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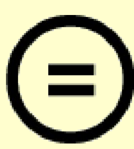

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치의학석사 학위논문

Negative regulation of osteoblast differentiation by cellular retinoic acid binding protein-II (CRABP-II)

Cellular retinoic acid binding protein-II의
조골세포 분화 저해 기작 규명

2015 년 2 월

서울대학교 대학원
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이 재 영

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2014년 11월

서울대학교 대학원
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ABSTRACT

Negative regulation of osteoblast differentiation by cellular retinoic acid binding protein-II (CRABP-II)

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(Directed by Prof. Hong-Hee Kim, Ph.D)

Cellular retinoic acid binding protein (CRABP) is a family of intracellular lipid-binding proteins (iLBPs). CRABP-II is known to bind retinoic acid (RA) in the cytoplasm and transport RA and RAR complex into the nucleus for gene transcription. In bone metabolism, there are two main types of cells called osteoblasts and osteoclasts. Osteoblast is the bone forming cell while osteoclast is a bone resorbing cell. Vitamin A is known to negatively affect osteoblasts. One of the study reported that high dose of vitamin A increases bone fracture in both animal and human. However, according to another study, the active form of vitamin A forms a complex with RA receptor (RAR) and stimulates the differentiation of ostoblastogenesis from

mesenchymal cells. In addition, the level of CRABP-II was shown to increase as osteoblast differentiates in osteoblast gene array study. The purpose of this study was to verify the exact role of CRABP-II in osteoblastogenesis.

For the study, primary mouse calvarial osteoblast was mainly used along with the MC3T3-E1 cell. The level of CRABP- II was measured by RT-PCR and western blot. The effect of knockdown and overexpression of CRABP-II in osteoblasts was analyzed by ALP staining and Alizarin Red S staining. To see the proliferation of the overexpressed CRABP-II in osteoblasts, CCK and BrdU were used.

The mRNA level and the protein expression of CRABP-II was observed in both primary osteoblasts and MC3T3-E1 cells as the cells become differentiated. However, CRABP-II was not expressed in osteoclasts. Knockdown of CRABP-II in both osteoblasts and MC3TC-E1 cells resulted in an increase in the intensity of ALP staining. A significant increase of mineralization was detected in CRABP-II knockdown cells. To determine the effect of CRABP-II overexpression in primary osteoblasts, mouse calvarial osteoblasts were transduced with a retroviral system. CRABP-II overexpressing cells had larger mineralized area that was stained with Alizarin Red S. Both BrdU and CCK assays presented similar results of no difference between the control cells and cells stably overexpressing CRABP-II. This study suggests the

possible negative regulation by CRABP-II of osteoblastogenesis.

Keywords : Osteoblast Differentiation, Retinoic Acid, CRABP-II,

Mineralization

Student Number: 2014-21813

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I. Introduction

The bone tissue is constantly remodeled by bone-resorbing osteoclasts and bone-forming osteoblasts. Osteoblast is differentiated from mesenchymal stem cells (MSCs) and plays a crucial role in the regulation of mineral homeostasis and maintenance of bone mass. These bone forming cells produce proteins for the extracellular bone matrix and regulate mineralization under the control of several transcriptional factors and signaling cascades (Komori 2010). Expression of Runx2 (Runt-related transcription factor 2) is the master regulator of bone formation and it controls a complex gene-regulatory network during osteoblastogenesis (Vaes, Ducy et al. 2006, Hecht, Seitz et al. 2007, Long 2012). Ascorbic acid has been used in cell culture studies for osteoblasts. During osteoblast differentiation, osteoblast phenotype related genes, such as alkaline phosphatase, osteopontin and osteocalcin, are up-regulated (Beck, Zerler et al. 2001). When mammalian calvarial cells are exposed to ascorbic acid, the collagenous extracellular matrix is deposited (Beck, Zerler et al. 2001).

Retinoic acid is an active form of vitamin A and known to regulate the gene expression through its receptor. RARs and RXRs are the two main retinoic acid receptors existing in three different isotypes (α, β, and γ). All-trans retinoic

acid (ATRA) is the most abundant form of retinoic acid that binds RXR and forms RAR/RXR heterodimers to bind DNA and directly regulate transcription of the target genes (Bastien and Rochette-Egly 2004). ATRA also plays important roles in regulating cell proliferation and differentiation. It is described that long half-life of vitamin A might cause toxicity by staying long time in the body (Binkley and Krueger 2000). In laboratory animals, chronic administration of high-dose vitamin A induces skeletal fracture and osteopenia, resulting in bone loss and increased bone resoprtion (Moore and Wang 1945, Binkley and Krueger 2000). Also excessive vitamin A spontaneously induces the long bone fracture in the animals with bone thinning and reduced diaphyseal radial growth (Binkley and Krueger 2000, Conaway, Henning et al. 2013). However, human study shows conflicting results with hypervitaminosis A. One case reported that hypervitaminosis A caused radiographic osteopenia and severe bone pain with high-dosage of vitamin A consumption (Gerber, Raab et al. 1954). The other case reported that there was no correlation between vitamin A consumption and the outcome of osteoporosis (Margolis, Attie et al. 1996). Moreover, vitamin A intake results in anorexia and reduced weight gain (Lind, Sundqvist et al. 2013).

One recent study showed that vitamin A negatively regulates mineralization of osteoblasts (Lind, Sundqvist et al. 2013). This study used the

murine preostoblastic cell line (MC3T3-E1). The result showed that the active metabolite of vitamin A suppressed in vivo mineralization through the RA receptor by reducing the protein level of Runx2 (Lind, Sundqvist et al. 2013). While it is still unclear how RA alters level of Runx2, one study suggested a possible mechanism for RA-induced proteasomal degradation of Runx2 via phosphorylated Smad1 (Sheng, Xie et al. 2010).

CRABP-I and CRABP-II are family of intracellular lipid-binding proteins (iLBPs) that bind RA with a high affinity (Dong, Ruuska et al. 1999). Although CRABP-I and CRABP-II exhibit a high degree of homology, these two proteins are encoded by different genes (Astrom, Tavakkol et al. 1991). Also, the tissue distributions during embryonic development predicted different isoelectric points (Bailey and Siu 1988). It has been suggested that CRABP-I regulates the metabolic fate of its ligand by directly affecting the activities of RA-metabolizing enzymes and dampens the response of F9 teratocarcinoma cells to RA by enhancing the degradation of the hormone (Boylan and Gudas 1991, Boylan and Gudas 1992).

CRABP-II is induced by RA and transport RA from the cytosol to the nucleus associating with RAR-α or RXR-α in a ligand-dependent manner. This heterodimer complex acts as a transcriptional regulator in RA-mediated signaling (Noy 2000, Bastie, Despouy et al. 2001). CRABP-II expression has

been reported in a wide variety of cancers in human. Comparing to normal breast tissues, both primary breast tumors and breast cancer cell lines had higher expression of CRABP-II (Bertucci, Houlgatte et al. 2000). In addition, overexpression of CRABP-II showed poor clinical outcome in Wilms tumors. Furthermore, overexpression of CRABP-II has been reported in other cancers, such as uterine leiomyoma (Tsibris, Segars et al. 2002), promyleocytic leukemia (Tsibris, Segars et al. 2002), ovarian cancer (Hibbs, Skubitz et al. 2004), gastric cancer (Nishigaki, Aoyagi et al. 2005), and head and neck squamous cell carcinoma (Vo and Crowe 1998). Taken together, these studies are indicating that CRABP-II may play a significant role in cancer development, yet the complex and detailed role of CRABP-II remains unclear.

Runx2 is a well known master regulator of osteoblasts for bone formation and controls gene-regulatory network during osteoblastogenesis (Hecht, Seitz et al. 2007, Komori 2011). Therefore, osteoblast differentiation is dependent on the appropriate expression level of Runx2 (Lian, Stein et al. 2006). Runx2 controls not only osteoblast lineage-specific genes such as Osx (osterix), Ocn (osteocalcin), and Bsp (bone sialoprotein), but also regulates non-osteoblast lineage-specific genes, including MyoD (myogenic differenctiation) and PPAR-γ (peroxisome proliferator-activated receptor gamma) that are related to myogenesis and adipogenesis, respectively (Jeon, Kim et al. 2003,

Gersbach, Byers et al. 2004, Zhang, Li et al. 2012). It is also reported that CRABP-II is upregulated during osteoblastogenesis (Beck, Zerler et al. 2001). One of recent studies showed constitutive occupancy of Runx2 in CRABP-II locus at the first intron (Wu, Whitfield et al. 2014). This binding increases in upstream and downstream of CRABP-II gene body correlated with enhanced differentiation (Wu, Whitfield et al. 2014). This data is supported by the observation of upregulation of CRABP-II during MC3T3-E1 cell differentiation and decrease of CRABP-II in Runx2 knockdown cells (Wu, Whitfield et al. 2014).

In several previous studies, it was suggested that Runx2 binds to the non-promoter region of CRABP-II in MC3T3-E1 preosteoblasts (Wu, Whitfield et al. 2014). Also CRABP-II expression parallels an increased expression of TGF-β in MC3T3-E1 (Beck, Zerler et al. 2001). In this study, expression of CRABP-II was only detected in osteoblasts out of several bone regulating cells. Therefore, I hypothesized that osteoblast is directly regulated by CRABP-II.

II. Materials and Method

1. Reagents

Recombinant human soluble RANKL and M-CSF were purchased from PeproTech (Rocky Hill, NJ, USA). Antibody against CRABP-I and CRABP-II and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Cell counting kit (CCK) was obtained from Dojindo (Kumamoto, Japan).

2. Cell culture and preparation of primary osteoblasts

To obtain primary osteoblast cells, calvaria from newborn mice were dissected free of surrounding muscles and soft tissues and washed in PBS containing penicillin and streptomycin. Isolated calvaria were sequentially digested in α-MEM (WelGENE Inc., Daegu, Korea). Cells were collected by centrifugation and resuspended in α-MEM supplemented with 10% FCS. To study the differentiation of osteoblasts, cells were cultured for up to 12 days in growth media containing ascorbic acid (AA; 100 μg/ml) and β-glycerophosphate (10 mM) and medium was changed every 3 days. PlatE cells were cultured in DMEM supplemented with 10% v/v heat-inactivated FBS, 100

units/ml of penicillin, and 100 μg/ml of streptomycin, and incubated at 37°C in 5% CO_2. Primary osteoblast cells were plated in 48-well culture plates at 1 x 10^4 cells per well and PlatE cells were plated in 60 mm culture dishes at $4x10^5$ cells per dish. Bone marrow-derived macrophages (BMMs) were cultured in α-MEM supplemented with 10% v/v heat-inactivated FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin, and incubated at 37°C in 5% CO_2. BMMs were plated in 6 mm culture dishes at $5x10^5$ cells per dish.

3. Retroviral gene transduction

In order to reproduce retroviral particles, Plat-E cells were transfected with pMX-PURO or pMX-flag-CRABPII plasmid using Ployfect transfection reagent (QIAGEN, Hilden, Germany). Viral supernatant was harvested after 48 hours and passed through 0.45 μm syringe filter (Sartorious, Goettingen, Germany). Primary osteoblast cells were infected with retroviral supernatant mixed with β-glycerophosphate (10 mM) and 8 μg/ml of hexadimethrine bromide (polybrene, Sigma Aldrich) for 12 hours. After overnight incubation, infected primary osteoblasts were cultured in ascorbic acid (AA; 100 mg/ml) and β-glycerophosphate (10 mM) for 12 days. The ectopic expression of transduced constructs and the formation of osteoblasts were determined by alkaline phosphase staining and Alizarine Red staining.

4. Reverse transcription-polymerase chain reaction (RT-PCR)

Primary osteoblast cells were incubated with 10 mM β-glycerophosphate and 100 μg/ml ascorbic acid for 0, 7 and 15 days. Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' specification. After isolation of total RNA, 3 μg of RNA was used for reverse transcription using Superscript II reverse transcriptase (Invitrogen) to synthesize cDNA. PCR reaction was performed with collected RNA samples. Initial step was denaturation at 95°C for 5 minutes and following reaction cycles consisted of denaturation at 95°C for 30 seconds. After denaturation, annealing step at 56°C for 30 seconds and extension step at 72°C for 30 seconds followed. Primers that were used for PCR reactions: CRABP I, CRABP II, Osteocalcin, Collagen Type I, m18s, ALP, TRAP. Final PCR products were analyzed by 1.5% agarose gel electrophoresis containing ethidium bromide and visualized under an UV illuminator.

BMMs cells were incubated with M-CSF (30 ng/ml) plus RANKL (100 ng/ml) for 0, 2, and 4 days. After isolation of total RNA using TRIzol, 3μg of RNA was used for the reverse transcription using Superscript II reverse transcriptase. Rest of the PCR steps are same as the primary osteoblast cell analysis.

5. siRNA transfection

In order to verify the role of CRABP-II in osteoblastogenesis, primary osteoblast cells were trasfected with 40 nM CRABP-I and CRABP-II specific siRNA. The double strand RNA oligomer targeting CRABP-I, CRABP-II and negative universal control siRNA oligonucleotides were purchased from Santa Cruz. The sequence of CRABP-I siRNA oligomer and CRABP-II siRNA oligomer and HiPerFect Transfection Reagent (QIAGEN, Hilden, Germany) were mixed with serum and antibiotics-free α-MEM. The mixtures were incubated for 20 minutes at room temperature before transfecting into the cells. Transfection complexes were dropped evenly over the cells and incubated for 24 hours. After the set time, cell culture medium was changed to serum-containing α-MEM.

6. Cell staining (ALP staining and Alizarin Red staining)

To verify osteoblast differentiation, cells were stained with Alkaline Phosphatase Kit (Sigma Aldrich, Cat. No. 86R-1KT) as per the manufacturer's instruction. In order to identify the presence of calcium after mineralization, alizarin red S staining was used. 100 mL of distilled water was missed with 2 g of alizarin red S solution. The mixture of the solution was adjusted to the pH of

4.1 to 4.3 with 10% ammonium hydroxide. Fixed cells were incubated with 2% alizarin red S solution for 3-5 minutes and washed three times with distilled water. Cells were washed with PBS to remove nonspecific staining. For quantitative evaluation, the densities of scanned images of stained plates were measured using Image J software.

7. Immunoblotting

Primary osteoblasts were cultured with 10 mM β-glycerophosphate and 100 μg/ml ascorbic acid for 0, 2, 5, 7, 9 and 12 days. Cells were lysed with a lysis buffer containing 120 nM Tris-HCl (pH 7.5), 2.5 mM sodium pyrophosphate, 1nM Na_2EDTA, 0.5% NP40, 1nM β-glycerophosphate, 150nM NaCl, 1nM Na_3VO_4, 1 mM NaF, 1nM EGTA, and protease inhibitors (Roche, Mannheim, Germany). Harvested proteins were evaluated by Dc protein assay kit (Bio-Rad, Hercules, California, USA) and the equal amount of protein was loaded onto 12 % SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Whatman GmbH, Dassell, Germany). The membrane was blocked with 5% nonfat skim milk (Sigma Aldrich) and primary antibodies were added for overnight incubation at 4°C. The immunoreactivity was detected with ECL reagents (Pierce, Rockford, IL, USA) after incubation with HRP-conjugated secondary antibodies for 2 hours in 1% skim milk. Comparable loading was

confirmed by probing the same membranes with anti-β-actin antibody.

8. Cell viability and proliferation assays

The proliferation of primary osteoblast stably expressing a control (pMX-PURO) or pMX-flag-CRABP-II construct was assassed using a colorimetric BrdU cell proliferation assay kit (Calbiochem, La Jolla, CA) according to the manufacturer's protocol. To examine cell viability, control and CRABP-II overexpressing primary osteoblast cells were treated with 10 mM β-glycerophosphate and 100 μg/ml ascorbic acid for up to 3 days. Cells were incubated with 10% CKK solution in cell culture medium for 30 min at 37°C. After incubation, optical density was measured with an ELISA reader at 450 nm.

III. Results

1. Upregulation of CRABP-I and CRABP-II during osteoblastogenesis

To investigate whether CRABP-I and CRABP-II were expressed during osteoblastogenesis, mouse calvarial osteoblasts were isolated and cultured with ascorbic acid and β-glycerophosphate for maturation. Cells were incubated with ascorbic acid and β-glycerophosphate for 7 and 15 days. As previously reported, stimulation increased the mRNA expression of osteocalcin, collagen type I and ALP. Similarly, CRABP-I and CRABP-II mRNA expression level was increased along with the osteoblast maturation (Fig. 1A). In order to investigate whether CRABP-I and CRABP-II are regulated during osteoclastogenesis, BMM were isolated and cultured with M-CSF and RANKL for 4 days. As reported earlier, RANKL treatment increased the mRNA expression of TRAP, the transcription factor associated with osteoclast differentiation. However, mRNA level of CRABP I and CRABP-II were not detected in BMM (Fig. 1B). CRABP-I and CRABP-II protein level was significantly increased from day 5 to day 9, when the calvarial osteoblast started to mineralize (Fig. 1C). MC3T3-E1 cells were also cultured in the presence of 10 mM β-glycerophosphate and

100 μg/ml ascorbic acid for 12 days. Significantly increased CRABP-I and CRABP-II protein levels were detected from day 2 to day 9 (Fig. 1D). These results suggest that ascorbic acid and β-glycerophosphate upregulates CRABP-I and CRABP-II mRNA expression level and protein level during osteoblastogenesis from primary osteoblasts and MC3T3-E1 cells.

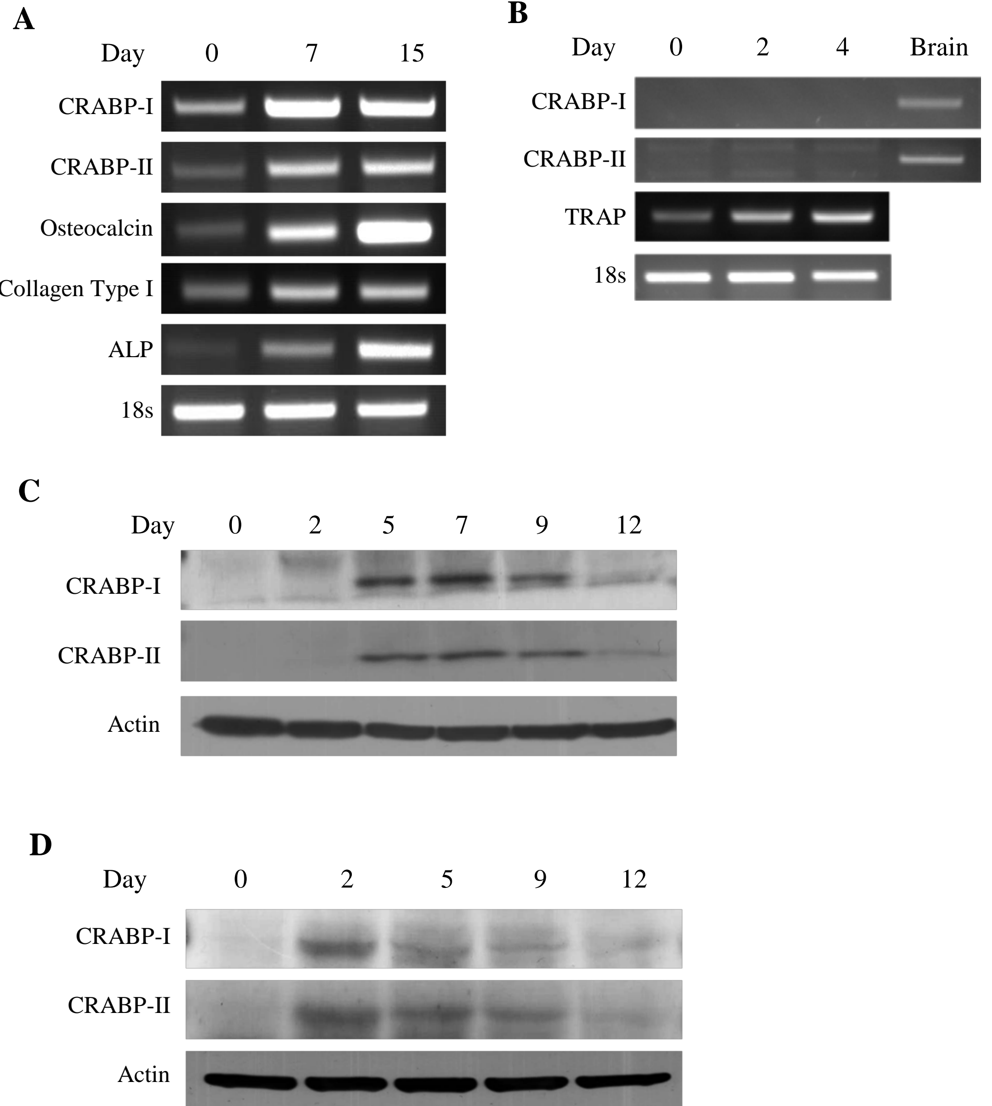

Figure 1. Expression of CRABP-II in calvarial osteoblast and MC3T3-E1 cells.

(A) Increased expression of CRABP-I and CRABP-II in murine calvarial osteoblasts. Cells were cultured for up to 15 days in growth media containing ascorbic acid (100 μg/ml) and β-glycerophosphate (10 mM). mRNA levels of

CRABP-I, CRABP-II , osteocalcin, collagen type I and ALP were analyzed by RT-PCR. m18s was used as a loading control. (B) CRABP-II is not expressed during osteoclastogenesis. BMMs were treated with M-CSF and RANKL for the indicated times. Cells were harvested at days 0, 2, and 4. mRNA expression for CRABP-I, CRABP-II and TRAP was examined by RT-PCR. m18s was used as loading controls. (C) Protein expression level of CRABP-I and CRABP-II in primary osteoblasts. Primary osteoblasts were treated with ascorbic acid and β-glycerophosphate for the indicated times. Cells were harvested at day 0, 2, 5, 7, 9 and 12. Protein expression of CRABP-I and CRABP-II was detected by Western blotting. Actin was used as loading controls. (D) The protein expression level of CRABP-I and CRABP-II in MC3T3-E1 cell. MC3T3-E1 cells were cultured in the presence of 10 mM β-glycerophosphate and 100 μg/ml ascorbic acid for 12 days. Cells were collected at indicated day and cell lysates were subjected to Western blotting

2. Negative regulation of osteoblast differentiation and mineralization by CRABP-II

Next, I investigated whether CRABP-II has a role in osteoblastogenesis. Primary calvarial osteoblast cells were transfected with CRABP-II siRNA oligonucleotides. Down-regulation of CRABP-II mRNA was confirmed by RT-PCR analysis (Fig. 2A). When osteoblastic differentiation was assessed by ALP staining, cells transfected with CRABP-II siRNA clearly showed enhanced staining intensity (Fig. 2B). To determine the effect of the mineralization by osteoblasts, CRABP-II knockdown primary osteoblast cells were cultured in osteogenic medium for 8 days and stained with alizarin red S. A significant increase of mineralization was detected in CRABP-II knockdown cell cultures (Fig. 2C). Hence, presence of CRABP-II negatively regulates osteoblast maturation and matrix mineralization by osteoblasts.

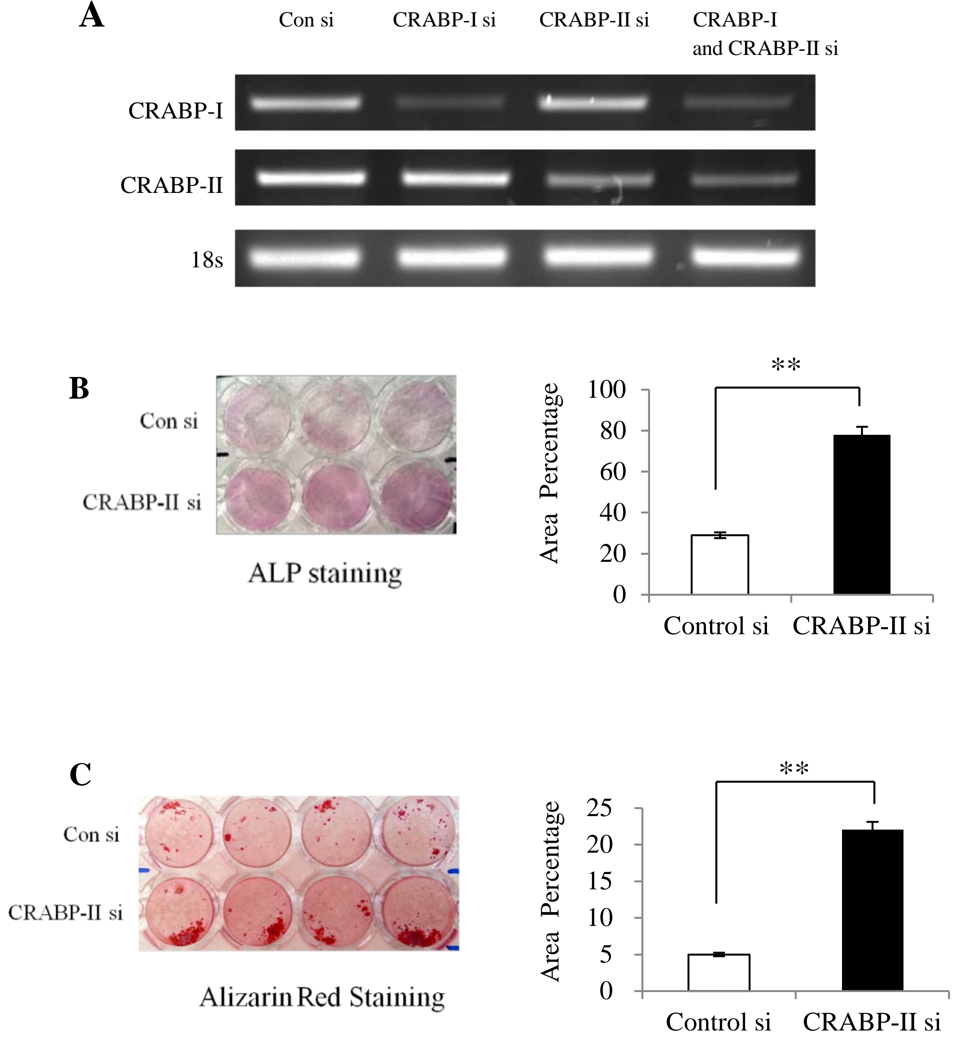

Figure 2. Down-regulation of CRABP-II promotes osteoblastogenesis.

(A) Murine primary osteoblast cells were transfected 40 nM CRABP-I and CRABP-II siRNA for 2 days. Cells were harvested at day 8. mRNA expression

for CRABP-I and CRABP-II was examined by RT-PCR. m18s was used as loading controls. (B) Murine primary osteoblast cells were transfected with CRABP-II siRNA and further cultured in the presence of 10 mM β-glycerophosphate and 100 μg/ml ascorbic acid for 3 days. After staining with ALP, the intensity of staining was assessed using the Image J program. (C) Murine primary osteoblast cells were transfected with CRABP-II siRNA and further cultured in the presence of 10 mM β-glycerophosphate and 100 μg/ml ascorbic acid 8 days. Each well was stained with alizarin red S. The intensity of staining was assessed using the Image J program.

3. Overexpression of CRABP-II in calvarial osteoblasts

To determine the effect of overexpression of CRABP-II, mouse calvarial primary osteoblasts was transduced with a retrovirus system. Overexpresssion of CRABP-II was clearly achieved as shown by Western blotting. The level of the CRABP-II protein was determined by both a CRABP-II antibody and a flag antibody (Fig. 3A). To obtain further evidence for the negative function of CRABP-II in osteoblast mineralization, primary osteoblast was cultured in osteogenic medium for up to 10 days. At days 6, 8 and 10, the cells were stained with alizarin red S to visualize the mineralization (Fig. 3B). As seen in Fig. 3C, CRABP-II-overexpressing cells had larger mineralized area that was stained with alizarin red S.

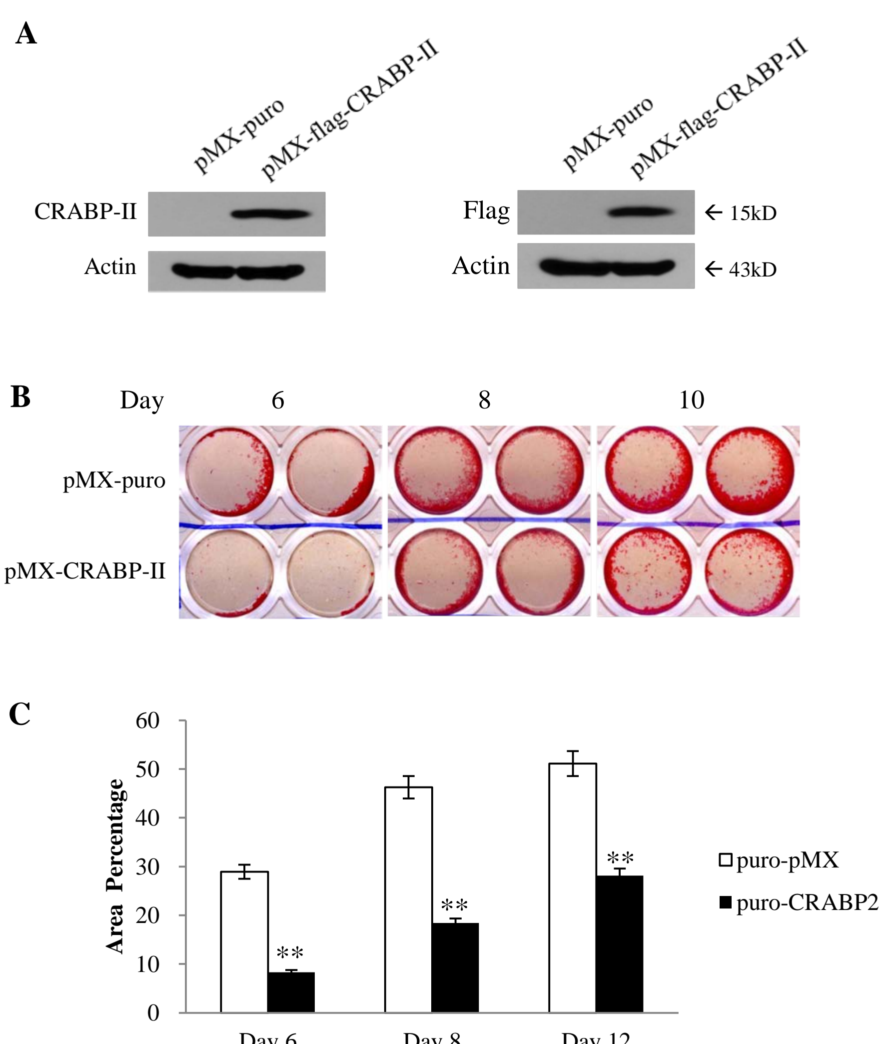

Figure 3. Overexpression of CRABP-II inhibits osteoblastogenesis.

(A) Primary mouse calvarial osteoblasts were infected with CRABP-II retrovirus and cultured with the osteogenic medium. Figure 3 A shows clearly expressed protein level of CRABP-II. pMX-puro was used as control. (B) Overexpression of CRABP-II inhibits osteoblastogenesis with stimulation.

Primary mouse calvarial osteoblasts were cultured for 6, 8 and 10 days with 100 μg/ml ascorbic acid and 10 mM β-glycerophosphate. Collected cells were stained with alizarin red S to detect mineralization by osteoblasts. (C) The intensity of alilzarin red S staining was assessed using the Image J program.

4. No effect of CRABP-II overexpression on cell proliferation

To investigate the effect on cell proliferation in the early stage of osteoblast maturation, primary murine calvaria osteoblasts were manipulated to overepxress CRABP-II. Cell proliferation was assessed by performing CCK assays at days 0, 1, 2 and 3. CRABP-II overexpressing primary osteoblasts and control cells showed no difference in CCK assays (Fig. 4A). Cell proliferation was also analyzed by BrdU assays. Similar to the result of CCK assays, BrdU assays showed no difference between control cell and CRABP-II overexpressing osteoblast cells (Fig. 4B). Taken together, these results suggest that CRABP-II negatively regulates osteoblastogenesis, without affecting proliferation rate of cells.

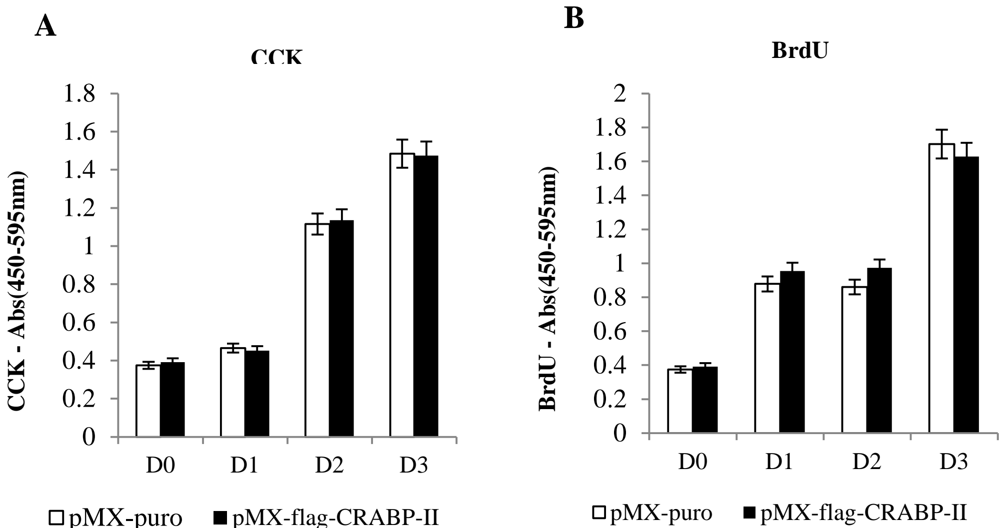

Figure 4. No difference in cell proliferation of calvarial osteoblasts by CRABP-II overexpression.

(A) Primary osteoblast cells infected with CRABP-II retrovirus were cultured with ascorbic acid and β-glycerophosphate for the indicated times. Cell proliferation was measured at days 0, 1, 2, and 3 with CCK reagents. (B) Primary osteoblast cells infected with CRABP-II retrovirus were cultured with ascorbic acid and β-glycerophosphate for the indicated times. BrdU assay was used to measure the cell proliferation as described in the Material and Method.

IV. Discussion

The role of CRABP-II has been studied in several cell types. However, its role in bone metabolism has been poorly studied. Recently, Wu et al. reported the increasing expression of CRABP-II during osteoblastogenesis and the binding of Runx2 on the CRABP-II non-promoter region in MC3T3-E1 cells (Wu, Whitfield et al. 2014). The study showed a relationship between CRABP-II and osteoblast. In the present study, I detected CRABP-II expression in primary mouse calvarial osteoblasts. However, CRABP-II was not detected in osteoclast, which is another major bone regulating cell.

In this study, I discovered a novel role of CRABP-II in the mouse calvarial osteoblast and an osteoblast precursor cell line MC3T3-E1. CRABP-II expression was upregulated during osteoblastic differentiation of mouse calvarial osteoblast and MC3T3-E1 (Fig. 1A, C, D). Also several gene array data were reported the increased level of CRABP-II during MC3T3-E1 cell differentiation (Beck, Zerler et al. 2001, Wu, Whitfield et al. 2014). Therefore, my results are consistent with the published data. CRABP-II was not expressed in osteoclasts derived from hematopoietic stem cell (Fig. 1B). However, it has been only reported that RXR-α and RAR-α were expressed in human osteoblast (Kindmark, Torma et al. 1993).

In a the recent report, Runx2, the osteoblast regulator, was suggested to

constitutively occupy the CRABP-II locus in the first intron (Wu, Whitfield et al. 2014). Down regulation of CRABP-II induced mineralization of mouse calvarial osteoblast (Fig. 2). The ALP staining and alizarin red staining showed more intense staining in CRABP-II down-regulated cells compared to the control cells (Fig. 2B and Fig. 2C). Wu et al. recently reported that knockdown of Runx2 in MC3T3-E1 decreased mRNA level of CRABP-II. In contrast, there was no difference in mineralization after CRABP-I knockdown (data not shown). This result indicates that CRABP-II specifically plays a negative role in osteoblast differentiation perhaps at the downstream of Runx2.

In *in vitro* conditions, overexpression of CRABP-II has only been studied in lesional and nonlesional psoriatic skins (Siegenthaler, Tomatis et al. 1992). In this study, overexpression of CRABP-II was achieved for the first time in mouse primary osteoblast cells. Overexpressed protein level of CRABP-II was confirmed with both a CRABP-II antibody and a flag tag antibody by Western blotting (Fig. 3A). Up-regulated CRABP-II significantly down-regulated matrix mineralization in osteoblast (Fig. 3B). This result correlated with the result from down-regulation of CRABP-II (Fig. 2B and 2C). These data indicated that osteoblastogenesis is negatively regulated by CRABP-II.

CRABP-II overexpression in calvarial osteoblasts did not cause any difference in the proliferation rate as shown by both CCK and BrdU assays (Fig.

4). It can be concluded that CRABP-II does not give any effect on cell proliferation during the period of osteoblastogenesis.

In conclusion, I revealed that a novel function of CRABP-II as an important regulator of bone metabolism. Further studies are required to understand molecular mechanism for the negative regulation of osteoblast differentiation by CRABP-II.

V. References

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국문 초록

Cellular retinoic acid binding protein-II 의 조골세포 분화 저해 기작 규명

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이 재 영

CRABP(cellular retinoic acid binding protein)는 CRABP-I과 II로 두 종류가 존재하는데 그 중 CRABP-II는 레티노인산 (retinoic acid)과 결합하여 레티노인산의 세포핵 내로의 수송을 조절하는 역할을 한다고 알려져 있다. 뼈를 구성하는 세포에는 조골세포와 파골세포가 있는데 뼈를 생성하는 조골세포는 골기질을 합성하고 분비하며 기질에 무기염을 침착시킴으로써 골조직을 석회화 시키는 능력을 가지고 있다. 활발한 골 형성이 진행된 후에는 골 조직에 묻혀 골세포가 된다. 조골세포가 분화하는 과정 중에 비타민 A의 활성체인 레티노인산도 영향을 주는데, 이는

조골세포의 분화를 억제 한다고 밝혀졌으며 동물에게 비타민 A를 다량 투어 하였을 경우 뼈의 밀도가 낮아 졌다는 연구 결과도 있다. 이 논문에 의하면 레티노인산이 이의 수용체와 결합하면서 조골세포의 형성을 억제할 때 조골세포의 분화 표시자인 Runx2도 함께 줄어든다고 한다. 최근 논문에 의하면 Runx2가 CRABP-II의 첫 번째 인트론에 결합하여 CRABP-II의 형성을 조절 한다고 알려졌으나 정확하게 어떠한 기전으로 조절을 하는지는 밝혀지지 않았다.

마우스 유래 조골세포 전구세포를 가지고 CRABP-II의 작용기전을 알아보려고 하였다. 조골세포의 분화 정도에 따른 CRABP-II의 mRNA와 단백질의 발현 정도를 측정하였으며, 아울러 sh-RNA 기법을 이용한 CRABP-II 유전자의 knockdown 방법과 과발현을 시킨 후 조골세포의 분화정도와 무기질화 작용을 alkaline phosphatase 염색 방법과 alizarin red 염색 방법을 통하여 확인하였다.

CRABP-II는 파골세포에는 발현이 되지 않았으며 murine preostoblast와 마우스 유래 조골세포주(MC3T3-E1)에서 세포가 분화 될수록 CRABP-II의 mRNA와 단백질의 발현이 높아지는 것을 볼 수 있었다. CRABP-II를 선택적으로 저해하였을 때 alkaline phosphatase가 증가 하였고, 무기질화

작용이 더 많이 일어난 것을 볼 수 있었다. 반면에, CRABP-II를 과발현 (overexpression) 하였을 경우 무기질화 작용이 줄어든 것을 확인 할 수 있었다. 또한, CRABP-II를 과발현해서 세포증식을 확인 하였을 경우 CKK와 BrdU 분석법 모두에서 차이가 없는 것으로 보아 CRABP-II는 세포증식에 관여 하지 않음을 추측 할 수 있다. CRABP-II와 Runx2의 관계를 실험적으로 직접 알 수는 없었지만 그동안 발표된 여러 보고와 본 실험결과를 비교하여 추측 하였을 경우 CRABP-II는 Runx2와 밀접한 상호작용을 통해 조골세포의 분화에 영향을 줄 수 있다고 할 수 있다.

주요어 : CRABP-II, 레티노인산, 조골세포, 무기질화 작용

학 번 : 2013-21813