Therapeutic Effect of Nanogel-Based Delivery of Soluble FGFR2 with S252W Mutation on Craniosynostosis

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Abstract

Apert syndrome is an autosomal dominantly inherited disorder caused by missense mutations in fibroblast growth factor receptor 2 (FGFR2). Surgical procedures are frequently required to reduce morphological and functional defects in patients with Apert syndrome; therefore, the development of noninvasive procedures to treat Apert syndrome is critical. Here we aimed to clarify the etiological mechanisms of craniosynostosis in mouse models of Apert syndrome and verify the effects of purified soluble FGFR2 harboring the S252W mutation (sFGFR2IIIcS252W) on calvarial sutures in Apert syndrome mice in vitro. We observed increased expression of Fgf10, Esrp1, and Fgfr2IIIb, which are indispensable for epidermal development, in coronal sutures in Apert syndrome mice. Purified sFGFR2IIIcS252W exhibited binding affinity for fibroblast growth factor (Fgf) 2 but also formed heterodimers with FGFR2IIIc, FGFR2IIIcS252W, and FGFR2IIIbS252W. Administration of sFGFR2IIIcS252W also inhibited Fgfr2-dependent proliferation, phosphorylation of intracellular signaling molecules, and mineralization of Fgfr2S252W-overexpressing MC3T3-E1 osteoblasts. sFGFR2IIIcS252W complexed with nanogels maintained the patency of coronal sutures, whereas synostosis was observed where the nanogel without sFGFR2S252W was applied. Thus, based on our current data, we suggest that increased Fgf10 and Fgfr2IIIb expression may induce the onset of craniosynostosis in patients with Apert syndrome and that the appropriate delivery of purified sFGFR2IIIcS252W could be effective for treating this disorder.

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Introduction

Genetic mutations in fibroblast growth factor receptor 2 (FGFR2) 1–3 cause several types of syndromic craniosynostosis, including Apert syndrome (AS; OMIM #101200) [1,2,3,4,5]. Most cases of AS are caused by either one of the two missense mutations in FGFR2 exon 7, i.e., C934G or C937G leading to the amino acid substitutions S252W or P253R, respectively [5]. These mutations are known to enhance ligand-dependent activation of FGFR2 by ligands produced by osteoblasts [6] but also formed heterodimers with FGFR2IIIc, FGFR2IIIcS252W, and FGFR2IIIbS252W. Administration of sFGFR2IIIcS252W also inhibited Fgfr2-dependent proliferation, phosphorylation of intracellular signaling molecules, and mineralization of sFGFR2IIIcS252W-overexpressing MC3T3-E1 osteoblasts. sFGFR2IIIcS252W complexed with nanogels maintained the patency of coronal sutures, whereas synostosis was observed where the nanogel without sFGFR2S252W was applied. Thus, based on our current data, we suggest that increased Fgf10 and Fgfr2IIIb expression may induce the onset of craniosynostosis in patients with Apert syndrome and that the appropriate delivery of purified sFGFR2IIIcS252W could be effective for treating this disorder.

Cholesteryl-bearing pullulan (CHP), which is composed of hydrophilic polysaccharides partially modified with hydrophobic cholesteryl groups, self-assembles in water and forms stable nanogels with a diameter of 30 nm [11,12]. The CHP nanogel has two unique characteristics as follows: 1) a high loading capacity for bioactive molecules inside their nano spaces with polymer networks and 2) a chaperone-like activity which enables the delivery of a variety of molecules to the targeted sites. To date, the CHP nanogel has been utilized as a drug delivery system for vaccines [17], and cytokine therapy [18]. More recently, the CHP nanogel has been utilized as a drug delivery system for multiple purposes, such as cancer treatment [15,14,15,16], nasal vaccines [17], and cytokine therapy [18]. More recently, the nanogel-crosslinked hydrogels were also developed by Michael addition of acryloyl-bearing CHP (CHPOA) to PEG containing four branched terminal thiol groups (PEGSH). These macrogels have been utilized as a scaffold material for controlled drug release in regenerative medicine [19,20].
We hypothesize that sFGFR2S252W may act as a suppressor for hyperdifferntiation of osteoblasts in the coronal sutures of AS mice. The aim of this study was to elucidate the pathogenic mechanisms of craniosynostosis in AS and verify the therapeutic applicability of purified sFGFR2IIIcS252W delivered via a polycarboxylate nanogel as a protein carrier in Apert calvarial sutures.

Materials and Methods

Animals

All animal experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (Permission number: 0110009B). All samples were extracted from mouse embryos at embryonic day 15.5 (E15.5). All efforts were made to minimize animal suffering. Pairs of AS mice (Fgfr2<sup>−/−</sup><sub>S252W</sub>) were generated by mating male Fgfr2<sup>−/−</sup>/Ella-Cre mice and female S252W/Ella-Cre mice. Polymerase chain reaction (PCR) genotyping of progeny mice was performed using tail genomic DNA isolated from the pups using a DNAeasy Blood and Tissue Kit (Qiagen, Crawley, UK); KOD Plus Polymerase (Toyobo, Osaka, Japan), and TaqMan probes were designed for the following genes: Runx2<sup>−/−</sup>, 5'-TGGATCCACTGGATGTTGGGGC-3' (forward) and 5'-TGTCAACCATGCAGAGTGAAAG-3' (reverse); Fgfr2<sup>−/−</sup>, 5'-AGCTTACATAGGAAAGTGTGGGC-3' (forward) and 5'-TCATTGGAGGAGGACATGAGCC-3' (reverse); Runx2<sup>−/−</sup>, 5'-GCCAGCAACACTTGTCTCTT-3' (forward) and 5'-TGTCAGCACTGGGAGAATGGA-3' (reverse); Esrp1, 5'-GCCCTCGCAGTATTTCAACA-3' (forward) and 5'-TGCCACATTTCAACTTGTTTAC-3' (reverse); β-actin, 5'-TGCCCTGACAGTTAAAGAGG-3' (forward) and 5'-GATGCACAGATTGATATA-3' (reverse). All samples were assayed in triplicate according to the manufacturer's recommendations.

RNA preparation, reverse transcription (RT)-PCR, and real-time PCR

Embryonic calvarial coronal sutures (E15.5) were dissected from AS mice and control littermates under a stereoscopic microscope. Both sutures from each calvarium were collected and combined. To isolate total RNA, tissues were lysed in Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Reverse transcription was performed using a PrimeScript First Strand cDNA Synthesis Kit (Takara, Shiga, Japan). Real-time PCR was performed using TaqMan gene expression assays on a 7300 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Primers and TaqMan probes were designed for the following genes: Runx2<sup>−/−</sup> (encoding mouse Runx2; Mm00501580_m1) and Opm (encoding mouse Osteopontin; Mm01611440_mH). Relative expression levels were calculated using the level of β-actin mRNA in each sample as a reference. The expression levels of Fgfr2IIb, IIc, Fig10, and Esp1 mRNAs were analyzed by real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems) with the following primer pairs: Fgfr2IIb, 5'-AGCTTACATAGGAAAGTGTGGGC-3' (forward) and 5'-TCATTGGAGGAGGACATGAGCC-3' (reverse); Fgfr2IIb, 5'-GCCAGCAACACTTGTCTCTT-3' (forward) and 5'-TGTCAGCACTGGGAGAATGGA-3' (reverse); Fgfr2, 5'-GCCCTCGCAGTATTTCAACA-3' (forward) and 5'-TGCCACATTTCAACTTGTTTAC-3' (reverse); β-actin, 5'-TGCCCTGACAGTTAAAGAGG-3' (forward) and 5'-GATGCACAGATTGATATA-3' (reverse).

Analysis of intracellular signaling

Embryonic calvarial coronal sutures (E15.5) were lysed in RIPA buffer supplemented with protease inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, IN, USA). Protein extracts (10 μg each) from paired sutures from embryos were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were then incubated with specific primary antibodies for 12 h at 4°C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, UK) and visualized using an LAS 4000 system (Fujifilm, Tokyo, Japan). Primary antibodies, including anti-extracellular signal-regulated kinase (ERK, 9102), anti-phospho (p)-ERK (9101), anti-MAPK kinase (MEK, 9122), anti-stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK, 9252), anti-p-SAPK/JNK (9251), anti-p38 (9321), anti-p-p38 (9211), anti-Akt (9272), anti-p-Akt (9271), and anti-Bax (2772), were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-Esp1/2 monoclonal antibodies (201-301-C31S) and anti-β-actin monoclonal antibodies (A1978) were purchased from Rockland (Gilbertsville, PA, USA) and Sigma-Aldrich (Poole, UK), respectively.

Purification of sFGFR2IIc and sFGFR2IIIcS252W

The culture medium from Cos-7 cells stably expressing Fgfr2IIc-FLAG or Fgfr2IIIcS252W-FLAG [8] was concentrated using an Amicon Ultra-4 Centrifugal Filter Unit (Millipore, Billerica, MA, USA). Both sFGFR2IIc and sFGFR2IIIcS252W proteins were purified using the FLAG M Purification Kit (Sigma-Aldrich), and purification was subsequently verified by western blot analysis with an anti-FLAG antibody (F3165; Sigma-Aldrich).

Pull-down Assay

First, we produced His-tagged Fg2 protein as follows. Murine primary lung cells were obtained from E15.5 wild-type mice, and RNA was extracted using Isogen (Nippon Gene). RT-PCR was performed using the following primer pair: Fig2, 5'-TGGTGCAACGGCCGATCCCTC-3' and 5'-GTCTTTCAGCAAGACGTGGAA-3'. PCR products of the Fig2 (459-bp) ampiclon were extracted and purified using a Gel Extraction Kit (Qiagen), followed by ligation into the TOPO II vector (Invitrogen, Carlsbad, CA, USA) using a Quick Ligation Kit (New England Biolabs, Ipswich, MA, USA). Using generated plasmids as templates, cDNA samples of Fig2 were subjected to PCR amplification with the following specific primer pair: Fig2, 5'-GACTAGTCCATGGGTGCAAGGACATCAGGCA-3' and 5'-GGAAATTCGTCCTTACAGCAGCAGGCTTTAAGAA-3' (with SpeI/EcoRI sites). The amplified product digested with SpeI/EcoRI was subcloned in frame into the pTracer-EF/V5-His expression vector (Invitrogen). After the sequences of the resulting expression vectors were confirmed by sequencing, these vectors were used to express Fg2-His proteins. Purification of Fg2-His proteins was then performed using the MagneHis Protein Purification System (Promega, Southampont, UK). With purified sFGFR2IIc and sFGFR2IIIcS252W as bait proteins and Fgf2-His as a prey protein, pull-down assays were carried out using a Pull-down PolyHis Protein-Protein Interaction Kit (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

Immunoprecipitation and western blot analysis

After Cos-7 cells reached 80% confluence in 10-cm culture dishes, cells were transfected with 4 μg of the following plasmids using Attractene Transfection Reagent (Qiagen): (1) FLAG-MOCK; (2) FGR2IIb<sup>S252W</sup>-Zeo(-) (full-length FGR2IIb with the S252W mutation subcloned into pcDNA<sup>3.1</sup>-Zeo(-) from Invitrogen); (3) FGR2IIb<sup>S252W</sup>-FLAG and sFGFR2IIc<sup>S252W</sup>.
Effects of sFGFR2IIIc and sFGFR2IIcS252W on the phosphorylation of intracellular signaling molecules

MC3T3-Ap cells were cultured in the presence of FGF2 (25 ng/ml), U0126 (20 μM), SB203580 (20 μM), sFGFR2IIc, or sFGFR2IIcS252W (60 ng/ml) for 4 h at 37°C in an atmosphere containing 5% CO2, as described before. Cells were then lysed in RIPA buffer and sonicated for 5 s. Proteins (10 μg) were loaded onto 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Amersham Biosciences), followed by incubation with primary antibodies specific for various intracellular signaling molecules as described above.

Matrix mineralization

Matrix mineralization was induced in vitro using MEM-α containing 10% FBS, ascorbic acid (50 μg/ml), dexamethasone (1×10−8 M), and β-glycerophosphate (10 mM) in the presence of U0126 (20 μM), SB203580 (20 μM), or sFGFR2IIcS252W (60 ng/ml) for the indicated culture periods (1–3 weeks). These concentrations were based on those used in previous reports [9]. The mineralized matrix was then stained with Alizarin Red S (AR-S) and washed five times with IP buffer, mixed with 100 μl of TrueBlot Anti-Rabbit IgG HRP (Rockland) for 2 h at 4°C. Cells were then incubated for 20 min with 40 mM AR-S with shaking. To minimize any nonspecific staining, cells were rinsed 5 times with deionized water and once with PBS for 20 min. Finally, AR-S staining of extracellular matrix mineralization was photographed.

Preparation of nanogel-crosslinked hydrogels complexed with sFGFR2IIcS252W

CHPOA was synthesized by reacting CHP (MW, 1.0×10^5 Da; 1,2 diethyl groups per 100 glucose units) with 2-(acryloyloxy)ethyl isocyanate. The degree of substitution, as determined by 1H nuclear magnetic resonance (NMR), was 10.5 per 100 anhydrous glucoside units. Nanogel-crosslinked hydrogels were prepared by Michael addition of acryloyl groups in CHPOA and terminal thiol groups in PEGSH (MW, 1.0×10^5; NOF Corporation) as follows: Rhodamine-labeled CHPOA nanogels with or without sFGFR2IIcS252W were prepared separately. CHPOA nanogels were mixed with PEGSH with a 1:8 molar ratio of acryloyl groups to thiol groups. Five-microliter aliquots of CHPOA/PEGSH mixtures were then placed between two glass slides coated with Parafilm and incubated for 2 h at 37°C in a humidified atmosphere to obtain disc-shaped nanogel-crosslinked hydrogels incorporating FGF2S252W (thickness and diameter, approximately 0.5 mm and 2.5 mm, respectively; sFGFR2IIcS252W, 60 ng).

Calvarial tissue culture

Calvarial bones (E15.5) were dissected from underlying mouse brains, and the skin was peeled off. Tails were used for genotyping as described above. The calvaria from each embryo was bisected in two along the midline of interfrontal and sagittal sutures using ophthalmic instruments under a stereoscopic microscope, taking extreme care not to damage the coronal suture. Bones were then placed in two separate dishes. Explants were cultured using a Trowell-type organ culture system (the grid method) [21]. Briefly, the explants were placed on filters (pore size, 0.1 μm; Whatman, Maidstone, UK) supported by a metal mesh, with the brain side oriented down and the skin side oriented up. The explants were then cultured in 10% FBS-DMEM containing 100 μg/ml ascorbic acid at 37°C in an atmosphere containing 5% CO2 under humidified conditions for 4 days, in the presence of either nanogel-crosslinked hydrogels complexed with sFGFR2IIcS252W.
or vehicle nanogels placed across the coronal suture (Figure S1). 5-
Bromo-2-deoxyuridine (BrdU) was added to the media at a final
concentration of 10 μM for 3 h prior to fixation. S-phase cells
were then detected using an In Situ Cell Proliferation Kit (Roche),
according to the manufacturer’s instructions. At least four explants
from AS mice were sectioned, and at least four sections from each
explant were used to quantify the number of BrdU-positive cells.

Results

Calvarial coronal suture cells of AS mice showed
enhanced osteoblastic differentiation and increased
Fgf21/1b, Fgf10, and Esrp1 expression

Previous reports have shown that the coronal sutures of AS mice
display premature fusion at approximately E18.5 [23,24,25]. To elucidate
the mechanisms underlying the premature closure of
suture tissue, we first investigated the expression of osteoblastic
marker genes in the embryonic calvarial coronal suture of AS mice
at E15.5. Real-time PCR analysis showed higher levels of Runx-
related transcription factor 2 (Runx2) and Osteopontin (Opn)
mRNA (Figure 1A) in the calvarial coronal sutures of AS mice
compared to that in control mice. Interestingly, we observed
significantly increased mRNA expression of Fgf21/1b, the epithelial
splicing form of Fgf2, in the coronal sutures of AS mice compared
with those of littermate control mice. In contrast, uniform Fgf21/1b-
mRNA expression was observed in the tissues of both AS and
control mice (Figure 1B). The expression of epithelial splicing
regulatory protein 1 (Esr1), which plays an important role in the
regulation of FGF21/1b expression, was detected only in AS mice
(Figure 1B). In addition, the Fgf10 protein, a ligand of FGF21/1b,
was also exclusively observed in the suture tissue of AS mice
(Figure 1B). From our western blot analysis, we found increased
phosphorylation of intracellular signaling molecules, such as
ERK1/2, MEK, and SAPK/JNK, and higher expression of Bax
in the coronal sutures of AS mice, compared to those of littermate
controls (Figure 1C). Therefore, these data suggested that the
coronal sutures of AS mice exhibited enhanced phosphorylation of
MAPK signaling molecules following activation of FGF signaling
and induction of apoptosis. Taken together, these results not only
confirmed that osteoblastic differentiation was strongly enhanced
in the coronal suture tissues of AS mice but also suggested that the
S252W mutation in the FGF2 gene may activate both
mesenchymal and epithelial signal transduction pathways
through FGF-FGFR in these tissues.

sFGFR2IIIcS252W binds to Fgf2

To identify which ligands bound to sFGFR2IIIcS252W and verify
whether the corresponding receptors dimerized with
sFGFR2IIIcS252W, sFGFR2IIIcS252W, and the soluble form of
wild-type FGF2 (sFGFR2IIc) were purified. A single band of
approximately 50 kDa was observed in each protein preparation
in the western blot analysis (Figure 2A). In addition, we also
generated Fgf2-His proteins as ligands for FGF2IIc (Figure 2B).
The results of the pull-down assays showed that both purified
sFGFR2IIc and sFGFR2IIcS252W bound to Fgf2 (Figure 2B).
Next, we examined whether sFGFR2IIcS252W formed heterodi-
mers with membrane-bound or cytoplasmic FGF2 to abolish
downstream signaling. Incomplete assembly of the intracellular
signaling complex formed by dimerization of sFGFR2IIcS252W
on the cell membrane or cytoplasmic FGF2 would in turn lead to
inhibition of subsequent signaling because of the formation of
incomplete dimers. Since both FGF2IIc and FGF2IIb
isomers were present in the calvarial coronal sutures of AS mice
(Figure 1B), we hypothesized that sFGFR2IIcS252W may dimerize
with both isomers and exert inhibitory effects on the activation of

Statistical analysis

Analysis of variance (ANOVA) and the Student-Newman-Keuls
test were used to analyze the results of proliferation assays. The
Mann-Whitney U-test with Bonferroni correction was used to
analyze the results of real-time PCR. P values of less than 0.01
were considered significant.
Soluble FGFR2IIIcS252W Inhibits Craniosynostosis

A. Runx2 and Ocn mRNA expression levels.

B. Fgfr2Iib, Fgfr2Iic, Fgf10, Esrp-1, and β-actin protein expression levels.

C. p-Erk1/2, Erk1/2, p-MEK, MEK, p-SAPK/JNK, SAPK/JNK, Bax, and β-actin protein expression levels.
FGF signaling as a decoy receptor due to the loss of ligand specificity. To confirm this hypothesis, double transfection of Cos-7 cells with various plasmid pairs of FGFRs and ligands was performed, followed by immunoprecipitation assays (Figure 2C). Our results clearly demonstrated that sFGFR2IIIcS252W formed heterodimers with FGFR2IIc, FGFR2IIIcS252W, and FGFR2IIIIcS252W (Figure 2C).

sFGFR2IIICs252W inhibited FGF2-stimulated phosphorylation of intracellular signaling and mineralization of MC3T3-E1 cells overexpressing FGFR2IIICs252W (MC3T3-Ap cells)

Western blot analysis using anti-FLAG polyclonal antibodies confirmed the expression of FGFR2IIICs252W-FLAG in MC3T3-Ap cells (Figure 3A). Morphological examination indicated that MC3T3-Ap cells had a somewhat cuboidal shape, unlike the controls (Figure 3A). Application of FGF2 promoted the proliferation of MC3T3-E1 cells, but not that of MC3T3-Ap cells (Figure 3B), most likely because of the aberrant activation of FGF signaling in mutant cells. Basal proliferation activity of MC3T3-Ap cells appeared to be lower than that of MC3T3-E1 cells, but no significant differences were found. Additionally, administration of U0126 and SB203580 (ERK and p38 inhibitors, respectively) inhibited the FGF2-stimulated proliferation of MC3T3-E1 cells. Cell proliferation was significantly decreased by adding sFGFR2IIICs252W in both cell lines (Figure 3B), suggesting that sFGFR2IIICs252W acted as a potential inhibitor for proliferation of osteoblasts with enhanced FGF signaling. Western blot analysis using antibodies against phosphorylated MAPK signaling molecules showed that addition of FGF2 stimulated the phosphorylation of Erk1/2, MEK, SAPK/JNK, p38, and Akt in MC3T3-E1 cells (Figure 4A). On the other hand, MC3T3-Ap cells exhibited spontaneous phosphorylation of all of these molecules, with the exception of Akt (Figure 4A). While U0126 was found to inhibit the phosphorylation of Erk1/2, MEK, SAPK/JNK, p38, and Akt, SB203580 inhibited the phosphorylation of p38 and Akt, but not Erk1/2, MEK, or SAPK/JNK (Figure 4A). SB203580 is a specific inhibitor of p38 MAPK and also inhibits the phosphorylation of Akt when used at higher concentrations than required to inhibit p38 MAPK [26]. Thus, these data suggested that the concentration we used was sufficient to inhibit the phosphorylation of Akt. In addition, treatment with either sFGFR2IIIC or sFGFR2IIICs252W inhibited the phosphorylation of all of these molecules; in particular, sFGFR2IIICs252W had more potent inhibitory effects than sFGFR2IIIC on Erk1/2, SAPK/JNK, and p38 (Figure 4A). These results suggested that sFGFR2IIICs252W had an inhibitory effect on activated FGF signaling by universally suppressing the phosphorylation of MAPK signaling molecules. We found that mineralization of MC3T3-E1 cells occurred within 3 weeks, whereas mineralization of MC3T3-Ap cells was observed within 1 week (Figure 4B). In addition, sFGFR2IIICs252W inhibited the mineralization capacity of both cell types, similar to the effects of U0126 and SB203580. Together, these data indicated that sFGFR2IIICs252W had an inhibitory effect on differentiation and mineralization of MC3T3-E1 cells overexpressing mutated FGFR2IIIC.

Application of sFGFR2IIICs252W with nanogel-crosslinked hydrogels maintained the patency of cranial coronal sutures in AS mice

Finally, to confirm the therapeutic applicability of sFGFR2IIICs252W for craniosynostosis, we applied a tissue culture system that used calvarial tissues dissected from AS mice and littermate controls. We performed preliminary experiments in which organs were cultured in the absence of nanogel. Using HE staining of serial sections, we confirmed that coronal sutures remained patent in control mice (n = 4/4), while AS mice exhibited synostosis of the coronal sutures (n = 4/4) after 4 days of culture in this system (Figure S1). Next, nanogel-crosslinked hydrogels complexed with or without sFGFR2IIICs252W were placed on separate sides of the coronal sutures of the calvarial bone (Figure S1). We found that nanogel-crosslinked hydrogels incorporating sFGFR2IIICs252W were able to maintain the patency of coronal sutures in AS mice (n = 4/4); however, synostosis was observed on the side where only the nanogel was applied (n = 4/4), as demonstrated by in situ hybridization for the bone sialoprotein (Bsp) gene (Figure 5A). The ratio of BrdU-positive cells to total cells tended to be lower on the side where the complex was applied even though without significant difference. Notably, in coronal sutures from AS mice (#9–#12), the ratio of BrdU-positive cells was smaller than that in the controls (#8 and #9; Figure 5B).

Discussion

In this study, we characterized the phenotypes and FGF/FGFR signaling pathways of the coronal suture in AS mice. Application of the purified soluble form of FGF2 carrying the S252W mutation suppressed the aberrant characteristics of osteoblasts overexpressing FGFR2IIICs252W and prevented premature fusion of the coronal suture in cultures of calvarial tissue from AS mice. Mesenchymal tissue from coronal sutures from AS mice exhibited enhanced differentiation of osteoblasts (Fig. 1A) and increased phosphorylation of ERK, MEK, and SAPK/JNK (Figure 1C). Previously, we reported marked premature ossification of the calvarium during the waiting period of distraction osteogenesis for the treatment of the deformed thumb in a patient with Apert syndrome (S252W) [27]. Consistent with previous reports showing that FGFR2-dependent osteogenesis was promoted by the Apert mutation [28,29], the clinical findings suggested that FGFR2 bearing the S252W mutation promotes osteogenic ability. We have reported the accelerated mineralization of osteoblast-like cells in two patients with Apert syndrome [8], MG63 osteosarcoma clones (n = 4) stably expressing FGFR2IIICs252W (the Apert mutation) [8], and transgenic mice overexpressing FGFR2IIICs252W in vivo and in vitro [9]. Mirai
et al. reported that FGFR2IIIcS252W promoted osteogenic gene expression through ERK1/2 and protein kinase C alpha (PKCα) signaling and accelerated mineralization of murine mesenchymal C3H10T1/2 [30]. Apoptosis has been proposed as the underlying mechanism of suture fusion [23]. However, Holmes et al. concluded that apoptosis is likely to be a consequence rather than a cause of synostosis even though they showed apoptotic cells are present in the limited osteoid contact in the Apert mutant coronal sutures after E16.5 [24]. Meanwhile, recent study reported a high percentage of apoptotic chondrocytes in Axin2 (−/−) posterior-frontal suture which showed significant closure delay at the early postnatal stage [31]. As mentioned above, the relationship between apoptosis and pathological premature suture closure or closure insufficiency does not reach a definitive conclusion. In this study, we observed an increased expression of Bax in the coronal suture of AS mice at E15.5 (Figure 1C), suggesting the early incidence of apoptosis in the coronal suture of Apert mice. Given our previous studies [8,9], we believe that the main causative mechanism is the enhanced osteoblast differentiation in the osteogenic front and sutural mesenchymal cells of the prematurely fused suture. This phenomenon could conductive to lower cell proliferation and apoptosis. Further studies are needed in order to clarify the detail role of apoptosis in the pathological suture development. We also observed increased expression of Esrpl, FGFR2IIIb, and Fgf10 mRNAs in AS mouse coronal sutures (Figure 1B). Previous studies have shown that ESRP1 and ESRP2 are required for the expression of epithelial FGFR2IIIb [32]. The Fgfr2IIIb isoform is known to be expressed in epithelial cells and has been shown to bind to Fgf7 and/or Fgf10 with high affinity [33]. FGFR2IIIb expression in the maxillofacial region of AS patients has not been examined; however, fibroblast cells obtained from the limbs of AS patients carrying an Alu-element insertion in

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Figure 2. Purification of soluble forms of FGFR2 and their biological activities in vitro. (A, B) The expression levels of purified sFGFR2IIIc and sFGFR2IIIcS252W proteins (A), as well as that of Fgf2-His (B), were analyzed by western blot analysis. (B) Pull-down assay for the binding of Fgf2-His with sFGFR2IIIc-FLAG and sFGFR2IIIcS252W-FLAG. Fgf2 interacted with sFGFR2IIIc-FLAG and sFGFR2IIIcS252W-FLAG. (C) Dimerization between sFGFR2IIIcS252W and FGFR2IIIc, FGFR2IIIcS252W, or FGFR2IIIbS252W was determined by immunoprecipitation assays using anti-FGFR2 antibodies that recognized the cytoplasmic region of Fgfr2, followed by western blot analysis using anti-FLAG antibodies. The formation of heterodimers between sFGFR2IIIcS252W-FLAG and membrane-bound FGFR2IIIc-FLAG, FGFR2IIIc-FLAG, FGFR2IIIcS252W-FLAG, or FGFR2IIIbS252W-FLAG was confirmed.

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FGFR2 have been reported to exhibit ectopic expression of FGFR2IIIb [34]. Molecular studies have shown that Alu insertions affect the alternative splicing of FGFR2, resulting in ectopic expression of FGFR2IIIb in mesenchyme-derived cell lineages that normally express FGFR2IIIc [34]. Hajihosseini and colleagues [35] reported that mice with heterozygotic abrogation of Fgfr2-exon 9 (IIIc) exhibit craniosynostosis and that Fgfr2IIIb is strongly expressed in calvarial sutures. The authors hypothesized that there is less Fgfr2IIIc because of the Cre deletion in these calvarial sutures, leading to increased FGFR2IIIb expression. The S252W or P253R mutation in FGFR2 allows FGFR2IIIc to be activated by FGF7 or FGF10 [6]. Based on these findings, we suggest that increased Fgfr2IIIb/Fgf10 expression contributes to the onset of craniosynostosis in AS mice. Rice et al. reported that the expression of Fgf2IIib mRNA was detected by in situ hybridization in the cranial base perichondrium and periosteum at very low levels [36]. In addition, in situ hybridization revealed minute amounts of Fgf2IIib mRNA at the osteogenic front of the sagittal suture at E15 and E17 [37] and at the developing primordia of the frontal bone [38]. Veistinen et al. showed low expression of Fgf10 mRNA in the developing primordia of the frontal bone by in situ hybridization [38]. These results prompted us to perform real-time PCR to assess the expression levels of Fgf2IIib, Fgf10, and Esrp1 in the coronal sutures of our AS mice (E15.5). We detected the expression of Fgf2IIib, Fgf10, and Esrp1 mRNA by real-time PCR; however, Fgf10 and Esrp1 proteins were not detected in control mice in our western blot analysis. Because the expression levels of these genes at the wild-type calvarial suture were low, the

Figure 3. Analysis of the inhibitory effects of sFGFR2IIIC252S252W on the proliferation of MC3T3-E1 cells. (A) Protein expression of FGFR2IIIC252S252W-FLAG in the established MC3T3-E1 cell clone (MC3T3-Ap) was confirmed by Western blot analysis using anti-FLAG antibodies. Morphological examination of MC3T3-E1-MOCK (left) and MC3T3-Ap (right) indicated that MC3T3-Ap had a somewhat cuboidal shape, unlike the controls. (B) MTT assays were used to measure the proliferation of parental MC3T3-E1 and MC3T3-Ap cells upon administration of FGF2 (25 ng/mL) in the presence of U0126 (20 μM), SB203580 (20 μM), or sFGFR2IIIC252S252W (60 ng/mL). FGF2 was found to promote the proliferation of parental MC3T3-E1 cells, but not MC3T3-Ap cells. Basal proliferation levels of MC3T3-Ap cells were lower than those of parental MC3T3-E1 cells. Additionally, U0126 and SB203580 (ERK and p38 inhibitors, respectively) inhibited the proliferation of MC3T3-E1 cells. The proliferation of all cell lines was significantly decreased by addition of sFGFR2IIIC252S252W. Statistical analysis was performed using ANOVA and the Student-Newman-Keuls test. *p<0.01.

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sFGFR2IIIcS252W, U0126, or SB203580 was visualized by Alizarin Red S staining. MC3T3-E1 cells and MC3T3-Ap cells in the presence or absence of sFGFR2IIIc on Erk1/2, SAPK/JNK, and p38. (B) Mineralization of control SB203580, or sFGFR2IIIcS252W inhibited mineralization. Compared to control MC3T3-E1 cells, and administration of U0126, MC3T3-Ap cells showed enhanced mineralization at 1 week, whereas the cells treated with sFGFR2IIIcS252W showed decreased proliferation and differentiation. These data suggested that sFGFR2IIIcS252W acted as an inhibitor for FGF2-stimulated proliferation. Our data also suggested that MC3T3-Ap mimicked the phenotypes of AS calvarial osteoblasts, exhibiting enhanced osteoblastic differentiation and phosphorylation of intracellular signaling molecules (Figures 1A, 1C and 4A, and 4B). These observations are consistent with those of previous studies showing that the S252W mutation increases osteoblast differentiation [7,9,24,25]. The differentiation capacity of MC3T3-AP cells was significantly increased compared to intact MC3T3-E1 cells, whereas the cells treated with sFGFR2IIIcS252W showed decreased proliferation and differentiation (Figures 3B and 4B). Increased differentiation is possibly a specific effect of the S252W mutation [7,9,24,25].

Figure 4. Analysis of the inhibitory effects of sFGFR2IIICS252W on intracellular signaling and in vitro mineralization. (A) Phosphorylation of Erk1/2, p38, MEK, SAPK/JNK, and Akt in parental MC3T3-E1 or MC3T3-Ap cells upon addition of FGF and sFGFR2IIICS252W was determined by western blot analysis. FGF2 stimulated the phosphorylation of Erk1/2, MEK, p38, and Akt, whereas SB203580 inhibited p38 and Akt, but not Erk1/2, MEK, or SAPK/JNK. Treatment with either sFGFR2IIIC or sFGFR2IIICS252W inhibited the phosphorylation of all of these molecules; in particular, sFGFR2IIICS252W had stronger inhibitory effects than sFGFR2IIIC on Erk1/2, SAPK/JNK, and p38. (B) Mineralization of control MC3T3-E1 cells and MC3T3-Ap cells in the presence or absence of sFGFR2IIICS252W, U0126, or SB203580 was visualized by Alizarin Red S staining. MC3T3-Ap cells showed enhanced mineralization at 1 week, compared to control MC3T3-E1 cells, and administration of U0126, SB203580, or sFGFR2IIICS252W inhibited mineralization.

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Figure 5. Analysis of the inhibitory effects of sFGFR2IIIcS252W on the premature fusion of coronal sutures. (A) The effects of nanogel-crosslinked hydrogels incorporating sFGFR2IIIcS252W on the coronal sutures of AS mice were determined using a tissue culture system. Mature bones are shown in purple, resulting from in situ hybridization using the bone sialoprotein (Bsp) probe. Yellow lines on HE-stained images show the contours of the parietal and frontal bony edges, whereas a black arrowhead shows the existence of FLAG-tagged proteins in the calvarial tissue, as determined by immunohistochemistry using anti-FLAG antibodies. The images of blue fluorescence (4', 6-diamidino-2-phenylindole [DAPI], staining the nucleic acids), green fluorescence (bromodeoxyuridine [BrdU], incorporated into cellular DNA during proliferation), and red fluorescence (rhodamine-stained nanogels) were merged. Administration of sFGFR2IIIcS252W/nanogel complex maintained the patency of the coronal sutures in AS mice (n = 4/4; AS mice Nos. 9 and 10 are shown as representative examples); however, synostosis was observed on the side where only the nanogel was applied (n = 4/4). Control mice (n = 2/2; control mouse No. 8 is shown as a representative example) did not show any fusion between the frontal and parietal bones. Scale bar = 200 μm. f, frontal bone; p, parietal bone. (B) The ratios of BrdU-positive cells in AS mice and littermate controls are shown. Although no significant difference was observed, the ratio tended to be decreased in the suture tissue of AS mice, compared to the control littermates. Statistical analysis was performed using ANOVA and the Student-Newman-Keuls test.

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interference and inhibition of MEK/ERK signaling also rescued craniosynostosis in AS mice carrying the S252W mutation [42]. Specifically, shRNAs targeting to Fgfr2S252W relieved premature fusion of coronal sutures in vivo [42]. In addition, recombinant Noggin has been reported to act as an inhibitor of craniosynostosis in rat calvarial coronal sutures with transplantation of human AS osteoblasts [43]. However, these strategies are currently distant from clinical applications. Therefore, because surgical intervention is the only current treatment available to reduce morphological and function defects in patients with AS, noninvasive procedures for treatment of AS need to be developed. To this end, our group has focused on the soluble form of FGFR2 and its dominant-negative effect on FGFRs [8,9,10]. We found that sFGFR2IIIcS252W was able to maintain the patency of the AS mouse coronal sutures in the calvarial tissue culture system (Figure 5A). Some basic studies have examined the proficiency of soluble receptors to function as decoy receptors in clinical applications [44,45]. Our purified sFGFR2IIIcS252W is unique for several reasons. First, we speculate that soluble FGFR2IIIcS252W can spread to the whole body through the bloodstream because of its endogenous expression in human tissues. Second, it has potent inhibitory effects towards the pathological conditions of AS because of the S252W mutation, which causes loss of ligand specificity and reduces ligand dissociation rates; this study is the first to report the use of a soluble receptor carrying a mutation associated with a human congenital anomaly. Third, purified sFGFR2IIIcS252W not only has binding affinity for Fgfr2, but was also shown to dimerize with membrane-bound or cytoplasmic monomeric FGFR2 in the presence or absence of the S252W mutation (Figure 2C), resulting in incomplete dimers that block subsequent intercellular signaling. Our current strategy, which applied the purified protein to the calvarial tissue by using a nanogel-crosslinked hydrogel system as the protein carrier has several advantages; individual nanogels can store proteins inside their nano spaces and then gradually release proteins locally without any significant change in their biological activities; they do not contain antigens; they are inexpensive. However, many problems must be resolved before this protein is applied in clinical settings for the treatment of AS patients. Because craniosynostosis typically occurs during fetal life [46], the safety and effectiveness of FGFR2-based treatment must be tested and an appropriate administration technique should be established using animal experiments in vivo. Some patients with craniosynostosis display recrudescence of bony fusion just after craniectomy; the complex might help block these unfavorable phenomena caused by aberrant GFG/FGFR signaling. Taken together, our data suggested that the appropriate delivery of purified sFGFR2IIIcS252W could be an effective method for treating not only AS but also other types of craniosynostosis resulting from aberrant FGFR/FGFR signaling.

Supporting Information

Figure S1 Diagram of the calvarial tissue culture system in this study and reproducibility of the premature fusion of coronal sutures in AS mice. (A) Calvarial bones (E15.5) were dissected from underlying mouse brains, and the skin was peeled off. The calvarial bone unit from each embryo was divided into two pieces along the midline interfrontal and sagittal sutures, and sectioned bones were placed in two separate dishes. The explants were then placed on filters supported by a metal mesh, with the brain side oriented down and the skin side oriented up. Explants were cultured in 10% FBS-DMEM containing 100 μg/mL ascorbic acid at 37°C in an atmosphere containing 5% CO2 under humidified conditions for 4 days in the presence of either nanogel-crosslinked hydrogels complexed with sFGFR2IIIcS252W or vehicle nanogels placed across the coronal suture. (B, C) We performed preliminary experiments to assess the incidence of premature fusion of the coronal sutures in AS mice during the 4-days tissue culture period in the absence of nanogel. Using HE, staining of serial sections, we confirmed that coronal sutures remained patent in control mice (n = 4/4), while AS mice exhibit synostosis of the coronal sutures (n = 4/4) in this system. Scale bar = 100 μm. cs, coronal suture; f, frontal bone; of, osteogenic front; p, parietal bone.

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Author Contributions

Conceived and designed the experiments: YM YK. Performed the experiments: MY YH YS KA. Analyzed the data: MY YK. Contributed reagents/materials/analysis tools: JM HS YH YS KA. Wrote the paper: YM YK YS YA KM.

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9. Tanimoto Y, Yokozeki M, Hiura K, Matsumoto K, Nakanishi H, et al. (2004) A negative effect on FGFs [8,9,10]. We found that sFGFR2IIIcS252W has focused on the soluble form of FGFR2 and its dominant-negative effect on FGFRs [8,9,10]. We found that sFGFR2IIIcS252W was able to maintain the patency of the AS mouse coronal sutures in the calvarial tissue culture system (Figure 5A). Some basic studies have examined the proficiency of soluble receptors to function as decoy receptors in clinical applications [44,45]. Our purified sFGFR2IIIcS252W is unique for several reasons. First, we speculate that soluble FGFR2IIIcS252W can spread to the whole body through the bloodstream because of its endogenous expression in human tissues. Second, it has potent inhibitory effects towards the pathological conditions of AS because of the S252W mutation, which causes loss of ligand specificity and reduces ligand dissociation rates; this study is the first to report the use of a soluble receptor carrying a mutation associated with a human congenital anomaly. Third, purified sFGFR2IIIcS252W not only has binding affinity for Fgfr2, but was also shown to dimerize with membrane-bound or cytoplasmic monomeric FGFR2 in the presence or absence of the S252W mutation (Figure 2C), resulting in incomplete dimers that block subsequent intercellular signaling. Our current strategy, which applied the purified protein to the calvarial tissue by using a nanogel-crosslinked hydrogel system as the protein carrier has several advantages; individual nanogels can store proteins inside their nano spaces and then gradually release proteins locally without any significant change in their biological activities; they do not contain antigens; they are inexpensive. However, many problems must be resolved before this protein is applied in clinical settings for the treatment of AS patients. Because craniosynostosis typically occurs during fetal life [46], the safety and effectiveness of FGFR2-based treatment must be tested and an appropriate administration technique should be established using animal experiments in vivo. Some patients with craniosynostosis display recrudescence of bony fusion just after craniectomy; the complex might help block these unfavorable phenomena caused by aberrant GFG/FGFR signaling. Taken together, our data suggested that the appropriate delivery of purified sFGFR2IIIcS252W could be an effective method for treating not only AS but also other types of craniosynostosis resulting from aberrant FGFR/FGFR signaling.

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Author Contributions

Conceived and designed the experiments: YM YK. Performed the experiments: MY YH YS KA. Analyzed the data: MY YK. Contributed reagents/materials/analysis tools: JM HS YH YS KA. Wrote the paper: YM YK YS YA KM.
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