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Microarray Analysis of the Developing Rat Mandible

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Abstract To analyze the molecular events that occur in the developing mandible, we examined the expression of 8803 genes from samples taken at different time points during rat postnatal mandible development. Total RNA was extracted from the mandibles of 1-day-old, 1-week-old, and 2-week-old rats. Complementary RNA (cRNA) was synthesized from cDNA and biotinylated. Fragmented cRNA was hybridized to RG-U34A GeneChip arrays. Among the 8803 genes tested, 4344 were detectable. We identified 148 genes with significantly increased expression, and 19 genes with significantly decreased expression. A comprehensive analysis appears to be an effective method of studying the complex process of development.

Keywords gene expression; microarray; rat mandible

Periodontal disease is an inflammatory disorder that damages tooth-supporting structures through complex interactions between periodontopathic bacteria and host defense systems [1–3]. The regeneration of tooth-supporting structures destroyed by periodontal disease is a major goal of periodontology, and products based on developmental biology are being tested in clinical trials for use in regenerative therapies [4–6]. These clinical therapies, however, are not predictable. One requirement for developing more predictable clinical procedures is an increased understanding of the cellular and molecular mechanisms regulating the development of tooth-supporting structures.

The development of tooth-supporting structures is a complex process that requires the interactions of large groups of genes and is accompanied by changes in gene expression [7–12]. Several analyses of gene expression have been carried out using hybridization and polymerase chain reaction (PCR) methods, but due to the limitations in the assay capability, the number of genes analyzed has been limited. To address this issue, we applied a gene array technique based on hybridization to survey a larger number of genes (1176 genes) [13].

In this paper, we analyze the expression of 8803 genes from samples taken at different time points during rat postnatal mandible development.

Materials and Methods

Animals

Wistar rats (1-day-, 1-week-, and 2-week-old) were obtained from Sankyo Labo Service (Tokyo, Japan). Six or seven rats of each age from the same mother were used for each microarray analysis. The animals were euthanized with a pentobarbital overdose while under anesthesia, and their mandibles were dissected. As much soft tissue as possible was removed with forceps under a microscope.

RNA processing methods

Total RNA was extracted from the mandible using Trizol reagent (Gibco BRL, Grand Island, USA), according to the manufacturer's instructions. Subsequent RNA processing procedures followed the protocols in the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, USA). Briefly, complementary DNA (cDNA) was

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prepared from 2 μg of total RNA using T7-dT24 primer. The cDNA was cleaned with phase-lock gels, extracted using phenol/chloroform, ethanol precipitated, and used for cRNA preparation. cRNA was synthesized from cDNA, then biotinylated using the BioArray HighYield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, USA). Typical yields of target cRNA were 60 μg. The cRNA (15 μg) was fragmented by heating at 94 ºC for 35 min in fragmentation buffer [40 mM Tris-acetate (pH 8.1), 125 mM KOAc, and 30 mM MgOAc] before hybridization. Then 15 μg of fragmented cRNA (including control cRNA and grid alignment oligonucleotides) was hybridized for 16 h to RG-U34A GeneChip arrays at 45 ºC under constant rotation in hybridization buffer. The arrays were washed and stained, followed by signal amplification with anti-streptavidin antibody using the Affymetrix Fluidics Station 400. Fluorescent signals were measured on the arrays using the Agilent Gene Array Laser Scanner (Agilent Technologies, Palo Alto, USA).

Statistical methods

The data were analyzed using GeneChip Analysis Suite (Affymetrix) and GeneSpring 4.2.1 (Silicon Genetics, Redwood City, USA) bioinformatics algorithms. Per-chip and per-gene normalization were carried out to β-actin in intensity and detection efficiency between spots. The data presented are the average of three separate experiments. Data from each time point were scaled and the relative mRNA expression levels were expressed as plus or minus fold changes with hierarchical clustering using GeneSpring 4.2.1 software.

Real-time PCR

RNA (3 μg per reaction) was reverse-transcribed using a First-Strand kit (Amersham Pharmacia Biotech, Piscataway, USA) at 42 ºC for 60 min. Following cDNA synthesis, 1 μl of cDNA template was used for each PCR. Real-time PCR was conducted using Assay-on-Demand Gene Expression Products (Applied Biosystems, Foster City, USA). PCR amplifications for the target genes and internal control β-actin were carried out in a capped 96-well optical plate. The reaction conditions were as follows: 5 min at 50 ºC, 10 min at 95 ºC, and 40 cycles of 15 s at 95 ºC and 1 min at 60 ºC. The gene-specific PCR products were measured continuously using the ABI PRISM 7700 detection system (Applied Biosystems). Samples were normalized using an internal control, and the results are expressed as the relative fold increase. The results represent the mean value of two independent experiments, and each experiment was run in triplicate.

Results

The data were analyzed with GeneChip Analysis Suite and GeneSpring 4.2.1 software. Using a DNA microarray containing 8803 genes, 4344 genes were detectable under at least one of the conditions tested.

First, we applied hierarchical clustering to the detectable genes to visualize the patterns of gene expression. The clustering algorithm grouped 1-day-old and 1-week-old rats separately from 2-week-old rats (**Fig. 1**).

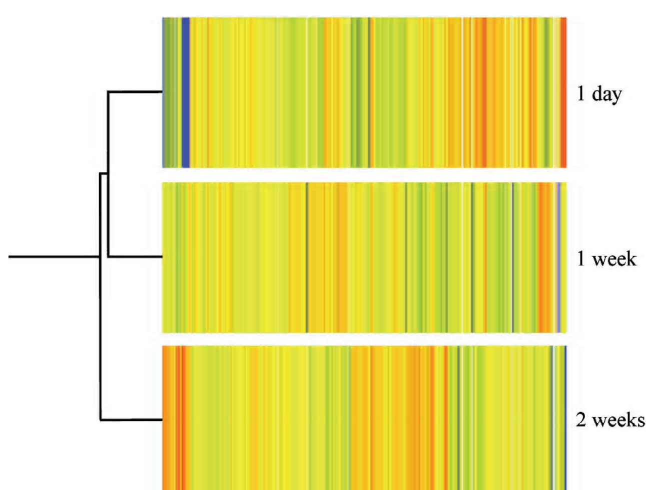

Fig. 1 Hierarchical clustering analysis of detectable genes in the developing rat mandible
Experimental tree clustering using standard correlations was applied to the microarray data. The lines indicate potential interrelationships between the experimental conditions.

The fold-change ratios between the 1-day-old and 2-week-old groups were used to select genes with increased and decreased expression. Welch's *t*-test was also applied, with $P<0.05$ denoting statistical significance between the two groups. **Tables 1–3** show the genes with expression increased or decreased by at least threefold after Welch's *t*-test. Using this strategy, we identified 148 genes that increased significantly, and 19 genes that decreased significantly. Of these, the known genes were classified according to their biological processes, cellular components, and molecular functions.

Ten genes (three ESTs) were up- or down-regulated by at least fivefold. Of these, we selected seven known genes

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Table 1 Genes in developing mandibles of rats (1 day, 1 week, and 2 weeks old) with increased or decreased expression, by biological process class

Biological process class		Gene	GenBank accession No.
Cell communication			
Cell adhesion	Increase	Embigin	AJ009698
Cell growth and maintenance	Increase	High affinity *L*-proline transporter	M88111
	Increase	Neuronal high affinity glutamate transporter	D63772
	Increase	PC3	M60921
	Increase	FGF receptor-1	D12498
	Increase	Tricarboxylate transport protein	L12016
	Increase	Proton channel of vacuolar H^+-ATPase	D10874
	Increase	Myelin protein SR13	S55427
	Decrease	JP 1986234785-A/1	E00988
Signal transduction			
Cell surface-linked signal transduction	Increase	Neuronal high affinity glutamate transporter	D63772
	Increase	Serine/threonine protein kinase	L01624
	Increase	Neuropeptide Y	M15880
	Increase	IAP	AF017437
	Increase	Calcium-binding protein	K00994
	Increase	NMDA receptor	S61973
	Increase	MRP14	L18948
	Decrease	Unknown effector coupling	L10073
Developmental processes	Increase	Glycoprotein 130	M92340
	Increase	Immunoglobulin K	M18530
Intracellular signalling	Increase	Immunoglobulin K	M18530
Physiological processes	Decrease	Light meromyosin	M12098
	Decrease	MHC	X04267

Table 2 Genes in developing mandibles of rats (1 day, 1 week, and 2 weeks old) with increased or decreased expression, by cellular component class

Cellular component class		Gene	GenBank accession No.
Intracellular			
Cytoplasm	Increase	Tau	X79321
	Increase	PEX11	AJ224120
	Increase	MHC	L13606
	Increase	MHC	S68736
	Increase	Myosin regulatory light chain	D14688
	Decrease	Troponin T	M80829
	Decrease	α-Actin cardiac	X80130
	Decrease	Light meromysin	M12098
	Decrease	MHC	X04267
Mitochondrion	Increase	Mitochondrial very-long-chain acyl-CoA thioesterase	Y09333
	Increase	Ubiquitous mitochondrial creatine kinase	X59737
Nuclear	Increase	HNF-3G	AB017044
Peroxisome	Increase	PEX11	AJ224120
Vacuole	Increase	Proton channel of vacuolar H^+-ATPase	D10874
Membrane			
Integral membrane	Increase	MHC class I RT1.C-type protein	L40362
	Increase	Cell surface glycoprotein	M15562
	Increase	MHC RT1.Aa α-chain precursor	M31018
Peroxisomal membrane	Increase	PEX11	AJ224120

MHC, myosin heavy chain.

Table 3 Genes in developing mandibles of rats (1 day, 1 week, and 2 weeks old) with increased or decreased expression, by molecular functional class

Molecular function class		Gene	GenBank accession No.
Enzyme	Increase	PEX11	AJ224120
	Increase	RMCP-3	U67888
	Increase	Mitochondrial very-long-chain acyl-CoA thioesterase	Y09333
	Increase	Src related tyosine kinase	U09583
	Increase	KAL	M22922
	Increase	MEKK1	U48596
	Increase	Competitive inhibitor of the cAMP-dependent protein kinase	M64092
	Increase	ADP-ribosylarginine hydrolase	M86341
	Increase	Plap	U17901
	Increase	Stearyl-CoA desaturase	J02585
	Increase	Protein kinase C C-terminal region	X04139
	Increase	Serine/threonine	L01624
	Increase	EST	AA684929
	Increase	CNP II	L16532
	Increase	E217kB	U13177
	Increase	α-KG-E2	D90401
	Increase	Glutathione transferase	X62660
	Increase	GTP cyclohydrolase I	M58364
	Increase	CP-2	S85184
	Increase	Uroporphyrinogen decarboxylase	Y00350
	Increase	Glutathione transferase	J03752
	Increase	RMCP-8	U67911
	Increase	Proton channel of vacuolar H^+-ATPase	D10874
	Increase	Na, K-ATPase	M28647
	Increase	ALAS2	D86297
	Increase	Na, K-ATPase β-3 subunit	D84450
Immunity protein	Increase	Anti-idiotype immunoglobulin M light chain	U75411
	Increase	λ-5	Z68145
	Increase	Immunoglobulin K	M18530
	Increase	Glycoprotein	X13044
	Increase	Embigin	AJ009698
	Increase	RT1.EC3	AF074609
	Increase	Cell surface antigen	M24026
	Increase	Myosin heavy chain RT1.Aa alpha-chain precursor	M31018
Microtubular dynamics	Increase	Tau	X79321
Other groups	Increase	E217kB	U13177
	Increase	Embigin	AJ009698
	Increase	Antioxidant	U06099
Signal transducer	Increase	Neuropeptide Y	M15880
	Increase	CX3C	AF030358
	Increase	FGF receptor-1	D12498
	Increase	CD9	X76489
Transport	Increase	Neuronal high affinity glutamate transporter	D63772
	Increase	Acyl carrier protein (405AA)	X13527
	Increase	Tricarboxylate	L12016
	Increase	CD9	X76489
	Increase	Proton channel of vacular H^+-ATPase	D10874

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Table 4 Sequences of the oligonucleotide primer pairs of seven genes found in the developing rat mandible, and control, used for real-time quantitative polymerase chain reaction

Gene	GenBank accession No.	Oligonucleotide sequence (5′→3′)
Calcium-binding protein (ICaBP)	K00994	(F)/ CAAAGAAGGCGATCCAAACC
		(R)/ GATTGTCTAGAGTACTTGAAGCCTTACG
		(P)/ CTGCTGATTCAGTCAGAGTTCCCCAA
Adipsin	M92059	(F)/ GATGTCCTGCAGCAACTGACA
		(R)/ CATGGTACGTGCGCAGATTG
		(P)/ TGTCAATCATGGACCGGAACACCT
Apolipoprotein E (ApoE)	S76779	(F)/ TTCTGACCAGGTCCAGGAAGAG
		(R)/ TTACTTCCGTCATAGTGTCCTCCAT
		(P)/ CCCAAGTCACACAGGAACTGACGG
Erythroid-specific δ-aminolevulinate synthase (rat ALAS-E)	D86297	(F)/ CCCTGACCTTCGTGGATGAA
		(R)/ GCGATATAGCCACCGACACA
		(P)/ CATCATCTCTGGAACTCTTGGCAAGGCC
Calcium-binding protein (MRP14)	L18948	(F)/ CAGCTGGAGCGCAGCAT
		(R)/ GGGTGTCAGGATGTCCATACTTC
		(P)/ AGCACCATCATCAATGTTTTCCATCAG
12-lipoxygenase	S69383	(F)/ AGCTGATGCCTGATGGACAAC
		(R)/ GGCTAGGAGCCAGTCCATTG
		(P)/ CCATAGCCATCCAGCTTGAACTTCCCA
Defensin 3a (Rat NP3a)	U16683	(F)/ GCACTAAGGAAGCTCCAGATGAG
		(R)/ TCCAAAACGACAGCTTGAGGTT
		(P)/ CTCTCCAAGATGCAGCTGTGAAGGCA
β-actin	VO1217	(F)/ TACTGCCCTGGCTCCTAGCA
		(R)/ GCCAGGATAGAGCCACCATC
		(P)/ AGATCATTGCTCCTCCTGAGCGCAAG

β-actin was used as the internal control. (F), forward primer; (P), FAM labeled probe; (R), reverse primer.

for real-time PCR to confirm the findings of the microarray analysis (**Table 4**). These genes were defensin 3a, 12-lipoxygenase (12-LOX), apolipoprotein E, calcium-binding proteins, adipsin, and erythroid-specific δ-aminolevulinate synthase. The trends in the fold changes were well correlated with the corresponding microarray data (**Table 5**).

Table 6 shows the fold-change ratios of the 1-week-old and 2-week-old groups. Five genes out of seven were less than threefold. These observations might support the

Table 5 Comparison of fold changes in gene expression in developing mandibles of 1-day-old and 2-week-old rats observed using GeneChip array and real-time polymerase chain reaction (PCR)

Gene†	GenBank accession No.	*P*-value	GeneChip	Real-time PCR
Defensin 3a	U50353	0.04974	12.1	19.3
12-lipoxygenase	S69383	0.03993	61.3	48.1
Apolipoprotein E (rApoE)	S76779	0.03294	6.5	4.9
Calcium-binding protein (ICaBP)	K00994	0.03191	12.9	17.4
Calcium-binding protein (MRP14)	L18948	0.01309	13.9	8.9
Adipsin	M92059	0.00243	7.8	4.4
Erythroid-specific δ-aminolevulinate synthase	D86297	0.00849	5.8	6.1

† The expression levels of these genes increased up to fivefold.

Table 6 Comparison of fold changes in gene expression in developing mandibles of 1-week-old and 2-week-old rats observed using GeneChip array

Gene	GenBank accession No.	GeneChip 1 week	GeneChip 2 weeks
Defensin 3a	U50353	6.86	12.1
12-lipoxygenase	S69383	28.69	61.3
apolipoprotein E (rApoE)	S76779	1.28	6.5
Calcium-binding protein (ICaBP)	K00994	2.80	12.9
Calcium-binding protein (MRP14)	L18948	2.04	13.9
Adipsin	M92059	2.47	7.8
Erythroid-specific δ-aminolevulinate synthase	D86297	2.30	5.8

results of the clustering algorithm.

Discussion

Previous studies indicate that the first two weeks of the postnatal period is critical for development of mandible [14–16]. Therefore, we analyzed the gene expression profiles from 1-day-old, 1-week-old, and 2-week-old rat mandibles using DNA microarrays. Welch's *t*-test was also applied, with $P<0.05$ indicating statistical significance between the 1-day-old and 2-week-old mandibles. This approach identified genes whose expression was altered at 2 weeks ($P<0.05$). These genes comprise numerous functional pathways involved in cell adhesion, cell growth and maintenance, signal transduction, immune responses, and enzymes. The list of differentially expressed genes provides an extensive, valuable tool for identifying the genes and pathways that are critically important for mandible development, or potential mechanisms involved in periodontal regeneration.

In this study, 12-LOX mRNA increased significantly, suggesting a potential role of this pathway in mediating mandible development; the 12-LOX pathway plays a key role in the actions of effector molecules, such as angiotensin and platelet-derived growth factor [17]. Angiotensin increased IP_3 and mitogen-activated protein kinase activity through the AT_1 receptor in osteoblasts [18].

MRP14 (S100A9) is the heavy chain of calprotectin, involved in recruiting inflammatory cells through interactions with endothelial cells [19]. In periodontitis, higher levels of calprotectin are found in gingival crevical fluid and tissue specimens [20–22]. The α-defensins, human neutrophil defensins-1, -2, and -3, have been detected in saliva and might be derived from neutrophils. The defensins have broad antimicrobial activity against Gram-negative and Gram-positive bacteria and are effective against oral microorganisms such as *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Actinobacillus actinomycetemcomitans* [23,24]. These results suggest that the inflammatory response is of particular importance in mandible development.

Previous study has shown that a wedge-shaped cartilage, venous lakes, teeth and bone were present in the mandibles from 1-day-old rats [25]. The symphyseal cartilage is undergoing endochondral-like bone formation. One week after birth, the cartilage becomes narrower and elongated to occupy the anterior-posterior space of the anterior segment of the mandible. Two weeks after birth, endochondral-like bone formation is still active in the posterior two thirds of the symphyseal region. In the anterior portion, thick collagenous fibers are demonstrable, forming a meshwork of interweaving fibers in the cartilaginous matrix. At this time, incisor teeth erupt and the cartilaginous plate is sealed off by bone [25]. The differentially expressed genes in our study might reflect the molecular events of these mandible developmental changes.

In conclusion, gene array technology appears to be an effective tool for studying the complex process of development. A more comprehensive analysis will help to define the developmental changes that occur in the mandible.

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