

CYR61 Regulates BMP-2-dependent Osteoblast Differentiation through the $\alpha_v\beta_3$ Integrin/Integrin-linked Kinase/ERK Pathway^{*[5]}

Received for publication, November 21, 2009, and in revised form, June 25, 2010. Published, JBC Papers in Press, July 30, 2010, DOI 10.1074/jbc.M109.087122

Jen-Liang Su,^{a,b,c,d1} Jean Chiou,^{a,b1} Chih-Hsin Tang,^{e,f1} Ming Zhao,^g Chun-Hao Tsai,^h Pai-Sheng Chen,ⁱ Yi-Wen Chang,^b Ming-Hsien Chien,^j Chu-Ying Peng,^c Michael Hsiao,^j Ming-Liang Kuo,ⁱ and Men-Luh Yen^{k,12}

From the ^aCancer Biology and Drug Discovery Ph.D. Degree Program, the ^cGraduate Institute of Cancer Biology and the ^fDepartment of Pharmacology, College of Medicine, and the ^eGraduate Institute of Basic Medical Science, China Medical University and the ^bCenter for Molecular Medicine and the ^hDepartment of Orthopedics, China Medical University Hospital, Taichung 404, Taiwan, the ^dDepartment of Biotechnology, Asia University, Taichung 41354, Taiwan, the ^gDepartment of Basic Medical Sciences, School of Medicine, University of Missouri, Kansas City, Missouri 64108, the ⁱInstitute of Toxicology, College of Medicine and Angiogenesis Research Center, the ^kDepartment of Primary Care Medicine, and the ^lDepartment of Obstetrics/Gynecology, National Taiwan University Hospital and College of Medicine, National Taiwan University, Taipei 100, Taiwan, and the ^jGenomics Research Center, Academia Sinica, Taipei 115, Taiwan

Osteoporosis is one of the most common bone pathologies. A number of novel molecules have been reported to increase bone formation including cysteine-rich protein 61 (CYR61), a ligand of integrin receptor, but mechanisms remain unclear. It is known that bone morphogenetic proteins (BMPs), especially BMP-2, are crucial regulators of osteogenesis. However, the interaction between CYR61 and BMP-2 is unclear. We found that CYR61 significantly increases proliferation and osteoblastic differentiation in MC3T3-E1 osteoblasts and primary cultured osteoblasts. CYR61 enhances mRNA and protein expression of BMP-2 in a time- and dose-dependent manner. Moreover, CYR61-mediated proliferation and osteoblastic differentiation are significantly decreased by knockdown of BMP-2 expression or inhibition of BMP-2 activity. In this study we found integrin $\alpha_v\beta_3$ is critical for CYR61-mediated BMP-2 expression and osteoblastic differentiation. We also found that integrin-linked kinase, which is downstream of the $\alpha_v\beta_3$ receptor, is involved in CYR61-induced BMP-2 expression and subsequent osteoblastic differentiation through an ERK-dependent pathway. Taken together, our results show that CYR61 up-regulates BMP-2 mRNA and protein expression, resulting in enhanced cell proliferation and osteoblastic differentiation through activation of the $\alpha_v\beta_3$ integrin/integrin-linked kinase/ERK signaling pathway.

Bone is a mineralized tissue that underlies multiple mechanical and metabolic functions of the skeleton (1). Bone functions include maintaining blood calcium levels, providing mechanical support to soft tissues and serving as levers for muscle action, supporting hematopoiesis, and housing the brain and spinal cord (2). Formation and maintenance of bone tissue are regulated in a sophisticated fashion by bone-forming osteoblasts and bone-resorbing osteoclasts (3). Development and differentiation of these two cell types are under tight regulation by a number of endogenous substances such as hormones, growth factors, and cytokines (4). These factors are individually secreted through endocrine, paracrine/autocrine, and neurocrine systems, with subsequent interaction essential to the delicate balance between bone-forming and -resorbing cells in the marrow microenvironment. An imbalance between the two cell types leads to pathogenesis of certain bone diseases including osteopetrosis and osteoporosis (5, 6).

Osteoporosis is the most common human metabolic bone disorder characterized by progressive and age-dependent bone loss and increasing bone fracture risk. It is an important public health issue in postmenopausal women; if untreated, more than half of white women will experience fractures during their lifetime. Between 30 and 50% of women and 15–30% of men will suffer a fracture related to osteoporosis in their lifetime (7). Fractures increase morbidity and mortality and impose a financial burden on the community (8). A most compelling therapeutic need for osteoporosis at the present time is a drug that will substantially increase bone formation. The use of an anabolic agent that stimulates bone formation, restores trabecular bone microarchitecture, and rebuilds bone that has been lost, possibly in conjunction with an inhibitor of bone resorption, would be an ideal therapeutic approach for patients. Therefore, it is clearly necessary to identify novel molecular targets to develop bone-forming drugs aimed to combat osteoporosis by rebuilding the lost bone.

Multiple anabolic signaling pathways are positively involved in controlling bone formation, such as bone morphogenetic

* This work was supported in part by National Science Council Grants NSC 97-2314-B-002-060-MY3, NSC 98-3111-B-002-009 (to M.-L. Y.), National Science Council Grants NSC 96-2320-B-004-MY2, NSC 97-2320-B-039-039-MY3, and NSC 98-2815-C-039-082-B, Taiwan Merit Scholarship TMS-094-2-B-023 from the National Science Council of Taiwan, National Health Research Institutes Grant NHRI-EX97-9712BC from Taiwan, Department of Health Executive Yuan Grant DOH97-TD-G-111-024 from Taiwan, China Medical University Grants CMU96-220, CMU96-189, and CMU97-277, and an Odyssey Scholarship from M. D. Anderson Cancer Center (to J.-L. S.).

[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–2.

¹ These authors equally contributed to this work.

² To whom correspondence should be addressed: Dept. of Primary Care Medicine, National Taiwan University Hospital and National Taiwan University, College of Medicine, No. 7, Chung Shan S. Rd., Taipei 100, Taiwan. Tel.: 886-2-23123456 (ext. 5122); Fax: 886-2-2391-1302; E-mail: mlyen@ntu.edu.tw.

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protein (BMP),³ Wnt, and Runx2 pathways. Among the BMP family, BMP2 is the best documented bone growth factor that stimulates osteoblast differentiation and bone formation (9). Genetic manipulation studies suggest that BMP2 is critical for postnatal bone formation (10). Aging studies have found that production and bone-forming activity of BMP2 are both significantly decreased in aged bones (11, 12). In human, BMP2 was recently recognized as an osteoporosis-associated gene through human polymorphism studies (13). Collectively, these findings suggest that decay of BMP2 function, which leads to an inhibition of bone formation, is one molecular mechanism that contributes to the development of osteoporosis with aging. Therefore, BMP2 has become an ideal target for drug development (14–17). BMP2 gene expression is transcriptionally regulated through various intracellular mechanisms that constitute a complex cross-talk between multiple signaling pathways, including estrogen receptors (18), PGE2 (19), HOXA13 (20), retinoic acid (21), 1,25(OH) vitamin D3 (19), hedgehog/Gli (22), Wnt/ β -catenin (23) and BMP/Smad (24), responsible for regulation of osteoblast functions. However, the precise mechanisms by which some of these pathways, such as 1,25-(OH) vitamin D3 and Wnt/ β -catenin, regulate BMP2 expression in osteoblasts need to be well elucidated. Recent evidence has indicated that cysteine-rich protein 61 (CYR61), a member of the connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family (25), may play an important role in regulating BMP2 expression in osteoblasts by mediating the effects of 1,25-(OH) vitamin D3 and Wnt/ β -catenin on BMP2 genes.

CYR61 is the first cloned member of the CCN family, an immediate early gene family consisting of six members with homologous DNA sequence that exhibit diverse cellular functions such as regulation of cell division, chemotaxis, adhesion, motility, tumorigenesis, angiogenesis, and ion transport (26–34). Interestingly, *in vitro* evidence has suggested that CYR61, as an extracellular signaling molecule in bone (35), plays an essential role in maintaining normal osteoblast functions, including osteogenic commitment of mesenchymal cells (36, 37), proliferation and maturation of osteoblast precursor cells (37, 38), and migration of osteoblastic cells (37, 39). In osteoblastic cells, CYR61 mRNA expression is up-regulated by 1,25-(OH) vitamin D3 (36) and the canonical Wnt signaling (37). Both 1,25-(OH) vitamin D3 and Wnt signaling are important for osteoblast differentiation of mesenchymal stem cells. Because these two mechanisms were found to induce BMP2 gene expression in osteoblasts (19, 23) and BMP2 is a critical factor responsible for osteoblast differentiation, we hypothesized that, as a positive extracellular signaling protein, CYR61 controls osteoblast functions by regulating BMP2 gene expression in osteoblasts. This study was designed to test this hypothesis and also determine the precise signaling

mechanisms involved in CYR61 regulation of BMP2 transcription in osteoblasts.

In the current work we found that CYR61 increases cell proliferation and osteoblastic differentiation; these results fit in with previous studies (36–39). We also provide the novel molecular mechanisms involved in CYR61-mediated osteogenic effects in this study. BMP-2-dependent phenomenon is critical for CYR61-induced osteogenic effects in fetal mouse pre-osteoblast MC3T3-E1 cells and primary cultured osteoblast cells. In addition, $\alpha_v\beta_3$ integrin/integrin-linked kinase (ILK)/extracellular signal-regulated kinase (ERK) signaling pathways are involved in CYR61-mediated induction of BMP-2 expression and subsequent cell proliferation and osteoblastic differentiation.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Anti-mouse BMP-2 antibody, BMP-2 ELISA kit, and noggin were purchased from R&D Systems (Minneapolis, MN). Anti- α -tubulin antibody, anti-pERK1/2 or ERK1 antibodies, anti-pAKT1/2/3 (ser-473) or total AKT-1 antibodies, anti-pJNK or JNK antibodies, anti-p-p38 or total p38 antibodies, and protein A/G beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant CYR61 protein was purchased from Abnova (Taipei, Taiwan). Chemicals, anti- β -actin antibody, and an Alkaline Phosphatase activity kit were purchased from Sigma. An *in vitro* osteogenesis kit and $\alpha_v\beta_3$ neutralizing antibody were purchased from Chemicom (Temecula, CA). Rabbit polyclonal antibody for ILK was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibody for glycogen synthase kinase 3 β (GSK3 β) and phosphor-GSK3 β were purchased from Cell Signaling Technology (Beverly, MA).

Cell Culture—MC3T3-E1 cells were purchased from ATCC (Manassas, VA) and grown in α MEM (Invitrogen, catalog no. 001008-3DJ, Invitrogen) containing 10% FBS, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Medium was changed every 48 h. Murine primary osteoblastic cells (pOB cells) were prepared as described previously (40). Calvaria were dissected from murine fetuses, divided into small pieces, and treated with 0.1% type I collagenase solution for 10 min at 37 °C. The next two 20-min sequential collagenase digestions were pooled and filtered through 70- μ m nylon filters (Falcon, NJ). Cells were grown on plastic cell culture dishes in 95% air, 5% CO₂ with α MEM that was supplemented with 20 mM HEPES and 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μ g/ml). The characteristics of osteoblasts were confirmed by morphology and expression of AP enzymatic activity.

MTT Assay—Growth rates of recombinant CYR61 (rCYR61)-treated MC3T3-E1 cells were determined using MTT as a substrate. The MTT assay is based on the activity of mitochondrial dehydrogenases, which reduce the water-soluble tetrazolium salt to a purple insoluble formazan product. The amount of MTT formazan product was analyzed spectrophotometrically at a wavelength of 570 nm. Each individual experiment was repeated at least three times.

Colony Formation Assay—Aliquots of 1000 cells were seeded into six-well culture dishes. The next day cells were exposed to

³ The abbreviations used are: BMP, bone morphogenetic protein; CCN family, connective tissue growth factor/cysteine-rich 61/nephroblastoma-overexpressed family; CYR61, cysteine-rich protein 61; rCYR61, recombinant CYR61; ILK, integrin-linked kinase; GSK3 β , glycogen synthase kinase 3 β ; qPCR, quantitative real-time PCR; α MEM, α -minimal essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ALP, alkaline phosphatase.

recombinant CYR61 protein in serum-free medium at the appropriate times. Cells were washed and further incubated with 2% FCS medium until further analysis. After incubation for 7 days, cells were washed with $1 \times$ PBS and fixed with methanol at room temperature for 10–20 min. After washing with $1 \times$ PBS again, cells were stained with 0.1% crystal violet, PBS, and colonies were counted. All experiments were carried out in triplicate.

Boyden Chamber Assay—Migration assays were performed using modified Boyden chambers with filter inserts for 24-well dishes containing 8- μ m pores (Nucleopore Corp, Pleasanton, CA). Cells (2×10^4) were plated into 100 μ l of complete α MEM in the upper chamber, and the lower chamber was filled with 1 ml of α MEM with various doses of rCYR61 or PBS. After 12 h in culture, cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side of filters were removed with cotton-tipped swabs, and filters were washed in PBS. Cells on the underside of filters were viewed and counted under a microscope (type 090-135.001, Leica Microsystems, Wetzlar, Germany). Each clone was plated in triplicate in each experiment, and each experiment was repeated at least three times.

Wound-healing Migration Assay—For the wound-healing migration assay, cells were seeded on 12-well plates at a density of 1×10^5 cells/well in culture medium. At 24 h after seeding, the confluent monolayer of culture was scratched with a fine pipette tip, and migration was visualized by microscope and magnification. The rate of wound closure was observed at the indicated time.

Differentiation Assay—To induce differentiation, confluent MC3T3-E1 cells were transferred to differentiation medium (α MEM supplemented with 10% FCS, 100 units/ml penicillin, 100 μ g/ml streptomycin, 0.2 mM ascorbic acid (Sigma), and 10 mM β -glycerol phosphate (Sigma)) Osteogenesis was determined by staining with Alizarin red solution. Cells were washed with PBS or Hanks' balanced salt solution and then fixed with 70% ethanol for 15 min. After fixation, cells were rinsed three times with distilled water for 10–15 min each time. One ml of Alizarin red solution was added into the well followed by incubation at room temperature for at least 20 min. Excess dye was removed, and then cells were washed four times with deionized water. Differentiated cells containing mineral deposits stained bright red with Alizarin red solution. After photography, bound staining was eluted with 10% (w/v) cetylpyridinium chloride, and Alizarin red solution in samples was quantified by measuring absorbance at 550 nm and calculated according to a standard curve. One molar Alizarin solution selectively binds about 2 mol of calcium.

ALP Activity Assay—MC3T3-E1 cells were cultured in α MEM containing 50 μ g/ml vitamin C and 10 mM β -glycerophosphate for 2 weeks, with medium changed every 3 days. After 14 days, cells were harvested in 1 ml of 0.2% Nonidet P-40, and the cell suspension was disrupted by sonication. After centrifugation at $1500 \times g$ for 5 min, ALP activity in the supernatant was measured using the method of Lowry *et al.* (41).

Total RNA Extraction and RT-PCR—Total RNA was isolated using Trizol reagent according to the manufacturer's instructions. Total RNA (5 μ g) was reverse-transcribed into single-

stranded cDNA using a Moloney murine leukemia-virus reverse transcriptase and random hexamers (Promega, Madison, WI). Primers were used at a final concentration of 0.5 μ M. The reaction mixture was first denatured at 95 $^{\circ}$ C for 5 min. The cDNAs were amplified with the forward (F) and reverse (R) primers by PCR as described. The primer sequences for *BMP-2* were 5'-CCAAGAGACATGTGAGGATT-3' (F) and 5'-TTA-GTGGAGTTCAGGTGGTC-3' (R). The primer sequences for *BMP-4* were 5'-ACGACTACTGGACACCAGAC-3' (F) and 5'-GTTGGTTGAGTTGAGGTGAT-3' (R). The primer sequences for *BMP-6* were 5'-GACATCACAGCAACTAGCAA-3' (F) and 5'-AGGAACACTCTCCATCACAG-3' (R). The primer sequences for *BMP-7* were 5'-GCTTACAGCTCTCTGTGGAG-3' (F) and 5'-GGTTGATGAAGTGAACAAGT-3' (R). The primer sequences for *BMP-9* were 5'-TACC-ACTATGAGGGGATGAG-3' (F) and 5'-CTATGAGCCACAGGAGAGTC-3' (R). The primer sequences for *GAPDH* were 5'-CTCACTCAAGATTGTCAGCA-3' (F) and 5'-GTCATC-ATACTTGGCAGGTT-3' (R). PCR conditions were 30 cycles of 95 $^{\circ}$ C for 30 s, 52 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 1 min followed by 72 $^{\circ}$ C for 10 min. PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

Quantitative Real-time PCR—Quantitative real-time PCR (qPCR) analysis was carried out using Taqman one-step PCR Master Mix (Applied Biosystems, Foster City, CA). One hundred nanograms of total cDNA were added per 25 μ l reaction with sequence-specific primers and Taqman probes. Sequences for all target gene primers and probes were purchased commercially (GAPDH was used as internal control) (Applied Biosystems). The qPCR assays were carried out in triplicate on an ABI Prism 7900 sequence detection system. Cycling conditions were 10 min of polymerase activation at 95 $^{\circ}$ C followed by 40 cycles at 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which transcript was detected (denoted C_T).

Western Blot Analysis—MC3T3-E1 cells were incubated in serum-free α MEM during treatment with rCYR61 (100 ng/ml), and cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin) for 15 min on ice. Cellular lysates were prepared as described previously (48). An equal quantity of protein from cell lysates was resuspended in gel sample buffer, resolved by 10% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Millipore). After blocking, blots were incubated with specific primary antibodies, and after washing and incubating with secondary antibodies, immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (GE Healthcare). Where indicated, membranes were stripped and reprobed with another antibody.

Assay of BMP-2—BMP-2 ELISA kits were used to detect BMP-2 levels. Briefly, cells were treated with various concentrations of rCYR61 for the indicated time. Culture medium was collected for measurement of BMP-2. Samples were placed in 96-well microtiter plates coated with monoclonal detective antibodies and incubated for 2 h at room tempera-

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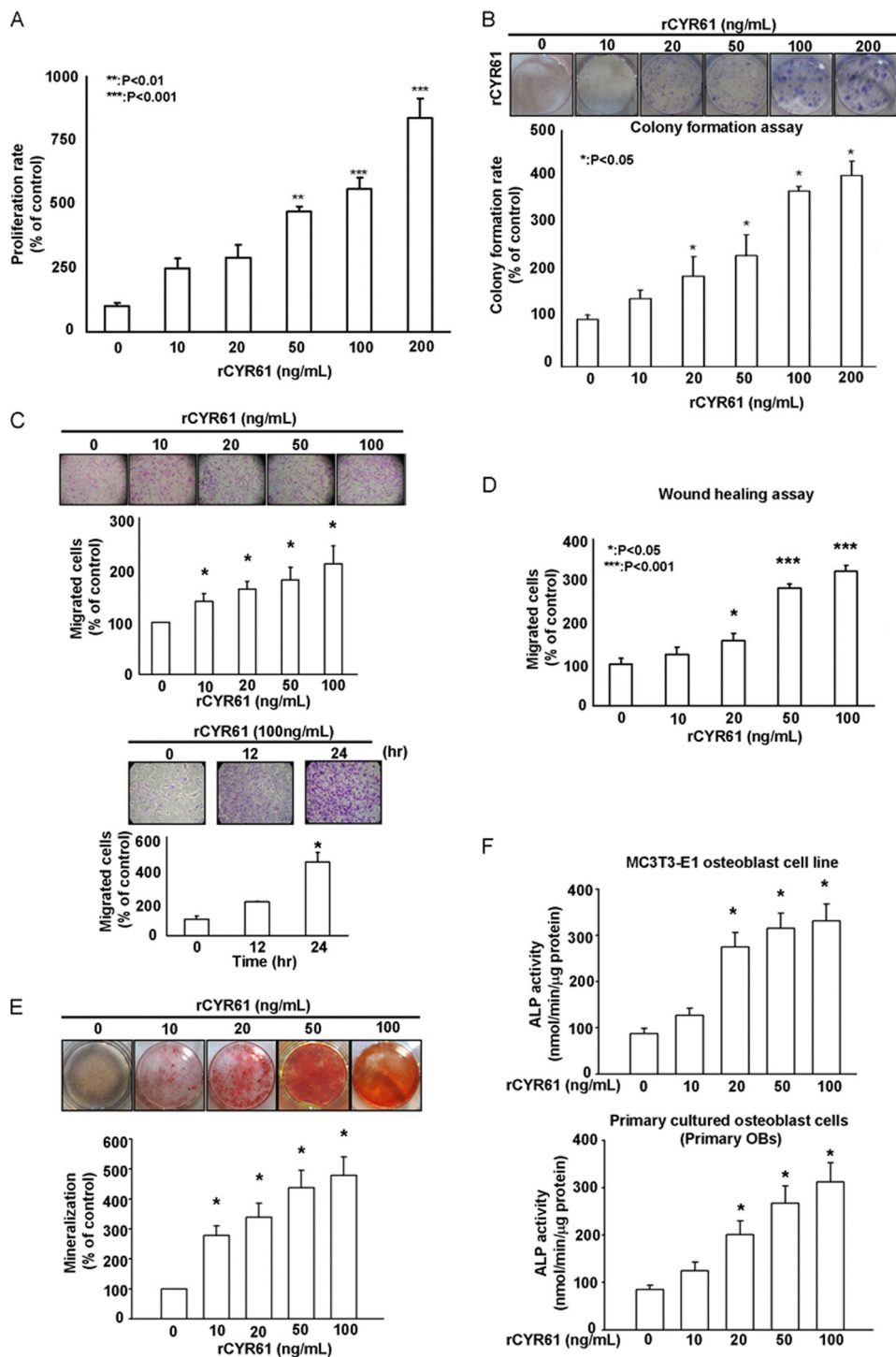


FIGURE 1. Recombinant CYR61 protein induces proliferation, migration, and osteoblastic differentiation of MC3T3-E1 cells. *A*, treatment with rCYR61 increased proliferation per MTT assay is shown. After treatment at various doses, growth rates were measured by MTT assay. *B*, treatment with rCYR61 increased proliferation per colony counts. After 7 days colonies were stained with crystal violet and counted. *C*, migration ability of MC3T3-E1 cells increased after rCYR61 treatment. Cells that migrated were stained with crystal violet and counted (upper). Cells were incubated with 100 ng/ml rCYR61 in Transwell plates for indicated times. Cells that migrated were stained with crystal violet and counted (lower). *D*, treatment with rCYR61 increased wound-healing migration. Still images were captured at the indicated times after wounding, and then cells were counted. *E*, cells were incubated with rCYR61 in the indicated doses. After 14 days cells were stained with Alizarin red (upper). The quantitative data of mineralization ability are shown in the lower panel. *F*, ALP activity assay identified osteoblastic differentiation of MC3T3-E1 and primary cultured osteoblasts after rCYR61 treatment. Cells were incubated with rCYR61 in the indicated doses. After 14 days, cells were collected to determine ALP activity. The upper panel indicates enzyme activity of MC3T3-E1 cells; the lower panel indicates enzyme activity of primary cultured osteoblasts (OB). Each experiment was performed in triplicate, and results represent the mean \pm S.D. of three independent experiments. The asterisks indicate a significant difference (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) between rCYR61 treatment and vehicle treatment cells.

ture. After removing unbound material by washing with washing buffer (50 mM Tris, 200 mM NaCl, and 0.2% Tween 20), horseradish peroxidase-conjugated streptavidin was added to bind to the antibodies. Horseradish peroxidase catalyzed conversion of the chromogenic substrate (tetramethylbenzidine) to a colored solution, with color intensity proportional to the amount of protein present in the sample. The absorbance of each well was measured at 450 nm. Results are presented as the percentage of change in activity compared with activity in untreated controls.

ILK Kinase Assay—ILK enzymatic activity was assayed in MC3T3-E1 cells lysed in Nonidet P-40 buffer (0.5% sodium deoxycholate, 1% Nonidet P-40, 50 mM HEPES, pH 7.4, 150 mM NaCl) as previous reported (42). Briefly, ILK was immunoprecipitated with ILK antibody overnight at 4 °C from 250 μ g of lysate. After immunoprecipitation, beads were resuspended in 30 liters of kinase buffer containing 1 mg of recombinant substrate (GSK3 fusion protein) and 200 μ M cold ATP, and the reaction was carried out for 30 min at 30 °C. Phosphorylated substrate was visualized by Western blot with phosphor-GSK3 β antibody. Total GSK3 β was detected with the appropriate antibody.

Statistical Analysis—Data are presented as the mean \pm S.D. Student's *t* test was used to compare data between groups. All statistical tests included two-way analysis of variance. *p* values of less than 0.05 were considered to be statistically significant.

RESULTS

Recombinant CYR61 Protein Induced Differentiation in MC3T3-E1 Cells—In this study we investigated the role of CYR61 in osteoblast proliferation and differentiation. We found that treatment with rCYR61 protein significantly increased proliferation of mouse osteoblast MC3T3-E1 cells in a dose-dependent manner to \sim 200 ng/ml (Fig. 1A), after which

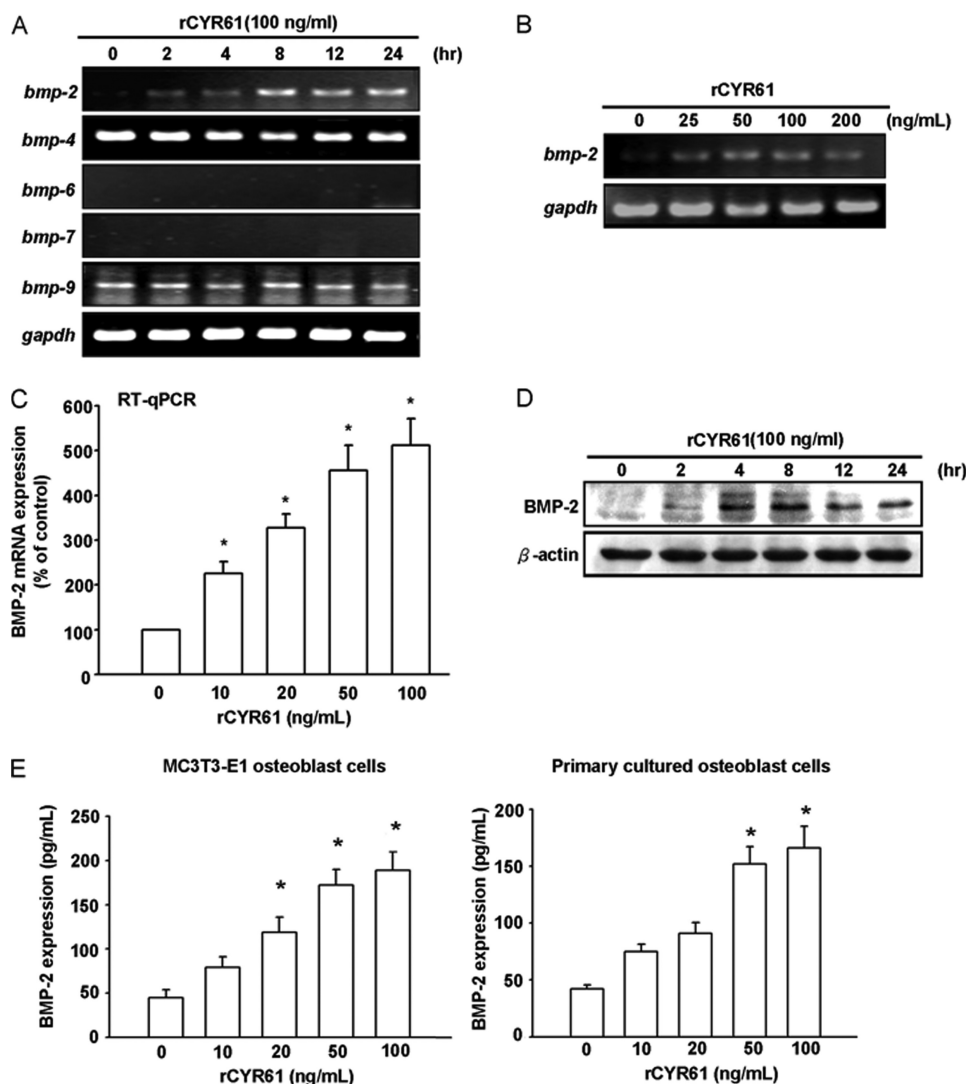


FIGURE 2. Recombinant CYR61 induces BMP-2 expression. *A* and *B*, RT-PCR for mRNA of different BMP family members is shown. The coding regions of cDNA were used as probes as indicated under "Experimental Procedures." *A* GAPDH probe was used as an internal control for RNA quantity. *C*, MC3T3-E1 cells were incubated with rCYR61 for 8 h, after which mRNA expression of BMP-2 was determined using qPCR. *D*, shown is Western blot analysis of BMP-2 protein expression in MC3T3-E1 cells. Total proteins were extracted from cells and probed with polyclonal antibody specific for BMP-2. Each lane contains 80 μ g of total protein. The internal loading control was β -actin. *E*, shown is an ELISA assay of BMP-2 protein expression in MC3T3-E1 cells (*left*) and primary cultured osteoblasts (*right*). MC3T3-E1 cells were incubated with the indicated dose of rCYR61 for 24 h. The protein secreted into conditioned medium was determined by BMP-2 ELISA assay kit. All the results except those in *D* are expressed as the means \pm S.E. Each assay was performed in three separate experiments. The asterisk indicates $p < 0.05$ between rCYR61 treatment and vehicle treatment cells.

we found that higher concentrations of rCYR61 decrease the proliferation of MC3T3-E1 cells ([supplemental Fig. 1A](#)). The above data imply that CYR61 may have a biphasic effect on MC3T3-E1 cell proliferation. The different results of CYR61 on MC3T3-E1 cell proliferation reported by our study and that *Craig et al.* (61) may be caused by the different experimental conditions and the biphasic effect of CYR61. Colony formation assay produced similar results (*Fig. 1B*). We also treated MC3T3-E1 cells with rCYR61 in a time-dependent manner (0, 24, 48, 72 h) and found that CYR61-induced MC3T3-E1 cells proliferation was detectable at 48 h as well as 72 h ([supplemental Fig. 1B](#)). Because osteoblastic differentiation is a complicated process that includes prolifer-

ation and migration of osteoblasts, we also tested the migrative ability of MC3T3-E1 cells after treatment with rCYR61. As shown in *Fig. 1, C* and *D*, rCYR61 significantly induced migration of MC3T3-E1 cells in both a Boyden chamber assay and in wound healing analyses in a time- and dose-dependent manner. In the *in vitro* differentiation assay, mineralization of MC3T3-E1 osteoblasts and primary cultured osteoblasts was demonstrated by Alizarin red staining assay and ALP activity assay after rCYR61 treatment (*Fig. 1, E* and *F*). These results indicate that CYR61 induces osteogenic differentiation in MC3T3-E1 osteoblasts and primary osteoblasts.

CYR61 Induced BMP-2 Production in Osteoblast Cells—Given the crucial role of BMPs in osteoblastic differentiation, we tested whether CYR61 mediated alteration of osteoblast proliferation and differentiation through regulation of BMP expression. According to *Cheng et al.* (43), BMP-2, -6, and -9 may be the most potent molecules that induce osteoblast lineage-specific differentiation. Therefore, we explored possible target gene expression during rCYR61-induced osteoblastic differentiation. We examined expression levels of members of the BMP family by RT-PCR in MC3T3-E1 osteoblast cells in response to rCYR61 treatment. Treatment with rCYR61 stimulated BMP-2 expression in a time-dependent manner by RT-PCR and Western blot analysis (*Fig. 2, A* and *D*). A significant increase in BMP-2 mRNA could be detected as early as 2 h after rCYR61 treatment. Elevation of BMP-2

mRNA was maximal at 8 h and lasted until 24 h after treatment. Expression level of other BMP mRNAs, such as BMP-4, -6, -7, and -9, were not significantly affected by rCYR61 (*Fig. 2A*). In addition, the stimulatory effect of rCYR61 on BMP-2 expression was dose-dependent in the range between 10 and 200 ng/ml (*Fig. 2, B* and *C*). The BMP-2 protein expression level in response to rCYR61 treatment was increased at 4~24 h after treatment (*Fig. 2D*). Furthermore, we found rCYR61 induced BMP-2 secretion into cultured medium of both MC3T3-E1 cells and primary osteoblasts by BMP-2 ELISA assay (*Fig. 2E*). These data indicate that rCYR61 induces expression of BMP-2 in osteoblast cells in a time- and dose-dependent manner.

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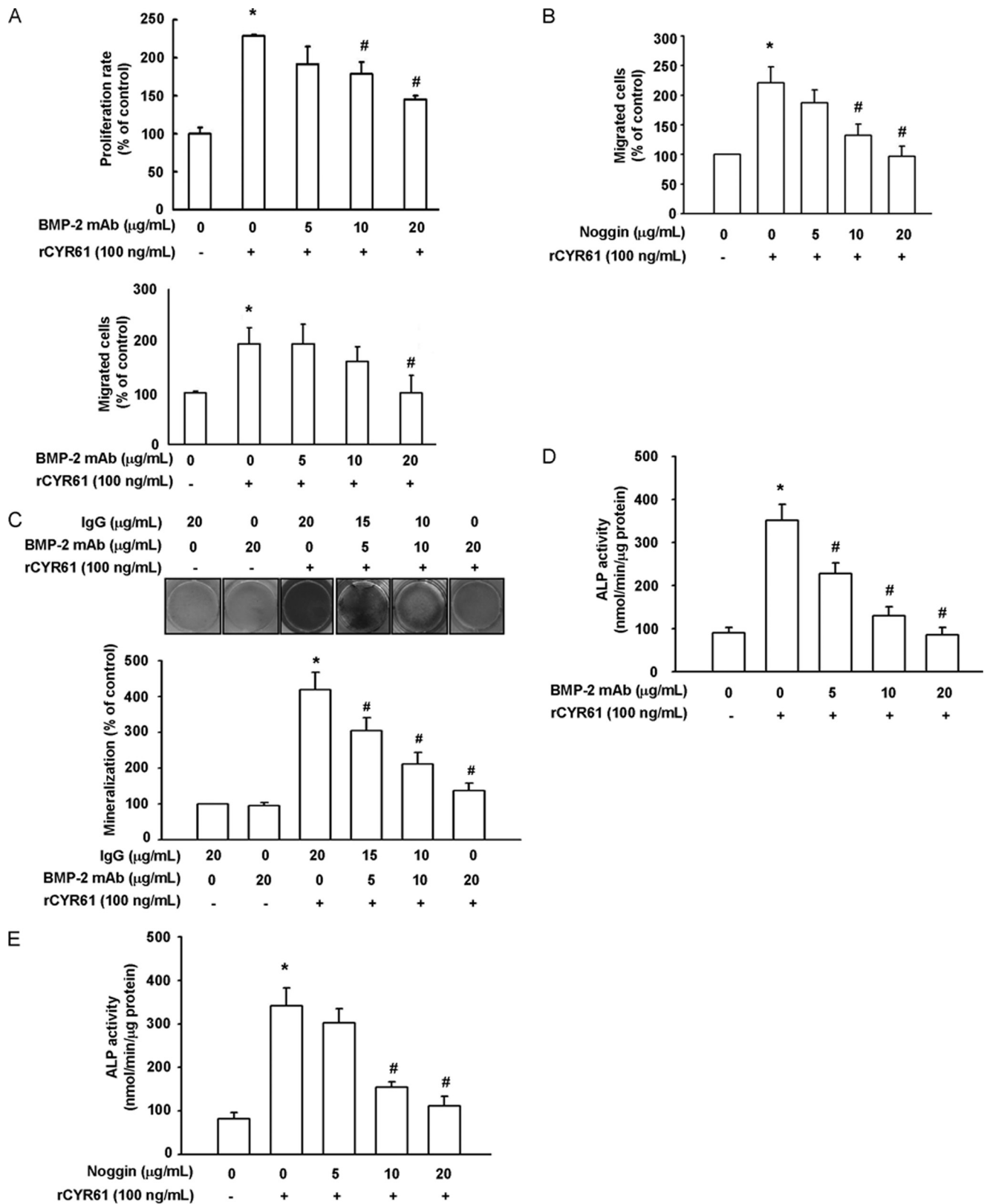


FIGURE 3. BMP-2 is critical for CYR61-mediated osteoblastic differentiation. *A*, cells were incubated with rCYR61 and BMP-2 neutralizing antibodies for the indicated times in 24-well plates. Growth rates were measured by MTT assay. *B*, cells were incubated with rCYR61- and BMP-2-neutralizing antibodies or with Noggin for the indicated doses in Transwell plates. Migrated cells were stained and counted. *C*, MC3T3-E1 cells were incubated with rCYR61- and BMP-2-neutralizing antibodies in the indicated doses. Cells were stained with Alizarin red (upper). The quantitative data are shown in the lower panel. *D*, MC3T3-E1 cells were incubated with rCYR61- and BMP-2-neutralizing antibodies in indicated doses. Cells were assayed with ALP activity assay kit. *E*, MC3T3-E1 cells were incubated with rCYR61 and Noggin in the indicated doses. Cells were assayed with ALP activity assay kit. Each assay was performed in three independent experiments. The asterisk indicates $p < 0.05$ between rCYR61 treatment and vehicle treatment cells. The # symbol indicates a significant difference ($p < 0.05$) between BMP-2 monoclonal antibody treatment and rCYR61 treatment cells.

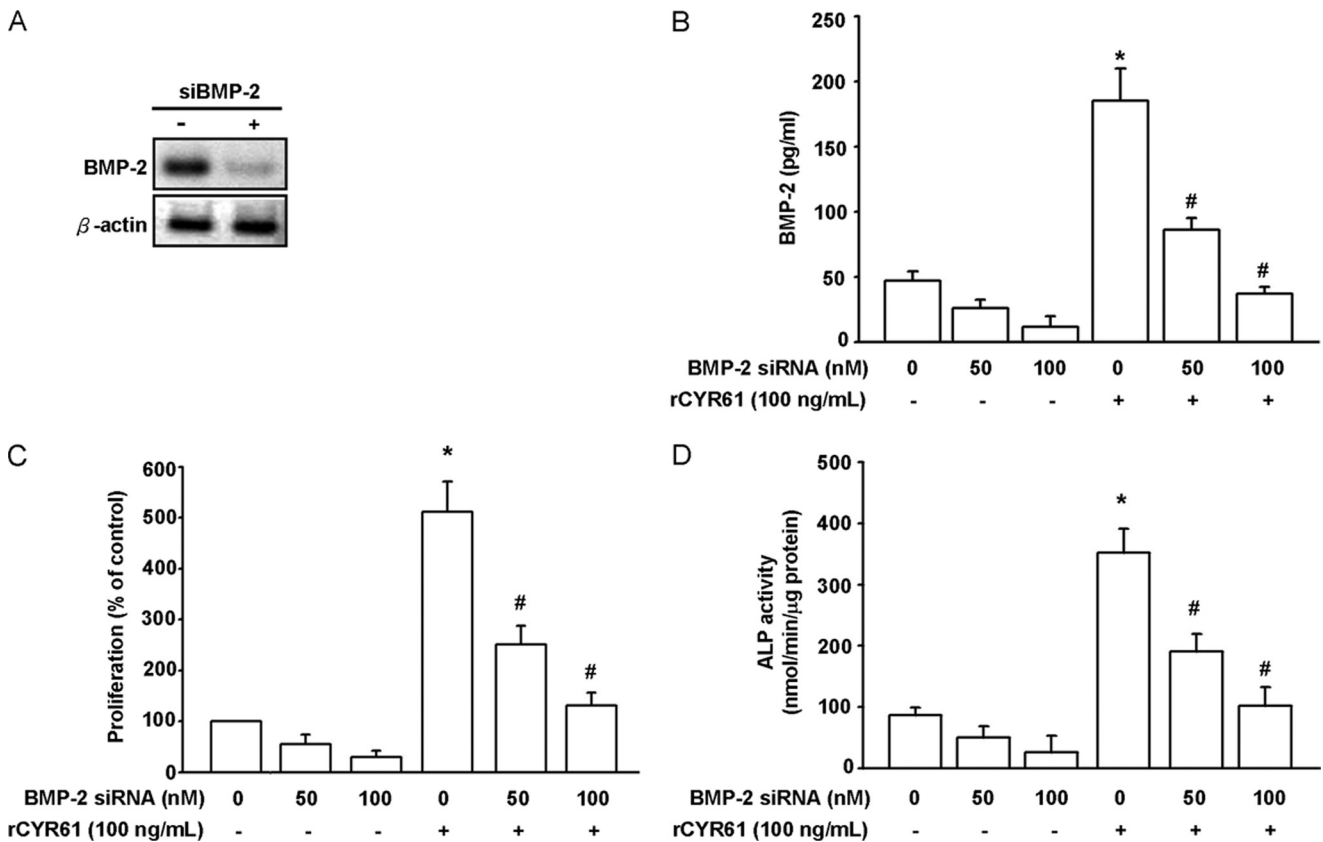


FIGURE 4. Knockdown of BMP-2 inhibits CYR61-induced osteoblastic differentiation. *A*, MC3T3-E1 cells were transfected with BMP-2 or control siRNA for 24 h, after which protein levels of BMP-2 were examined using Western blot analysis. *B*, MC3T3-E1 cells were transfected with BMP-2 or control siRNA for 24 h followed by stimulation with CYR61 for 24 h, after which secreted protein levels of BMP-2 were examined using an ELISA assay. *C*, MC3T3-E1 cells were transfected with BMP-2 or control siRNA for 24 h followed by stimulation with CYR61 for 24 h, then proliferation was measured with an MTT assay. *D*, MC3T3-E1 cells were transfected with BMP-2 or control siRNA for 24 h followed by stimulation with CYR61 for 24 h, after which osteoblastic differentiation was measured with ALP activity assay after 14 days. Each assay was performed in three independent experiments. The asterisk indicates $p < 0.05$ between rCYR61 treatment and vehicle treatment cells. The # symbol indicates a significant difference ($p < 0.05$) between BMP-2 monoclonal antibody treatment and rCYR61 treatment cells.

BMP-2 Is Critical for CYR61-mediated Osteoblastic Differentiation—To determine whether induction of BMP-2 is critical for CYR61-mediated osteogenesis, the inhibitory effect of BMP-2 neutralizing antibody on rCYR61-induced MC3T3-E1 osteoblast cell migration, proliferation, and differentiation was investigated. Our data showed that rCYR61-induced osteoblast proliferation and migration activity were significantly decreased after treatment with neutralizing BMP-2 antibodies for indicated times and dosages (Fig. 3*A*). Further evidence showed that CYR61-induced migration of osteoblast cells was also inhibited by the BMP-2 inhibitor Noggin (Fig. 3*B*). These observations suggest that CYR61-induced osteoblast proliferation and migration may be due to BMP-2 expression. In the *in vitro* differentiation assay, CYR61-induced bone mineralization was inhibited by neutralizing BMP-2 antibodies in a dose-dependent manner (Fig. 3*C*). In an ALP activity assay, both BMP-2 neutralizing antibody and Noggin inhibited rCYR61-induced ALP activity in osteoblast cells (Fig. 3, *D* and *E*). To further confirm the role of BMP-2 in rCYR61-mediated osteoblastic function, MC3T3-E1 cells were treated with BMP-2-specific siRNA or control siRNA. Western blot and ELISA analysis showed that expression levels of BMP-2 protein were significantly suppressed by transfection with BMP-2 siRNA (Fig. 4, *A* and *B*). Treatment with BMP-2 siRNA also reduced

CYR61-induced proliferation and differentiation of MC3T3-E1 osteoblast cells (Fig. 4, *C* and *D*). The above data show that rCYR61 induces proliferation, migration, and differentiation of osteoblast cells via a BMP-2-dependent pathway.

CYR61-induced BMP-2 Expression and Subsequent Osteogenesis Were Dependent on Integrin $\alpha_v\beta_3$ Receptor—According to previous study, MC3T3-E1 cells express integrin receptor α_v , α_2 , β_1 , and β_3 , and CYR61 is the ligand of integrin $\alpha_6\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_{11b}\beta_3$ (44). This implies that CYR61 may affect MC3T3-E1 cells through integrins $\alpha_v\beta_3$ or $\alpha_v\beta_5$. To examine this hypothesis, MC3T3-E1 cells were treated with anti- $\alpha_v\beta_3$ and anti- $\alpha_v\beta_5$ inhibitory monoclonal antibodies to evaluate which integrin was involved in CYR61-induced BMP-2 expression and subsequent osteogenesis. Pretreatment of cells for 30 min with anti- $\alpha_v\beta_3$ but not anti- $\alpha_v\beta_5$ inhibitory monoclonal antibody markedly inhibited CYR61-induced BMP-2 mRNA and protein expression using qPCR analysis (Fig. 5*A*), ELISA assay, and Western blot analysis (Fig. 5*B*). Treatment with cyclic (RGD) peptides, which has been reported to bind $\alpha_v\beta_3$ at high affinity and block its function (45), but not treatment with low $\alpha_v\beta_3$ binding affinity (RAD) peptides, also inhibited CYR61-induced BMP-2 protein expression (supplemental Fig. 2). CYR61-induced increases in proliferation and migration ability as well as differentiation activity of MC3T3-E1 osteo-

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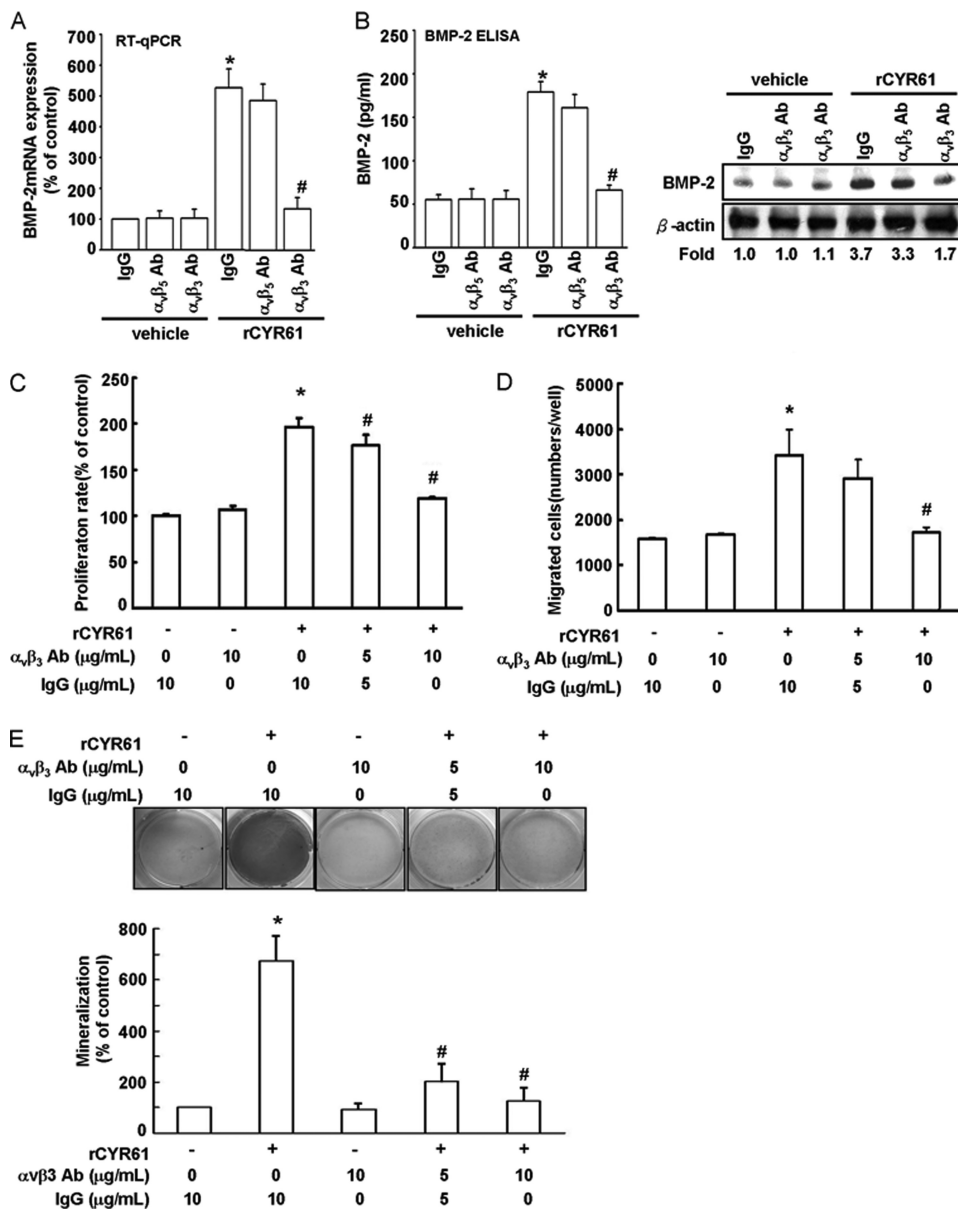


FIGURE 5. Integrin $\alpha_v\beta_3$ is involved in CYR61-induced BMP-2 expression and osteoblastic differentiation. A, cells were pretreated with $\alpha_v\beta_3$, $\alpha_v\beta_5$, and IgG control antibody (10 μ g/ml) for 30 min followed by stimulation with CYR61 (100 ng/ml) for 24 h, then mRNA levels of BMP-2 were determined using qPCR assay. B, cells were pretreated with $\alpha_v\beta_3$, $\alpha_v\beta_5$, and IgG control antibody (10 μ g/ml) for 30 min followed by stimulation with CYR61 (100 ng/ml) for 24 h, then protein levels of BMP-2 were determined using ELISA assay and Western blot analysis. C, cells were incubated with rCYR61 and $\alpha_v\beta_3$ neutralizing antibodies at the indicated doses in 24-well plates. Growth rates were measured by MTT assay. D, cells were incubated with rCYR61 and $\alpha_v\beta_3$ neutralizing antibodies for the indicated times in Transwell plates. Migrated cells were stained and counted. E, cells were incubated with rCYR61 and $\alpha_v\beta_3$ neutralizing antibodies for the indicated number of days. Cells were stained with Alizarin red. The quantitative data of mineralization ability are shown in the lower panel. Each assay was performed in three independent experiments. The asterisk indicates $p < 0.05$ between rCYR61 treatment and vehicle treatment cells. The # indicates a significant difference ($p < 0.05$) between $\alpha_v\beta_3$ antibody treatment and rCYR61 treatment cells.

blast cells were decreased by treatment with integrin $\alpha_v\beta_3$ inhibitory antibody in a dose-dependent manner (Fig. 5, C–E). This evidence taken together shows that $\alpha_v\beta_3$ integrin is critical for CYR61-induced bone differentiation through a BMP-2-dependent pathway.

ILK/ERK Pathway Is Required for CYR61-induced BMP-2 Expression and Osteoblastic Differentiation—ILK has been shown to be capable of regulating integrin-mediated signaling and biological functions such as cell spreading, migration, inva-

sion, and proliferation (46). To see whether ILK is involved in CYR61-mediated signaling and osteogenesis, we first examined ILK activity after CYR61 treatment with use of an ILK kinase assay. As shown in Fig. 6A, using GSK3 β as substrate (47), ILK kinase activity increased in a time-dependent manner in response to rCYR61 stimulation, reaching a maximum between 30 to 60 min. Pretreatment with integrin $\alpha_v\beta_3$ inhibitory antibody or RGD peptide significantly decreased CYR61-induced ILK activity (Fig. 6B). Treatment with the ILK inhibitor KP392 decreased BMP-2 protein and mRNA expression (Fig. 6C). KP392 also dramatically inhibited CYR61-induced osteoblast-specific ALP activity (Fig. 6D). Afterward, we performed genetic knockdown of ILK expression using ILK-specific siRNA in MC3T3-E1 cells; we found knockdown of ILK had an inhibitory effect on rCYR61-induced BMP-2 protein secretion (Fig. 6E) and ALP activity (Fig. 6F).

It has been reported that BMP-2 expression in osteoblasts is regulated by MAPK and PI3K/Akt pathways (48, 49). Thus, we used Western blotting assay to check the phosphorylation status of some candidate signaling molecules, including JNK, p38, ERK, and Akt. As shown in Fig. 7A, treatment of MC3T3-E1 cells with rCYR61 increased ERK phosphorylation but not phosphorylation of JNK, p38, or Akt. Moreover, pretreatment with U0126 and PD98059 (MEK inhibitor) decreased CYR61-stimulated BMP-2 protein expression by ELISA assay (Fig. 7B). We further examined whether the ERK pathway is involved in CYR61-mediated osteoblastic function of MC3T3-E1 cells. CYR61-induced MC3T3-E1 osteo-

blast differentiation was inhibited not only by treatment with ERK inhibitors but also by transfection of an ERK inactive mutant form-expressing vector (Fig. 7, C and D). Furthermore, we found that CYR61-mediated ERK phosphorylation was inhibited by treatment with ILK-specific siRNA or the ILK chemical inhibitor KP392 (Fig. 7E). These results indicate that the $\alpha_v\beta_3$ integrin/ILK/ERK pathways are involved in CYR61-induced BMP-2 up-regulation and subsequent osteoblastic differentiation in MC3T3-E1 osteoblast cells.

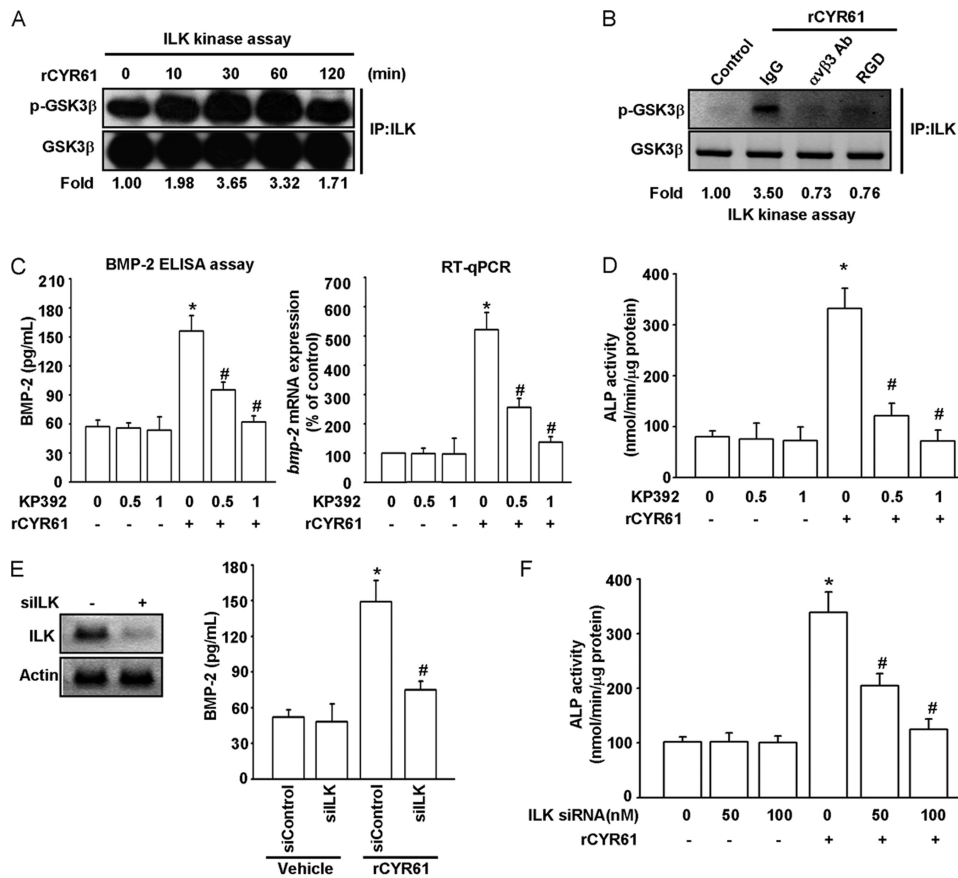


FIGURE 6. ILK activity is involved in CYR61-induced BMP-2 expression and osteoblastic differentiation. *A*, cells were incubated with rCYR61 (100 ng/ml) for the indicated times. Cell lysates were collected and analyzed by immunoprecipitation (IP)/immunoblotting (anti-ILK/anti-p-GSK3 β). The internal loading control was GSK3 β . The quantitative data are shown in the *bottom*. *B*, cells were pretreated with $\alpha_v\beta_3$ antibody (10 μ g/ml) and RGD (100 nM) for 30 min followed by stimulation with CYR61 (100 ng/ml) for the indicated times. Cell lysates were then collected and analyzed by immunoprecipitation/immunoblotting (anti-ILK/anti-p-GSK3 β). The internal loading control was GSK3 β . Quantitative data are shown in the *bottom*. *C*, cells were incubated with rCYR61 (100 ng/ml) and ILK inhibitor KP392 at the indicated doses. The mRNA levels of BMP-2 were determined using qPCR (*left*), and the protein levels were determined using ELISA assay (*right*). *D*, cells were incubated with rCYR61 (100 ng/ml) and ILK inhibitor KP392 at the indicated doses. Osteoblastic differentiation was examined using an ALP activity assay. *E*, cells were transfected with control and ILK siRNA for 24 h. ILK protein level was determined using Western blot analysis (*left panel*). BMP-2 protein level was determined using an ELISA assay (*right panel*). *F*, cells were transfected with ILK siRNA at indicated doses and incubated with CYR61 (100 ng/ml) for the indicated times. Osteoblastic differentiation was determined using an ALP activity assay. Each assay was performed in at least three independent experiments. The asterisk indicates $p < 0.05$ between rCYR61 treatment and vehicle treatment cells. The # symbol indicates significant difference ($p < 0.05$) between KP392 or siILK treatment and rCYR61 treatment cells.

DISCUSSION

In this study with osteoblasts, elevation of BMP-2 mRNA and protein levels followed recombinant CYR61 protein treatment through integrin $\alpha_v\beta_3$ receptor induction of the ILK and ERK signaling pathway. Our findings provide the first evidence that BMP-2-dependent osteoblastic differentiation may be regulated by CYR61, providing a link and molecular mechanism between CCN family and BMP family proteins in the physiology of bone.

CCN proteins are known to be involved in development, homeostasis, and repair of mesenchymal tissues. CCN proteins have four modules; they are an insulin-like growth factor-binding protein domain (module I), a Von Willebrand factor domain (module II), a thrombospondin-homology domain (module III), and a cysteine knot, heparin binding domain (module IV). Because the amino acid sequences

of human CYR61 protein are very similar to mouse CYR61 protein (35), our experimental model seems to be appropriate. It has been reported that BMP-2 can induce expression of several CCN family proteins such as CYR61, Connective Tissue Growth Factor, WISP1, and WISP2 (50). Our results show for the first time that CYR61 can also act as an inducer to enhance BMP-2 expression in osteoblast cells (Fig. 2). Taking together our data and the data of others, we hypothesize there might be a feedback regulatory loop between CYR61 and BMP-2 or further CCN family and BMP family members. Feedback mechanism and other biological functions require further examination.

BMPs play an important role in bone tissue formation and remodeling (51). It has been well documented that stimulation of osteoblastic differentiation is characterized mainly by increased expression of AP, type I collagen, and osteocalcin (51). BMP-2 has been shown to activate SMAD signaling, but it inhibits the p38 MAPK and PI3K/p70 S6K signaling pathway, which is involved in osteoblastic differentiation (52, 53). Our study indicates that production of BMP-2 increased in rCYR61-treated MC3T3-E1 cells and that treatment with BMP-2-neutralizing antibody or BMP-2-specific siRNA can inhibit MC3T3-E1 osteoblastic cell proliferation, migration, and differentiation.

Integrin-linked kinase (4) was discovered in 1996; its kinase activity is stimulated by integrins and soluble mediators including growth factors and chemokines (46). The same paper reported that the function of ILK in bone formation is promotion of chondrocyte proliferation, adhesion, and spreading. However, the role of ILK in osteoblastic differentiation is still unclear (46). We present in this study the first evidence to show that ILK also plays a critical role in CYR61-mediated BMP-2 expression and subsequent osteoblastic differentiation (Fig. 6). Previous research showed that ILK was not required for some specific conditions, such as cyclic strain-mediated ERK1/2 activation (54). In our study we found ILK is involved in CYR61-mediated ERK activation. This discrepancy may be due to different cell types as well as different experimental conditions. It has been reported that ILK modulates cell spreading, migration, and cytoskeletal organization by activating p21-Activated Kinase interactive exchange factor (α -PIX, also known as ARHGAP6),

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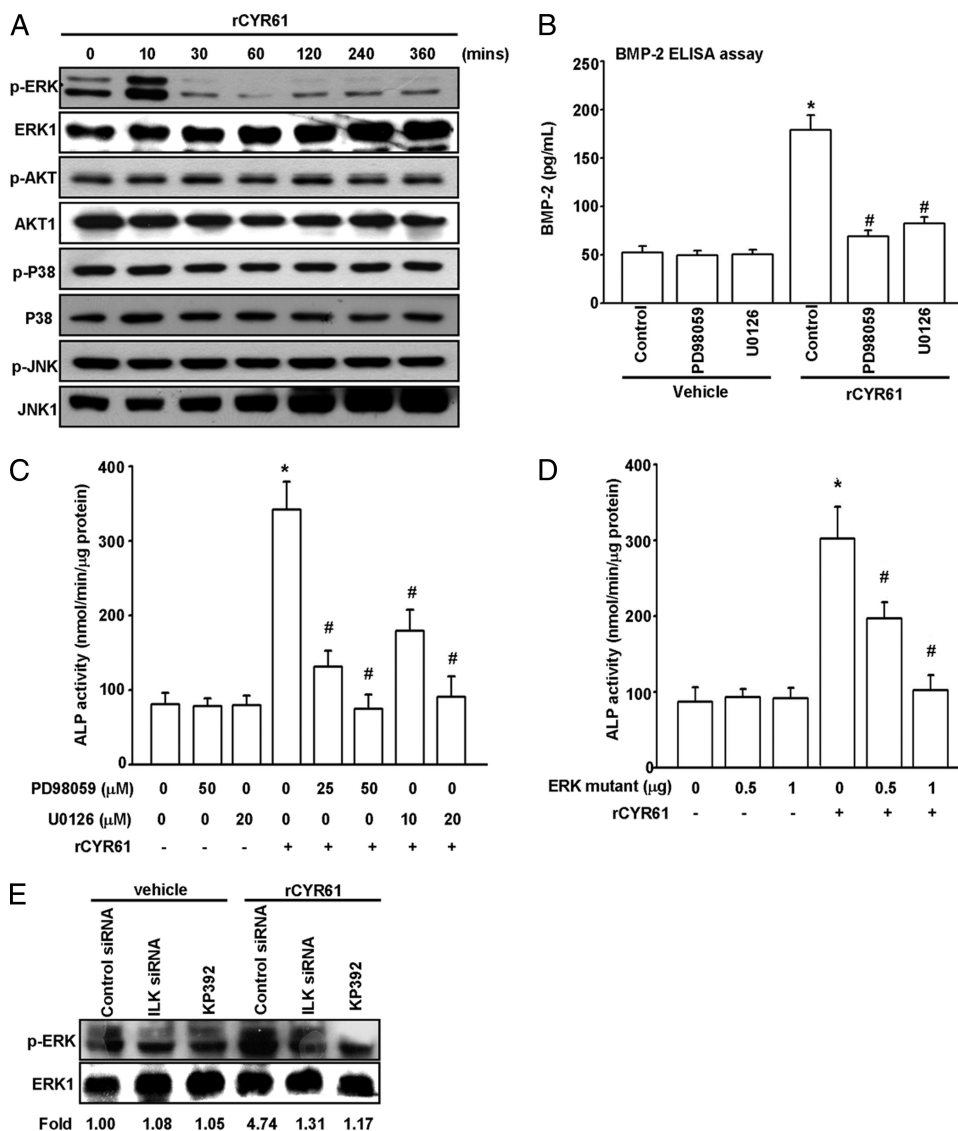


FIGURE 7. CYR61-induced ERK phosphorylation is essential for BMP-2 expression and osteoblastic differentiation. *A*, cells were plated at 5×10^5 in 6-cm plates. After a 24-h starvation, rCYR61 at 100 ng/ml was added to MC3T3-E1 cells for the indicated times. Whole cell extracts were prepared, and 40 μ g total protein of lysate was subjected to Western blot analysis using antibody against phosphorylated ERK (*p-ERK*), phosphorylated AKT (*p-Akt*), phosphorylated JNK (*p-JNK*), and phosphorylated p38 (*p-p38*). The Western blot was stripped and probed with antibody against total ERK, AKT, JNK, p38 (ERK1, AKT1, JNK1, and p38) to confirm that the same amounts of whole-cell extracts were analyzed. *B*, cells were incubated with rCYR61 (100 ng/ml) and MEK inhibitor PD98059 and U0126 at the indicated doses. BMP-2 protein level was determined using an ELISA assay. *C*, cells were incubated with rCYR61 (100 ng/ml) and MEK inhibitor PD98059 and U0126 at the indicated doses. Osteoblastic differentiation was determined using an ALP activity assay. *D*, cells were transfected with ERK-mutated vector at indicated doses followed by stimulation with rCYR61 (100 ng/ml). Osteoblastic differentiation was determined using an ALP activity assay. *E*, cells were transfected with ILK siRNA or KP392 at the indicated doses and incubated with CYR61 (100 ng/ml) for the indicated times. The phosphorylation activity of ERK was determined using Western blot analysis. Each assay was performed in at least three independent experiments. The asterisk indicates $p < 0.05$ between rCYR61 treatment and vehicle treatment cells. The # symbol indicates significant difference ($p < 0.05$) between inhibitors or ERK mutant vector treatment and rCYR61 treatment cells.

a guanine-nucleotide exchange factor for Rac1 and Cdc42 (55). Other studies have indicated that Rac/Cdc42 activates the ERK pathway and promotes cell migration (56). Considering previous studies and our current data raises the possibility that ILK might regulate ERK through indirect interaction.

The ERK1/2 signaling pathway is important in osteoblast cell proliferation and differentiation (57, 58). A number of studies have reported that ERK is an important mediator of BMP-2-

induced osteoblastic differentiation and that inhibition of ERK1/2 results in suppression of differentiation markers (59). Suzuki *et al.* (67) reported that ERKs play an essential role in cell replication during osteoblastic differentiation. In our study phosphorylation of ERK1/2 rapidly increased within 10 min of rCYR61 treatment and then decreased by 30 min (Fig. 7A). Furthermore, MEK inhibitors PD98059 and U0126 both inhibited BMP-2 protein production (Fig. 7B). Thus, we demonstrated that CYR61-induced BMP-2 expression is regulated via the ERK pathway.

The murine homologue of CYR61 is an extracellular matrix-associated protein that modulates basic fibroblast growth factor signaling, angiogenesis, and binds to integrin $\alpha_v\beta_3$ (44). In the human breast cancer cell line MCF-7, overexpression of CCN1/Cyr61 up-regulates $\alpha_v\beta_3$ and binds to it to activate ERK1/2 MAPK, thus promoting cell proliferation and survival (60). In the present study, integrin $\alpha_v\beta_3$ was proven able to regulate rCYR61-induced BMP-2 expression, osteoblast cell proliferation and migration, and osteoblastic differentiation (Fig. 5). Furthermore, blockade of integrin $\alpha_v\beta_3$ inhibited ERK1/2 phosphorylation and BMP-2 protein expression. Thus, these results indicate that rCYR61-induced BMP-2-dependent osteoblastic differentiation is mediated through integrin $\alpha_v\beta_3$.

In addition to the BMP pathway that we have demonstrated here to be involved in CYR61 action on osteoblasts, the canonical Wnt pathway is also an important regulator of CYR61 activity in bone cells, as we described in the Introduction (37, 39). It has been reported that Wnt3a treatment significantly increased CYR61 mRNA levels in osteoblast-like cells (37), suggesting that CYR61 is potential downstream target of the Wnt signaling in controlling osteoblast maturation. Previously, we have found that the Wnt/ β -catenin signaling pathway is a powerful enhancer of BMP2 gene expression in osteoblasts (23). However, we were not able to identify the functional binding site(s) in the BMP2 promoter responsible for β -catenin/TCF4 transactivation. Based on the results of current study, we reason that the stimulation of BMP2

expression by Wnt signaling in osteoblasts is likely mediated through CYR61. Not only regulating CYR61 gene expression, the recent report of Craig *et al.* (61) suggests that Wnt pathway may also directly bind to CYR61 and modulate CYR61 activity on osteoblasts. They found that Sclerostin, a proven endogenous antagonist that blocks both BMP and Wnt signaling pathways by binding to BMP ligands (62, 63) or Wnt receptor LRP5 (64, 65), can directly interact with CYR61 protein and affect proliferation of mature MC3T3/E1 osteoblast cells. The results from another study have suggested that Wnt induces osteoblast differentiation through BMPs, which is blocked by BMP antagonist Sclerostin, and the expression of BMP proteins in this autocrine loop is essential for Wnt-3A-induced osteoblast differentiation (66). Given the fact that both Wnt and BMP signaling pathways are extremely critical for osteoblast differentiation and bone formation, we think that, as an active coordinator in the cross-talk between these anabolic pathways, CYR61 and its signaling mechanism are a potential molecular target for identifying drugs that stimulate bone formation.

In summary, BMP-2-dependent osteoblastic differentiation may be regulated by CYR61 in pre-osteoblastic cells. The effects of rCYR61 on osteoblast cell maturation are strongly associated with BMP-2 production followed by ERK1/2 activation. Therefore, data suggest that rCYR61 may be beneficial in stimulating osteoblastic activity and promoting formation of bone tissue through integrin $\alpha_v\beta_3$ /ILK/ERK signaling.

Acknowledgment—We thank RNAi Core (Academia Sinica, Taiwan) for providing specific siRNAs.

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