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Original Article

Detection of differentially expressed genes in the early developmental stage of the mouse mandible

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ABSTRACT We previously examined the development of the mouse mandible, and demonstrated that odontogenesis occurs between embryonic day 10.5 (E10.5) and E12. Based on the histological findings, we performed cDNA subtraction between the E10.5 and E12 mandibles to detect any differentially expressed genes which might be involved in the initiation of odontogenesis. By sequencing, homology search and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), we thus found *Pgk-1*, *Ccte*, *Hsp86*, *Nucleolin*, *Hsc73*, *Frg1*, N-*ras*, *Set alpha* and *Hsj2* from the E10.5 mandible, and *E25*, *ATPase6*, *Mum2*, *Thymosin beta4* and *L21* from the E12 mandible to be differentially expressed genes. These genes are functionally related to protein transport, signal transduction, transcription, translation and molecular chaperon activity. *In situ* hybridization analyses of *Set alpha* and *E25* showed that *Set alpha* was detected in the tooth germ at E12 and E14.5, thus indicating a close relationship of this gene to odontogenesis. Meanwhile, the *in situ* signal of *E25* was found in the muscular layer of the tongue, thus suggesting *E25* to be related to the differentiation of muscular tissue. In conclusion, we found 15 differentially expressed genes in the course of the early developmental stage of the mouse mandible using a combination of the cDNA subtraction and semi-quantitative RT-PCR methods, while in addition, two genes were demonstrated to be related to the initiation and the development of both tooth germ and the tongue according to the *in situ* hybridization technique.

KEY WORDS: *cDNA subtraction, semi-quantitative RT-PCR, differentially expressed genes, mandibular development, odontogenesis*

Introduction

In embryogenesis, various organs share many common morphological features and molecular mechanisms, and are regulated by sequential and inductive epithelial-mesenchymal interactions. During the early developmental stage of organogenesis, a thickening of the epithelial tissue is observed in the organ forming area and then the epithelium invaginates into the underlying mesenchyme to form the epithelial bud in association with mesenchymal cell condensation around the bud. Many kinds of molecules including growth factors, transcription factors, components of the cell surface proteins, extracellular matrices, and intracellular molecules have been shown to play important roles in organogenesis (Thesleff *et al.*, 1995).

In previous studies, various genes such as *Bmp4*, *Barx1* (Tucker *et al.*, 1998), *Fgf8*, *Fgf9* (Kettunen and Thesleff, 1998), *Msx1*, *Msx2* (Jowett *et al.*, 1993), *Pax9* (Neubuser *et al.*, 1997) and *Bmp7* (Wang *et al.*, 1999) were expressed in the early developmental stage of the mouse mandible including the predictable tooth germ formation area, thus showing that these genes might be related to the initiation of tooth germ formation. In addition, they are constantly expressed from the initiation of tooth germ formation through the cap stage. Though the constant expression of the genes throughout a specific developmental phase may be reliable evidence for their involvement in the developmental process, there

Abbreviations used in this paper: E10.5, embryonic day 10.5; RT-PCR, reverse transcription-polymerase chain reaction; DEPC, diethylpyrocarbonate; cDNA, DNA complementary to RNA; ds cDNA, double-stranded cDNA; LD, long distance; DIG, digoxigenin; SSC, standard saline citrate; E value, Expect value; EST, expressed sequence tag; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; bp, basepair(s)

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might be alterative and temporal expressions, and on/off switching of other genes that are specifically involved in the initiation of odontogenesis, and therefore play an important role in the regulatory process. Biological events such as cellular growth and organogenesis are mediated by strictly controlled programs of differentially expressed genes (Mally *et al.*, 1994). To understand the molecular regulation of these events, the relevant subsets of differentially expressed genes must be identified, cloned and studied (Diatchenko *et al.*, 1996).

We have already presented the minute histological features of the developing lower first molar tooth germ in the mouse (Shigemura *et al.*, 1999). No remarkable change is observed in the oral epithelial layer and the underlying ectomesenchyme of the predictable first molar tooth germ formation area in the E10.5 mandible. Meanwhile the focal thickening of the oral epithelium corresponding to the predictable first molar tooth germ formation area is recognized in the E12 mandible. The underlying ectomesenchymal tissue does not change in the E12 mandible, either. Based on the histological findings, we performed PCR-based cDNA subtraction using the total RNAs isolated directly from the mouse E10.5 and E12 mandibles to identify the differentially expressed genes, which might be related to the initiation of tooth germ formation as well as those in the other events of the early development of the whole mandible.

Results

Detection of the differentially expressed genes from the E10.5 and E12 mandibles

To detect a differentially expressed gene, we constructed the subtracted cDNA libraries. We picked up 450 and 453 single colonies from the subtracted cDNA libraries of the E10.5 and E12 mandibles, respectively. Among them, 35 positive clones were obtained from the E10.5 mandible by means of colony array screening. In addition, 47 positive clones were also obtained from the E12 mandible. Both sequencing and a homology search showed these clones to contain 25 genes at the E10.5 mandibles and 37 genes at the E12 mandibles which thus were identical to those that appeared in the sequence databases. Seven and 6 uncharacterized genes were identified from the E10.5 and E12 mandibles, respectively.

Semi-quantitative RT-PCR was performed on selected genes to determine whether or not they were differentially expressed. *Pgk-1*, *Ccte*, *Hsp86*, *Nucleolin*, *Hsc73*, *Frg1*, N-*ras*, *Set alpha* and *Hsj2* were more highly expressed in the E10.5 mandible than in the E12 mandible (Fig. 1). In addition, it was confirmed that one uncharacterized gene (subtraction clone no. 15) was more predominantly expressed in the E10.5 mandible than in the E12 mandible (Fig. 1). Regarding the E12 mandible, the expression levels of *E25*, *ATPase6*, *Mum2*, *Thymosin beta4* and *L21* were higher than those in the E10.5 mandible (Fig. 1). The principle characterizations of the differentially expressed genes identified by semi-quantitative RT-PCR are summarized in Table 1. Regarding the other analyzed genes, significant differences between the E10.5 and E12 mandibles could not be verified by the semi-quantitative RT-PCR.

In situ detection of Set alpha and E25 during mandible development

We have tried to examine the *in situ* expression of differentially expressed genes shown in the Figure 1. Among these genes, *Set alpha* and *E25* were found to be clearly expressed in the tissue sections. *Set alpha* was widely expressed in the epithelial and ectomesenchymal cells of the mandible at E10.5 (Fig. 2A). At E12, *Set alpha* was also expressed in the epithelium and the ectomesenchyme. However the expression was restricted in the epithelial and ectomesenchymal cells corresponding to the predictive lower first molar region (Fig. 2B). At E14.5, the expression of *Set alpha* was localized in the outer layer of the odontogenetic epithelium, but no such expression was found in the primary

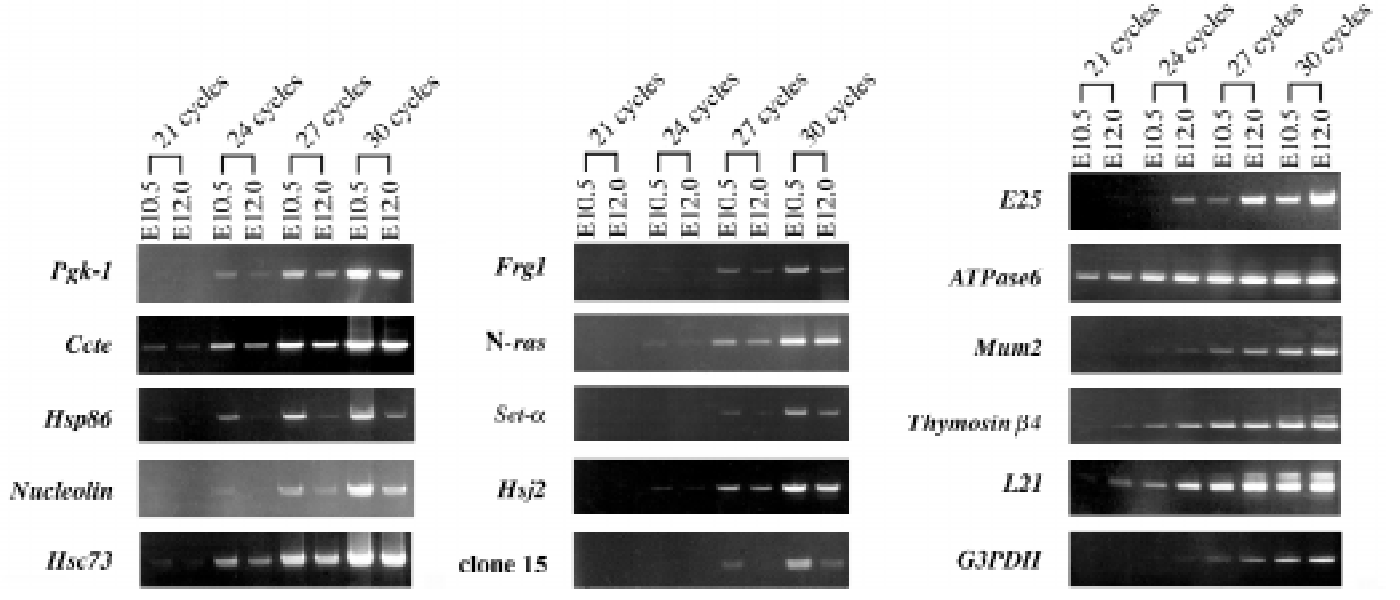

Fig. 1. Representative results of semi-quantitative RT-PCR analysis of the differentially expressed genes from the E10.5 and E12 mandibles. *Five μl of RT-PCR products at different cycles were loaded in each lane of the agarose gel. The gel was stained with ethidium bromide. Pgk-1, Ccte, Hsp86, Nucleolin, Hsc73, Frg1, N-ras, Set alpha, Hsj2 and subtraction clone no. 15 are differentially expressed in the E10.5 mandible. Meanwhile E25, ATPase6, Mum2, Thymosin beta4 and L21 are predominantly expressed in the E12 mandible. The expression level of G3PDH is the same in both E10.5 and E12 mandibles.*

TABLE 1

PRINCIPAL CHARACTERIZATION OF THE DIFFERENTIALLY EXPRESSED GENES

GENE	ASSOCIATED FUNCTION AND PROPERTY
Differentially expressed genes in the E10.5 mandible	
Frg1	hydrophobic molecule transport; candidate gene for facioscapulohumeral muscular dystrophy
N-*ras*	small GTP binding protein; regulation of the transduction of physiological signals from the cell membrane to the nucleus
Nucleolin	major nucleolar phosphoprotein of exponentially growing eukaryotic cell; regulation of ribosome biogenesis and maturation
Set alpha	protein phosphatase 2A inhibitor; chromatin remodeling factor
Ccte	subunit of TCP-1 (tailless-complex polypeptide 1), which is member of the chaperonin family; hetero-oligomeric molecular chaperone mediating protein folding
Hsp86	a part of the *Hsp90* multigene family chaperone protein
Hsc73	a part of the *Hsp70* multigene family chaperone protein; developmental regulation in the murine mammary gland
Hsj2	a part of the DnaJ-like protein family; regulatory factor modulating the chaperone activity of different members of the Hsp70 family
Pgk-1	enzyme in glycolysis; X chromosome-linked gene inactivated by genomic imprinting
Differentially expressed genes in the E12 mandible	
E25	membrane-associated protein chondro-osteogenic differentiation marker
ATPase6	subunit of the mitochondrial inner membrane-spanning F^0 part of ATP synthase mitochondrial encoding gene
Mum2	vesicular transport of proteins from the endoplasmic reticulum to the Golgi
Thymosin beta4	actin monomer binding protein mediation of the dynamics of actin polymerization and depolymerization
L21	ribosomal protein

enamel knot in the tooth germ. *In situ* signal was also weakly found in ectomesenchymal cells in the dental papilla (Fig. 2C).

E25 was also expressed in the ectomesenchyme in the E10.5 and E12 mandibles. However, no signal was found in the predictive lower first molar region (Fig. 2 D,E). Instead, *E25* expressing cells, especially at E12, were often detected in the central area of the mandible which corresponded to the lateral lingual swelling that is a part of the tongue primordium (Fig. 2E). At E14.5, the *in situ* signal of *E25* was expressed in the muscular layer of the tongue (Fig. 2F).

No hybridization signals were detected using the control sense probes at any of the investigated developmental stages (data not shown).

Discussion

Various genes such as *Bmp4*, *Bmp7*, *Barx1*, *Fgf8*, *Fgf9*, *Msx1*, *Msx2* and *Pax9* have been examined for their involvement in the early developmental stages of odontogenesis. However, almost all these genes are members of families and/or homologs of the genes which have already been isolated and well characterized in other organs and species such as the kidney, limb, *Drosophila melanogaster* and *Caenorhabditis elegans*. Therefore, such genes are expected to be involved in mouse odontogenesis as well as the morphogenesis of others organs. During the screening of the subtracted cDNA libraries of the E10.5 and E12 mandibles, we could not detect such genes as *Bmp4*, *Bmp7*, *Barx1*, *Fgf8*, *Fgf9*, *Msx1*, *Msx2* and *Pax9*. The reason for this is that these genes are constantly expressed, or their expression levels may not be so different between the E10.5 and E12 mandibles to allow them to be detected by the cDNA subtraction method. We, however, tried to isolate the potential genes involved in the initiation of tooth germ formation directly from the mouse embryonic mandible, just focusing on the differential expressions of genes.

In the early developmental stage of the mouse mandible, it is hard to isolate the tooth germ formation area from the whole mandible. Even if it could be done, the amount of total RNA from the area would not be enough for cDNA subtraction and the subsequent experiments. Since the E10.5 mandible is not histologically different from the E12 mandible except for the thickening of the oral epithelium in the predictable tooth germ formation area and the simple increase in the whole mandible size, it seems reasonable to suppose that we isolated the genes related to the initiation of tooth germ formation by subtraction between the E10.5 and E12 whole mandible-derived cDNAs. The subtraction method used here is a powerful approach to identify and isolate cDNAs of differentially expressed genes, while it also allows us to detect both high- and low-abundance transcripts by introducing the equalization of concentrations (normalization) of specific transcriptions during the subtraction process. By incorporating PCR into the subtraction procedure, the sensitivity of the subtraction is increased and it is also possible to use a small amount of RNA (Gurskaya *et al.*, 1996; Diatchenko *et al.*, 1996; Diatchenko *et al.*, 1999). Using this method, the targeted genes, including low expressing transcriptions, can all be detected efficiently.

For the purpose of recognizing that the detected genes are differentially expressed in each mandible, we performed semi-quantitative RT-PCR. The semi-quantitative RT-PCR provides a rapid method to estimate the difference in the amounts of a message in various RNA populations with only a trace of RNA. Moreover, it is much more sensitive to detect a low quantity of transcribed mRNA than the traditional methods such as Northern blotting and a ribonuclease protection assay (Santagati *et al.*, 1997). Using semi-quantitative RT-PCR, we recognized 10 cloned genes including an uncharacterized one from the E10.5 mandibles and 5 cloned genes from the E12 mandibles as the differentially expressed genes. However, several genes could not be recognized by the assay used here, because a two- or three-fold difference in the amount of a target RNA between two samples may not be accurately determined by semi-quantitative RT-PCR (Santagati *et al.*, 1997). As for those which could not be confirmed to be differentially expressed genes, a further analysis is necessary using another method such as competitive RT-PCR and the use of radioactive or fluorescence-labeled nucleotide.

We examined the *in situ* expression of the genes shown in Fig. 1. Among these genes, both *Set alpha* and *E25* were clearly expressed in frozen sections. The expression of *Set alpha* was detected in the epithelial and ectomesenchymal cells of the predictable lower first molar region at E10.5 and E12. Interestingly, the expression was localized in the outer layer of the odontogenetic epithelium of the tooth germ at E14.5. We previously reported the minute distribution pattern of BrdU- and TUNEL-positive cells in the developing mouse lower first molar (Shigemura *et al.*, 1999). The *in situ* expression pattern of *Set alpha* in the odontogenic epithelial cells corresponded with the localization of BrdU-positive cells. Furthermore we also previously reported that no BrdU-positive cells were found in the

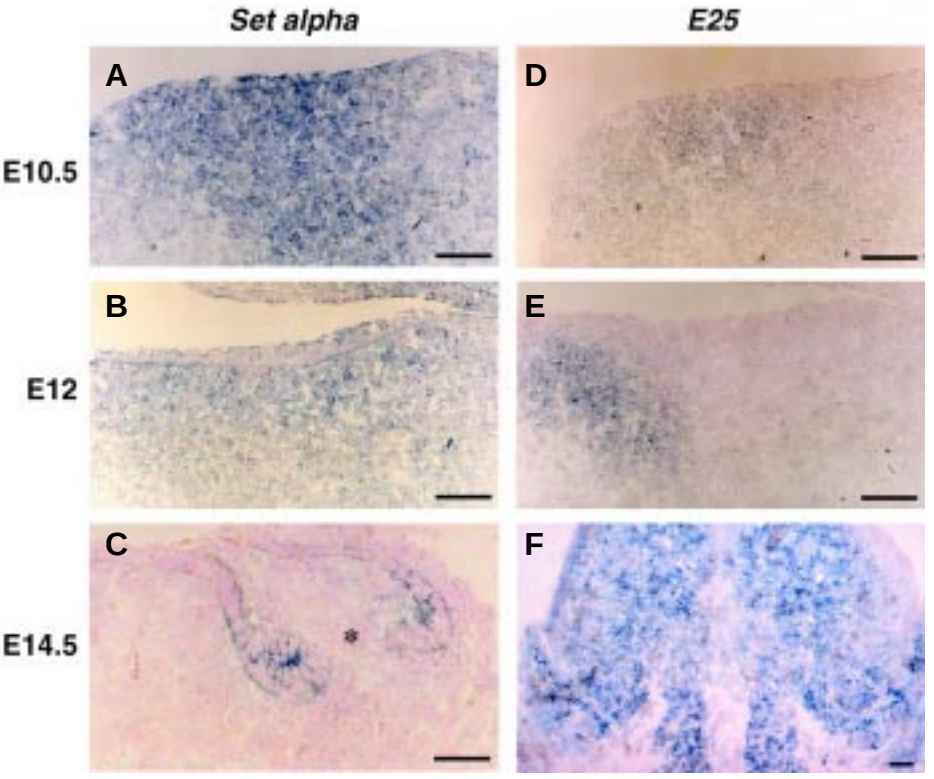

Fig. 2. The expression pattern of *Set alpha* (A,B,C) and *E25* (D,E,F) in the developing mouse mandible by *in situ* hybridization. (A) *Set alpha is widely expressed in the epithelial and ectomesenchymal cells of the mandible at E10.5.* **(B)** *At E12, Set alpha is also expressed in the epithelium and the ectomesenchyme. However, the expression of Set alpha is restricted in the epithelial and ectomesenchymal cells corresponding to the predictable lower first molar region.* **(C)** *At E14.5, the expression of Set alpha is localized in the outer layer of the odontogenetic epithelium, but no such expression is found in the primary enamel knot (indicated by asterisk).* **(D,E)** *E25 is also expressed in the mesenchyme in the E10.5 and E12 mandibles, but not in the predictable first molar tooth germ . At E12, the in situ signals of E25 are found in the lateral lingual swelling.* **(F)** *At E14.5, E25 is predominantly expressed in the developing skeletal muscle of the tongue . Bar, 50 μm.*

primary enamel knot. In the present study, we did not detect the *in situ* signal of *Set alpha* in the primary enamel knot. These findings of both our present and previous studies suggest that the expression of *Set alpha* is related to cell proliferation, and therefore is involved in the initiation and the morphogenesis of tooth germ because cell proliferation is the essential biological event of morphogenesis.

E25 was also expressed in the mesenchymal cells in both E10.5 and E12 mandibles. However, the expression pattern differed from that of *Set alpha*, and tended to be localized in the central region of the mandible which corresponded to the lateral lingual swelling. At E14.5, the gene was expressed in the muscular layer of the tongue. These findings therefore suggest that *E25* is related to the differentiation of mesenchymal cells to striated muscle cells. No *in situ* signals of *E25* were found in the tooth germ at E14.5, and therefore this gene does not seem to be involved in the initiation or development of tooth germ formation.

Regarding the other genes listed in Table 1, no reliable data on *in situ* expression could be obtained because the probes designed for those genes did not work well, thus resulting in either no signal or nonspecific hybridization. Redesigning the probes and further optimizing the parameters for their *in situ* hybridization are presently underway in our laboratory.

In this study, we focused on the differential expression of the genes to detect the genes involved in the initiation and morphogenesis of the tooth and other parts of the embryonic mouse mandible. As a result, we obtained 15 differentially expressed genes including several uncharacterized genes whose information is absent in the sequence databases. Among these genes, we were able to demonstrate the *in situ* expression of *Set alpha* and *E25*, while also showing that they might be related to the early morphogenesis of tooth germ and the tongue, respectively. Although *in situ* hybridization analyses of other differentially expressed genes are now in progress, some of these differentially expressed genes, except for *Set alpha* and *E25*, might be also involved in the initiation and/or early morphogenetic events in the mouse embryonic mandible.

Materials and Methods

Animals

BALB/c mice were obtained from the Animal Center of Kyushu University (Fukuoka, Japan). Female BALB/c mice (10~30 weeks) were caged together with male mice overnight. After 12 hours, the insemination was judged based on the presence of a postcopulatory plug in the vagina. The embryonic day was defined as E0.5 after the plug was recognized. The embryos were removed from the parent mice on days under anesthesia. The experimental procedures were performed according with the guidelines of the Animal Center of Kyushu University.

RNA isolation

The embryos were immersed in diethylpyrocarbonate (DEPC)-treated phosphate-buffer saline (PBS, pH 7.4) in a 30 mm-culture dish placed on the deeply cooled metal plate to avoid any damage to the RNAs. The mandibles were removed from the E10.5 and E12 embryos using 27 Gauge needles under a stereomicroscope. They were then transferred into tubes containing guanidine thiocyanate-based lysis buffer (SV Total RNA Isolation System, Promega, Madison, WI), and thereafter were homogenized rapidly by grinding with a tight-fitting pestle. Total RNAs were isolated from the mandibles according to the instructions of the SV Total RNA Isolation System. The pools of the total RNAs from each population were stored at -80°C until use.

Subtraction cloning of differentially expressed genes in the E10.5 and E12 mandibles

The following experiments were performed using a SMART™ PCR cDNA Synthesis Kit (Clontech Laboratories, Inc., Palo Alto, CA), CLONTECH PCR-Select™ cDNA Subtraction Kit (Clontech) and CLONEAMP® pAMP1 System (GibcoBRL, Gaithersburg, MD).

Briefly, 1 µg of total RNA was converted into first-stranded cDNA using SuperScript II Reverse Transcriptase (GibcoBRL). The double-stranded cDNA (ds cDNA) was then synthesized by long-distance (LD) PCR amplification using Advantage® cDNA Polymerase Mix (Clontech). The synthesized ds cDNAs were purified with CHROMA-SPIN™-1000 column supplied by the SMART™ PCR cDNA Synthesis Kit.

For the subtraction cloning of differentially expressed genes in the E10.5 and E12 mandibles, the purified ds cDNA from each population was digested with *Rsa*I to convert into short and blunt-ended ds cDNAs. One of the two populations was named "tester" cDNA, and the other as "driver" cDNA (CLONTECH PCR-Select™ cDNA Subtraction Kit User Manual, PT1117-1, Clontech) (Gurskaya *et al.*, 1996; Diatchenko *et al.*, 1996; Diatchenko *et al.*, 1999). The tester cDNA was subdivided into two portions and then ligated to a different adaptor (adaptor 1 or adaptor 2R), respectively. After the first and second hybridizations which were performed according to the manufacturer's instructions, the subtracted tester cDNAs with adaptors were exclusively amplified by first and second PCR using the Advantage® cDNA Polymerase Mix (Clontech) and the modified nested primers designed for the CLONEAMP® pAMP1 System (GibcoBRL). The PCR products were purified using NucleoTrap® DNA Purification kits (Clontech), and then were cloned into the pAMP1 vector to construct the subtracted cDNA libraries.

Differential screening of the subtracted cDNA libraries of E10.5 and E12 mandibles

Colony array screening was performed based on the protocol of the PCR-Select differential Screening Kit (Clontech), substituting digoxigenin (DIG)-labeled probes for ^{32}P-labeled ones. Four kinds of probes were prepared using "forward-subtracted", "reverse-subtracted", and "unsubtracted" E10.5 and E12 cDNAs as a template, respectively, according to the User Manual of the PCR-Select differential Screening Kit (PT3138-1, Clontech) and the instructions of the DIG High Prime DNA Labeling and Detection Stater Kit II (Roche Molecular Biochemicals, Mannheim, Germany).

Each single colony from the subtracted cDNA libraries was cultured in 200 µl of 2 X YT medium at 37°C for 12 hours, and then was divided into four groups and transferred onto four different pieces of nylon membranes (Hybond N+, Amersham Pharmacia Biotech, Cleveland, OH) corresponding to the appropriate category of the probes. After incubation at 37°C for 12 hours, each bacterial genomic DNA including a portion of cDNA was fixed to the membranes by baking at 80°C for 2 hours, and then was hybridized in Church buffer (Church and Gilbert, 1984) containing one of the four DIG-labeled probes at 68°C for 16 hours. After washing with SSC-based buffers, the hybridized probe was detected using the DIG High Prime DNA Labeling and Detection Stater Kit II (Roche Molecular Biochemicals).

Our criteria for selecting a "positive" clone, which means a differentially expressed gene in each population, were detrmined according to the User Manual of the PCR-Select differential Screening Kit (PT3138-1, Clontech).

DNA sequencing and homology search

The plasmids from the screened positive clones were prepared using QIAprep Spin Miniprep Kits (QIAGEN, Hilden, Germany). Their DNA sequences were then determined with an ABI autosequencer model 373 (Perkin-Elmer, Norwalk, CT) by the dideoxynucleotide chain-termination method using Thermo Sequnase II dye terminator cycle sequencing premix Kit (Amersham Pharmacia Biotech)

A nucleic acid homology search was performed using the blastn in the BLAST 2.0.10 program (BLAST service at National Center for Biotechnology Information© NCBI, National Library of Medicine, Bethesda, MD) (Altschul *et al.*, 1997). We evaluated the BLAST report based on the Expect value (E value). When any candidate gene comes out with an E value of < 0.001, we accept it as a gene identical to our cloned gene and ignore the genes with an E value of ≥ 0.001. If the BLAST report does not include any gene with an E value of < 0.001, we then consider such a gene to be an "uncharacterized gene", which has not been submitted to any of the sequence databases yet.

EST sequence analysis

Regarding the several genes whose information on mouse homolog was absent in the sequence databases at the NCBI, the mouse expressed sequence tag (EST) database was screened using the mouse ests in the BLAST 2.0.10 program and the Mus musculus UniGene database (NCBI) (Wheeler *et al.*, 2000) to design the mouse-specific primers of each gene. An alignment of the EST clones was carried out using MACVECTOR™ (version 6.5, Oxford Molecular Ltd., Oxford, UK).

Semi-quantitative RT-PCR analysis

A semi-quantitative RT-PCR analysis was performed to confirm that each selected gene was differentially expressed between the E10.5 and E12 mandibles. As a template, we used the same cDNAs which had been used for the construction of the subtracted cDNA libraries. We first carried out PCR amplification on one of the most popular "housekeeping genes", glyceraldehyde-3-phosphate dehydrogenase (G3PDH), at 21, 24, 27 and 30 cycles using the specific primers for G3PDH gene:
(5'-ACC ACA GTC CAT GCC ATC AC-3' and
5'-TCC ACC ACC CTG TTG CTG T A-3', Clontech) and thus confirmed the expression level of the G3PDH gene in the E10.5 mandible to be

TABLE 2

MACVECTOR DESIGNED PRIMERS USED IN PCR

Gene	Forward Primer	Reverse Primer
Pgk-1	5'-ATG TCG CTT TCC AAC AAG-3'	5'-CTA AAC ATT GCT GAG AGC ATC C-3'
Ccte	5'-ATG GCG TCC GTG GGG ACC CTC-3'	5'-TTA TTC TTC AGA TTC TCC AGG-3'
Hsp86	5'-ATG CCT GAG GAA ACC CAG ACC CAA-3'	5'-TTA GTC TAC TTC TTC CAT GC-3'
Nucleolin	5'-ATG GTG AAG CTC GCA AAG G-3'	5'-GGA AAG AAT GGG ATG GAA GG-3'
Hsc73	5'-ATG TCT AAG GGA CCT GCA GTT G-3'	5'-CCC TGT GGA ACA AAG CTA CAC C-3'
CAP-E	5'-ATG TAT GTT AAA TCA ATC ATT C-3'	5'-TCA TAC ATC CTG GTT CCA GC-3'
SMARCA5	5'-AGG TTG GAT GGA CAG ACA CC-3'	5'-GCA TCA ACA GCA TAG TTG GC-3'
Frg1	5'-ATG GCC GAA TAT TCC TAT GT-3'	5'-TCA CTT GCA GTA TCG GTC AGC TTT C-3'
N-*ras*	5'-ATG ACT GAG TAC AAA CTG GTG GTG G-3'	5'-TCA AAG TGT CTT ACA CAT CAG CAC-3'
Copg1	5'-ATG TTG AAG AAA TTC GAC AAG AAG GAC-3'	5'-CTA GCC CAC GGA TGC CAA GAT G-3'
Set alpha	5'-ATG GCC CCG AAG CGG CAA TCT G-3'	5'-TGA GAC CTC AAA AAC AGG GCG-3'
Set beta	5'-ATG TCT GCG CCG ACG GCC AAA G-3'	5'-TGA GAC CTC AAA AAC AGG GCG-3'
Ptp	5'-ATG TCG GCA ACC ATC GAG-3'	5'-TTA GGT GTC TGT CAA TCT TGG C-3'
SPF45	5'-ATG TCC CTA TAT GAT GAC CTG-3'	5'-TCA AAC TTG CTC TGC TAG ATC C-3'
Hsj2	5'-ATG GTG AAA GAA ACC ACT TAC-3'	5'-ATG GTG AAA GAA ACC ACT TAC-3'
clone 15	5'-CAG AAC TGT GCT GAG TAG TGC CTG G-3'	5'-GCC CAC TAA CAC TCC AGA AGC GAG-3'
E25	5'-ATG GTG AAG ATC GCC TTC AAC-3'	5'-ATG GTG AAG ATC GCC TTC AAC-3'
Gamma actin	5'-ATG GAA GAA GAA ATC GCC GCA CTC-3'	5'-CTA GAA GCA TTT GCG GTG GAC G-3'
Tcof1	5'-GCA GAG GAT GAT GAG ACC CTT CAG-3'	5'-GCA GAG GAT GAT GAG ACC CTT CAG-3'
Oscp	5'-ATG GCC GCG CCT GCA GCG TCC G-3'	5'-TCA GAG CAT CTC CCG CAT GGC CTT GC-3'
16S rRNA	5'-GCA GCC ACC AAT AAA GAA AGC G-3'	5'-TCT CCG AGG TCA CCC CAA CC-3'
Ub11	5'-ATG TCT GAC CAG GAG GCA AAA C-3'	5'-ATG TCT GAC CAG GAG GCA AAA C-3'
ATPase6	5'-ATG AAC GAA AAT CTA TTT GC-3'	5'-TTA TGT ATT ATC ATG TAG ATA TAG GC-3'
Mum2	5'-CCG ATG ACT GTC CAC AAT CTG-3'	5'-TCA GCC AGC TCT GGC AGA GAA G-3'
OSF-3	5'-ATG TCT TCA GGA AAT GCA AAA ATT G-3'	5'-GGC AGA AAA ATG GTC CAG TGC-3'
LSm3	5'-ATG GCG GAC GAC GTA GAT CAG C-3'	5'-TCA GCC AAC TCT CAA TGG AGG G-3'
Hsp84	5'-ATG CCT GAG GAA GTG CAC CAT GGC-3'	5'-ACC CAA CCC TGC TAT TCT GTG G-3'
AOE372	5'-ATG GAG GCG CGG TCC AAG-3'	5'-TCA GTT TAG CTT GTC GAA ATA C-3'
Thymosin beta4	5'-ATG TCT GAC AAA CCC GAT ATG-3'	5'-CTC TCT ATT TCA TCA TCT CCC-3'
L21	5'-ATG ACG AAC ACA AAG GGA AAG AG-3'	5'-TTA GGC CAT GAA TTC GTA TGG-3'
SDF-1 beta	5'-ATG GAC GCC AAG GTC GTC GC-3'	5'-GGC TTT TCC TTC TTG CTG TCC C-3'
Nedd-4	5'-ATG AGT GGA ATC CTT ACC AGC G-3'	5'-CTA ATC AAC GCC ATC AAA GCC C-3'
H(0)1	5'-ATG ACC GAG AAC TCC ACC TCC G-3'	5'-AGG AAG GAG TGT CCC CAA GC-3'
UBcM4	5'-ATG GCG GCC AGC AGG AGG CTG ATG-3'	5'-GGC AGA TTT TAG TCC ACA GGT CG-3'
L26	5'-ATG AAG TTC AAT CCC TTC GTG AC-3'	5'-TGC ATG GGA TGT CTC TAC TC-3'
S27-1	5'-ATG CCT CTC GCA AAG GAT CTC-3'	5'-CAA TCA GGG GCT TTC AGT GC-3'
H19	5'-ATG GGA TCC AGC AAG AAC AG-3'	5'-AGA GCA GCA GAG AAG TGT TAG-3'

equivalent to that in the E12 mandible (Fig. 1). We then proceeded to analyze several selected genes at 18, 21, 24, 27 and 30 cycles. The specific primer pairs for each gene were designed with MACVECTOR™ and are illustrated in Table 2. To verify the differential expression of each selected gene, we compared the brightness of each band at every cycle on the 1.2% agarose gel, especially focusing on the PCR products amplified at the cycles within the exponential phase of the PCR amplification for each template.

In situ hybridization

The embryos at 10.5, 12, 12.5, 13.5, 14.5, 15.5, 16.5 and 18 days of age (E10.5-E18) after gestation were isolated and fixed in 4% paraformaldehyde in diethylpyrocarbonate (DEPC)-treated PBS (pH 7.4) for 12 h at 4°C, and the embedded in OCT compound (Sakura Finetechnical Co. Ltd, Tokyo Japan). Serial cryosections, 8 μm thick, were mounted on silane-coated glass slides and dried.

The murine *Set alpha* cDNA (1006 bp) and the murine *E25* cDNA (291 bp) were subcloned into the pGEM-3Z (Promega) to synthesize both antisense and sense probes labeled with DIG-UTP. The sections were fixed with 4 % paraformaldehyde in DEPC-treated PBS (pH 7.4) for 10 min, and then were treated with 20 μg/ml proteinase K for 2 min and 0.25% (vol/vol) acetic anhydride for 10 min at room temperature to reduce the background signals. Hybridization was carried out overnight in a humidified chamber at 55°C. The sections were then washed twice in 2X SSC containing 50 % formamide for 30 min at 65°C and treated with 20 μg/ml RNase A for 30 min at 37°C to remove any nonspecifically bound probe. These slides were washed twice in 2X SSC containing 50 % formamide for 20 min at 65°C, followed by a further wash for 15 min in 2X SSC and 0.1X SSC at 37°C. They were washed in PBST (PBS + 0.1% Tween 20) for 15 min at room temperature and treated with 10% normal goat serum for 1 h at room temperature. The DIG-labeled probes were detected with alkaline phosphatase-conjugated anti-DIG antibodies using BM purple (Roche Molecular Biochemicals) as the color substrate. After undergoing the reaction, the sections were briefly counterstained with nuclear fast red.

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