Fgf9 Repression of Msx2 Gene Expression Enhances Transcriptional Activity of Osteocalcin Gene

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기술적 도움을 기꺼이 주었던 Dr. David Diefenderfer 에게 감사의 말씀을 전합니다. Adenovirus 를 제공해주신 Dr. Lillian Shum, luciferase assay 를 위한 construct 를 제공해주신 Dr. Masahiro Iwamoto 에도 지면상으로 감사의 말씀을 전합니다. 자칫 외롭고 힘들어질 수 있는 유학생활 중 힘이 되어준 펜실베이니아 치과대학 교정학교실 교수님 여러분과 수련의 친구들에게 감사드립니다. 또한 연세대학교 교정학 교실의 수련의 여러분과 특히 논문의 진행과 기타 행정적인 일들에 자신의 일보다 더 적극적으로 도움을 주신 차정열 선생과 논문 일정에 차질이 없도록 힘쓴 주억 선생에게 감사의 말씀을 전합니다.

이국에 있는 동안 편안히 학업에 정진할 수 있도록 헤아릴 수 없는 정성으로 감싸주신 어머니, 그리고 아이를 돌보듯 내내 저희 내외를 굽어 보살피신 장인어른과 장모님께 깊은 감사의 말씀을 드립니다. 끝으로 외로운 이국 생활에서도 내조에 힘쓰며 든든한 버팀이 되어준 나의 사랑하는 아내 최영윤 선생과 탈없이 건강하게 자라준 두 자녀 준영, 채영에게 사랑과 고마움의 마음이 담긴 이 논문을 드립니다.

2003 년 12 월

저자 씀

Table of Contents

List of Figures

ABSTRACT

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Mutations of either FGFR2 or MSX2 genes cause craniosynostosis via similar gain-of-function mechanism. Both genes are known to play crucial roles in the calvarial bone formation and are expressed in the early osteoblast stage in the developing cranial suture. However, the regulatory function between FGF/FGFR and MSX2 is not known. Thus, we asked if Fgf/Fgfr signaling is an upstream regulator of Msx2 gene expression in the same genetic pathway and wished to elucidate the possible regulatory role of these genes in differentiating osteoblasts. To investigate further the molecular events occurring in the cell level, we used an in vitro culture with primary mouse calvarial osteoblasts. We demonstrated the expression pattern of murine Fgfr2 and Msx2 gene in different maturation stage, with peak expression of Msx2 gene preceding that of Fgfr2. To examine the effect of Fgf/Fgfr signaling on the Msx2 gene expression, primary osteoblasts were treated with Fgf9, a cognate ligand for Fgfr2, and Msx2 gene expression was significantly

suppressed via a protein synthesis. To gain further insight into the significance of this down-regulation, Msx2 was overexpressed in the osteoblasts using adenovirus containing Msx2 gene tagged with hemagglutinin (HA) epitope. An RT-PCR analysis showed that the overexpression of Msx2 inhibited the increase of the endogenous osteocalcin gene expression induced by Fgf9. Subsequent gel-shift assays revealed that Msx2 overexpression blocked the binding of Cbfa1/Runx2 on the cognate sequence (OSE2) in the presence of Fgf9. Moreover, overexpression of Msx2 followed by transfection with 6XOSE2-luciferase construct resulted in reduced transcriptional activity of the promoter in the presence of Fgf9. From these results, we propose a possible model of osteoblast differentiation with integrated Fgf/Fgfr and Msx2 function, implicating the suppressive role of Fgf/Fgfr on Msx2 expression by modulating the Cbfa1/Runx2, leading to increased osteocalcin transcription.

Key words; calvarial bone formation, FGF/FGFR, MSX2, mouse, osteoblast, Cbfa1/Runx2, osteocalcin

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I. INTRODUCTION

Critical molecular clues to the regulation of calvarial osteoblast differentiation have been revealed in the pursuit of finding a cause of craniosynostosis, i.e. premature fusion of cranial sutures, which leads to a variety of craniofacial deformities (Opperman 2000, Kim et al. 1998, Rice et al. 2000). Mutations in the genes encoding MSX2, fibroblast growth factor receptors (FGFRs) and TWIST are known causes of various syndromic / non-

syndromic craniosynostosis (Hehr and Muenke 1999, Jabs et al. 1993, Liu et al. 1995, El Ghouzzi et al. 1997). Since premature fusion of sutures results from disrupted regulation of balanced proliferation and/or differentiation of osteoblasts at the osteogenic front, these genes are thought to play crucial roles for the regulation of calvarial osteoblast differentiation.

Point mutations in FGFR2 have been reported to cause human craniosynsotosis syndromes, such as Apert, Pffeifer, Jackson-Weiss, and Crouzon syndromes (Wilkie 1997, Wilkie et al. 1995, Jabs et al. 1994, Reardon et al. 1994, Lajeunie et al. 1995, Rutland et al. 1995). Fgfrs are transmembrane tyrosine receptor kinases and activated by a number of ligands, Fgfs, showing complex ligand-binding specificity (Ornitz et al. 1996, Shimoaka et al. 2002). Fgfr2 is a major isoform that is expressed at the osteogenic fronts in the developing suture (Iseki et al. 1999), implicating important roles in the calvarial bone formation (Eswarakumar et al. 2002, Rice et al. 2003).

The net effect of Fgf/Fgfr signaling in the bone cells is complicated. Fgf2 is known to stimulate osteoblast proliferation and inhibit differentiation markers (Canalis and Raisz 1980, Hurley et al. 1992). In contrast, FGF induces expression and transactivation of Cbfa1/Runx2 (Kim et al. 2003, Xiao et al. 2002) and osteocalcin (Boudreaux and Towler 1996). Cbfa1/Runx2 is a runt domain transcription factor that has been shown to be exclusively essential for

the maturation of bone cells (Harada et al. 1999, Komori et al. 1997). Osteocalcin is a small, calcium binding marker protein for mature osteoblasts consisting of 49 amino acids and is expressed only in bones and teeth undergoing mineralization (Newberry et al. 1998, Bidder et al. 1998). Gain-of-function mechanisms of FGFR2 by either constitutive activation of receptors or loss of ligand-binding specificity have been proposed (Neilson and Friesel 1996, Yu et al. 2000, Anderson et al. 1998) to explain the pathogenesis of craniosynsotosis..

The first molecular clue to the mechanism of craniosynostosis was the Pro148His mutation in the human MSX2 gene that caused Boston-type craniosynostosis (Jabs et al. 1993). Similarly, early fusion of sutures and ectopic bone formation was reproduced in the transgenic mice models expressing same mutant Msx2 (Ignelzi et al. 1995, Liu et al. 1995, Liu et al. 1999).

Msx2 is a homeodomain-containing transcription factor and is crucial for the cranial bone formation (Davidson 1995, Satokata et al. 2000). In the developing skull, Msx2 is mainly expressed in the sutural mesenchyme, underlying dura mater and extreme ends of the osteogenic fronts (Rice et al. 2003, Kim et al. 1998, Opperman et al. 1993, Ma et al. 1996), implying its significant role during early differentiation of osteoblasts (Towler et al. 1994). Dodig et al. reported that Msx2 retarded differentiation of the calvarial

osteogenic cells and maintained cells in a proliferative state (Dodig et al. 1996, Dodig et al. 1999), contributing to the increase in the bone mass. Msx2 is also a known suppressor of the osteocalcin promoter likely by protein-protein interaction (Newberry et al. 1997). Like in the mutations of FGFR2, Maxson et al. proposed a gain-of-function mechanism of the Pro148His mutation of Msx2, demonstrating the increased DNA binding of mutant gene-encoded Msx2 protein (Ma et al. 1996).

Normal Fgfr2 and Msx2 have been shown to be co-expressed in a variety of sites in the developing mouse embryo as well as in the calvarial suture (Orr-Urtreger et al. 1991, Orr-Urtreger et al. 1993, Rice et al. 2003) and mutations of these genes lead to early fusion of sutures via similar gain-of-function of the protein. The question then arises if FGF/FGFR2 and MSX2 lie in the same genetic pathway in the differentiation of osteoblasts. Previous studies, utilizing the in situ hybridization approach in the suture tissue, attempted to answer this question only to show appreciable but still controversial results regarding the effects of Fgf on the Msx2 gene expression (Kettunen and Thesleff 1998, Kim et al. 1998, Ignelzi et al. 2003). However, individual events occurring in the bone cells may be better understood by investigating the molecular functions at the cell level.

We hypothesized that Fgf/Fgfr2 may be an upstream regulator of Msx2 in the same genetic pathway in differentiating osteoblasts and wished to elucidate

further the role of these molecules during initial differentiation of calvarial osteoblasts. We used in vitro culture system for primary mouse calvarial bone cells. Expression of Fgfrs, Msx2 and some other bone marker genes were first analyzed to characterize the phenotype of the cells obtained through serial digestion of calvarial bones. We demonstrated that Fgf9 (a cognate ligand for Fgfr2) treatment of calvarial osteoblasts significantly suppressed Msx2 gene expression via a protein synthesis.

To gain further insight into the significance of this Fgf9-induced down-regulation of Msx2, we overexpressed the Msx2 gene with an adenovirus containing Msx2 tagged with hemagglutinin (HA) epitope (Takahashi et al. 1998). We then analyzed the effects of overexpression of Msx2 on the transcriptional activity of endogenous osteocalcin gene through RT-PCR, on the DNA binding of Cbfa1/Runx2 through gel-shift assays, and on the transcriptional activity of osteocalcin promoter through transfection and luciferase assays using 6XOSE2 luciferase construct, in the presence or absence of Fgf9. we propose a possible model of osteoblast differentiation with integrated Fgf/Fgfr and Msx2 function, implicating the suppressive role of Fgf/Fgfr on Msx2 expression by modulating the Cbfa1/Runx2, leading to increased osteocalcin transcription. This study proposes an important role of Fgf/Fgfr signaling in the regulation of Msx2 for enhanced Cbfa1/Runx2 function, which has been unnoticed until now.

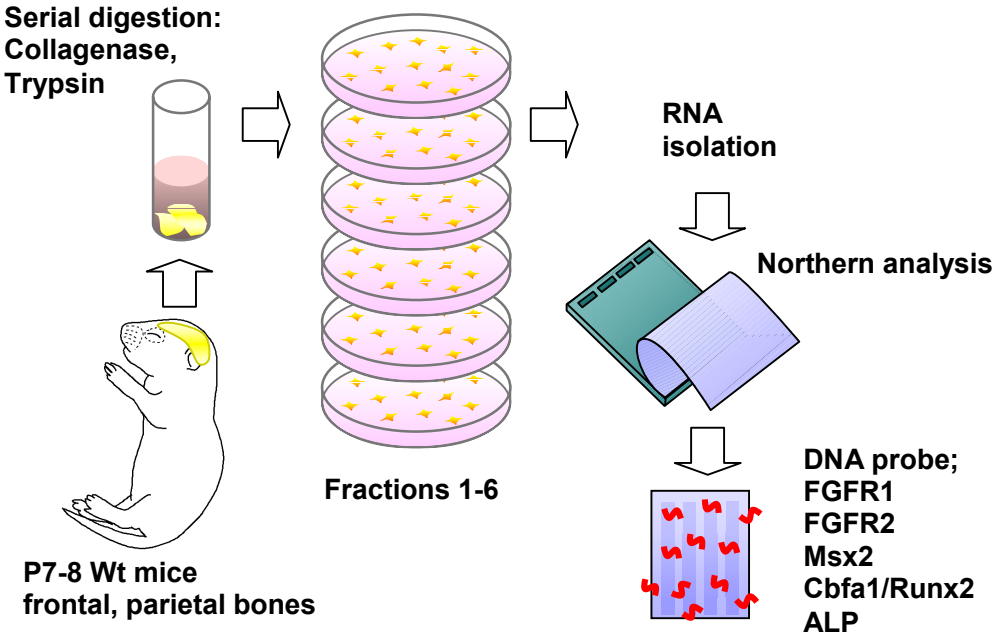
II. MATERIALS AND METHODS

A. Primary Mouse Calvarial Bone Cell Culture

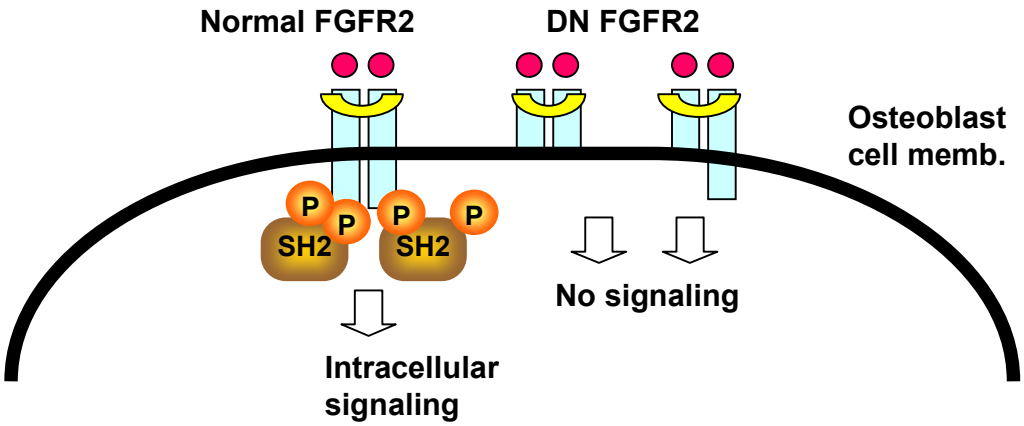
Primary mouse calvarial bone cells were cultured as previously described (Dodig et al. 1996, Wong and Cohn 1974). Briefly, calvariae isolated from 7-8-day-old mice were cleaned by gently removing fibrous tissue tags as well as dura mater and were subjected to sequential enzymatic digestion in 1mg/ml bacterial collagenase (Sigma Aldrich Co., St. Louis, MO) and 0.25% trypsin (Invitrogen Corp., Carlsbad, CA) at 37^{o}C for 20 min per digestion cycle. Digestion was repeated to obtain the cells corresponding to each fraction 1 to 6. 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^{o}C incubator with 5% CO_2. Medium was renewed every 48 hours. After 1 week, when the cells became confluent, the first two fractions were discarded and the cells from fractions 3-6 were released and pooled, centrifuged and resuspended. Viable cells were re-plated at a density of 3.3 x 10^4 cells/cm^2 plate in α-MEM containing 10% FCS for subsequent expreiments. Cells were given 24 hours for attachment and appropriate Fgf9 (R&D System, Minneapolis, MN) treatment was followed.

Primary mouse calvarial osteoblasts with dominant negative (DN) Fgfr2 were obtained from the transgenic mice expressing DN Fgfr2, in the same way. (Ratisoontorn et al. 2003)

Schematic diagram of primary mouse calvarial bone cell culture and northern blot analysis



Schematic diagram of normal and dominant negative (DN) Fgfr and the functions



B. RNA Isolation and Northern Blot Analysis

Total RNA was isolated using TRIzol Reagent (Invitrogen life Tech., Carlsbad, CA) as previously described (Chomczynski 1993, Sambrook et al. 1989). 10μg of RNA samples were separated on a 1% denaturing agarose gel, containing 2.2 M formaldehyde and 1X MOPS, and transferred overnight onto a nylon membrane (Maximum Strength Nytran, Schleicher & Schuell, Keene, NH). The membrane was prehybridized in a solution containing 50% formamide, 5X SSPE (0.75 M NaCl, 50mM NaH_2PO_4, 5mM EDTA, pH 7.4), 1.5% SDS, 5X Denhardt's solution and 0.5mg/ml of denatured salmon sperm DNA for two hours.

cDNA probes for mouse Fgfr1 and Fgfr2, Msx2, Cbfa1 and alkaline phosphatase were labeled with [α-^{32}P]dCTP (Amersham Biosciences,

Piscataway, NJ) using random primer labeling method (Rediprime^{TM}II, Amersham Biosciences) and were hybridized onto the nylon membranes at 42^{o}C overnight. The blots were rinsed three times with 2X SSC (0.3 M NaCl and 30 mM sodium citrate, pH 7.0) and 0.5% SDS for 10 min per wash at room temperatue, and again washed three times in 0.2X SSC and 0.5% SDS for 30 min per wash at 55 ^{o}C. X-ray films with intensifying screens were exposed to the membrane for reading.

C. Recombinant Adenovirus Infection

Adenovirus containing Msx2 tagged with hemagglutinin (HA) epitope under the control of EF-1 promoter was a kind gift from Dr. Lillian Shum. (National Institutes of Health, Bethesda, MD) (Takahashi et al. 1998, Mizushima and Nagata 1990)

For the isolation of the RNA or nuclear extract, primary mouse calvarial bone cells were cultured in α-MEM supplemented with 10% FCS 24 hours before the viral infection. Virus (3 x 10^6 pfu/10^5 cells) was added in the medium and infected cells were grown for 24 hours before proceeding to the next step.

Viral infectivity of the primary mouse osteoblasts was determined by both immunoblot and immunostaining using an antibody specific for hemagglutinin (rabbit anti-HA IgG; Santa Cruz Biotechnology Inc., Santa Cruz, CA).

D. Immunoblot Assay

Immunoblot analysis with nuclear extracts to detect virally produced Msx2-HA proteins was performed as previously described (Takahashi et al. 1998). The procedure for obtaining nuclear extracts is explained below. 20 μg of nuclear extracts isolated from virus-infected or control cells were resolved on a 10% (w/v) SDS-polyacrylamide gel and electrotransferred onto a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech, Piscataway, NJ). Following blocking in a 5% nonfat milk solution (1X TBS with 0.1% Tween20 ; TBST) for 1 hour and washing (4 times for 5 min. each with 1X TBST), the blot was incubated with the primary antibody against HA at 1:1000 dilution in 1X TBST with 5% nonfat milk overnight at 4^{o}C. The blot was again washed 4 times. A horseradish peroxidase (HRP)-conjugated goat anti-rabbit or mouse IgG was used as secondary antibody (Amersham Biosciences, Buckinghamshire, England) and incubation was continued for 1 hour at room temperature. Then, the antigen-antibody reaction was detected using a chemiluminescent detection kit (Western Lightning Chemilumi-nescence Reagent 1, Perkin Elmer Life Sciences, Boston, MA).

E. Immunostaining for Msx2-HA

Immnostaining was designed to localize the virally produced Msx2-HA proteins in the cell culture system. Primary mouse osteoblasts were plated on

tissue culture slides (Falcon Cultureslide, Becton Dickinson, Franklin Lakes, NJ), and were infected with adenovirus containing Msx2-HA gene. Both infected and uninfected cells were fixed in 4% paraformaldehyde for 15 min. and again in 70% ethanol for 30 min. Fixed cells were dried and re-hydrated with PBS, followed by blocking in 10% normal goat serum for 1 hour. The cells were then incubated with anti-HA polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 3 hours at 37^{o}C at 1:100 dilution in PBS. After washing three times for 2 min. with PBS, subsequent incubation with secondary biotin-goat anti-rabbit IgG (Zymed Laboratories, San Francisco, CA) at 1:500 dilution for 30 min., and with streptavidin-HRP conjugate at 1:250 dilution for 10 min. were carried out. Substrate-chromogen mixture (DAB) was added for 5 min. After washing with distilled water, the cells were counterstained with hematoxylin.

F. Reverese Transcriptase-Polymerase Chain Reaction (RT-PCR)

A reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to determine the expression of the endogenous osteocalcin gene. Total RNA was reverse transcribed to obtain cDNA, using the SuperScript^{TM} First-Strand Synthesis System (Invitrogen Life Technologies, Carlsbad, CA). The cDNAs were amplified through 40 cycles of PCR. Each cycle consisted of 1 min. of

denaturation at 94°C, 1 min. of annealing at 55°C and 1 min. of extension at 72°C, using the taqDNA polymerase (Promega, Madison, WI).

Since 2 different types of mouse (rat) osteocalcin gene with 1557bp and 1848bp are known, we used the overlapping sequences in the two genes as the osteocalcin primer, as follows;

forward primer 5'-TCTCTGACCTCACAGGTATG-3' and

reverse primer 5'-ACACTGTACAAGAGGCTCCAG-3'.

The size of PCR product was anticipated to be 517 and 521bp, respectively. As an internal control, a PCR was performed for 20 cycles using GAPDH primer.

G. Extraction of Nuclear Protein and Gel Mobility Shift Assay

Nuclear extracts were isolated from the control and the virus-infected cells with or without treatment of Fgf9. Cells were washed, harvested in TBS, centrifuged for 10 min. at 4000 rpm at 4°C and then incubated for 15 min. on ice in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 1 mM NaVO3, cocktail of proteinase inhibitor (Sigma, St Louis, MI) at 1:100 dilution). After adding 25μl of nonidet P-40, lysis solution was vortexed and centrifuged for 5 min. at 4000 rpm at 4°C.

Supernatant was discarded and the remaining nuclei pellet was dissolved in ice-cold buffer C (20 mM HEPES, 1 mM EDTA, 0.4 M NaCl, 2 mM PMSF,

0.1 mM TPCK, 10 mM NEM, 1 mM NaVO3, cocktail of proteinase inhibitor at 1:100 dilution) for 1 hour at 4^{o}C with gentle agitation. Protein concentration was determined by Bradford assay using bovine serum albumin as a standard.

Gel mobility shift assays were carried out using commercially available Nushift kit (Geneka Biotechnology Inc.,Montreal, Canada). According to the manufacturer's instruction, double-stranded oligonucleotides containing Cbfa1 binding element (OSE2) in the (human) osteocalcin promoter oligonucleotides (5'-CCCGTATTAACCACAATAAAACTCG - 3') was radio-labeled with [α-^{32}P]dCTP. Mixtures of radio-labeled oligonucleotides (1μl) and nuclear extracts were incubated for 20 min. at room temperature. Unlabeled oligonucleotide was used as a competitor. Anti-Cbfa1/Runx2 rabbit IgG for observation of super-shift were incubated with nuclear extracts in the same way.

Prepared samples were resolved on 5% non-denaturing polyacrylamide gel for 2 hours at 12.5V/cm. Subsequently, the gel was dried at 80^{o}C for 90 min. under vacuum and was exposed to X-ray film overnight.

H. Transfection and Luciferase Assay

A reporter plasmid containing 6-repeats of the consensus Cbfa1 binding site (6XOSE2) was kindly provided by Dr. Masahiro Iwamoto (Thomas Jefferson University Medical School, Philadelphia, PA).

Both wild type and mutant (dominant negative, DN) mouse osteoblasts were plated at 75,000/well density in a 12-well plate and infected with either control virus or virus containing Msx2-HA gene. 24 hours after infection, cells were cotransfected with 6XOSE2 Luc reporter vector (1μg/well) and PRL luciferase (100ng/well) in a 10:1 ratio using the Fugene6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) in Opti minimum essential medium (Invitrogen Corp.). 24 hours after transfection, Fgf9 (10 ng/ml) was added in the medium (1% FCS) and cells were incubated for 12 hours. Luciferase activities were read on a luminometer using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

III. RESULTS

A. Phenotypic Characterization of Calvarial Osteoblasts; Expression Pattern of Fgfr2 and Msx2 mRNA with Alternative Peaks.

Since osteoblastic cells do not show any remarkable morphological changes during differentiation (Aubin et al. 1999), observation of gene expression pattern is necessary to identify the differentiation status of the cells. Enzymatic digestion appears to release cells in the superficial layer of the bone, thus the cell population collected during the first digestion cycle will contain less mature osteogenic cells while more mature bone cells are collected during the final digestion cycle. Cells obtained from each digestion cycle were grown on 10 cm plates for 1 week until they become confluent and RNAs were isolated.

Northern analysis in Fig.1 demonstrated the basal level expression of Fgfr1, Fgfr1, Msx2 and Cbfa1/Runx2, along with alkaline phosphatase as a differentiation marker.

First, significant Cbfa1 expression in fractions 2-6 is noted, which indicates that the cells in fractions 2-6 are committed osteogenic cells. Second, gradual increase of Msx2 mRNA was shown from fraction 1 to 3, with peak expression at fraction 3 and 4 and decrease afterwards. The Fgfr2 expression increased from fraction 1 to 5 in a similar pattern, and began to decrease at

fraction 6. Thus, Fgfr2 and Msx2 genes displayed alternative peak expression. Fgfr1 gene expression was detected at a much lower level than that of Fgfr2 but also showed gradual increase. The alkaline phosphatase gene expression pattern represents the gradient of differentiation of osteoblastic cells.

From these results, cells from fraction 3-6 were regarded appropriate for investigation of the relationship between Msx2 and Fgfr2 because they were committed osteogenic cells and they showed considerable FGFR2 and Msx2 gene expression.

B. Msx2 Gene is Repressed by Fgf9 Treatment, Which Requires Protein Synthesis.

To examine the time-dependent effect of Fgf9-induced signaling on Msx2 gene expression, primary mouse calvarial bone cells plated at a density of 2 million/10cm plate were treated with Fgf9 (10ng/ml) in α-MEM containing 1% FCS. RNAs were collected at 3, 6, 9, 12 and 24 hours, respectively. Northern blot hybridization was conducted using the rat Msx2 probes.

Msx2 mRNA level was gradually reduced within 3 hours and reached the lowest level after 12 hours of treatment. In spite of the continuous Fgf9 treatment, level of expression began to recover between 12 and 24 hours (Fig.

2 (A)). Since the down-regulation was the most apparent at 12 hours, we applied 12 hours of treatment time to the subsequent experiments.

When the cells were treated with different concentrations of Fgf9 (1, 5, 10, 20 ng/ml), the effect of Fgf9 on the reduction in the Msx2 gene expression was similar at concentrations ranging from 5-20 ng/ml (data not shown).

Next, the cells were preincubated with cyclohexamide (50, 100 μg/ml), an inhibitor for protein synthesis, for 1 hour and again treated with Fgf9 for additional 6 hours. At both concentrations of cyclohexamide, Msx2 level was maintained even in the presence of Fg9, indicating that Fgf9-mediated down-regulation of Msx2 expression requires protein synthesis (Fig 2 (B)).

Primary calvarial bone cells, obtained from wild type (wt) and dominant negative (DN) mutant mice littermates were cultured in the presence or absence of FGF9. A dominant negative receptor forms a dimer with normal or mutant receptor and inhibits regular signal transduction induced by Fgfr2 activation (Ratisoontorn et al. 2003). Northern analysis was performed to assess the change in the Msx2 gene expression.

Unlike the wild type cells, DN cells didn't show any apparent change in the Msx2 gene expression in the presence of Fgf9 (Fig 2 (C)). This indicates that the down-regulation of Msx2 by Fgf9 is mediated through Fgfr.

C. Msx2 Overexpression Blocks Fgf9-induced Increase in the Endogeneous Osteocalcin Gene Expression.

It has been shown that Fgf/Fgfr signaling increases DNA binding of the Cbfa1/Runx2, as well as its mRNA levels (Kim et al. 2003). Cbfa1/Runx2 has its binding cognate (osteocalcin-specific cis acting element, OSE2) on the osteocalcin promoter and upon binding, it increases the transcriptional activation of osteocalcin gene (Xiao et al. 2002, Kim et al. 2003). On the contrary, Msx2 is a known suppressor of the rat osteocalcin promoter (Towler et al. 1994, Newberry et al. 1997) possibly by inhibiting the Cbfa1/Runx2 function (Shirakabe et al. 2001). Therefore, we next asked whether the Fgf9/Fgfr2-mediated downregulation of Msx2 might help the Fgfr2-mediated up-regulation of the transcription factor Cbfa1/Runx2 in the mouse bone cells. To examine this, we overexpressed Msx2 in mouse primary osteoblasts with adenovirus containing Msx2-HA and observed Cbfa1/Runx2 function regarding osteocalcin gene expression. We hypothesized that the overexpression of Msx2 may inhibit Fgf9–induced Cbfa1/Runx2 activity and reduce endogenous osteocalcin gene expression.

To confirm the proper production of the virally encoded protein in the primary mouse osteoblasts, immunohistochemical analysis and immunoblot analysis were carried out. After 24 hours of infection, cells were fixed and

immunostained with antibody specific for HA epitope. (Santa Cruz Biotechnology, Santa Cruz, CA) The adenovirus-encoded Msx2 was distinctly expressed and localized in the nuclei (Fig.3 (A)), while this was not observed in the uninfected cells. An immunoblot analysis using the nuclear extract and anti-HA antibody showed the specific band at 35 kD corresponding to the Msx2-HA protein (Fig.3 (B)). Cytoplasmic expression of the Msx2-HA was hardly detectable.

Primary calvarial bone cells were infected with control adenovirus or adenovirus containing Msx2-HA for 24 hours, treated with 10ng/ml Fgf9 for 12 hours in α-MEM with 1% FCS. Isolated RNAs were reverse transcribed and amplified through 40 cycles of PCR. The sizes of the PCR products were estimated from the relative migration compared to the DNA ladder (Lambda DNA/Hind III Markers, Promega, Madison, WI).

Endogenous osteocalcin gene was significantly increased by Fgf9 treatment in uninfected cells and in cells treated with control adenovirus. In contrast, this up-regulation was blocked in cells overexpressing Msx2. Basal level expression was generally weak and didn't seem to display any notable change. GAPDH was used as internal control and the intensity of signal was almost the same, regardless of Fgf9 treatment. Thus, Msx2 overexpression blocks the Fgf9-induced increase in the endogenous osteocalcin gene expression.

D. Msx2 Overexpression Suppresses Fgf9-induced Increase in the DNA Binding of Cbfa1/Runx2.

We next asked if the inhibition of the endogenous osteocalcin gene expression by overexpressed Msx2 is associated with altered DNA binding of Cbfa1/Runx2. Msx2 suppression of osteocalcin promoter is mainly mediated by protein-protein interaction (Willis et al. 2002, Towler et al. 1994, Hoffmann et al. 1994).

To investigate the effect of overexpression of Msx2 on the DNA binding of Cbfa1, control or virally infected primary mouse osteoblasts were cultured with or without Fgf9 treatment for 16-24 hours in α-MEM containing 1% FCS and nuclear extracts were isolated. Electrophoretic mobility shift assays (EMSA) were performed using radio-labeled Cbfa1 binding element (OSE2) in the (human) osteocalcin promoter. This radioactive probe contains the cognate binding sequence (GCAGTCA).

Diminished signal with the competitor and apparent supershift with the anti-Cbfa1/Runx2 IgG indicates the specific DNA-Cbfa1/Runx2 complex (Fig.4 (A)). The signal was notably enhanced with the treatment of Fgf9 in uninfected cells and cells infected with control virus. In contrast, the increase was not found in cells overexpressing Msx2 (Fig.4). Although the exact

mechanism is not clear, overexpression of Msx2 inhibited the Fgf-induced DNA binding of Cbfa1/Runx2.

E. Msx2 Overexpression Abrogates Fgf9-induced Increase in the Osteocalcin Transcriptional Activity.

Since binding of transcription factor may stimulate or suppress transcription of the gene, actual change of the transcriptional activity needed to be examined. Therefore, the effect of overexpressed Msx2 on the transcriptional activity needed to be measured as luciferase activity using 6XOSE2 Luc reporter vector. We hypothesized that overexpression of Msx2 may change osteocalcin transcriptional activity of Cbfa1/Runx2.

Control or virally (either control virus or Msx2 virus) infected wild type or dominant negative primary mouse osteoblasts were co-transfected with 6XOSE2 Luc reporter vector (1μg/well) and PRL luciferase (100ng/well) for 24 hours and Fgf9 treatment (10ng/ml) was followed in α-MEM containing 1% FCS. Luciferase activities were measured in 12-16 hours after Fgf9 treatment. The luciferase activity was normalized using the Renilla luciferase activity.

Uninfected cells showed almost 8-fold increase in the luciferase activity when treated with Fgf9. However, this increase was not observed in the cells

overexpressing Msx2 (Fig.5 (A)). Particularly, DN cells did not show any significant increase in the luciferase activity even in the presence of Fgf9 (Fig.5 (B)), indicating that the increase in the transcriptional activity induced by Fgf9 is mediated through Fgfr signaling.

To summarize the results, overexpression of Msx2 in the primary mouse calvarial osteoblasts inhibits Fgf9-stimulated Cbfa1/Runx2 function. Consequently, this leads to reduced osteocalcin gene expression and transcriptional activity by decreased DNA binding of Cbfa1. We also speculate that Fgf9/Fgfr2 signaling suppresses the Msx2 expression and this downregulation appears to help activation of the Cbfa1 function to increase the OC gene expression.

IV. DISCUSSION

Cranial Suture Development and Its Importance.

Cranial sutures serve as major growth sites of intramembranous bones during mammalian skull development (Opperman 2000). For this function, sutures need to remain patent until maturity to encompass the growing neurocranium, while allowing new bone formation at the edges of approximating bone fronts. Wide-open fontanels or osseous obliteration of the sutures are inferred as the results of disturbed regulation of bone cell differentiation at the osteogenic fronts (Cohen 1993, Opperman 2000).

Craniosynostosis has been an important subject in bone biology because it represents a serious craniofacial deformity, and thereby it provides a good model system for studying the gene and their functions in the regulation of bone formation (Wilkie 1997).

Reciprocal Temporal Expression pattern of Fgfr2 and Msx2 in the Calvarial Osteoblasts.

Fgfr2 and Msx2 are both expressed in the early stage of osteoblast differentiation and are involved in the pathogenesis of craniosynostosis. In fact,

FGF-impregnated bead (Kim et al. 1998, Ignelzi et al. 2003) induced premature fusion of sutures, similarly in the transgenic mice with Fgfr2 knock-in (Chen et al. 2003) or with overexpression of Msx2 (Liu et al. 1995). In contrast, Bmp4, a potent inducer of osteogenesis, didn't facilitate sutural growth (Kim et al. 1998). This indicates that the early regulatory mechanism is important in the pathogenesis of craniosynostosis.

In the same context, phenotypic assessment of the primary calvarial cells is important to define the level of differentiation of the cells used for the study. Besides, the action of Fgf on osteoblasts is known to be biphasic and stage-specific (Rice et al. 2000). Fig.1 demonstrates that the majority of the cells are likely to be proliferating osteoblast precursors. This can be inferred from the low expression level of Fgfr1 relative to the significant expression of Fgfr2 and Msx2. This is consistent with previous studies (Iseki et al. 1999). Cells in fraction 6 showed high expression of alkaline phosphatase and also reduced but still significant level of Fgfr2 and Msx2. Therefore, these primary cells were considered suitable for studying the interaction of Fgfr2 and Msx2.

Fgf9 suppresses Msx2 mRNA.

Each Fgfr1-4 shows complicated ligand-binding specificity to individual ligands. In particular, Fgf9 is a highly specific ligand for Fgfr2(IIIc) isoform,

while Fgf1 activates all Fgfr splice variants (Ornitz et al. 1996). In this study, we used Fgf9 in order to evaluate mainly the function of signaling mediated by Fgfr2.

It is noteworthy that the Msx2 gene expression was suppressed by Fgf9 treatment. This is in contrast with previous reports where Msx2 increased or showed no change in the presence of FGF (Ignelzi et al. 2003, Kim et al. 1998). Another interesting reaction is the recovery of Msx2 mRNA after 24 hours of FGgf9 treatment. This fluctuation pattern in 24 hours encouraged us to define that the immediate and direct effect of Fgf9 is a suppression of Msx2. This finding is also coincident with the results shown in Fig.1. Msx2 level peaked at fraction 3 and 4 and then was reduced thereafter, while Fgfr2 peaked at fraction 5.

Obviously, Msx2 expression peaked before Fgfr2 gene reached its highest level, implying certain reciprocal action. Since Fgf9 is a known cognate ligand for Fgfr2, and the relatively high expression of Fgfr2 compared to Fgfr1 encouraged us to speculate that the suppression action may be mainly mediated through Fgfr2. Changes in Msx2 mRNA in the cells expressing DN Fgfr2 substantiate this finding; when the Fgfr function was blocked, the downregulation did not occur.

In spite of the continuous Fgf9 treatment, Msx2 returned to the initial level between 12-24 hours. Recovery of Msx2 mRNA level may be mediated

through another molecule. We speculate that Bmp2 may be involved in the recovery, since it is known to upregulate Msx2 in the developing suture (Rice et al. 2003, Vainio et al. 1993) and in our culture cells Fgf9 significantly increased Bmp2 mRNA expression (Preliminary study; Data not shown). However, this downregulation of Msx2 by Fgf9 still requires another protein synthesis (Fig 2(B)). The main intracellular pathway and the type of protein involved in the regulation of Msx2 need to be identified.

Role of Overexpressed Msx2 on Fgf9-induced Endogenous Osteocalcin Gene Transcription.

Osteocalcin is indeed a powerful tool for studying nuclear protein-DNA interaction that modulates transcriptional activities in the osteoblasts, due to the identified selective binding sites for the key transcription factors (Newberry et al. 1998).

Since the concentration of Fgf9 in this study was relatively low (10ng/ml), compared to other bead implantation studies (Ignelzi et al. 2003), we speculate this downregulation of Msx2 reflects the possible molecular event occurring in vivo. Fgf is a known inducer of Cbfa1/Runx2, (Kim et al. 2003) that itself is a direct inducer of osteocalcin gene transcription (Geoffroy et al. 1997, Ducy and Karsenty 1995). Although Fgf stimulated expression of Cbfa1/Runx2

(Kim et al. 2003), the actual osteocalcin transcriptional activity appears to be associated with the functional activity of Cbfa1/Runx2 as well. Therefore, we examined both the endogenous osteocalcin mRNA expression and the functional activity of osteocalcin promoter in the presence of Fgf9, with or without infecting the cells with adenovirus containing Msx2 gene.

First, endogenous osteocalcin mRNA expression was not increased by Fgf9 treatment when Msx2 was overexpressed. This is in agreement with previous studies. Although Msx2 is known to suppress both basal (Newberry et al. 1998) and Fgf2/Fsk-induced osteocalcin promoter activity (Newberry et al. 1997), the change in the basal level osteocalcin gene expression was hardly noticeable in our study. Presumably, our primary cells were still undergoing early differentiation but osteocalcin is a known late differentiation marker. Therefore, Msx2 may not influence the scarce endogenous osteocalcin level at this stage of cells.

Moreover, Fgf9 treatment itself was sufficient to increase the osteocalcin mRNA level, without the synergistic effect of cyclic AMP demonstrated in the Towler's study. This might be a characteristic response of the primary cells at the early differentiation stage, or the specific effect of the ligand-Fgf9. Since Fgf9 selectively activates Fgfr2 isoform (Ornitz et al. 1996), it possibly represents the effects of selective activation of Fgfr2 in osteoblasts.

We confirmed that the external Msx2 introduced via viral infection functionally produces protein that is localized in the nucleus, but we couldn't quantitate the actual infection efficiency. According to Towler's study, inhibitory action of the Msx2 on the osteocalcin promoter was constant, regardless of the amount of plasmid, and did not follow a dose-dependent manner (Newberry et al. 1997). Thus, the amount of Msx2 protein doesn't appear to be critical in terms of osteocalcin gene regulation.

Inhibition of Fgf9-induced Osteocalcin Promoter Activation is Mediated through Reduced DNA-protein Binding at OSE2.

There have been several sites of interest in the osteocalcin promoter that implicate the Msx2 action for the modulation of the osteocalcin transcriptional activity (Hoffmann et al. 1994). However, as already has been reported, Msx2 binding on the HOXBOX cognate is not required. Instead, direct binding onto Cbfa1/Runx2, (Shirakabe et al. 2001) Msx2 interaction with Dlx5, (Newberry1998) and inhibition of protein binding on the OCFRE site (Willis et al. 2002) have been proposed to be a regulatory mechanisms. We first wished to focus on the Cbfa1/Runx2 function, because it is a known potent stimulator of osteocalcin and the Cbfa1/Runx2 cognate DNA oligonucleotide

(OSE2) could be easily recognized. Thereby we examined the effects of Msx2 overexpression on the Cbfa1/Runx2-OSE2 binding.

Interestingly, Fgf9-induced increase in the DNA binding of Cbfa1/Runx2 was abrogated under forced expression of Msx2. Our oligonucleotide sequence contained the minimal sequence for Cbfa1/Runx2 binding, that is ACCACA, but not the OCFRE sequence (GCAGTCA, Willis et al. 2002). This means that modulation of Cbfa1 binding may not be mediated through another protein. From this result, we inferred that direct binding or direct interaction with Cbfa1/Runx2 protein is likely to be a mechanism by which Msx2 modulates the Cbfa1/Runx2 binding.

Next, luciferase assays additionally confirmed the effects of DNA binding on the actual transactivation of osteocalcin gene. Msx2 infected cells didn't show any increase in the luciferase activity in the presence of Fgf9, implying that the inhibited protein-DNA binding may have caused the actual suppression of transcriptional activity. That is, the Msx2-induced transcriptional suppression in the osteocalcin promoter is due to the reduced binding of Cbfa1/Runx2 on the binding cognate on the promoter. We couldn't find any change in the basal osteocalcin transcriptional activity even in the presence of Msx2, likely because of the differentiation stage of the primary cells.

Integration of Msx2 and Fgf/Fgfr in the Early Phase of Osteoblast Differentiation.

Fgfr2 and Msx2 exhibited overlapping expression pattern and apparently there is an interaction, which appear to be a reciprocal suppressive effect, not synergistic effect. This can be figured in the initial ontogeny of calvarial osteoblasts.

As a whole, these results substantiate the speculation that Ffg/Ffgr(2) is an upstream regulator of Msx2 gene in mouse calvarial bone cells and coordinated activity between FGF/FGFR and Msx2 may have a crucial role in the modulation of the Cbfa1 function, regulating the differentiation of the cells.

At the early stage of differentiation, relatively high level of Msx2 appears to inhibit Cbfa1/Runx2 function. Therefore, the osteocalcin expression and differentiation are also blocked and thus proliferation is sustained. However, in cells expressing high level of Fgfr2, Msx2 is suppressed and thereby Cbfa1/Runx2 function is enhanced. This will lead to increased osteocalcin transcription that leads to osteoblast differentiation. Briefly, Fgf/Fgfr signaling suppresses Msx2 gene expression in order to facialitate transcription of osteocalcin, leading to differentiation of osteoblasts (Fig 6).

Fgf/Fgfr is known to stimulate both proliferation and differentiation in the osteoblasts, but with regard to the transcriptional activity of osteocalcin, Fgf

obviously facilitates the differentiation of still immature osteoblast. Msx2 apparently retards the differentiation procedure. In this context, Fgf and Msx2 may have different roles in the pathogenesis of craniosynostosis; Fgfr, through increased early differentiation and Msx2, through suppressed differentiation. This assumption needs to be further evaluated, involving ample information on the differences in the clinical phenotypes depending on the type of mutations.

V. CONCLUSION

The purpose of this study was to investigate if Fgf/Fgfr signaling is an upstream regulator of Msx2 gene expression in the same genetic pathway and to elucidate the possible regulatory role of these genes in differentiating osteoblasts. For analysis of the molecular events occurring in the cell level, we used an in vitro culture with primary mouse calvarial osteoblasts.

We demonstrated the expression pattern of Fgfr2 and Msx2 gene in different maturation stage, with peak expression of Msx2 gene preceding that of Fgfr2.

To examine the effect of Fgf/Fgfr signaling on the Msx2 gene expression, primary osteoblasts were treated with Fgf9, a cognate ligand for Fgfr2, and Msx2 gene expression was significantly suppressed via a protein synthesis. To gain further insight into the significance of this down-regulation, Msx2 was

overexpressed in the osteoblasts using adenovirus containing Msx2 gene tagged with hemagglutinin (HA) epitope. Subsequent series of RT-PCR, gel-shift assay and transfection & luciferase assay showed that the overexpression of Msx2 inhibited the Fgf9–induced increase in the endogenous osteocalcin gene expression, likely by altering the binding of Cbfa1/Runx2 on the OSE2 sequence in the osteocalcin promoter. From these results, we propose a possible model of osteoblast differentiation with integrated Fgf/Fgfr and Msx2 function, implicating the suppressive role of Fgf/Fgfr on Msx2 expression by modulating the Cbfa1/Runx2, leading to increased osteocalcin transcription.

REFERENCES

Anderson J, Burns HD, Enriquez-Harris P, Wilkie AO, Heath JK. Apert syndrome mutations in fibroblast growth factor receptor 2 exhibit increased affinity for FGF ligand. Hum Mol Genet. 1998;7:1475-83.

Aubin JE, Candeliere GA, Bonnelye E. Heterogeneity of the osteoblast phenotype. Endocrinologist 1999;9:25-31

Bidder M, Latifi T, Towler DA. Reciprocal temporospatial patterns of Msx2 and Osteocalcin gene expression during murine odontogenesis. J Bone Miner Res. 1998;13:609-19

Boudreaux JM, Towler DA. Synergistic induction of osteocalcin gene expression: identification of a bipartite element conferring fibroblast growth factor 2 and cyclic AMP responsiveness in the rat osteocalcin promoter. J Biol Chem. 1996;271:7508-15

Canalis E, Raisz LG. Effect of fibroblast growth factor on cultured fetal rat calvaria. Metabolism. 1980;29:108-14

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 Aug;33:169-78.

Chomczynski P. Bio Techniques 1993;15:532-535

Cohen MM Jr. Sutural biology and the correlates of the craniosynostosis. Am. J. Med. Genet. 1993;47:581-616

Davidson D. The function and evolution of Msx genes: pointers and paradoxes. Trends Genet. 1995;11:405-11

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

Dodig M, Tadic T, Kronenberg MS, Dacic S, Liu YH, Maxson R, Rowe DW, Lichtler AC. Ectopic Msx2 overexpression inhibits and Msx2 antisense stimulates calvarial osteoblast differentiation. Dev Biol 1999;209:298-307

Ducy P, Karsenty G. Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. Mol Cell Biol 1995;15:1858-69

El Ghouzzi V, Le Merrer M, Perrin-Schmitt F, Lajeunie E, Benit P, Renier D, Bourgeois P, Bolcato-Bellemin AL, Munnich A, Bonaventure J. Mutations of the TWIST gene in the Saethre-Chotzen syndrome. Nat Genet 1997;15:42-6

Eswarakumar VP, Monsonego-Ornan E, Pines M, Antonopoulou I, Morriss-Kay GM, Lonai P. The IIIc alternative of Fgfr2 is a positive regulator of bone formation. Development 2002;129:3783-93

Geoffroy V, Ducy P, Karsenty G. A PEBP2 alpha/AML-1-related factor increases osteocalcin promoter activity through its binding to an osteoblast-specific cis-acting element. J Biol Chem 1995;270:30973-9

Harada H, Tagashira S, Fujiwara M, Ogawa S, Katsumata T, Yamaguchi A, Komori T, Nakatsuka M. Cbfa1 isoforms exert functional differences in osteoblast differentiation. J Biol Chem 1999;274:6972-8

Hehr U, Muenke M. Craniosynostosis syndromes: from genes to premature fusion of skull bones. Mol Genet Metab 1999;68:139-51

Hoffmann HM, Catron KM, van Wijnen AJ, McCabe LR, Lian JB, Stein GS, Stein JL. Transcriptional control of the tissue-specific, developmentally regulated osteocalcin gene requires a binding motif for the Msx family of homeodomain proteins. Proc Natl Acad Sci U S A 1994;91:12887-91

Hurley MM, Kessler M, Gronowicz G, Raisz LG. The interaction of heparin and basic fibroblast growth factor on collagen synthesis in 21-day fetal rat calvariae. Endocrinology 1992;130:2675-82

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

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

Iseki S, Wilkie AO, Morriss-Kay GM. Fgfr1 and Fgfr2 have distinct differentiation- and proliferation-related roles in the developing mouse skull vault. Development. 1999;126:5611-20

Jabs EW, Li X, Scott AF, Meyers G, Chen W, Eccles M, Mao JI, Charnas LR, Jackson CE, Jaye M. Jackson-Weiss and Crouzon syndromes are allelic with mutations in fibroblast growth factor receptor 2. Nat Genet 1994;8:275-9

Jabs EW, Muller U, Li X, Ma L, Luo W, Haworth IS, Klisak I, Sparkes R, Warman ML, Mulliken JB, et al. A mutation in the homeodomain of the human MSX2 gene in a family affected with autosomal dominant craniosynostosis. Cell 1993;75:443-50

Kettunen P, Thesleff I. Expression and function of FGFs-4, -8, and -9 suggest functional redundancy and repetitive use as epithelial signals during tooth morphogenesis. Dev Dyn 1998;211:256-68.

Kim HJ, Kim JH, Bae SC, Choi JY, Kim HJ, Ryoo HM. The protein kinase C pathway plays a central role in the fibroblast growth factor-stimulated expression and transactivation activity of Runx2. J Biol Chem 2003;278:319-

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.

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

Lajeunie E, Ma HW, Bonaventure J, Munnich A, Le Merrer M, Renier D. FGFR2 mutations in Pfeiffer syndrome. Nat Genet 1995;9:108

Liu YH, Kundu R, Wu L, Luo W, Ignelzi MA Jr, Snead ML, Maxson RE Jr. Premature suture closure and ectopic cranial bone in mice expressing Msx2 transgenes in the developing skull. Proc Natl Acad Sci U S A 1995;92:6137-41

Liu YH, Tang Z, Kundu RK, Wu L, Luo W, Zhu D, Sangiorgi F, Snead ML, Maxson RE. Msx2 gene dosage influences the number of proliferative osteogenic cells in growth centers of the developing murine skull: a possible mechanism for MSX2-mediated craniosynostosis in humans. Dev Biol 1999;205:260-74

Ma L, Golden S, Wu L, Maxson R. The molecular basis of Boston-type craniosynostosis: the Pro148-->His mutation in the N-terminal arm of the MSX2 homeodomain stabilizes DNA binding without altering nucleotide sequence preferences. Hum Mol Genet 1996;5:1915-20

Mizushima S, Nagata S. pEF-BOS, a powerful mammalian expression vector. Nucleic Acids Res 1990;18:5322

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

Newberry EP, Boudreaux JM, Towler DA. Stimulus-selective inhibition of rat osteocalcin promoter induction and protein-DNA interactions by the homeodomain repressor Msx2. J Biol Chem 1997;272:29607-13

Newberry EP, Latifi T, Towler DA. Reciprocal regulation of osteocalcin transcription by the homeodomain proteins Msx2 and Dlx5. Biochemistry 1998;37:16360-8

Opperman LA, Sweeney TM, Redmon J, Persing JA, Ogle RC. Tissue interactions with underlying dura mater inhibit osseous obliteration of developing cranial sutures. Dev Dyn 1993;198:312-22

Opperman LA. Cranial sutures as intramembranous bone growth sites. Dev Dyn 2000;219:472-85

Ornitz DM, Xu J, Colvin JS, McEwen DG, MacArthur CA, Coulier F, Gao G, Goldfarb M. Receptor specificity of the fibroblast growth factor family. J Biol Chem 1996;271:15292-7

Orr-Urtreger A, Bedford MT, Burakova T, Arman E, Zimmer Y, Yayon A, Givol D, Lonai P. Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). Dev Biol 1993;158:475-86

Orr-Urtreger A, Givol D, Yayon A, Yarden Y, Lonai P. Developmental expression of two murine fibroblast growth factor receptors, flg and bek. Development 1991;113:1419-34

Ratisoontorn C, Fan GF, McEntee K, Nah HD. Activating (P253R, C278F) and dominant negative mutations of FGFR2: differential effects on calvarial bone cell proliferation, differentiation, and mineralization. Connect Tissue Res 2003;44 Suppl 1:292-7

Reardon W, Winter RM, Rutland P, Pulleyn LJ, Jones BM, Malcolm S. Mutations in the fibroblast growth factor receptor 2 gene cause Crouzon syndrome. Nat Genet 1994;8:98-103

Rice DP, Aberg T, Chan Y, Tang Z, Kettunen PJ, Pakarinen L, Maxson RE, Thesleff I. Integration of FGF and TWIST in calvarial bone and suture development. Development 2000;127:1845-55

Rice DP, Rice R, Thesleff I. Fgfr mRNA isoforms in craniofacial bone development. Bone 2003;33:14-27

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

Rutland P, Pulleyn LJ, Reardon W, Baraitser M, Hayward R, Jones B, Malcolm S, Winter RM, Oldridge M, Slaney SF, et al. Identical mutations in the FGFR2 gene cause both Pfeiffer and Crouzon syndrome phenotypes. Nat Genet 1995;9:173-6

Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1989

Satokata I, Ma L, Ohshima H, Bei M, Woo I, Nishizawa K, Maeda T, Takano Y, Uchiyama M, Heaney S, Peters H, Tang Z, Maxson R, Maas R. Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. Nat Genet 2000;24:391-5

Shimoaka T, Ogasawara T, Yonamine A, Chikazu D, Kawano H, Nakamura K, Itoh N, Kawaguchi H. Regulation of osteoblast, chondrocyte, and osteoclast

functions by fibroblast growth factor (FGF)-18 in comparison with FGF-2 and FGF-10. J Biol Chem 2002;277:7493-500

Shirakabe K, Terasawa K, Miyama K, Shibuya H, Nishida E. Regulation of the activity of the transcription factor Runx2 by two homeobox proteins, Msx2 and Dlx5. Genes Cells 2001;6:851-6

Takahashi K, Nuckolls GH, Tanaka O, Semba I, Takahashi I, Dashner R, Shum L, Slavkin HC. Adenovirus-mediated ectopic expression of Msx2 in even-numbered rhombomeres induces apoptotic elimination of cranial neural crest cells in ovo. Development 1998;125:1627-35

Towler DA, Rutledge SJ, Rodan GA. Msx-2/Hox 8.1: a transcriptional regulator of the rat osteocalcin promoter. Mol Endocrinol 1994;8:1484-93

Vainio S, Karavanova I, Jowett A, Thesleff I. Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. Cell 1993;75:45-58

Wilkie A. O., Slaney S. F, Oldridge M., Poole M. D., Ashworth G. J., Hockley A. D., Hayward R. D., David D. J., Pulleyn L. J., Rutland P., et al. Apert

syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome. Nat Genet 1995;9:165-72

Wilkie AO. Craniosynostosis: genes and mechanisms. Hum Mol Genet 1997;6:1647-56

Willis DM, Loewy AP, Charlton-Kachigian N, Shao JS, Ornitz DM, Towler DA. Regulation of osteocalcin gene expression by a novel Ku antigen transcription factor complex. J Biol Chem 2002;277:37280-91

Wong GL, Cohn DV. Separation of parathyroid hormone and calcitonin-sensitive cells from non-responsive bone cells. Nature 1974;252:713-5

Xiao G, Jiang D, Gopalakrishnan R, Franceschi RT. Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, Cbfa1/Runx2. J Biol Chem 2002;277:36181-7

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

Legends

Fig.1 Phenotypic Characterization of Calvarial Osteoblasts; Expression Pattern of Fgfr2 and Msx2 mRNA with Alternative Peaks.

Cells were obtained from repeated digestion of the mouse calvarial bones. Early fraction cells were released from the superficial layer, representing less mature phenotype, than the late fraction cells. Significant Cbfa1 expression in fractions 2-6 is noted. Gradual increase of Msx2 mRNA was shown from fraction 1 to 3, with peak expression at fraction 3 and 4 and decrease afterwards. The Fgfr2 expression increased from fraction 1 to 5 in a similar pattern, and began to decrease at fraction 6. Fgfr1 mRNA is still scarce. Alkaline phsphatase (ALP) gene level shows the gradual differentiation pattern.

Fig.2 Fgf9 Suppresses Msx2 Gene Expression and This Down-regulation Requires Protein Synthesis.

(A) Primary mouse calvarial bone cells plated at a density of 2 million/10 cm plate were treated with Fgf9 (10ng/ml) in α-MEM containing 1% FCS. RNAs were collected at 3, 6, 9, 12 and 24 hours, respectively. Fgf9 reduced Msx2 gene within 3 hours. Msx2 gene level reached the lowest level at 12 hours and began to recover between 12 and 24 hours.

(B) Primary mouse osteoblasts were preincubated with cyclohexamide (50, 100 μg/ml) for 1 hour and were treated with Fgf9 for additional 6 hours. It is noticed that either concentration of cyclohexamide blocked Fgf9-induced downregulation of Msx2, indicating that the Fgf9-mediated down-regulation of Msx2 expression requires a protein synthesis.

(C) Cells obtained from wild type (wt) and dominant negative mutant (DN) mice littermates were cultured in the presence or absence of Fgf9. Down-regulation of Msx2 gene expression is found in the presence of Fgf9 in wt cells, but not in DN cells where normal Fgfr function is scarce. This confirms that the Msx2 down-regulation is mediated through FGF/FGFR signaling.

Fig.3 Msx2 Overexpression Blocks Fgf9-induced Increase in the Endogeneous Osteocalcin Gene Expression.

(A) 24 hours after infection with adenovirus containing Msx2, cells were fixed and immunostained with antibody specific for HA epitope. The adenovirus-encoded Msx2 was distinctly expressed and localized in the nuclei (arrow), while this was not observed in the uninfected cells.

(B) An immunoblot analysis using the nuclear extract and anti-HA IgG showed the specific band at 35 kD corresponding to the Msx2-HA protein.

(C) Primary calvarial bone cells were infected with either control adenovirus or adenovirus containing Msx2-HA for 24 hours, then treated with 10ng/ml

Fgf9 for 12 hours in α-MEM with 1% FCS. RNAs were amplified by RT-PCR using mouse osteocalcin primer. Endogenous osteocalcin gene was significantly increased by Fgf9 treatment in uninfected cells and in cells infected with control adenovirus. To the contrary, this up-regulation was blocked in cells overexpressing Msx2 (left panel). GAPDH was used as internal controls (right panel).

Fig.4 Msx2 Overexpression Suppresses Fgf9-induced Increase in the DNA Binding of Cbfa1/Runx2.

Control or virally infected primary mouse osteoblasts were cultured with or without Fgf9 treatment for 16-24 hours in α-MEM containing 1% FCS and nuclear extracts were isolated. Diminished signal with the competitor (unlabeled probe) and apparent supershift with the anti-Cbfa1/Runx2 IgG indicate that the arrowed band is the specific DNA-Cbfa1/Runx2 complex (left panel). The signal was notably enhanced with the treatment of Fgf9 in uninfected cells as well as in cells infected with control virus (middle and right panel). To the contrary, the increase was not found in cells overexpressing Msx2 (middle panel).

(Ad-Cont ; cells infected with control adenovirus

Ad-Msx2 ; cells infected with Msx2 adenovirus)

Fig.5 Msx2 Overexpression Abrogates Fgf9-induced Increase in the Osteocalcin Transcriptional Activity.

Control or virally (Msx2 adenovirus) infected wild type or DN primary mouse osteoblasts were co-transfected with 6XOSE2 luciferase reporter vector (1μg/well) and PRL luciferase (100ng/well) vector for 24 hours and Fgf9 treatment (10ng/ml) was followed. Bars are mean ± SD.

(A) Uninfected cells showed almost 8-fold increase in the luciferase activity when treated with Fgf9, however, this increase was not observed in the cells overexpressing Msx2.

(B) DN cells did not show any significant increase in the luciferase activity even in the presence of Fgf9.

Fig. 6 A model of osteoblast differentiation with integrated Fgf/Fgfr and Msx2 with regard to Cbfa1/Runx2 function and transcription of osteocalcin.

Fgf/Fgfr is an upstream regulator of Msx2 gene, and a coordinated activity between the two appears to play an important role during differentiation of committed osteoblastic cells. At the early stage of differentiation, relatively high level of Msx2 appears to inhibit Cbfa1/Runx2 function. Therefore, the osteocalcin expression and differentiation are also blocked and thus proliferation may be sustained. However, when cells start to express high level

of Fgfr2, Msx2 is suppressed and thereby suppressed Cbfa1/Runx2 function is relieved. This will lead to increased osteocalcin transcription that leads to osteoblast differentiation.

Figures

Figure 1.

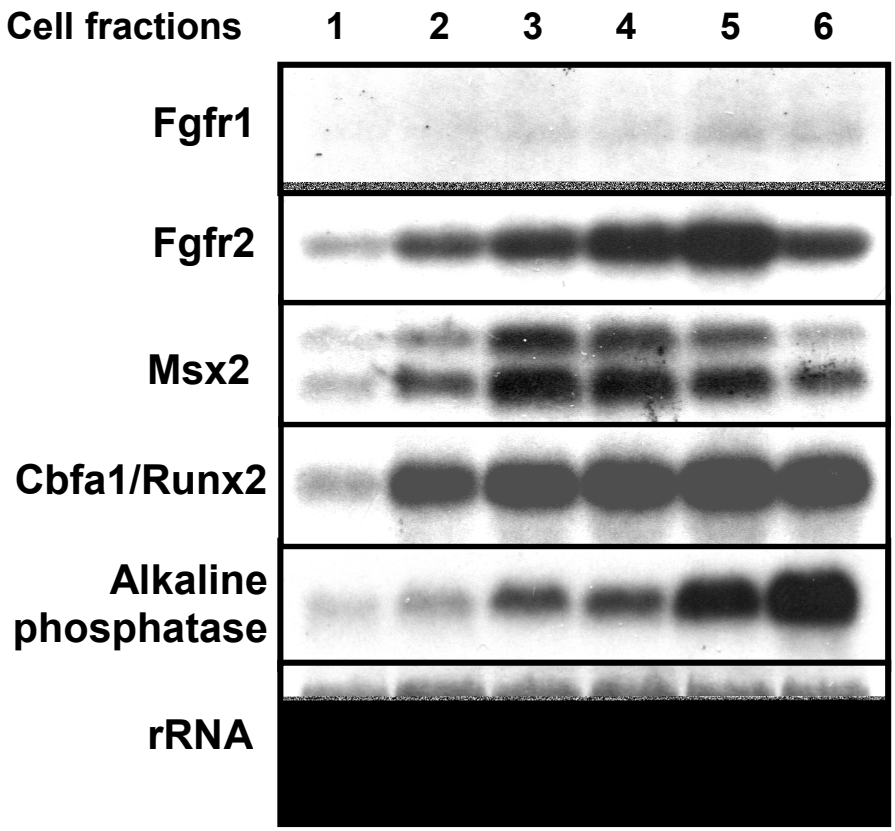
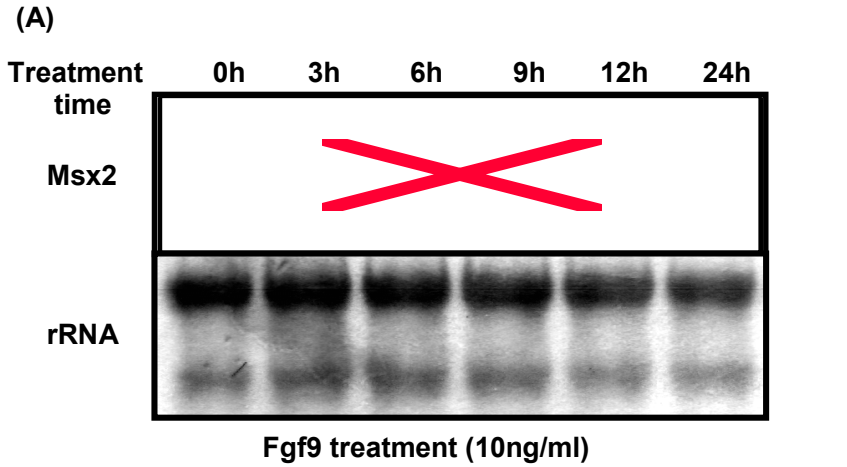
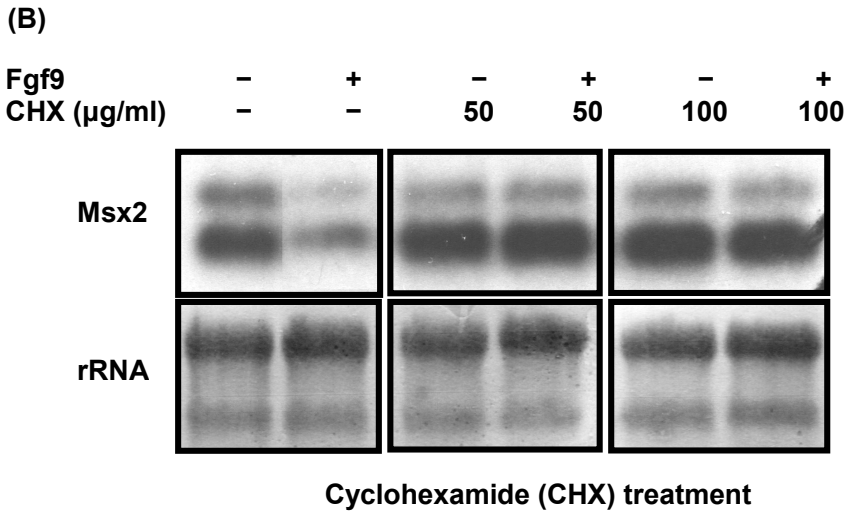


Figure 2.


(A)
Treatment time
0h
3h
6h
9h
12h
24h
Msx2
rRNA
Fgf9 treatment (10ng/ml)


(B)
Fgf9
CHX (μg/ml)
−
+
−
+
−
+
−
−
50
50
100
100
Msx2
rRNA
Cyclohexamide (CHX) treatment

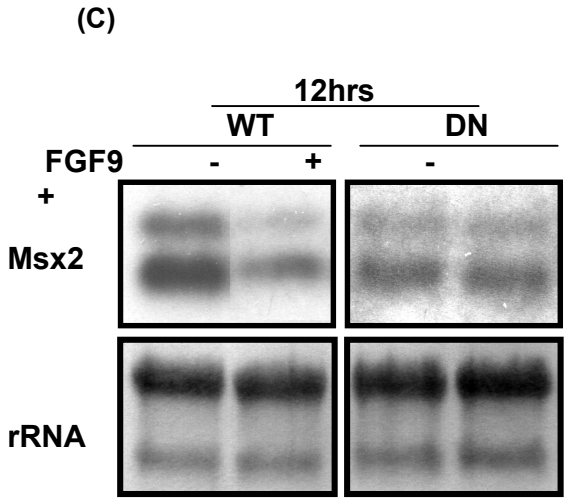
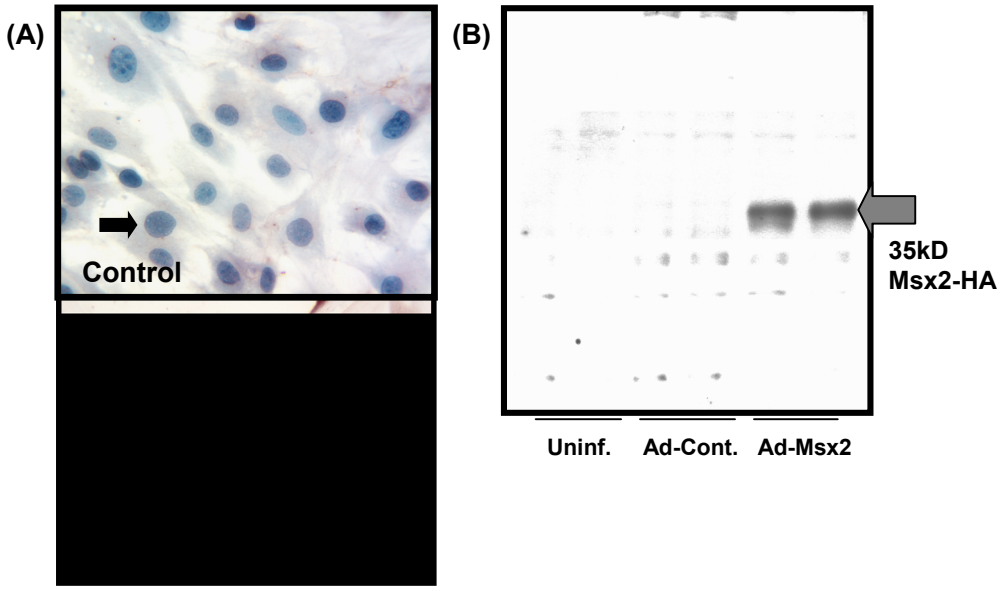

(C)
12hrs
WT
DN
FGF9
-
+
-
+
Msx2
rRNA

Figure 3.


(A)
Control
(B)
35kD
Msx2-HA
Uninf.
Ad-Cont.
Ad-Msx2

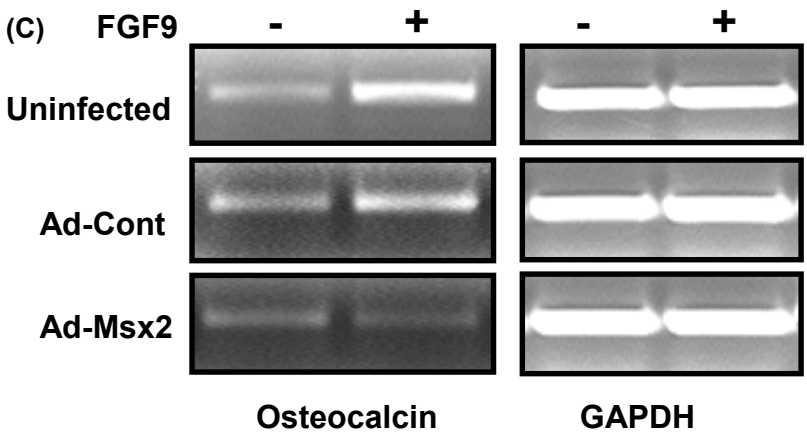

(C)
FGF9
-
+
-
+
Uninfected
Ad-Cont
Ad-Msx2
Osteocalcin
GAPDH

Figure 4.

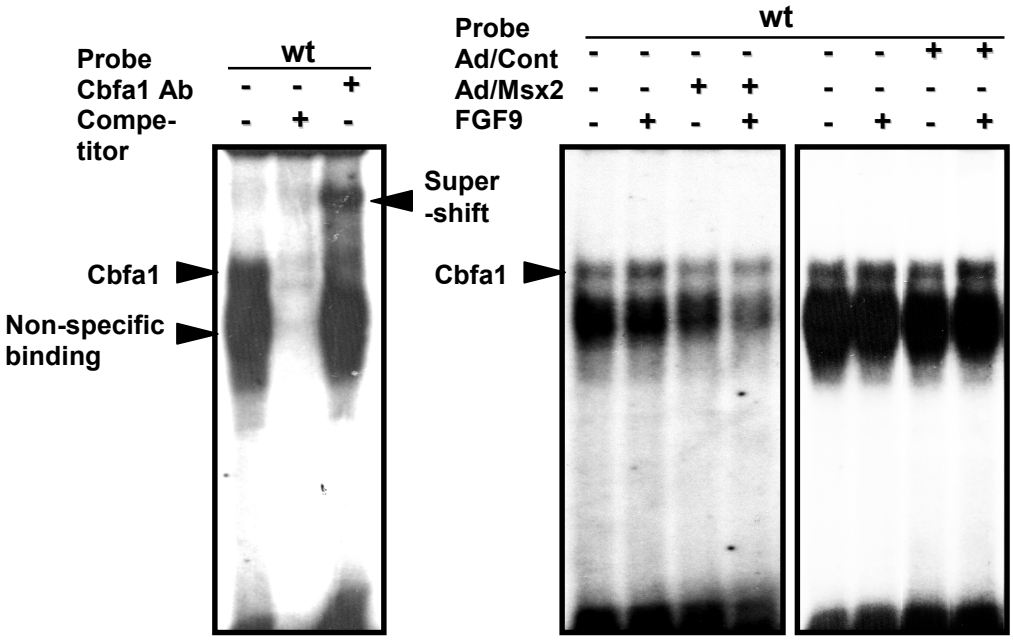

Probe
wt
Cbfa1 Ab
Compe-
titor
Super
-shift
Cbfa1
Non-specific
binding
Probe
wt
Ad/Cont
Ad/Msx2
FGF9
Cbfa1

Figure 5.

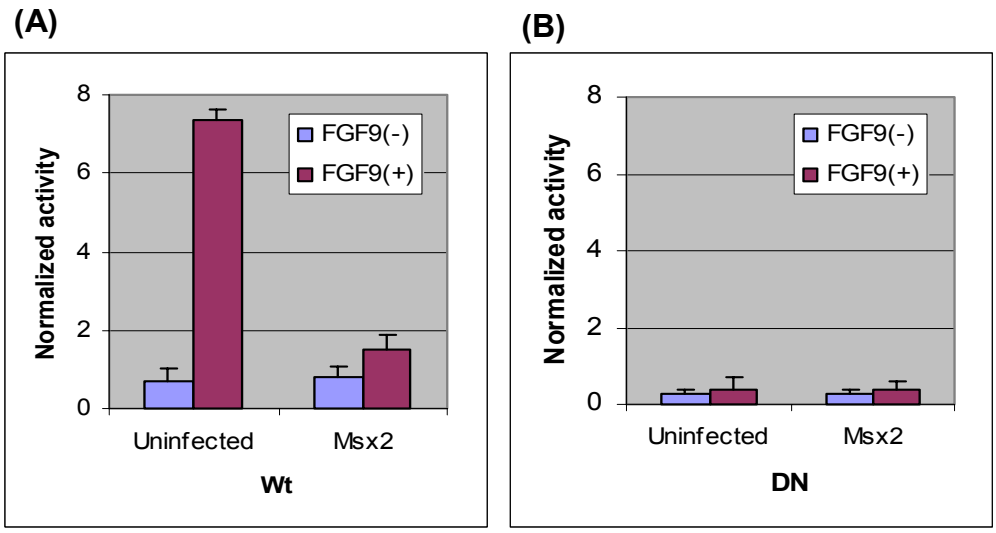
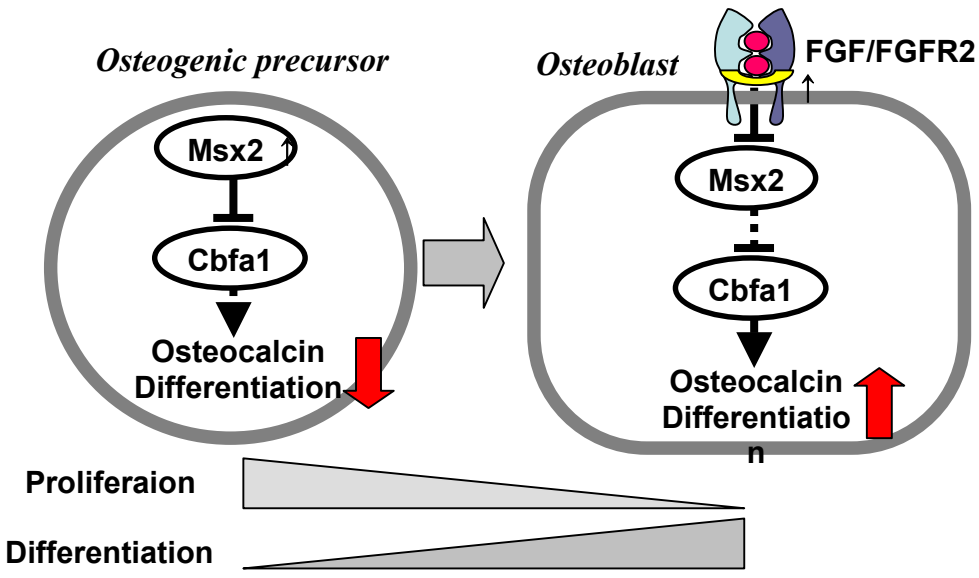


Figure 6.



국문요약

조골세포 분화기전에서 Fibroblast Growth Factor 9 (FGF9) 의 Msx2 발현 억제에 의한 Osteocalcin Gene 의 전사 유도

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이 기 준

섬유아세포 성장인자 수용체 2 (FGFR2) 혹은 엠에스엑스 2 (MSX2) 유전자의 돌연변이는 각 단백질의 기능항진을 통해 두개골 유합증을 유발한다. 두 가지 유전자 모두 두개관의 골형성에 매우 중요하며 발육중인 두개봉합의 초기 조골세포에서 발현하는 것으로 알려져 있으나 이 두 요소의 조절기전에 대해서는 알려진 바가 미미하다. 따라서 본 연구에서 FGF/FGFR 신호전달계가 MSX2 유전자 발현의 상부 조절자인지 여부를 알아보고 분화중인 조골세포에서의 이들 유전자의 가능한 조절기능을 알아보고자 하였다. 세포 수준에서 일어나는 분자생물학적 반응을 조사하기 위해 쥐 두개관에서 얻은 일차 조골세포를 배양하였다. 각각 다른 성숙 단계에 있는 조골세포에서 Fgfr2 와 Msx2 의 표현 양상을 관찰한 결과 Msx2 의 고도의

발현이 Fgfr2 보다 앞서 나타나는 것으로 나타났다. Msx2 유전자의 발현에 대한 Fgf/Fgfr 신호전달계의 영향을 알아보기 위해 일차 조골세포를 Fgf9 으로 처리한 결과 Msx2 유전자의 발현이 어떤 단백질 합성 경로를 통해 유의한 수준으로 감소하는 것을 발견하였다. 이러한 하향 조절의 의미를 알아보기 위해 헴어글루티닌 (hemagglutinin) 표식자가 부착된 Msx2 유전자를 함유한 아데노바이러스 (adenovirus) 를 이용하여 Msx2 유전자를 과발현시켰다. 역전사효소-중합효소 연쇄반응 (RT-PCR) 결과 과발현된 Msx2 하에서 내인성 오스테오칼신 (osteocalcin) 유전자가 Fgf9 처리후에도 억제됨을 관찰하였다. 또한 젤 전이실험 (gel-shift assay) 을 통해 Msx2 의 과발현된 경우 Fgf9 하에서도 코어결합인자알파 1 (Cbfa1/Runx2) 의 OSE2 서열과의 결합이 저하됨을 발견하였다. Msx2 의 과발현 후 6XOSE2 – 루시퍼라아제 (luciferase) 유전자를 주입한 경우 역시 Fgf9 처리시에도 전사활성이 떨어짐을 확인하였다. 이상의 결과에 따라 본 연구에서 Fgf/Fgfr 이 Msx2 의 억제자로 작용함으로써 Cbfa1/Runx2 의 기능을 변환시켜 궁극적으로 조골세포의 분화를 촉진하는 조골세포 분화모델을 제시하였다.

핵심되는 말; 두개관 골형성, 섬유아세포 성장인자/수용체 (Fgf/Fgfr), 엠에스엑스 2 (MSX2), 쥐, 조골세포, 코어결합인자알파 1 (Cbfa1/Runx2), 오스테오칼신 (osteocalcin)