

## Microarray analysis of osteogenesis gene of human dental follicle cells stimulated with dexamethasone in vitro

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**Purpose:** To examine osteoblastic genes expression of freshly isolated human dental follicle cells during osteogenic differentiation in vitro using cDNA microarrays.

**Materials and Methods:** The dental follicle cells isolated from dental follicle were cultured in DMEM media with  $10^{-8}$  mol/L Dex for 4 weeks, the control without Dex. Total RNA was extracted from the cultured follicle cells with Trizol. An analysis of osteoblastic genes expression of dental follicle cells treated with Dex and the control using cDNA microarrays. The image file was inverted and spots were digitized using ScanAlyze software.

**Results:** There were differences in the expression of 28 genes by human dental follicle cells cultured in the presence of Dex. Among the 28 genes, the expression of 20 genes up-regulated and eight genes down-regulated in Dex-treated cells than control.

**Conclusion:** Our work demonstrates differential osteoblastic genetic expression of dental follicle cells with a Dex-based protocol by microarray for the first time. Some of these selective changes in gene activity might thus reflect the fundamental events that underlie osteoblastic differentiation of human dental follicle cells in vitro. (*Int Chin J Dent 2006; 6: 115-122.*)

**Key Words:** cDNA microarray, dental follicle cell, dexamethasone, differentiation, osteoblast.

### Introduction

The dental follicle or dental sac is a loose ectomesenchymally derived connective tissue surrounding the enamel organ and the dental papilla of the developing tooth germ prior to eruption.<sup>1</sup> Cells within the dental follicle region, a loose connective tissue surrounding the developing tooth, harbours progenitor cells for the periodontium. The periodontium as the supporting tissue of the tooth is composed of the periodontal ligament (PDL), alveolar bone and the mineralized bone-like cementum covering the tooth root surfaces. Substantial evidence exists indicating that follicle cells are progenitors of periodontal cells including cementoblasts, PDL fibroblasts, and alveolar osteoblasts.<sup>1,2</sup> When triggered appropriately, dental follicle cells are considered to be able to differentiate toward a cementoblast/osteoblast phenotype. The differentiation capacity of these follicle cells was proven by *in vivo* tests with SCID mice. Here, cells formed a cementumlike matrix as opposed to bovine PDL fibroblasts or bovine alveolar osteoblasts.<sup>3,4</sup> Zhao et al. reported that BMP-2 promotes differentiation of immortalized murine dental follicle cells towards an osteoblast or cementoblast phenotype.<sup>5</sup>

Recently, precursor cells (PCs) were isolated from dental follicle of human third molar teeth and demonstrated that cultured PCs are unique undifferentiated lineage committed cells residing in the periodontium prior or during tooth eruption. PCs differentially express osteocalcin (OCN) and bone sialoprotein (BS) after transplantation in immunocompromised mice but without any sign of cementum or bone formation.<sup>6</sup> Gene expressions of osteocalcin (OCN), bone morphogenic protein (BMP)-2 and nestin were increased during the both differentiation approaches.<sup>7</sup>

Bone formation is a complex process involving proliferation of osteoprogenitors and/or pre-osteoblasts and expansion of the osteoblast population followed by osteoblast differentiation resulting in matrix maturation and mineralization. This sequence of events requires the up-regulation and inhibition of several genes that encode phenotypic proteins, e.g. collagen type I, alkaline phosphatase (ALP), and osteocalcin.<sup>8</sup> Transcription factors

such as c-fos, c-jun, Cbfa1 are also important for osteoblast growth and differentiation.<sup>9,10</sup> The expression of these key genes is further modulated by several factors including growth factors, peptide and steroid hormones.

Although several target genes of which relate to bone formation on human dental follicle cells are known, a detailed analysis of target genes involved in the osteoblastic differentiation of human dental follicle cells is still lacking. In this study, the osteogenic gene expression changes in human dental follicle cells treated with dexamethasone were investigated by functional microarray technique.

## Materials and Methods

### Materials

Expression profiling was performed with the use of the GEArray DNA Q Series Microarrays: Human Osteogenesis Gene Array containing 96 genes correlated with bone formation (Superarray Bioscience Corp., Bethesda, MD, USA). This particular array was selected because it contains genes involved in genes of osteoblastic differentiation, some of which are makers of bone formation, including Growth factor and associated molecule, Matrix and its associated protein, Cell adhesion molecule.

### Cell culture

Human dental follicles from third mandibular molars were surgically removed from patients who need orthodontic treatment and informed consent, the age of the patients varied between 12 and 13 years. Dental follicles below the crown were separated from the tissue. The surfaces of the follicle tissues were cleaned and minced by using a sterilized scalpel. Also dental papilla tissue was discarded. Then tissues were digested and cultured as previously described.<sup>6</sup> Minced and digested tissues of dental follicle explants were seeded into 60 mm plates in DMEM media (Gibco, Los Angeles, CA, USA) at 37°C in 5% CO<sub>2</sub> in a humidified atmosphere. After single cells had attached on the plastic surface, non-adherent cells were removed by change of medium. Plastic adherent cells were continuously propagated until confluence. The dental follicle cells isolated from dental follicle were cultured at an initial density of approximately 5,000 cells/cm<sup>2</sup> in DMEM media (Gibco) until first passage.

### Cell exposure to Dex

For dexamethasone analysis, cells were cultured as described previously.<sup>7</sup> Dex was dissolved in DMEM media with 10% FBS at a concentration of 10<sup>-8</sup> mol/L, the control without Dex, Long-term cultures were made with follicle cells at passage 5 for 4 weeks with media change twice a week, but without cell passaging. After 4 weeks, the culture medium was removed from the cells, which were then washed twice with PBS and then for RNA extraction.

### RNA extraction

Total RNA was extracted from the cultured follicle cells with Trizol. The pellet of total RNA was washed briefly with 75% ethanol, resuspended in 20 µL DEPC-treated water. The total RNA was then treated with DNase I (Gibco) for removal of any DNA contamination. The quantity and purity of the RNA were measured by means of a spectrophotometer.

### Expression profiling and data analysis

A GEArray Q series human osteogenesis gene array kit (SuperArray Inc., www.superarray.com, Bethesda, MD, USA) was obtained. RNA (1.5 mg) from Dex-treated and the control were used as a template to generate Biotin-16-dUTP labeled cDNA probes, according to the manufacturer's instructions. The cDNA probes were

denatured and hybridized at 60°C with the osteogenesis SuperArray membrane. The membrane was then washed, exposed using a chemiluminescent substrate, and analyzed by scanning the X-ray film and importing the image into Adobe Photoshop as a TIFF file. The image file was inverted and spots were digitized using ScanAlyze software, and normalized by subtracting the background as the average intensity value of three spots containing plasmid DNA (PUC18). The average of two GAPDH spots were used as positive controls and set as baseline values to which the signal intensity of other spots was compared. Using this normalized data, the signal intensity from the membranes was compared using the GEArray Analyzer program. This analysis was repeated three times.

The detection parameters were arbitrarily set to report greater than two-fold changes in expression levels either up or down. Differences in gene expression greater than two-fold are presented as the ratios of gene expression at DEX treated cells compared with the control cells. To assess the reliability and repeatability of the methods, we performed the expression profiling experiments twice, each time point in duplicate and each set of RNA labeled and hybridized twice. Comparison of the individual replicate filters showed that there were no differences between them within the threshold limits set as described above (data not shown). For the final analysis, the replicate images were therefore merged to produce a composite image.

## Results

Using the GEArray Analyzer software settings, Global expression analysis of genes associated with bone formation regulated by Dex. The representative array image of overall expression of the 96 genes in Dex-treated cells (Fig. 1) and control (Fig. 2) was shown. Each gene was deposited as a duplicate for statistic analysis and quality control. We obtained evidence that there were differences in the expression of 28 genes by human dental follicle cells cultured in the presence of Dex. Among the 28 genes, the expression of 20 genes was identified as Dex-promoted genes which showed an up-regulation at least 2-fold in Dex-treated cells than control. And the expression of eight genes was identified as Dex-suppressed genes which showed a down-regulation of more than 50% in Dex-treated cells than control. Among those, the expressions of five genes were inhibited more than 5-fold (Table 1). In Table 1, these 28 differentially expressed genes have been assigned to two groups based on the functional activity of the corresponding protein; growth factor and associated molecule, matrix and its associated protein. Cell adhesion molecules were not different between two groups.

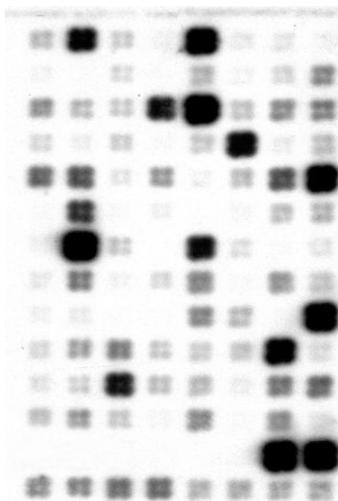


Fig. 1. The representative array image of in Dex-treated cells.

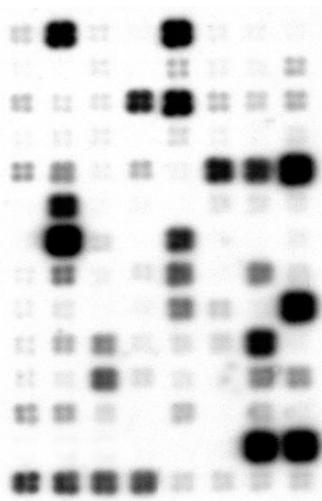


Fig. 2. The representative array image of in control cells.

**Table 1.** The 28 genes differentially expressed by human dental follicle cells in response to Dex.

GenBank	Description	Gene Name	Ratio
NM_000478	Growth factor and associated molecule alkaline phosphatase	ALP	2.252E+0
NM_000711	Bone gamma-carboxyglutamate (gla) protein (Osteocalcin)	Osteocalcin	5.857E+0
NM_006128	Bone morphogenetic protein 1	BMP1	2.303E+0
NM_001200	Bone morphogenetic protein 2	BMP2	3.336E+0
NM_001201	Bone morphogenetic protein 3 (Osteogenic)	BMP3	2.910E+0
NM_001202	Bone morphogenetic protein 4	BMP4	3.474E+0
NM_021073	Bone morphogenetic protein 5	BMP5	4.818E+0
NM_001718	Bone morphogenetic protein 6	BMP6	3.109E+0
NM_001719	Bone morphogenetic protein 7 (Osteogenic protein 1)	BMP7	#DIV/0!
NM_001720	Bone morphogenetic protein receptor type IA	ALK-3	2.353E+0
NM_002006	Fibroblast growth factor 2 (Basic)	bFGF2	2.682E+0
NM_000660	Transforming growth factor, beta 1 1 (Camurati-Engelmann disease)	TGF $\beta$ 1	3.097E+0
NM_000759	Colony stimulating factor 3 (Granulocyte)	G-CSF	1.744E-1
NM_000800	Fibroblast growth factor 1 (Acidic)	FGF1	4.720E-1
NM_005900	MAD, Mothers against decapentaplegic homolog 1 (Drosophila)	Smad1	2.650E+0
NM_005585	MAD, Mothers against decapentaplegic homolog 6 (Drosophila)	Smad6	2.326E-1
NM_005904	MAD, Mothers against decapentaplegic homolog 7 (Drosophila)	Smad7	1.057E-1
NM_003238	Transforming growth factor, beta 2	TGF $\beta$ 2	4.016E-1
NM_005247	Fibroblast growth factor 3	FGF3	2.205E-1
NM_000604	(Murine mammary tumor virus integration site (v-int-2) oncogene homolog) Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome) Matrix and its associated proteins	FGFR1	1.662E-1
NM_000088	Collagen, type I, alpha 1	COL1A1	1.074E+1
NM_000493	Collagen, type X, alpha 1 (Schmid metaphyseal chondrodysplasia)	COL10A1	2.980E+0
NM_001855	Collagen, type XV, alpha 1	COL15A1	2.928E+0
NM_001856	Collagen, type XVI, alpha 1	COL16A1	2.007E+0
NM_001858	Collagen, type XIX, alpha 1	COL19A1	4.378E+0
NM_000090	Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	COL3A1	2.646E-2
NM_033380	Collagen, type IV, alpha 5 (Alport syndrome)	COL4A5	2.114E+0
NM_000094	Collagen, type VII, alpha 1 (Epidermolysis bullosa, dystrophic, dominant and recessive)	COL7A1	2.204E+0

## Discussion

Morsczeck and colleagues documented isolation of dental follicle cells from two individual human dental follicles by their ability to adhere on a plastic surface. Both isolated cell lines were able to differentiate into a membrane-like structure with mineralized foci.<sup>6</sup> We isolated dental follicle cells from human dental follicle before the root began its formation using Morsczeck's methods and applied osteogenic differentiation protocols with dexamethasone. The process of osteogenic differentiation can be divided into several steps, consisting of cell proliferation, extracellular matrix synthesis, maturation and mineralization. The process is complex, the regulators and participants consist of growth factor and associated molecule, matrix and its associated protein, and cell adhesion molecule. The TGF- $\beta$  superfamily of growth factors includes BMPs, the TGF- $\beta$ s, activins, inhibins, myostatin, and others.<sup>11,12</sup>

BMPs are potent osteoblast differentiation factors in vitro. Various BMPs, including BMP-2, BMP-4, and BMP-7, induce the differentiation of multipotential mesenchymal cells (e.g., C3H10T1/2 cells) into both osteochondrogenic lineage cells and osteoblast precursor cells.<sup>13-16</sup> The expression patterns of several of BMPs, particularly BMP-2, BMP-4, and BMP-7, suggest a role in the generation of facial bone.<sup>17,18</sup> BMP-2 is expressed during facial skeletogenesis, in the developing tooth germ, and in the facial ectomesenchyme and Meckel's cartilage.<sup>19</sup> BMP-2 and BMP-4 also play important roles in mandibular induction<sup>20</sup> and in other oral

and facial structures.<sup>21</sup> We performed microarray analysis on human dental follicle cells in which BMP1-BMP8 were included to obtain more insight into the mechanisms that govern Dex-induced differentiation of dental follicle cells. In the present study, we observed BMP-1, BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7 transcripts up-regulated, and only BMP-8 not in dental follicle cells treated with Dex for 4 weeks. Morsczeck and colleague<sup>7</sup> also reported up-regulation of expression of BMP-2, the differentiation-related gene within osteoblast differentiation, followed by commitment and establishment of the bone phenotype in human dental follicle cells of Dex treatment. Zhao et al. reported that BMP-2, in a dose- and time-dependent manner, induced cementoblast/osteoblast differentiation of follicle cells in CD-1 mice, as reflected by enhanced core binding factor a1 (Cbfα1), bone sialoprotein (BSP), and osteocalcin (OCN) mRNA expression and enhanced mineral formation.<sup>5</sup> It suggests the BMPs play an important role in the differentiation of dental follicle cells to osteoblast-like cells. It was reported that BMP-3 is a negative determinant of bone density.<sup>22</sup> But in our experiment, BMP-3 was also up-regulated.

Smad1 is expressed mainly in the inner enamel organ epithelium and the adjacent dental mesenchyme, suggesting that Smad1 mediated BMP signaling is critical for epithelial-mesenchymal interaction during tooth morphogenesis.<sup>23</sup> Smad6 and Smad7 are inhibitors of TGF-β, activin and BMP signaling. One of the mechanisms proposed to explain the inhibitory effects of Smad6 and Smad7 is that each of these Smads can bind to diverse TGF-β family receptors and interfere with phosphorylation of R-Smads. For instance, Smad7 associates stably with the TGF-β receptor complex and inhibits TGF-β-mediated phosphorylation of Smad2 and Smad3. Alternatively, another mechanism which may help to explain the selective inhibition of BMP signaling by Smad6 suggests that, at low concentration, Smad6 can compete with Smad4 for binding to activated Smad1 and block the BMP signaling pathway.<sup>24</sup> In our experiments, a significant increase in BMPs, Bone morphogenetic protein receptor and Smad1, moreover decreased in Smad 6, Smad 7 of Dex-treated dental follicle cells than controls. This suggests that the Smads play an important role in differentiation of human dental follicle cells to osteoblasts, and Dex induced osteoblastic differentiation involved in BMP and Smad pathways.

While in vitro studies on TGF-β2 and TGF-β3 expression are poorly documented, the rapid up-regulation of TGF-β1 mRNA has previously been shown to occur in bonelike cells as a result of fluid flow-induced shear stress<sup>25</sup> and also during mechanical stretching of osteoblasts.<sup>26</sup> Recombinant TGF-β1 alone could not replace the components and its inclusion in cultures resulted only in stimulation of matrix secretion by existing cells of the dental papillae and no differentiation of odontoblasts. However, when recombinant TGF-β1 was combined with either an inactive preparation of dentine extracellular-matrix components, heparin or fibronectin, odontoblast differentiation was induced.<sup>27</sup> Dex not only increased the gene expression of TGF-β1 in our experiments, but also enhanced the expression of the Fibronectin in the works of Morsczeck and colleague. It suggests that TGF-β1 plays an important role in the process of differentiation of dental follicle cells to osteoblast-like cells. Signals for TGF-β1 and TGF-β3 transcripts in the mouse increase significantly as odontoblasts differentiated, whereas TGF-β2 transcripts were high in preodontoblasts and diminished with polarization during odontoblast cytodifferentiation.<sup>28</sup> Other studies also show only weak detection of TGF-β2 transcripts in mouse and human odontoblasts.<sup>29</sup> Using microarray technique, we found that Dex up-regulated the TGF-β1 and down-regulated TGF-β2, but not the TGF-β3 on the human dental follicle cells at first time.

ALP is not a bone matrix protein, yet it is well known that it is closely related to bone matrix mineralization.

During the late stage, osteocalcin (OC) production is observed. OC is thought to be associated with matrix calcification and is regarded as a late maturation marker. OPN is an extracellular matrix protein and a cytokine that has been reported to act in a number of physiological and pathological events including bone remodeling.<sup>30-33</sup> The results of our experiments revealed that ALP, OCN of the human dental follicle cells up-regulated after being treated with Dex for 4 weeks, but OPN not. OCN transcripts increased by about 5-fold at day 28 of differentiation in Dex-treated cells, compared to untreated cells. This is consistent with previous reports.<sup>7</sup> Dexamethasone affects the differentiation of bone marrow stromal cells to osteoblastic cells. It stimulates ALP activity,<sup>34-36</sup> osteonectin<sup>34</sup> and osteocalcin<sup>28,34</sup> the type I collagen synthesis.<sup>34</sup> These results were consistent with our experiments.

Ninety percent of the organic bone matrix is collagen, mostly type I. Our study showed that Dex increased the mRNA levels for type I, type X, type XV, type XVI, type XIX, type IV and type VII respectively, and decreased the mRNA level of Collagen III for the analysis of the microarray data. A similar up-regulation of COL X mRNA has been reported with the differentiation of embryonic stem cells into chondrocytes, hypertrophic chondrocytes, and osteoblasts.<sup>37</sup>

Collagen III and bone sialoprotein are useful markers in that they aid in differentiating among cementum, dentin, and bone (absent or low collagen III, modest bone sialoprotein expression).<sup>38,39</sup> Both insulin-like growth factors and receptors (IGF-RI and IGF-RII) have also been implicated as regulators of bone turnover,<sup>40</sup> and IFG-I did not affect the proliferation or early osteogenic differentiation of human marrow stromal cells. The beneficial effects of IGF-I on the skeleton are not mediated primarily via an effect on osteoprogenitor fraction and are thus consistent with the hypothesis that the effects of IGF-I are differentiation dependent and restricted largely to the more mature cells of the osteoblast lineage.<sup>41</sup> In this study, we demonstrated by microarray that Dex increased the expression of IGF-IR, but decreased the expression of IFG-II, but not IGF-I in human dental follicle cells. The lack of effect of Dex on the expression of the expression IGF-I is consistent with the results of earlier studies using human marrow stromal cells.<sup>42</sup> This suggests that IGF-I does not play a major role in directing dental follicle cells toward the osteogenic pathway of differentiation.

Cam et al.<sup>43</sup> and Russo et al.<sup>44</sup> elucidated the expression of FGF-2 in the cells differentiating from the dental papilla and dental follicle in the region of the crown during early tooth development. They showed that FGF-2 expression is specific to the epithelium and ectomesenchyme associated with the developing crown and showed progression with time. The results of our experiment revealed that Dex up-regulated the expression of FGF-2, but down-regulated the expression of FGF-1, FGF-3 and FGFR-1. It suggested that FGF-1, FGF-2, FGF-3 and FGFR-1 were involved the differentiation of human dental follicle cells.

Our results revealed that the expression of G-CSF was decreased by Dex, which suggested G-CSF was involved in the osteoblastic differentiation progress of human dental follicle cells. Osteoblast activity, as measured by histomorphometry and osteocalcin expression, is strongly down-regulated during G-CSF treatment. However, the G-CSF receptor is not expressed on osteoblasts; accordingly, G-CSF had no direct effect on osteoblast function.<sup>45</sup>

In conclusion, using a microarray approach, we have identified a number of genes that are associated with the process of human dental follicle cells differentiation at first time. In our own studies, we observed 20 transcripts up-regulated and eight transcripts down-regulated in human dental follicle cells treated with Dex for 4 weeks. Our results presented here demonstrate conclusively that human dental follicle cells display osteoblastic feature

after treated with Dex for 4 weeks, and the progress involved a number of osteoblastic genes. It might be highly interesting to examine what role these gene products play in differentiation and how they work together.

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