CONSTRUCTION OF RECOMBINANT PLASMID pcDNA3.1/*BMP*-2 AND ITS INVOLVEMENT IN DIFFERENTIATION OF HUMAN DENTAL PULP-DERIVED CELLS INTO AN ODONTOBLASTIC LINEAGE

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Abstract

Experimental studies have shown that dental pulp tissue has potential to regenerate dentine in response to adverse stimuli, such as caries and associated operative procedures. However, the potential of dental pulp regeneration seems to be limited by regenerative capacity of the cell involved. In this study, we report the effect of transfection of a recombinant plasmid containing human *BMP*-2 gene in proliferation and differentiation of dental pulp tissue in vitro. The regenerative capacity was analyzed by ALP production and calcium content. Results showed that the transfected dental pulp cell was able to differentiate into the odontoblast phenotype, indicating the presence of odontoblast progentitor cells in dental pulp tissue.

Keywords: bmp-2, dental pulp, odontoblast, pcDNA 3.1, recombinant plasmid

1. Introduction

The goal of modern restorative dentistry is to functionally and cosmetically restore lost tooth structure which is currently restored using restorative materials. Although it has proven to be highly effective at preserving teeth, they have a limited life-span and ultimately require replacement or either tooth extraction or endodontic treatment.[1] Therefore, development of novel techniques to regenerate, as opposed to repairing, lost tooth structure would have significant benefits.

The stem cell–based, bioengineered dental tissue is a promising technique for regenerative dentistry that will hopefully replace injured teeth.[2] Thus, tissue engineering with the triad of dental pulp progenitor/stem cells, growth factors, and extracellular matrix may provide a useful alternative method for pulp-capping and root canal treatment.[3] However, the technique for manipulation of the growth of the progenitor/stem cells *in vitro* needs to be developed. In this study, the regenerative capacity of dental pulp cells was investigated by detecting the presence of STRO-1^+ population cells and its odontogenic differentiation activity after transfected with recombinant plasmid containing a growth factor (*bmp*-2) gene.

2. Methods

mRNA axtraction and RT-PCR. Total RNA from osteoblast cell line (MG 63) was prepared by using the guanidium thiocyanate-phenol-chloroform extraction method (TRIzol reagent) following the instruction provided by the supplier (Roche). First-strand cDNA was synthesized by 5µg of total RNA reverse transcriptase synthesis kit (Fermentas). PCR was carried out using oligonucleotides, which was modified from the sequences published by Kochanowska.[4] The forward and backward direction of the primers were: acgagatctatggattcgtggtggaagtg, and gcaag atctgtggagttcagatgatcacg, respectively. The reaction mixture were denatured at 94^{o}C for 30s, annealed at temperature between 66-55^{o}C (long PCR program) for 30s and extended at 72^{o}C for 30s for 30 cycles. The amplification products were checked by using 2% agarose gel alectrophoresis with molecular weigh markers.

Construction of recombinant plasmid pcDNA-*bmp*2. For construction of a recombinant plasmid bearing human *bmp*2 gene, the purified products (390 bp) and plasmid eukaryotic expression vector (PcDNA3.1) were digested with *BglII*. The ligation reaction system was conducted according to protocol provided by kit supplier (Fermentas). The final plasmid pcDNA-*BMP*2 was transformed into *E. coli* JM 2163. The ligation

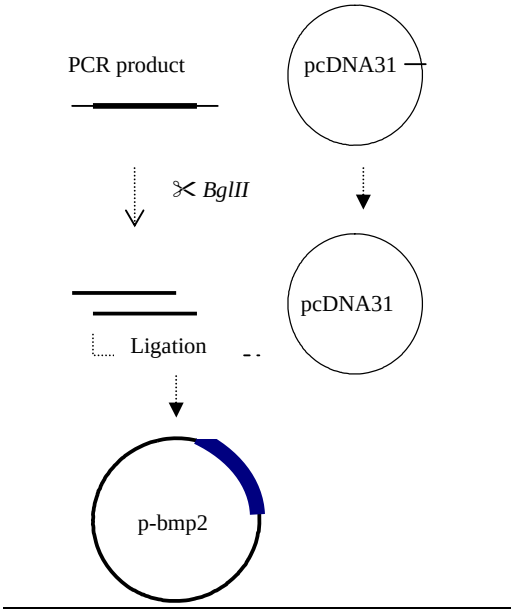

Figure 1. A Schematic Procedure of Reconstruction of pcDNA/*BMP*-2. The Recombinant Plasmid was Cloned in *E. coli* JM2163 as Mentioned in Text

products were cultured with Luria-Bertani (L-B) medium containing ampicillin (100 µg/mL) overnight. Afterward, the recombinant plasmid, which has been propagated in bacteria was extracted from colony transformants prior to be identified by digesting with *BglII*, and confirmed by agarose gel electrophoresis.

Culture and propagation of cells and alkaline hosphatase (ALP) activity. Primary human dental pulp cells (DPSCs) were isolated and cultured as previously reported in details.[5] Briefly, human dental pulp tissue was removed from third molars, which were extracted for oral surgery reason. The tooth were immediately cracked opened and the coronal pulp tissue was removed, minced into explants (<1 mm^3) and placed in wells containing Dubelco Modified Essential Medium (DMEM) supplemented with 1% glutamine, penicillin/ streptomycin (100 IU/mL – 100 µg/mL), and 10% FCS in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The medium was removed every two days. After 15 days, STRO-1-positive human DPSCs (6^{th} passage) detected using immunocytochemistry staining were expanded in DMEM medium as mentioned above. These cells were used for all experiments.

Odontogenic potential of the DPSCs was tested for the production of ALP. Briefly, the cultured cells were scraped, and seeded in 24-well culture plates at a density of 10^5 cells per well and cultured in DMEM medium. Time intervals were set up so after 5, 7, and 14 days in culture medium, cell layer was rinsed with PBS, scraped into 1ml of buffer (10mM Tris-HCl, 5mM $MgSO_4$, 0.1% Triton X100, 0.1% $NaNO_3$). ALP activity was determined by the hydrolysis of *p*-nitrophenyl phosphate in 2-amino-2-methyl-1-propanol buffer (pH 10.4) at 37°C for 30 minutes. Absorbance at 405nm was measured with microplate reader.

Von kossa staining. The mineralisized cells were detected according to methods reported elsewhere.[6] Briefly, culture media in tissue culture disk from each of the time periods was removed and the cells were rinsed with PBS. Subsequently, selver nitrat solution (5%) was added and the cells were exposed to UV light for 20 minutes. The cells were washed in distilled water three times, 5% sodium thiosulfat was added for 5 minutes, then were washed three times with distilled water. The cells were then dehydrated and cleared. The staining results were analyzed by counting the number of cell that containing calcium deposit. Under a 10X magnification of a light microscope.

Statistical analysis. Data derived from one representative experiment out of three performed with similar results. Results are expressed as mean ± standard error of triplicate determinations. Comparative studies of means were performed using *t* test analysis with a statistical significance at $p<0.05$.

3. Results and Discussion

Construction of pcDNA3.1-*BMP*2. The recombinant plasmid pcDNA3.1-BMP2 was successfully constructed (Fig. 1) and identified by restriction endonuclease digestion (Fig. 2) STRO-1^+ Dental pulp cell (Fig. 3) could be effectively transfected by the recombinant plasmid using kit containing lipofectamine in vitro. The stable expression of BMP-2 in pcDNA3.1–*BMP2* transfection cells was confirmed by detecting the BMP-2 protein using SDS-PAGE analysis (data is not shown), according to the experimental time course.

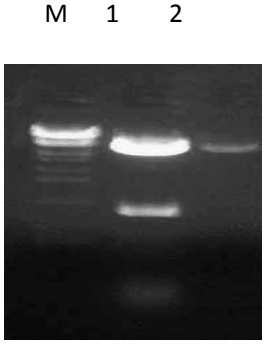

Figure 2. Confirmation of pcDNA3.1–*BMP2*. The Recombinant Plasmid was Digested with *BglIII*. Lanes 1 and 2 are pcDNA3.1 (5000 bp) and P*BMP*2 (5349 bp) M, Molecular Marker

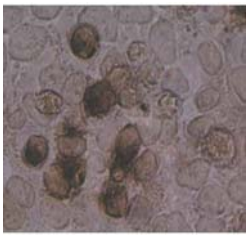

Figure 3. von Kossa Staining of DPSCs, The Black Area Represents a Positive Appearance of Mature Mineralization

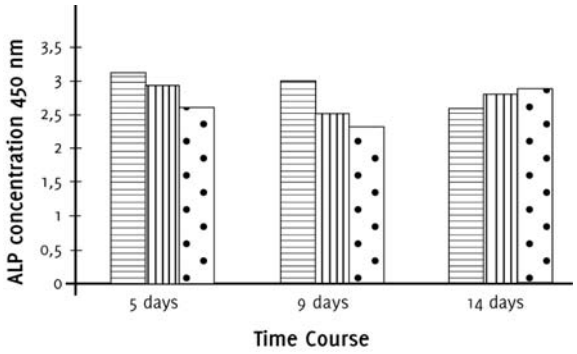

Figure 4. ALP Activity at Various Culture Times. The Results are Expressed as the Means± S.D. (*P* < 0.001), After Three Times Repeated Experiments

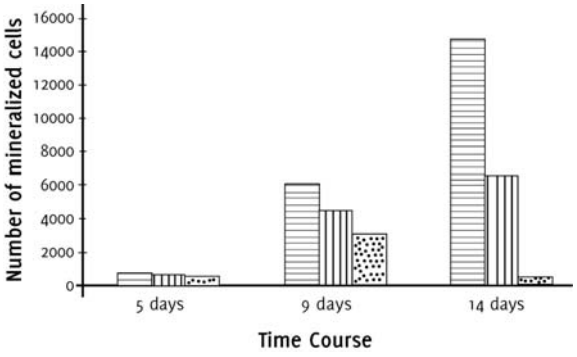

Figure 5. Effect of Tranfection p-*BMP*2 on Mineralizion of Odontoblast-Like Cells. Vertical Bars Indicate Mean ± SD (n = 3). The Significant Difference (p < 0.05) between Treated Cells and Control was Calculated by t Test

Effect of pcDNA3.1–*BMP2* transfection on ALP activity calcium deposite. To investigate the possibility that pcDNA3.1–*BMP2* induces the production of ALP, we examined the ALP activity in cultured cells. Our data revealed that continuous treatment with the recombinant plasmid caused a significant increase in the concentration of ALP and the number of mineralized nodules, in culture medium and cultured cells respectively (Fig. 3 and Fig. 4). This increase reflects the involvement of pcDNA3.1–*BMP2* in proliferation/ differentiation and maturation process of odontoblast lineage within the cultured human dental pulp cells.

Several studies have looked at the odontogenic potential of adult human dental pulp cells by using cell-culture methods and noted the ability of such cells to differentiate and mineralize.[7-8] Our data showed that when p-*BMP2* was introduced into human dental pulp cells, this cells are capable to express the high-level of ALP. This result indicates that the exogenous *bmp*2 gene could stimulate the proliferation, differentiation and function of odontoblast-like cells. This experiment also demonstrates, the number of cells with calcium deposit increased when the cultured cells were exposed with p-*BMP2* in a specific time point, which demonstrating the involving of recombinant plasmid in the maturation of the odontoblast-like cells. The maturation process was characterized first by early differentiated odontoblast marker such as ALP.

ALP is a membrane-bound enzyme that is abundant early in odonblast formation [9] and the production of ALP in this study showed considerable ALP activity in all cultures after transduced with p-*BMP*2 within experimental periods, indicating early gene upregulation by *BMP*-2 and induction toward the odontogenic phenotype, a phenomenon that has been reported by other in vitro experiments using osteoblast.[9-11]

4. Conclusion

A recombinant plasmid bearing human cDNA *bmp*2 was successfully constructed. This plasmid was used as genetic material to be transfected into human dental pulp cell, which contain a population of mesenchimal stem cells. The expression of ALP in culture medium and mineralization of the cultured cell could be relevant to the process of odontogenesis. Although it may be clear that p*BMP*2 participates in this process, in remain to be elucidated the role of extracellular matrix as well as other growth factors in the transfection efficiency. Therefore, much works are still needed to improve our understanding in the role of dental pulp tissue as a sources of mesenchymal adult stem cells.

Acknowledgement

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