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A novel enhancer of *Shh* in tooth development

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Sonic hedgehog (*Shh*) is a highly regulated gene that plays a major role in pattern formation in the developmental process. The Regulation of *Shh* is dependent on a long-range regulatory mechanism consisting of multiple enhancers which are scattered over nearly 850 kb in the mouse genome. Nervous system enhancers have been uncovered between intron and 430 kb upstream of *Shh*. The intergenic region from 430 kb to 850 kb has been reported to be a residence for enhancers in the oral cavity, pharynx, lungs, gut, and limbs. However, the intergenic region from 430 kb to 550 kb upstream of *Shh* have not been studied in detail.

In this study, we found a novel long-range enhancer located 502 kb upstream of *Shh* in mouse. The enhancer named MRCS2 showed a physical interaction with the *Shh* promoter in the developing tooth. The pattern of MRCS2 activation did not match the pattern of *Shh* expression, indicating that MRCS2 does not determine the localization of *Shh* expression. Phenotypic change was not observed in the MRCS2-deleted mice, indicating that deletion of MRCS2 alone could not cause morphological changes in teeth. These results suggest that although MRCS2 regulates the expression level of *Shh*, the deficiency of MRCS2 is sufficiently compensated by other enhancers.

Keywords: Tooth Germ, Development, Enhancer, Shh

Introduction

Morphogenesis and pattern formation during development are largely governed by precise regulation of gene expression patterns and levels. Because these fine controls are driven by enhancers, identification of these enhancers is a key step in understanding these gene regulatory networks. Enhancers are cis-regulatory elements that mediate spatiotemporal activation,

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or inhibition, of gene transcription [1].

Sonic hedgehog (*Shh*) plays a major role in pattern formation in many ectodermal and endodermal organs. A number of previous studies have investigated the regulatory domain of Shh and demonstrated the machinery of *Shh* regulatory network. *Shh* is one of the genes that are dependent on the long-range regulatory mechanisms for its spatiotemporal expression pattern [2]. The genomic regulatory domain of *Shh* is expanded over 850 kb long in mice.

Since enhancers located 9 kb upstream of the Shh transcription start site (TSS) showed their activity in the spinal cord and brain [3], many brain enhancers were discovered 120 kb to 430 kb upstream of *Shh* [2, 4]. Another four cis-regulatory enhancers, which were identified in a cluster from 610 to 740 kb upstream of the *Shh*, were mammal reptile conserved sequence 1 (MRCS1), mammal fish conserved sequence 4 (MFCS4), and mammal amphibian conserved sequence 1 (MACS1), placental conserved sequence 1 (PCS1). These enhancers regulate *Shh* expression in developing tooth and tongue papillae [1, 5]. Zone of polarizing activity regulatory sequence (ZRS) is the enhancer to regulate *Shh* expression in the limb bud over a distance of 850 kb away from the *Shh* [6, 7, 8, 9]. However, still many conserved noncoding sequences (CNCSs) in the upstream of the *Shh* TSS remains to be elucidated.

In the present study, we explored CNCSs in the genomic region from 430 to 550 kb upstream of *Shh* TSS by comparing the genomes of vertebrate species and discovered a new CNCS named mammal reptile conserved sequence 2 (MRCS2). By utilizing chromosome conformation capture (3C) assay, MRCS2-*lacZ* reporter mouse and MRCS2-deleted mice, we found that the MRCS2 interacts physically with the *Shh* TSS and that MRCS2 deletion was compensated by other enhancers.

Materials and methods

DNA sequence Analysis

Human genome hg19 from chromosome 7:156191755 to 156193325 was used as the base genome, and Evolutionary conservation and DNA sequence similarities were analyzed by using the web tool ECR Browser (http://ecrbrowser.dcode.org).

Animals

All animal experiments in this study were approved by the Animal Care and Use Committee of the National Institute of Genetics and the Yonsei University College of Dentistry, Intramural Animal Use, and Care Committee.

Chromosome Conformation Capture (3C) analysis

3C assay was conducted in cells isolated from molars of wild-type ICR mice at E14.5 as described previously [5]. Developing molars were harvested from maxillae and mandibles of E14.5 wild-type ICR mouse embryos. Tissues were homogenized to get a homogeneous single-cell suspension ($2x10^7$ cells). Cells were crosslinked with 2 % formaldehyde for 20 minutes at room temperature, and the reaction was quenched with glycine (0.125 M). Then the cells were pelleted, washed with PBS, and lysed in 5 ml ice-cold lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM NaCl, 0.2% NP-40 [pH 8.0]) containing complete protease inhibitors (Roche, Mannheim, Germa-

ny). Isolated nuclei were washed with the restriction enzyme buffer and resuspended in the restriction enzyme buffer (0.5 mL per 1×10^7 cells) containing 0.3% SDS and incubated for 1 hour at 37°C in a thermomixer at 1400 rpm. Triton X-100 (1.8%) was added and further incubated for 1 hour at 37°C. The crosslinked DNA was digested overnight with the BglII restriction endonuclease (400 U per 1×10^7 cells, Takara, Shiga, Japan). The restriction enzyme was inactivated by adding SDS (1.6% final) and incubation for 20 min at 65°C. The reaction mixture was diluted with ligase buffer (50 mM Tris-Cl [pH 7.5], 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP), Triton X-100 was added (1% final), and incubated at 37°C for 1 hour. The DNA was ligated using T4 ligase 8000 cohesive end units (NEB, Beverly, MA, USA) for 4 hours at 16 °C followed by 30 minutes at room temperature. Crosslinks were reversed by adding Proteinase K and incubated overnight at 65°C in the thermomixer at 1400 rpm. The following day, DNA templates were incubated for 30 minutes at 37 °C with 30 μL of 10 mg/mL RNase A, and the DNA was purified by phenol extraction followed by ethanol precipitation. The resulting 3C template represents a library of all possible ligations, which are then detected by using PCR (Forward Primer- GTCCTCAGTGTGCTGGGAAGATCG, Reverse Primer- CTGGCTGTGGAAGCAGGTTTCG) and the DNA sequence is confirmed by the sequencing of the PCR product.

Production of enhancer-driven reporter mice

MRCS2-lacZ reporter mice were generated on the C57BL/6 genetic background to visualize the *in-vivo* activity of MRCS2. The upstream enhancer 502 kb away from the *Shh* transcription start site (TSS) was cloned into upstream of the Hsp68 promoter and β-galactosidase (β-gal) gene in the pHSF51 plasmid [10]. Briefly, the enhancer DNA were amplified by PCR from mouse genomic DNA as the template and cloned into a HindIII site of the pHSF51 plasmid. The plasmid constructs were microinjected into the pronuclei of C57BL/6 mouse eggs, and the fertilized mouse eggs were transferred to surrogate mothers. To visualize lacZ-positive cells, enhancer-driven lacZ reporter mouse embryos at E13.5 (n = 12) were stained for β-gal enzyme activity with 5-bromo-4- chloro-3-indolyl-β-D-galactopyranoside (x-gal) substrate.

Production of enhancer-deleted mice

A targeted deletion of the genomic region of MRCS2 (mm10: chr5:28,969,630-28,970,104) was achieved by utilizing CRISPR/Cas9 in C57BL/6 zygotes to investigate the role of the enhancer in gene expression and tooth patterning. Two sgRNAs were designed beside the MRCS2 sequence (sgRNA1- TCTCAAAGGTTGTGTAGGTCAGG, sgRNA2-TTGAAAGTCCACTGTCCAGCTGG) and sgRNAs and Cas9 enzyme were microinjected into the pronuclei of C57BL/6 mouse eggs, and the fertilized eggs were transferred into the uterus of surrogate mothers (Macrogen Clinical Laboratory, Seoul, Korea). A successful deletion was recognized by genotyping the newborns with PCR (Forward Primer-GAGCAAGTTGGGAGCAGGAG, Reverse Primer-CCCTGGGGTACTTGGTTTCT)

Micro-computed tomography (Micro CT)

$MRCS2^{\Delta/\Delta}$ and $MRCS2^{+/+}$ mice skulls were collected at 14 weeks old (Homo n = 6, wild type n =

6) and micro-computed tomography images were obtained (Skyscan1173, Bruker, Belgium). Skull micro-CT images were 3D reconstructed using the software Ondemand3d (Cybermed, Korea)

Results

A novel CNCS located 502 kb upstream of *Shh* physically interacts with the *Shh* promoter.

To identify CNCSs in the sequence 430 to 550 kb upstream of the *Shh* TSS, we analyzed sequence homology among ten different animal species, including zebrafish, fugu, frog, chicken, opossum, rat, mouse, cow, dog, and human, using the ECR Browser (Fig. 1A–B). A CNCS, which is located 502 kb upstream of the mouse *Shh* TSS, was selected as an enhancer candidate based upon the conservation level, as well as the availability of endonuclease recognition sites for 3C assays (MRCS2 in Fig. 1A).

Enhancer candidate was conserved mostly among the mammals, reptiles and aves, but not in the *Xenopus* of Amphibia, and the fugu of bony fish (Fig. 1B), so it was named as Mammal Reptile Conserved Sequence 2 (MRCS2). In the 3C assay, the MRCS2 demonstrated a physical interaction with the *Shh* promoter in mouse tooth germs at E13.5 (Fig. 2A).

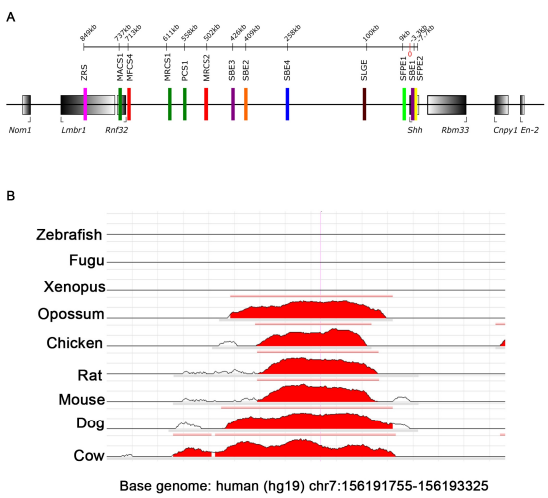

Fig. 1. A novel enhancer of *Shh* located 502 kb upstream of *Shh*. (A) The relative genomic location of the known *Shh* enhancers, the distance from the *Shh* TSS.(B) The evolutionarily conserved region (ECR) browser snapshot of the novel enhancer region from the human (hg19) base genome chromosome 7:156191755 to 156193325. The conservation across 9 animal species including cow, dog, mouse, rat, chicken, opossum, xenopus, fugu, and zebrafish are shown in peaks.

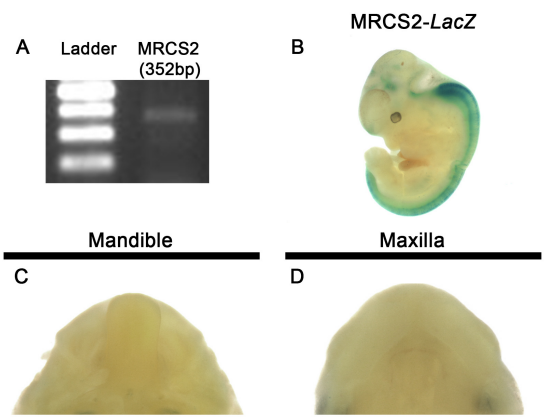

Fig. 2. Physical interaction of novel enhancer with *Shh* promoter and the activity of the mammal reptile conserved sequence 2 (MRCS2). (A) Chromosome conformation capture assay results using developing molars from maxillae and mandibles of E14.5 mouse embryos indicate the physical interaction between novel enhancer MRCS2 with the *Shh* promoter in tooth germs. (B-D) MRCS2-*lacZ* mouse embryos show *lacZ*-positive cells after X-gal staining in the neural tube, forehead, and brain. Localization of *lacZ*-positive cells is not observed in incisors and molars, fungiform papilla, whiskers, and hairs in either the mandible or maxilla.

MRCS2 activity and *Shh* expression were not co-localized in developing tooth germs, fungiform papillae, and hairs *in vivo.*

To investigate the regulatory activity of MRCS2 *in vivo*, we generated transgenic mice with a MRCS2-*lacZ* reporter construct (MRCS2-*lacZ*). In x-gal staining, *lacZ*-positive cells were observed in the neural tube, forehead and brain (Fig. 2B). The localization of *lacZ*-positive cells was not observed in incisors and molars, fungiform papilla, whiskers and hairs in either the mandible or maxilla (Fig. 2C–D). Three lines out of the five MRCS2-*lacZ* mouse lines showed the identical localization pattern of *lacZ*-positive cells. This result indicates that MRCS2 does not determine the localization of *Shh* expression.

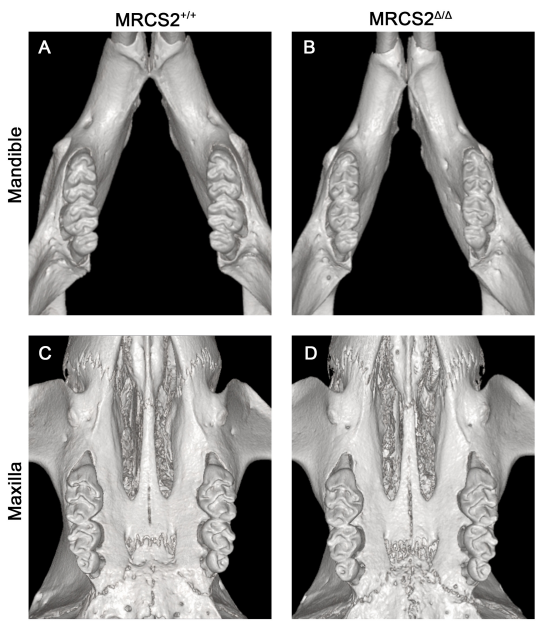

Fig. 3. Phenotypic changes in mutant mice with deletion of MRCS2. (A, C) Three-dimensional (3D) reconstructed micro-CT images of the mandible and maxilla of wild-type mice, and (B, D) MRCS2$^{\Delta/\Delta}$ mice at 14 weeks old. The size and shape of teeth are the same in both the mandible and maxilla of MRCS2$^{\Delta/\Delta}$ mice with the wild-type mice, and there is no change in the arrangement of the cusps.

Phenotypic change was not observed in mice with deletion of the MRCS2.

Next, we tried to find out the extent to which MRCS2 regulates Shh expression level. We generated the mutant mice with genomic deletion of MRCS2 (MRCS2$^{\Delta/\Delta}$). MRCS2$^{\Delta/\Delta}$ mice survived after birth and were fertile. Additionally, there was no significant reduction in life expectancy. As results, the tooth morphology of MRCS2$^{\Delta/\Delta}$ mice and that of the wild-type mice was not significantly different (Fig. 3A–D). The size and shape of the teeth were the same in both the lower and upper jaws, and there was no change in the arrangement of the cusps. These results indicate that deletion of MRCS2 alone could not cause morphological changes in teeth. Morphological change was not observed in other parts of the body.

Discussion

A novel CNCS located 502 kb upstream of *Shh*

Previous studies have already reported three enhancers, such as MRCS1, MFCS4 and PCS1, in developing teeth [1, 5, 11]. In the present study, we discovered a fourth enhancer MRCS2 which have the physical interaction with *Shh* promoter. This suggests that many enhancers are required to control the localization and intensity of *Shh* expression in the developing teeth.

Spatial inconsistency between MRCS2 activity and *Shh* expression

We investigated whether MRCS2 regulates the localization and intensity of Shh expression in

developing teeth. At first, the pattern of MRCS2-induced reporter was different from the pattern of *Shh* expression. This spatial discrepancy between the *Shh* expression and MRCS2 activation indicates that MRCS2 is not involved in determining the pattern of *Shh* expression in teeth. Among all the enhancers reported in previous studies, there was no enhancer of which activation pattern was completely consistent with *Shh* expression pattern [1, 5, 11]. Therefore, it is suggested that MRCS2 is involved in regulating the level of *Shh* expression rather than determining the localization of *Shh* expression.

The role of MRCS2 in contributing to *Shh* transcription

Tooth morphology of MRCS2$^{\Delta/\Delta}$ mice was not significantly different from that of the wild-type mice. Similar to this, neither MRCS1$^{\Delta/\Delta}$ mice nor MFCS4$^{\Delta/\Delta}$ mice showed any morphological changes, whereas MRCS1 $^{\Delta/\Delta}$; MFCS4$^{\Delta/\Delta}$ mice showed supernumerary molar [11]. These results suggest that the combined absence of enhancers synergistically contributes to the morphological change and that a single deletion of MRCS2 enhancer is not sufficient to induce morphological changes in teeth due to the compensation of other enhancers.

In conclusion, it is suggested that MRCS2 is involved in *Shh* transcription, but the role of MRCS2 in tooth patterning is not clear. In order to confirm the role of each Shh enhancer in the developing teeth, morphological and molecular analysis of the compound enhancer-deleted mice is essential.

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한글초록

치아발생 중 *Shh*의 새로운 인핸서

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Sonic Hedgehog (***Shh***)는 발달 과정에서 패턴 형성에 중요한 역할을 하는 유전자이다. ***Shh***의 조절은 마우스에서 거의 850 kb에 흩어져 있는 여러 인핸서에 의해 조절된다. ***Shh***로부터 인트론과 430 kb 사이에서 신경계 인핸서가 발견되었고, 430에서 850 kb 떨어진 곳에서는 구강, 인두, 폐, 내장 및 팔다리의 인핸서가 보고되었다.

이 연구에서 우리는 마우스에서 ***Shh***의 502 kb 업스트림에 위치한 새로운 장거리 인핸서 MRCS2를 발견하였다. 우리는 치배에서 MRCS2 인핸서가 ***Shh*** 프로모터 부분과 물리적으로 상호작용을 한다는 것을 확인하였다. MRCS2 활성화를 확인하기 위한 *lacZ*-reporter 마우스에서 MRCS2는 구강 내에서 활성을 보이지 않았다. 이는 MRCS2가 ***Shh*** 발현 위치를 결정하지 않음을 의미한다. MRCS2가 ***Shh*** 발현의 정도를 조절하는지 여부를 확인하기 위해 제작한 MRCS2-결손 마우스에서도 표현형의 변화는 관찰되지 않았다. 이전 결과에서도 단일 인핸서가 결손된 마우스에서는 구강구조의 변화가 없었다. 이러한 결과로 보아, MRCS2가 ***Shh***의 발현에 관여하긴 하지만, MRCS2의 결핍이 다른 인핸서에 의해 충분히 보상되는 것으로 판단된다.

주제어: 치배, 발생, 인핸서, Shh