Elimination of a long-range cis-regulatory module causes complete loss of limb-specific *Shh* expression and truncation of the mouse limb

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Summary

Mutations in a conserved non-coding region in intron 5 of the *Lmbr1* locus, which is 1 Mb away from the sonic hedgehog (*Shh*) coding sequence, are responsible for mouse and human preaxial polydactyly with mirror-image digit duplications. In the mouse mutants, ectopic *Shh* expression is observed in the anterior mesenchyme of limb buds. Furthermore, a transgenic reporter gene flanked with this conserved non-coding region shows normal polarized expression in mouse limb buds. This conserved sequence has therefore been proposed to act as a long-range, cis-acting regulator of limb-specific *Shh* expression. Previous phylogenetic studies have also shown that this sequence is highly conserved among tetrapods, and even in teleost fishes. Paired fins of teleost fishes and tetrapod limbs have evolved from common ancestral appendages, and polarized *Shh* expression is commonly observed in fins. In this study, we first show that this conserved sequence motif is also physically linked to the *Shh* coding sequence in a teleost fish, the medaka, by homology search of a newly available genomic sequence database. Next, we show that deletion of this conserved intronic sequence by targeted mutation in the mouse results in a complete loss of *Shh* expression in the limb bud and degeneration of skeletal elements distal to the stylopod/zygopod junction. This sequence contains a major limb-specific *Shh* enhancer that is necessary for distal limb development. These results suggest that the conserved intronic sequence evolved in a common ancestor of fishes and tetrapods to control fin and limb development.

Key words: Limb development, *Shh*, cis-acting regulator, Medaka, Mouse

Introduction

Anteroposterior pattern formation in tetrapod limb development requires polarized expression of *Shh* in posterior limb bud mesenchyme (Johnson and Tabin, 1997; Neumann et al., 1999; Riddle et al., 1993). Preaxial polydactyly (PPD) with mirror-image digit duplication, a common human congenital anomaly, maps to chromosome 7q36 and is closely linked to *Shh* (Heutink et al., 1994; Tsukurov et al., 1994). Preaxial polydactyly mutations with similar mirror-image digit duplication also map to the syntenic region on mouse chromosome 5 (Martin et al., 1990; Sharpe et al., 1999). Mutations identified in four unrelated families with PPD and two mouse mutations, *Hx* and *M100081*, are caused by a single base substitution in the conserved sequence of intron 5 of the *Lmbr1* locus (Lettice et al., 2003; Sagai et al., 2004). In mutant mice, ectopic expression of *Shh* is observed in the anterior margin of the limb bud mesenchyme (Masuya et al., 1995), suggesting that the mutations disrupt the system regulating polarized *Shh* expression in the posterior limb bud. A transgenic reporter gene controlled by this sequence is expressed in the posterior mesenchyme of developing mouse limb buds (Lettice et al., 2003). This also suggests that the conserved intronic sequence contains a limb-specific *Shh* enhancer. Furthermore, previous cis-trans assays using a *Shh* knockout (KO) allele indicated that the phenotype of preaxial polydactyly disappears when the mutant alleles are placed in a cis position relative to the *Shh* KO allele (Lettice et al., 2002; Sagai et al., 2004). All these studies suggest that the conserved intronic sequence functions as a cis-acting regulator for limb-specific expression of *Shh*.

Use of a comparative genomic approach for identifying functional units within genomic sequences has increasingly gained support as a result of several recent reports (Boffelli et al., 2004). The paired fins of teleost fishes and tetrapod limbs have evolved from common ancestral appendages (Grandel and Schulte-Merker, 1998; Sordino et al., 1995; Ruvinsky and Gibson-Brown, 2000). Previous phylogenetic studies have revealed that the intronic sequence of *Lmbr1* is highly conserved among tetrapods and teleost fishes (Sagai et al., 2004). In this study, we first examined the physical linkage of *Shh* and the conserved intronic sequence in a teleost fish, medaka, because the genome sequence of the medaka fish is now available. The conserved sequence in the medaka fish is located in the same scaffold as the medaka *Shh* gene, and is placed in intron 5 of the medaka *Lmbr1* homologue. This indicates that the physical linkage of the *Shh* coding sequence and the conserved intronic sequence evolved prior to the divergence of teleost fishes and tetrapods.

Based on these data, we wished to examine directly the role of the conserved sequence in mouse limb development by gene targeting. The knockout mouse showed a complete loss of *Shh* expression in the limb buds and severe amputation of distal elements of the limbs, a phenotype similar to the *Shh* KO mouse (Chiang et al., 1996; Chiang et al., 2001) and the human congenital deformity acheiropodia (Ianakiev et al., 2001). All results provided unequivocal evidence that the intronic sequence contains a major enhancer for limb-specific expression of *Shh* and is essential for distal limb development.

Materials and methods

Phylogenic analysis of the conserved sequence

The primer pairs used for the amplification of the conserved sequence were previously described (Sagai et al., 2004). The clustalW system (http://ortholog.nig.ac.jp/homology/clustalw-e.shtml) and VISTA program (http://www-gsd.lbl.gov/vista/index.shtml) were used for sequence alignment and homology comparison. Draft genome sequence of medaka fish (scaffold 35 form) was obtained from the database of the NIG DNA sequencing center (http://dolphin.lab.nig.ac.jp/medaka/index.php).

Animals

Two preaxial polydactylous mutants, *Hx*, *M100081,* were described previously (Sagai et al., 2004). The *Shh* knockout mouse was kindly provided by Dr P. Beachy (Chiang et al., 1996). The animal experiments in this study were approved by the Animal Care and Use Committee of National Institute of Genetics.

ES targeting

The basic targeting vector was constructed by inserting pKO Neo and pKO DT cassettes into the pKO Scrambler V901 vector (Lexicon Genetics Incorporated). The long arm (5652 bp) and the short arm (2357 bp) fragments derived from BAC clone BAC311J12 (129/Sv origin) were ligated into the basic vector to replace the conserved sequence (1167 bp) with the Neo cassette. The targeting vector was electroporated into R1 ES cells, which originated from the 129/Sv strain. Recombined cells were screened with the following PCR primer pairs: p1; 5′-TTAAGCATGCTTGTCTCTCG-3′ and p2; 5′-CATCGCATTGTCTGAGTAGG-3′. Positive clones were aggregated with 8-cell embryos from (DBA/2x C57BL/6J) F1 mice and transplanted into surrogate females. Male chimeras were mated with C57BL/6J females, and germline transmission of the knockout allele was confirmed by presence of the Agouti coat color. Genotyping of mice was carried out using the following PCR primer pairs: p3; 5′-GACCAATTATCCAAACCATC-3′ and p4; 5′-TAACACTAAGCA-GCACTTCC-3′, p5; 5′-GGCTATTCGGCTATGACTGG-3′ and p6; 5′-GAGATGACAGGAGATCCTGC-3′.

Skeletal preparation

Mouse skeletons were stained by alizarin red and alcian blue as described previously (Wallin et al., 1994). For E14 embryos, cartilage was stained as described previously (Jegalian and De Robertis, 1992).

Whole-mount in situ hybridization

Whole-mount in situ hybridization of embryos was performed according to the method described by Wilkinson (Wilkinson, 1992). Briefly, digoxygenin-labeled riboprobes were transcribed in vitro according to the manufacturer's protocol (Roche). The following probes were used: *Shh* (A. McMahon), *Gli3* (C. C. Hui), *Fgf4* (G. Martin) and *dHand* (*Hand2* – Mouse Genome Informatics) [generated from the entire coding region of murine *dHand* (Srivastava et al., 1995)].

Results

Three blocks of conserved sequences are located in the same scaffold as the *Shh* coding sequence in the genome of medaka fish

We conducted a database search for putative cis-regulators in the intervening sequence between the *Shh* and *Lmbr1* genes in the genome of medaka fish. We found three clustering blocks of conserved non-coding sequences between mammals and medaka fish, and the medaka sequences are located in the same scaffold as the *Shh* coding sequence (Fig. 1). The ordering of the three blocks is conserved between medaka fish and mammals, with the most distant block found in intron 5 of *Lmbr1*. Notably, the distances between the *Shh* coding sequence and the conserved non-coding sequence blocks are similar in both the human and mouse genomes, but the distance in medaka is just one-tenth of that observed in the mammalian genomes. We designated the most distant sequence, in which human PPD and mouse mutations were identified, mammals-fishes-conserved-sequence 1 (MFCS1), and the other two sequences, MFCS2 and MFCS3, respectively (Fig. 1). The alignment of the core sequences of the three MFCS blocks is shown Fig. S1 in the supplementary material. VISTA data for the MFCS1 sequence is shown in Fig. 2. The core sequence (160 bp in length) of MFCS1 shows a high degree of sequence

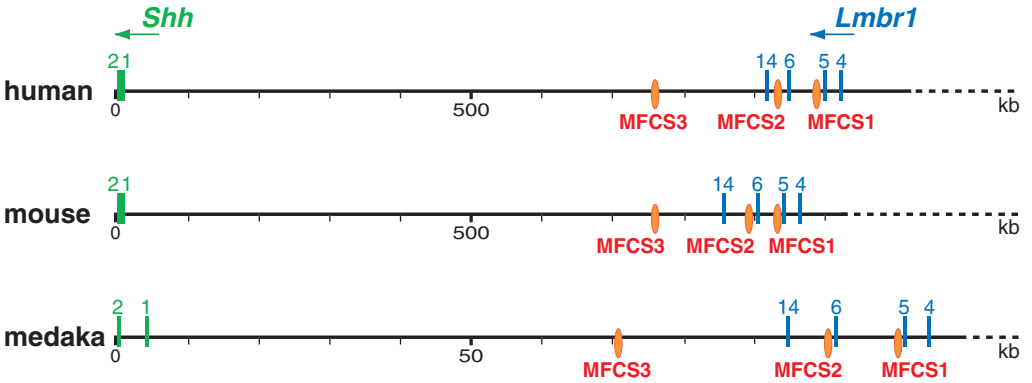


Fig. 1. Three blocks of non-coding sequence are conserved among mammals and the teleost fish medaka. Intervening sequences between the *Shh* coding region and the *Lmbr1* gene are compared between human, mouse and medaka genomes using the VISTA program. The genomic sequences used for comparison of the three species are 1,088,638 bp of human sequence (Ensemble, chromosome 7, 155013840-156102478), 992,498 bp of mouse sequence (Ensemble, chromosome 5, 26711297-27703795), and 203,199 bp of medaka fish sequence (scaffold 35). The boxes and circles depict the conserved fragments that show more than 75% identity over 100 bp of sequence among the three species. The green boxes depict the exons of *Shh*, and blue boxes the exons of *Lmbr1*. The orange circles depict conserved non-coding sequences. Notably, the *Shh* coding region and the conserved sequence blocks are physically linked on the same chromosomes in mouse, human, and medaka fish.

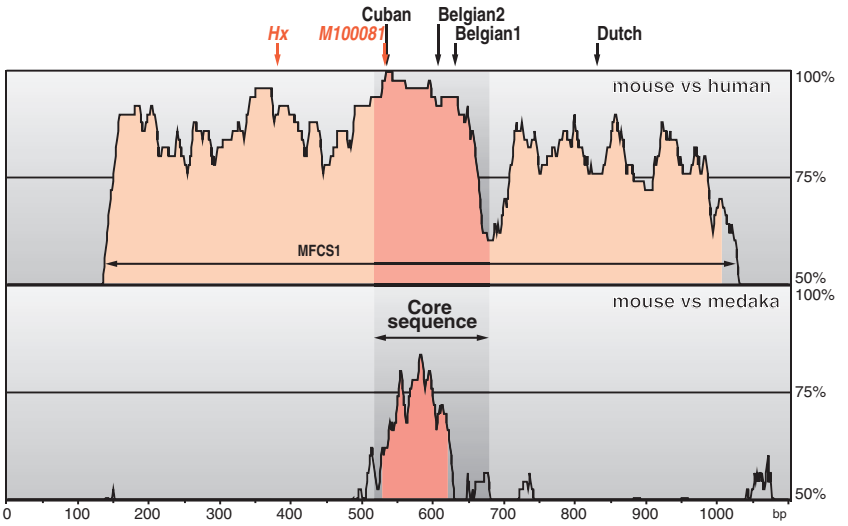


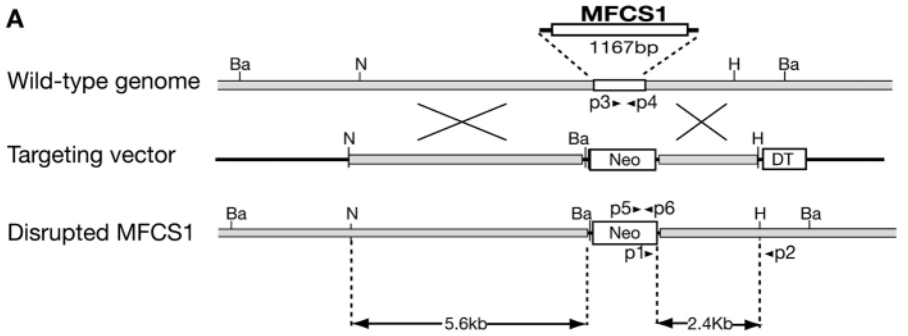
Fig. 2. VISTA data of MFCS1. The VISTA search for mouse-human and mouse-medaka homologous sequence revealed the full extent and core sequence of the MFCS1. In the VISTA search, we used 50 bp window length and 75% conservation level. The sites of two mouse mutations (red arrows), *Hx* and *M100081*, and four human PPD mutations (black arrows) are superimposed on MFCS1. A DNA segment (1167 bp in length) that includes the entire MFCS1 was eliminated by gene targeting in this study.

similarity, exceeding 60-75% identity, between mouse and medaka (Fig. 2). The flanking sequences of the MFCS1 core show low sequence conservation between mouse and medaka, but are highly conserved between mouse and human (Fig. 2). The sites of the human and mouse mutations that lead to preaxial polydactyly are superimposed on the same figure (Lettice et al., 2003; Sagai et al., 2004). Four mutations are confined to the core sequence, but the remaining two map to the flanking sequence of the MFCS1 core. We found no sequence motifs commonly shared among the three MFCS sequence blocks. Moreover, we failed to identify any consensus sequences for known transcription factors.

Since polarized expression of *Shh* in posterior mesoderm is essential for anteroposterior patterning in the appendages of both teleost fish and tetrapods (Johnson and Tabin, 1997; Neumann et al., 1999; Tanaka et al., 2002), conservation of physical linkage between the conserved sequence region and *Shh*-coding sequence in medaka fish suggests that the cis-acting regulator for *Shh* was established prior to the divergence of teleost fishes and mammals.

Disruption of MFCS1 causes severe defects in limb development

We carried out gene targeting experiments to examine directly the role of MFCS1 in limb development. To generate a deletion mutant in the mouse, a targeting vector was designed to replace a 1167 bp DNA fragment that includes the entire MFCS1 sequence in *Lmbr1* intron 5 by a Neo cassette (Fig. 2, Fig. 3A). We obtained two lines of germline chimeras from two targeted ES clones (Fig. 3B), and obtained MFCS1-deleted KO homozygotes by intercrossing the progeny carrying the KO allele (Fig. 3C). Genotyping revealed that KO homozygotes segregated in a Mendelian fashion (data not shown). Although homozygous KO embryos are approximately the same size as their wild-type littermates at E12.5, they display distally truncated, vestigial limbs (Fig. 4A,B). By E14.5, mutant embryos form thin, stick-like appendages that are indistinguishable from those of *Shh* KO embryos (Fig. 4D) (Chiang et al., 1996; Chiang et al., 2001). However, in contrast to the *Shh* KO mutants, which die at around E18.5 with severe central nervous system defects, the MFCS1 KO homozygotes are viable, and survive at least three months after birth (Fig. 4F). Although the body size of the mutant mice is slightly smaller than wild-type mice, they appear healthy and can move freely using their shortened appendages, suggesting that the deleted MFCS1 has a limb-specific function. The hindlimbs have one hard digit with a nail on the dorsal side and a pad-like structure on the ventral side (Fig. 4H,J), indicating that the dorsoventral axis is properly established.



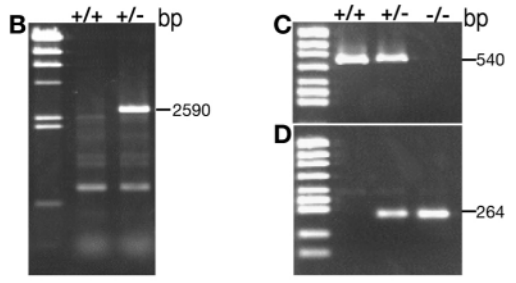


Fig. 3. Generation of mutant mice lacking MFCS1. (A) Design of the targeting vector. A neomycin (Neo) cassette used for positive selection was flanked by a 5652 bp long arm (AB114903 98833-104485) on the 5′ end, a 2357 bp short arm (AB114903 95309-97666), and a diphteria toxin (DT) cassette used for negative selection on the 3′ end. Homologous recombination results in deletion of 1167 bp of MFCS1. (B) PCR primers p1 and p2 were used for screening recombined ES clones. (C) Genotyping of mice was carried out using the p3 and p4 primers for the wild-type allele, and (D) the p5 and p6 primers for the MFCS1 KO allele. Ba, *Bam*H1; N, *Not*1; H, *Hpa*1.

MFCS1 sequence is essential for the development of distal limb skeletal elements

The skeletal phenotype of MFCS1 mutant

limbs resembles that of the *Shh* KO mouse (Chiang et al., 2001) (Fig. 5B,C). In both mutants, skeletal elements distal to the stylopod/zeugopod junction are severely affected, but more proximal structures are normal. For example, the pectoral girdle, scapula and stylopod (humerus) of the forelimbs appear to be normal in MFCS1 KO homozygotes. However, the distal portion of the humerus is fused with a single zeugopod element, and there is no joint at the elbow (Fig. 5D,E). The deformity of the zeugopod elements is often asymmetric, with the right arm straight but the left arm curved at the junction between the stylopod and zeugopod. In the hindlimb, the pelvic girdle and femur appear normal, but skeletal elements distal to the knee joints exhibit a complex phenotype (Fig. 5G,H). The two distinct but incomplete zeugopod elements, representing the tibia and fibula, are fused in the midline, and their proximal ends form a knee joint. A series of ossified rods separated by joints follows the distal ends of the zeugopod. The most proximal rod, which may represent the tarsal bone, emerges from between the two zeugopodal elements, followed by the metatarsus and the two phalanges. At the end of the autopod, we observed an arrowhead-shaped ossified terminal phalanx. Thus the autopod of the mutant hindlimb is essentially composed a single digit with well-formed interphalangeal joints.

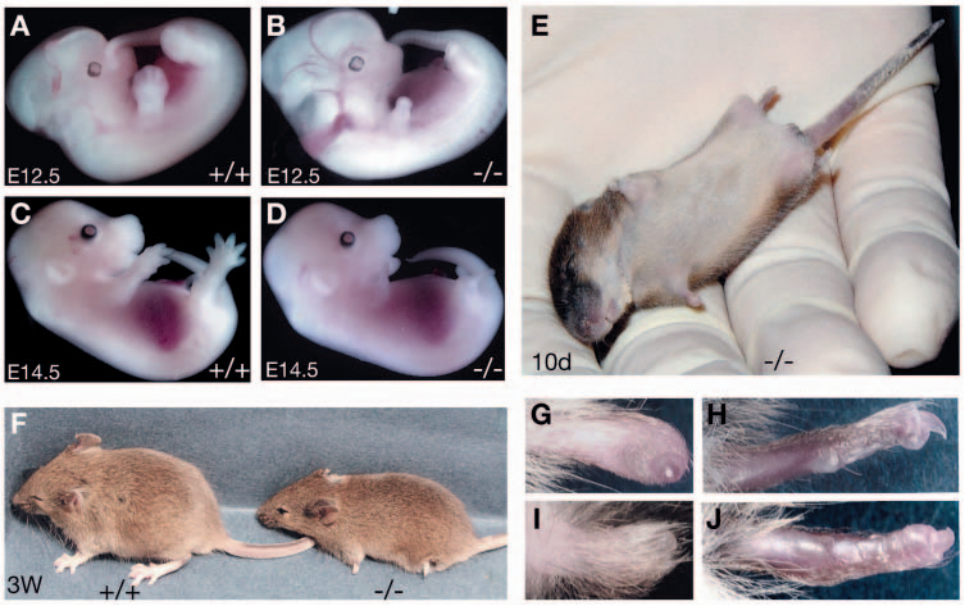


Fig. 4. External phenotype of the MFCS1 KO mouse. (A,B) The limbs of the MFCS1 KO embryos at E12.5 are extremely reduced. (C,D) In the KO embryos at E14.5, the distal ends of the limbs becomes very thin. (E) A 10-day post-natal MFCS1 KO homozygote appears quite healthy, except for the limb abnormality. (F) The body size of a 3-week-old MFCS1 KO homozygote (right) is comparable to that of wild-type (left). (G-J) Magnified pictures of the forelimbs (G,I) and hindlimbs (H,J) in the MFCS1 KO homozygote. The autopod is not recognizable in the forelimbs. In the hindlimbs, only one digit with a nail is observed (H). On the surface of the ventral side of the digit, pad-like tissue is observed (J).

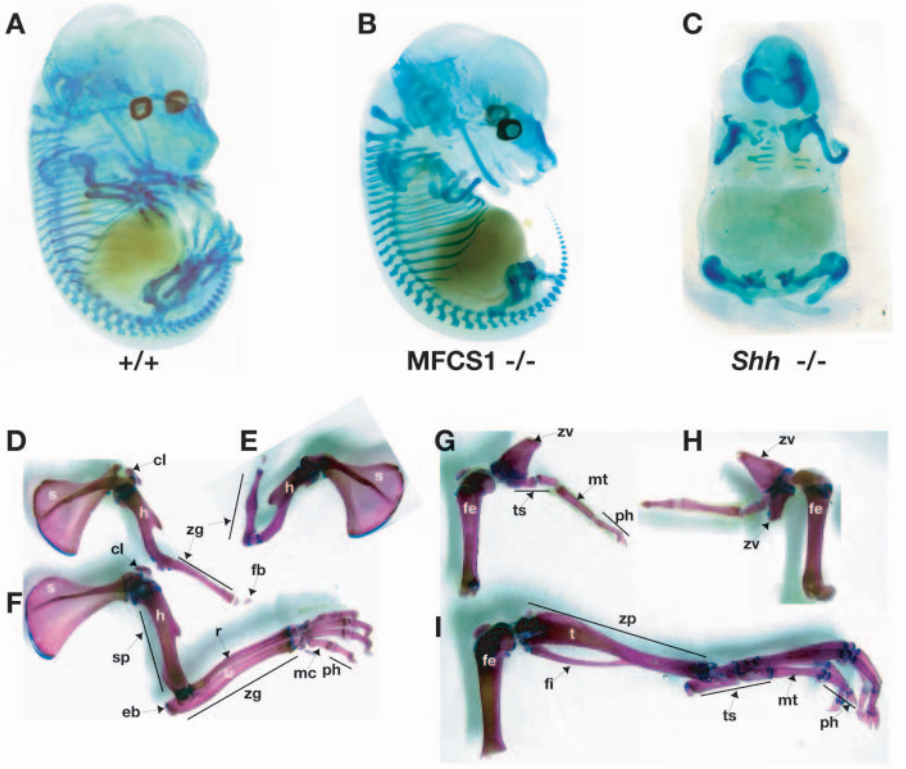


Fig. 5. Skeletal phenotypes of MFCS1 and *Shh* KO mutants. (A-C) Skeletal preparations of E14.5 embryos were stained with alcian blue. (A,B) Lateral view of a wild-type and MFCS1 KO homozygote. (C) Dorsal view of a *Shh* KO homozygote. (D-I) Skeletal preparations of 3-week-old mice were stained with alizarin red and alcian blue. The forelimb and hindlimb of the wild-type mouse are shown in (F) and (I) respectively. (D,E) Forelimbs of MFCS1 KO homozygotes. (G,H) Hindlimbs of MFCS1 KO homozygotes. cl, clavicle; eb, elbow joint; fb, floating skeletal element; fe, femur; fi, fibula; h, humerus; mc, metacarpals; mt; metatarsal bones; ph, phalanges; r, radius; s, scapla; sp, stylopod; t, tibia; ts, tarsal bones; u, ulna; zg, zeugopod; zv, zeugopodial vestiges.

Expression of *Shh* is abolished in the limb buds of MFCS1 KO mice

The similarity in phenotype between MFCS1 and *Shh* KO mice suggests that the MFCS1 KO phenotype is caused by a loss of *Shh* expression in the limb buds. To examine this possibility we carried out whole-mount in situ hybridization on mutant embryos. At E10.5, *Shh* expression in the limb buds of MFCS1 KO homozygotes is completely lost (Fig. 6G-I), but expression in the neural tube and notochord is unaffected (data not shown). To examine whether loss of *Shh* expression in the limb buds is due to an alteration in the expression of genes upstream or independent of *Shh* signaling (Buscher et al., 1997; Charite et al., 2000), we analyzed the expression patterns of *dHand*, *Gli3*, *Fgf8* and *Fgf4*. At E10.5, *dHand* expression is not detected in the forelimb bud of MFCS1 KO homozygotes, but weak expression is observed in posterior mesoderm of the hindlimb buds (Fig. 6L,M), as is the case in *Shh* KO embryos (Litingtung et al., 2002). The expression pattern and level of *Gli3*, which controls *Shh* expression, is comparable in the hindlimb of MFCS1 KO homozygotes to that of wild-type embryos (Fig. 6O,Q). MFCS1 KO embryos also have reduced *Fgf8* expression, an AER marker, in the forelimb bud (Fig. 6T), as previously reported for *Shh* KO homozygous embryos (Chiang et al., 2001; Kraus et al., 2001). At the same stage, *Fgf4* is not detected in the AER of MFCS1 KO homozygous embryos in either the fore- or hindlimb buds (Fig. 6X,Y).

Discussion

MFCS1 functions in two distinct manners

The similar limb phenotypes of MFCS1 KO and *Shh* KO mice indicate that the limb defects in MFCS1 KO mutant mice are directly caused by abolishing *Shh* expression. Thus MFCS1 contains a long-range, cis-acting enhancer that specifically upregulates *Shh* expression in the posterior mesenchyme of developing limb buds.

It is notable that the point mutations responsible for preaxial polydactyly in humans and mice are scattered throughout the entire MFCS1 motif (Lettice et al., 2003; Sagai et al., 2004). In the two mouse mutants, *Hx* and *M100081*, ectopic *Shh* expression is observed in anterior limb bud mesenchyme (Masuya et al., 1995; Sagai et al., 2004). This suggests that there are multiple consensus motifs controlling *Shh* expression, some of which are involved in the repression of *Shh* expression in the anterior mesenchyme of limb buds, and others that are responsible for its activation in the posterior mesenchyme of limb buds. Mutations in the former probably lead to ectopic *Shh* expression in the anterior margin of the limb buds, and preaxial polydactyly with mirror-image digit duplication, whereas deletion of the entire conserved sequence results in complete loss of *Shh* expression, as shown in the present study. It is unclear why so many motifs are required for repressing *Shh* expression in the anterior mesenchyme of developing limb buds. As yet, no known consensus binding sequences have been identified in the MFCS1. Moreover, no transcription factors are known to bind to the MFCS1. Characterization of additional point mutations in the conserved sequence will be necessary to elucidate the molecular mechanism responsible for long-range cis-acting regulation.

In this study, we found that two other sequence blocks, MFCS2 and MFCS3, are highly conserved between medaka and mammals. The ordering of the three blocks relative to the *Shh* coding sequence is also conserved among the three species. It is possible that all three MFCS blocks cooperatively function in the cis-regulation of *Shh* expression in fins or limbs, although we cannot exclude the possibility that MFCS2 and MFCS3 are both involved in regulating *Lmbr1* expression, or some other function altogether. Since there are no sequence motifs commonly shared among the three MFCS blocks, different trans-factors may bind to each MFCS block. At present, there is no evidence that human and mouse preaxial polydacytyly is caused by mutations in the MFCS2 and MFCS3 blocks. It would be of interest to examine the limb phenotype when these two sequence blocks are deleted in mice.

A notable feature of MFCS1 is its long distance from the coding sequence of *Shh*. Another long-range cis-regulatory element, the local control region (LCR) of the β-globin locus, has been identified and well characterized (Li et al., 1999). A recent report showed that the LCR is in close physical proximity to the β-globin gene during active transcription, suggesting a long-range interaction of the cis-regulatory element and the transcription unit (Harrow et al., 2004). It has been reported that a *LacZ* reporter transgene, in which MFCS1 was linked to a minimal β-globin promoter, is sufficient to

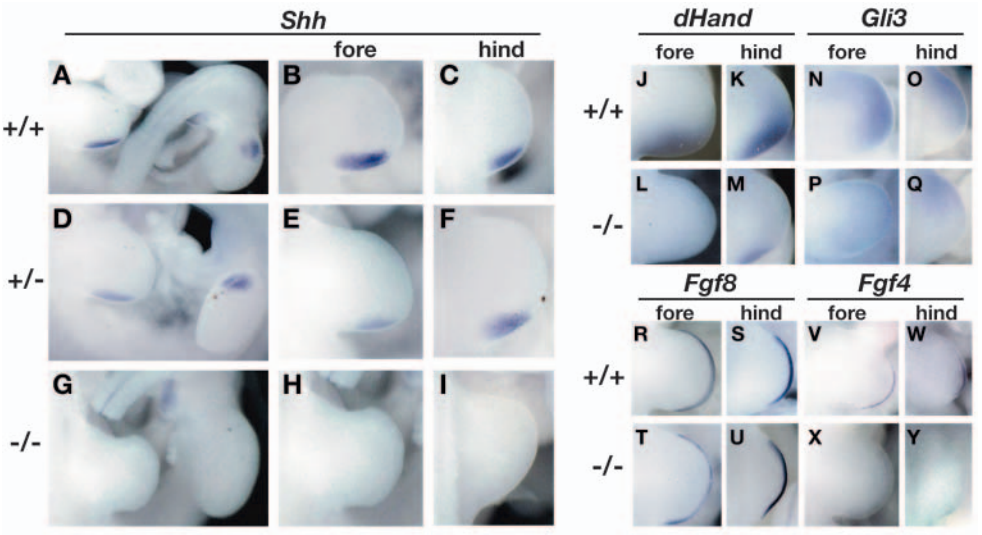


Fig. 6. Expression patterns of marker genes in the limb buds at E10.5. In all figures except for A, D and G, anterior side is top. In wild-type (A-C) and MFCS1 KO heterozygous (D-F) embryos, *Shh* expression is observed in the posterior mesenchyme of the limb buds. In the limb buds of MFCS1 KO homozygous embryos (G-I), *Shh* expression is not detected. Expression of marker genes, *dHand* (J-M) and *Gli3* (N-Q), *Fgf8* (R-U) and *Fgf4* (V-Y) were examined at a stage prior to *Shh* expression.

initiate limb-specific expression at the proper time (Lettice et al., 2003). However, the transgenic construct failed to terminate gene expression at the appropriate time, suggesting that while the physical distance between the cis-regulatory element and the *Shh* coding sequence is not essential for the initiation of limb-specific *Shh* expression, proper termination of transcription may require some distance between the two elements (Lettice et al., 2003). At present, however, it is still unknown whether direct communication is required between the long-range cis-regulatory element and the *Shh* coding sequence.

MFCS1 KO mouse is a model for human acheiropodia

Severe truncation of distal skeletal elements of human limbs, resembling the phenotype of the present MFCS1 KO mouse and the *Shh* KO mouse, is known as acheiropodia, which maps to chromosome 7q36 (Ianakiev et al., 2001). Acheiropodia is caused by a deletion of a 4-6 kb fragment including exon 4 of human *LMBR1* and its flanking region (Ianakiev et al., 2001). We searched sequences conserved between human and mouse genomes in the corresponding region, but failed to identify conserved non-coding regions with sequence homology comparable with the three blocks of MFCS. Since the overall phenotype of acheiropodia resembles that of MFCS1 KO homozygotes, it is possible that in the human genome there is a human-specific cis-regulatory element controlling limb-specific *Shh* expression in the vicinity of exon 4 of *LMBR1*. Considering the similarity of the map position and the phenotype, the MFCS1 KO mutant mouse is probably a useful animal model for studying acheiropodia.

Limbless species have lost MFCS1

We have demonstrated that MFCS1 in the mouse contains a major limb-specific enhancer of *Shh* that is essential for proper limb development. MFCS1 is also present in reptiles and amphibians as well as the teleost fish Medaka (Sagai et al., 2004). A key role of the cis-regulator for fin- and limb-specific *Shh* expression may have placed constraints on MFCS1 to remain unchanged during the evolution of the tetrapod and teleost lineages. Interestingly, we have previously found that this sequence is lost in certain limbless species of reptiles and amphibians, such as snakes and a limbless newt (Sagai et al., 2004). Given our present data showing the importance of this enhancer in limb development, it is possible that loss of the conserved intronic sequence MFCS1 represents one way by which limblessness may have evolved in vertebrate species.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/4/797/DC1

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