Original Article

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Effect of Basic Fibroblast Growth Factor on Expression of Let-7 MicroRNA in Proliferation of Human Dental Pulp Cells

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Abstract:

Objective: Basic fibroblast growth factor (bFGF) plays a pivotal role in cell proliferation, differentiation and extracellular matrix turnover in various tissues. In human dental pulp cells (HDPCs), let-7 microRNA is involved in cell proliferation and differentiation. There is little information on the effect of bFGF-induced cell proliferation on let-7 microRNA in HDPCs. This study investigated the effect of bFGF on let-7g, let-7f, and let-7i microRNAs and some of the genes involved in cell proliferation including p53 and Ki67 in HDPCs.

Material and Methods: HDPCs were cultured and treated with bFGF at 0, 1, and 5 ng/mL. Cell proliferation was examined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay at 24 and 48 hours. Additionally, gene expressions of let-7g, let-7f, let-7i microRNAs, and p53 and Ki67 were examined by quantitative real-time polymerase chain reaction at 24 hours. All experiments were performed in triplicate.

Results: The results showed that let-7g, let-7f, and let-7i microRNAs were expressed in HDPCs. MTT assays showed that bFGF induced greater cell proliferation than the controls at 24 and 48 hours (p-value<0.050). HDPCs treated with bFGF showed a decrease in p53 expression (p-value<0.001) while Ki67 expression increased (p-value<0.001). The expression of let-7g microRNA decreased under the influence of bFGF (p-value<0.050). However, bFGF had no effect on expression of let-7f and let-7i microRNAs (p-value>0.050).

Conclusion: Our preliminary study showed that exogenous bFGF could decrease let-7g microRNA expression suggesting that let-7g microRNA may be involved in bFGF-induced HDPCs proliferation.

Keywords: basic fibroblast growth factor, cell cycle, cell proliferation, human dental pulp cells, let-7 microRNA

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Introduction

Basic fibroblast growth factor (bFGF) is a member of the FGF family. bFGF plays a role in various cell processes including proliferation, migration, and differentiation.[1] A previous report found that bFGF could increase the total mice dental pulp cell numbers in an in vivo study.[2] Another study found that bFGF also induced human dental pulp cell (HDPC) proliferation.[3] bFGF signaling has also been reported to be involved in dental pulp cell proliferation.[4]

Let-7 microRNA were originally discovered in *Caenorhabditis elegans*, and have been found to play a significant role in cell proliferation, differentiation, and oncogenesis.[5,6] It controls timing and cellular proliferation.[7] Previous studies have reported the involvement of let-7 microRNA in various cancers. Let-7b and let-7c microRNAs were found to inhibit cell proliferation in human hepatocellular carcinoma.[8,9] Overexpression of let-7f microRNA was found to inhibit proliferation of papillary thyroid carcinoma cells.[10] In another study, let-7a, let-7b, let-7c, let-7d, and let-7g microRNAs were found to repress cell proliferation in lung cancer cells.[11] These data indicate a role of let-7 microRNA in cancer cell proliferation. However, there are few studies to date examining the role of let-7 microRNA in cell proliferation of HDPCs.

A prior study reported expression of let-7c microRNA in human dental pulp cells.[12] Yue and colleagues also reported that dental pulp cells expressed let-7a, let-7c, let-7d, let-7f, let-7g, and let-7i microRNAs. The study reported that expression of let-7a, let-7c, and let-7g microRNAs decreased in differentiated dental pulp cells. Additionally, the expressions of let-7d, let-7f, and let-7i microRNAs were higher in differentiated cells than undifferentiated cells. They suggested that these microRNAs may play a role in differentiation in HDPCs.[13] However, the study did not explore the role of let-7 microRNA in cell proliferation.

The effect of bFGF on microRNA expression in many cell types has been studied previously. bFGF significantly upregulated miR-132 in cultured astroglial cells, which are involved in brain function.[14] However, exogenous bFGF has been shown to reduce miR-381 expression in human chondrosarcoma cell lines leading to endothelial cell tube formation and cell migration.[15] Another study reported that bFGF secreted by endothelial cells can interact with adipose-derived stem cells through bFGF-receptors, which causes downregulation of miR-145 and promotion of tube-like formations.[16] Therefore, bFGF may have an influence on microRNA expression. However, there are limited data on let-7 microRNA expression in HDPCs under the effect of bFGF. Thus, we aimed to investigate the effect of bFGF on gene expressions of let-7g, let-7f, let-7i microRNAs, and p53 and Ki67 in HDPCs.

Material and Methods

Cell isolation and culture

This work was approved by the Human Research Ethics Committee, Srinakharinwirot University (Approval number SWUEC-086/2562E). Cell lines from four different donors were used in this experiment. Briefly, healthy third molars were extracted from patients under informed consent. First, the teeth were rinsed with sterile phosphate buffer saline. The pulp tissues were then removed from the pulp chamber. HDPCs were isolated from dental pulp tissue and cultured in media composed of Dulbecco's modified Eagle's medium (DMEM; Hyclone, South Logan, Utah, USA) supplemented with 10.0% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 2 mM L-glutamine (Gibco, Carlsbad, CA, USA), 100 units/mL penicillin (Gibco, Carlsbad, CA, USA), 100 µg/mL streptomycin (Gibco, Carlsbad, CA, USA) and 5 µg/mL amphotericin B (Gibco, Carlsbad, CA, USA). HDPCs were maintained in a humidified chamber at 37°C in 5.0% carbon dioxide. The 3rd to the 6th passages of HDPCs were used in the study. All experiments were performed in triplicate.

Treatment of cells

The experiments were performed as follows: for the cell proliferation assays, HDPCs were plated into 96-well plates at 5,000 cells per well and maintained in the growth medium for 24 hours. For the quantitative real-time polymerase chain reaction (qRT-PCR) in this experiment, HDPCs were seeded at a concentration of 3×10^5 cells per well in 6-well plates and maintained in the growth medium for 24 hours. After 24 hours, the cells were treated with 0, 1, and 5 ng/mL recombinant bFGF (Gibco, Carlsbad, CA, USA). The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich Corp., St. Louis, MO, USA) assays were performed at 24 and 48 hours. For the qRT-PCR, the cells were collected after 24 hours.

MTT assays

HDPCs were seeded at a density of 5,000 cells/well in 96-well plates and maintained in the growth medium for 24 hours. HDPCs were cultured in the absence or presence of recombinant bFGF at concentrations of 1 and 5 ng/ml for 24 hours and 48 hours. Cell viability was analyzed using MTT assays. The cells were treated with MTT solution for 4 hours at 37 °C. Formazan crystals were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich Corp., St. Louis, MO, USA). The final products were determined by spectrophotometer at a wavelength of 570 nm.

Extraction of total RNA and reverse transcription for qRT-PCR

HDPCs were seeded at 3×10^5 cells per well in 6-well plates and maintained in the growth medium for 24 hours. The cells were treated with bFGF (0, 1, and 5 ng/mL) for 24 hours. The cultured HDPCs were collected, and purified RNA was extracted using a miRNeasy kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. A miScript reverse transcription kit (QIAGEN, Hilden, Germany) was used to convert RNA to cDNA.

Quantitative RT-PCR for microRNA expression

Quantitative RT-PCR for microRNA was performed using a LightCycler®480 Instrument (Roche, Indianapolis, IN, USA) with a miScript SYBR Green PCR Kit and miScript Primer Assays according to the manufacturer's instructions (QIAGEN, Hilden, Germany). The miScript Primer Assays were specific to the miRNAs of interest as follows: Hs_let-7i (Cat. No. MS00008351), Hs_let-7f (Cat. No. MS00008330), Hs_let-7g (Cat. No. MS00008337), and Hs_RNU6 (Cat. No. MS00033740). The reaction products were quantified with RNU-6 as the reference gene.

Quantitative RT-PCR for gene expression analysis

Quantitative RT-PCR for gene expression was performed using a LightCycler®480 SYBR Green I Master kit (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. Relative gene expression was quantified with GAPDH as the reference gene. The oligonucleotide sequences were as follows: p53 forward, 5'-CAG CCA AGT CTG TGA CTT GCA CGT AC-3' and reverse, 5'-CTA TGT CGA AAA GTG TTT CTG TCA TC-3'; Ki67 forward, 5'-CGT TTG TTT CCC CAG TGT CT-3' and reverse, 5'-CTC CCT GCC CCT TTC TAT TC-3' GAPDH forward, 5'-GAA GGT GAA GGT CGG AGT C-3' and reverse, 5-'GAA GAT GGT GAT GGG ATT TC-3'.

Statistical analysis

Data were reported as the mean±standard deviation (S.D.) of at least three separate experiments. Statistical analysis between two groups was performed using independent student's t-test. A p-value less than 0.050 was considered statistically significant. All analyses were performed with SPSS Statistics version 20.0 (IBM SPSS Statistics, IBM Corp, Somers, NY, USA).

Results

Expression of Let-7g, let-7f, and let-7i microRNAs in HDPCs

Initially, we investigated the expression of let-7g, let-7f, and let-7i microRNAs in HDPCs by qRT-PCR. The microRNA expression of let-7 was normalized to the expression level of RNU-6. The qRT-PCR results showed the expression of let-7g, let-7f, and let-7i microRNAs in HDPCs. Interestingly, the expression of let-7g was higher than that of let-7f and let-7i microRNAs as shown in Figure 1.

bFGF-induced cell proliferation in HDPCs

Next, we investigated the effect of bFGF-induced cell proliferation in HDPCs. The cells were seeded at a density of 5,000 cells/well and grown to subconfluence. After 24 hours, the HDPCs were divided into two groups, with one group treated with bFGF (1, 5 ng/mL) and the other group not treated as a control group for 24 and 48 hours. The results of the MTT assays and phase contrast microscopy indicated that the treated group showed an increased number of HDPCs in a dose-dependent manner. Significant differences were found between the 5 ng/mL and control groups at 24 hours (p-value=0.026) and 48 hours (p-value=0.042) (Figure 2A, 2B).

Because p53 and Ki67 are markers related to cell proliferation, we further investigated the level of these genes on bFGF-induced cell proliferation in HDPCs by qRT-PCR. We observed that 1 and 5 ng/mL of bFGF significantly decreased the p53 levels and increased the Ki67 levels (p-value<0.001) compared to the untreated cells (Figure 3). These results indicate that bFGF could promote cell proliferation in HDPCs.

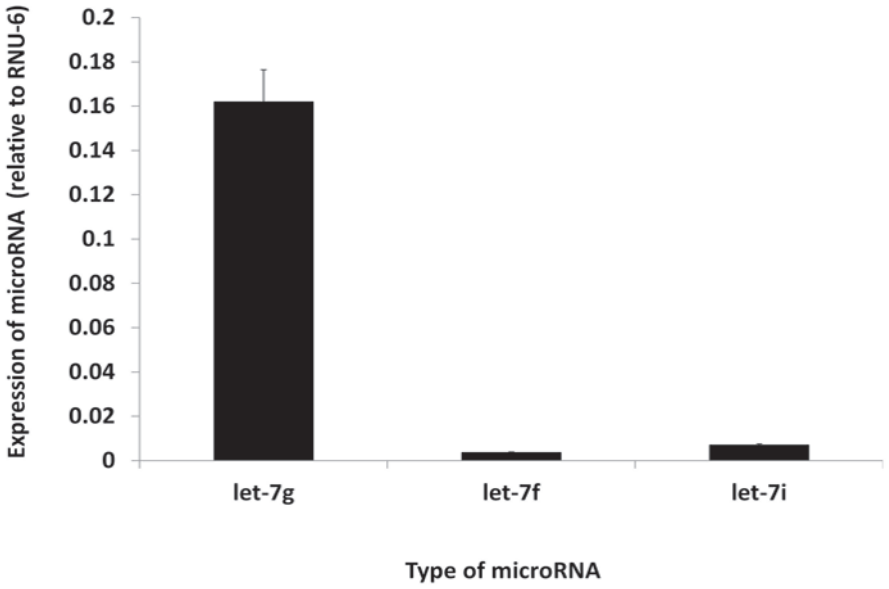

Figure 1 Expression of let-7g, let-7f and let-7i microRNAs in HDPCs. Cells were harvested at 80.0% confluency. The graph represents the products from qRT-PCR analysis when normalized with RNU-6, showing the mean±S.D. from three separate experiments.

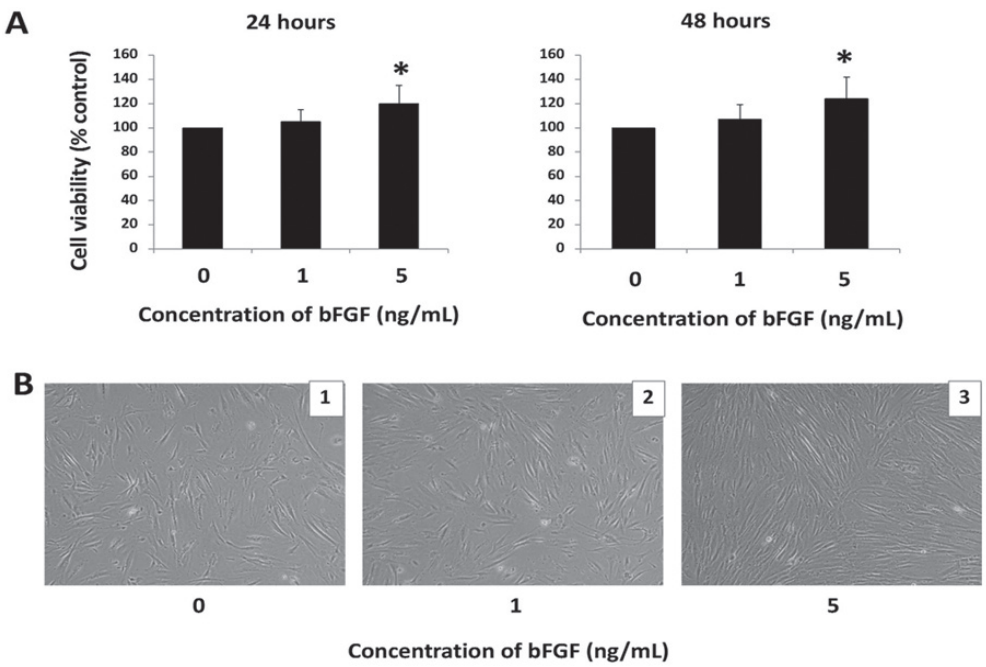

Figure 2 bFGF-induced cell proliferation in HDPCs. (A) HDPCs were maintained in culture medium with bFGF 0, 1, and 5 ng/mL and collected at 24 and 48 hours. The graph represents the OD density from MTT analysis when the control was marked as 100. (B) The pictures are phase-contrast microscope pictures of the HDPCs. HDPCs were treated with bFGF (B1) 0 ng/mL (B2) 1 ng/mL and (B3) 5 ng/mL for 48 hours.

Effect of bFGF on let-7g, let-7f and let-7i microRNA expression in HDPCs

Finally, to confirm the effect of bFGF-induced cell proliferation on let-7 microRNA expression, HDPCs were treated with bFGF (1, 5 ng/mL) and not treated with bFGF as a control group for 24 hours. We investigated the effect of bFGF on let-7 microRNA expression level by qRT-PCR. Interestingly, we observed that let-7g microRNA expression significantly decreased when treated with both 1 and 5 ng/mL of bFGF (p-value=0.001 and p-value<0.001, respectively) as shown in Figure 4. However, there were no significant differences in let-7f and let-7i microRNA expression between cells treated with bFGF at concentrations of 1 and 5 ng/mL and the controls (p-value=0.116 and p-value=0.123, respectively and p-value=0.060 and p-value=0.199, respectively). These results indicate that bFGF-induced cell proliferation had an influence on let-7g microRNA expression suggesting that let-7g microRNA may play a role in cell proliferation of HDPCs.

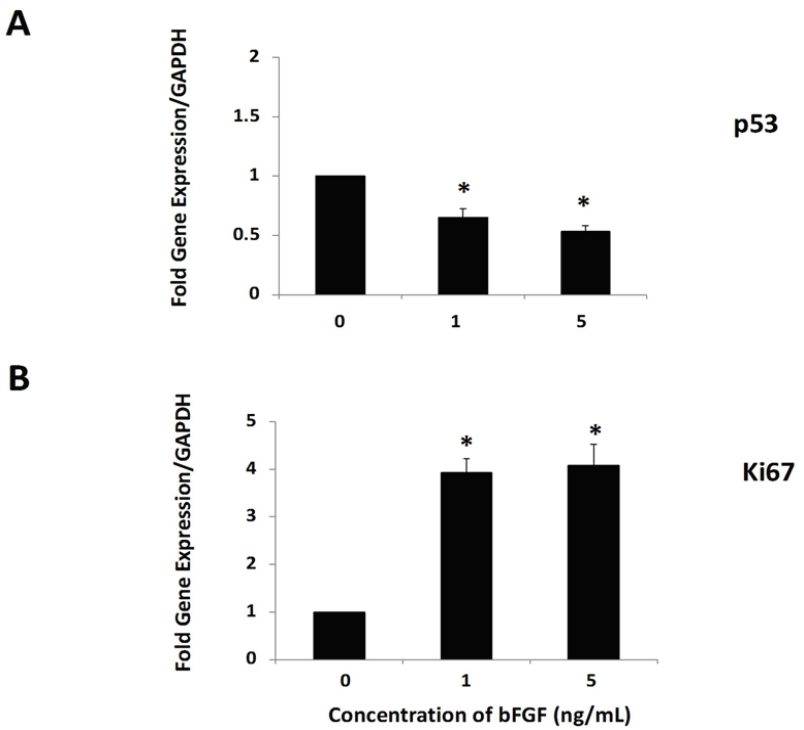

Figure 3 The expression of p53 and Ki67 under bFGF treatment. Cells were maintained in culture media with 0, 1, 5 ng/mL of bFGF and collected at 24 hours. The graph shows p53 and Ki67 expression when normalized with GAPDH. All the graphs show the mean±S.D. from three different experiments. The asterisks (*) indicate statistical significance, p-value<0.050 when compared with the controls.

Discussion

In this study, we found that let-7g, let-7f, and let-7i microRNAs were expressed in the HDPCs. We found that bFGF could induce cell proliferation leading to an increase in Ki67 and a decrease in p53 expression. Our study also found that exogenous bFGF could decrease the expression of let-7g but not let-7f and let-7i microRNAs. These results indicate that let-7g microRNA may be involved in bFGF-induced cell proliferation in HDPCs.

bFGF plays important roles in cell proliferation, differentiation, and survival in many tissues. Previous studies demonstrated the influence of bFGF on cell proliferation in various cell types including hemangioblasts, neuronal stem cells, and HDPCs.[17-19] The amount of bFGF- induced cell proliferation varied among dental pulp stem cells, human periodontal ligament tissues, human gingival fibroblasts, and stem cells isolated from human exfoliated deciduous teeth (SHEDs). Previous studies reported that the concentrations of bFGF required to enhance cell proliferation of HDPCs, human periodontal ligament tissues, human gingival fibroblasts, and SHEDs were 100 ng/mL, 40 ng/mL, 40 ng/mL, and 20 ng/mL, respectively.[20,21] bFGF at a concentration of 5 ng/mL has been shown to provide a greater effect on apical papilla stem cell proliferation than 1, and 3 ng/mL.[22] However, lower concentrations of bFGF (1,4, and 5 ng/ml) have been shown to induce cell proliferation in HDPCs.[23-25] Another study reported that bFGF at a concentration of 4 ng/mL increased HDPC proliferation by 2.4-fold when

compared to the control group at 24 hours.[23] Another prior study reported a 1.75-fold increase in HDPC proliferation when induced with bFGF at a concentration of 5 ng/mL at day 4.[24] Another study reported that bFGF at a concentration of 1 ng/mL resulted in an increase of dental pulp stem cells of 1.4-fold when compared to the control group at day 3.[25] These findings demonstrate the effect of bFGF on increasing the number of HDPCs at different concentrations and exposure times. Our study also found that bFGF at concentrations of 1 and 5 ng/mL induced cell proliferation in HDPCs at 24 and 48 hours as shown in Figure 2A. The differences in the responses of HDPCs to bFGF at various concentrations may be due to the conditions used in each experiment such as time of bFGF exposure and source of bFGF.

bFGF was reported to be involved in controlling cell cycle progression. It was shown to repress the tumor suppressor gene p53 leading to cell proliferation.[26] Ki67 is a proliferative marker and is expressed during the cell cycle. A previous study demonstrated that bFGF upregulated Ki67 protein expression in stem cells from human SHEDs, and bFGF treatment significantly increased the G2/M and decreased the G0/G1 population in SHEDs.[21] In our study, we observed an increase in the level of Ki67 and a decrease in the p53 level under the influence of bFGF-induced cell proliferation in HDPCs. In addition,

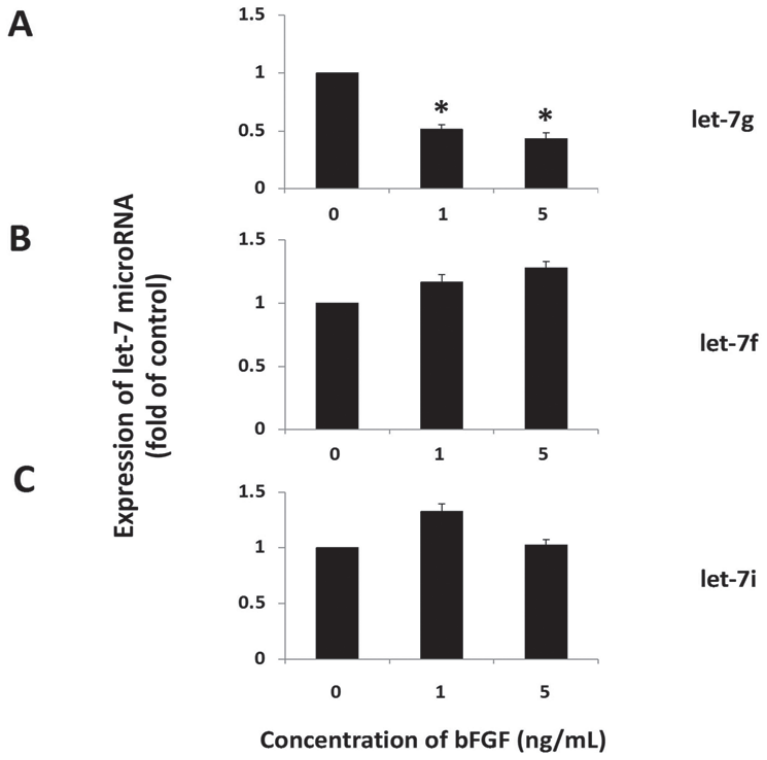

Figure 4 The effect of bFGF on let-7 microRNA expression in HDPCs. The HDPCs were treated with bFGF at 0, 1, and 5 ng/mL. The levels of let-7g, let-7f, and let-7i microRNAs were recorded at 24 hours for qRT-PCR. The results showed that bFGF decreased the expression of (A) let-7g but not (B) let-7f or (C) let-7i microRNA. The graph shows the mean±S.D. from three independent experiments using three established cell lines. *Significant difference, p-value<0.050.

the expressions of let-7g, let-7f, and let-7i microRNAs were detected in HDPCs in this study. Prior studies supported the role of microRNAs in cell proliferation of HDPCs.[27] Although various studies have reported several isoforms of let-7 microRNA being expressed in HDPCs, the functional effects of let-7 microRNA may be mediated by one or more of these isoforms.[6,28,29] In agreement with these studies, our study found that bFGF decreased the expression of let-7g microRNA corresponding with the cell proliferation but the bFGF had no effect on let-7f and let-7i microRNAs in the HDPCs (Figure 4). However, the signaling pathway of bFGF-induced let7 microRNA in HDPCs remains uncertain. Previous study demonstrated that FGF regulates transforming growth factor beta (TGF-β) ligands and receptors expression via control of let-7 microRNA levels by TGF-β signaling in primary mouse endothelial cells.[30] In addition, knock-down of fibroblast growth factor receptor substrate 2 lead to increases in MYCN and LMO1 expression through let-7 microRNA and TGF-β1 signaling in neuroblastomas.[31] Therefore, let-7 microRNA may be regulated by bFGF in HDPC proliferation.

Let-7g microRNA has been shown to play various roles in cancer growth.[32] Our results are consistent with several cancer studies indicating that let-7 family members may act as tumour suppressors and suppress metastasis.[5,33,34] Lan and colleagues reported that let-7g microRNA may act as a tumor suppressor gene that inhibits hepatocellular carcinoma cells proliferation.[33] Wu and colleagues also reported that overexpression of let-7g microRNA significantly inhibited cell proliferation in hepatoma cells.[34] However, in contrast to our results, Yue and coworkers reported that expression of let-7g microRNA decreased while the expression of let-7f and let-7i microRNAs increased in differentiated HDPCs.[13] We believe that differences in the genetic characteristics of the HDPCs in our study and the previous study could have contributed to these inconsistent results.

The molecular mechanism of let-7 microRNA associated with cell proliferation in cancer cells has been previously reported. Ricarte-Filho and colleagues reported that let-7f microRNA overexpression markedly inhibited proliferation of human papillary thyroid carcinoma cell lines. One of the effects of let-7f microRNA on thyroid growth and differentiation could be attenuation of the neoplastic process through impairment of mitogen-activated protein kinase (MAPK) signaling pathway activation.[35] Gao and colleagues reported that let-7b microRNA suppressed cell growth in oral squamous cell carcinoma. These findings also suggest that let-7b microRNA regulate cell cycle progression through downregulation of the insulin-like growth factor 1 receptor (IGF-1R) and insulin receptor substrate-2 (IRS-2), mediated by activation of the protein kinase B (AKT) and MAPK pathways in oral squamous cell carcinoma.[36] A study of Li and colleagues also found that let-7b microRNA inhibited tumor growth and metastasis through MAPK/extracellular signal-regulated kinase (ERK) pathway in human lung adenocarcinoma.[37] This evidence suggests that let-7 microRNA regulates cell proliferation at a molecular level through the MAPK/ERK pathway in various cancer cells. However, the signaling pathway of let-7g microRNA-induced cell proliferation in HDPCs remains unclear. Further studies are required to further elucidate the role of let-7 microRNA in proliferation and differentiation of HDPCs.

Conclusion

In conclusion, the results from our study suggest the possibility of the let-7g microRNA being involved in the control of cell proliferation in HDPCs. The let-7g microRNA may be involved in bFGF-induced HDPCs proliferation. In this study we only investigated let-7 microRNA expression level, but in the future we plan to investigate the signaling pathway that is involved in let-7g microRNA-induced cell proliferation in HDPCs.

Acknowledgement

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Conflict of interest

The authors declare no conflicts of interest.

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