to the non-supplemented PLC-MSCs culture (81 hrs). Furthermore, bFGF impelled PLC-MSCs into cell cycle machinery where a substantial fraction of cells was driven to S and G2/M phases. Amongst, 36 screened cytokines, bFGF had only altered the secretion of IL-8, IL-6, TNFR1, MMP3 and VEGF. **Conclusion:** The present study showed that bFGF supplementation promotes the growth of PLC-MSCs without significantly deviating from the standard criteria of MSCs. Thus, bFGF could be considered as a potential mitogen to facilitate the large-scale production of PLC-MSCs.

Keywords: Basic fibroblast growth factor, Placenta, Mesenchymal stem cells, Cell cycle, Cytokine secretion

INTRODUCTION

There is a growing demand for large-scale production of mesenchymal stem cells as they encompass a great therapeutic potential in regenerative medicine, tissue engineering and also in transplantation settings. However, the manufacturing of MSCs to meet the escalating clinical needs and also research remains an issue. Hence, an efficient system is required to warrant a rapid expansion of MSCs without compromising the quality of universally accepted MSC's criteria, and to achieve an adequate quantity for the end use. To date, several studies have been conducted to obtain an optimal condition that allows a rapid harvest of MSCs by carefully deciphering and also combining various factors/parameters to ensure the reproducibility and safety of the cellular products. These include factors such as optimal physiochemical culture environment and bioreactors that are automated and standardised to obtain high cell densities. However, too high a cell density could result in loss of cell properties for some stem cell types.

The optimisation of MSC culture for the clinical utilisation as well as research use involves multiple phases that cover from procurement of the samples for production till the packaging of MSCs for the end use. Among all

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stages, the optimisation process during the *in vitro* expansion of MSCs is pivotal since the number and quality of cells harvested for the final use critically rely on this stage. In this case, the expansion procedure is aimed at low cell densities and frequent passage. In the expansion phase, parameters such as the selection of basal culture medium, glucose concentration, stable glutamine, selection of serum, seeding density, passaging density and the quality of plastic surface (collagen or fibronectin-coated plates) are carefully picked to cater an optimal growth of MSCs (1-7). Besides, supplementation of single or combination of growth factors is also required when the basal media which is a cocktail of essential nutrients, unable to support the growth of a particular cell type (1-7). In line with this, several crucial growth factors such as basic fibroblast growth factor (bFGF) (8), platelet-derived growth factor (PDGF) (9), epidermal growth factor (EGF) (10), insulin-like growth factor (IGF-1) (11) and vascular endothelial growth factor (VEGF) (12) were tested for their capability to enhance the growth of MSCs.

Among these growth factors, bFGF (a.k.a fibroblast growth factor-2, or FGF-2) is commonly used to culture MSCs from various sources (1). It was shown that bFGF acts as a mitogen to increase MSC's proliferation while maintaining MSC's multipotential differentiation ability (13). Also, it was reported that bFGF appears as a cell growth factor that enhances angiogenesis and tissue repair, and is believed to prevent the senescence of bone marrow stromal cells by maintaining them in an immature state during the *in vitro* expansion (14). However, few other studies have also reported bFGF supplementation affects the multilineage differentiation ability of MSCs by enhancing the osteogenic (15), chondrogenic (16), adipogenic (17) and neurogenic (18) differentiation capacity. Nonetheless, no adverse or tumorigenic effect of bFGF have been reported in the available *in vitro* and *in vivo* studies (13,19).

Similar to the umbilical cord tissue and cord blood, placenta as well a clinical waste product that resulted from labour wards. However, the use of placenta to generate MSCs is not as extensive as bone marrow and umbilical cord counterparts. The placenta is naturally a fibrous tissue that supports a developing foetus during the gestational period by allowing nutrients and gas exchange via the umbilical cord. For many years, placenta after the delivery was returned to the respective parents as to respect the local custom and rituals that entail burial of placenta. However, with increasing awareness of the usability of the placenta in therapeutic and aesthetic purposes, donation of placenta for research become more apparent. Although several research groups had already documented the possibility of placental tissue to yield MSCs, yet as observed in other sources, placenta as well reaches cellular senescence at *in vitro* culture above passage 15 onwards (20, 21). Thus, the present study has explored the role of bFGF in promoting PLC-MSCs culture by gauging the changes inflicted in morphology, growth kinetics, cell cycle and cytokine secretion parameters.

MATERIALS AND METHODS

Generation and characterisation of placenta-derived MSCs (PLC-MSCs)

Human placenta samples were collected from post-delivery of normal full term pregnancies with the assistance of gynaecologists from Britannia Women & Children Specialist Centre. Samples were obtained with written, informed consent from respective parents and by ethical committee requirements of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Placenta tissues were processed; subjected for mesenchymal stem cell generation and characterisation by adhering to our published protocols (21, 22).

Morphology study

The morphology and growth of the primary cultures of PLC-MSCs with and without bFGF supplementation were observed from passage 0. Images were captured; the morphological changes were assessed and compared between bFGF supplemented and non-supplemented PLC-MSCs in terms of cell size, shape and granularity. The cell size and complexity were further confirmed by flow cytometry (BD, USA) analysis using scatter plot display (Cell Quest Pro software).

Growth kinetic analysis

The growth kinetics of PLC-MSC was determined by the trypan blue exclusion cell count method. Cells (4×10^3 cells/well) were plated into 6-well plates and incubated at 37 °C in a 5% CO₂ humidified incubator. The triplicates of MSCs were harvested at every 2 days using 0.05% trypsin-EDTA, and cell count was performed using Trypan blue dye. This step was carried out until day 14 for both bFGF treated and non-treated PLC-MSCs. Doubling time for PLC-MSCs was determined using Patterson's Formula as shown below. Trypan blue cell count was performed at every passage when the cells attained cellular confluency. The initial seeding number and days in culture were recorded.

Patterson's Formula: $Td = T \lg 2 / \lg(Nt/N0)$

Td is doubling time (hour), T is time required for cells to proliferate from N0 to Nt (hour),

N0 is initial seeding number & Nt is cell count at time T

Tritiated thymidine proliferation assay

The proliferation of PLC-MSC was accessed by tritiated thymidine ($^3\text{H-TdR}$) uptake during the cell cycle. Early passages of PLC-MSCs from P2-P4 were used for this assay. Cells at 5×10^3 cells/well were seeded into 96-well plates and treated with bFGF at various concentrations (0, 5, 10, 20 and 40 ng/ml). Cultures were incubated for 72 hours and pulsed with tritiated thymidine ($^3\text{H-TdR}$) (0.037MBq/well [0.5 μCi /well] (Perkin Elmer) at the final 18 hours of incubation. At 72 hours, cells were harvested onto 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 the Microbeta Trilux beta counter (Perkin Elmer, USA).

Cell cycle assay

Cells at 0.2×10^6 cells/25cm² flasks in the presence and absence of bFGF supplementation (40 ng/ml) were seeded and incubated at 37 °C in 5% CO₂ humidified incubator. Upon reaching 90% confluence, cells were harvested and continued with the PI (Sigma-Aldrich, St. Louis, MO, USA) staining of the intracellular DNA. The stained cells were acquired using flow (BD, USA) and the proportion of cells in G₀/G₁, S and G₂/M phases were determined with De Novo Software.

Human cytokine antibody array

Conditioned media of MSCs cultures that grown with and without bFGF supplementation (40 ng/ml) were collected after 48 hours of incubation. Human cytokine antibody array (Panomics, Fremont, CA, USA) was incubated with 2 ml of conditioned media and processed according to the manufacturer's instructions. The absorbance readings were obtained using a MRX II microplate reader (Dynex Technologies, Virginia, USA). Images were captured by exposing the membranes for 12-15 minutes using FluorChem Imager (Alpha Innotech Corp, California, USA).

VEGF assay

Conditioned media were harvested from MSC cultures supplemented with and without bFGF supplementation for 48 hours. The Human Vascular Endothelial Growth Factor (Hu VEGF) ELISA (Invitrogen, Canada) was used for the *in vitro* quantitative determination of human VEGF in the MSC conditioned media. All standards, controls and samples were run in triplicate, and the absorbance was read at 450 nm using a microtiter plate reader (DYNEX TECHNOLOGIES, MRX II).

RESULTS

bFGF supplementation enhances PLC-MSC's expansion in a dose-dependent manner

The effect of bFGF supplementation on PLC-MSC proliferation was assessed by Tritiated thymidine ($^3\text{H-TdR}$) assay. bFGF concentrations up to 40 ng/ml were used for optimisation as 40 ng/ml was the highest concentration reported in previous studies. The results showed that bFGF at lower concentrations, 5 and 10 ng/ml are not inducing PLC-MSCs into rapid cellular expansion as compared non-supplemented PLC-MSCs (0 ng/ml). However, the mitogenic potential of bFGF was noticeable from 20 ng/ml onward, and the highest cell proliferation was noted at 40 ng/ml (Figure 1). Hence, bFGF supplementation at 40 ng/ml concentration was maintained to expand PLC-MSCs throughout this study.

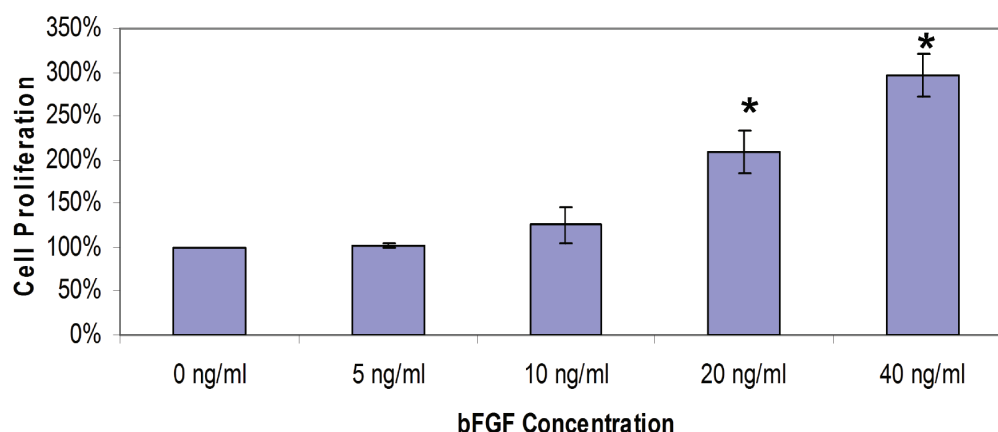


Figure 1: bFGF increases MSC's proliferation in a dose-dependent manner.

The proliferation of PLC-MSCs in the presence or absence of bFGF was evaluated by tritiated thymidine assay. bFGF stimulated the proliferation of PLC-MSCs in a dose-dependent manner. The means represent the average percentage of MSC proliferation \pm SD of 3 repeated experiments; * significant at $p < 0.05$ compared to control (non-treated).

bFGF supplementation stabilises the morphology of PLC-MSCs

The morphology of PLC-MSCs that expanded with and without bFGF supplementation was examined using an inverted phase contrast microscope and flow cytometer. The morphology and level of confluency upon culture at various days were evaluated by photomicrograph images while the cell size and complexity were confirmed by scatter plots of flow cytometer analysis. Placental cells cultured without bFGF for 7 days, the number of adherent cells with fibroblastic morphology was low, however upon media change, the adherent cells were started to expand and reached confluency approximately on day 26 (Figure 2A, 2B & 2C). Besides, a slower growth rate, non-bFGF supplemented PLC-MSCs also displayed a slightly bigger size, irregular & polygonal shaped cells. Whereas, bFGF supplemented placental cells showed comparably high adherent cells on day 7 and reached cellular confluency at day 14 (Figure 2D, 2E & 2F). In line with a greater proliferative rate, bFGF supplemented PLC exhibited cells with small in size and well-defined spindle-shaped cells. On average, PLC-MSCs cultured with 40 ng/ml bFGF supplementation took approximately 2 weeks to reach full confluence, while the non-supplemented cultures acquired 3 to 4 weeks to reach full confluence at P0. The scatter plot further confirmed that bFGF supplemented PLC-MSCs were relatively smaller in size and less complex as compared to the non-supplemented samples (Figure 3).

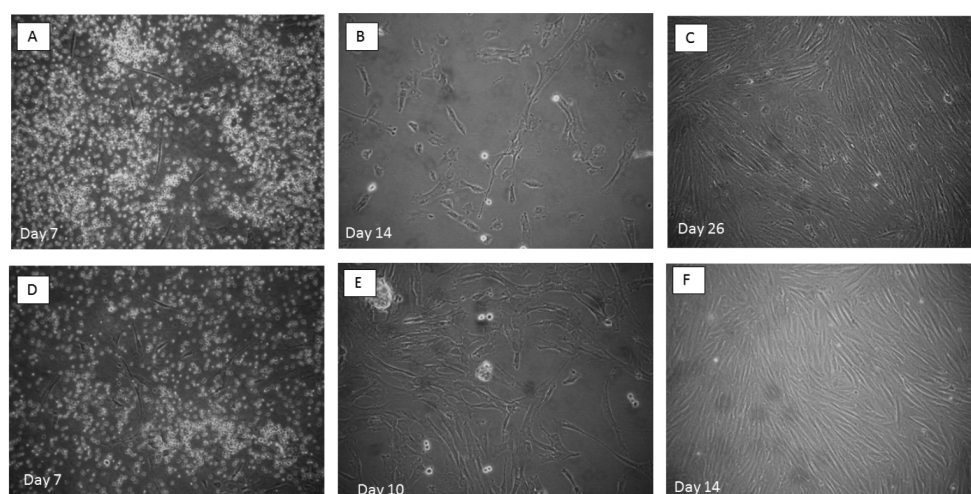


Figure 2: bFGF induces colony formation and enhance expansion of PLC-MSCs.

Single cells from the placenta tissue were cultured in the presence or absence of bFGF. In the absence of bFGF, adherent cells formed fewer colonies and took a longer period to attain confluence (A-C). Whereas, bFGF supplemented PLC-MSCs expanded rapidly and reached cellular confluence within 14 days (D-F). Photomicrographs were captured using phase contrast microscope at 100x magnification.

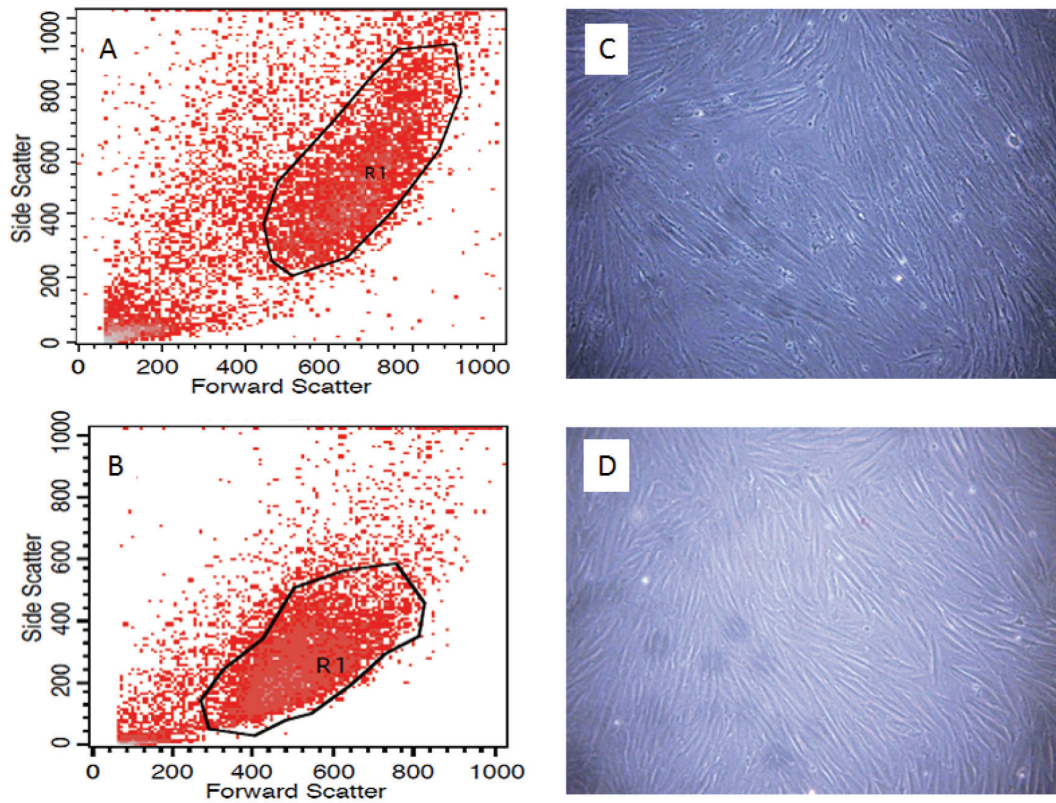


Figure 3: bFGF preserve a stable morphology of PLC-MSCs

Adherent cells from P0 were harvested and subjected to the flow cytometer analysis. PLC-MSCs cultured without bFGF acquired a larger size with increased amount of granules (A) and irregular edges (C). PLC-MSCs that supplemented with bFGF appeared relatively smaller (B) and well defined fibroblastic-like cells (D). Photomicrographs were captured using phase contrast microscope at 100x magnification.

Supplementation of bFGF accelerates growth kinetics of PLC-MSC

Growth kinetics and doubling time of PLC-MSCs were determined by plotting the growth curve for both non-supplemented and those supplemented with 40 ng/ml bFGF. It was noted that the growth kinetics of PLC-MSCs with bFGF supplementation showed a shorter lag phase from day 1-6, followed by a longer and aggressive exponential log-phase at day 6-12 till a plateau phase was reached. Whereas the non-supplemented PLC-MSCs showed a rather slower growth curve with having a longer lag phase from day 1-10, a shorter exponential lag phase at day 10-12 and then a plateau phase (Figure 4A). The doubling time of bFGF supplemented PLC-MSCs was within a range of 30-50 hours which was approximately 2-3 folds shorter than the non-supplemented UC-MSC's doubling time, 50-90 hours. The mean doubling time of bFGF supplemented and non-supplemented PLC-MSCs were 45 hours and 81 hours, respectively (Figure 4B).

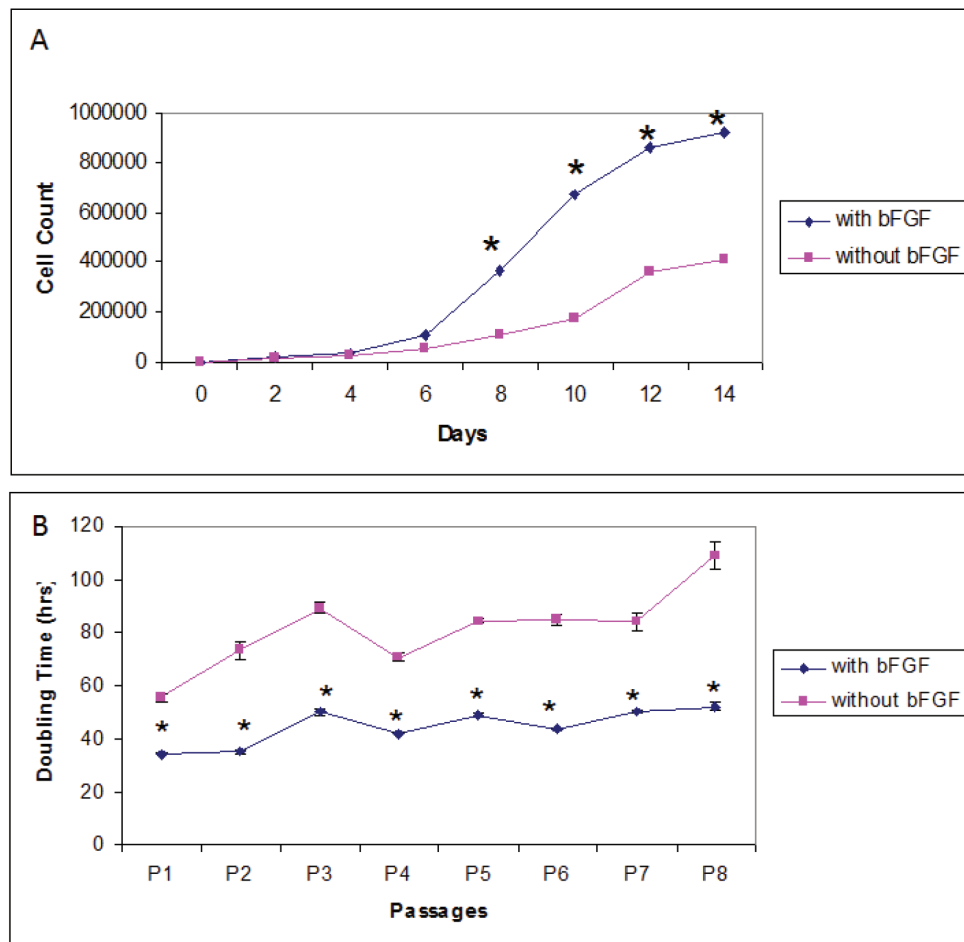


Figure 4: bFGF advances the growth kinetics of PLC-MSCs

PLC-MSCs at P3 were seeded in 6-wells plate, and the growth of cells was determined by manual haemocytometer counting. PLC-MSCs supplemented with bFGF showed a robust growth curve (A) and shorter doubling time (B). Values are means of 3 independent experiments, with \pm SD; results significant at $p < 0.05$.

bFGF drives PLC-MSCs into active cell cycle

To further understand the mitogenic nature of bFGF on PLC-MSCs, cell cycle assays were performed by assessing the intracellular DNA during the cellular expansion phase. Upon reaching 90% confluence, cultures of PLC-MSCs with and without bFGF supplementation were harvested for cell cycle analysis. When PLC-MSCs were grown without bFGF, the major fraction of cells remained in G_0/G_1 phase (87.24%) with a small population entered into S phase (7.48%) and G_2/M phase (5.28%) (Figure 5A). However, the cell cycle machinery of PLC-MSCs was activated in the presence of bFGF supplementation where more cells were committed to cell cycle with a reduced G_0/G_1 phase (71.62%) and greater S phase (17.12%) and G_2/M phase (11.27%) (Figure 5B).

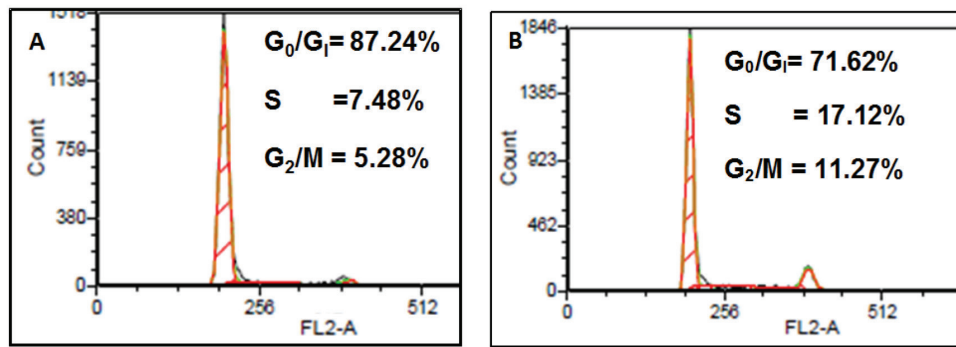


Figure 5: bFGF drives PLC-MSC into active cell cycle

Single cells of PLC-MSCs after trypsinization were subjected to the cell cycle analysis using flow cytometer. In the absence of bFGF, most of the PLC-MSC remained at G₀/G₁ phase (> 85%) (A). However, supplementation of bFGF induced PLC-MSCs into S phase (B). DNA contents were quantified using PI dye. Fluorescence-activated cell sorting (FACS) histogram plot results are representative of 4 experiments

bFGF retains the normal cytokine secretion profile of PLC-MSC

The cytokine secretion of both bFGF supplemented and non-supplemented PLC-MSCs were analysed qualitatively using Human Cytokine Antibody Array kit. The results showed that PLC-MSCs without bFGF supplementation constantly secreted high levels of IL6 and IL8; moderate level of MMP3; and low level of TNFRI (Figure 6A). However, the level of IL-8, IL-6, TNFR1 and MMP3 were changed upon bFGF supplementation where the IL-8 level was found to be elevated, and other cytokines' levels were slightly reduced. PLC-MSCs cultured with bFGF secreted significantly higher VEGF (117 pg/ml) compared to non-supplemented PLC-MSCs (11 pg/ml) (Figure 6D).

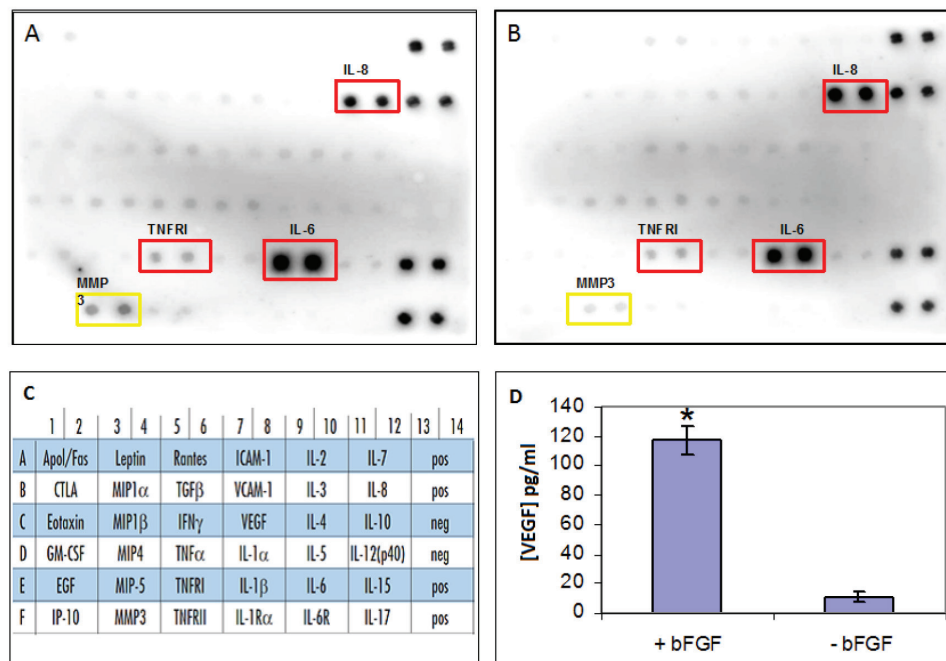


Figure 6: bFGF slightly modify the cytokine profile of PLC-MSCs

Conditioned media from bFGF supplemented and non-supplemented PLC-MSCs were collected after 6 days of culture for antibody array analysis. In non-supplemented culture, PLC-MSCs showed a standard cytokines profile with the distinctive secretion of IL-6 and IL-8 (A). Upon bFGF supplementation, only fewer cytokines were modulated by bFGF (B). Manufacturer's template shows the list of cytokines (C). Cytokine analysis includes 36 human cytokines; images were collected using a Fluochem™ imager (Alpha Innotech). Human VEGF was significantly induced in bFGF supplemented and captured by ELISA technique (D). Results are expressed as the mean of VEGF concentration (pg/ml) ± SD. P < 0.05 were compared to control (without bFGF supplemented samples); results are representative of 3 repeated experiments.

DISCUSSION

Mesenchymal stem cells were traditionally isolated from the bone marrow culture and had been extensively studied in many laboratory and animal models. However, the nature of harvesting procedures (bone marrow aspiration) and status of the donor (age and disease conditions) had become limiting factors as the invasive bone marrow aspiration procedure causes pain, high risk for infections, excessive bleeding and discomfort to the donor (4, 23). All these issues necessitate a need for an alternative source of MSCs that complies ethical requirements and readily available at low cost. Although MSCs derived from embryonic and aborted foetal tissues are excellent sources of MSCs, and provide a sufficient number of cells, yet their usage in clinical application and research is severely hindered by ethical concerns.

Human delivery wastes such as umbilical cord tissue, cord blood, placenta and amniotic fluid are the attractive sources for many postnatal stem cells such as mesenchymal stem cells, haematopoietic stem cells and endothelial stem cells. As compared to the classical bone marrow, these tissues considered as clinical waste, readily available, require a non-invasive technique and free from major ethical issues (24). The human placenta is a fetomaternal organ, formed by both foetal (chorion and amnion) and maternal tissues (decidua basalis and decidua parietalis). Formation of the placenta is one of the important processes during embryogenesis, for the well-being of the foetus and is discarded at postpartum (25). In this study, MSCs were successfully generated from placenta by using a novel method that combines enzymatic digestion and mechanical dissociation. This is a new method which was found to be an efficient approach in generating MSCs from placenta compared to the explant method or the enzymatic digestion method using combination of collagenase II and DNase1 (26, 27).

There were significant differences between the bFGF-supplemented and non-supplemented MSC cultures regarding their appearance and colony formation ability. The addition of bFGF in culture media resulted in the significant maintenance of MSC appearance and morphological properties as compared to the control (without bFGF). When examined under a phase contrast microscope, the primary PLC-MS culture supplemented with 40 ng/ml bFGF appeared as spindle-shaped fibroblastic-like cells, smaller in size, well-defined, strongly adhered to the plastic surface and took approximately 2 weeks to reach confluence. In contrast, the non-supplemented primary MSC samples appeared to be slightly bigger in size, less defined, polygonal in shape and required a longer time (3 to 4 weeks) to reach confluence. Consequently, the non-supplemented PLC-MS cultures could only be expanded until P7-P9. Beyond this, PLC-MS appeared as isolated colonies, unhealthy and most probably undergoing the *in vitro* culture exhausted cellular senescence. Although cellular senescence assay through detection of a specific marker was not conducted, the changes in cytoskeleton structure that organise organelles, and determines the cell's size had reflected a common feature of cellular senescence. Similarly Li et al. indicated that upon passaging, there was no morphological alteration in human placenta-derived MSCs treated with growth factor (28). The enhanced proliferation of MSCs and prevention of senescence upon bFGF supplementation were reported as a consequence of increased telomere length and telomerase activity (29, 30).

The morphological stability of PLC-MSCs induced by bFGF was also accompanied by a steady progression of cell cycle. The mitogenic activity of bFGF culminates at 40 ng/ml although other lower concentration had significantly increased the proliferation. It was evident that a higher proportion of bFGF-supplemented PLC-MSCs were synthesising DNA (S phase) and entering into the cell cycle, whereas non-supplemented MSCs showed a slower cell cycle progression, with most of the MSCs accumulated in the G₀/G₁ phase. In agreement with this, Ito et al. had also discovered that bFGF suppresses cellular senescence in MSCs by downregulating TGF- β 2 expression and preventing the cell growth arrest in the G₁ phase (31). The robustness of bFGF in driving cells into cell cycle was clearly depicted via growth kinetic analysis. Although both supplemented and non-supplemented PLC-MSCs had a similar period of lag phase, the log phase of bFGF supplemented PLC-MSCs had greatly surged and achieved approximately more than twice of non-supplemented cell's proliferation. More to the point, a similar pattern of doubling time as well was noted when comparing both bFGF supplemented and non-supplemented PLC-MSCs. The mean doubling time, time taken for cell replication for bFGF supplemented PLC-MSCs was 45 hrs, whereas the non-supplemented PLC-MSCs depicted 81 hrs. These two parameters have undoubtedly advocated the positive effect of bFGF supplementation in initiating and executing PLC-MSCs cellular expansion.

The secretory profile of PLC-MSCs was screened using a standard cytokine screening kit that screens upto 36 most common cytokines. In general, bFGF supplementation did not alter the cytokine secretory profile of PLC-MSCs. However, the levels of several cytokines namely IL-8, TNF, MMP3 and IL-6 were variably modulated in the presence of bFGF. The level of IL-8 was found to be raised whereas the levels of IL-6, TNFR1; MMP3 were decreased when PLC-MSCs cultured with bFGF. However, it will be impossible to deduce the biological significance imposed by the noted changes of cytokines levels as the screening method utilised is unable to provide a quantitative measurement of cytokines. Both IL-8 and IL-6 are important cytokines that support haematopoiesis in bone marrow. Although, PLC-MSCs are irrelevant to haematopoiesis, a few scientific reports suggesting the role of IL-6, IL-8 and (TNFR1) as pro-inflammatory cytokines (32-34). The expression of pro-inflammatory cytokines allows human MSCs

to inhibit the proliferation, apoptosis and cell cycle of immune cells (35-38). Nasef et al. have demonstrated that MSC-derived IL-8 along with other soluble inhibitory factors were responsible for MSC induced immunosuppression (39). However, the current study was unable to evaluate the impact of bFGF on the immunosuppressive activity of PLC-MSCs. Since the changes in cytokine level were qualitative, it was difficult to determine whether such changes are sufficient to perpetrate a functional enhancement or reduction on PLC-MSC-mediated immunosuppression.

In the present study, two pro-angiogenic factors namely VEGF and MMP3 were detected. The cytokine screening assay had failed to detect the level of VEGF due to the limitation of the test kit. However, the ELISA method was able to capture the differences in VEGF level at both bFGF supplemented and non-supplemented PLC-MSC cultures. VEGF is known as a potent angiogenic factor and plays a significant role in angiogenesis (40). Besides, VEGF has also been shown to improve the cell growth, prevent stress-induced apoptosis of endothelial cells, activate survival signalling pathways in MSCs and mediate endothelial and stem cell migration (41-43). In parallel, the present study clearly showed that PLC-MSCs with bFGF supplementation significantly increased VEGF expression than the non-supplemented PLC-MSC that might facilitate the angiogenesis and cell migration properties of PLC-MSCs.

CONCLUSION

In conclusion, supplementation of bFGF into PLC-MSC cultures enhanced the proliferation by inducing an exponential growth rate, shorter doubling time, progressive cell cycle without any novel alteration in the cytokine profile as compared to the non-supplemented PLC-MSCs samples. The current findings suggest that PLC-MSC with bFGF supplementation may serve as a potential source to accommodate the increasing demand for large-scale expansion of MSCs for clinical applications.

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