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Generation of a transgenic mouse model to study cranial suture development; Apert syndrome

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The form and function of the craniofacial structure critically depend on genetic information. With recent advances in the molecular technology, genes that are important for normal growth and morphogenesis of the craniofacial skeleton are being rapidly uncovered, shaping up modern craniofacial biology. One of them is fibroblast growth factor receptor 2 (FGFR2). Specific point mutations in the FGFR2 gene have been linked to Apert syndrome, which is characterized by premature closure of cranial sutures and craniofacial anomalies as well as limb deformities. To study pathogenic mechanisms underlying craniosynostosis phenotype of Apert syndrome, we used a transgenic approach; an FGFR2 minigene construct containing an Apert mutation (a point mutation that substitute proline at the position 253 to arginine; P253R) was introduced into fertilized mouse germ cells by DNA microinjection. The injected cells were then allowed to develop into transgenic mice. We used a bone-specific promoter (a DNA fragment from the type I collagen gene) to confine the expression of mutant FGFR2 gene to the bone tissue, and asked whether expression of mutant FGFR2 in bone is sufficient to cause the craniosynostosis phenotype in mice. Initial characterization of these mice shows prematurely closed cranial sutures with facial deformities expected from Apert patients. We also demonstrate that the transgene produces mutant FGFR2 protein with increased functional activities. Having this useful mouse model, we now can ask questions regarding the role of FGFR2 in normal and abnormal development of cranial bones and sutures.

Key words : FGFR2, Apert syndrome, Transgenic mice, Bone-specific promoter

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Craniofacial growth is influenced by both genes and environmental cues[1-4]. Although many have already recognized the crucial role of the genes and their functions in the formation of the head structure, majority of the studies on this topic in the past had to rely on

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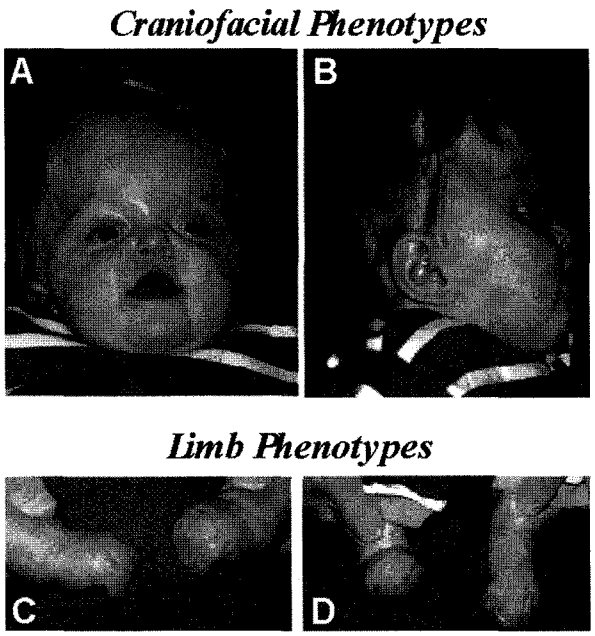


Fig. 1. Craniofacial phenotypes (A,B) and limb phenotypes (C,D) of the Apert syndrome (Adopted from Nah HD. Clin Orthod Res 2000;3:37-45)
A 6 months old infant with increased head height, shortened head length, exophthalmosis, midface deficiency and total fusion of digits (syndactyly)

clinical data due to the limited information on the genes and the lack of available materials and tools[5,6]. However, with rapid advances in biotechnology and molecular biology in the past decade, numerous research methodologies have emerged. Consequently, a number of genes associated with congenital or developmental craniofacial malformations have been identified[7] and in particular, mutations in genes causing various syndromic craniosynostoses[8-11] have been a main area of interest in the craniofacial biology because of the clinical implications.

Craniosynostosis refers to premature fusion of cranial suture and is a relatively common birth defect, occurring 1 in 2500 live births[12]. While normal cranial sutures need to remain patent until maturity to allow for the growth of the underlying neurocranium, premature fusion of sutures inhibits the bone growth at right angle to the fused suture and results in a variety of deformities depending on the location of the involved sutures and the timing of premature fusion[13,14]. Syndromic

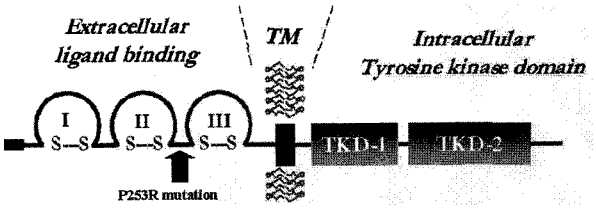


Fig. 2. Schematic diagram of FGFR2
FGFR2 is composed of 3 immunoglobulin-like loops (I-III), transmembrane domain (TM) and intracellular tyrosine kinase domain (TKD-1 & 2). The Apert mutation (P253R) is located between immunoglobulin-like loop II and III and is indicated with an arrow.

craniosynostosis has been found to be associated with a number of monogenic mutations in fibroblast growth factor receptors 1-3 (FGFR1-3), MSX2 or TWIST[7,11,15]. Among them, mutations associated with craniosynostosis syndromes, including Apert syndrome, are most frequently found in the FGFR2 gene[8,11].

Apert syndrome is an autosomal-dominant syndromic craniosynostosis, accounting for 4.5% of all craniosynostoses[12]. Apert syndrome is characterized by typical tower-shaped head, midface deficiency, hypertelorism, exophthalmia and occasional neurological complications due to the fusion of coronal suture and varying degrees of fused digits and/or toes (syndactyly) (Fig. 1)[15,16]. In Apert patients, a point mutation in the gene encoding FGFR2 leads to a single amino acid substitution either from serine to tryptophan (S252W), or from proline to arginine, (P253R) (Fig. 2)[9,17]. FGFR2 is a membrane-bound receptor that responds to various fibroblast growth factor (FGF) and is known to modulate the differentiation of bone cells in the developing cranial suture[15]. Identification of the mutations in the FGFR2 allowed formulating mechanistic questions at the tissue and cellular levels. Calvarial bone cells obtained from Apert patients with either S252W or P253R mutations appear to have more differentiated phenotype than those from age-matched normal individuals[18]. However, the rarity of clinical samples has greatly limited the experimental design for a broader scope of studies, demanding for well-characterized and stable experimental animal models[19].

Genetically engineered animal models having the same type of mutation as in human can be a powerful tool for studying the molecular mechanism of the genetic disorders[20]. A transgenic animal refers to the animal whose genome contains DNA of exogenous origin that has been introduced through experimental manipulation. Transgenics and knock-in/out are two main areas in the generation of genetically engineered animal models[21]. In transgenics, pre-designed transgene constructs are randomly inserted and incorporated in the mouse genome[22], and in knock-in/out, only the specific (target) locus of the endogenous gene is altered by homologous recombination[23,24]. Among the model animals used to study human head development, rodents are regarded as animal of choice because they have comparable head structures such as bony palate and cranial sutures that fish, amphibians and birds don't have[7].

The main purpose of this study is to demonstrate how to establish a stable transgenic mouse model that displays the human cranial phenotypes in Apert syndrome, i.e. craniosynostosis, through tissue-targeted transgenics. Previous in situ hybridization studies have localized intensive expression of FGFR2 in the osteogenic fronts of the developing suture, while it was not noticeably detected in the sutural mesenchyme or underlying dura mater[26,27]. Therefore we hypothesized that the tissue-targeted expression of mutant FGFR2 gene (P253R) in the bone tissue is sufficient to cause premature fusion of the cranial suture in mice, resembling the human craniosynostosis phenotype. According to this hypothesis, we produced a transgene construct that contains one of the Apert mutations (P253R) in FGFR2 and induced the expression of this transgene selectively in the developing bone tissue, using a bone-specific collagen type I promoter (Col1A1)[25].

MATERIALS AND METHODS

Generation of Transgenic Mice Expressing a P253R Apert Mutation of FGFR2

The transgene construct was prepared using a multi-step subcloning strategy[22] (Fig.3) First, a hemagglutinin (HA)-epitope tag (YPYDVPDYA) was attached at the 3' end of the mouse FGFR2 coding sequence by a polymerase chain reaction (PCR) to distinguish the transgene product from endogenous FGFR2. Second, an Apert mutation was introduced to FGFR2 construct; Proline 253 (CCA) of FGFR2 was substituted with Arginine (CGA) by site-directed mutagenesis. Third, the polyadenylation sequence (PA) of the bovine growth hormone gene was added to the 3' end of FGFR2-HA to produce a functional mRNA. Finally, the FGFR2-HA-PA sequence was ligated to the 3' end of the promoter complex. A bone-specific 3.6kb rat type I collagen promoter (Col1A1 promoter provided by Dr. D Rowe, Univ. of Connecticut, Farmington, CT) contiguous with the first exon and intron from the rat type I collagen gene, was used for tissue-targeted

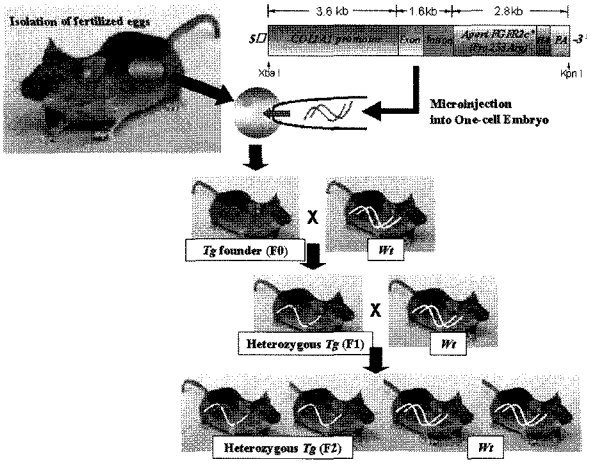


Fig. 3. Schematic diagrams of transgenic FGFR2 constructs with Apert mutation (P253R), microinjection method and breeding strategy.
Transgene construct consists of bone tissue-specific Col1A1 promoter, contiguous first exon and intron, P253R mutant FGFR2c (mesenchymal tissue-specific form of FGFR2) gene, hemagglutinin (HA) gene and polyadenylation (PA) tail.
Transgene construct is inserted into the pronucleus of fertilized one-cell embryo and the embryo is grown to be a transgenic founder (F0). Repeated mating with wild type mice yields offspring (F2) composed of wild type and heterozygous transgenic mice.
(Tg; transgenic, Wt; wild type)

expression of the transgene construct.

The linearized transgene constructs were injected into the B6SJL strain of mouse embryos. DNA injection and generation of the founder mice were done at the University of Pennsylvania Transgenic Core Facility, Philadelphia, USA. The tail DNA from F0 and F1 generations were analyzed by genomic Southern blot hybridization to identify transgenic mice having positive germ line transmission.

Heterozygous transgenic mice carrying the P253R FGFR2 transgene were crossed with wild type mice and the offspring was analyzed for this study.

RNA Isolation and Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

An RT-PCR was performed to determine tissue-specific expression of the transgene. RNAs were isolated from calvarial bone, brain, heart, liver, lung, skeketal muscle and kidney tissues removed from postnatal day 10 transgenic mice, using Trizol reagent (Invitrogen Life Technologies. Carlsbad, CA), according to manufacturer's recommendation. RNAs were reverse transcribed, using the SuperScript™ First-Strand Synthesis System (Invitrogen Life Technologies, Carlsbad, CA), and the cDNAs were amplified with the taqDNA polymerase (Promega, Madison, WI) in 40 cycles of polymerase chain reaction. Each cycle consisted of 1 min. of denaturaion at 94℃, 1 min of annealing at 60℃ and 1 min. of extension at 72℃. The transgene-specific primers used for PCR are 5'-CGATGTCGTTGAACGG TCACGACACCGGCCCATCCTCCAAGC-3' (forward) and 5'-GCTTGGAGGATGGGCCGGTGTCGTGACCG TTCAACGACATCG-3' (reverse)

Primary Mouse Calvarial Osteoblast Culture

Primary mouse calvarial bone cells were isolated from parietal and frontal bones from postnatal day 7-10 mice. Bones were cleaned by gently removing fibrous tissues and subjected to a sequential enzymatic digestion in 1mg/ml bacterial collagenease (Sigma Aldrich Co. St. Louis, MO) and 0.25% trypsin (Invitrogen Corp. Carlsbad, CA) at 37℃ for 6 cycles with each lasting for 20 min. The cells were plated and grown in α-minimum essential medium (α-MEM, Gibco Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS, Atlanta Biologicals, Norcross, GA), 100IU/ml penicillin and 100μg/ml streptomycin (Invitrogen Corp.) at 37℃ incubator with 5% CO_2 for 1 week until they become confluent. Medium was renewed every 48 hours.

Preparation of Total Cell Lysates and Immunoblot Analysis

Calvarial osteoblast cultures were washed with PBS and lysed in RIPA buffer (157mM NaCl, 1.044% NP-40, 0.522% Deoxycholate, 0.1044% SDS, 525mM Tris-HCl, 5mM EDTA, 10mM NaF, 10mM Sodium pyrophosphate). The protein concentration of the lysate was determined by Bradford assay by measuring OD at a 595nm wavelength. Bone-specific expression of the transgene was determined by immunoblotting, using an antibody specific for the hemagglutinin(HA) epitope. 40 μg of total cell lysates were resolved on a 10% (w/v) SDS-polyacrylamide denaturating gel and electro-transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were blocked with a 5% nonfat milk solution in TBST (1X TBS containing 0.1% Tween[20]) for 1 hour, washed 4 times with 1X TBST for 5 min.ea., and incubated with the primary antibodies at 1:1000 dilution (anti-HA or anti-FGFR2 IgG, Santa Cruz Biotechnology, Santa Cruz, CA) in 1X TBST with 5% nonfat milk overnight at 4℃. The blot was rinsed 4 times and incubated with secondary antibodies (horseradish peroxidase (HRP)-conjugated goat anti-rabbit or mouse IgG; Amersham Biosciences, Buckinghamshire, England) for 1 hour at room temperature. The antibody-antigen reaction was detected using a chemiluminescent detection kit (Western Lightning Chemiluminescence Reagent 1; Perkin Elmer Life Sciences, Boston, MA).

Immnuhistochemistry

The heads were dissected out from newborn mice (postnatal day 1), immediately embedded in OCT compound (Tissue Tek, Sakura Finetek USA Inc., Torrance, CA) and frozen at −70℃. 10㎛ thick parasagittal sections were prepared from frozen specimens and fixed in ice-cold acetone for 10 min. Tissue sections were blocked with 10% normal goat serum for 1 hour at room temperature, incubated with the anti-HA rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 3 hours at 37℃, washed three times with PBS and incubated with biotin-goat anti-rabbit IgG (Zymed, San Francisco, CA) for 30 min. After washing with PBS three times, streptavidin-HRP conjugate at 1:250 dilution was applied for 10 min. Antobody-antigen reaction was visualized using substrate-chromogen mixture (DAB). Sections were counterstained with hematoxylin.

Alkaline Phosphatase and Alizarin Red S Staining for the Tissue Section

Alkaline phosphatase buffer solution was prepared for alkaline phosphatase staining. 33 ㎕ of 4-nitro blue tetrazolium chloride (NBT, Boehringer Mannheim Corp., Indianapolis, IN) and 33 ㎕ of X-phosphate/5-Bromo-4-chloro-3-indolyl-phosphate (BCIP, Boehringer Mannheim Corp., Indianapolis, IN) were sequentially mixed in 10 ml of alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl, 100 mM Tris, pH 9.5). Prepared solution was applied onto acetone-fixed parasagittal sections of newborn mice for 5 min. Sections were washed with distilled water. For alizarin red S staining, fixed tissue sections were treated with 0.5% of alizarin red S (Sigma, St Louis, MO) solution for 5 min. and rinsed with 70% ethyl alcohol. Stained sections were mounted using 60% glycerol.

Whole Mount Head Staining with Alcian Blue and Alizarin Red S

Heads were removed from 4-week-old-mice and cleaned by removing soft tissues. The skulls were fixed in 95% ethanol for 2-5 days and stained in a 0.015% alcian blue solution for 1-2 days. The skulls were dehydrated in 100% ethanol for 2 days and cleared in 1% KOH solution for 2 days. Mineralized bone was stained with 0.001% alizarin red S in 1% KOH solution for 2 days and further cleared in a series of glycerin solutions (25, 50, 80 %) for 24 hours per solution. The stained skulls were stored in 100% glycerin were observed under a dissection microscope (Zeiss, Germany).

RESULTS

The FGFR2 transgene driven under a bone-specific promoter is correctly expressed in transgnic mice

To ascertain that our construct is predominantly expressed in the bone tissue, we have examined various tissue types, such as calvarial bone, brain, kidney, heart, liver, lung, and skeletal muscle, by RT-PCR of RNAs isolated from these tissue types. The set of PCR primers used in this RT-PCR recognize RNA produced from the transgene but not that from the endogenous FGFR2 gene. As anticipated, the transgene was predominantly expressed in the bone, confirming that the Col1A1 promoter is mainly active in the bone tissue (Fig. 4A).

To confirm that RNA produced from the transgene is translated into protein, whole cell lysates isolated from transgenic and wild type primary mouse (postnatal day 7-8) calvarial bone cells were analyzed by immunoblotting, using the antibody specific for hemagglutinin (HA). The FGFR2 transgene was tagged with an HA epitope to facilitate the detection of mutant FGFR2 proteins produced from the transgene. The antibody specific for the HA epitope tag recognized a band with a molecular size of 120kD. As expected, the same antibody did not detect any protein in the wild type littermates (Fig. 4B). We have confirmed that the band recognized by the HA-specific antibody is FGFR2, using the FGFR2-specific antibody on duplicate blots. This antibody strongly recognized the

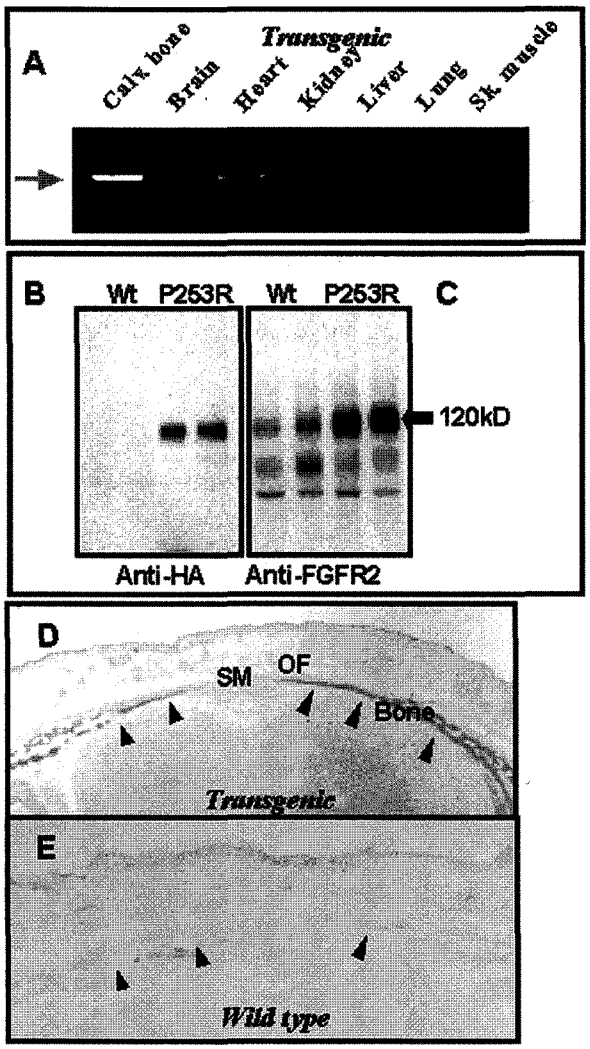


Fig. 4. Transgene expression driven by bone-specific primer in transgenic mice

(A) RT-PCR analysis of RNAs extracted from various tissues demonstrate the most intensive transcriptional activity induced by the Col1A1 promoter in the calvarial bone among all sampled tissues.

(B, C) Immunoblot analysis of calvarial bone cell lysates with antibody specific for HA (B) and FGFR2 (C). Anti-HA IgG detected 120kD proteins only in the transgenic mice (B). Anti-FGFR2 IgG recognized proteins with same molecular weight in the wild type lysates (C), indicating that the expressed proteins in (B) are correct transgenic FGFR2.

(D, E) Immunostaining with anti-hemagglutinin (HA) IgG for frontal section of neonatal (p1) transgenic and wild type mouse (X100). In the sagittal suture, only calvarial bones expressed transgene-encoded protein tagged with HA epitope (D), unlike the wild type (E). Sutural mesenchymes and epithelial tissues showed negative staining.

(SM; suture mesenchyme, OF; osteogenic front)

120kDa band in lysates from transgenic mouse bone cells (Fig. 4C). It also recognized endogenous FGFR2 of 120 kDa in lysates from wild type mice. Thus, the transgene encoded mutant FGFR2 is expressed as protein at a high level in transgenic mice.

We performed immunohistochemical analysis of transgenic mouse calvarial bone section for spatial localization of the transgene-derived FGFR2 portein. As shown in Fig. 4D, the antibody specific for HA epitope stained the osteoblasts along the bone surface, verifying that expression of the FGFR2 transgene product is bone-specific. Similar sections from wild type mice are not stained by the HA-specific antibody (Fig. 4E).

Bone-specific expression of Apert mutation causes craniosynostosis

Distinct craniofacial features observed in the P253R FGFR2 transgenic mice were dome-shaped head with vertically increased dimension and anterior crossbite. P253R transgenic mice commonly displayed turribrachycephalic features that are characterized by frontal bossing, high forehead with shortened anteroposterior dimension of the face. In particular, as a result of mid-face deficiency, lower anterior teeth elongation, anterior cross bite and relatively protruded mandible were noted. These features imply the presence of prematurely fused cranial sutures that inhibit the elongation of the face in the sagittal plane. Occasional occipital bossing indicates that a compensatory brain growth has occurred to the posterior direction (Fig. 5).

Parasagittal sections of newborn (postnatal day 1) mouse heads from both P253R and wild type littermates were stained for active alkaline phosphatase or mineralized bone in the coronal suture. Osteogenic fronts of the coronal sutures in P253R mice showed a considerably flattened bony overlap while those of the wild type were still widely separated, as shown in sections stained for alkaline phosphatase activity (Fig 6A, C). Alizarin red S staining demonstrated more elongated and approximated osteogenic fronts in the Apert (P253R) than in the wild type mice (Fig. 6B, D).

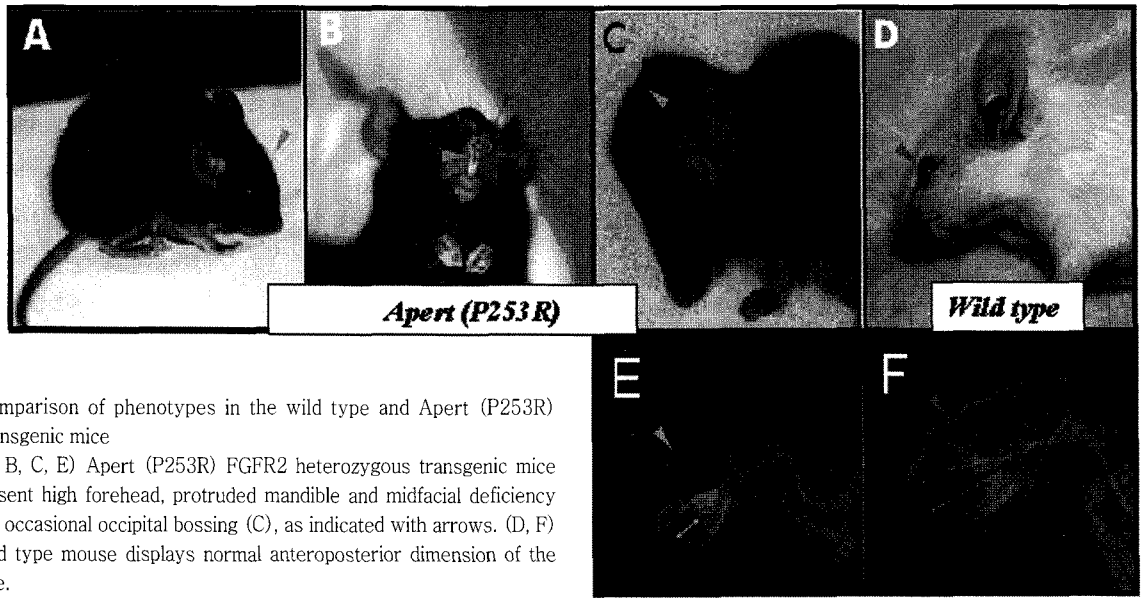
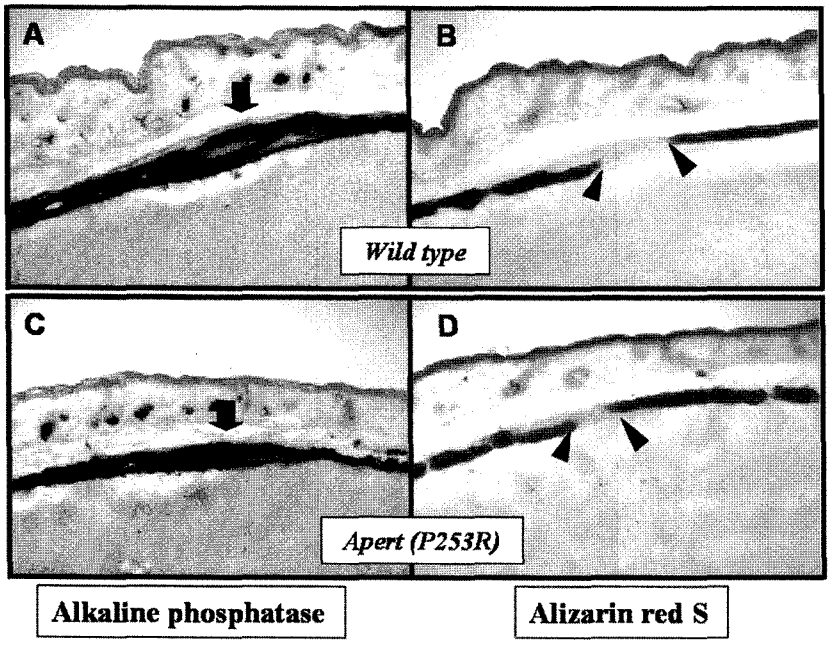


Fig. 5. Comparison of phenotypes in the wild type and Apert (P253R) transgenic mice
(A, B, C, E) Apert (P253R) FGFR2 heterozygous transgenic mice present high forehead, protruded mandible and midfacial deficiency and occasional occipital bossing (C), as indicated with arrows. (D, F) Wild type mouse displays normal anteroposterior dimension of the face.



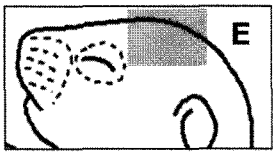


Fig. 6. Development of the coronal suture in the wild type and Apert (P253R) FGFR2 transgenic littermates at post-natal day 1. Alkaline phosphatase staining (A, C) and alizarin red S staining (B, D) (X100).
(A - D) Compared to the wild type coronal suture (A, B), P253R mouse demonstrates more flattened and advanced suture formation (C, D). Osteogenic fronts are more closely approximated in the Apert (P253R) coronal suture (D).
(E) Location of the parasagittal section for observation of the coronal suture.

Examination of adult P253R transgenic mice (4 months old) clearly shows fusion of the coronal and sometimes sagittal suture (Fig. 7B). However, we do not know how early fusion occurs in these mice. These mice show the anterior crossbite as early as day 7, at the time when incisors emerge into the oral cavity. Further detailed studies are needed to determine the onset and location of cranial suture fusion.

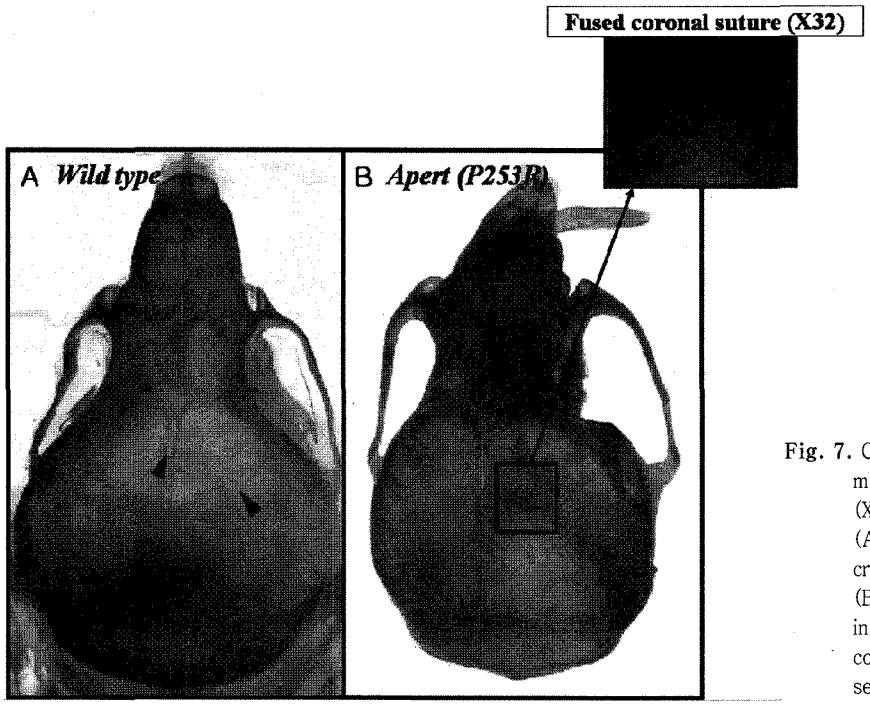


Fig. 7. Calvarial suture formation in the adult (4 months) wild type / Apert (P253R) mice (X12). Alizarin red S staining.
(A) Wild type mouse skull displays patent cranial suture.
(B) Premature fusion of coronal suture is found in Apert (P253R) mouse. Anteroposteriorly constricted face, extruded upper incisor and severe facial asymmetry are also noticed.

DISCUSSION

Syndromic or non-syndromic craniosynostosis is an area of interest in the craniofacial biology for two reasons. First, it exhibits serious medical problems, such as cerebral dysfunction, neurological complications and lethality, as well as morphological deformities. Second, it is a good disease model to study the gene functions in the regulation of bone formation.

Fused cranial sutures are normally managed by surgical separation of the sutures to prevent serious complications. However, these once surgically separated sutures tend to fuse again, requiring repetitive surgical approaches. An affected patient is expected to face their first surgery within the first year of life and the last surgery in their early twenties[28]. Even repeated surgical interventions cannot change the overall cranial pattern after all. Thus a thorough understanding of the pathogenesis is necessary to design effective ways to inhibit reunification of synostotic sutures.

Genetically engineered mouse models enable us to design experiments to prove genotypic/phenotypic associations, and ultimately develop new approaches to treatment and prevention of diseases[29]. There are many different strategies in making an appropriate model for individual study and one should be well aware of the characteristics of the target gene, as well as the main purpose of a specific animal model. As has already been explained, two main approaches to making genetically engineered mice are transgenics and knock-in/out, depending on the way transgene construct is finally incorporated in the mouse genome. The knock-in/out appears to represent the human genetic disorder better than the other one, but transgenics is relatively less complicated, less costly and less time-consuming than the knock-in/out. As long as one could obtain a transgenic mouse model that represents the human genetic disorder and phenotype, transgenics is considered to be an efficient and economic way to the same goal.

In the transgenic mice, however, when the transgene happened to be inserted in the critical part of the genome, normal development or vital function would be interrupted[20]. Unfortunately, it is not easy to confirm the

actual site where the transgene is mingled. This could be overcome by having a number of different transgenic lineages. A lineage with common and representative characteristics would be an appropriate model. We produced several different lineages and obtained some lineages that represented the typical dome-shaped head in common. Subsequent in-vitro studies were carried out in these lineages. As in Fig. 3, individual mating was repeated through several generations to obtain stable lineages. When heterozygous mice were mated with wild type mice, offspring littermates were nearly 1:1 between wild type and transgenic mice, providing an age-matched group suitable for many comparative studies.

The main purpose of this study was to establish a well-characterized and stable transgenic mouse model that reproduces the cranial phenotype, i.e. premature fusion of the coronal suture, in Apert syndrome. To be a well-characterized model, an appropriate type of mutation of the gene needs to be expressed in the critical region to simulate human phenotype. Stable model means that the animals should survive after birth and perform normal reproduction, so as to provide with ample resources such as cells, tissues or organs. Thus a lethal or infertile model would be less helpful from that standpoint, which was the case in many transgenic approaches[30-32].

According to these rationale, we designed a tissue-specific expression of transgene from two standpoints; first, we hypothesized that expression of the mutant gene exclusively in the bone tissue may be sufficient to reproduce cranial phenotype in Apert syndrome, according to the previous studies [26,27], since the area of our interest was the cranial phenotype, not that of the extremities. Second, we speculated that the tissue-specific expression of the transgene may yield more stable transgenic lineages, by avoiding universal or indiscriminative expression of the mutant gene.

In order to avoid effects of the expression of the inserted transgene in other tissues, selection of appropriate promoters is essential[24]. A promoter is the part that is stimulated by a transcription factor to initiate the transcription of the gene. Expression of the gene depends on the presence of the transcription factor that stimulates the promoter. A universal promoter such as human cytomegalovirus promoter[33], would have induced the transgene expression in a wide variety of mouse tissues throughout development. Similarly, a knock-in approach, where the mutation is introduced in the endogenous FGFR2, was another alternative. However, these alternatives were expected to complicate the interpretation of the phenotype by broad expression of the mutant gene in other tissues and increase the risk of the premature death, which will greatly limit the type of feasible experimental design. The 3.6kb Col1A1 promoter used in this study has been shown to be active in osteogenic progenitor cells as well as in differentiated osteoblasts[34]. In this study, bone tissue-specific expression of the P253R FGFR2 transgenes was demonstrated with RT-PCR, immunohistochemistry and immunoblot (Fig.4). Thus we conclude that rat Col1A1 promoter is an effective and functional promoter to induce the selective expression of the transgene in the oseoblasts in the transgenic mouse model.

In terms of the characteristics of the transgenic mouse, cranial phenotype of the Apert mice was similar to that of human. A remarkable feature was the antero-posteriorly constricted face. Some variation in the phenotype was also noticed as in human, probably depending on the time and primary site where the fusion first takes place. The alizarin red S staining of Apert (P253R) mouse head aged 4 months demonstrated complete fusion of both coronal sutures (Fig.7). A significant facial asymmetry was also found. This is likely to have been formed during growth, because the asymmetry was not apparent in their early life. The cranial phenotype became more apparent as the mice grew older compared to the normal litteramates. Additional researches on the microscopic features to clarify the exact time point of the suture fusion need to be performed later on.

Human Apert patients display an overall skull shape that is constricted anteroposteriorly and elongated

upward, decreased maxillary height and length, with little change in the mandible shape[35]. It appears that early fusion of the anterior cranial base greatly restricts the anterior growth of maxilla at the cranial base level, thus requiring the Le Fort III procedure in many Apert patients in their early life[36]. Apert patients have been reported to show premature fusion of coronal suture at 5 months[37] but at an earlier age, significantly large defects along the sagittal suture have also been observed[28]. An abrupt transition from the large defect to the early closure of the suture implies a critical event occurring at a specific time point during calvarial development. Since we observed the mice at two remote time points, i.e. postnatal day 1 and 4 months, it is hard to pinpoint the time when the fusion takes place. We assume that this is an early event in their life like in human, because the Apert mice already showed more approximated suture development at birth(Fig. 6). A systemized study has to be conducted to clarify the onset and location of fusion, using this mice model.

In terms of the stability of the transgenic mouse, we anticipated that the limited expression of the transgene in the bone tissue would reduce or eliminate the limb phenotype and/or any unnecessary systemic disorders that are not our area of interest. This was partly proven in our study since no limb phenotype was found in these mice. However, a latest report on the knock-in mouse with S252W mutation in FGFR2 did not show any syndactyly either[32]. Further investigation on the detailed mechanism of formation of each deformity needs to be carried out.

Obviously, homozygous offspring showed variable lethality, depending on the severity of phenotype. In contrast, majority of the heterozygous transgenic offspring appeared normal at birth with no apparent lethality and survived to adulthood. However, the Apert (P253R) mice showed generally reduced fertility with great variation between individuals, compared to the wild type littermates. Moreover, occasional deaths of adult transgenic mice due to serious illness in the internal organs were also observed. Poor health and malnutrition due to the malocclusion in Apert mice might be one of the causative factors in the reduced fertility. Development of anterior crossbite and facial asymmetry may greatly have disturbed normal feeding. Although we couldn't clearly judge if the tissue-specific expression of mutant gene increased the survivability of the transgenic mice, heterozygous P253R mice produced many batches of offspring and were considered a stable model to study the sutural pathology.

The findings from the sagittal sections at P1 (Fig.6) where Apert mice showed more approximated suture formation imply various mechanisms such as reduced proliferation, advanced maturation and/or increased apoptosis of the bone cells, or the combination of these, resulted from the change of receptor functions[18,32,39-43]. This is coincident with the findings that the FGFR2 is a key regulator of the proliferation, differentiation and survival of the osteoblasts on the developing osteogenic front[32,39]. A crucial role of the dura as a regulator of suture development has also been proposed to answer why particularly coronal sutures are involved in the Apert syndrome[38]. This study does not provide any information on the molecular mechanism of the osteoblast differentiation. We expect that this animal model will be useful for the upcoming investigation on the detailed events occurring at the cellular level when the normal functions of the receptor are deteriorated due to specific types of mutation.

CONCLUSION

We have prepared a transgenic mouse model to study the function of FGF/FGFR2 during normal and abnormal development of the cranial sutures. In these mice the mutant FGFR2 harboring an Apert mutation (P253R) was expressed in the bone tissue, using the rat Col1A1 promoter. Analyses of these mice reveal that:

1. P253R FGFR2 transgene is predominantly expressed in bone at both RNA and protein levels.
2. P253R FGFR2 transgenic mice show advanced coronal suture development at postnatal day 1, and cranial deformity and complete fusion of the coronal

sutures in the adulthood, similar to the human phenotype.

These results assure that these transgenic mice will be able to serve as a useful animal model to study the pathogenic mechanisms of the craniosynostosis phenotype of Apert syndrome.

REFERENCES

1. Proffit WR, Fields HW. Contemporary orthodontics. St Louis, Mosby 2000 : 113-147
2. Nuckolls GH, Shum L, Slavkin HC. Progress toward understanding craniofacial malformations. Cleft Palate Craniofac J 1999 : 36 : 12-26
3. Moss JP. The soft tissue environment of teeth and jaws. An experimental and clinical study : part 1. Br J Orthod 1980 : 7 : 127-37
4. Moss JP. The soft tissue environment of teeth and jaws. Experimental malocclusion : Parts 2 and 3. Br J Orthod 1980 : 7 : 205-16
5. Chung CS, Niswander JD, Runck DW, Bilben SE, Kau MC. Genetic and epidemiologic studies of oral characteristics in Hawaii's schoolchildren. II. Malocclusion. Am J Hum Genet 1971 : 23 : 471-95
6. Litton SF, Ackermann LV, Isaacson RJ, Shapiro BL. A genetic study of Class 3 malocclusion. Am J Orthod 1970 : 58 : 565-77
7. Wilkie AO, Morriss-Kay GM. Genetics of craniofacial development and malformation. Nat Rev Genet 2001 : 2 : 458-68
8. Hehr U, Muenke M. Craniosynostosis syndromes : from gene to premature fusion of skull bones. Minireview. Mol Genet and Metabol 1999 : 68 : 139-51
9. Wilkie AO, Slaney SF, Oldridge M, Poole MD, Ashworth GJ, Hockley AD, Hayward RD, David DJ, Pulleyn LJ, Rutland P, et al. Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome. Nat Genet 1995 : 9 : 165-72
10. Kan S-H, Elanko N, Johnson D, Cornejo-Roldan L, Cook J, Reich et al. Genomic screening of fibroblast growth-factor receptor 2 reveals a wide spectrum of mutations in patients with syndromic craniosynostosis. Am J Hum Genet 2002 : 70 : 472-86
11. Wilkie AO. Craniosynostosis : genes and mechanisms. Hum Mol Genet 1997 : 6 : 1647-56
12. Gorlin RJ, Cohen MM Jr, Levin LS. Syndromes of the head and neck. New York, Oxford University Press 1990 : 519
13. Cohen MM Jr. Sutural biology and the correlates of craniosynostosis. Am J Med Genet 1993 : 47 : 581-616
14. Opperman LA. Cranial sutures as intramembranous bone growth sites. Dev Dyn 2000 Dec : 219 : 472-85
15. Nah H. Suture biology : Lessons from molecular genetics of craniosynostosis syndromes. Clin Orthod Res 2000 : 3 : 37-45
16. von Gernet S, Golla A, Ehrenfels Y, Schuffenhauer S, Fairley JD. Genotype-phenotype analysis in Apert syndrome suggests opposite effects of the two recurrent mutations on syndactyly and outcome of craniofacial surgery. Clin Genet 2000 : 57 : 137-9
17. Park WJ, Theda C, Maestri NE, Meyers GA, Fryburg JS, Dufresne C, Cohen MM Jr, Jabs EW. Analysis of phenotypic features and FGFR2 mutations in Apert syndrome. Am J Hum Genet 1995 : 57 : 321-8
18. Lomri A, Lemonnier J, Hott M, de Parseval N, Lajeunie E, Munnich A, Renier D, Marie PJ. Increased calvaria cell differentiation and bone matrix formation induced by fibroblast growth factor receptor 2 mutations in Apert syndrome. J Clin Invest 1998 : 101 : 1310-7
19. Liu YH, Snead ML, Maxson RE Jr. Transgenic mouse models of craniofacial disorders. Methods Mol Biol 2000 : 137 : 499-512
20. Jacenko O. Strategies in generating transgenic mammals. Methods Mol Biol 1997 : 62 : 399-424
21. Linder CC. The influence of genetic background on spontaneous and genetically engineered mouse models of complex diseases. Lab Anim 2001 May : 30 : 34-9
22. Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH. Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci 1980 : 77 : 7380-4
23. Smithies O, Gregg RG, Boggs SS, Koralewski MA, Kucherlapati RS. Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. Nature 1985 : 317 : 230-4
24. Ignelzi MA Jr, Liu YH, Maxson RE Jr, Snead ML. Genetically engineered mice : tools to understand craniofacial development. Crit Rev Oral Biol Med 1995 : 6 : 181-201
25. Dodig M, Kronenberg MS, Bedalov A, Kream BE, Gronowicz G, Clark SH, Mack K, Liu YH, Maxon R, Pan ZZ, Upholt WB, Rowe DW, Lichtler AC. Identification of a TAAT-containing motif required for high level expression of the COL1A1 promoter in differentiated osteoblasts of transgenic mice. J Biol Chem 1996 : 271 : 16422-9
26. Kim HJ, Rice DP, Kettunen PJ, Thesleff I. FGF-, BMP- and Shh-mediated signalling pathways in the regulation of cranial suture morphogenesis and calvarial bone development. Development 1998 : 125 : 1241-51
27. Rice DP, Rice R, Thesleff I. Molecular mechanisms in calvarial bone and suture development, and their relation to craniosynostosis. Eur J Orthod 2003 : 25 : 139-48
28. Cohen MM Jr, Maclean RE. Anatomic, genetic, nosologic, diagnostic, and phychosocial considerations; in Craniosynostosis - diagnosis, evaluation and management. New York, Oxford University Press 2000 : 119-143
29. Murray KA. Issues to consider when phenotyping mutant mouse models. Lab Anim 2002 : 31 : 25-9
30. Winograd J, Reilly MP, Roe R, Lutz J, Laughner E, Xu X, Hu L, Asakura T, vander Kolk C, Strandberg JD, Semenza GL. Perinatal lethality and multiple craniofacial malformations in MSX2 transgenic mice. Hum Mol Genet 1997 : 6 : 369-79
31. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997 : 89 : 755-64

31. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997 : 89 : 755-64
32. Chen L, Li D, Li C, Engel A, Deng CX. A Ser250Trp substitution in mouse fibroblast growth factor receptor 2 (FGFR2) results in craniosynostosis. Bone 2003 : 33 : 169-78
33. Dressler GR, Wilkinson JE, Rothenpieler UW, Patterson LT, Williams-Simons L, Westphal H. Deregulation of Pax-2 expression in transgenic mice generates severe kidney abnormalities. Nature 1993;362(6415) : 65-7
34. Visnjic D, Kalajzic I, Gronowicz G, Aguila HL, Clark SH, Lichtler AC, Rowe DW. Conditional ablation of the osteoblast lineage in Col2.3deltatk transgenic mice. J Bone Miner Res 2001 : 16 : 2222-31
35. Kreiborg S, Aduss H, Cohen MM Jr. Cephalometric study of the Apert syndrome in adolescence and adulthood. J Craniofac Genet Dev Biol 1999 : 19 : 1-11
36. Bachmayer DI, Ross RB, Munro IR. Maxillary growth following LeFort III advancement surgery in Crouzon, Apert, and Pfeiffer syndromes. Am J Orthod Dentofac Orthop 1986 : 90 : 420-30
37. Cinalli G, Renier D, Sebag G, Sainte-Rose C, Arnaud E, Pierre-Kahn A. Chronic tonsillar herniation in Crouzon's and Apert's syndromes : the role of premature synostosis of the lambdoid suture. J Neurosurg 1995 : 83 : 575-82
38. Ignelzi MA Jr, Wang W, Young AT. Fibroblast growth factors lead to increased Msx2 expression and fusion in calvarial sutures. J Bone Miner Res 2003 : 18 : 751-9
39. Lemonnier J, Delannoy P, Hott M, Lomri A, Modrowski D, Marie PJ. The Ser252Trp fibroblast growth factor receptor-2 (FGFR-2) mutation induces PKC-independent downregulation of FGFR-2 associated with premature calvaria osteoblast differentiation. Exp Cell Res 2000 : 256 : 158-67
40. Lemonnier J, Hay E, Delannoy P, Fromigue O, Lomri A, Modrowski D, Marie PJ. Increased osteoblast apoptosis in apert craniosynostosis : role of protein kinase C and interleukin-1. Am J Pathol 2001 : 158 : 1833-42
41. Neilson KM, Friesel R. Ligand-independent activation of fibroblast growth factor receptors by point mutations in the extracellular, transmembrane, and kinase domains. J Biol Chem 1996 : 271 : 25049-57
42. Yu K, Herr AB, Waksman G, Ornitz DM. Loss of fibroblast growth factor receptor 2 ligand-binding specificity in Apert syndrome. Proc Natl Acad Sci U S A 2000 : 97 : 14536-41
43. Anderson, J., H.D. Burns, P. Enriquez-Harris, A.M. Wilkie, and J.K. Heath. Apert syndrome mutations in fibroblast growth factor receptor 2 exhibit increased affinity for FGF ligand. Hum Mol genet 1998 : 7 : 1475-1483

국문초록

두개봉합 발육 연구를 위한 형질변환 쥐의 개발 ; 어퍼트 신드롬

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악안면 구조의 형태와 기능은 대개 유전자 정보에 의해 결정된다. 분자생물학의 발달로 인해 정상 성장과 형태 형성에 중요한 유전자에 대한 정보가 밝혀지고 있고 이는 현대 두개안면 생물학의 근간이 되고 있다. 밝혀진 사실들 중 주목할 만한 것은 섬유아세포 성장인자2 (FGFR2)에서의 특이한 돌연변이가 어퍼트 증후군 (Apert syndrome) 의 발생과 관련이 있다는 것이다. 어퍼트 증후군은 두개 관상봉합의 조기 유합과 사지의 기형으로 특징지워진다. 그 중 특히 두개골 유합증의 병인과 형성기전을 연구하기 위해 본 연구에서 유전자 변환기법을 시도하여 어퍼트 증후군의 유발인자로 알려진 FGFR2에서의 단일 아미노산 치환 돌연변이를 재연한 인위 유전자구조물을 제작하고 이를 미세주입법으로 쥐의수정란에 삽입하여 형질변환 쥐를 제작하였다. 본 연구에서는 전체 조직이 아닌 골조직에서 특이하게 활성화되는 전사촉진자 (promoter, 제 I형 교원질 유전자의 전사촉진자) 를 이용하여 골조직에서만 돌연변이 유전자의 발현을 재현함으로써 이 시도가 쥐에서 두개골유합증을 유발하는지 검증하고자 하였다. 초기 표현형 분석을 통해 어퍼트 환자에서 기대되는 두개골 유합증을 확인하였다. 또한 본 연구에서 삽입된 변환유전자가 원활히 돌연변이 단백질을 생산하고 기능의 증가를 보임을 확인하였다. 이러한 동물 모델을 이용함으로써 이제 정상적 혹은 비정상적 두개골 및 봉합 발육에서의 FGFR2의 역할을 연구하는 것이 가능하리라 사료된다.

주요 단어 : 섬유아세포 성장인자2, 어퍼트 증후군, 형질변환 쥐, 골조직 특이 전사촉진자