The role of Foxc2 on osteoblast differentiation

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The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the
degree of Doctor of Medical Science

Se-Hwa Kim

December 2008

This certifies that the Doctoral Dissertation of Se-Hwa Kim is approved

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December 2008

Acknowledgements

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저를 이해해 주시고 따뜻한 사랑으로 지켜봐 주신 사랑하는 부모님과 남편, 그리고 항상 엄마가 최고인 사랑하는 인기와 지인이에게 이 논문을 바칩니다.

2008년 12월

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ABSTRACT

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The forkhead box C2 (Foxc2) protein is a member of the family of winged helix/forkhead transcription factors. Mice deficient in the Foxc2 gene display defective formation of the aortic arches, multiple craniofacial bones, and the vertebral column. However, little is known about how this transcriptional factor functions in the proliferation of cells and osteoblast differentiation. Therefore, we investigated the role of Foxc2 in osteoblast differentiation using the developing cranial suture model and the established mesenchyme and preosteoblast cell line.

Calvaria of E13.5 mice were dissected and cultured in a Trowell-type culture dish. Plasmid DNA containing Foxc2 was transfected into the suture mesenchymal cells by electroporation. Foxc2-siRNA transfection was performed using transfection reagents. Immunohistochemical staining was performed after 48h of culture. Alkaline phosphatase (ALP) was stained strongly in the suture mesenchymal cells in the Foxc2 overexpressed calvaria, whereas the control revealed weak ALP staining in the suture mesenchymal cells. Moreover, we found that bone sialoprotein (BSP), a marker for mature osteoblasts, was weakly expressed in the suture mesenchymal cells in the Foxc2 overexpressed calvaria. There was no BSP staining in the suture mesenchymal cells of the control. These findings suggested that Foxc2 over-expression was able to stimulate osteoblast differentiation of suture mesenchymal cells. In addition, we examined whether Foxc2 plays a role in the differentiation of MC3T3-E1 preosteoblasts. Foxc2-siRNA treated cells showed decreased ALP staining and Alizarin red staining compared to the control cells during induction of osteoblast differentiation in MC3T3-E1 cells.

We examined whether Foxc2 affects the Wnt-β-catenin signaling

pathway to elucidate the molecular mechanism by which Foxc2 stimulates osteoblast differentiation. Foxc2 over-expression increased active β-catenin levels and stimulated the T cell factor/lymphoid enhancer factor (TCF/LEF) transcriptional activity in MC3T3-E1 preosteoblasts and C3H10T1/2 cells. Moreover, Wnt3a (50ng/mL) increased Foxc2 protein levels in MC3T3-E1 and C3H10T1/2 cells. A protein kinase A (PKA) inhibitor, H-89, suppressed Foxc2-mediated increase of TCF/LEF transcriptional activity in the C3H10T1/2 cells (~40%, $P < 0.01$). In addition, Foxc2 enhanced Runx2 transcriptional activity without any change of the Runx2 expression in the preosteoblast cells.

In conclusion, our results demonstrated that Foxc2 stimulated osteoblast differentiation of mesenchymal cells and preosteoblasts. The activation of canonical Wnt-β-catenin signals might be involved in the Foxc2-mediated stimulation of osteoblast differentiation. At least, activation of Runx2 plays a role in the stimulation of differentiation by Foxc2 in the preosteoblasts.

Key words: Foxc2, Wnt, β-catenin, Runx2, Osteoblast differentiation

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I. INTRODUCTION

Osteoblastogenesis is a temporally controlled, multistep process, starting around E13.5 of mouse development in both membranous and endochondral skeletal elements. It requires sequential activity of two transcription factors, Runx2/Cbfa1,[1-3] a member of the runt family, and Osterix,[4] encoding a Zn finger transcription factor of the Kruppel-like family, which acts downstream of Runx2. Canonical Wnt-β-catenin signaling has recently been implicated in osteoblastogenesis.[5,6] Wnts activates the canonical pathway through interaction with the receptors of the Frizzled family and the co-receptors of

the Lrp5/6 family.[7,8] The cytoplasmic stabilization and accumulation of β-catenin, via canonical Wnt signaling, leads to β-catenin entering the nucleus and heterodimerizing with T cell factor/lymphoid enhancer factor (TCF/LEF) to regulate Wnt target genes. Loss-of- function mutations in the Lrp5 gene, encoding a co-receptor of canonical Wnt signaling, result in a decrease in bone density associated with defects in osteoblast differentiation and proliferation.[5,9] On the other hand, Lrp5 gain-of-function mutations are associated with an increase in bone mass.[10]

Forkhead box C2/ Mesenchyme forkhead-1 (Foxc2/MFH-1) is a member of the family of winged helix/forkhead transcription factors.[11] Previously, Foxc2 was isolated as a 62-KDa protein, and shown to be expressed in developing cartilaginous tissues, the kidneys and the arch arteries of mice. [12,13] Mice deficient in the Foxc2 gene display defective formation of the aortic arches, multiple craniofacial bones, and vertebral column, thus implying that it plays an essential role in the normal development of the axial skeleton and aortic arch in mice.[14-16] However, it is not clear how this transcription factor functions during osteoblast differentiation and cell proliferation.

Furthermore, Foxc2 is known as a key regulator of adipocyte

metabolism.[17] Transgenic mice with adipocyte-specific overexpression of Foxc2 manifested a lean, insulin-sensitive phenotype.[17] In these mice, deposition of white adipose tissue in the abdomen was reduced, whereas interscapular brown adipose tissue was hypertrophic. Besides, increased Foxc2 expression, induced by a high fat diet in wild-type mice, appeared to counteract most of the signs associated with obesity and diet-induced insulin resistance.[17] Some reports have shown that Foxc2 inhibits white adipocyte differentiation by inhibiting PPARγ promotion of adipogenic gene expression.[18] It is well known that both osteoblasts and adipocytes share a common progenitor, derived from stromal cells in the bone marrow, and that bone loss is associated with an expansion of adipose tissue in the bone marrow.[19,20] Taken together, we hypothesized that Foxc2 takes a role in osteoblast differentiation of the mesenchymal cells.

To prove the hypothesis, we investigated the role of Foxc2 in osteoblast differentiation of mesenchymal cells using the developing cranial suture model and the established mesenchymal- and preosteoblast cell lines. Here, We found that Foxc2 responded to Wnt3a and subsequently promoted osteoblast differentiation of calvaria mesenchymal cells and MC3T3-E1 preosteoblasts.

II. MATERIALS AND METHODS

1. Materials

C3H10T1/2 cells were purchased from ATCC (Manassas, VA, USA) and MC3T3-E1 cells were provided by HM Ryoo (Seoul National University, Seoul, Korea). Anti-Foxc2 and Anti-active β catenin antibody were obtained from Sigma (St. Louis, MO, USA) and BD Biosciences (San Jose, CA, USA), respectively. Anti-Runx2 and anti-myc antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RhWnt3a was purchased from R&D system (Minneapolis, MN, USA). TOPflash or FOPflash were obtained from Upstate biotechonology (Lake Placid, NY, USA). H-89 was purchased from Sigma (St. Louis, MO, USA). Six copies of osteoblast specific element (OSE) was kindly provided by HM Ryoo. Lipofectamine plus was from Invitrogen (Carlsbad, CA, USA).

2. Cell culture and plasmid constructions

Mouse osteoblastic cell line, MC3T3-E1 cells, and C3H10T1/2 cells (Stem cell like fibroblasts) were cultured in α-MEM or Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, respectively. The medium was changed twice a

week. We amplified the full-length Foxc2 by PCR. PCR primers for mouse Foxc2 were forward 5'-CGTCTCGAGCGCACGATGCAG-3' and reverse 5'-TGCGGTCTAGAGGCTGGAGCGGA-3'. For pCS2+MT, the PCR product of Foxc2 and vector were digested with *Xba1* and *XhoI*. The digested gene fragments were inserted into pCS2+MT vector to generate the expression constructs pCS2+MT-Foxc2.

3. Calvaria organ culture and immunohistochemistry

Calvaria of E13.5 mice were dissected and cultured in a Trowell-type culture dish. The dura mater beneath the sagittal suture area was removed. Explants were placed on filter membranes (Tracketch, 1.0-μm pore; Whatman Nucleopore), supported on stainless steel grids in sterile culture dishes, and cultured at the air-medium interface at 37°C and 5% CO_2 for 48h. Ascorbic acid, 100 μg/ml, was supplemented daily and culture medium was changed daily. After 48h of culture, calvaria were harvested, fixed in 4% paraformaldehyde (PFA) and dehydrated through a graded ethanol series, embedded in paraffin wax and sectioned to a thickness of 7 μm. Sections were stained with Hematoxylin-Eosin (HE). These sections were used for immunohistochemistry or stained with alkaline phosphatase (ALP) or 0.1% Alcian blue or 0.1% Alizarin red for 1 min, after they were washed in

running water for 5 min. The specimens were embedded in optimal cutting temperature (OCT) compound using conventional methods. Sections of the specimens were incubated at 4 °C overnight with the primary mouse monoclonal antibody against bone sialoprotein (BSP; Chemicon Inc; Temecula, CA, USA). After washing with phosphate-buffered saline (PBS), the specimens were allowed to react with biotinylated goat antimouse immunoglobulins and streptavidin peroxidase at room temperature for two consecutive 10 minute incubations. Finally, the specimens were visualized using a 3,3′-diaminobenzidine (DAB) reagent kit (Zymed; San Francisco, CA, USA).

4. Electroporation and small interfering RNA treatment

Plasmid DNA containing Foxc2 was purified by using a plasmid purification kit (Qiagen; Valencia, CA, USA) and dissolved in T1/4# (10mM Tris-HCL, pH 8.0/0.25 mM EDTA). Fast Green (diluted 1:10,000; Sigma) was added to the DNA solution for visualization within the tissue [Fig. 1]. A microcapillary needle was used to inject 1 μg/μl DNA into the suture mesenchymal cells, after which 20-ms current pulses of 25V were applied with an electroporator. Foxc2-siRNA and negative control-siRNA obtained from Bioneer (Daejeon, Korea). The optimal oligonucleotide siRNA

sequences for Foxc2 were 5'-CGCAGGUAACUUAUCCGCA-3' and 5'-UGCGGAUAAGUUACCUGCG-3'. A 1 nM siRNA solution was prepared as follows. A siRNA stock was diluted with DMEM/F12 medium containing transfection reagent (siPORT^{TM}*NeoFX*^{TM}, Ambion; Austin, TX, USA) and then incubated for 10min at room temperature. Foxc2-siRNA and negative control-siRNA and were applied to each culture plate containing 10 sets of calvaria.

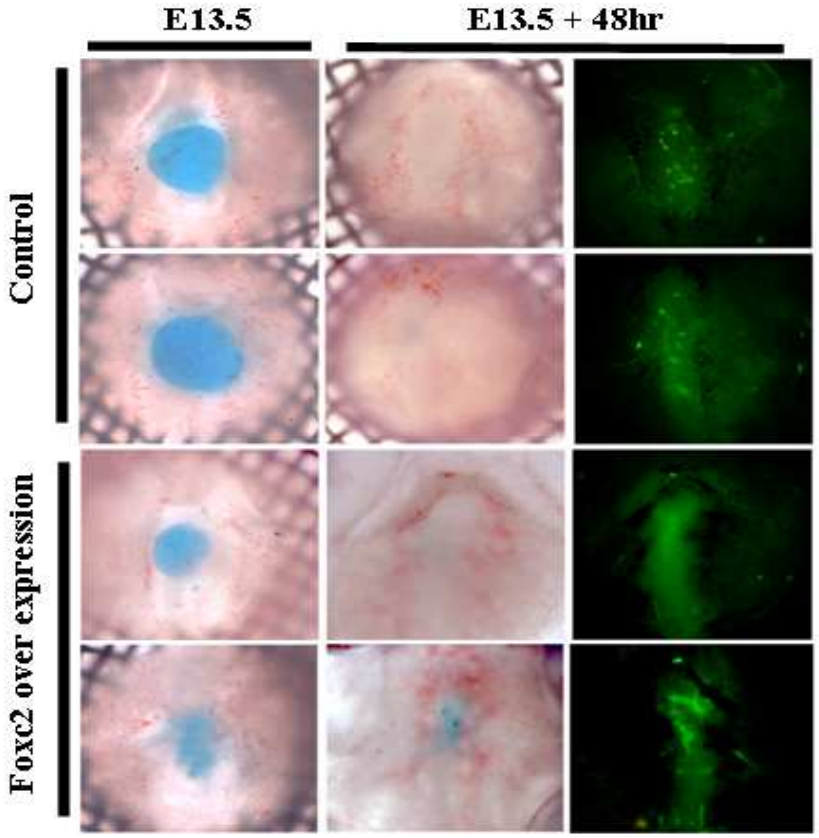


Figure 1. Visualization of GFP-Foxc2 transfection using electroporation in the calvaria organ culture. (Left) Fast Green (diluted 1:10,000) added to Foxc2 DNA solution was seen in the suture mesenchyme (in blue). (Right) GFP signal was observed in the suture mesenchymal area after 48hr of culture under a fluorescence microscope (in green).

5. Western blot analysis

Samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore; Bedford, MA, USA). The membrane was incubated with anti-Foxc2 antibody, anti-myc antibody, anti-Runx2 antibody, anti-active β catenin antibody or anti-β actin antibody. After washing, the membrane was incubated with second antibody for 1 h. Immunoreactive proteins on the membrane were visualized using ECL detection kit (Santa Cruz, CA, USA)

6. Transfection and luciferase reporter gene assay

C3H10T1/2 and MC3T3-E1 cells were plated at a density of $0.5x10^5$ to $1x10^5$ cells /ml into 6-well plates. After overnight culture, the cells were transfected with lipofectamine plus reagent according to the manufacturer's recommendation (Invitrogen; Carlsbad, CA, USA). Each transfection assay was performed with Foxc2-expressing or empty pCS2+MT plasmid, TOPflash- or FOPflash-luciferase reporter plasmid and OSE-luciferase reporter plasmid. Three hours after transfection, the medium was changed and the cells were cultured for an additional 48h. The cells were then harvested and luciferase activity was determined by using a Luciferase assay kit (Promega; Madison, WI, USA). Luminescence was measured in a

Dynatech MLX luminometer, and light integration was measured at 5 s as summed relative luminescence. Each transfection was performed in triplicate and replicated at least three times.

7. RNA isolation and semiquantitative or real-time quantitative RT-PCR

Cells were transfected with 1μg of the Foxc2 expressing vector or pCS2+MT vector in 6-well plates for 3h. After 2 days, semiquantitative or real-time quantitative RT-PCR were performed. Total RNA was isolated from the cells using Trizol reagent (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instruction. cDNA was synthesized from 2 μg total RNA using M-MuLV reverse transcriptase (Promega; Madison, WI, USA). Real-time PCR quantitation of mRNA of Runx2, osteocalcin, and β-catenin was performed in iCycler IQ detection system by using SYBR® Green I as a double-stand DNA-specific binding dye. Primer pairs were as follows: Runx2, forward 5'CCGCACGACAACCGCACCAT-3', reverse 5'-CGC TCCGGCCCACAAATCTC-3'; osteocalcin, forward 5'-TGAGTC TGACAAAGCCTTCA-3', reverse 5'-GGACTGAGGCTCCAAGGTAG-3'; β-catenin, forward 5'-AGTGACTCACGCAGTGAAGA-3', reverse 5'-AAA CAAAGAACAAGCAAGGC-3'; PPARγ, forward 5'-CACTTCACAAGA AATTACCAT-3', reverse 5'-GAAGGACTTTATGTATGAGTC-3';

adipocyte fatty acid binding protein (aP2), forward 5'-TCACCTGGAAG ACAGCTCCT-3', reverse 5'-TCGACTTTCCATCCCACTTC-3'; β-actin, forward 5'-GATAGCGATATCGCTGCGCT-3', reverse 5'-GCTCATTGCC GATAGTGACCT-3'; Adiponectin, forward 5'-GTTGCAAGCTCTCCTG TTCC-3', reverse 5'-TCTCCAGGAGTGCCATCTCT-3'.

8. Osteoblast differentiation of MC3T3-E1 cells

MC3T3-E1 cells were plated at a density of $1x10^5$ cells /ml into 6-well plates. 24h later, at ~80% confluency, the cells were transfected with 100pmol of Foxc2-siRNA or control-siRNA using lipofectamine plus reagent. Induction of differentiation was achieved by treatment with the standard induction mixture containing β-glycerophosphate (10mM), ascorbic acid (50μg/ml), and dexamethasone (10nM) on 1 day post-confluent cells. This differentiation mixture was changed every other day throughout the study.

9. Alkaline phosphatase (ALP) and Alizarin red staining

ALP staining was performed to monitor osteoblastic differentiation. In vitro bone nodule formation was determined by Alizarin red staining. Cells were washed with PBS twice, fixed with 2% paraformaldehyde, and stained

for alkaline phosphatase according to the manufacturer's instructions (Sigma; St. Louis, MO, USA). For the Alizarin red staining, cell were fixed in 10% formalin/PBS and stained with 2% Alizarin red S (pH 4.0) solution.

10. Statistical analysis

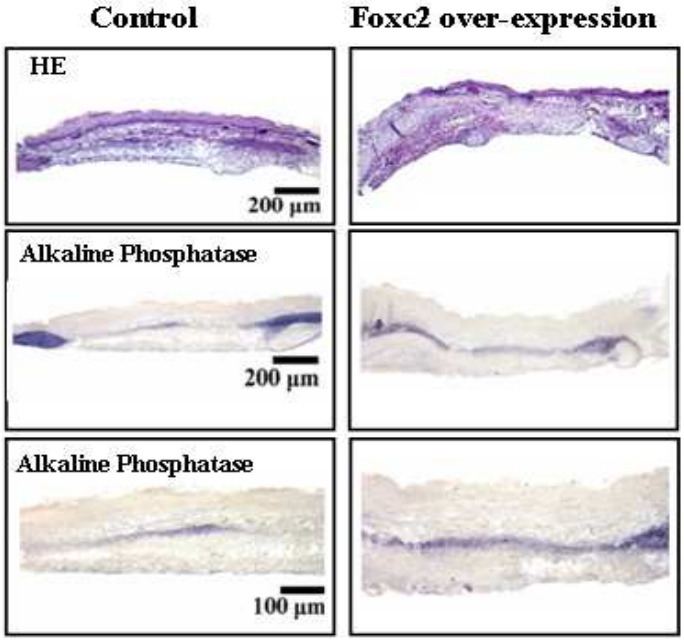
The results are expressed as mean ± SD. Statistical analysis of differences between two means was assessed by Student's *t*-test. *P* values < 0.05 were considered statistically significant.

III. RESULTS

1. Effect of Foxc2 on mesenchymal cell differentiation in the calvaria organ culture

To determine the role of Foxc2 in osteoblast differentiation of suture mesenchymal cells, we examined ALP and BSP expression in calvaria organ cultures. ALP expression was observed in the suture mesenchymal cells in the Foxc2 over-expression calvaria, whereas ALP was weakly expressed in the suture mesenchymal cells in the control [Fig. 2A]. These findings suggested that Foxc2 over-expression might stimulate osteoblast differentiation of suture mesenchymal cells. Moreover, we found that BSP, a marker for mature osteoblasts, was weakly expressed in the suture mesenchymal cells in the Foxc2 over-expression calvaria as indicated in the arrow. This finding indicated that mesenchymal cells in the suture mesenchyme were differentiating into mature osteoblasts. There was no BSP staining in the suture mesenchymal cells of the control as indicated in the arrow head [Fig. 2B].

A.


Control
Foxc2 over-expression
HE
200 μm
Alkaline Phosphatase
200 μm
Alkaline Phosphatase
100 μm

B

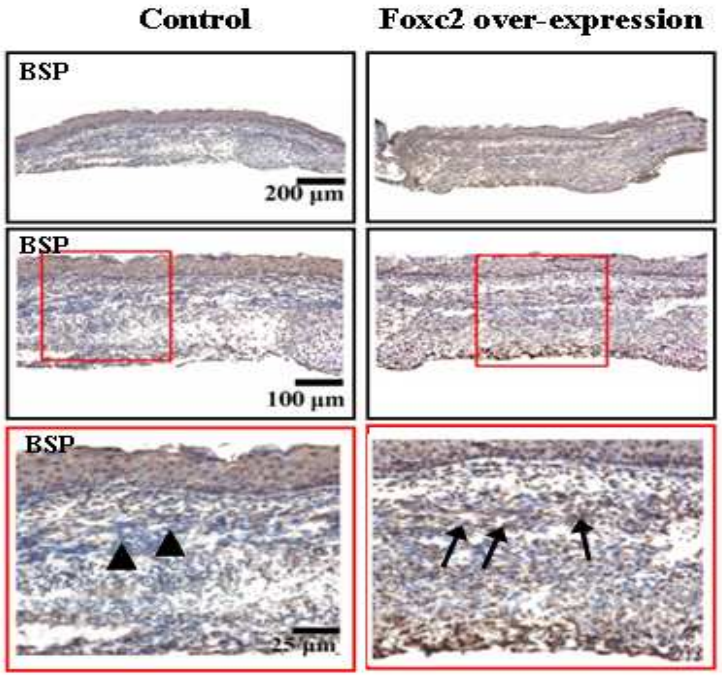

Control
Foxc2 over-expression
BSP
200 μm
BSP
100 μm
BSP
25 μm

Figure 2. Foxc2 over-expression stimulated suture mesenchymal cell differentiation. The calvaria of E13.5 mice were dissected and cultured in the Trowell-type culture dish. The Foxc2 construct was transfected into the suture mesenchymal cells by electroporation. After 48h of culture, the calvaria was harvested and fixed. (A) Hematoxylin-Eosin (H&E), Alkaline phosphatase (ALP) staining and (B) Bone sialoprotein (BSP) staining were performed in each sample. Positive BSP expression (in brown) was indicated by the arrow in the Foxc2-siRNA calvaria.

After 72h of Foxc2-siRNA transfection, ALP staining was rarely observed in the suture mesenchymal cells and the adjacent parietal bone of the calvaria. However, the control calvaria showed ALP staining in the parietal bone as indicated in the arrow head [Fig. 3]. Alizarin red staining, which represents mineralized nodule, rarely observed in the both Foxc2-siRNA and control-siRNA calvaria. Alcian blue positive cells were seen in the suture mesenchyme area of the Foxc2-siRNA treated calvaria as indicated in the arrow [Fig. 3]. The thinning of epidermis and dermis was also observed as well as the reduction of hair follicle in the Foxc2-siRNA treated calvaria than did in the control.

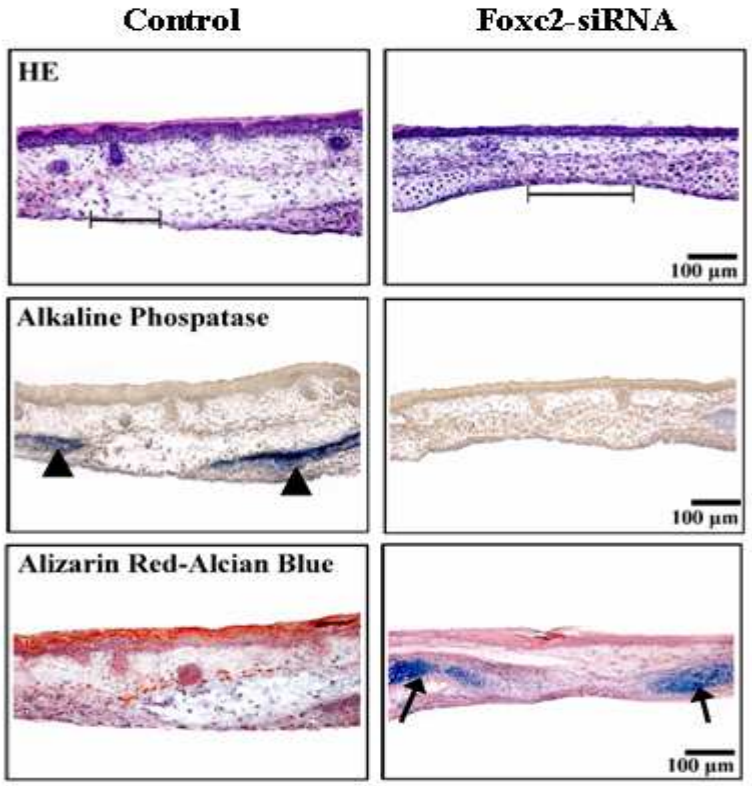


Figure 3. Effect of Foxc2-siRNA in the calvaria organ culture. The calvaria of E13.5 mice were dissected and cultured in the Trowell-type culture dish. The Foxc2-siRNA or control-siRNA was transfected into the suture mesenchymal cells using the transfection reagents as described in the Materials and Methods section. After 72h in culture, the calvaria was harvested and fixed. Hematoxylin-Eosin (H&E), Alkaline phosphatase (ALP), Alizarin red, and Alcian blue staining were performed in each sample.

2. Foxc2-siRNA inhibited osteoblast differentiation of MC3T3-E1 cells

We examined whether Foxc2 plays a role in the differentiation of MC3T3-E1 preosteoblasts. The cells were transfected with Foxc2-siRNA or control-siRNA using the lipofectamine plus reagent. The cells were induced to differentiate into osteoblast with the osteoblast induction media containing ascorbic acid(50μg/ml), dexamethasone (10nM) and β-glycerophosphate (10mM) at a post-confluent state. On differentiation day 4, the control cells showed weak positive ALP staining whereas the Foxc2-siRNA treated cells did not [Fig. 4A].

Similarly, *in vitro* matrix mineralization visualized by Alizarin red staining was significantly decreased in the cells after Foxc2-siRNA treatment [Fig. 4B]. These results suggest that the Foxc2 may stimulate osteoblast differentiation of the MC3T3-E1 preosteoblasts.

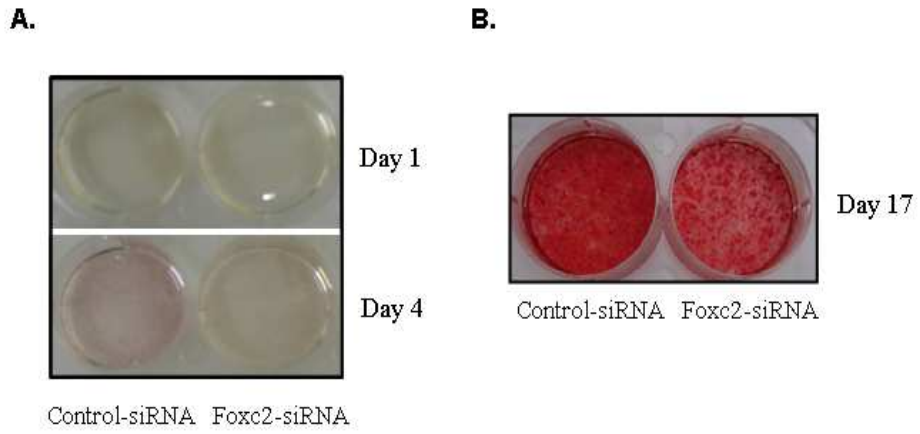
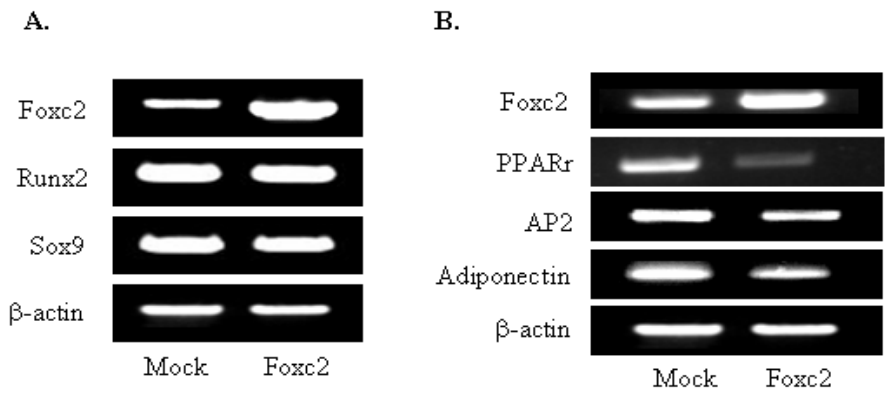


Figure 4. Foxc2-siRNA inhibited osteoblast differentiation of MC3T3-E1 cells. The cells were transfected with Foxc2-siRNA or control-siRNA using the lipofectamine plus reagent. The post-confluent cells were induced to differentiate as noted in the Materials and Methods section. (A) ALP staining was performed on day 1, 4, and 12 of differentiation induction. (B) Alizarin red staining was performed on day 9 and 17.

3. Effect of Foxc2 on Runx2 and Wnt-β-catenin signaling in C3H10T1/2 cells

To better understand the molecular mechanisms by which Foxc2 stimulates osteoblast differentiation, we over-expressed Foxc2 in C3H10T1/2 mesenchymal cells and MC3T3-E1 preosteoblast cells. Foxc2 over-expression did not influence the Runx2 and Sox9 mRNA expression [Fig 5A]. However, Foxc2 decreased PPARγ, which is an essential transcription factor for adipocyte differentiation. In addition, Foxc2 transfection decreased adipocyte gene markers such as aP2 and adiponectin [Fig 5B]. Interestingly, Wnt3a enhanced the Foxc2 protein levels up to 140 % after twelve hours while it decreased 25% after twenty four hours [Fig 5C].



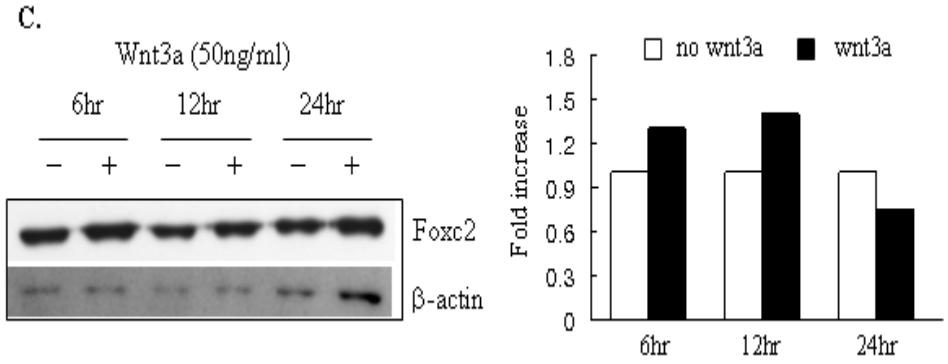


Figure 5. Effect of Foxc2 on osteoblast and adipocyte gene expression in C3H10T1/2 cells. The cells were transfected with Foxc2-expressing (0.7μg) or an empty (0.7μg) pCS2+MT plasmid. After 2 days, total RNA was prepared and analyzed for osteoblast (A) and adipocyte (B) marker gene expression by RT-PCR. (C) Wnt3a (50ng/mL) was administrated at a given time. The cells were harvested for Foxc2 protein expression.

To investigate whether Foxc2 affects the transcriptional activity stimulated by the Wnt-β-catenin pathway in C3H10T1/2 cells, we used the TOPflash luciferase reporter plasmid that has T-cell factor/lymphoid enhancer factor (TCF/LEF) binding elements in the promoter for the experiments. Foxc2 increased TOPflash luciferase activity up to 4-fold [Fig. 6A]. To investigate the possible mechanism by which Foxc2 activates Wnt-β-catenin signaling, we studied the effects of H-89, protein kinase A (PKA) inhibitor. Previous results demonstrated cross talk between cAMP/PKA and the Wnt pathway. Moreover, Foxc2 is known to activate PKA.[16] We examined whether PKA inhibition regulated Foxc2-mediated increase of TOPflash luciferase activity. We found that H-89 moderately decreased the TOPflash luciferase activity by Foxc2 (~ 40%, $P < 0.01$) [Fig. 6B]. These results suggest that the effects of Foxc2 on the canonical Wnt-β-catenin signaling pathway occur at least in part via the cAMP/PKA pathway.

To examine the effects on the transcriptional activity of Runx2 by Foxc2, a luciferase reporter gene construct containing an osteoblast-specific *cis*-acing element (OSE) including the Runx2 binding sites was transfected into C3H10T1/2 cells. Foxc2 over-expression inhibited Runx2 transcriptional activity when Foxc2- and Runx2-expressing plasmids were co-transfected in a dose dependent manner [Fig. 7].

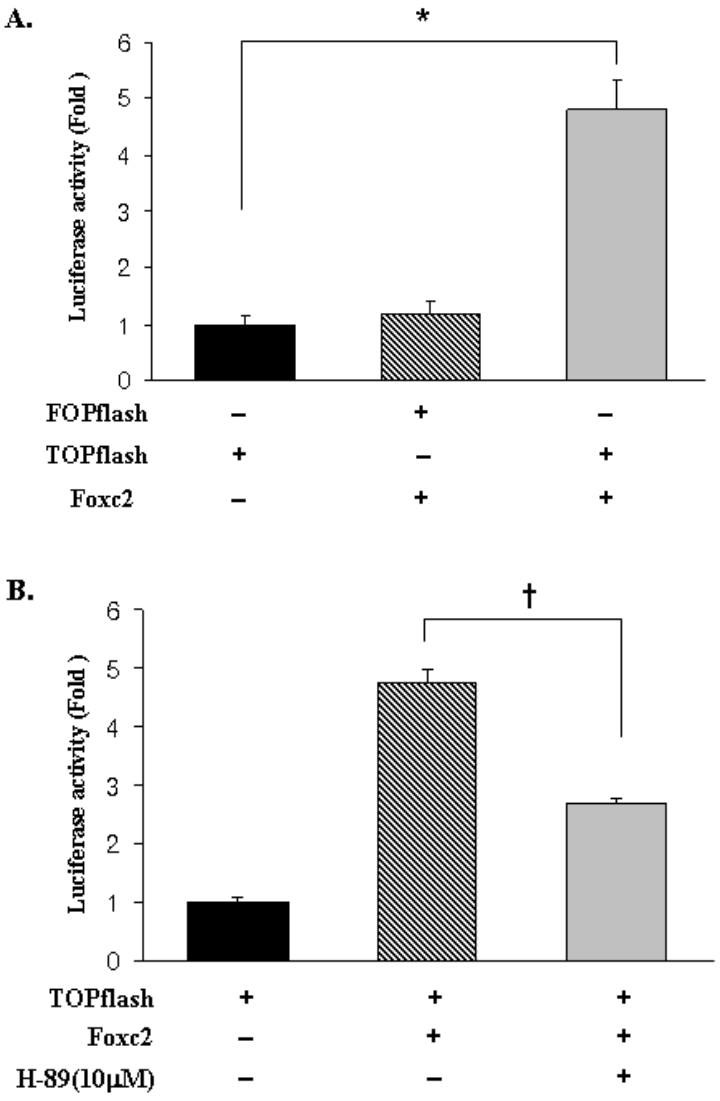


Figure 6. Effect of Foxc2 on TOPflash reporter activity in C3H10T1/2 cells. (A) The cells were transfected with plasmids containing TOPflash, FOPflash, and Foxc2. (B) The cells were transfected with plasmids containing

TOPflash and Foxc2 in the presence or absence of PKA inhibitor, H-89 (10μM). The cells were harvested 24-48 h later and luciferase activity was measured and normalized by the protein concentration in the cell lysates. Values are the mean ± SD. * $P < 0.01$ compared with control group. † $P < 0.01$ compared Foxc2 only group.

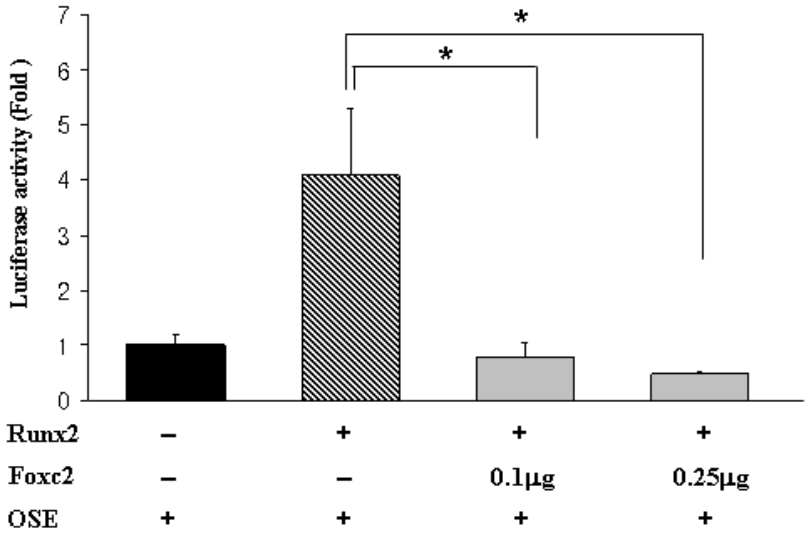


Figure 7. Effect of Foxc2 on OSE reporter activity in C3H10T1/2 cells. The cells were transfected with plasmids containing Runx2, Foxc2, and OSE-luciferase reporter gene. The cells were harvested 24-48 h later and luciferase activity was measured and normalized by the protein concentration in the cell lysates. Values are the mean ± SD. * $P < 0.01$ compared with Runx2 only group.

4. Effect of Foxc2 on Runx2 expression and OSE reporter activity in MC3T3-E1 cells

We demonstrated that Wnt3a markedly enhanced the Foxc2 protein levels more in the MC3T3-E1 cells compared to the C3H10T1/2 cells [Fig. 8A]. However, PTH (10^{-8}M) did not influence the Foxc2 levels [Fig. 8B]. We also examined the effects of Foxc2 on the level of osteoblast marker gene expression. Real-time RT-PCR analysis revealed that the expression of Runx2 and osteocalcin were not affected [Fig. 9A]. Moreover, Foxc2 transfection had no effect on the Runx2 protein levels [Fig. 9B].

To examine the effects on the transcriptional activity of Runx2 by Foxc2, a luciferase reporter gene construct containing an osteoblast-specific *cis*-acing element (OSE) including the Runx2 binding sites was transfected into the MC3T3-E1 cells. We found that Foxc2 transient transfection increased OSE luciferase activity up to 2 fold [Fig. 10]. Collectively, these results demonstrated that Foxc2 stimulated Runx2 transcriptional activity.

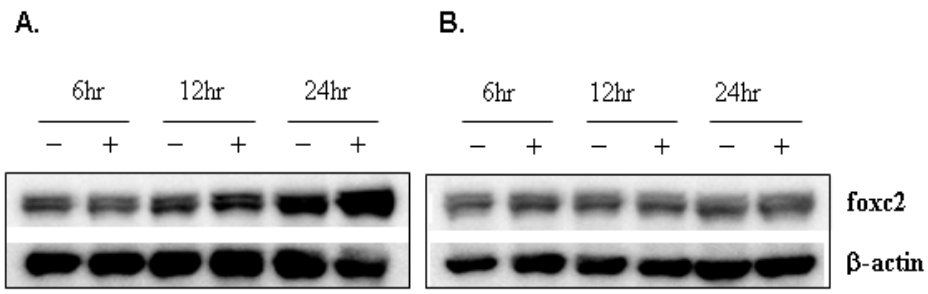


Figure 8. Effect of Wnt3a and PTH on Foxc2 protein expression in MC3T3-E1 cells. (A) Wnt3a (50ng/mL) or (B) PTH (10^{-8}M) was administrated at a given time. The cells were harvested for Western blot analysis.

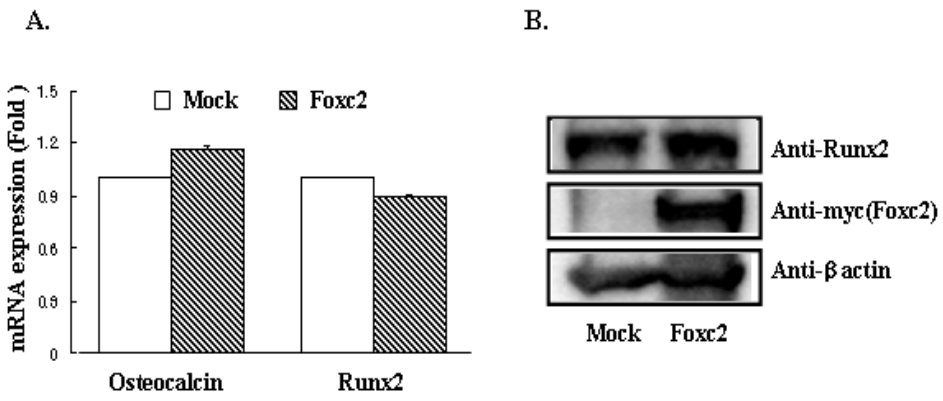


Figure 9. Effect of Foxc2 on Runx2 expression and OSE reporter activity in MC3T3-E1 cells. The MC3T3-E1 cells were transiently transfected with Foxc2-expressing (0.7ug) or an empty (0.7ug) pCS2+MT plasmid. (A) Osteoblast marker gene expression including Runx2 was determined by real-time RT-PCR. (B) Runx2 protein levels were assessed by Western blot analysis.

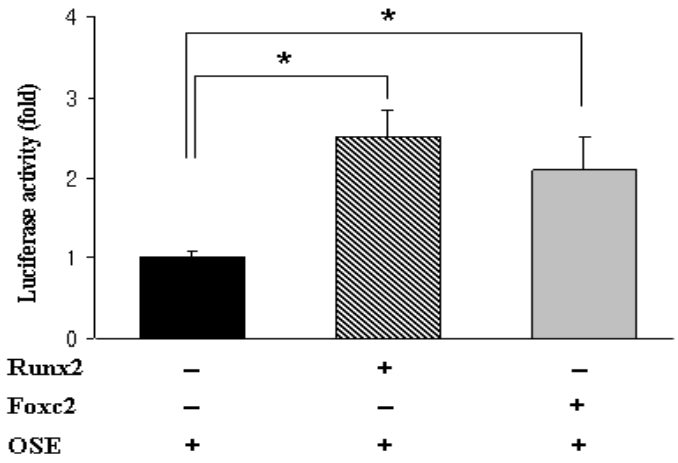


Figure 10. Effect of Foxc2 on OSE reporter activity in MC3T3-E1 cells. The cells were transfected with plasmid containing OSE luciferase reporter gene alone or with the plasmid containing Runx2 or Foxc2, and cultured for 2 days. Luciferase activity was measured and normalized by protein concentration in the cell lysates. * $P < 0.05$ vs. OSE luciferase reporter gene alone.

5. Effect of Foxc2 on levels of β-catenin and TCF/LEF luciferase activity in MC3T3-E1 cells

We examined the effects of Foxc2 on the level of β-catenin protein in MC3T3-E1 cells by Western blot analysis. Foxc2 significantly increased active β-catenin levels after 48h of transfection [Fig. 11A]. However, Foxc2 did not affect the levels of β-catenin mRNA assessed by real-time quantitative PCR [Fig. 11B].

We next investigated whether Foxc2 affects the transcriptional activity stimulated by the Wnt-β-catenin pathway in the MC3T3-E1 cells. Foxc2 increased TOPflash luciferase activity up to 2.5 fold. Meanwhile, there was no increase in the mock DNA transfected control cells [Fig. 12].

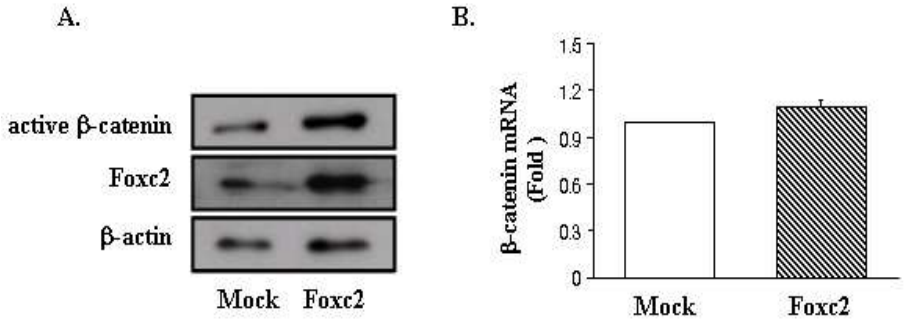


Figure 11. Effect of Foxc2 on β-catenin protein and mRNA expression in MC3T3-E1 cells. MC3T3-E1 cells were transiently transfected with Foxc2-expressing (0.7μg) or an empty (0.7μg) pCS2+MT plasmid. (A) Forty-eight hours later, protein extraction of the cells and Western blot analysis were performed. (B) RNA extraction and real-time quantitative RT-PCR analyses were performed. Values are the mean ± SD.

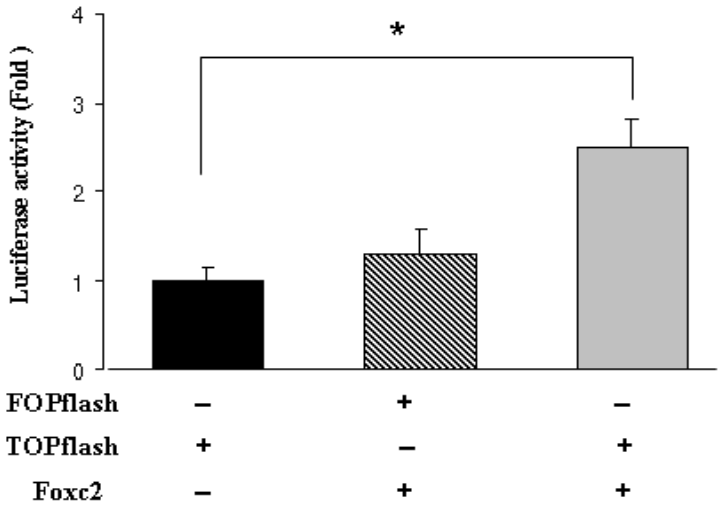


Figure 12. Effect of Foxc2 on TCF/LEF luciferase activity in MC3T3-E1 cells. MC3T3-E1 cells were transfected with plasmid containing TOPflash, FOPflash, and Foxc2. Forty-eight hours later, the cells were harvested. Luciferase activity was measured and normalized by the protein concentration in the cell lysates. Values are the mean ± SD. * $P < 0.01$ compared with control.

IV. DISCUSSION

In this study, we showed that Foxc2 stimulated osteoblast differentiation of suture mesenchymal cells and preosteoblast cells, and activation of the Wnt-β-catenin signaling played a significant role in the Foxc2-mediated osteoblast differentiation.

In Foxc2-deficient mice, hypoplasia of the basio and ali-sphenoid bones, absence of several ossification centers in the neurocranium, deformities of the middle ear ossicles, and cleft plate are reproducibly observed.[14,15] Mice lack of Foxc2 also exhibit hypoplasia of the vertebrae and insufficient chondrification or ossification of the medial structures.[21] These findings suggest that Foxc2 plays an essential role in the normal development of the axial skeleton and the skull of mice. However, the role of Foxc2 in skeletogenesis and osteoblast differentiation has not yet been studied.

The developing cranial suture is a good model for the study of osteoblast differentiation. The developing cranial suture contains cells at all stages of osteoblast differentiation, namely undifferentiated mesenchymal stem cells in the suture mesenchyme, committed and actively differentiating

preosteoblasts at the osteogenic fronts, and fully differentiated active osteoblasts in the parietal bones.[22] To investigate the role of Foxc2 in osteoblast differentiation of mesenchymal cells, we transfected a Foxc2-expressing plasmid into the suture mesenchyme using the electroporation method [Fig. 1]. Foxc2 over-expression showed more ALP positive cells in the suture mesenchyme than the control did. The expression of bone sialoprotein, a marker for mature osteoblasts, was weakly seen in the suture mesenchymal cells in the Foxc2 overexpressed calvaria but not expressed in the control. Foxc2-siRNA transfection showed an increase in the suture width, distance between both parietal bones, compared to the control group. Interestingly, Alcian blue positive cell clusters were observed in the suture mesenchyme area of the Foxc2-siRNA treated calvaria. Taken together, these findings suggested that Foxc2 stimulated osteoblast differentiation of mesenchymal cells, whereas mesenchymal cells or osteoblast precursors lacking Foxc2 had their differentiation blocked and developed into chondrocyte instead. *In vitro* assay also revealed that Foxc2-siRNA transfection inhibited osteoblast differentiation in MC3T3-E1 cells. Our results are consistent with the findings of the previous report by Yang et al. Their study showed that both ALP activity and osteocalcin production by bone morphogenetic protein-2 (BMP-2) decreased markedly in antisense

Foxc2 cell lines when compared to the control cell lines.[23] However, they did not elucidate the molecular mechanism explaining Foxc2 mediated osteoblast differentiation.

Activated β-catenin induces osteoblast differentiation of mouse pluripotent C3H10T1/2 cells.[24] Hill TP *et al*. reported that β-catenin is required for osteoblast lineage differentiation by conditionally deleting β-catenin in limb and head mesenchymal tissue.[25] Osteoblast precursors lack of β-catenin are blocked from further differentiation and it develops into chondrocytes instead.[25] We have demonstrated the ability of Foxc2 to increase the levels of β-catenin protein, and have shown functional activation of the downstream Wnt pathway as assessed by the TCF/LEF binding element fused to the luciferase gene in MC3T3-E1 cells. The results of the present study demonstrated that Foxc2 over-expression increased the level of active β-catenin in mouse osteoblast cells. Moreover, Foxc2 over-expression enhanced the TOPflash luciferase activity in C3H10T1/2 and MC3T3-E1 cells. Our findings suggest that Foxc2 induces the canonical Wnt-β-catenin pathway in mesenchymal and preosteoblast cells. However, we are not able to search out the precise mechanism how Foxc2 activates Wnt-β-catenin signaling.

Many previous studies have demonstrated cross talk between cAMP/PKA and the Wnt pathway.[26-28] It has been shown that phosphorylation of glycogen synthase kinase 3 beta (GSK3β), a downstream component of the Wnt signaling pathway, by cAMP-dependent PKA leads to inhibition of GSK3β, thus resulting in stimulation of the Wnt pathway.[26,28] Moreover, a recent study demonstrated that PKA activation by parathyroid hormone related protein (PTHrP) activated β-catenin through phosphorylation [ASBMR 2008]. A previous study reported that Foxc2 up-regulated the transcription of the RI subunit of PKA to augment the β-adrenergic-cAMP-PKA response, which leads to increase sensitivity to insulin.[16] Therefore, we hypothesized that Foxc2 could stimulate the Wnt-β-catenin pathway through inhibition of GSK3β by augmenting the cAMP/PKA signaling with various extracellular stimuli. Here, we showed that activation of TCF/LEF luciferase reporter activity by Foxc2 was inhibited by the PKA inhibitor, H-89, in the C3H10T1/2 cells. However, the PKA inhibitor did not have any influence on the MC3T3-E1 cells. We have no explanation for this discrepancy and additional studies are needed to clarify this issue. However, these results suggest that the effects of Foxc2 on the canonical Wnt-β-catenin signaling pathway occur at least in part via the cAMP/PKA pathway in mesenchymal cells.

Runx2, a member of the runt homology domain transcription factor family, is essential for osteoblast differentiation.[1] Mutations in the Runx2 gene in mice and humans are associated with cleidocranial dysplasia and significant defects in bone formation.[3,29] We examined whether Foxc2 influences Runx2 protein level or transcriptional activity to illustrate how Foxc2 enhances osteoblast differentiation. As expected, Runx2 transcriptional activity was increased by Foxc2 without any change of Runx2 expression in the preosteoblasts. However, we did not observe any physical interaction between Runx2 and Foxc2 in the immunoprecipitation experiments (data not shown). Therefore, Runx2 activation might be one of the mechanisms of Foxc2 mediated stimulation of osteoblast differentiation. In this study, we also performed an experiment on a different mesenchymal cell, C2C12. Here, as similar to the preosteoblasts, Foxc2 enhanced the Runx2 activity (data no shown). However, the Foxc2 decreased the Runx2 activity in the C3H10T1/2 cells. An exact explanation cannot be made for the reason of why C3H10T1/2 showed a different result compared to the other cell line. We could probably explain this situation if Foxc2 indirectly increase Runx2 activity through another factor that might be expressed differently in each cell line. Further study should be done for this topic.

In summary, our results suggest that Foxc2 plays a regulatory role in osteoblast differentiation of mesenchymal cells and differentiation of preosteoblasts. Furthermore, we showed activation of canonical Wnt-β-catenin signaling and Runx2 could be involved in the stimulation of osteoblast differentiation by Foxc2.

V. CONCLUSION

The results of the present study demonstrated the role of Foxc2 in the calvaria organ culture and preosteoblast cell line. We discussed the possible mechanisms involved in osteoblast differentiation.

1. In the calvaria organ culture, Alkaline phosphatase (ALP) was stained strongly in the suture mesenchymal cells in the Foxc2 overexpressed calvaria, whereas the control revealed weak ALP staining in the suture mesenchymal cells.

2. We found that bone sialoprotein (BSP), a marker for mature osteoblasts, was weakly expressed in the suture mesenchymal cells of the Foxc2 overexpressed calvaria. The control, by contrast, did not express BSP in the suture mesenchymal cells.

3. Both ALP activity and Alizarin red staining were decreased in the Foxc2-siRNA treated cells compared to the control-siRNA cells during osteoblast differentiation in MC3T3-E1 cells.

4. Wnt3a increased Foxc2 protein levels in mesenchymal cells and preosteoblast cells.

5. Foxc2 over-expression increased active β-catenin levels and stimulated the TCF/LEF transcriptional activity in C3H10T1/2 cells and preosteoblast cells. Moreover, protein kinase A (PKA) inhibitor suppressed Foxc2-mediated increase of TCF/LEF transcriptional activity in the C3H10T1/2 cells.

6. Foxc2 enhanced Runx2 transcriptional activity but not Runx2 protein levels in the preosteoblast cells.

The above results suggest that Foxc2 plays a regulatory role in the osteoblast differentiation of mesenchymal cell and preosteoblasts. The activation of canonical Wnt-β-catenin signaling and Runx2 could be involved in the stimulation of osteoblast differentiation by Foxc2.

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ABSTRACT (IN KOREAN)

전사인자 Foxc2 가 조골세포의 분화에 미치는 영향

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배아발생과 세포분화에 중요한 조절인자로 알려진 Foxc2는 forkhead/ winged helix 전사인자 family중의 하나로, Foxc2 결핍 쥐에서 두개 안면골격 및 척추의 일부 결손이 나타나고 대동맥 형성의 장애를 보여, 골격과 혈관 생성의 정상적인 발달에 중요한 역할을 하는 것으로 알려졌다. 최근에는 Foxc2가 지방세포에 표현되며 Foxc2를 과 발현시킨 쥐에서 고지방식이에 의한 체중증가 및 인슐린 저항성이 감소되었고, 일부 보고에서 Foxc2가 간엽세포에서 지방세포로의 분화를 억제하였다고 하였다. 조골세포와 지방세포가 하나의 간엽세포에서 유래한다는 사실을 고려할 때 Foxc2가 간엽세포에서 조골세포로의 분화에 중요한 역할을 할 것이란 가설을 세웠다.

본 연구에서는 배아발생 13.5일째 두개골을 배양 시키고, 전기천공법을 이용하여 Foxc2가 측두골 사이의 간엽세포에서 과발현 되도록 하였다. Foxc2 과발현 시켰을 때 조골세포로의 초기분화 표지자인 알칼라인 포스파타제에 염색되는 세포들이 대조군에 비해 많이 관찰되었다. 성숙한 조골세포의 표지자인 bone sialoprotein은 Foxc2 과발현 군에서 일부 표현되었으나, 대조군에서는 거의 표현되지 않았다. 전구 조골세포에서 조골세포로의 분화를 유도하는 동안 Foxc2-siRNA를 처리하여 분화정도에 차이가 있는지 조사하였다. 대조군에서는 분화 4일째 알칼라인 포스파타제 염색에서 양성을 보였으나, Foxc2-siRNA군에서는 거의 염색되지 않았다. 또한 무기질화 결절을 의미하는 알리자린레드 염색도 분화 17일째 대조군에 비해서 Foxc2-siRNA군에서 약하게 염색됨을 관찰하였다. 이상의 결과로 Foxc2가 간엽세포에서 조골세포로의 분화 및 전구 조골세포에서 조골세포로의 분화를 촉진시킴을 알 수 있었다.

Foxc2가 조골세포 분화를 촉진시키는 기전을 규명하기 위해서, 조골세포의 분화에 중요한 Wnt-β-catenin 신호전달과 전사인자 Runx2와의 관계를 C3H10T1/2 세포와 MC3T3-E1 전구 조골세포에서 조사하였다. 두 세포에서 Wnt3a를 처리하였

을 때 Foxc2 단백표현이 의미있게 증가하였다. Foxc2 과발현을 시켰을 때 β-catenin mRNA 표현은 증가하지 않았으나 활성형 β-catenin 단백이 증가하였다. Foxc2에 의해 증가된 활성화 β-catenin이 실제 전사인자를 조절하는지 알아보기 위해 TOPflash reporter 플라스미드 (TCF/LEF 결합부위 포함)를 이용하여 luciferase 활성도를 조사하였다. Foxc2 를 과발현 시켰을 때 대조군에 비해 TCF/LEF 활성도를 4배 정도 의미있게 증가시켰다. 또한 protein kinase A (PKA) 억제제를 처리한 후에는 Foxc2에 의해 증가된 TCF/LEF 활성도가 40% 정도 억제됨을 관찰하였다. 또한 Foxc2 과발현이 전구조골세포에서 Runx2 mRNA 및 단백 표현에는 영향을 미치지 않았으나 Runx2 활성도를 2배 정도 증가시켰다.

결론적으로 Foxc2는 간엽세포와 전구조골세포에서 조골세포로의 분화를 촉진하는 작용을 나타냈다. Foxc2가 조골세포 분화를 촉진하는 가능한 기전으로 Foxc2가 cAMP/PKA 신호전달을 증가시켜서 β-catenin을 활성화시키고, Wnt 신호전달을 활성화시키는 것으로 생각되며, 일부에서 Runx2 활성도를 증가시킴으로써 작용하는 것으로 보인다.

핵심되는 말: Foxc2, Wnt, β-catenin, Runx2, 조골세포 분화