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*Lef*1 may contribute to agenesis of the third molars in mice

Takehiko Shimizu[1,2], Eri Yokoi[1], Takahiro Ichinosawa[1], Yuri Kiguchi[1], Fusae Ishida[1], Takahide Maeda[1,2]

[1]Department of Pediatric Dentistry, Nihon University School of Dentistry at Matsudo, Chiba, Japan
[2]Nihon University Research Institute of Oral Science, Chiba, Japan
Email: shimizu.takehiko@nihon-u.ac.jp

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ABSTRACT

Tooth agenesis is the most common developmental anomaly of the human dentition. Epilepsy-like disorder (EL) mice, which have a 100% incidence of agenesis of the third molars, may be a good model for the genetic study of human tooth agenesis. Our previous congenic breeding strategy using EL mice confined a major locus for agenesis of M3, designated *am*3, within an approximately 1 Mega base pair (Mbp) interval on chromosome 3, which contains five known genes; *Lef*1, *Hadh*, *Cyp*2*u*1, *Sgms*2 and *Papss*1. The aim of this study was to identify the strongest candidate for *am*3 among the five genes using real-time PCR analysis. The tooth germs of M3 in the bud stage of EL and control mice were dissected out, and total RNA was extracted. In real-time PCR analysis, a significantly low level of expression of *Lef*1, which is one of the essential transcription factors for early tooth development, was observed in M3 of EL mice. In addition, a significantly low level of expression of *Fgf*4, which is a direct transcriptional target for LEF1 in early tooth development, was observed in M3 of EL mice. Our results suggest that the cause of M3 agenesis of EL mice may be a low level of *Lef*1 expression in M3 in the bud stage of EL mice.

Keywords: Hypodontia; Gene Expression; EL Mice

1. INTRODUCTION

Tooth agenesis, the congenital absence of one or more teeth, is one of the most common craniofacial anomalies in humans. Its prevalence varies from 1.6% to 9.6% when the third molars are not considered [1]. The most frequently missing teeth are the third molars, which are absent in around 20% of the population, followed by the mandibular second premolars and maxillary lateral incisors [2]. It appears as both syndromic and non-syndromic/isolated features. The genes *SHH* [3], *PITX*2 [4], *IRF*6 [5] and *p*63 [6] have been associated with syndromic tooth agenesis. Mutations in *MSX*1 [7], *PAX*9 [8], *AXIN*2 [9], *WNT*10*A* [10] and the ectodermal dysplasia genes *EDA* [11], *EDAR* [12] and *EDARADD* [13] have been associated with non-syndromic tooth agenesis. However, in the majority of cases of tooth agenesis, the causes remain unknown [14-18], implying that other genes must be involved.

Inbred mice with homozygous alleles and a high degree of homology with human genes are frequently used in studies seeking to identify disease loci. Mice have a short life cycle that enables easy transgenerational observation and can be reared under the same conditions so that environmental factors can be kept almost constant, making them useful for genetic research. The normal mouse dentition is composed of one continuously growing incisor and three molars of limited growth in each quadrant. Congenital tooth agenesis is rarely observed in inbred mouse strains. However, the epilepsy-like disorder (EL) mouse, which was developed as an animal model for the study of epilepsy [19], has a 100% incidence of absence of M3 without any generalized craniofacial anomalies [20]. EL mice therefore may be a good model for the genetic study of agenesis of the third molar or other types of tooth agenesis in humans. Our previous genome-wide scan using F_2 progeny from intercrosses between EL mice and the wild-type mice identified that alleles on chromosome 3 contribute to M3 agenesis in EL mice, suggesting independence from alleles of epilepsy [21]. Previous genes causing isolated tooth agenesis; *Msx*1, *Pax*9, *Axin*2, *Wnt*10*a*, *Eda*, *Edar* and *Edaradd* were clearly ruled out from the candidates for M3 agenesis in EL mice [22].

Recently, a major locus for agenesis of M3 (allele symbol *am*3) was defined in approximately the 1 Mbp region of chromosome 3 using a congenic breeding strategy [23]. The region contained five known genes; *Lef*1,

Hadh, *Cyp2u*1, *Sgms*2 and *Papss*1. The purpose of this study was to identify the strongest candidate for *am*3 among the five genes by gene expression analysis.

2. MATERIALS AND METHODS

2.1. Mice

EL mice were purchased from the Laboratory Animal Resource Bank at National Institute of Biomedical Innovation (Osaka, Japan) and MSM/Msf mice, a wild-type strain derived from *Mus musculus molossinus* progenitors, were obtained from the animal facilities at the National Institute of Genetics (Mishima, Japan). All animals were maintained and used in accordance with the guidelines of the Nihon University Intramural Animal Use. The experimental protocol was approved by the Institutional Animal Experiment Committee (No. AP11 MD029).

2.2. Microdissection of Tooth Germ of M3 and Total RNA Isolation

Ten EL mice and ten MSM mice were sacrificed under anesthesia on postnatal Day 3 (P3) when the tooth germ of M3 is in the bud stage. The heads were immediately embedded in Tissue-Tek OCT compound (Sakura Fineteck Japan, Tokyo, Japan). Serial sections of 30 μm thickness were prepared using a Leica cryostat (Leica Microsystems, Wetzlar, Germany). Sections were dehydrated in 70% ethanol for 15 sec and stained with 0.25% toluidine blue for 15 sec. The upper and lower tooth germs of M3 were dissected out using a needle under a dissection microscope, avoiding the tissues surrounding the tooth follicle (**Figure 1**). A total of 40 M3s from each strain were collected and stored in RNAlater™ RNA Stabilization Reagent (Qiagen, Tokyo, Japan) and total RNA from sections was isolated using an RNeasy Total RNA kit (Qiagen, Tokyo, Japan), in accordance with the manufacturer's instructions. The quality and integrity of the RNA were checked by means of spectrophotometry and agarose-gel electrophoresis.

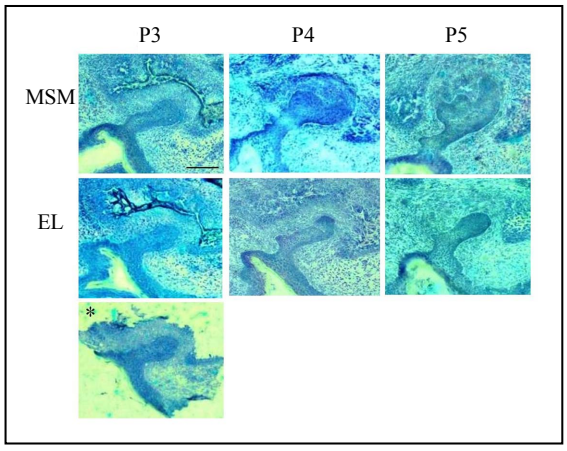

Figure 1. The third molar (M3) in EL and MSM/ms (MSM) at postnatal 3-day (P3), P4 and P5 (frontal section). *M3s were dissected out and collected from the sections for total RNA extraction. Bar indicates 100 μm.

2.3. Reverse-Transcriptase Polymerase Chain-Reaction (RT-PCR)

Total RNA (100 ng) from tooth germs of M3 of EL mice and MSM mice was reverse-transcribed with the PrimeScript® High Fidelity RT-PCR kit (Takara, Tokyo, Japan). The reverse-transcribed cDNA was amplified with nine pairs for the nine genes of the PCR primers listed in **Table 1**. The 130.73 - 131.69 Mbp region on chromosome 3 for *am*3 defined in EL congenic mice [23] contains five known genes; *Lef*1, *Hadh*, *Cyp2u*1, *Sgms*2 and *Papss*1, according to the information from Ensembl (http://asia.ensembl.org/index.html). In addition to these five genes, *Wnt*10*a*, which is upstream signaling molecule for *Lef*1 in early tooth development, and *Fgf*4 and *Fgf*3, which are downstream signaling molecules for *Lef*1 [24], were included in the analysis. *Glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) was used as control. cDNAs were amplified by 30 cycles at 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec in the GeneAmp® PCR System 9700 (Applied Biosystems) and were analyzed by agarose gel electrophoresis. Products spanned at least one intron, so that cDNA products could be distinguished from potential genomic DNA products. The presence/absence and intensity of the PCR products for each gene were compared using the KODAK Molecular Imaging System (Kodak).

2.4. Quantitative Real-Time PCR

PCR amplification of cDNA was performed using Thermo

Table 1. Primer sets for RT-PCR and real-time PCR.

Gene	Forward/ Reverse Primer (5'-3')	Size (bp)
*Lef*1	aaggcgatccccagaaggag	
	agggtgttctctggccttgt	190
Hadh	ttgcgctccatgtcctcctc	
	gactctcctcaattcccttc	194
*Cyp2u*1	ggctgaagtgttcagtgacc	
	cgtatgcaaactcctcgatg	188
*Sgms*2	tcgaccgggtcaaatgggca	
	gcattccgggcacaggtaac	190
*Papss*1	gggatgcagagagcaaccaa	
	gtgtagcacggaatgccgtg	194
*Wnt*10*a*	tggagactcggaacaaagtc	
	agcttccgacggaaagcttc	193
*Fgf*4	gctgggcctcaaaaggcttc	
	ctgctcatggccacgaagaa	192
*Fgf*3	ctggccatgaacaagagagg	
	tgccattcaccgacacgtac	190
Gapdh	ggaagcccatcaccatcttc	
	cgtggttcacacccatcaca	203

Scientific DyNAmo SYBR Green qPCR kits (Thermo Fisher Scientific, Kanagawa, Japan). The primers for real-time RCR were the same as those used for RT-PCR. The PCR program was as follows: initial denaturation at 95˚C for 5 min, followed by 40 cycles at 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec in the DNA Engine OPTICON® Continuous Fluorescence Detector (BioRad). Gene expression levels were normalized according to the level of *Gapdh* expression. Relative amounts of *Gapdh* mRNA in each sample were calculated from standard curves obtained by sequential dilution of total RNA prepared from M3 at P3. We used the Mann-Whitney test to compare the expression levels of five experiments in EL and MSM mice.

3. RESULT

Significant decreases in the quantity of *Lef*1 mRNA in M3 of EL mice compared to that of MSM mice were detected in both RT-PCR and real-time PCR analysis (**Figure 2**). Significantly lower level of expression of *Fgf*4 and *Fgf*3 from M3 of EL mice than that of MSM mice was detected in both RT-PCR and real-time PCR analysis (**Figure 3**).

4. DISCUSSION

In a previous study, to determine the location of the *am*3 locus *in vivo*, we produced EL congenic strains for *am*3 in which the restricted interval on chromosome 3 of EL mice was replaced by a wild-type-derived homologue. The congenic mice that were either heterozygous or homozygous for the wild-type-derived interval exhibited a significant decrease in the incidence of M3 agenesis. The results confined the *am*3 locus to an approximately 1 Mbp region flanked by *AC*114668.1 at 130.73 Mbp and *Dkk*2 at 131.69 Mbp on chromosome 3, demonstrating the five candidate genes for *am*3; *Lef*1, *Hadh*, *Cyp*2*u*1, *Sgms*2 and *Papss*1 based on Ensembl information [23].

*Lef*1 is a cell-type-specific transcription factor that participates in the Wnt signaling pathway and *Lef*1 has a critical role in regulating tooth morphogenesis [25,26]. *Lef*$^{-/-}$ mouse embryos exhibited that the absence of LEF1 resulted in complete lack of tooth development [24]. LEF1 directly regulates *Fgf*4 gene expression, and FGF4 regulates the expression of *Fgf*3 in the dental mesenchyme to mediate the critical epithelial-mesenchymal interaction [27]. In the present study, significant decreases in the quantity of *Lef*1 mRNA in M3 of EL mice compared to that of MSM mice were found. In addition, a significantly lower level of expression of *Fgf*4 and *Fgf*3 from M3 of EL mice than that of MSM mice was detected, while there was no difference between EL and MSM mice in expression of *Wnt*10*a*, which is a direct upstream signaling molecule for *Lef*1 in the bud stage.

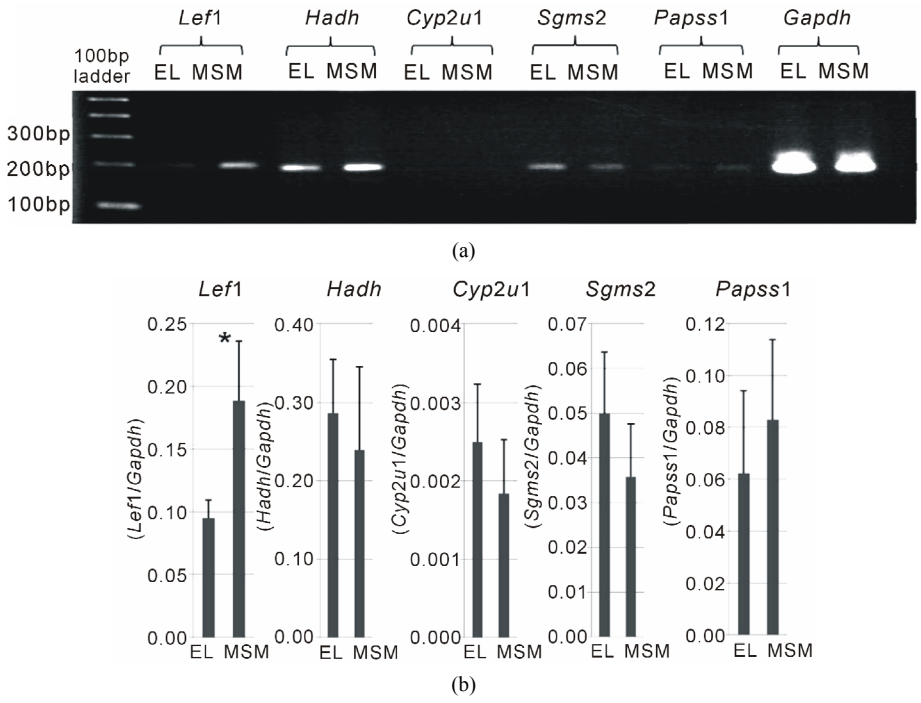

Figure 2. mRNA expression of *Lef*1, *Hadh*, *Cyp*2*u*1, *Sgms*2 and *Papss*1 in the third molar (M3) in the bud stage at postnatal 3-day. (a) RT-PCR analysis; (b) Real-time PCR analysis. The relative amounts of *Lef*1, *Hadh*, *Cyp*2*u*1, *Sgms*2 and *Papss*1 mRNA were divided by that of *Gapdh*. Results are expressed as the means of five experiments ±SD. Significantly lower level of expression of *Lef*1 from M3 of EL mice than that of MSM mice was detected. $^{*}p < 0.05$.

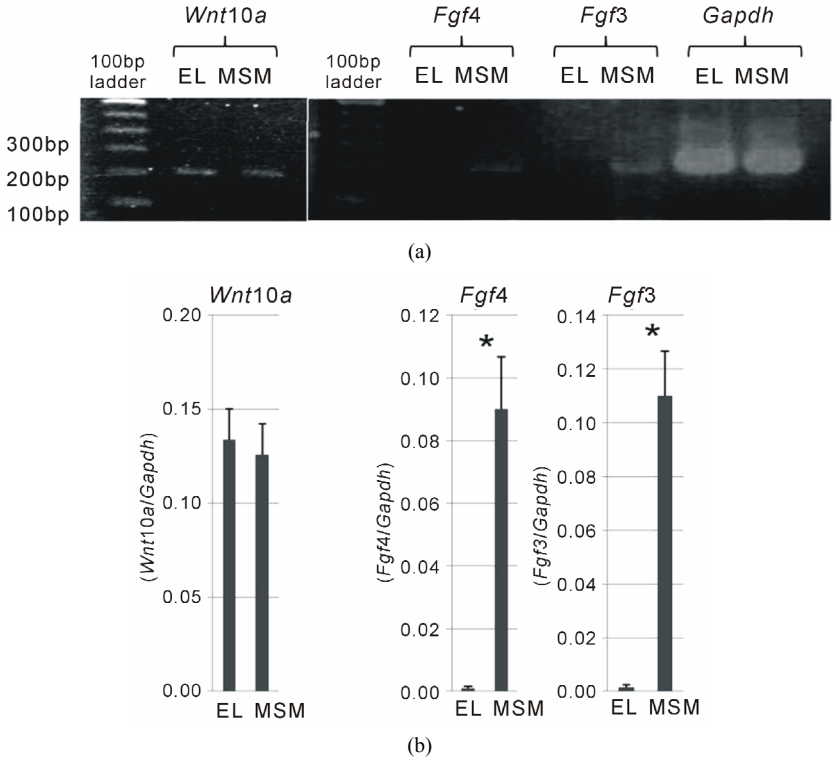

Figure 3. mRNA expression of *Wnt*10*a*, *Fgf*4 and *Fgf*3 in the third molar (M3) in the bud stage at postnatal 3-day. (a) RT-PCR analysis; (b) Real-time PCR analysis. The relative amounts of *Wnt*10*a*, *Fgf*4 and *Fgf*3 mRNA were divided by that of *Gapdh*. Results are expressed as the means of five experiments ±SD. Significantly lower level of expression of *Fgf*4 and *Fgf*3 from M3 of EL mice than that of MSM mice was detected. $^{*}p < 0.05$.

Interestingly, expression of these genes encoding signaling molecules in M3 of EL mice in the bud stage was reported to be very similar to that in the first molar (M1) in the bud stage of *Lef*1$^{-/-}$ mice. M1s in the late bud stage of *Lef*1$^{-/-}$ embryos showed absence of expression of *Fgf*4 and *Fgf*3, but showed expression of *Wnt*10*a* [24]. This resemblance with previous findings suggested that *Fgf*4 might not be activated by LEF1 from M3 of EL mice, similar to the failure of *Fgf*4 activation in M1s of *Lef*1$^{-/-}$ embryos. The decrease in *Lef*1 may inhibit the activation of *Fgf*4 and *Fgf*3 in M3 in the bud stage of EL mice. *Fgf*4 and *Fgf*3, which are located on chromosome 7, had been excluded from the list of potential candidates for *am*3 based on the results of previous linkage analysis [21]. Our previous mutation analysis for the candidate genes did not find any mutation in the coding sequence of the five candidate genes from EL mice, and suggested that gene mutation is not responsible for M3 agenesis in EL mice [23]. Polymorphism in the other region of *Lef*1 may be the cause of the decreased expression of *Lef*1 in M3 of EL mice.

Other candidates for *am*3 including *Hadh* (hydroxyacyl-coenzyme A dehydrogenase), *Sgms*2 (sphingomyelin synthase 2), *Papss*1 (3'-phosphoadenosine 5'-phosphosulfate synthase 1) and *Cyp*2*u*1 (cytochrome P450, family 2, subfamily u, polypeptide 1), show no evidence of association with hypodontia or early odontogenesis. *Hadh* plays a critical role in the mitochondrial beta-oxidation of short chain fatty acids and the gene mutation causes familial hyperinsulinemic hypoglycemia [28]. *Sgms*2 catalyzes the synthesis of sphingomyelin and diacylglycerol from phosphatidylcholine and ceramide, and *Sgms*2$^{-/-}$ mice exhibited difficulty of this conversion [29]. *Papss*1 is a bifunctional enzyme with both adenosine triphosphate sulfurylase and adenosine 5'-phosphosulfate kinase activity [30]. *Cyp*2*u*1 catalyzes the hydroxylation of arachidonic acid, docosahexaenoic acid and other long chain fatty acids [31]. In present study, we detected no significant difference in mRNA expression for *Hadh*, *Sgms*2, *Cyp*2*u*1 and *Papss*1 in M3 between EL and wild-type mice, suggesting no association with M3 agenesis.

Based on our gene expression analysis, we conclude that *Lef*1 is the strongest candidate for *am*3, although the cause of the decrease of *Lef*1 expression in M3 of EL mice is still unclear. Unknown genes must be involved in various types of human tooth agenesis; therefore, *Lef*1 might contribute to a form of tooth agenesis. Mutational analysis of LEF1 in non-syndromic tooth agenesis will

be needed in subsequent experiments. The identification of *am*3 in EL mice may provide clues to understanding a new mechanism of hypodontia, especially agenesis of the third molars, in humans. In the future, it may also be possible to apply the mechanism to the field of tooth regeneration.

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