Identification of *cis*-elements regulating expression of *Fgf10* during limb development

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ABSTRACT **Fibroblast growth factor 10 (FGF10) is known to be expressed in limb mesenchymal cells and to function as a mesenchymal signaling factor involved in epithelial-mesenchymal interactions during limb development. To elucidate regulation of *Fgf10* expression, we isolated the promoter region of *Fgf10* containing its 2.0 kb upstream 5'-fragment from the initiation codon and its 0.9 kb downstream fragment. Transcriptional activity of the fragment was examined with transgenic mice, using a *lacZ*-reporter system. Although no significant expression of the reporter gene was observed for the 0.2 kb 5'-fragment, expression was detected in the apical ectodermal ridge of the limb bud and developing cartilage of the limb for the 2.0 kb and 0.7 kb 5'-fragments, respectively. From comparison of the mouse sequences of the 2.0 kb fragment with corresponding sequences of human and chicken *Fgf10*, we identified 17 conserved putative enhancer motifs for AER expression and other unidentified expressions. For limb cartilage expression, we found putative enhancer sequences conserved among the three species in the 0.7 kb 5'-fragment. In the fragment, three DNA binding motifs were identified in the mouse and human sequence, although they are not conserved in the corresponding chicken sequence.**

KEY WORDS: *Fgf10, enhancer analysis, mouse, transgenic mouse, developing cartilage*

Introduction

Limb morphogenesis proceeds upon the regulation of growth and differentiation mediated by epithelial-mesenchymal interaction (Reviews: Ohuchi et al., 1999, Dahn and Fallon, 1999, Martin, 2001, Tickle and Munsterberg, 2001, Capdevila and Izpisua Belmonte, 2001). Especially, interaction between apical ectodermal ridge (AER) and mesenchymal cells underneath the AER is essential for limb development. Both the AER and mesenchymal cells produce various signaling factors including fibroblast growth factors (FGFs) (Martin, 1998, Lewandoski et al., 2000), Wnts, bone morphogenetic proteins (BMPs), etc (References are cited in the above reviews.). In these factors, FGF10 is known to be one of mesenchymal signaling factors expressed in limb mesenchymal cells (Ohuchi et al., 1997, Yonei-Tamura et al., 1999). It has been reported that FGF10 can induce formation of an additional limb ectopically between the fore and hind limbs in chick embryos (Ohuchi et al., 1997, For other FGF, Cohn et al., 1995, Ohuchi et al., 1995, Crossley et al., 1996, Vogel et al., 1996), while the

Fgf10-null mutant mice exhibited complete truncation of the fore and hind limbs (Min et al., 1998, Sekine et al., 1999). It was also reported that *Fgf10* is co-expressed with *SnR* and *twist* in presumptive limb territories of early chick embryos (Isaac et al., 2000) and its expression is regulated by WNT signals in limb initiation and AER induction in the chick embryo (Kawakami et al., 2001). These results clearly indicated that FGF10 is one of the essential factors for limb formation. In addition to the limb, it was found that the mutant mice also had severe malformations in various organs including the lung, salivary glands, teeth, etc. (Kato and Sekine, 1999, Ohuchi et al., 2000, Suzuki et al., 2000, Makarenkova et al., 2000). Although it has been already clarified that *FGF10* has a pivotal role in limb formation and other organogenesis, its transcriptional regulation has not been elucidated yet so far. Here, we isolated the 2.0 kb 5'-upstream region of *Fgf10*, and analyzed its enhancer activity with transgenic mice. From comparison of the

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mouse sequences of the 2.0 kb fragment with corresponding sequences of the human and chicken *Fgf10*, we identified 17 conserved putative enhancer motifs for the AER expression and other unidentified expressions. For the limb cartilage expression, we found putative enhancer sequences conserved among the three species in 0.7 kb 5'-fragment. In the fragment, three DNA binding motifs were identified in the mouse and human sequence, although they are not conserved in the corresponding chicken sequence.

Results and Discussion

Sequence Analyses and Localization of Conserved Regions in the Fgf10 *Promoter Regions among Mouse, Chicken and Human*

To identify transcriptional regulatory regions of the vertebrate *Fgf10*, we compared sequences of the mouse 2.0-kb 5'-fragment of the *Fgf10* promoter region with the corresponding regions of the human and chicken *Fgf10*, using the percent identity plot (pip) obtained with PipMaker, which shows both the position in one sequence and the degree of similarity for each aligning segment between the two sequences (Schwartz et al., 2000). The results are shown in Fig. 1 A,B, where several highly conserved sequences (more than 75%) among the three species are identified (Fig. 1 A,B). There are three regions where mouse sequences are highly homologous to the corresponding human and chicken ones, as indicated by the number 1 to 3, while a region indicated by P may contain promoter sequences. The three regions conserved among human, mouse and chicken may contain some enhancer motifs, regulating expression of *Fgf10*.
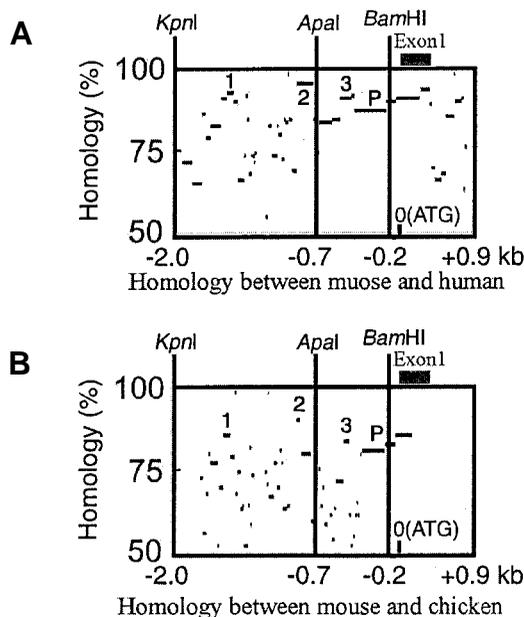

Fig. 1. Homology analysis of 5'-fragment of *Fgf10* between mouse and human (A) and between mouse and chicken (B), illustrated by PipMakerA *(Schwartz* et al., *2000). There are three conserved regions indicated by the number in the fragment. P indicates the promoter region of Fgf10.*
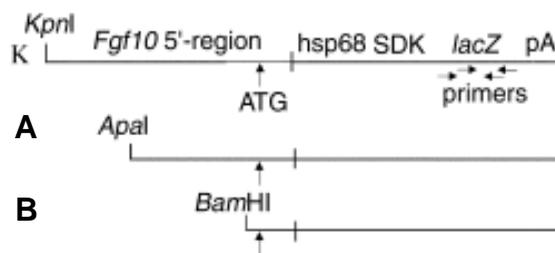

Fig. 2. Schematic illustration of the constructs for generating transgenic mice. *The 5'- fragment (a Kpn*I *fragment) was digested by restriction enzymes into two smaller fragments with **(A)** Apa*I *(0.7 kb) and **(B)** Bam*HI *(0.2 kb) restriction enzymes. Each fragment was inserted in a lacZ reporter vector containing a promoter of the heat shock protein 68 (hsp68) and Shine-Dalgarno-Kozak (SDK) sequence. PCR primers generated in the lacZ cassette are indicated by arrows.*

Identification of Mouse Cis-*Control Elements of* Fgf10 *for Transgene Expression in the Developing Limb*

In order to identify the mouse cis-control elements regulating *Fgf10* expression in the developing limb, we generated transgenic mice which had a *lacZ* reporter gene under the control of 2.0 (designated as construct K), 0.7 (A), or 0.2 (B) kb 5'-fragment of *Fgf10* (Fig. 2). In embryos (generation 0 (F0)) injected with the construct K (K-transgenic embryos), the transient transgene expression was detected at the apical ectodermal ridge (AER) of the limb bud in E11.5 embryos (n = 2 out of 9)(Fig. 3 A,B). So far, we have not generated any stable transgenic line with the construct K. In the transient transgenic E10 embryos with the construct A (A-transgenic embryos), the transgene was not expressed in the limb bud (Fig. 3 C,D), while the expression was detected clearly in the developing somites (Fig. 3 C,D). However, with the progress of limb development, the expression was observed in the distal tips of the limb bud (E12.5) and then in limb cartilage (from E13.0 to neonate at least). In order to confirm the results obtained in the A-transient F0 mice, we generated two stable transgenic lines with the construct A and analyzed expression patterns more precisely. The transgene was expressed in the distal tip of the limb buds as spots in E12.5 (Fig. 4A). This spotty expression was similar to the expression pattern of *Fgf10* in the limb bud of E12.5 embryos (Fig. 4B). Then, the transgene expression was observed in cartilage of the developing limb. In E14.5 embryos, the transgene expression was localized in cartilage of the zoygopod (Fig. 4 C,D). In the limb of neonates, X-gal stained regions were observed in the epiphyseal cartilage of the autopod. Their sections revealed that the stained cells are localized in the part of the proliferating cartilage cells of the epiphyseal growth plate. The expression in somites, brain, midgut loop, and cardiac vesicle remained unchanged in the A-transgenic mice, as observed in the K-transgenic mice. These results indicated that the enhancers for expression of the epiphyseal growth plate are involved in the 0.7 kb 5'-fragment.

On the other hand, in the transgenic mice with the shortest construct including 0.2 kb *Fgf10* 5'-fragment (B-transgenic mice), significant transient expression of the transgene was not detected except for the posterior narrow region of the limb bud and somites (Fig. 3 E,F). This result suggested that the 0.2 kb 5'-region which is a direct upstream of the first codon of *Fgf10* and 0.9 kb downstream region do not contain strong regulatory elements for expression in the limb bud. These results indicated that the

enhancers for expression of limb carti-lage are involved in the 500 bp-fragment localized between - 0.7 and - 0.2 kb 5' fragment of mouse *Fgf10*.

Conservation of Putative Regulatory Regions in the Mouse Cis-*Element, regulating Expression of* Fgf10 *in the Developing Limb*

To identify DNA-binding motifs in the 2.0 kb sequences of *Fgf10,* we used MatInd and MatInspector as tools for detection of consensus matches in nucle-otide sequence data, proposed by Quandt et al. (1995). The nucleotide sequences of the identified regulatory elements re-veal several DNA-binding motifs of tran-scription factors which are highly con-served among mouse, human, and chicken, as shown in Fig. 5. There are potential enhancer sequences in the -2.0 – -0.7 kb (*Kpn*I-*Apa*I) region, containing NFAT, MYT1, COUP, CMYB, VMYB, ETSF, GFI1, PIT1, AP1F, MZF1, EGRF, AREB, AP4R, PAX5, OCT1, COMP, and FKHD, which are all conserved in the mouse, human, and chicken sequences. De-tailed information is listed in Table 1. Although *Fgf10* is a mesen-chymal factor and not expressed in the AER of developing normal limb bud, the transgene expression under the regulation of the construct K (2.0 kb upstream of the first codon of *Fgf10*) was detected in the AER. On the other hand, this AER expression was not observed in the A-transgenic mice, indicating there are
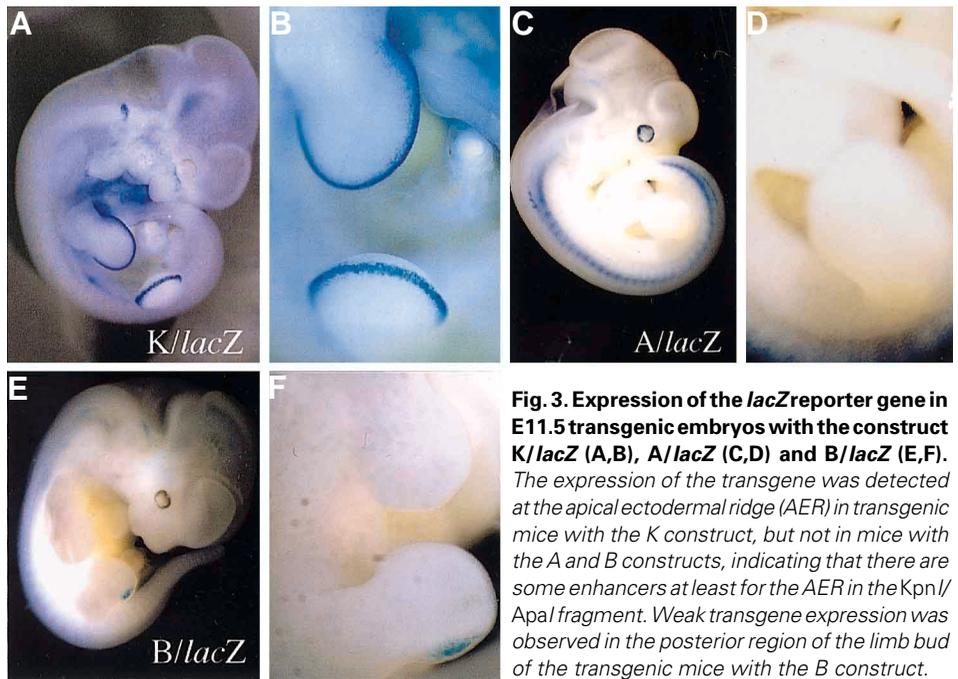
Fig. 3. Expression of the *lacZ* reporter gene in E11.5 transgenic embryos with the construct K/*lacZ* (A,B), A/*lacZ* (C,D) and B/*lacZ* (E,F). *The expression of the transgene was detected at the apical ectodermal ridge (AER) in transgenic mice with the K construct, but not in mice with the A and B constructs, indicating that there are some enhancers at least for the AER in the* Kpn*I/* Apa*I fragment. Weak transgene expression was observed in the posterior region of the limb bud of the transgenic mice with the B construct.*

enhancers for the AER in the 1.3 kb *Kpn*I/*Apa*I 5'-fragment of *Fgf10*. This result suggested that the enhancer activity for the AER in this fragment is suppressed somehow in normal develop-ment. In the 1.3 kb *Kpn*I/*Apa*I fragment, a candidate region for enhancers including the AER enhancer should be a high homol-ogy region conserved among mouse, human and chicken. In this region, there are 17 DNA-binding motifs as listed in Table 1.

For the AER enhancer, Liu et al. (1994) found that 439 bp of 5' flanking sequence of the *Msx2* homeobox gene contains regulatory elements for its exclusive expression in the AER of the developing limb. Recently, Pan et al. (2002) found that the B-TAAT site of the four potential homeodomain binding TAAT sites in a 348-bp fragment of the chicken *Msx2* gene is critical for AER enhancer activity. However, there is not such se-quence in the *Kpn*I/*Apa*I fragment. Thus, we can not determine which is the AER enhancer in the conserved 17 motifs. More precise experiment with the short fragment containing the several motifs is necessary to identify enhancer motifs in this region.

The 500-bp *Apa*I/*Bam*HI murine frag-ment responsible for the cartilage expres-sion of *Fgf10* contains a 100 bp sequence conserved highly among mouse, human, and chicken (Fig. 5), suggesting that the sequence may act as cis-element regulat-ing cartilage-specific expression of the *Fgf10* gene in developing limb. The 100-bp mouse and human sequences contain three conserved DNA-binding motifs: RREB, GKLF, IRFF, and EVI1 (see Table
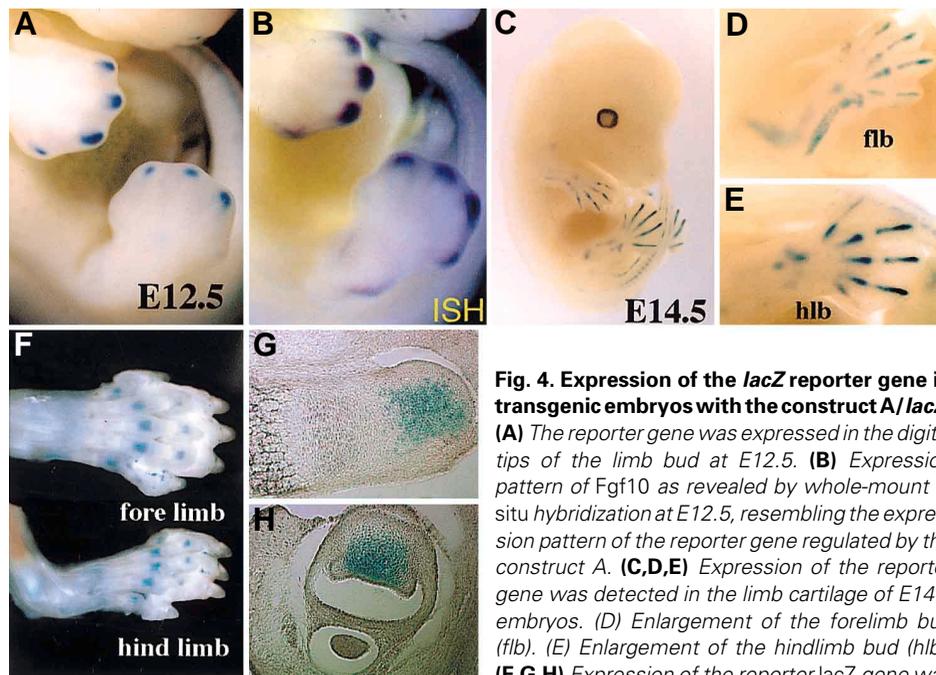
Fig. 4. Expression of the *lacZ* reporter gene in transgenic embryos with the construct A/*lacZ*. **(A)** *The reporter gene was expressed in the digital tips of the limb bud at E12.5.* **(B)** *Expression pattern of* Fgf10 *as revealed by whole-mount* in situ *hybridization at E12.5, resembling the expres-sion pattern of the reporter gene regulated by the construct A.* **(C,D,E)** *Expression of the reporter gene was detected in the limb cartilage of E14.5 embryos. (D) Enlargement of the forelimb bud (flb). (E) Enlargement of the hindlimb bud (hlb).* **(F,G,H)** *Expression of the reporter* lacZ *gene was observed in the epiphyseal growth plate of the limb cartilage of the new born limb. Semisagittal and transverse sections of the stained limb were shown in G and H, respectively.*

TABLE 1

CONSERVED ELEMENTS IN THE 5' REGION OF THE FGF10 PROMOTER BETWEEN MOUSE, HUMAN AND CHICKEN SEQUENCE

Name of family	Further Information	Conserved	RE	Opt.	Position	Strand	Core sim.	Matrix sim.	Sequence
(1) *Kpn*I / *Apa*I region									
NFAT	Nuclear factor of activated T-cells	C	1.91	0.95	220 - 231	(+)	1.000	0.967	caaagGAAAttg
MYT1	MyT1 zinc finger transcription factor involved in primary neurogenesis	C	0.21	0.88	464 - 474	(-)	1.000	0.894	aaaAAGTtcac
COUP	COUP antagonizes HNF-4 by binding site competition or synergizes by direct protein - protein interaction with HNF-4	C	0.19	0.80	465 - 478	(+)	0.977	0.875	tGAACttttactt
CMYB	c-Myb, important in hematopoesis, cellular equivalent to avian myoblastosis virus oncogene v-myb	C	2.02	0.91	478 - 495	(-)	1.000	0.917	tctcgcttccGTTGctga
VMYB	v-Myb	C	1.04	0.89	480 - 488	(+)	1.000	0.970	agcAACGga
ETSF	nuclear respiratory factor 2	C	0.16	0.86	483 - 492	(+)	1.000	0.947	aacGGAAgcg
GFI1	growth factor independence 1 zinc finger protein acts as transcriptional repressor	C	1.57	0.93	488 - 511	(+)	1.000	0.946	aagcgagaAATCaatgattcatta
PIT1	Pit1, GHF-1 pituitary specific pou domain transcription factor	C	0.55	0.85	500 - 509	(+)	1.000	0.951	aatgATTCat
AP1F	AP1 binding site	C	1.08	0.95	501 - 509	(-)	0.934	0.960	aTGAATCAt
MZF1	MZF1	C	3.84	0.97	903 - 910	(+)	1.000	0.981	ggaGGGGa
EGRF	nerve growth factor-induced protein C	C	0.03	0.79	958 - 969	(+)	0.763	0.793	tcGCGCtggtgg
AREB	AREB6 (Atp1a1 regulatory element binding factor 6)	C	1.76	0.97	1175 - 1183	(+)	1.000	0.976	ggGTTTcag
AP4R	Tal-1beta/ITF-2 heterodimer	C	0.06	0.84	1176 - 1191	(+)	1.000	0.846	ggtttCAGAtgtccca
PAX5	zebrafish PAX9 binding sites	C	0.02	0.77	1184 - 1207	(+)	0.933	0.780	atgtCCCAccgccgtttgacccccc
OCT1	octamer-binding factor 1	C	4.82	0.83	1222 - 1236	(+)	1.000	0.881	acccctgcAAATgag
COMP	COMP1, cooperates with myogenic proteins in multicomponent complex	C	0.66	0.75	1231 - 1254	(+)	0.791	0.805	aatgaggTTTGaccagcagaggca
FKHD	Fork head Related ACtivator-2	C	0.11	0.83	1302 - 1317	(-)	1.000	0.917	cgcttgTAAAcaggtt
(2) *Apa*I / *Bam*HI region									
FKHD	Xenopus fork head domain factor 3		0.04	0.82	1606 - 1619	(-)	1.000	0.870	acggtgAACAaaac
AP4R	Tal-1beta/ITF-2 heterodimer		0.06	0.84	1620 - 1635	(-)	1.000	0.892	aaaaaCAGAtgacagg
EVI1	ectopic viral integration site 1 encoded factor		<0.01	0.71	1671 - 1686	(+)	1.000	0.717	agtgAAGAagagaaca

In Conserved column, letter C represents conserved elements among mouse, human and chicken.
Position is shown by the number of nucleotide in the mouse sequence starting from the first nucleotide of the *Kpn*I site.
RE (random expectation): The RE-value for each individual matrix gives an expectation value for the number of matches per 1000 bps of random DNA sequence.
Opt. (optimized matrix threshold): This matrix similarity is the optimized value defined in a way that at most 3 matches are found in 10000 bps of non-regulatory test sequences.
Core sim. (core similarity): The "core sequence" of a matrix is defined as the (usually 4) consecutive highest conserved positions of the matrix. The core similarity is calculated as described in the MatInspector paper. The maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix match exactly in the sequence.
Matrix sim. (matrix similarity): The matrix similarity is calculated as described in the MatInspector paper. A perfect match to the matrix gets a score of 1.00 (each sequence position corresponds to the highest conserved nucleotide at that position in the matrix), a "good" match to the matrix usually has a similarity of >0.80.
Basepairs marked red are important, i.e. they appear in a position where the matrix exhibits a high conservation profile (ci-value > 60).
Basepairs in capital letters denote the core sequence used by MatInspector.

1). Since all of them is not exactly conserved in the corresponding chicken sequence, we can not exclude a possibility that unknown trans-acting factors may bind to these motifs.
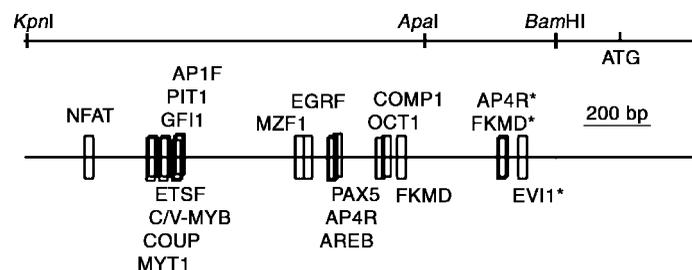

Fig. 5. Position of DNA binding motifs conserved among mouse, human and chicken in the 2.0 kb 5'-fragment of mouse *Fgf10*, *analyzed with MatInd and MatInspector (Quandt* et al.*, 1995). For details, see Table 1.* indicates that the motif is not conserved in the chicken sequence.*

Materials and Methods

Isolation of the 5'-Flanking Region of Fgf10

Mouse *Fgf10* genomic clones were isolated from a TT2 ES cell genomic library by plaque hybridization using the full- length rat *Fgf10* cDNA as a probe(Sekine et al., 1998). Chicken *Fgf10* genomic clones were obtained by hybridization screening of the genomic library in lambda phage (Clontech, USA) and used as a template for sequencing after subcloning in pBluescript.

Constructs and Generation of Transgenic Mice

We isolated a mouse genomic clone containing a 3.2 kb 5'upstream fragment of *Fgf10.* The 3.2 kb fragment was digested with *Kpn*I, *Apa*I, and *Bam*H I to 2.0 (K), 0.7 (A), 0.3 (B) kb fragments (Fig. 2). To construct transgenes, each fragment was inserted in a vector phsp68lacZpA containing the promoter of the heat shock protein 68 and *lacZ* reporter gene (Fig. 2) (Sasaki and Hogan, 1996). After excision of a transgene from the vector, the concentration of the transgenes was adjusted to 500

molecules/pl and the solution was microinjected into the male pronuclei of fertilized eggs derived from superovulated BDF1(C57BL/6 X DBA2 F1) female mice crossed with males of the same strain. Oviducts implantation of surviving injected embryos into pseudopregnant MCH/ICR female mice were carried out according to the standard protocol (Yamaoka et al., 1995). Pregnant mice after implantation were sacrificed and embryos from Embryonic day10 (E10) to E14.5 were treated by 2% paraphormaldehyde for 30 minutes. Reporter activity was analyzed by X-gal staining for over night in a CO_2 incubator at 37°C. The integration of the transgene into the mouse genome was detected by PCR, using primers established at the sequence of *lacZ* cassette and DNA extracted from tail snips of 3-week live offspring by the proteinase K/SDS method (Yamaoka et al., 1995). With the construct A, two stable transgenic lines were obtained. Newborn limbs were stained with X-gal after desquamated to enhance permeation of the X-gal, as described previously (Sasaki and Hogan, 1996).

Histology

X-gal stained newborn limbs were fixed in 2% paraphormaldehyde, dehydrated, placed in xylene, and paraffin embedded. The sections with 30μm thickness were prepared to analyze the signals at cellular level.

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