Developmental analysis of Limb development after temporal blocking of Hedgehog signaling

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Developmental analysis of Limb development after temporal blocking of Hedgehog signaling

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Ki-Bum Kim

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ABSTRACT

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The vertebrate limb skeleton develops through the endochondral bone formation, which involves in the substitution a cartilage primordium with the calcified bone matrix. The cartilage formation, called chondrogenesis, is crucial in determining the shape and size of definitive bones in vertebrate. During mouse limb chondrogenesis, mesenchymal progenitor cells are condensed differentiates into chondrocytes to form the cartilage primordium, and chondrocytes undergo sequential proliferation and differentiation along with secretion of extracellular matrix. Many studies have reported extensively the molecular mechanism on the endochondral bone formation rather than chondrogenesis. Especially, Indian hedgehog (Ihh) signaling is known to

control both hypertrophy of chondrocytes and bone replacement which is particularly important in postnatal endochondral bone formation. In this study, maternal transfer of 5E1 (a hedgehog-blocking antibody) to E12 mouse embryo causes an attenuation of the Indian hedgehog activity, and causes malformation of the mouse limb. We analysed genetic relationship during chondrogenesis in limb buds with mRNA microarray. Furthermore, we treated exogenous IHH protein into mesenchymal cells of limb bud in the micromass culture system. As a result, we found some genes interacting with *Ihh ; Glo1, Dkk4, Mapk8, Ltbp3, Col1a2, Wasl, Dmkn, Flnc, Eltd1, Egfl7, Col22a1, Krt1, Col22a1, Krt1, Col19a1, Krt10, Hspa8, Fbxo2, Ocrl, map3k4, Garnl4, Fgf7, Krt4, Klf7* were up-regulated, and *Hhip, Hoxd13, Casp1, Nrg4, Alpl, Rassf8, Foxc2, Bmp5, Ddef1, Ptch1, Sdc4, Tbx5, Runx2, Ikkbkg, Ubc, Nog, Runx3, Fgfr1op2, Nfia, Gh* were down-regulated. We suggested that *Ihh* increased condensation of mesenchymal cells and proliferation of prechondrocytes in early chondrogenesis. In conclusion, Ihh signaling positively regulates early chondrogenesis, as well as hypertrophy of chondrocytes in the mouse limb development.

Key words; mouse limb development, endochondral bone formation, 5E1, Sonic Hedgehog, Indian Hedgehog, chondrogenesis

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I. INTRODUCTION

1. Embryonic limb as a classic experimental model

The body plan is established in the early embryo by precise coordination of cell migration, proliferation and differentiation. The embryonic limb possesses two signaling centers, the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA), which produce signals responsible for

directing the proximo–distal outgrowth and anterior–posterior patterning of the limb skeletal elements, respectively. The embryonic limb has served for years as the model system for studying pattern formation and chondrogenesis[1]. Limb skeletal elements develop from a cartilage template, which is formed by the process termed chondrogenesis[2,3]. This process is crucial in determining the shape and size of definitive bones in vertebrates[4]. The development of the vertebrate limb as a functional three-dimensional structure is a complex process dependent on the interaction of various proteins, including growth factors such as fibroblast growth factors (FGFs)[5,6] and transforming growth factors (TGFs)[7-13], morphogens such as the Wnt family of secreted glycoproteins,[14-16] and transcription factors such as engrailed (En)[17], sonic hedgehog (Shh)[18,19], radical fringe (r-Frg)[20,21], and those encoded by the homeobox (Hox) gene family[22-24]. The limb develops along three axes (Fig. 1): proximodistal (shoulder/finger) defined by the FGFs[2-6], dorsoventral (knuckles/palm) defined by the interaction of Wnt-7A and the En-1 transcription factor domains[16,25], the anteroposterior (pinky/thumb) axis which is largely dependent on *Shh* and the *Hox* genes[22-34]. *Hox* genes are expressed throughout both the AER and the ZPA[23,32,35-39] and appear to respond to soluble signals emanating from the ZPA and the AER such as *Shh*[19,34], and *Fgfs*[3,4] and Indian hedgehog (Ihh), a member of the hedgehog family and a regulator of cartilage differentiation[40].

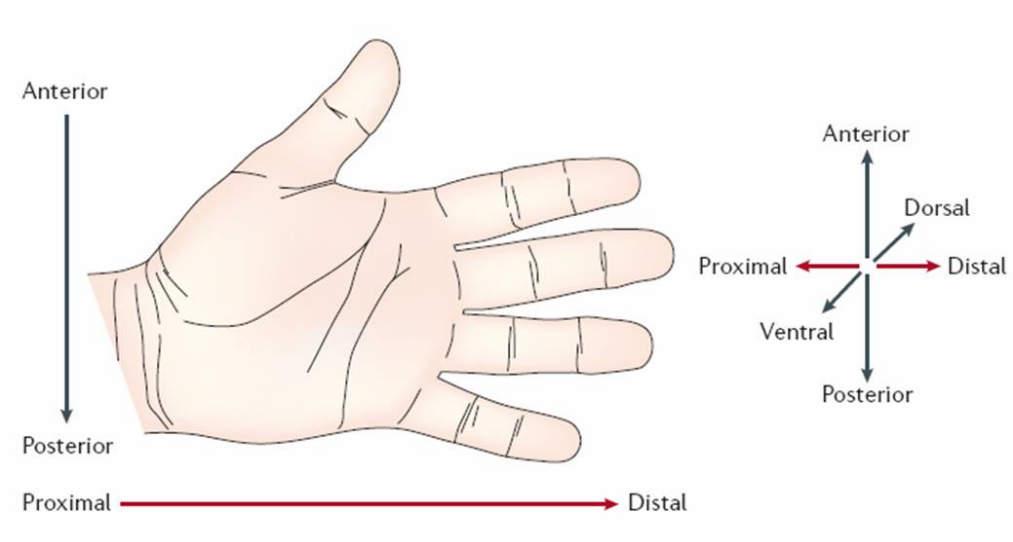

Figure 1. The three main axes of the human hand[40]. The diagram shows the three main axes, proximo-distal, antero-posterior and dorso-ventral, of a human hand. Tightly regulated processes during embryonic development ensure that the thumb arises at one edge of the hand, whereas the little finger arises at the other.

The first indication of cartilaginous condensations is apparent proximally at embryonic day 11 forelimb. The limb bud is expanding in the anterior-posterior dimension to form the presumptive hand/foot plate. The development and regression of the apical ectodermal ridge (AER) occurs time as well as the development of the skeletal structures. The marginal sinus is prominent distally and is closely associated with the AER at E12 limb bud. In this stage, alcian blue staining shows the presence of the stylopodial element (humerus/femur) and the posterior zeugopodial element (ulna/fibula) (Fig. 2).

2. Basic bone formation process

The skeletal tissues in the body are formed by either 1) intramembranous [42] or 2) endochondral ossification[43]. First, intramembranous ossification process means that the direct conversion of undifferentiated mesenchymal cells into bone. The majority of the bones of the face and skull form via intramembranous ossification. Mesenchymal cells proliferate and directly differentiate into pre-osteoblasts. As these cells mature into active osteoblasts, there is a parallel increase in alkaline phosphatase activity and synthesis of bone matrix proteins. Regulated proliferation and maturation of osteoblasts are critical features of the intramembranous ossification process. Second, events that characterize endochondral bone formation (Fig. 3): the axis, pelvis, and limbs form by endochondral ossification. The process follows; (1) during condensation of mesenchymal progenitor cell and determination of

mesenchymal cells to precursors to form pre-cartilaginous regions. The close proximity of the aggregated mesenchymal cells permits critical cell–cell surface interactions and signaling events that initiate intracellular changes culminating in the activation of overt chondrocyte differentiation. (2) Chondrocyte differentiates to form the cartilage anlagen. Differentiation of chondrogenic precursors to chondrocytes characterized by initiation of synthesis of type II collagen and cartilage-specific proteoglycans. (3) Proliferation and maturation of chondrocytes occur through periauricular chondrocyte, columnar chondrocyte, and hypertrophy. (4) Apoptosis means calcification of the cartilage matrix. Invasion of the matrix by osteoclasts, osteoblasts, and hematopoietic cells are replaced of the calcified matrix by bone. Since endochondral ossification plays a central role in the formation of the vertebrate skeleton, the numbers and shapes of the cartilaginous elements are key elements of the provisional skeleton.

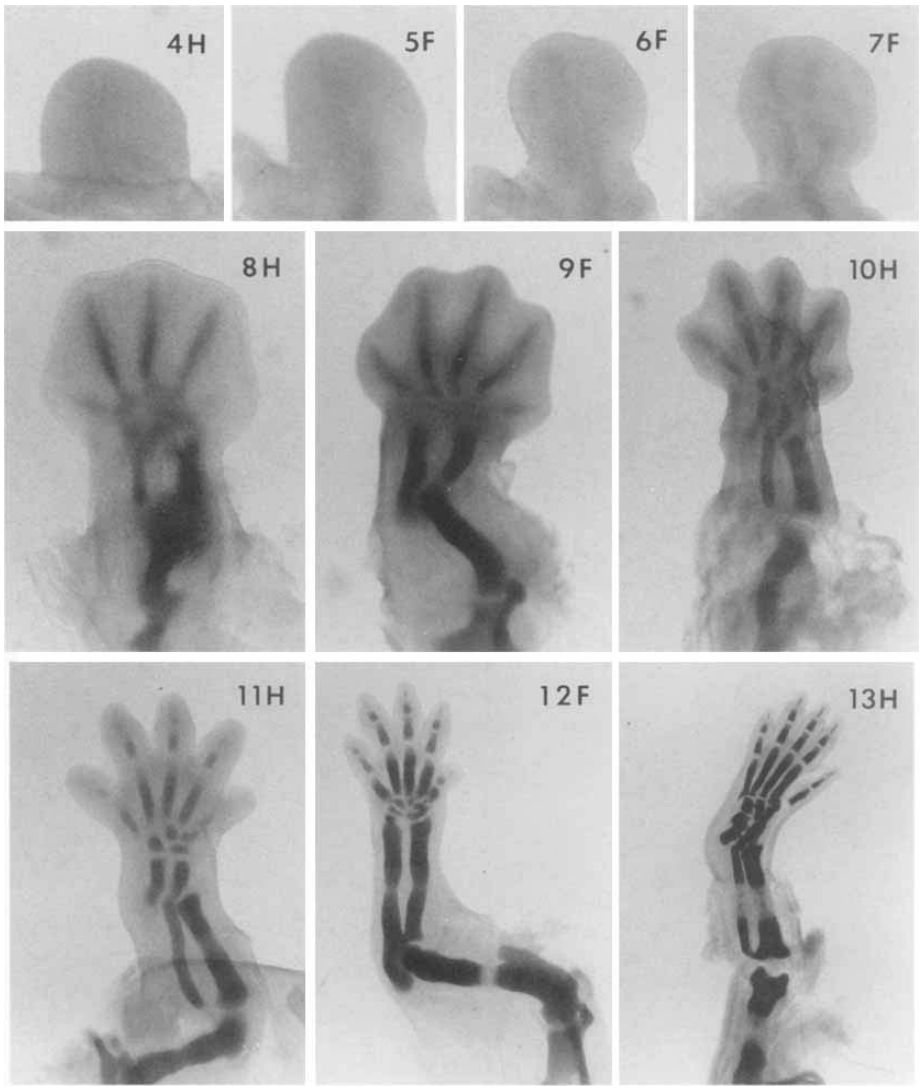

Figure 2. Alcian blue stained skeletal preparation[44]**.** Each is a dorsal view of a left limb with anterior to the right of each photograph. Numbers indicate the stage of each limb, and letters H or F denote hindlimb or forelimb, respectively. Magnifications are follows: 4H, x27; 5F, x28; 6F, x25; 7F, x22; 8H, x26, 9F, x21; 10H, x19; 11H, x17; 12F, x11, and 13H, x7.

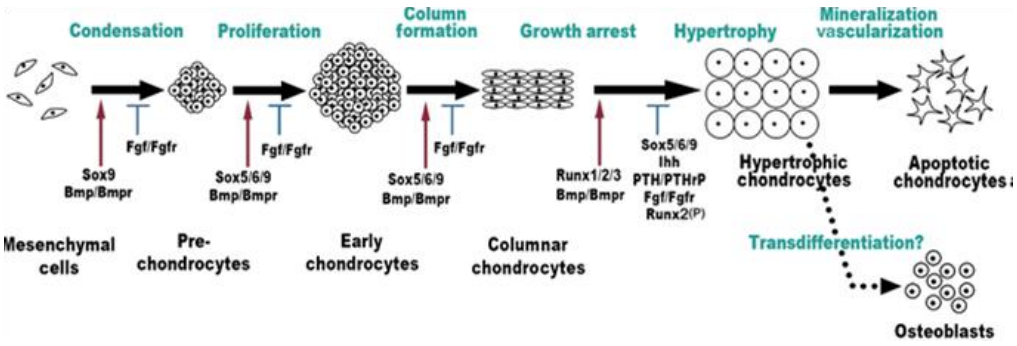

Figure 3. Schematic representation of the multistep process of chondrogenesis in the developing limb bud[45]**.** Undifferentiated mesenchymal cells derived from the lateral plate mesoderm aggregate to form condensations, which prefigure the future skeletal pattern. These cells differentiate into chondrocytes to undergo a series of differentiation processes including proliferation, hypertrophy and cell death. Proliferating chondrocytes are eventually arranged into parallel columns and subsequently exit the cell cycle to convert into hypertrophic chondrocytes. Following the onset of hypertrophy, chondrocytes direct mineralization and vascular invasion. Hypertrophic chondrocytes undergo programmed cell death and are replaced by the bone matrix. Runx2 (P) indicates the Runx2 expressed in the perichondrium.

3. Genetic interactions in the mouse limb bud

Limb bud outgrowth is driven by signals in a positive feedback loop involving fibroblast growth factor (Fgf) genes, sonic hedgehog (Shh) and Gremlin1 (Grem1) (Fig. 4). Precise termination of these signals is essential to restrict limb bud size[2-4]. A mechanism where the positive *Fgf/Shh* loop drives outgrowth and an increase in FGF signaling, which triggers the *Fgf/Grem1* inhibitory loop. The inhibitory loop then operates to terminate outgrowth signals in the order observed in either mouse or chick limb buds. This study unveils the concept of a self-promoting and self-terminating circuit that may be used to attain proper tissue size in a broad spectrum of developmental and regenerative settings[46].

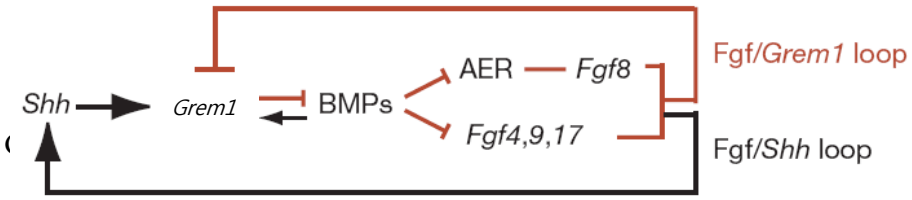

Figure 4. Model describing a self-promoting and self-terminating mechanism to control limb-bud outgrowth signals. The inhibitory loop (outlined in red) is shown in relation to the existing positive loop. Arrows indicate activation, whereas 'T'-shaped lines indicate inhibition. BMP regulation of AER architecture indirectly affects Fgf8 expression. Grem1 is also positively regulated by BMP signaling[47, 48].

4. The role of hedgehog proteins in skeletal formation

There are at least five homologous members of the hedgehog gene family: 1) Sonic hedgehog (Shh)[49-51], 2) Indian hedgehog (Ihh)[49,51], 3) Desert hedgehog (Dhh)[49] , 4) Tiggy-winkle (Twhh)[52], and 5) Echidna hedgehog (Ehh)[53]. Of these, Shh is the most well characterized gene. *Shh* plays an important role in limb patterning. *Shh* is thought to be an intrinsic product of ZPA (zone of polarizing activity) which determines the anterior-posterior axis of the limb. Chiang et al.[54] reported that loss of *Shh* function would result in simple mispatterning of the anterior-posterior limb axis. The loss of *Shh* impaired the continuous production of fibroblast growth factors (FGFs) required for the distal growth of the limb in the apical ectodermal ridge (AER)[3,4,55]. Recently hedgehog families have been shown to regulate skeletal formation in vertebrates (Fig. 5, 6)[40,56]. The amino terminal fragment of Sonic hedgehog (Shh-N), which is an active domain of *Shh***,** has the ability to induce ectopic cartilage and bone formation in vivo. *Shh*-N stimulates chondrogenic differentiation in cultures of chondrogenic cell line cells in vitro and inhibits chondrogenesis in primary limb bud cells. Indian hedgehog (Ihh) is prominently expressed in developing cartilage. Ectopic expression of *Ihh* decreases type X collagen expression and induces the up-regulation of parathyroid hormone-related peptide (PTHrP) gene expression in perichondrium cells. A negative feedback loop consisting of *Ihh* and *PTHrP,* induced by *Ihh*, appears to regulate the rate of chondrocyte maturation. *Shh* and *Ihh* stimulate alkaline

phosphatase activity in cultures of pluripotent mesenchymal cell line cells and osteoblastic cells and that these cells express putative receptors of hedgehog proteins. Hedgehog proteins seem to be significantly involved in skeletal formation through multiple actions on chondrogenic mesenchymal cells, chondrocytes, and osteogenic cells.

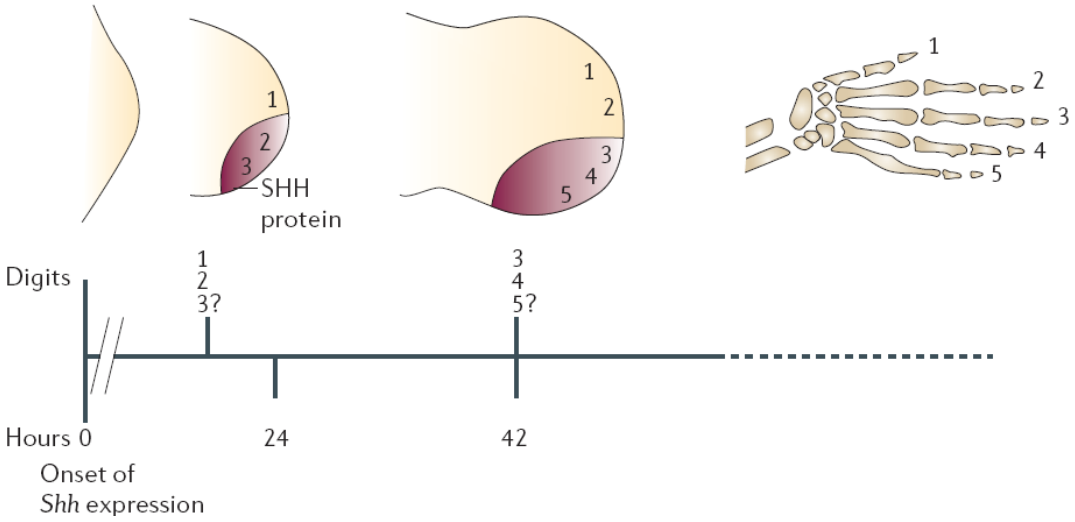

Figure 5. A new model for the specification of antero posterior values in the mouse limb that involves both the concentration and the length of exposure to SHH[40]. The numbers represent positional values for each mouse digit. Shh is expressed at embryonic day (E) 9.75. By E10.5, the SHH concentration gradient (shaded area) that was established in the early limb bud has led to the specification of the positional value of digit 2, and contributes to the specification of digit 3. The development of digit 1 is not dependent on SHH signaling. By E11.5, digit 3 is specified according to both the concentration and the length of exposure to SHH, but digits 4 and 5 are specified according to differences in the length of exposure to SHH. The timeline shows that, according to this model, posterior digits will be specified at later stages of development than anterior digits.

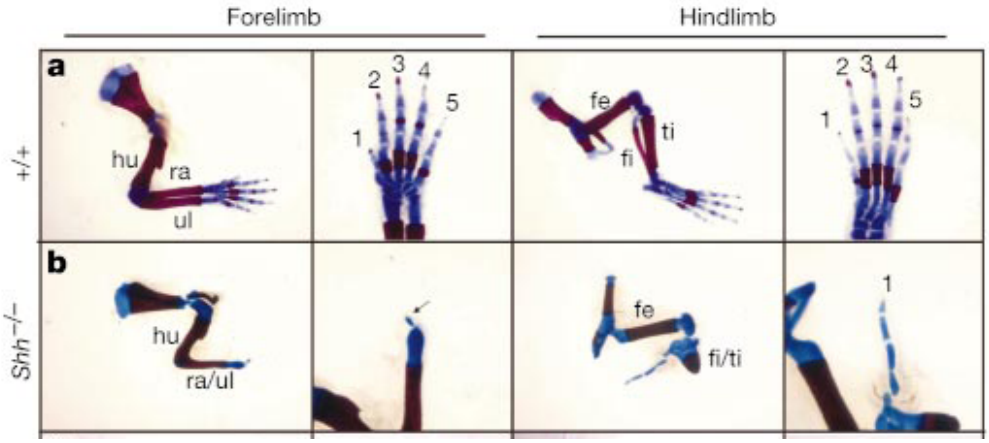

Figure 6. Skeletal analysis of E16.5 wild-type (a), Shh$^{-/-}$ (b)[56]. a, Wild-type stylopod (hu, humerus; fe, femur), zeugopod (ra, radius; ul, ulna; ti, tibia; fi, fibula) and autopod (digits 1–5) elements are labelled. b, Shh -/- stylopods are normal and zeugopods are reduced or fused. Autopods have a single unidentifiable element in the forelimb (arrow) and a digit 1 in the hindlimb.

5. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation

In *Ihh* null mouse, the abnormal long bones development can be seen (Fig 7, 8). And the chondrocyte proliferation reduced, that means proliferating chondrocytes are likely direct targets of Ihh signaling. Chondrocyte maturation and bone formation are abnormal and absence of mature osteoblasts in *Ihh* $^{-/-}$ long bones. *Ihh* acts independently of *PTHrP* to control chondrocyte proliferation, periarticular chondrocyte differentiation and osteoblast differentiation. It promotes also chondrocyte hypertrophy independently of PTHrP mediated by Bmp and Wnt/b-catenin signaling[57].

In this study, maternal transfer of 5E1 (a hedgehog-blocking antibody) to E12 mouse embryo causes an attenuation of the Indian hedgehog activity, and causes malformation of the mouse limb. We analyzed genetic relationship during chondrogenesis in limb buds with mRNA microarray and RT-qPCR. Furthermore, we treated exogenous Ihh protein into mesenchymal cells of limb bud in the micromass culture system. We found some genes interacting with *Ihh* and that *Ihh* increased condensation of mesenchymal cells and proliferation of prechondrocytes in early chondrogenesis. In conclusion, Ihh signaling positively regulates early chondrogenesis, as well as hypertrophy of chondrocytes in the mouse limb development.

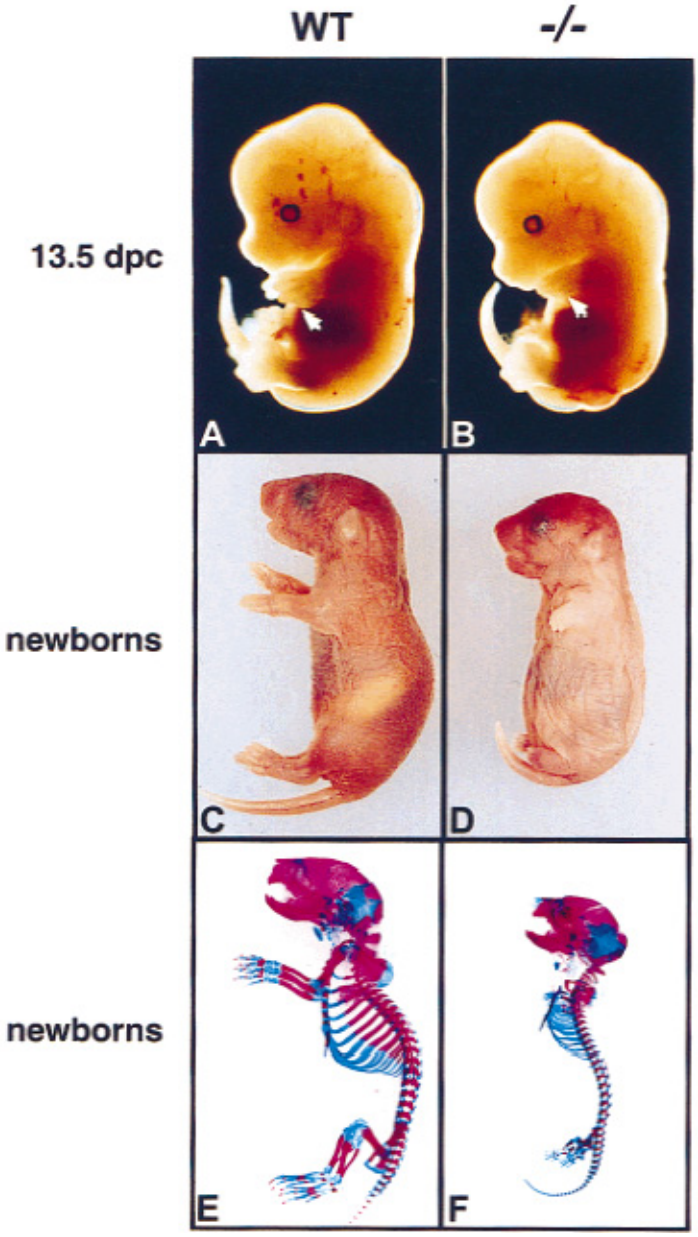
WT
-/-
13.5 dpc
newborns
newborns
A
B
C
D
E
F

Figure 7. Phenotype of *Ihh* mutant mice[57]**.** (A, C, E) Wild-type animals (*Ihh* $^{+/+}$); (B, D, F) mutant animals (*Ihh* $^{-/-}$). (A, B) 13.5-dpc embryos. The forelimb in the mutant embryo is significantly shorter than in the wildtype (white arrows). (C, D) Gross appearance of newborns. Note the rounded skull, shortened face and tail, and the severe dwarfism of the limbs in the mutant. (E, F) Skeletons of newborns stained with Alcian blue (cartilage) and Alizarin red (calcified tissue). General patterning of the skeleton is normal but all elements of the axial and appendicular skeletons are significantly reduced in size in the mutant.

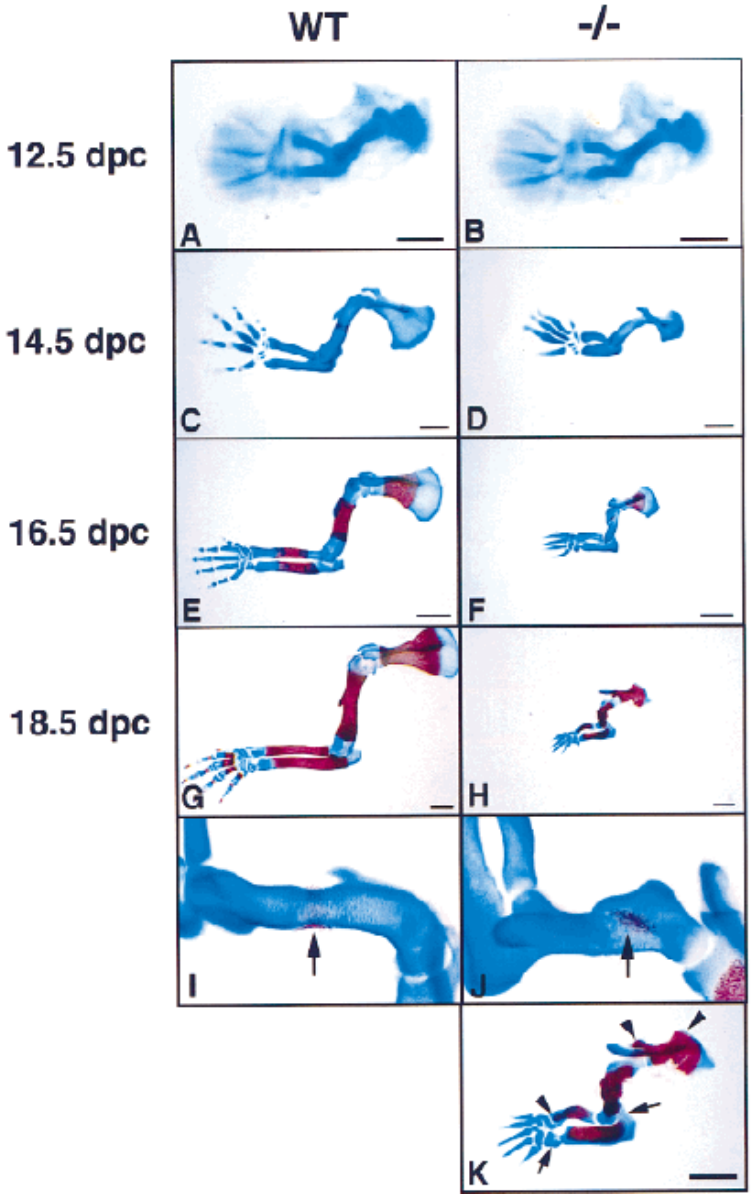
WT
-/-
12.5 dpc
14.5 dpc
16.5 dpc
18.5 dpc
A
B
C
D
E
F
G
H
I
J
K

Figure 8. Development of the forelimb skeleton[57]**.** (A, C, E, G, I) Wild-type forelimbs; (B, D, F, H, J, K) *Ihh* -/- forelimbs. In A and B forelimbs were stained with Alcian blue. In C–K forelimbs were stained with Alcian blue and Alizarin red. (A, B) 12.5-dpc limbs. Note that the initial cartilage elements form normally in the mutant. (C, D) 14.5-dpc limbs. (E, F) 16.5-dpc limbs. (G, H) 18.5- dpc limbs. An obvious difference in length of the long bones visible at 14.5-dpc becomes progressively more severe, indicating a failure of the growth process in the mutant. (I) Higher magnification of the wild-type humerus shown in C. Arrow indicates formation of a bone collar. (J) Higher magnification of the $Ihh^{-/-}$ humerus shown in F. Arrow indicates calcification in the center of the cartilage only. (K) 18.5-dpc $Ihh^{-/-}$ limb at higher magnification. Arrowheads indicate areas of ectopic calcification. Arrows indicate incomplete joint formation. (A–D) Bar 0.5 mm; (E–H, K) bar 1 mm.

II. MATERIALS AND METHODS

All experiments were performed according to the guidelines of the Yonsei University, College of Dentistry, Intramural Animal Use and Care Committee.

1. Experimental animals

Adult Institute of Cancer Research (ICR) mice were housed in a temperature-controlled room (22°C) under artificial illumination (lights on from 0500 to 1700 hours) and 55% relative humidity. The mice had access to food and water ad libitum. Embryos were obtained from time-mated pregnant mice. E0 was designated as the day a vaginal plug was confirmed. Embryos at developmental stages E10 to and E14 were used in this study.

2. Micromass culture

The limb buds from E12 were dissected in calcium-/magnesium-free saline (CMF), placed in 1.2 unit/ml dispase II (Roche) in CMF at 37°C for 25 mins, and then wash and ectoderm removed using tungsten needle. Mesenchymal cells were gently dissociated in 1-2 ml medium to produce a single cell suspension. Cells were suspended at 2 X 10^7 cells per ml in all mixture of 60% culture medium (Nutrient mixture F-12 Ham with 10% fetal calf serum, 1-4

mM-L-glutamine, 1% penicillin streptomycin and 200 ug/ml ascorbic acid with 40% CMF containing 10% newborn calf serum). A single 10 ul drop of this suspension was plated on to each 35 mm tissue culture dish, and incubated at 37°C in an atmosphere of 5% CO_2/95% air in a humidified incubator. Cells were allowed to attach for 50min and then flooded with 1-2 ml culture medium. Cultures received fresh medium daily.

3. Ihh protein overexpression in limb bud micromass cultures

For hedgehog inhibition experiments, cells were treated with 130 ug/ml 5E1. The Ihh overexpression experiments were carried out with 500 ng/mL IHH (R&D Systems) After 4days, the number of mesenchymal aggregates was assessed, and then, cultures were fixed in 4% PFA in CMF and stained with Alcian blue to assess the extent of chondrogenesis.

4. Alcian blue staining and quantitation of chondrogenesis

Micromass cultures were fixed with 2% PFA, washed in PBT and stained with 1% Alcian Blue 8GX (Sigma) in 0.1 N HCl, pH 1 for 5hours (Lev and Spicer, 1964). Cultures were then de-stained with 70% ethanol. Alcian blue incorporated into the cell matrix was extracted with 0.5 ml of 4 M guanidine HCl (pH 5.8), and quantified by measuring absorbance at OD600 nm (Lev and Spicer, 1964). The statistical significance of the difference in

Alcian blue staining between control and experimental micromass cultures was assessed using the nonparametric Wilcoxon signed rank test (Ostle, 1975). A value of $P<0.01$ was considered to reflect a statistically significant difference.

5. Whole mount *In Situ* Hybridization

5.1 Embryo processing

Samples which sacrificed at 1day after 5E1 and PBS (phosphate-buffered saline) injection were fixed 24 hours in 4% paraformaldehyde in PBS hand dechorionated and dehydrated overnight in methanol at -20°C.

5.2 Riboprobe synthesis

The plasmid was linealised, to allow transcription, using an appropriate restriction endonuclease. 50 µg of DNA was digested in a final volume of 100 µl of the appropriate 10 X buffer, 2~3 µl of restriction endonuclease and rest RNase free water. This was incubated at 37°C for 2 hours before running aliquot on a 1% agarose gel to check the DNA was completely linealised. The linealised DNA was cleaned by Qiagen Clean up Kit (Qiagen, USA)

Riboprobe were made in batches of 20 µl as follows:

4 µl 5 X buffer

4 µl 0.1 M DTT

x µl linealised DNA (to give approximately 5 µg DNA present)

1.5 µl Dig RNA

2 µl Polymerase

2 µl RNase inhibitor

RNase free water was added to a final volume of 20 µl

The mixture was incubated at 37°C for 2 hours before running aliquot on a 1% agarose gel to check the presence of an RNA band. Then, treat with 4 µl DNaseI RNase free and incubated at 37°C for 30 mins.

RNA purification

a) 15 µl 3 M sodium acetate and add 125 µl 00% cold EtOH

b) Incubate at -20°C for 30 mins

c) Centrifuge at 4°C, 1200 rpm for 10 mins

d) Discard the flowing through

e) Wash with 500 µl 70% cold EtOH

f) Discard the flowing through

g) Centrifuge at 4°C, 1200 rpm for 10 mins

h) Step e~g

i) Drying up all of EtOH

j) Redissolve in 40 µl RNase free water

k) Storage at 70°C

Before use Riboprobe, incubate at 85°C for 4 mins and 2 mins in ice.

5.3. Pre-hybridization treatment and hybridization

Then the samples were rehydrated stepwise in methanol/PBS and finally put back in 100% PBT (PBS with 0.1% Tween 20). Embryos older than the beginning of somitogenesis were treated with proteinase K (10 mg/ml in PBT). Samples were postfixed in 4% paraformaldehyde in PBS for 20 minutes and then rinsed in PBT 5 times for 5 mins each. The embryos were prehybridized at least 1 hour at 70°C in hybridization buffer [50% formamide, 5 X SSC, 50 mg/ml heparin, 500 mg/ml tRNA, 0.1% Tween 20]. The hybridization was done in the same buffer containing 200 ng of probe overnight at 70°C.

5.4 Post-hybridization treatment

Then the samples were washed at 70°C for 2 times 15 mins in 2 X SSC, 2 times 30 mins in 10% CHAPS, 2 X SSC, 2 times 15 minutes in 10% CHAPS, 1 X SSC, 2 times 15 mins in 10% CHAPS, 0.2 X SSC, 2 times 15 mins in 10% CHAPS, 0.1 X SSC. Further washes were performed at room temperature for 2 times 5 mins in TBT (1 M Tris (pH7.5), 5M NaCl, DEPC-H_2O), and then 3 hs in TBT with 2 mg/ml FBS (Fetal Bovine Serum (Clontech, USA)). Then the samples were incubated overnight at 4°C with the preabsorbed alkaline phosphatase-coupled anti-digoxigenin antiserum (described in Boehringer instruction manual) at a 1/5000 dilution in TBT buffer containing 2 mg/ml FBS. Finally the samples were washed 2 times for 15 minutes each in TBT at room temperature. Detection was performed in alkaline phosphatase reaction buffer described in the Boehringer instruction manual. When the color was developed, the reaction was stopped in 1 X PBS.

5.5 Solutions and buffers for wholemount *in situ* hybridization

All solutions were autoclaved where appropriate.

PBT

PBS, 0.1% (v/v) Tween-20 (Sigma, USA)

Prehybridization solution (for 50 ml)

25 ml deionized formamide (50% final)

12.5 ml 20X SSC, pH 4.5 (25% final)

50 µl 10 mg/ml Heparin stock (50 µg/ml final) (Sigma, USA)

50 µl 50 mg/ml Yeast-tRNA (50 µg/ml final) (Sigma, USA)

250 µl 1 M EDTA (5 mM final; ethylenediamine tetra acetic acid)

200 µl Tween-20 (0.4% final)

1 g Boheringer Blocking Powder (2% final)

0.5 ml CHAPS (0.1% final, 10% stock in RNase free water) (Sigma, USA)

RNase free water was added to a final volume of 50 ml

20X SSC

3 M NaCl, 0.3 M sodium citrate, pH 4.5 with citric acid

Blocking solution

PBT, 15% heat-inactivated lam serum

TBT (for 1 L)

50 ml 1 M Tris-Cl (pH 7.5)

33.33 ml 5 M NaCl

10 ml Tween-20

RNase free water was added to a final volume of 1 L

NTMT (for 50 ml)

5 ml 1 M Tris-Cl (pH 9.5)

1 ml 5 M NaCl

2.5 ml 1 M MgCl2

0.5 ml Tween-20

41 ml T.D.W.

6. Alcian blue and Alizarin red skeletal staining

Mice from 1day after 5E1 and PBS injection, PN 1day, PN 3week, and PN 6week with PBS and 5E1 maternal injection were fixed in 95% ethanol for 12-48 hs slowly rocking at room temperatures. All procedures were followed by Eddy M. De Robertis staining methods.

Alcian blue

0.03% Alcian blue

80% Ethanol

20% Acetic acid

Alizarin red

0.03% Alizarin red

1% KOH

Water

7. Microarray analysis

Gene-chip expression analysis was performed with RNA from limb bud from embryos of pregnant mice at 1day after injection (PBS, *n*=2; 5E1, *n*=2),

using a mouse gene microarray (Gene Chip Mouse Genome 430 2.0, Affymetrix, Santa Clara, CA, USA). A gene-chip scanner (Gene Chip Scanner 3000, Affymetrix) was used to measure the intensity of the fluorescence emitted by the labelled target. Raw image data were converted to cell-intensity (CEL) files using the Affymetrix Gene Chip Operating System, and these resultant CEL files were normalized using the MARS 5.0 algorithm. Following statistical analysis, differentially expressed genes were selected using GenePlex software version 3.0 (ISTECH, Korea). Differentially expressed genes with changes of 1.5- to 2.0-fold in the 5E1-treated group compared with the control group were selected, and then analysed statistically using Student's *t*-test with the level of statistical significance set at $P<0.01$.

8. 5E1 purification

A monoclonal antibody (mAb) to 5E1 (monoclonal anti-Shh antibody) and an mAb to 40-1a (anti-β-galactosidase antibody) were obtained from hybridoma cells at the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA, USA). A single injection of 5E1 (10 mg/kg body weight), or PBS (1 ml) was administered intraperitoneally to pregnant ICR mice at the stage of embryonic day (E) E12

9. Micro CT analysis

Specimens were PBS and 5E1 transferred mice limbs at PN 6week. Specimens were scanned with a micro computed tomography (Micro-CT, Skyscan 1076, Skyscan, Antwerp, Belgium). Serial 2D images gained by Micro-CT were cross-sectional 1968*1968 pixel images. From 2D images (pixel: 35X35 ㎛), a 3D structural image with voxels 35x35x35 ㎛ in size was reconstructed. After reconstructing the 3D image of the PN 6week limb, the specimens were measured bone density, trabecular separation, trabecular thickness, and trabecular number with an Image analysis software "Image-Pro®Plus version 4.0" automatically. These results were statistically analyses using "Microsoft Office Excel" in two groups: (1) PBS transferred group, and (2) 5E1 transferred group. The mean and standard deviation were calculated for each parameter, and a T-test was carried out to examine whether value differences between parameters, such as the presence of tooth and sex, were significant (at 95% confidence interval, $P<0.05$). The analysis of variance (ANOVA) was also used to determine whether differences between regions were statistically significant (at 95% confidence interval, $P<0.05$).

III. RESULTS

1. The mouse morphology of blocking hedgehog signaling

Maternal transfer of 5E1 (a hedgehog-blocking antibody) to E12 mouse embryo causes an attenuation of hedgehog activity, and causes malformation of multiple mouse organs. Hedgehog-blocked PN 1week mouse has very keratinized skin, bended tail and shorter limbs (Fig. 9 A). In addition, its eyes and ears developed with covered skin. Hedgehog-blocked PN 3 week mouse, compared with unblocked mouse which usually re-open its eyes at PN 12day, could not re-open its eyes and ears with covered with skin. Moreover, its hind limb and fore limb outgrew, but still shorter (Fig. 9 B). Hedgehog blocked PN 6week mouse has normal morphology of its limbs, except for their length. It has shorter forelimbs and hindlimbs. And one mouse could not re-open its eyes until this stage (Fig. 9 C). All of 5E1 maternal transferred mice has shorter limbs and are smaller than PBS transferred ones (data not shown).

2. *In situ* hybridization with Hedgehog families such as *Shh, Ihh, Ptch1, Gli1 and Gli3* from E10 to E14.

Shh expressed at ZPA region at E10, and disappeared from E11 (Fig. 10 A). *Ihh* expression could be found metacarpal region at E11 and in the middle part

of cartilage from E12 (Fig 10 B). Therefore, in this study, *Ihh* expression pattern not *Shh* expression pattern is proper for the condensation stage, E12. The maternal transferring of 5E1 leads to block hedgehog signaling, especially hedgehog signaling. *Ptch1* expressed in the posterior part of E10 limbs. At E11, the expression was appeared at metacarpal region and remained at posterior part. From E12, *Ptch1* expression was found at condensing mesenchyme region (Fig 10 C). *Gli1* was found positively at posterior part, and negatively at anterior part (ZPA region where *Shh* expressed) of E10 mouse limb, and was found metacarpal and phalange regions at E11. From E12 to E14, the expression of *Gli1* was found at phalange regions (Fig 10 D). *Gli3* was found at anterior part, and spread out hand plate. From E12, the expression of *Gli3* was shown at the tip of hand plate, and at E13 and E14, *Gli3* expressed around phalanges (Fig 10 E).

Figure 9. The morphology of hedgehog signaling blocked mouse. 5E1 (Hedgehog signaling blocking antibody) was injected at E12. (A) At PN 1week, keratinized skin, bended tail, and short limbs were shown. Moreover, their ears and eyes were covered with the skin. (B) At PN 3week, their limbs were grown, but still short. Their ears and eyes were not open, neither. (The mouse usually re-opens its eyes at PN 12 days) (C) At PN 6week, limbs were still shorter. And one mouse could not re-open its eyes until this stage.

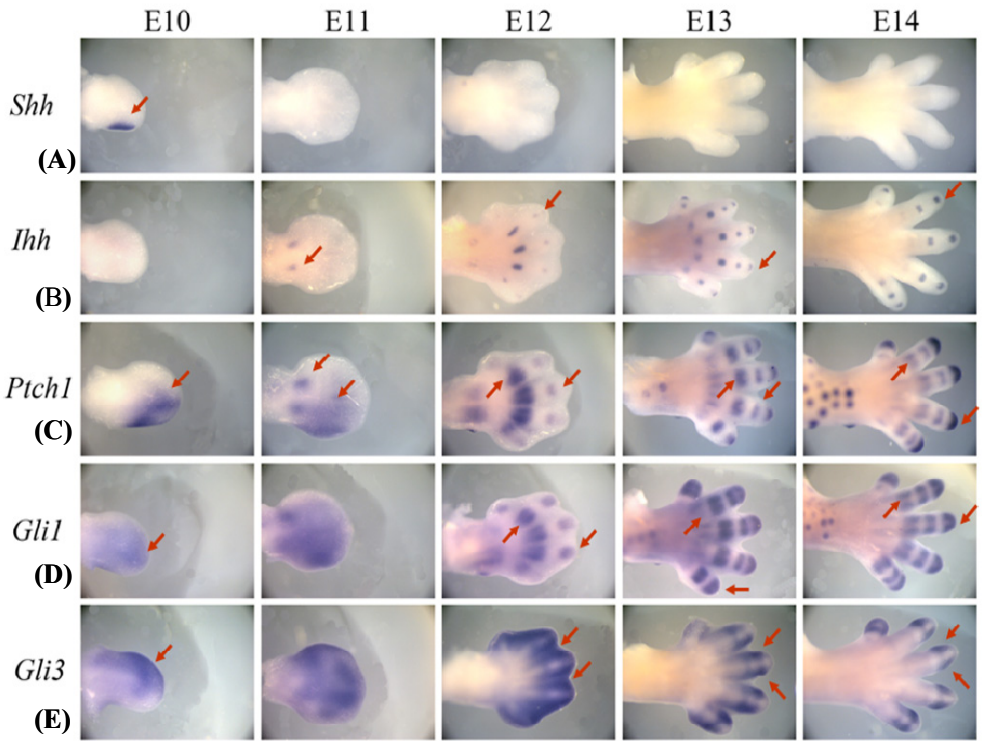

Figure 10. *In situ* hybridization with Hedgehog families from E10 to E14. (A) *Shh* expressed in ZPA region at E10 and disappeared from E11. (B) *Ihh* expression appeared at E11 metacarpal region. After E12 to E14, *Ihh* expression could be found at phalange regions. (C) *Ptch1* expression appeared at posterior part. At E11, the expression was shown at metacarpal and remained at posterior part. From E12, *Ptch1* expression was found at condensing mesenchyme region. (D) *Gli1* was positive at posterior part at E10 and metacarpal and phalange regions at E11. From E12 to E14, the expression of *Gli1* was found at phalange regions. (E) *Gli3* was found at anterior part, and spread out hand plate. From E12, the expression of *Gli3* was shown at the tip of hand plate, moreover, at E13 and E14, *Gli3* expressed around phalanges.

3.The limb analysis of Hedgehog blocking mouse; 5E1 injection at E12

3.1. Skeletal staining with alcian blue and alizarin red

Temporal hedgehog-blocked by 5E1 at E12 led to short limb. To analyze cartilage and bone of PBS injected and 5E1 injected mouse, alcian blue and alizarin red staining were performed. As a result, the ossification center already set in both PBS and 5E1 injection mice (Fig. 11 A) in 2days after hedgehog blocking at E12, however, they had surely shorter limb than control group. Their limbs of PN 1day, PN 3week and PN 6week by blocking *Ihh* were short but have 5 phalanges (Fig. 11 B-E).

3.2. MicroCT analysis

The pattern that the length of limb of 5E1 transferred was shorter than PBS injected ones as same skeletal staining results as the microCT analysis (Fig. 12 A). In *Ihh* blocked group, the length of humerous was shorter as 22.5% at PN 6week, the ulna as 15.4% and radius as 25.4% (Fig. 12 B). Moreover, bone density as 42.5%, trabecular separation as 37%, trabecular thickness as 28.5%, and trabecular number as 18.3% were down after blocked Ihh signaling (Fig. 12 C).

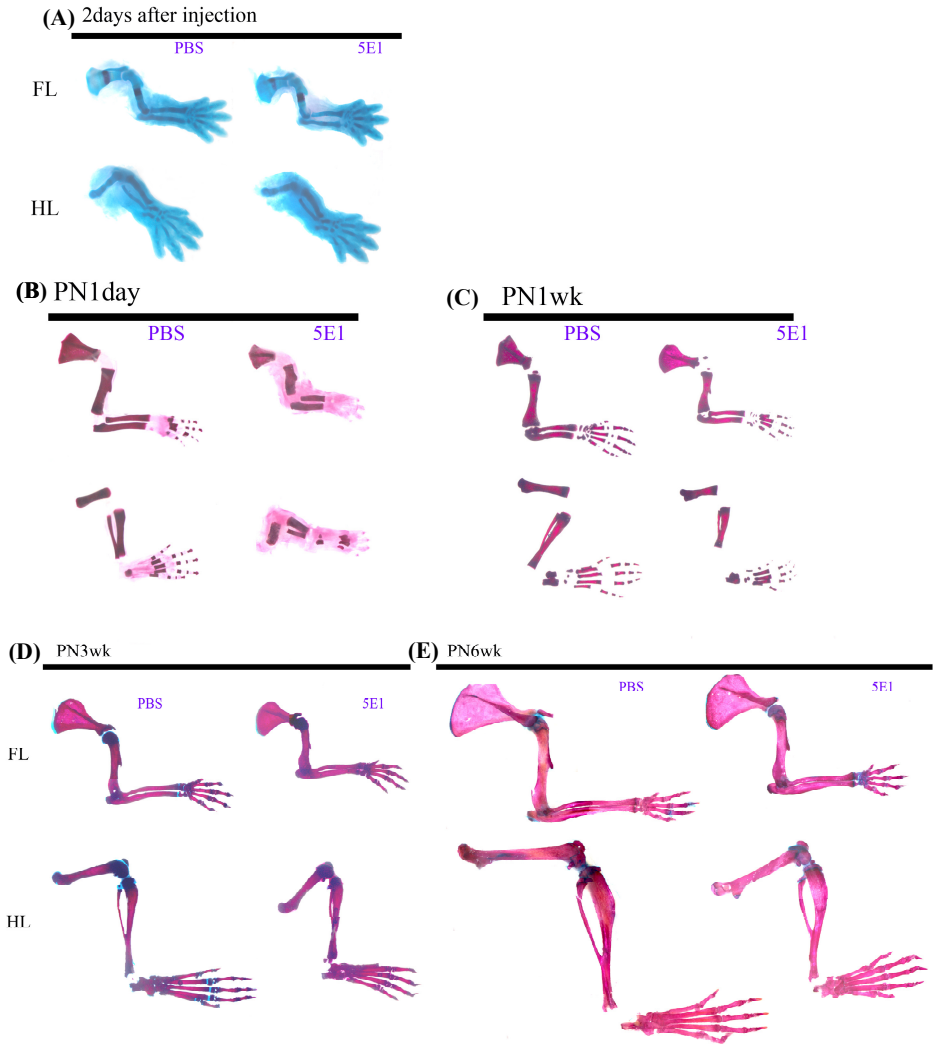

Figure 11. The skeletal staining with alcian blue and alizarin red at 2 days after 5E1 injection at E12 (A), PN 1day, (B) PN 1week, (C) PN 3week and (D) PN 6week (E). 5E1 transferred mice has shorter not only fore limb but also hind limb. The bones grow well like PBS injected mice.

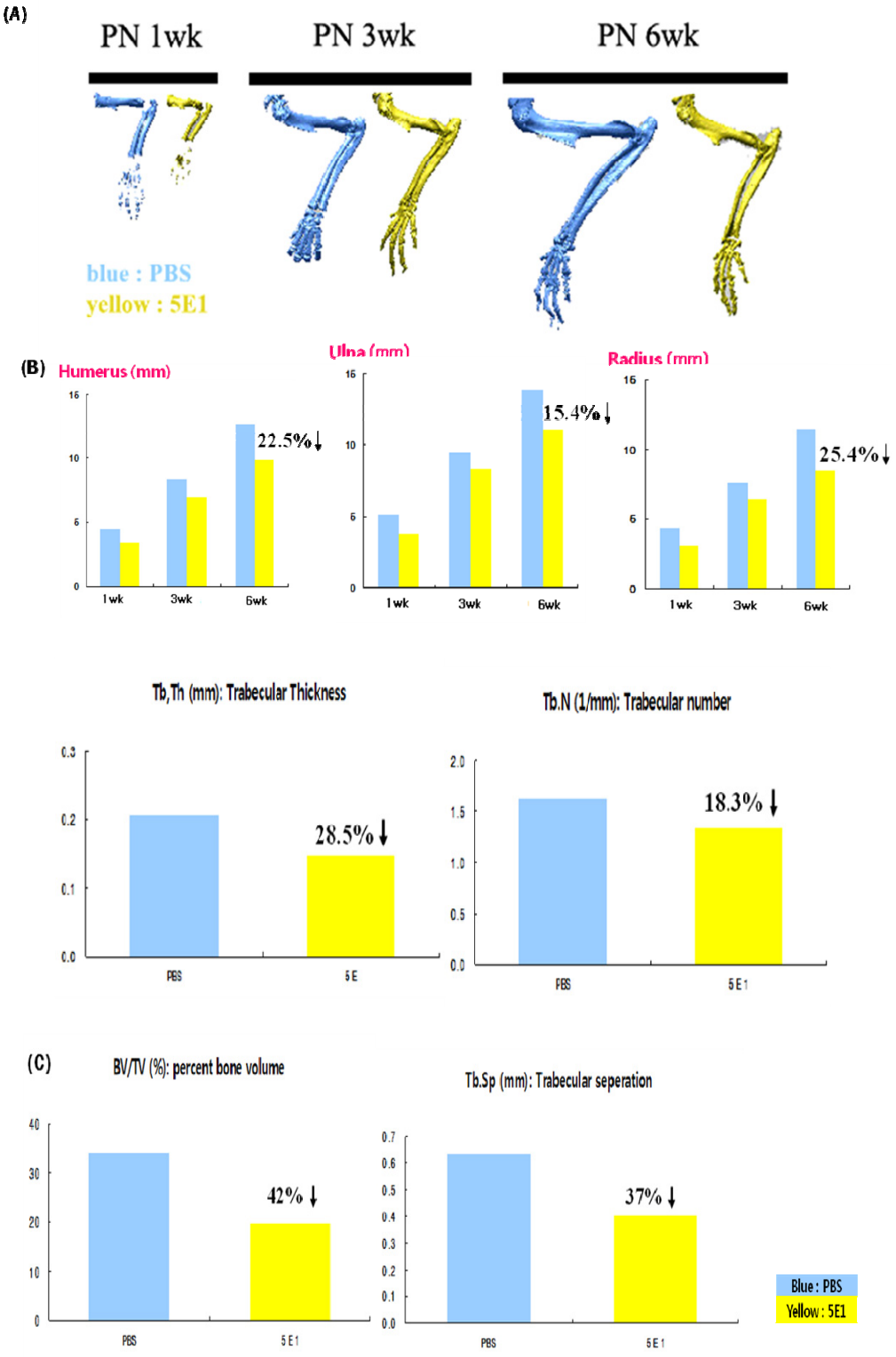
(A)
PN 1wk
PN 3wk
PN 6wk
blue : PBS
yellow : 5E1
(B)
Humerus (mm)
22.5%↓
Ulna (mm)
15.4%↓
Radius (mm)
25.4%↓
1wk
3wk
6wk
Tb,Th (mm): Trabecular Thickness
28.5%↓
Tb.N (1/mm): Trabecular number
18.3%↓
PBS
5E
5E1
(C)
BV/TV (%): percent bone volume
42%↓
Tb.Sp (mm): Trabecular seperation
37%↓
Blue : PBS
Yellow : 5E1

Figure 12. MicroCT analysis for length of the limb. (A) The length of limb of 5E1 transferred was shorten than PBS injected ones as same skeletal staining results as the microCT analysis. (B) In *Ihh* blocked group, the length of humerous was shorter as 22.5% at PN 6week, the ulna as 15.4% and radius as 25.4%. (C) Bone density, trabecular separation, trabecular thickness, and trabecular number were down as 42.5%, 37%, 28.5%, and 18.3%, respectively, after blocked Ihh signaling

4. The successful blocking Ihh signaling by 5E1

We can check the successful blocking Ihh signaling by 5E1 by in situ hybridization with Hedgehog family and its downstream genes such as *Ptch1, Gli1* and *Gli3* at 1day after blocking *Ihh* at E12. Because the 5E1 antibody can block Ihh protein, *Ihh* expression pattern was not changed (Fig 13 A, B). *Ptch1* and *Gli1* were dramatically down regulated by blocking Ihh protein activity (Fig 13 C-F). *Gli3* was not changed (Fig 13 G. H). Therefore, 5E1 antibody can block successfully Ihh signaling.

5. Role of Ihh protein on condensation of limb bud mesenchymal cells

By the micromass culture system, to investigate a role of Ihh protein, exogenous 500 ng/ml Ihh protein caused produce more cartilage nodules by increasing condensation of mesenchymal cells and proliferation of prechondrocytes in early chondrogenesis. 130 ug/ml 5E1 transferred mesenchymal cells of limb bud slightly decrease condensation and proliferation. (Fig 14)

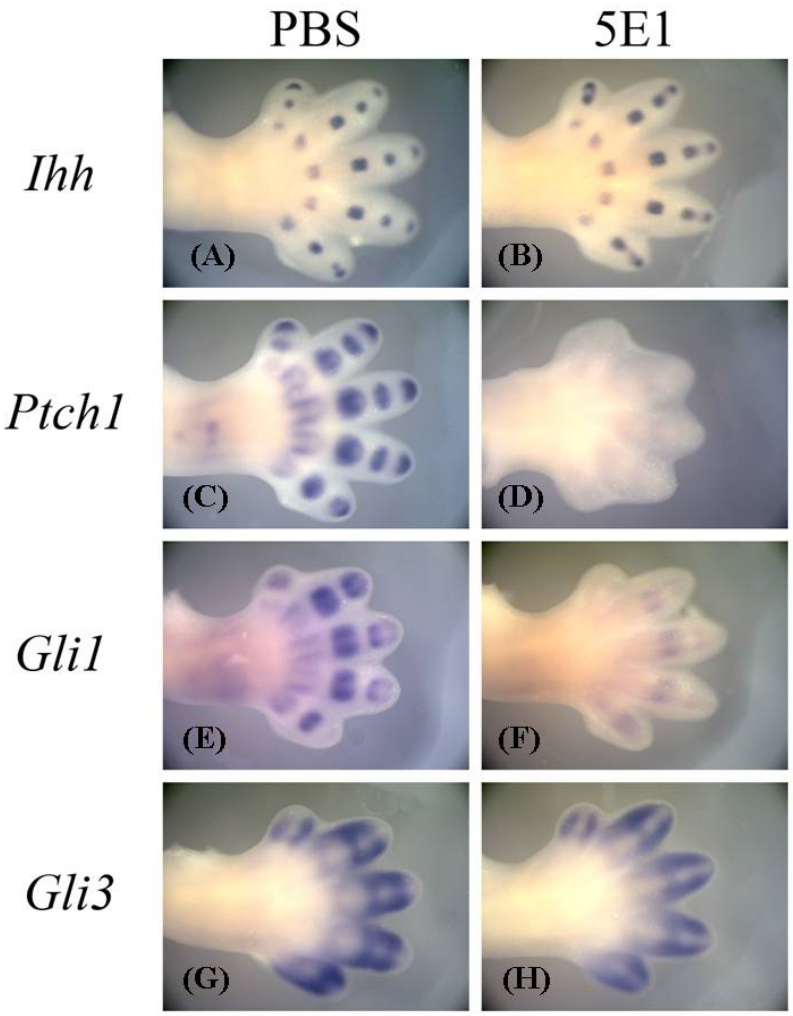

Figure 13. *In situ* hybridization of *Ihh, Ptch1, Gli3* and *Gli1* when blocking hedgehog signaling: after 1day of 5E1 injection at E12. (A, B) *Ihh* expression pattern was not changed. (C-F) *Ptch1* and *Gli1* were down regulated by blocking Ihh protein activity. *Gli3* did not change.

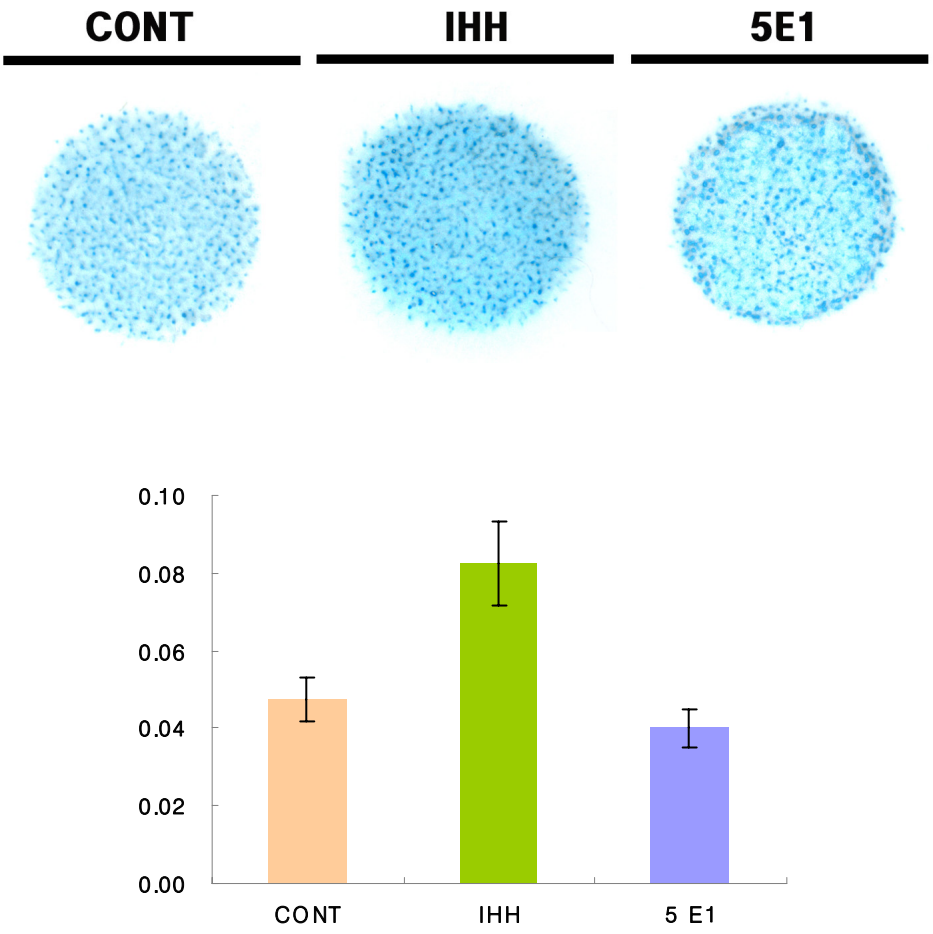

Figure 14. Micromass culture of E12 mesenchymal limb bud cells. Exogenous 500 ng/ml Ihh protein led to produce more cartilage nodules by increasing condensation of mesenchymal cells and proliferation of prechondrocytes in early chondrogenesis. 130 ug/ml 5E1 treated mesenchymal cells of limb bud slightly decrease condensation and proliferation.

6. Analysis of the relationship *Ihh* with other genes by microarray analysis : 5E1 injection at E12

The results of microarray analysis suggested that *Glo1, Dkk4, Mapk8, Ltbp3, Col1a2, Wasl, Dmkn, Flnc, Eltd1, Egfl7, Col22a1, Krt1, Col22a1, Krt1, Col19a1, Krt10, Hspa8, Fbxo2, Ocrl, map3k4, Garnl4, Fgf7, Krt4, Klf7* were up-regulated, and *Hhip, Hoxd13, Casp1, Nrg4, Alpl, Rassf8, Foxc2, Bmp5, Ddef1, Ptch1, Sdc4, Tbx5, Runx2, Ikkbkg, Ubc, Nog, Runx3, Fgfr1op2, Nfia, Gh* were down- regulated. Especially, in the down-regulated results, *Hhip* and *Ptch1,* which are the members of hedgehog signaling, were down-regulated, that means this microarray might be trustful and 5E1 inhibited successfully hedgehog signaling (Fig 15).

(A)

Up-regulated genes
Glo1, Dkk4, Mapk8, Ltbp3, Col1a2, Wasl, Dmkn, Flnc, Eltd1, Egfl7, Col22a1, Krt1, Col19a1, Krt10, Hspa8, Fbxo2, Ocrl, map3k4, Garnl4, Fgf7, Krt4, Klf7
Down-regulated genes
Hhip, Hoxd13, Casp1, Nrg4, Alpl, Rassf8, Foxc2, Bmp5, Ddef1, Ptch1, Sdc4, Tbx5, Runx2, Ikkbkg, Ubc, Nog, Runx3, Fgfr1op2, Nfia, Gh

(B)

gene_symbol	Gene_Title	Reference Sequence	limb 5E1 VS PBS
Up-regulated Genes			
Glo1	glyoxalase 1	NM_025374	4.067209485
Dkk4	dickkopf homolog 4 (Xenopus laevis)	NM_145592	2.52899827
Glo1	glyoxalase 1	NM_025374	1.89773099
Ltbp3	latent transforming growth factor beta binding protein 3	NM_008520	1.877562262
Col1a2	collagen, type I, alpha 2	NM_007743	1.815162362
Wasl	Wiskott-Aldrich syndrome-like (human)	NM_028459	1.762941375
Krt1	keratin 1	NM_008473	1.643410056
Krt10	keratin 10	NM_010660	1.613671595
Fgf7	fibroblast growth factor 7	NM_008008	1.533121941
Krt4	keratin 4	NM_008475	1.515976038
Down-regulated Genes			
Hhip	Hedgehog-interacting protein	NM_020259	-1.508230782
Hoxd13	homeobox D13	NM_008275	-1.509049678
Foxc2	forkhead box C2	NM_013519	-1.538395396
Ptch1	patched homolog 1	NM_008957	-1.572028638
Sdc4	syndecan 4	NM_011521	-1.595889763
Tbx5	T-box 5	NM_011537	-1.60623335
Runx2	runt related transcription factor 2	NM_009820	-1.707060867

Ubc	ubiquitin C	NM_019639 /// XM_001471699	-1.760649824
Nog	noggin	NM_008711	-1.800504743
Runx3	runt related transcription factor 3	NM_019732	-1.94216847
Fgfr1op2	FGFR1 oncogene partner 2	NM_026218	-2.017904991

Figure 15. The microarray analysis. (A) Up-regulated and down-regulated genes are shown. (B) *Hhip* and *Ptch1* (blue box) were down-regulated in 5E1 maternal transferred group.

IV. DISCUSSION

The axis, pelvis, and limbs are formed by endochondral ossification. During the condensation of the mesenchymal progenitor cells and their determination into precursors forming pre-cartilaginous regions, chondrocytes differentiate to form the cartilage anlagen. Proliferation and maturation of chondrocytes occur through periarticular chondrocyte, columnar chondrocyte, and hypertrophy. Apoptosis leads calcification of the cartilage matrix. Invasion of the matrix by osteoclasts, osteoblasts, and hematopoietic cells replaces the calcified matrix with bone. Since endochondral ossification plays a central role in the formation of the vertebrate skeleton, the numbers and shapes of the cartilaginous elements are key elements of the provisional skeleton[45]. When Hedgehog signaling in E12 mouse embryo is temporally blocked by 5E1, malformations of multiple organs were caused in mouse. 5E1-treated mice at PN 6week showed shorter and smaller limbs in length and in volume respectively. However, the overall shape of limbs is same between 5E1-treated and PBS-treated mice. Not only the shorter forelimbs and hindlimbs, but also eye-opening was retarded in 5E1-treated mice (Fig. 9 C). All 5E1-treated mice have shorter and smaller limbs than PBS transferred mice do.

Recently, hedgehog families have been suggested to regulate skeletal formation in vertebrates [56]. $Shh^{-/-}$ mice showed the normal stylopods, but the reduced or fused zeugopods. Autopods in these mice are consists of a single unidentifiable element in the forelimb and a digit in the hindlimb. On the other

hand, *Ihh*$^{-/-}$ mouse embryos at E13.5 have significantly smaller limbs than the wild-type mice at E13.5 do, and skeletons of mutant newborns are reduced especially in limb size[57].

Hedgehog proteins seem to be significantly involved in skeletal formation through multiple actions on chondrogenic mesenchymal cells, chondrocytes, and osteogenic cells[56]. Two kinds of hedgehog are expressed in limbs. Shh expression appeared until E11 ZPA region, while *Ihh* is expressed in metacarpals and phalange regions from E11 to E14 (Fig. 10 B). These two hedgehogs are also different in function. *Shh* is mainly known to be related in the pattern formation of limbs, while the *Ihh* was reported as the regulator in the endochondral bone formation. Since the cartilage primordium appeared at E12 stage in mouse[44], and *Shh* is not expressed in limbs from E12, maternal transfer of 5E1 to mouse embryo was applied at E12 in this study. As reported in the *Ihh*$^{-/-}$ mice, normal phalanges and smaller limbs were induced by the temporal blocking of hedgehog.

To analyze cartilage and bone of the PBS-transferred and 5E1-transferred mice, skeletal staining using alcian blue and alizarin red was performed. As a result, 5E1-transferred mice showed the shorter limb than PBS-transferred mice did. At PN 1day, PN 3week and PN 6week, the size of limbs were smaller in *Ihh*-blocked mice than PBS treated mice. However, the number of phalanges is the same in both kinds of mice (Fig. 11 A, B, C). The decreasement in limb length after 5E1 treatment was evident in the microCT

analysis as well as the skeletal staining (Fig. 12 A). In Ihh blocking group, the length of humerus was shorter as 22.5% at PN 6week, the ulna as 15.4% and radius as 25.4% (Fig. 12 B). Moreover, bone density, trabecular separation, trabecular thickness and trabecular number decreased as 42.5%, 37%, 28.5% and 18.3% respectively after blocking of Ihh signaling (Fig. 12 C). These results suggested that temporal blocking of hedgehog signaling using 5E1 maternal transfer to E12 embryos can cause dwarfism and short limbs. Furthermore, five phalanges in 5E1-treated mice showed that the 5E1 blocked Ihh signaling rather than Shh signaling in this stage of the mouse limb buds.

In order to identify whether the Ihh signaling in limb was blocked by 5E1 successfully, gene expression of *Ihh* and its downstream genes such as *Ptch1, Gli1* and *Gli3* were investigated by *in situ* hybridization method at 1day after blocking Ihh at E12. Because the 5E1 can block Ihh protein, *Ihh* mRNA expressing in chondrocytes and the prehypertrophic regions of limb and phalanges was not changed (Fig. 13 A, B), while *Ptch1* and *Gli1* were dramatically down regulated, which indicates the efficient blocking of Ihh protein activity (Fig. 13 C-F). Gli3, which suggested not to be regulated by Shh and Ihh, did not change (Fig. 13 G, H).

By the micromass culture system, to investigate a role of Ihh protein, exogenous 500 ng/ml Ihh protein led to produce more cartilage nodules. Therefore, overexpression of Ihh protein leads to produce more cartilage nodules by increasing condensation of mesenchymal cells and proliferation of

prechondrocytes in early chondrogenesis. Moreover, 5E1 transferred group were slightly decreased to condensed and proliferated. It will be required to investigate more.

The microarray experiment, to analyze the relationship *Ihh* and other genes, was performed. *Glo1, Dkk4, Mapk8, Ltbp3, Col1a2, Wasl Dmkn, Flnc, Eltd1, Egfl7, Col22a1, Krt1, Col22a1, Krt1, Col19a1, Krt10, Hspa8, Fbxo2, Ocrl, map3k4, Garnl4, Fgf7, Krt4, Klf7* were up-regulated, and *Hhip, Hoxd13, Casp1, Nrg4, Alpl, Rassf8, Foxc2, Bmp5, Ddef1, Ptch1, Sdc4, Tbx5, Runx2, Ikkbkg, Ubc, Nog, Runx3, Fgfr1op2, Nfia, Gh* were down-regulated. Especially, in the down-regulated results, *Hhip* and *Ptch1* which are the members of hedgehog signaling were down-regulated, that means 5E1 inhibited successfully hedgehog signaling. In the up-regulated genes, *Dkk4* is one of *Wnt* signaling, and *Ltbp3* is a Tgf beta signaling and related to skeletal development. *Krt10* is related to cytoskeleton organization and biogenesis, and *Fgf7* is a positive regulator of epithelial cell population.

In the down-regulation genes, *Hoxd13* and *Foxc2* are known to be related with skeletal development, and *Tbx5* plays an important role in limb development. *Noggin* is well known as a negative regulator of BMP signaling pathway, and is related with skeletal development, also. In this study, many genes are related to mouse limb cartilage formation with *Ihh*, and *Ihh* and its related genes co-operate and play an important role in early chondrogenesis.

Therefore, Ihh signaling positively regulates early chondrogenesis, as well as hypertrophy of chondrocytes in the mouse limb development.

Overall, vertebrate limb development is indeed very powerful model system to understand molecular networks as well as morphological changes due to skeletal deformity. Within limited approaches, several interesting results have been found after 5E1 treatment in this study. Moreover, Shh-Ihh signaling has been intensively observed by variable methods. Mutual interactions with putative molecules are started to open our sights clearly, which leads future aspects of bone development.

V. CONCLUSION

Temporal blocking of Hedgehog signaling using 5E1 to E12 mouse embryo, leading inhibition of the Indian hedgehog activity, causes malformation of the mouse limb. *Ihh* expression was shown in the chondrocytes and prehypertrophic regions. We could find the expression of *Ptch1* and *Gli1* decreased. Therefore, Ihh protein function was inhibited by 5E1 successfully.

Overexpression of Ihh protein leads to produce more cartilage nodules by increasing condensation of mesenchymal cells and proliferation of prechondrocytes in early chondrogenesis.

In this study, many genes are related to mouse limb cartilage formation with *Ihh*, and *Ihh* and its related genes co-operate and play an important role in early chondrogenesis

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ABSTRACT (in KOREAN)

생쥐 사지발생 초기에서의

Hedgehog 유전자의 역할

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척추동물의 사지 골격은 연골의 원기가 석회화된 뼈의 기질로 바뀌는 연골내뼈형성 과정을 거쳐 발생하게 된다. 그러므로, 연골의 형성은 척추동물에 있어서, 완전한 뼈의 모양과 크기를 결정하는데 중요한 역할을 한다. 생쥐 사지의 연골형성 동안, 간엽성 전구세포는 서로 밀집해서, 연골의 원기를 형성하기 위해 연골세포로 분화한다. 그리고, 이 연골세포는 세포외기질을 분비하면서 증식과 분화를 거친다. 최근까지는, 연골형성 과정보다는 연골내뼈형성 과정에 대해서 많은 연구가 되어 왔다. 특히, Indian Hedgehog (Ihh) 신호는 연골내뼈형성 과정에서 연골세포가 비대화되어 뼈로 대체되는 과정에서 중요한 역할을 한다고 알려져 있다. 본 연구에서는, hedgehog 을 억제하는 항체인 IgG I type 의 5E1 물질을 태생 12 일된 배아를 임신한 어미의 복강에 주입하여, Indian hedgehog 의 활성을 일시적으로 차단하였다. 그 결과 다른 부분보다 생쥐 사지의

길이가 현저히 짧아짐을 알 수 있었다. 이러한 사지 싹에서의 연골형성과정 동안 Ihh 와 상호작용하는 유전자들을 알아보기 위하여, 태생 12 일 배아에 5E1 을 처리한 후, mRNA microarray 를 이용하여 분석하였다. *Glo1, Dkk4, Mapk8, Ltbp3, Col1a2, Wasl, Dmkn, Flnc, Eltd1, Egfl7, Col22a1, Krt1, Col22a1, Krt1, Col19a1, Krt10, Hspa8, Fbxo2, Ocrl, map3k4, Garnl4, Fgf7, Krt4, Klf07* 등 유전자들의 발현 양이 높게 나타나고, *Hhip, Hoxd13, Casp1, Nrg4, Alpl, Rassf8, Foxc2, Bmp5, Ddef1, Ptch1, Sdc4, Tbx5, Runx2, Ikkbkg, Ubc, Nog, Runx3, Fgfr1op2, Nfia, Gh* 등의 발현 양이 떨어짐을 알 수 있었다. 또한, 태생 12 일 배아의 사지 싹 간엽세포를 Ihh 단백질을 처리하였다. Micromass culture system 을 이용하여 배양했을 때, Ihh 단백질을 처리한 군에서, 연골결절 형성이 더 많이 일어나는 것을 알 수 있었다. 결과적으로, *Ihh* 유전자 신호는 생쥐의 사지 발생에 있어서, 다른 유전자들과의 상호작용을 함으로써 연골세포의 비대화뿐만 아니라, 이른 시기의 연골형성 과정을 조절함을 알 수 있었다.

핵심 되는 말; 생쥐의 사지발생, 연골내뼈형성과정, 5E1, Sonic Hedgehog, Indian Hedgehog, 연골형성과정