Int J Clin Exp Pathol 2017;10(1):298-305
www.ijcep.com /ISSN:1936-2625/IJCEP0038284

Original Article

Overexpression of RBP4 promotes proliferation, differentiation and mineralization of MC3T3-E1

Abdulsamad Ghanem, Yunhe Lu, Tianyi Cai, Xiongzheng Mu

Department of Plastic Surgery, Huashan Hospital, Fudan University, Shanghai, China

Received August 20, 2016; Accepted August 28, 2016; Epub January 1, 2017; Published January 15, 2017

Abstract: Craniosynostosis is one of the most common congenital craniofacial deformities and could cause a series of diseases or defects. But the molecular mechanism underlying craniosynostosis is rarely reported. Retinal-binding protein 4 (RBP4) was shown to be involved in osteogenesis and decreased dramatically in prematurely fused suture. To investigate the role of RBP4 in osteogenesis, a cell line stably expressing RBP4 was constructed based on MC3T3-E1 cells and RBP4 overexpression was confirmed at both mRNA level and protein level. Then we found that RBP4 upregulation promoted proliferation of MC3T3-E1 cell and significantly increased alkaline phosphatase activity from day 7 to day 14 in MC3T3-E1 cells. Further, in alizarin red staining assay, RBP4 was shown to increase the deposit of calcium phosphate remarkably from day 14 to day 21 in MC3T3-E1 cells cultured in differentiated medium. This indicated that RBP4 prompted the mineralization of MC3T3-E1 cells. In accordance with this phenotype, we detected the typical osteo-differentiation markers by RT-qPCR and demonstrated that RUNX2, OC, OPN and COLL1 were greatly increased in RBP4-overexpressed MC3T3-E1 cells compared to the control. Therefore, our data suggest that RBP4 plays a positive role during osteogenesis and may be favorable for bone formation. This study provides us a new opinion about the role of RBP4 in craniosynostosis and RBP4 is worth of further study about its application on bone regeneration or bone development.

Keywords: Craniosynostosis, retinal-binding protein 4, osteogenesis, MC3T3-E1

Introduction

Human brain with intricate neural networks is known as the most complicated and sophisticated organ. Any disturbance during embryogenesis would cause malformation, severe disease, even death. Craniosynostosis, a birth defect that causes one or more sutures on an infant's skull to close earlier than usual, is one of the most common congenital craniofacial deformities [1]. It was reported to affect 1 in 2,500 live births and lead to abnormalities in skull shape and a series of neurological problems such as cognitive impairment, ophthalmic anomaly and mental impairment combined with a significant reduction in IQ [2, 3].

Retinoid signaling is vital to skeletal development and excessive exposure of non-human primates to retinoic acid could cause craniosynostosis [4]. Retinoic acid was the metabolites of retinoid. Retinal-binding protein 4 (RBP4) was reported to be a molecular chaperone of retinol and carried retinol from liver stores to target tissues [5]. It was a 21 kDa protein mainly synthesized in the liver and secreted into the bloodstream [6]. The expression pattern of RBP4 in chondrocytes of developing mouse long bones indicated its important role in formation of secondary ossification center [6]. Coussens et al found that RBP4 was localized to mesenchymal cells in human suture at the osteogenic front and to cells at the bone surface, which suggest novel roles of RBP4 in the maintenance of suture patency or in control of early osteoblast differentiation [7]. But unexpectedly, RBP4 was shown to be downregulated about 37 folds in pathological fused suture compared to normal unfused suture [7]. Further, RBP4 was demonstrated to be decreased during in vitro mineralization of human suture mesenchymal cells, which is critical for osteogenesis [8]. In all, RBP4 played important role in craniosynostosis and showed decreased expression in prematurely fused suture. But the

Table 1. Gene-specific primers used in PCR amplification

Runx2	Forward 5'-GCCGGGAATGATGAGAACTA-3'
	Reverse 5'-GGACCGTCCACTGTCACTTT-3'
OC	Forward 5'-CTGGCCCTGGCTGCGCTCTGT-3'
	Reverse 5'-GGTCCTAAATAGTGATACCGTAGATGCG-3'
OPN	Forward 5'-CTGCTAGTACACAAGCAGACA-3'
	Reverse 5'-CATGAGAAATTCGGAATTTCAG-3'
COLL1	Forward 5'-GAGGCATAAAGGGTCATCGTGG-3'
	Reverse 5'-CATTAGGCGCAGGAAGGTCAGC-3'

molecular mechanism underlying craniosynostosis and how RBP4 affect osteogenesis is rarely reported.

To investigate the role of RBP4 in osteogenesis, we forced expression of RBP4 in MC3T3-E1 cell which was derived from calvaria bone of mice and extensively studied for bone development [9]. Then we explored the effects of RBP4 overexpression on MC3T3-E1 cell proliferation, alkaline phosphatase activity, in vitro mineralization of MC3T3-E1 cells and expression pattern of differentiation markers. Our study would enlarge our knowledge about the function of RBP4 in osteogenesis and provide valuable information for clinical therapy of bone-development malformations.

Materials and methods

Cell culture

New born mouse calvaria-derived MC3T3-E1 cells were obtained from Riken Cell Bank (Tokyo, Japan). The cells were maintained in a-minimum essential medium (HyClone, Beijing, China) supplemented with 10% fetal bovine serum (Gibco, Australia), 100 U/ml penicillin and 100 mg/ml streptomycin (HyClone) in a humidified 5% CO_2 balanced-air incubator at 37°C, the media being changed every 3 days.

To induce differentiation and mineral deposition of MC3T3-E1, differentiation medium was prepared by the addition of 50 mg/ml ascorbic acid, 10 mM β-glycerophosphate and 50 nM dexamethasone to the growth medium mentioned above.

Lentivirus infection and cells group

To produce lentivirus, 293T cells were co-transfected with pLenO-GTP-3XFLAG-RBP4 (Biolink, Shanghai, China), which were amplified in E. coli DH5a, purified using a Plasmid Maxi Kit (Qiagen, Valencia, CA), and transfected into 70% confluent 293T cells using lipofectamine 2000 (Invitrogen). Lentiviral particles were harvested from the supernatant 72 hours after transfection and purified by ultracentrifugation. These particles were hereafter referred to as pLenO-GTP-3XFLAG-RBP4, and pLenO-GTP. Forty-eight hours after infection, the number of GFP positive MC3T3-E1 cells was scored by fluorescence-activated cell sorting (FACS) (Olympus, Japan) analysis to determine the titer. The cell line was divided into the following three experimental groups: control group (non-infected cells), negative control group (pLenO-GTP-infected cells), and RBP4 group (LENTI-RBP4-infected cells). Real-time PCR and Western blot was used to confirm the efficiency of RBP4 expression in MC3T3-E1 cells.

CCK8 assay

MC3T3-E1 cells were cultured at 5×10^3 cells/well in a 96-well plate in triplicate. Subsequently, the cells were cultured for 1, 3, 5 and 7 days. WST-8 solution (Dojindo, Japan) was added (1 part to 10 parts medium) to each well and the plate was incubated at 37°C for 2 h. Then, the OD value at 450 nm wavelength was quantified using Thermo Scientific Multiskan GO Microplate Spectrophotometer (Thermo Fisher, America).

Alkaline phosphatase activity assay

The cells were plated at a density of 2.0×10^4 cells/well in 48-well tissue culture plates in the growth medium. At full confluence, the medium was changed to the differentiation medium (regarded as day 0) and the incubation was continued to 7, 14 and 21 days. Then the medium was removed and the tissue was washed twice with PBS. The cells were detached from the culture dish after the addition of PBS and Triton X. Alkaline phosphatase (ALP) activity was then assayed using a commercial kit (Alkaline Phosphate Assay Kit, Beyotime, Nanjing, China). Alp activity was determined according to the manufacturer's protocol. The optical density at 405 nm was measured and compared with the value of a series of p-nitrophenol standards.

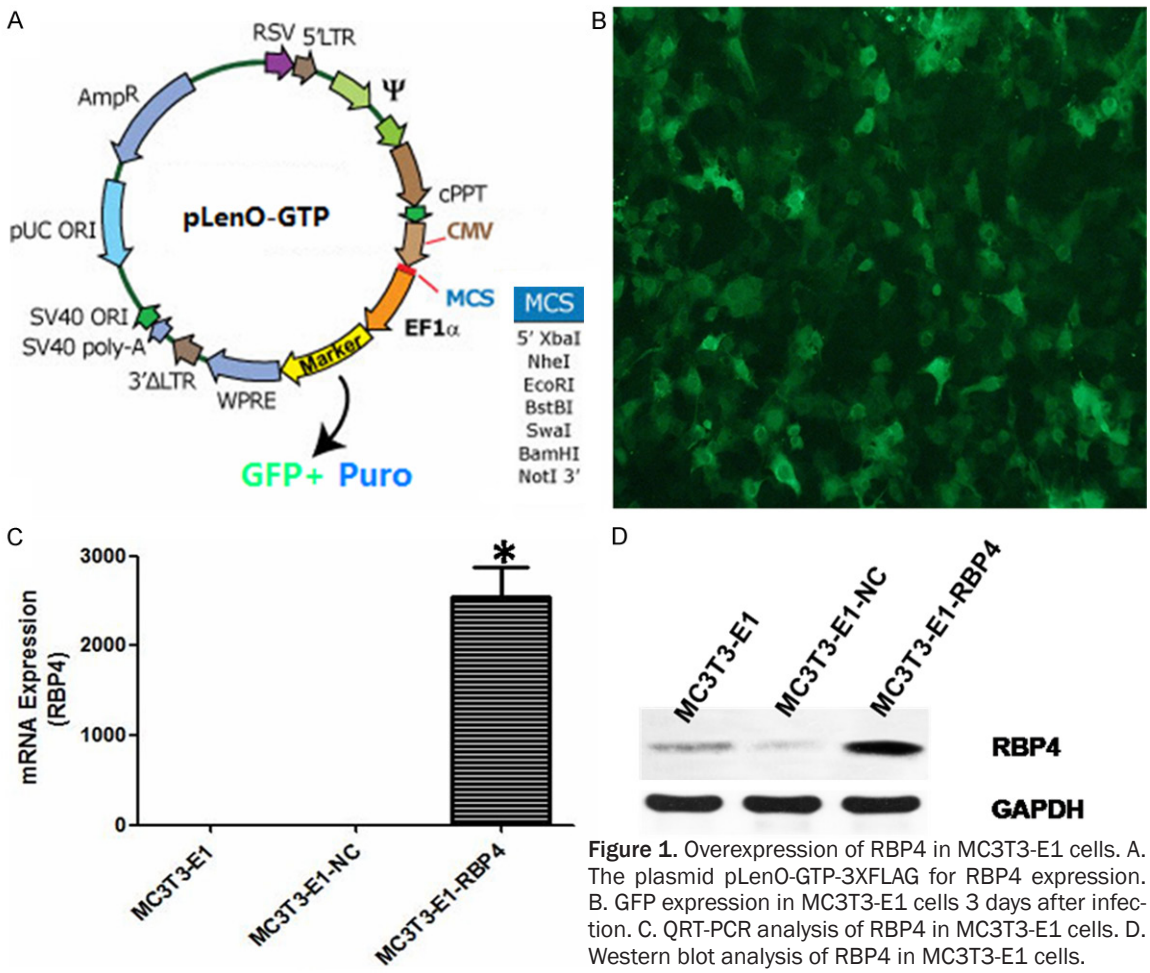

Figure 1. Overexpression of RBP4 in MC3T3-E1 cells. A. The plasmid pLenO-GTP-3XFLAG for RBP4 expression. B. GFP expression in MC3T3-E1 cells 3 days after infection. C. QRT-PCR analysis of RBP4 in MC3T3-E1 cells. D. Western blot analysis of RBP4 in MC3T3-E1 cells.

Mineralized bone-like tissue observation

After the cell culture (which was plated at 2.0×10^5 cells/well in the growth medium in 6-well tissue culture plates) reached confluence, incubation was continued in the differentiation medium (regarded as day 0). On day 7, 14 and 21, cells were fixed with 70% ethanol, and rinsed three times with ddH_2O to remove ethanol completely. The cells were then stained with 40 mM alizarin red stain (AR-S) solution (Sigma-Aldrich, China) for 15 minutes to label the calcium deposits. After that, cells were rinsed with ddH_2O five times to remove unbound AR-S. The stained cultures were observed under a dissecting microscope and the representative pictures were photographed. It was preliminarily confirmed that the well incubated without cells and subjected to a similar procedure of media changes did not show positive staining for calcium by Alizarin red S.

Quantitative real-time RT-PCR analysis

Expression of osteoblastic markers, Runt-related transcription factor2 (Runx2), osteocalcin (OC), osteopontin (OPN) and type I collagen (COLL1), were detected using RT-PCR analysis. The cells plated at 1.0×10^6 cells/dish in the growth medium in 100 mm cell culture dishes were cultured to confluence and was changed to the differentiation medium. On day 14, Total RNA was extracted from cells using TRIZOL reagent (Ambion; Carlsbad, CA) according to the manufacturer's instructions. Total RNA was reversely transcribed into cDNA by using PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa). The cDNA samples were subjected to PCR analysis using SYBR® Premix Ex TaqTM II (TaKaRa). PCR amplification of Runx2, OC, OPN and COLL1 cDNA was conducted using specific primers (**Table 1**). Amplifications were performed on an ABI7500 Real-time PCR system (USA). The optimal conditions were defined as

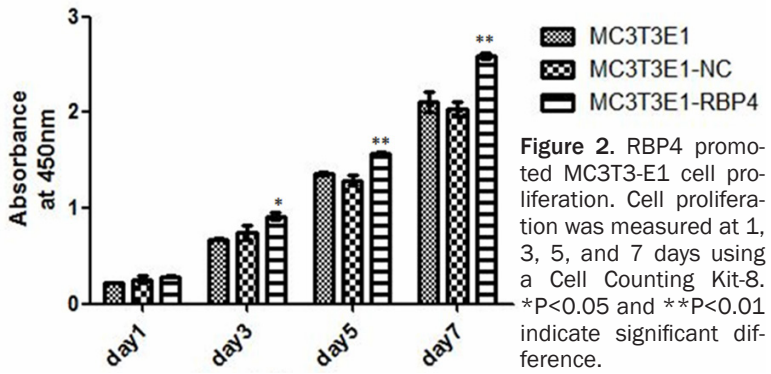

Figure 2. RBP4 promoted MC3T3-E1 cell proliferation. Cell proliferation was measured at 1, 3, 5, and 7 days using a Cell Counting Kit-8. *P<0.05 and **P<0.01 indicate significant difference.

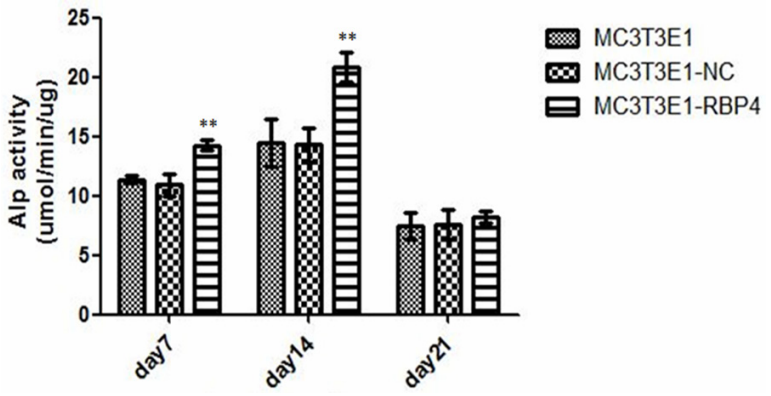

Figure 3. The effect of RBP4 on ALP activity in MC3T3-E1 cells. Cells were cultured under differentiated conditions up to 21 d. ALP activity was detected. Statistical significance was determined using t test. *P<0.05 and **P<0.01 indicate significant difference.

follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min, and melting curve analysis at 95°C for 15 s, at 60°C for 1 min, at 95°C for 15 s, and at 60°C for 15 s. The relative mRNA expression of the marker genes of osteoblastic differentiation was adjusted according to the expression of GAPDH.

Statistical analysis

Statistical analysis was performed using SPSS17.0 software (SPSS company, IN). Differences were evaluated using two-ways Anova/t-test. Data were presented as mean ± standard error and a minimum of three independent experiments were performed for each assay. *P<0.05 or **P<0.01 was considered as significant difference.

Results

RBP4 was successfully overexpressed in MC3T3-E1 cells

To investigate the function of RBP4 in MC3T3-E1 cells, RBP4 was overexpressed in MC3T3-E1 cells by lentivirus vector pLenO-GTP-3XFlag (**Figure 1A**). As shown in **Figure 1B**, at day 3 post-infection, high level of green fluorescence in a large number of cells was observed. And the mRNA level of RBP4 was greatly upregulated in RBP4-overexpressed MC3T3-E1 cells (MC3T3-E1-RBP4) compared to the group infected with empty lentivirus vector (MC3T3-E1-NC) or without any vector (MC3T3-E1) (**Figure 1C**). Further, by western blot analysis, RBP4 was shown to be significantly increased at protein level in MC3T3-E1-RBP4 cells (**Figure 1D**). Therefore, RBP4 was successfully introduced into MC3T3-E1 cells.

RBP4 promoted proliferation of MC3T3-E1 cells

Cell proliferation and differentiation are two different processes. Calcium phosphates such as α-TCP and TetCP were shown to have no effect on MC3T3-E1 proliferation but promoted cell differentiation [10]. Elevated retinoic acid inhibited calvarial osteoblast proliferation but improved differentiation [8]. We explored the effect of RBP4 on MC3T3-E1 cell proliferation from day 1 to day 7. As shown in **Figure 2**, MC3T3-E1 cells grew gradually from day 1 to day 7 in all three groups cultured in undifferentiated medium. There were no significant difference between control group and negative control group (P>0.05). On contrary, RBP4 overexpression significantly improved cell proliferation compared to other two groups (P<0.05). Conclusively, our data suggest that RBP4 did promote the growth of MC3T3-E1 cells.

RBP4 affected the ALP activity in MC3T3-E1 cells

ALP activation is one of markers in osteoblast phenotype and increased ALP activity often reflects a shift of bone cells to a more differentiated state [11]. TetCP was reported to induce MC3T3-E1 cell differentiation and increased

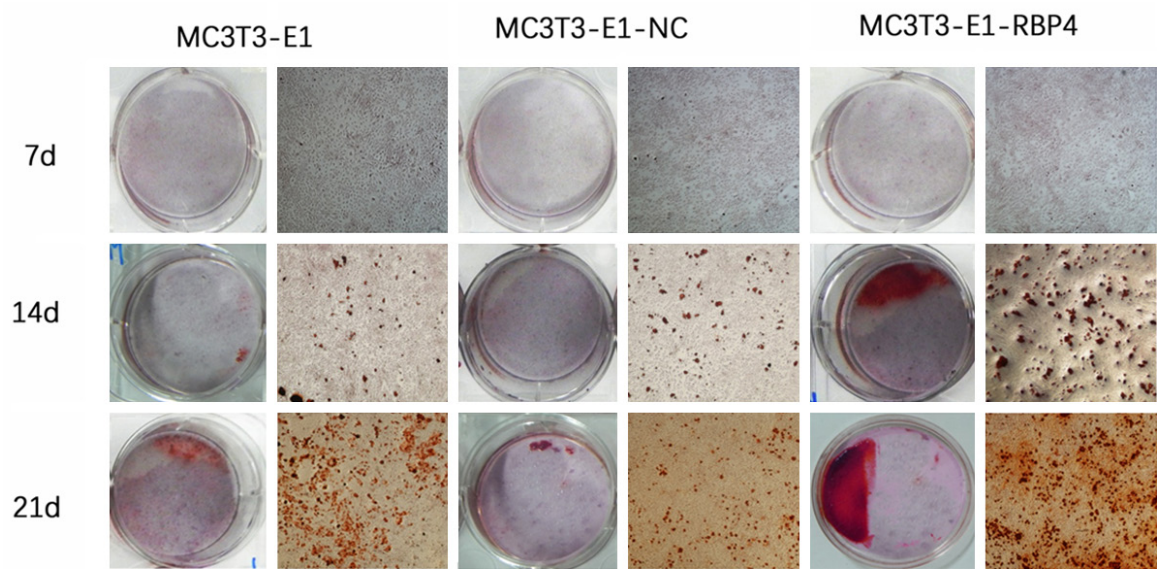

Figure 4. The effect of RBP4 on calcium phosphate deposit in MC3T3-E1 cells. Alizarin Red S staining was used to observe calcium phosphate deposit at 7, 14 and 21 days. The mineralized nodules in MC3T3-E1-RBP4 group were much more than that in the other two control groups.

ALP activity [10]. In this study, the effect of RBP4 on ALP activity in MC3T3-E1 was determined from day 7 to day 21 under differentiated condition. From the data in **Figure 3**, ALP activity increased during the first 14 days for all three groups, but decreased greatly at the 21st day. There was no difference in ALP activity between MC3T3-E1 and MC3T3-E1-NC group during the whole 21 days. But the ALP activity in MC3T3-E1-RBP4 group was much higher than that in the other two groups at day 7 and day 14 ($P<0.05$). Moreover, the ALP activity increased greatly from day 7 to day 14 in MC3T3-E1-RBP4 group. However, no significant difference was seen after 21 d of incubation between the three groups.

RBP4 promoted mineralization of MC3T3-E1 cells

To examine the effect of RBP4 on mineralization of MC3T3-E1 cells, Alizarin red staining assay was used to observe calcium phosphate deposits in day 7 to day 21 cultures. In **Figure 4**, we demonstrated that very little positive staining of mineralized tissue could be observed at day 7 and day 14 in MC3T3-E1 or MC3T3-E1-NC group. Even at day 21, very weak positive staining was observed. On the contrary, obvious mineralized tissue was formed at day 14 in MC3T3-E1-RBP4 group. But the amount of mineralization showed little further increase at day 21. The above data support the conclusion that RBP4 could promote the mineralization of MC3T3-E1 cells.

RBP4 enhanced expression of differentiation-related genes

There were three stages during osteoblast development including: cell proliferation, extracellular matrix production and mineralization [12]. Differentiation of osteo-progenitor cells relied on formation of extracellular matrix [13]. In one word, extracellular matrix formation was usually ahead of or accompanied by mineralization. In the study by Coussens, four genes including RUNX2, OC, OPN and COLL1 were selected as marker genes for osteoblastic differentiation [12]. In another study by Ehara A et al, COLL1 gene expression was markedly upregulated in mineralized MC3T3-E1 cells. Also, OC and OPN were increased at late stage of mineralization. Here as shown in **Figure 5**, these four genes were all dramatically upregulated when RBP4 was overexpressed in MC3T3-E1 cells at day 14 under differentiated culture condition. The mRNA level of RUNX2 and OPN was more than three folds inMC3T3-E1-RBP4 group compared to MC3T3-E1 group while the OC or COLL1 expression was upregulated about two folds in MC3T3-E1-RBP4 group, respectively.

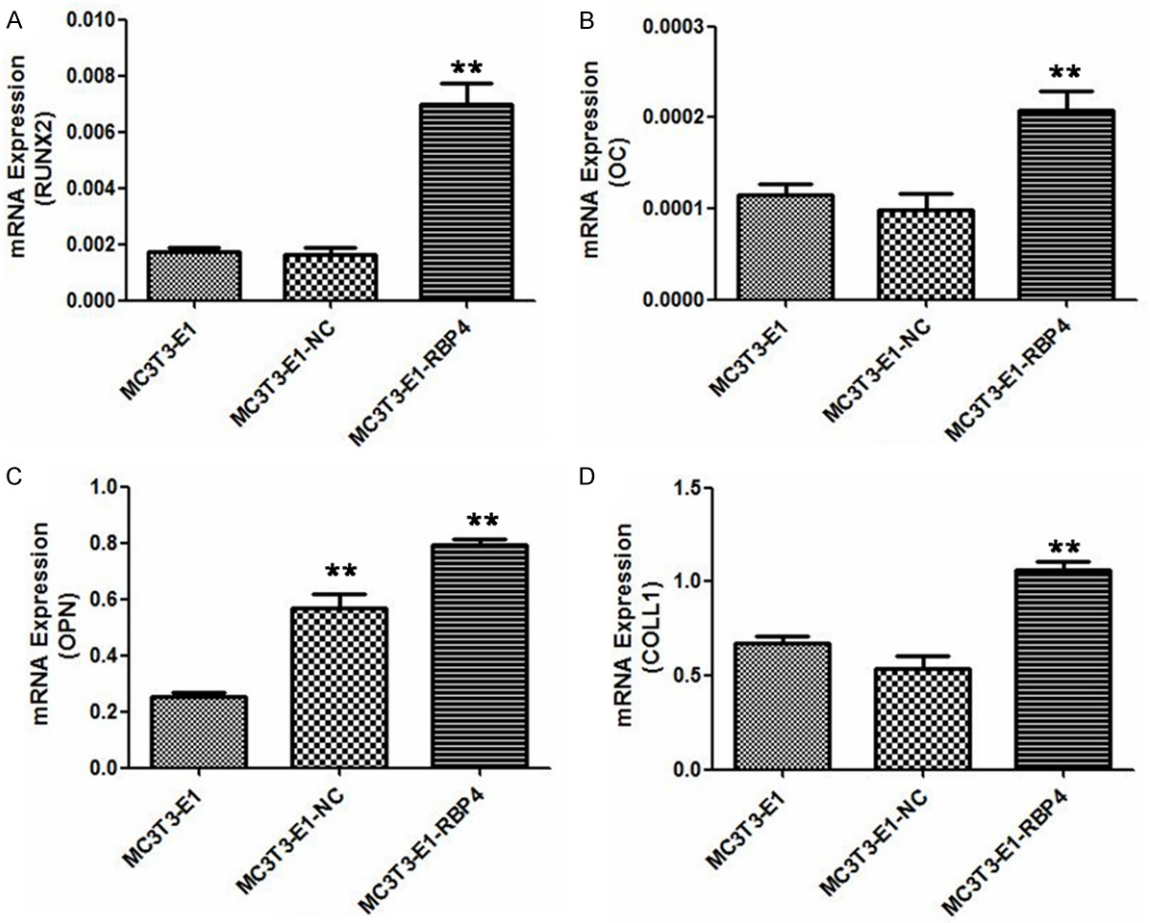

Figure 5. Effect of RBP4 on osteoblast-related gene markers in MC3T3-E1 cells after 2 weeks of mineralization culture. Runx2 (A), OC (B), OPN (C) and COLL1 (D) level were increased markedly in RBP4 overexpressed MC3T3-E1 cells. *P<0.05 and **P<0.01 indicate significant difference.

Discussion

Craniosynostosis was a development defect of skull bone and caused severe malformation [1]. But no confirmed molecular mechanism underpinning this process was reported to date. Here, we employed an in vitro cell culture system based on MC3T3-E1 cell to explore the effects of RBP4 overexpression on proliferation, differentiation and mineralization of osteoblast, which was representative of the three stages of osteogenesis. MC3T3-E1 cell was extensively used as a model of osteogenesis in previous studies.

RBP4 gene is located on chromosome 10 (10q23-q24) [14, 15], which encodes a protein of 201 amino acids and has a molecular mass of 21 kDa [16]. It is mainly produced by hepatocytes but also expressed in several extrahepatic tissues [17]. RBP4 protein belongs to the lipocalin family and was known as a transporter of retinol from liver store pool.

RBP4 was shown to express in human cranial suture tissue but was downregulated during suture fusion in craniosynostosis [7, 18]. In accordance, we determined low expression of RBP4 in MC3T3-E1 cells. So a cell line stably expressing RBP4 was constructed and we found that RBP4 had a significant effect on MC3T3-E1 proliferation. Simultaneously, the ALP activity was enhanced and osteo-differentiation markers including RUNX2, OC, OPN and COLL1 were greatly increased by RBP4. ALP activity is one of the indicators of the osteoblast phenotype and usually reflects a shift to a more differentiated state [11].

Runx2 was required for osteoblast formation and chondrocyte differentiation, so it was an essential regulator of intramembranous and

endochondral bone formation [19]. OC was the most abundant noncollagenous protein in bone and was expressed by osteoblasts during mineralization [20]. It was a late-stage marker of osteoblastic differentiation and was detected in fully developed mineralized matrix [21]. OPN was a major hydroxyapatite-binding protein synthesized by osteoblasts. OPN acted not only as a trigger for osteoblast early differentiation but activated osteoclast resorption. Collagen (COLL1), synthesized and secreted by osteoblasts, was the most abundant extracellular matrix protein in bone tissue and formed the structural support for osteoblast differentiation in bone formation.

The above data suggest that RBP4 pushed MC3T3-E1 into a more differentiated phenotype in differentiation condition. This was not expected and was contrary to previous studies in which RBP4 was examined to be markedly downregulated in fused suture compared to unfused suture. This was surprising and unbelievable. But in Alizarin red staining assay observing the calcium deposit, RBP4 overexpression were found to enhance the formation of mineral nodules and increase bone-like tissue formation in MC3T3-E1 in vitro. This may be partially due to the increased production of extracellular matrix by upregulation of OPN and OC. This data further support the deduction that RBP4 contributes to osteogenesis.

In conclusion, our data demonstrate that RBP4 overexpression has significant effect on the proliferation of MC3T3-E1 cells as well as it obviously promotes the differentiation of MC3T3E-1 into osteoblasts and accelerates extracellular matrix mineralization. This is an exited finding for both lab and clinical researchers and will do a great help for patients with various bone developmental formations.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (NSFC) (No.81401616).

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Xiongzheng Mu and Abdulsamad Ghanem, Department of Plastic Surgery, Huashan Hospital, Fudan University, 12 UrumuqiZhong Road, Shanghai 200040, China. Tel: (0086) 21-52889999; E-mail: cranio@vip.163.com (XZM); abdulsamad1982@hotmail.com (AG)

References

[1] Lattanzi W, Bukvic N, Barba M, Tamburrini G, Bernardini C, Michetti F, Di Rocco C. Genetic basis of single-suture synostoses: genes, chromosomes and clinical implications. Childs Nerv Syst 2012; 28: 1301-1310.

[2] Gray TL, Casey T, Selva D, Anderson PJ, David DJ. Ophthalmic Sequelae of Crouzon Syndrome. Ophthalmology 2005; 112: 1129-1134.

[3] Da Costa AC, Anderson VA, Holmes AD, Lo P, Wray AC, Chong DK, Greensmith AL, Meara JG. Longitudinal study of the neurodevelopmental characteristics of treated and untreated non-syndromic craniosynostosis in infancy. Childs Nerv Syst 2013; 29: 985-995.

[4] Weston AD, Hoffman LM, Underhill TM. Revisiting the role of retinoid signaling in skeletal development. Birth Defects Res C Embryo Today 2003; 69: 156-73.

[5] Napoli JL. Physiological insights into all-trans-retinoic acid biosynthesis. Biochim Biophys Acta 2012; 1821: 152-167.

[6] Hatfield JT, Anderson PJ, Powell BC. Retinol-binding protein 4 is expressed in chondrocytes of developing mouse long bones: implications for a local role in formation of the secondary ossification center. Histochem Cell Biol 2013; 139: 727-734.

[7] Coussens AK, Wilkinson CR, Hughes IP, Morris CP, van Daal A, Anderson PJ, Powell BC. Unravelling the molecular control of calvarial suture fusion in children with craniosynostosis. BMC Genomics 2007; 8: 458.

[8] Leitch VD, Dwivedi PP, Anderson PJ, Powell BC. Retinol-binding protein 4 downregulation during osteogenesis and its localization to non-endocytic vesicles in human cranial suture mesenchymal cells suggest a novel tissue function. Histochem Cell Biol 2013; 139: 75-87.

[9] Liu Y, Xu F, Pei HX, Zhu X, Lin X, Song CY, Liang QH, Liao EY, Yuan LQ. Vaspin regulates the osteogenic differentiation of MC3T3-E1 through the PI3K-Akt/miR-34c loop. Sci Rep 2016; 6: 25578.

[10] Ehara A, Ogata K, Imazato S, Ebisu S, Nakano T, Umakoshi Y. Effects of α-TCP and TetCP on MC3T3-E1 proliferation, differentiation and mineralization. Biomaterials 2003; 24: 831-836.

[11] Malaval L, Liu F, Roche P, Aubin JE. Kinetics of osteoprogenitor proliferation and osteoblast

differentiation in vitro. J Cell Biochem 1999; 74: 616-627.
[12] Coussens AK, Hughes IP, Morris CP, Powell BC, Anderson PJ. In vitro differentiation of human calvarial suture derived cells with and without dexamethasone does not induce in vivo-like expression. J Cell Physiol 2009; 218: 183-191.
[13] Franceschi RT, Iyer BS. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. J Bone Miner Res 1992; 7: 235-246.
[14] Meigs JB, Panhuysen CI, Myers RH, Wilson PW, Cupples LA. A Genome-Wide Scan for Loci Linked to Plasma Levels of Glucose and HbA1c in a Community-Based Sample of Caucasian Pedigrees The Framingham Offspring Study. Diabetes 2002; 51: 833-840.
[15] Saucedo R, Zarate A, Basurto L, Hernandez M, Puello E, Mendoza-Lorenzo P, Ostrosky-Wegman P. RBP4 gene variants are associated with insulin resistance in women with previous gestational diabetes. Dis Markers 2014; 2014: 269208.
[16] Jaconi S, Rose K, Hughes GJ, Saurat JH, Siegenthaler G. Characterization of two post-translationally processed forms of human serum retinol-binding protein: altered ratios in chronic renal failure. J Lipid Res 1995; 36: 1247-1253.
[17] Soprano DR, Soprano KJ, Goodman DS. Retinol-binding protein messenger RNA levels in the liver and in extrahepatic tissues of the rat. J Lipid Res 1986; 27: 166-171.
[18] James AW, Levi B, Xu Y, Carre AL, Longaker MT. Retinoic Acid Enhances Osteogenesis in Cranial Suture-Derived Mesenchymal Cells: Potential Mechanisms of Retinoid-Induced Craniosynostosis. Plast Reconstr Surg 2010; 125: 1352-61.
[19] Enomoto H, Enomoto-Iwamoto M, Iwamoto M, Nomura S, Himeno M, Kitamura Y, Kishimoto T, Komori T. Cbfa1 is a positive regulatory factor in chondrocyte maturation. J Biol Chem 2000; 275: 8695-8702.
[20] Hauschka PV, Lian JB, Cole DE, Gundberg CM. Osteocalcin and matrix Gla protein: vitamin K-dependent proteins in bone. Physiol Rev 1989; 69: 990-1047.
[21] Sato M, Yasui N, Nakase T, Kawahata H, Sugimoto M, Hirota S, Kitamura Y, Nomura S, Ochi T. Expression of bone matrix proteins mRNA during distraction osteogenesis. J Bone Miner Res 1998; 13: 1221-1231.