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Identification of the chondrogenic pathway in the mandibular condylar cartilage

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1. ABSTRACT

To identify genetic expression of mandibular condylar cartilage during natural growth and under mechanical strain as a result of mandibular advancement. One hundred and forty four 35 days old female Sprague-Dawley rats were sacrificed on 8 different experimental days. Total RNA was extracted for oligonucleotide microarray gene chips containing 15,923 transcripts. Sixteen genes representing the chondrogenesis pathway were identified. Five of them were novel and have never been identified in mandibular condyles before. Quantitative analysis using real-time PCR revealed that these genes were involved in different stages in chondrogenesis and played an important role in condylar growth.

2. INTRODUCTION

Recently, it has been reported that the growth of the condyle is regulated by a series of factors that are endogenously expressed by cells in the condyle (1). Sox9 plays a critical role in chondrocyte differentiation and regulates the synthesis of type II collagen (2, 3). PTHrP promotes the differentiation of mesenchymal cells into chondroblasts and retards their further maturation (4). Ihh regulates the proliferation of the chondrogenesis in mandibular condyle under mechanical strain (5). Cbfa1 controls the maturation of chondrocytes into hypertrophic chondrocytes and regulates the differentiation of mesenchymal cells into osteoblasts (6). Type X collagen, the expression marker of the onset of endochondral

ossification, forms the major component of the hypertrophic cartilage matrix in growing mandibular condyles (3). VEGF is produced by hypertrophic chondrocytes and is actively responsible for hypertrophic cartilage neovascularization in the mandibular condyle by inducing endothelial cell migration and proliferation (7). The mesenchymal cells carried by new blood vessel invasion differentiate into osteoblasts then lead to new bone formation in the condyle (3).

Even though the progress made over the past decade helped in identifying key factors that regulate condylar growth, the genes involved in regulating key steps in condylar growth remain unidentified. For example, are there any components in the condylar cartilage that give cartilage the ability to absorb the shock and prevent the injury? How would chondroadherin adhere to the matrix and maintain the cartilage structural integrity? Are there any regulatory factors that can interact with antagonists of BMPs so that they can attract more cells to the condylar cartilage? Are there any factors modulate the collagenous framework during cartilage formation? Are there any novel genes that can regulate bone growth during the pubertal growth period?

Therefore, this study was designed to identify gene expression, during induced condylar growth and relate their levels of expression to different relevant stages of condylar growth, with an aim to elucidate the factors involved in condylar growth regulation.

3. MATERIALS & METHODS

3.1. Experimental animals

One hundred and forty four 35 days female Sprague-Dawley rats were randomly divided into 8 experimental and 8 control groups. Acrylic bite-jumping appliances were fitted to the upper incisors of the experimental rats to produce a continuous 3.5-mm anterior and 3-mm inferior displacement of the mandible. All rats were fed with a soft diet. The appliances were worn 24 hours a day and were cemented with Panavia F20 (Kuraray Co Ltd, Osaka, Japan). Each group of rats was sacrificed on the following experimental days: 1, 3, 7, 9, 14, 21, 30 and 33.

3.2. Total RNA extraction

Total RNA was extracted from the mandibular condylar cartilage by means of the RNeasy Fibrous Tissue Midi Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The purified RNA was quantified by a spectrophotometer at 260nm, and RNA quality were evaluated by an RNA 6000 Nano Lab-on-a-chip Kit with a 2100-Bioanalyzer system (Agilent Techonologies). Only those RNA samples with 28S/18S ratio greater than 1.8 would be used for microarray analysis.

3.3. Microarray analysis

Experimental procedures for GeneChip microarray were conducted at the Genome Research Centre at The University of Hong Kong according to the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara). 2μg of total RNA was used to synthesize double-stranded cDNA and the biotinylated cRNAs were hybridized to Rat Expression Set 230A (RAE230A; Affymetrix), which contained 15,923 transcripts. The data were analyzed using Affymetrix® Microarray Suite (Version 5.0) and DNA-Chip Analyzer (dCHIP) softwares. The results were expressed as the genes expression ratio. Using a two-fold change criteria as the appropriate 'cut-off' value, we identified those genes that exhibited more than two-fold change related to the control group in this study.

3.4. Real-time quantitative RT-PCR

To confirm the expression levels of the genes indicated in the microarray data, we selected five genes (Mustang, CryAB, Noggin, NOV, and CHAD) involved in different steps of chondrogenesis for quantitative real-time PCR analysis. The total RNA extracted from rat condylar cartilage was reversed transcribed using Superscript First Strand synthesis system (Invitrogen) according to the manufacturer's instruction. The resulting cDNA solution was diluted 10 times for real-time quantitative PCR. Oligonucleotide primers sets for target genes and GAPDH for real time RT-PCR were designed by using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 _www.cgi). The primers are listed in Table1. Quantified control were constructed for all target genes by cloning the cDNA fragment (obtained by PCR amplification) into TOPO TA plasmid (invitrogen). Single colonies were grown up overnight, shaking at 200 rpm at 37^{o}C and plasmid was subsequently purified using the QIAfilter plasmid midiprep kit (Qiagen, Crawley, UK). DNA sequencing (Applied Biosystem) confirmed the sequences of the cloned products. A standard curve with five quantities ranging from 10^7 copies to 10^3 copies was generated using a 10-fold dilution series. Reactions were performed in triplicate using the Bio-Rad Real-time Detection System (Bio-Rad) according to the manufacturer's instructions. The cDNAs were amplified using the following thermal cycling conditions: one cycle at 95 ^{o}C for 3min, followed by 45 cycles of 30s at 95 ^{o}C and 40s at 53 ^{o}C and 30 sat 72 ^{o}C. The relative mounts of transcripts were normalized with GAPDH.

4. RESULTS

In the present microarray analysis, a total of 1,082 genes showing more-than-two-fold change expression in experimental group than in control group were identified. Among them, 666 genes showed an increasing expression and 416 genes showed a decreasing expression (Figure 1). 16 chondrogenic genes were found involved in condylar growth process (Table 2).

We used real-time RT-PCR to assess the quantities of the 5 chondrogenic genes to confirm the microarray analysis results. These genes were Mustn1, Cryab, Noggin, NOV and Chad, which were involved with different stages of chondrogenesis in mandibular condyle growth. Figure 2 displayed the gene expressions obtained by microarray and real-time RT-PCR and showed that the correlation was good.

Table 1. Primers used in real-time RT-PCR with sequence, length of amplification (bp) and number of cycles

Name	Primer squences		Length of Amplificon (bp)	Number of Cycles
	Sense 5'- 3'	Anti-sense 5'- 3'		
Mustang	GAGCTTGCTTGGCATCTACC	TTGGGCTTCTCGAAGACTGT	238	45
CryAB	TTCTTCGGAGAGCACCTGTT	TCCGGTACTTCCTGTGGAAC	292	45
Noggin	TCCAAGTCTGTGCACCTCAC	GCCAGGTCTCTGTAGCCAAG	261	45
NOV	TCTGTGGGATCTGCAGTGAC	ACACGGGACAACTCCTTCAC	303	45
CHAD	ACCTGGACAGGAACCAACTG	GAACTTGGCTGGTGAAGAGC	395	45
GAPDH	CATGTTCCAGTATGACTCTACCC	AGCATCACCCCATTTGATGT	136	45

Table 2. The candidate chondrogenic genes expression changes in differential experimental days.

Affymetrix ID	NAME	Fold Change						
	exp day1	exp day3	exp day7	exp day9	exp day14	exp day30	exp day33	
1368788_at	Chad	2.639	3.031	2	4.287	2.639	1.866	1.072
1376734_at	Nov	-1.414	1	1	1	1.741	1.973	2.297
1384931_at	Col11a1	1.32	-1.414	-1.072	-3.031	-1.149	-1.32	3.031
1390386_at	Casp3	-1.072	-1.072	1	1.072	1.072	2	4
1370026_at	Cryab	1.866	1.866	1.741	1.741	4	-1.414	1.231
1371166_at	Nos3	-1.231	1.072	1.231	-1.32	2.462	-1.32	1
1368668_at	Plaa	1.231	1	-1.072	-1.072	1	1.149	2.297
1373032_at	Mustang	1.414	1.231	1.414	1.516	2.462	1	-1.149
1368969_at	Sost	-1.072	-1.072	3.482	3.482	2.462	-1.231	1
1369320_at	Mia	1	-2.297	-2.144	-1.741	-1.625	-1.866	1
1368836_a_at	AGC1	-1.625	-3.482	-2	-1.32	-1.231	-1.625	1.149
1387137_at	Comp	-1.414	-4	-2.297	-1.625	-1.866	-2.297	1
1374870_at	Col27a1	-1.516	-2.639	-2.639	-1.866	-1.625	-2.144	1.072
1384211_at	Col11a1	1.32	-1.32	-1.414	-1.516	-2.639	-2	-1.072
1387164_at	Lect1	-1.32	-5.657	-3.249	-5.278	-6.964	-4.925	-1.32
1371052_at	Noggin	-1.414	-2	-1.149	-1.072	-2.297	-1.414	1.149

For every time point analyzed, differential gene expression changes for 16 candidate chondrogenic genes were shown by a fold change value (experimental group related to control group)

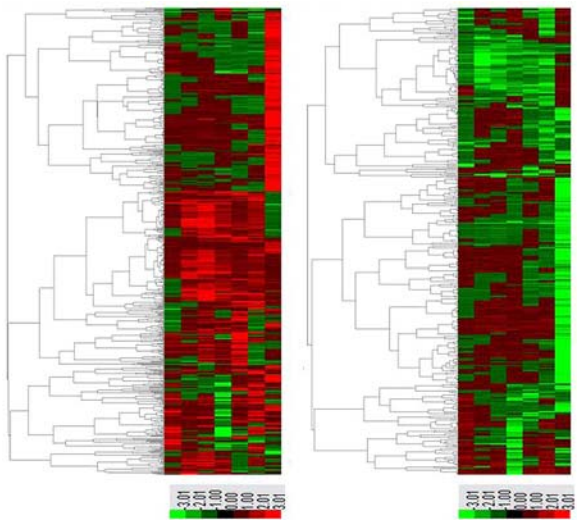

Figure 1. Gene expressions in different experimental days (1, 3, 7, 9,14, 30, 33 experimental days) were analyzed using hierarchical clustering and self-organizing maps different colors indicated different gene expression change directions (red: increase, green: decrease).

5. DISCUSSION

In the present study, we identified 16 genes involved in chondrogenesis of the mandibular condyles (Table 2). Five out of the 16 were novel genes that had never been identified in the condyles before. These genes were Mustang, CryAB, Noggin, NOV and CHAD.

Mustang is a novel gene that encodes for an 82 amino acid nuclear protein with no homology to any known protein family. It was identified in a study examining bone remodeling in rats after fracture. (8) The expression of Mustang was localized in osteoprogenitor cells of the periosteum, proliferating chondrocytes, and young active osteoblasts. Initial characterization of its activation during bone regeneration and embryogenesis revealed that it was involved in the development and regeneration of the musculoskeletal system. In this study, we did not detect significant variations in Mustang expression during natural growth. The spurts of rat growth exist on age day 31.5 (Luder, 1996), and the age of rats used in this study was 35 days at the beginning. It supports the finding of Lombardo (2004) that Mustang was inactivated in most adult tissues. Mandibular advancement, however, induced a significant increase in Mustang level on day 7 (Figure 2A). It is important to note that the increased Mustang expression was associated with the activation of mandibular condylar cartilage formation after mandibular advancement. In previous studies, mechanical strain produced by mandibular advancement enchanced growth of the mandibular condyle (3, 9). The number of replicating mesenchymal cells increased under mechanical strain, which was closely correlated with the mount of bone formation of mandibular condyle (9). Mustang is expressed by mesenchymal cells and periosteal osteoprogenitors (8), the high expression of Mustang in madibular condylar cartilage on the early stage of mandibular advancement implied that Mustang may play an important role in chondrogenic and osteogenic cell differentiation. Our data showed that the Mustang expression response to mechanical strain continuously increased and reached the highest level in day 14. Although the mustang expression

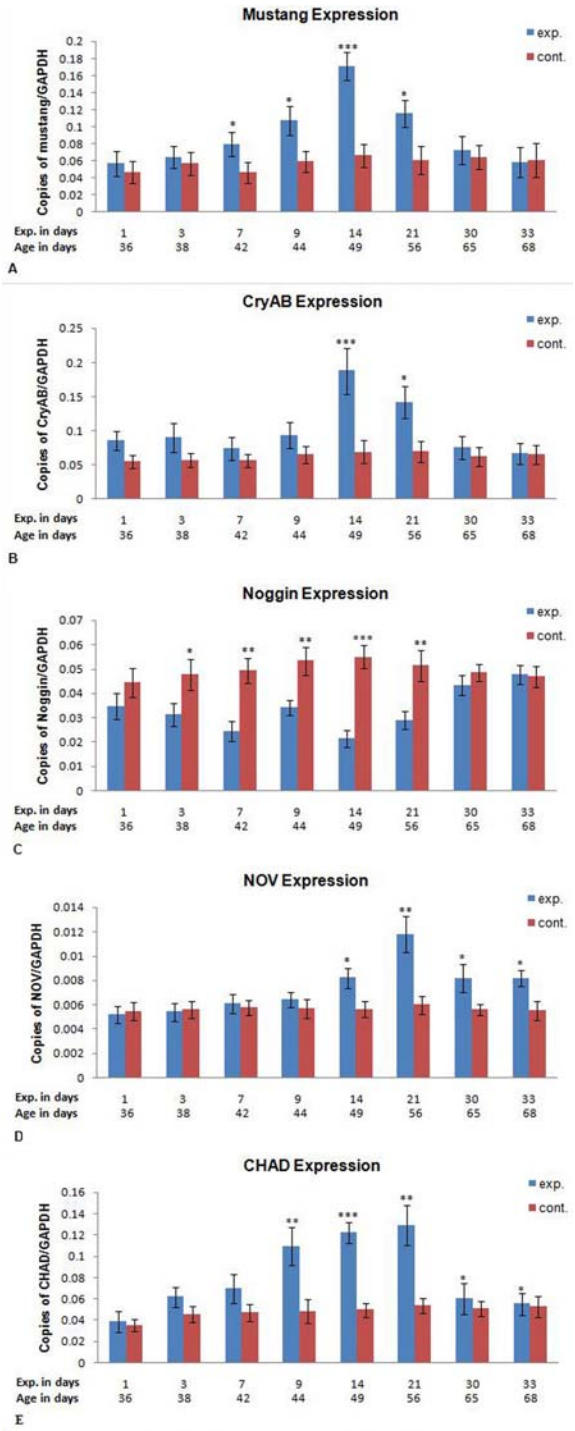

Figure 2. The mRNA expression quantified using real-time RT PCR in condylar cartilage during natural growth (cont.) and during mandibular advancement (exp.) (A: Mustang, B: CryAB, C: Noggin, D: NOV, E: chondroadherin). Values were mean ± SD. Significant difference between control and experimental animals was marked with asterisks (*p<0.05, ***p<0.001).

decreased in day 21, the level was still significantly higher than the expression during natural growth. (Figure 2A) The expression changes in Mustang during mandibular condylar growth suggested that Mustang maybe a novel marker of chondrogenesis and plays an important role in cartilage formation of mandibular condyle. Its exact function in chondrogenesis pathway needs to be confirmed in further studies.

Alpha B-crystallin (CryAB) belongs to the small heat shock protein family and was first identified as a structural component of the vertebrate eye lens (10). CryAB is also expressed in various non-lenticular tissues (11), including bone and in osteoblast-like cells (12). It is the first time to identify CryAB expression in mandibular condylar cartilage. CryAB can prevent cellular injury after biomechanical and physiological stress (13), furthermore, the transcript of CryAB increased in 6 hours after mechanical stretch *in vitro* study (14). Our data further supported the finding that CryAB expression changed in response to biomechanical strain. (Figure 2B) The expression of CryAB in mandibular advancement group maintained a higher level than natural growth from the beginning. In previous reports, mechanical strain stimulated osteoblasts proliferation and differentiation (15). The osteoblasts formation significantly increased on day 14 and day 21 during mandibular advancement. It is important to note that the increased CryAB expression was associated with the increase of these cells (Figure 2B). An *in vitro* study suggested CryAB played a role in conformational stability of the components in the cartilage extracellular matrix (16). In our study, we did not detect significant variations in CryAB expression during natural growth. Mandibular advancement, however, induced a significant increase on day 14 and day 21 (Figure 2B). Endochondral ossification requires a precise coordination of the activities of chondroclasts and osteoblasts to remove the cartilage and to deposit the osteoid. Kulterer (2007) suggested that CryAB involved in osteogenic differentiation since CryAB was significantly up-regulated during osteoblast differentiation. Our data further supported the findings and suggested that CryAB played a role in chondrogenesis and osteogenesis in mandibular condyle.

Noggin is a bone morphogenetic protein (BMP) antagonist expressed in condensing cartilage and immature chondrocytes (17). It was well-documented to regulate BMP activity during chondrogenesis to inhibit chondrocytes hypertrophy and proliferation (18, 19). The overexpression of Noggin decreased cartilage differentiation and caused shortening of the developing bones (17). In noggin knockout mice, the excess BMP activity enhanced the recruitment of cells into cartilage, resulting in oversized growth plates and cartilage hyperplasia (20). In a recent study, mechanical stress induced an significant decrease in Noggin expression (21). Our data supported the finding that Noggin expression could be down-regulated in response to biomechanical strain. In the present study, we did not detect significant variations in Noggin expression during natural growth (Figure 2C) because the rats passed the peak spurt of the growth which occur at age 31.5 days (Luder, 1996). In mandibular advancement group, however, the decrease of Noggin expression was first identified on day 3 and lasted

until day 21. (Figure 2C) Furthermore, Heller (2007) reported that a negative correlation existed between Noggin and Runx2 expression under mechanical strain (decreased in Noggin expression and increase in Runx2 expression). In our previous report, mandibular advancement significantly increased Runx2 expression, which regulated chondrocytes maturation and enhanced endochondral ossoficication in the condyle (15). The present study echoed our previous findings where mechanical strain produced by mandibular advancement changed the biophysical environment of mandibular joint which solicited cellular and molecular responses and finally enhanced cartilage and bone formation in mandibular condyle (22). Most interestingly, a recent study reported that downregulation in Noggin could enhance osteogenesis and suggested a novel approach to clinically accelerate bone formation by Noggin suppression (23). The decrease in Noggin expression elevated the BMPs activity to increase the recruitment of cells into cartilage and enhanced chondrocytes hypertrophy and proliferation in mandibular condylar cartilage.

Nephroblastoma overexpressed (NOV), also called CCN3, belongs to the CCN family of proteins, which also includes CYR61, CTGF, ELM1, RCOP1 and WISP3 (24). All these members are cysteine-rich secreted proteins and were reported to play roles in development, embryogenesis, differentiation, and matrix remodeling (25-28). Some reports suggested the function of NOV varied with specific cell type (29, 30), in the case of chondrocytes, an *in vitro* study suggested that NOV had a positive role in differentiation (31). In present study, no significant variations were detected in NOV expression during natural growth. Mandibular advancement, however, induced a significant increase in NOV expression on day 14 and day 21 (Figure 2D). A recent study indicated that NOV could support cartilage integrity by positively modulating the expression type X collagen (32). It was pointed out that a close correlation existed between the expression of the type X collagen and bone formation (3). Type X collagen acted as the marker of the hypertrophic chondrocytes and formed the collagenous framework of the hypertrophic cartilage destined for endochondral ossification. Our previous study demonstrated that type X collagen expression in rat condylar cartilage reached the highest level in the age of day 49 (3). It is important to note that the increase NOV expression was associated with the increase of type X collagen (experimental day 14). Our data further supported the hypothesis that NOV was involved in the transition phases between chondrogenesis and osteogenesis during endochondral ossification (33). These findings implied that NOV plays a role in collagenous framework formation in chondrogenesis process of mandibular condylar cartilage.

Chondroadherin (CHAD), a non-collagenous extracellular matrix protein in cartilage, is expressed by cartilaginous cells (34) and was identified as a member of the leucine-rich repeat (LRR) family of proteins (35). Localization of chondroadherin in the developing rats showed that the protein presented in the territorial matrix around late proliferative cells in the growth plate and the most prominent chondroadherin increase was seen between age day 20 and 33 (36). It supported our finding that no significant variations in chondroadherin expression were detected during natural growth because the rats used in this study were between the age of 35 and 65 days. However, the mechanical strain induced the significant increase in chondroadherin expression between experimental day 9 and day 21 (Figure 2E). Chondroadherin was shown to interact with type II collagen, the major constituents of the extracellular matrix of cartilage, *in vitro* and *in vivo (*37). In previous study, type II collagen expression was detected to increase on experimental day 14 and 21 during mandibular advancement (4). Thus, it is important to note that the increase chondroadherin expression was associated with the increase of the expression in type II collagen in response to mechanical strain. An *in vitro* study showed that chondroadherin adhered to chondrocytes via the integrin $\alpha_2\beta_1$ receptors (38), whereas integrin $\alpha_2\beta_1$ was also shown to bind collagen type II (37). Moreover, integrin $\alpha_2\beta_1$ was found that it could be upregulated during changes in cell-matrix interactions such as mechanical stress (39). This suggested that, by sharing the receptors on the chondrocyte and by interacting with each other, chondroadherin and type II collagen might set up a complex extracellular matrix system maintaining structural integrity in condylar cartilage during chondrogenesis.

In summary, this study is the first to analyze the gene expression of mandibular condyle during condylar natural growth and under mechanical strain stimulation. Novel genes involved in mandibular condylar growth were identified and their levels of expression were related to relevant stages of condylar growth. Further studies on their precise roles in condylar growth are necessary and will contribute to clarifying the gene regulatory process of mandibular condylar growth and the molecular mechanism of dentofacial orthopedic treatment.

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Abbreviations: Sox9: SRY(sex determining region Y)-box containing gene 9; PTHrP□parathyroid hormone-related protein; Cbfa1: core binding factor alpha1; VEGF: vascular endothelial growth factor; BMP: Bone morphogenetic protein; Mustang: musculoskeletal temporally activated novel; CryAB: Alpha B-crystallin; NOV: nephroblastoma overexpressed gene; CHAD: chondroadherin; RNA: ribonucleic acid; DNA: deoxyribonucleic acid; RT-PCR: reverse transcription-polymerase chain raction; cDNA: complementary deoxyribonucleic acid; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

Key Words: Mechanical, Stress, Cartilage, Mustang, CryAB, Noggin, NOV, CHAD, Microarray, Real time RT-PCR, condyles

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