

# CYR61/CCN1 stimulates proliferation and differentiation of osteoblasts *in vitro* and contributes to bone remodeling *in vivo* in myeloma bone disease

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**Abstract.** Cysteine-rich 61 (CYR61/CCN1), a secreted protein in bone marrow (BM) microenvironment, has diverse effects on many cellular activities such as growth and differentiation. However, the effect of CCN1 on osteoblasts (OBs) in myeloma bone disease remains unclear. In our study, the level of CCN1 in multiple myeloma (MM) patients was detected by ELISA and RT-PCR. The proliferation and differentiation of OBs from MM patients were observed after stimulated by CCN1 *in vitro*. The myeloma cells transduced with CYR61 gene (RPMI-8226/CYR61) were injected in a mouse model to evaluate the efficacy of CCN1 *in vivo* and compare with zoledronic acid. The results showed that CYR61/CCN1 levels in BM supernatant and OBs both elevated significantly in all newly diagnosed MM patients, especially in patients without bone disease ( $P=0.001$  and  $P<0.001$ ). After 30 ng/l CCN1 stimulation for 24 h, the quantity and mineralization of OBs increased significantly *in vitro* ( $P=0.046$  and  $0.048$ ). The transcription factors of Wnt pathway, runt-related transcription factor 2 (Runx2) and  $\beta$ -catenin were upregulated in OBs after CCN1 stimulation ( $P=0.012$  and  $0.011$ ). After injection of RPMI-8226 cells, bone lesions were observed obviously by microCT and histochemistry at 7 weeks. Radiographic analysis of the bones showed decreased resorption in CCN1 overexpression group and zoledronic acid group, while severe resorption in negative control. Furthermore, trabecular bone volume in CCN1 overexpression group ( $1.7539\pm0.16949$ ) was significantly higher than zoledronic acid group ( $1.2839\pm0.077$ ) ( $P=0.012$ ). In conclusion, CCN1 can stimulate the proliferation

and differentiation of OBs *in vitro* and contribute to bone remodeling *in vivo* in MBD.

## Introduction

Multiple myeloma (MM) is a hematologic malignancy of terminally differentiated plasma cells. A most common complication of MM is myeloma bone disease (MBD), occurring in >80% of MM patients. Bone lesions in MM are the result of imbalance between osteoclasts (OCs) and osteoblasts (OBs), the activation of OCs and inhibition of OBs (1,2). Many studies have focused on the function of OCs and found RANKL/RANK system, which became the target of treatment (3,4). However, osteolytic lesions still exist after the inhibition of OCs, which is associated with the inhibition of OBs. Recent research showed many cytokines in bone marrow (BM) microenvironment participate in the pathogenesis of MBD, such as CCL3 (5,6), activin A (7), alpha hypoxia-inducible factor (HIF-1 $\alpha$ ) (8), pim-2 (9), cysteine-rich protein 61 (CYR61) (10), which may be new targets in MBD.

CYR61 is the first cloned member of the CCN family, an immediate early gene family consisting of six members, CTGF, NOV, WISP-1, WISP-2 and WISP-3 (11,12). CYR61 is known to link cell surface and extracellular matrix and plays important roles on cell adhesion, proliferation, migration, differentiation and angiogenesis during normal developmental and pathophysiological processes (11). Clines *et al* (13) found that tumor-produced endothelin-1 increased osteoblasts proliferation and new bone formation by activating Wnt signaling pathway through suppression of Wnt pathway inhibitor DKK1 in murine primary OBs culture. Si *et al* (14) suggested that tightly regulated CCN1/CYR61 expression may play an important role in Wnt3A-induced OBs differentiation from mesenchymal stem cells. In MC3T3-E1 osteoblasts, CYR61 upregulated BMP-2, resulting in enhanced cell proliferation and osteoblastic differentiation through activation of the  $\alpha(v)\beta(3)$  integrin/integrin-linked kinase/ERK signaling pathway (15). The study of Chen *et al* (16) in osteoarthritis indicated that CCN1 heightened OSM expression via  $\alpha(v)\beta(3)$  receptor, FAK, c-Src, PI3K, and NF- $\kappa$ B pathway in osteoblastic cells. Johnson *et al* (10) found that recombinant CCN1 directly inhibited growth of MM cells *in vitro*, and overexpression of CYR61 in

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MM cells prevented bone destruction *in vivo*. However, still scarce research exists of CCN1 on OBs in MM patients. In our study, we cultured OBs from MBD patients with recombinant CCN1 and observed the effect of CCN1 on OBs *in vitro*, then further investigated the effect of CCN1 on bone remodeling *in vivo*.

## Patients and methods

**Study subjects.** Thirty-six newly diagnosed MM patients (20 males and 16 females) were enrolled in this study. The participants were selected as inpatients in the Hematology Department of Tianjin Medical University General Hospital from January 2015 to November 2016 according to the International Myeloma Workgroup criteria. According to X-ray scanning data obtained before treatment, bone disease was graded into three stages: stage A, no osteolytic lesions or osteoporosis alone; stage B, one to three osteolytic lesions; stage C, more than three osteolytic lesions and/or a pathological fracture (17). There were 9 patients in stage A and 27 in stage B/C in our study. The characteristics of the patients are shown in Table I. Eighteen healthy volunteers with a median age of 48 years (range, 42–69 years) were included as normal controls. Bone marrow aspirations were collected from all the patients diagnosed with MM and from normal controls. This study was approved by the Ethics Committee of the Tianjin Medical University. Written informed consent was obtained from the patients for the publication of this report and any accompanying images.

**Enzyme-linked immunosorbent assay.** The CCN1 level in the bone marrow supernatant was assessed using the CCN1 ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA). Human paraprotein (Lambda) level in the mouse model was detected using the Human Lambda ELISA kit (Bethyl Laboratories, Inc., Montgomery, TX, USA). Diluted standards and patient serum (100  $\mu$ l) were added in duplicate and incubated at 37°C for 1 h. After washing the plates 5 times, 100  $\mu$ l of antibody was added to each well and incubated at room temperature for 1 h. Next, HRP was added to each well. After incubation at 37°C for 30 min, the wells were washed 5 times. Then, TMB solution was added to each well, and the samples were incubated in the dark at room temperature for 20 min. Finally, a stop solution was added, and the OD was read at 450 nm within 15 min.

**Cell culture.** The bone marrow mononuclear cells (BMMNC) were separated using Ficoll-Hypaque density sedimentation. The BMMNCs were cultured in Dulbecco's modified Eagle's medium/F12 medium supplemented with 15% fetal bovine serum (Gibco, Darmstadt, Germany),  $1 \times 10^{-7}$  mol/l dexamethasone, 0.05 g/l vitamin C, 0.01 mol/l  $\beta$ -sodium glycerophosphate, 100 g/ml penicillin (Gibco), and 100 U/ml streptomycin (Gibco). Non-adherent cells were removed the next day, and the media were replaced every other day. Adherent BMMNCs were cultured at 37°C in an atmosphere containing 5% CO<sub>2</sub>. The OBs were counted and seeded in 24-well plates at a plating density of  $1 \times 10^4$  cells/cm<sup>2</sup>. Trypsin was used to detach three wells of OBs for cell count every second day. The number of cells was used to draw the OB growth curve. The OB doubling

time (DT) was calculated using the following formula:  $DT = t \times [\lg 2 / (\lg Nt - \lg No)]$ , where  $t$  is the culturing period (h);  $No$  is the cell density when the cells were seeded; and  $Nt$  is the cell density when the cells were cultured after  $t$  hours.

The OBs were divided into two groups: the blank group and CCN1 group (OBs were cultured with CCN1: 10 and 30 ng/l). The changes in the OB osteogenic potential and biological characteristics were observed after intervention.

**Quantitative real-time PCR.** The total RNA from the OBs of each group was extracted using the TRIzol reagent (Invitrogen). The TIANScript RT kit (Tiangen, Beijing, China) was utilized to reverse-transcribe 1  $\mu$ g of RNA. Table II presented the primer sequences of runt-related transcription factor 2 (Runx2),  $\beta$ -catenin, CYR61, bone morphogenetic protein-2 (BMP2) and GAPDH. These primer sequences were designed and synthesized by Sangon Biotech (Shanghai, China).

The quantitative real-time PCR was performed using the Bio-Rad iQ 5 Real-time system (Bio-Rad, Hercules, CA, USA). The SYBR Green (Invitrogen) was used as a double-strand DNA-specific dye. The amplification of CYR61 and Runx2 utilized 40 cycles at 95°C for 30 sec and 95°C for 5 sec with the extension at 57°C for 30 sec. The amplification of BMP2 and  $\beta$ -catenin both utilized 45 cycles at 95°C for 30 sec and 95°C for 5 sec with the extension at 55°C for 30 sec. GAPDH was employed as the housekeeping gene to standardize the targeted mRNA expression. The CYR61, Runx2,  $\beta$ -catenin and BMP2 levels were calculated using the  $2^{-\Delta\Delta Ct}$  method [ $(Ct, \text{target gene } Ct, \text{ GAPDH})_{\text{sample}} - (Ct, \text{target gene } Ct, \text{ GAPDH})_{\text{control}}$ ] after normalizing the data according to the GAPDH mRNA expression.

**ALP and Von Kossa staining.** The alkaline phosphatase (ALP) expression was an early osteoblast marker detected using the ALP Staining kit (Sigma-Aldrich, Taufkirchen, Germany). The expression was utilized to confirm the presence of OB. Von Kossa staining was performed to confirm that the OB synthesized and mineralized the extracellular matrix. The mineralized nodules were found in both groups cultured for 3 weeks. The amount of mineralized nodules in each patient was counted and used as an indicator of OB function.

**Lentiviral vector and myeloma cell transduction.** The sequence of cDNA for human CYR61 (GENE-ID 3491) was cloned into the Ubi-MCS-SV40-EGFP-IRES-puromycin vector. Lentiviral particles were generated by cotransfecting the expression vector Ubi-MCS-SV40-EGFP-IRES-puromycin and ViraPower Packaging Mix into 293T cells according to the Invitrogen ViraPower Lentiviral Expression Systems protocol (18).

Myeloma cells (RPMI-8226) were plated at a density of  $5 \times 10^4$  cells per well in a 6-well plate. For each of the following 3 days, cells were exposed to viral supernatant at  $1 \times 10^8$  TU/ml in the presence of polybrene (5  $\mu$ g/ml). Transduced RPMI-8226/CYR61 cells were used for experiments *in vivo*. The efficiency of transduction was determined using analysis of GFP expression. We detected the expression of CYR61/CCN1 by qRT-PCR, ELISA and western blotting. In addition, the proliferation of non-transduced RPMI-8226, transduced with CYR61 or EV RPMI-8226 cells was measured by CCK-8 kit.

Table I. Profiles of patients with MM enrolled in this study.

Total no. of patients	36
Gender	20 M-16 F
Age-median (range)	68 (43-80)
Type of MM	IgG (17), IgM (7), IgA (5), light-chain (6), non-secretory (1)
Stage at diagnosis (ISS)	
I	6 (17%)
II	8 (22%)
III	22 (61%)
Bone disease	
Stage A	9 (25%)
Stage B/C	27 (75%)
Parameters at baseline	
Creatinine clearance >30 ml/min	18 (50%)
Creatinine clearance ≤30 ml/min	18 (50%)
Hb ≥100 g/l	7 (19%)
Hb <100 g/l	29 (81%)
Ca >2.75 mmol/l	7 (19%)
Ca ≤2.75 mmol/l	29 (81%)

MM, multiple myeloma; Hb, hemoglobin; Ca, calcium.

**Animal model of myeloma bone disease.** Female NOD/SCID<sup>cc</sup> null mice at 5 weeks of age (the Chinese Academy of Medical Sciences Institute of Radiation Medicine) were acclimatized to the animal housing facility for 1 week before experiments. All of the experimental procedures on animals were carried out with strict adherence to the rules and guidelines for the ethical use of animals in research.

Fifteen microliter of the cell suspension ( $1 \times 10^6$  cells per inoculum) were injected directly into the marrow space of right tibia. The needle was inserted 3-5 mm down the diaphysis of the tibia through the cortex of the anterior tuberosity. The left contralateral tibia was injected with PBS alone as an internal control (19). Ten mice were investigated for myeloma cell infiltration and bone disease at 3, 5 and 7 weeks after transplantation, respectively.

In the next experiment, mice were randomized into three groups (bone disease group as a control, zoledronic acid group and CCN1 overexpression group) of 10 mice per group. For the control and zoledronic acid groups, RPMI-8226/EV myeloma cells were injected into the marrow space of right tibia. In parallel, RPMI-8226/CYR61 cells were injected to the mice of CCN1 overexpression group. From the fourth week after myeloma cell injection, mice of zoledronic acid group were injected subcutaneously with antiresorptive bisphosphonate zoledronic acid (Novartis Pharma) at 100  $\mu$ g/kg/dose once weekly for 4 weeks. All the mice were sacrificed at 7 weeks after myeloma cell implantation.

**Analysis of bone disease (microCT).** Tibiae were fixed in formalin and scanned using a microCT scanner (model 1172;

Table II. Primer sequences.

Target	Sense and antisense sequences	bp
Runx2	F: 5'-GACGAGGCAAGAGTTTCACC-3' R: 5'-GGTTCCTGAGGTCCATCTAC-3'	106
$\beta$ -catenin	F: 5'-GCTTTGTGTGCTCTTGAACG-3' R: 5'-TCAGCAATCCCTTTCTCACC-3'	122
CYR61	F: 5'-GAAGCGGCTCCCTGTTTTTG-3' R: 5'-CGGGTTTCTTTTCAAGGCG-3'	174
BMP2	F: 5'-TCAAGCCAAACACAAACAGC-3' R: 5'-AGCCACAATCCAGTCATTCC-3'	103
GAPDH	F: 5'-GCACCGTCAAGGCTGAGAAC-3' R: 5'-TGGTGAAGACGCCAGTGA-3'	100

bp, base pairs.

Skyscan) at 50 kV and 200 AA with a pixel size of 4.3  $\mu$ m, an image being captured every 0.7° through 180° rotation of each bone. During scanning, the samples were enclosed in a tightly fitting rigid plastic tube to prevent movement, scanned images were reconstructed and analyzed using the Skyscan Recon and Skyscan CT analysis software, respectively. The following three-dimensional (3D) parameters were measured: total bone volume/total volume, bone surface area/bone volume, trabecular thickness, trabecular number, trabecular spacing and trabecular bone volume (20).

**Immunohistochemistry and histochemistry in the mouse model.** Immunohistochemistry (IHC) staining was performed using formalin-fixed, complete decalcification was confirmed with 2 weeks of decalcification in 0.5 mol/l EDTA/0.5% paraformaldehyde in PBS (pH 8.0) at 4°C and tibiae were then paraffin-embedded. Longitudinal sections (5  $\mu$ m) were prepared and stained with IHC. Anti-CD138 antibody (sc-69833 Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200) was used. Tartrate-resistant acid phosphatase (TRAP) staining, a well-recognized marker of osteoclasts *in vivo*, was performed with an acid phosphatase kit (Sigma, St. Louis, MO, USA). Analysis was done on an Olympus CX41 microscope (21).

**Statistical analysis.** SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analysis. The data are expressed as the mean  $\pm$  SD or median. The unpaired t-test and one-way ANOVA analysis were used to analyze the significance between different groups. Non-parametric test was used when the data were of abnormal distribution. A value of  $P < 0.05$  was considered statistically significant.

## Results

**CYR61/CCN1 is elevated significantly in all newly diagnosed MM patients, especially in patients without bone disease (stage A).** We detected CCN1 level in BM supernatant by ELISA in newly diagnosed patients and normal controls. The levels of CCN1 in the patients in stage A and stage B/C were

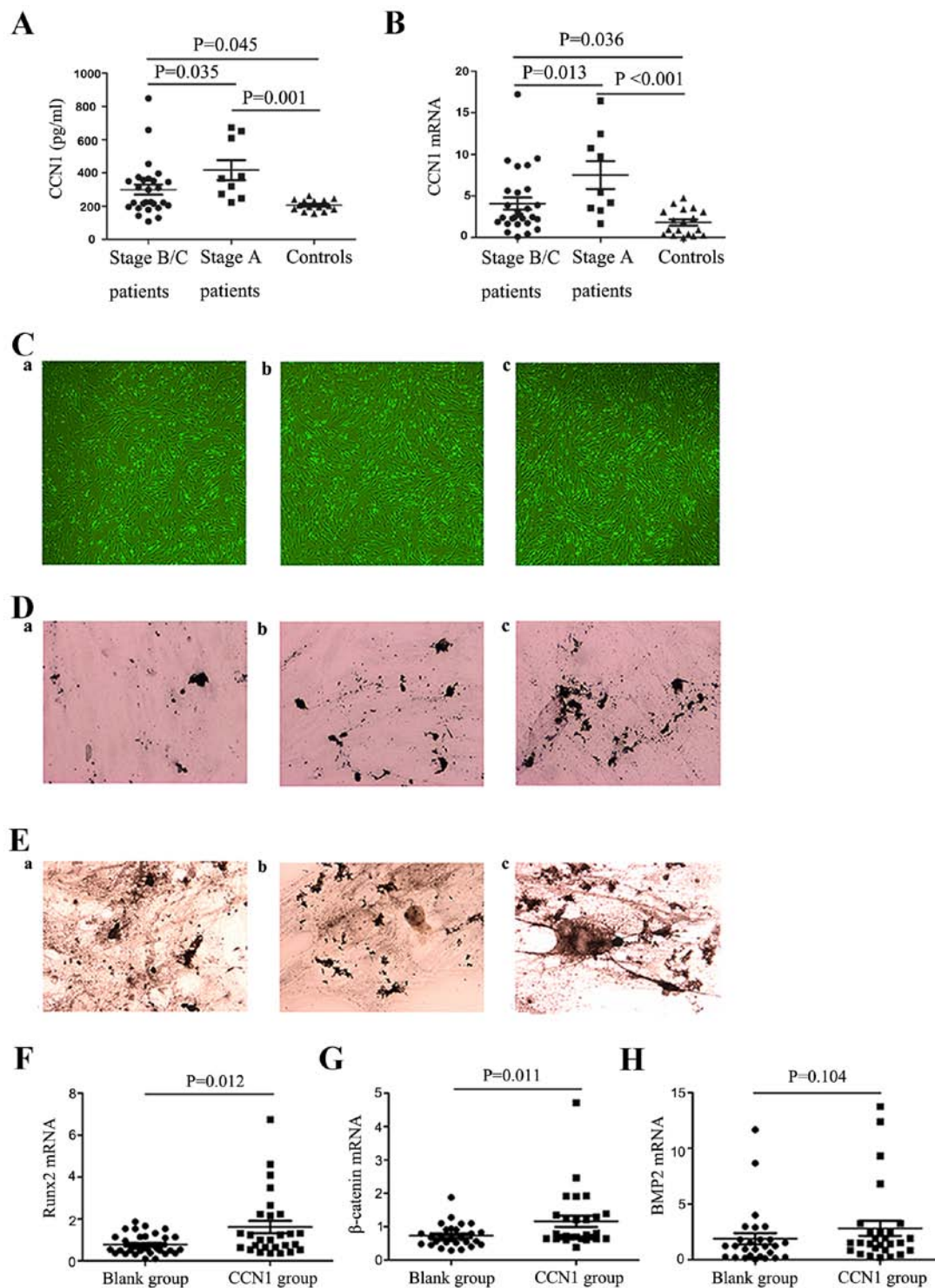


Figure 1. The level of CCN1 in newly diagnosed patients and the effect of CCN1 on OBs *in vitro*. (A) The level of CCN1 in BM supernatant in patients in stage A and B/C were both significantly higher than the normal controls. (B) The mRNA level of CYR61 in patients in different stages were significantly higher than the normal controls. (C) The quantity of OBs increased in 10 ng/ml CCN1 (b) and 30 ng/ml CCN1 (c) than the blank (a). (D) The amount of mineralized nodules cultured with 30 ng/ml CCN1 (c) was also significantly increased compared to the blank group (a), while there was no significant difference in 10 ng/ml CCN1 group (b). (E) ALP staining became strongly positive after CCN1 stimulation [10 ng/ml (b) and 30 ng/ml (c)] compared with control (a). Runx2 (F) and β-catenin (G) mRNA levels were significantly increased after 30 ng/ml CCN1 stimulation. While BMP2 level had no significant change (H).

416.90±179.78 and 299.48±160.65 pg/ml, which were both significantly higher than normal controls [202.12±41.53 pg/ml (P=0.001 and 0.045)] (Fig. 1A). Also, the level of CCN1 in stage A was significantly higher than stage B/C (P=0.035), indicating that CCN1 may have a promoting role in bone remodeling.

Previous research has shown that CYR61 was not expressed in normal plasma cells, MM cells, osteoclasts, but highly expressed in MSCs (10). We further detected the expression of CYR61 in OBs by real-time PCR. The median CYR61 mRNA levels in the patients in stage A and stage B/C were 5.45 (0.08-17.23) and 2.46 (1.68-16.48), both significantly increased

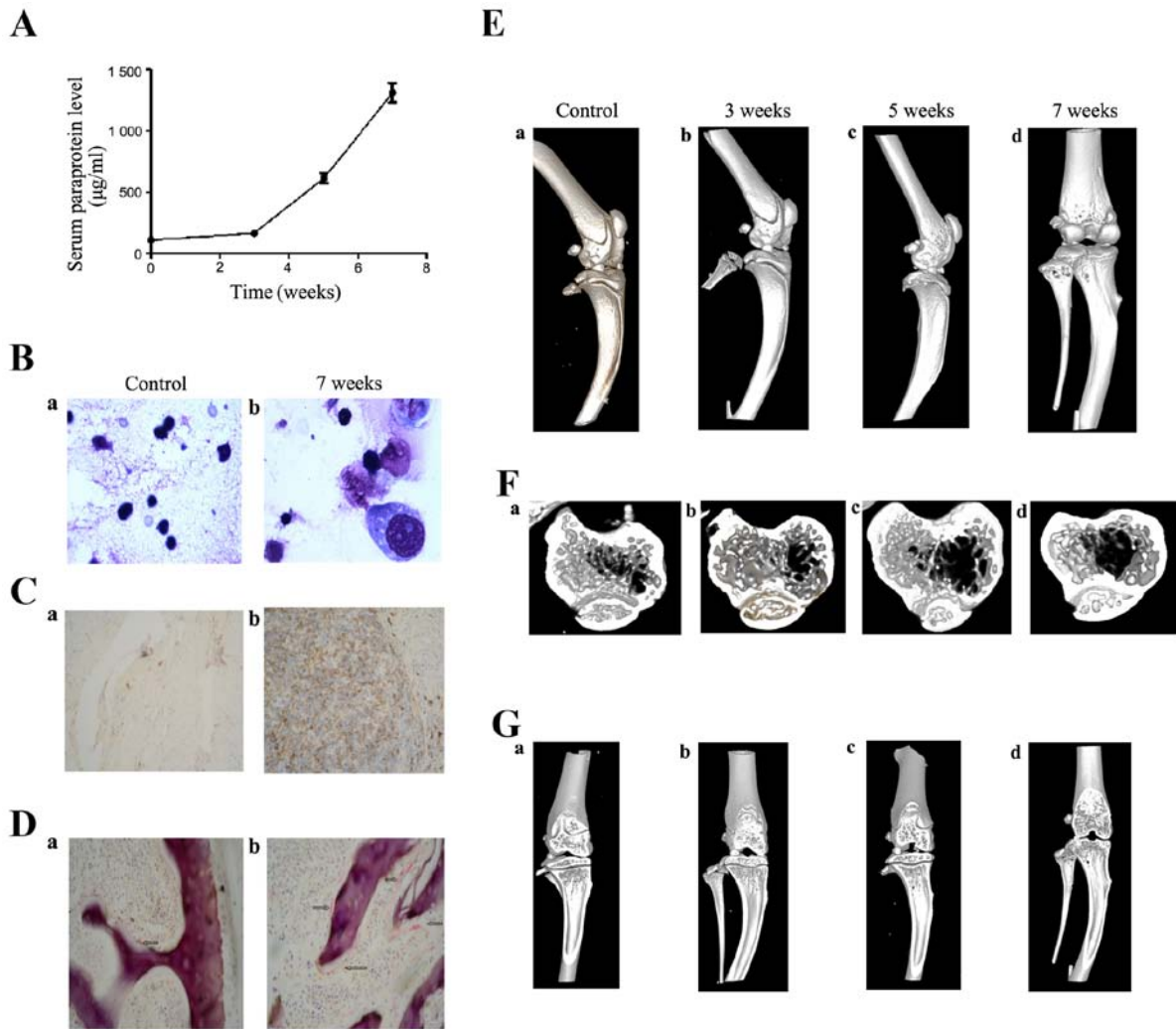


Figure 2. Myeloma bone disease mouse model. (A) Myeloma cells (RPMI-8226) were injected directly into the marrow space of right tibiae of NOD/SCID $\delta\delta$  null mice, PBS injected into mice as controls. Serum monoclonal Ig level of mice was detected by ELISA at 0, 3, 5 and 7 weeks after the myeloma cell injection. (B) Myeloma cells in BM were observed by H&E staining in the mouse model at 7 weeks after injection (b) compared with controls (a) (x400). (C) CD138 staining by immunohistochemistry positive in the mouse model (b), while negative in controls (a) at 7 weeks after injection (x400). (D) TRAP staining was positive as indicated by the arrows in mouse model (b) while negative in controls (a) 7 weeks later (x400). Bone damage by microCT were observed in the mouse model from lateral (E-b-d), coronal (F-b-d) and sagittal scan (G-b-d) at 3, 5 and 7 weeks after the myeloma cell injection, compared with the controls (E-a, F-a and G-a).

compared with normal controls (1.47, 0.01-4.76) ( $P<0.001$  and  $P=0.036$ ). Similarly, the level of stage A was significantly higher than stage B/C ( $P=0.013$ ) (Fig. 1B).

*CCN1 promotes proliferation and mineralization of OBs in vitro.* The OBs from the patients in stage B/C were incubated without or with CCN1 (10 and 30 ng/l) for 24 h *in vitro* (16) and the quantity and function of OBs were subsequently observed. Matrix formation and mineralization were also investigated by histochemistry. ALP and Von Kossa staining positively identified the OBs. The quantity and function of OBs both continuously increased with increasing CCN1 concentration (Fig. 1C-E). The OB quantity cultured with different CCN1 concentrations (10 and 30 ng/l) were  $3.01\pm0.49\times10^5/\text{ml}$  and  $3.39\pm1.21\times10^5/\text{ml}$ , while there was significant difference between the blank group and 30 ng/l CCN1 group ( $P=0.046$ ). The amount of mineralized nodules cultured with 30 ng/l CCN1 ( $14.33\pm5.72/\text{HPF}$ ) was also significantly increased compared to the blank group ( $9.11\pm0.97/\text{HPF}$ ) ( $P=0.048$ ),

Table III. Quantity and function of OBs cultured with different concentration of CCN1.

	No. of cells ( $10^5/\text{ml}$ )	No. of mineralized nodules (/HPF)
Blank	$2.72\pm0.24$	$9.11\pm0.97$
CCN1 (10 ng/l)	$3.01\pm0.49$	$11.82\pm3.76$
CCN1 (30 ng/l)	$3.39\pm1.21^a$	$14.33\pm5.72^a$

<sup>a</sup>Significantly higher than that in the blank group.

while there was no significant difference in 10 ng/l CCN1 group ( $11.82\pm3.76/\text{HPF}$ ) (Table III). According to these results, we found that suitable concentration of CCN1 stimulating the OBs was 30 ng/l. Therefore, we detected the Runx2,  $\beta$ -catenin and BMP2 by real-time PCR in 30 ng/l CCN1 incubation, not 10 ng/l CCN1.

Table IV. The three-dimensional (3D) parameters in myeloma bone disease mouse model.

	Total bone volume/ total volume	Bone surface area/ bone volume	Trabecular thickness	Trabecular number	Trabecular spacing	Trabecular bone volume (mm <sup>3</sup> )
Controls	0.5224±0.06294	24.5472±7.14071	0.0879±0.02992	5.4937±0.6331	0.0962±0.00779	1.533-0.20391
3 weeks	0.4959±0.01369	23.8545±2.32456	0.0844±0.00797	5.9045±0.41117	0.0855±0.00359	1.3563±0.0498
5 weeks	0.3650±0.06294 <sup>a</sup>	28.1016±1.16317	0.0713±0.00301	6.4713±0.27484	0.0835±0.00603	1.1042±0.07738 <sup>a</sup>
7 weeks	0.3218±0.06155 <sup>b</sup>	33.4926±8.32048	0.0620±0.01372	5.799±0.618	0.1118±0.03037	0.7912±0.12501 <sup>b</sup>

<sup>a</sup>Compared with controls, P=0.041 (5 weeks), P=0.027 (5 weeks). <sup>b</sup>Compared with controls, P=0.017 (7 weeks), P=0.004 (7 weeks).

Table V. The three-dimensional (3D) parameters in different groups.

	Total bone volume/ total volume	Bone surface area/ bone volume	Trabecular thickness	Trabecular number	Trabecular spacing	Trabecular bone volume (mm <sup>3</sup> )
Controls	0.2759±0.10325	43.1139±12.52269	0.0492±0.0148	5.5365±0.42373	0.1321±0.02678	0.7219±0.122
Zoledronic acid	0.4848±0.030 <sup>a</sup>	26.9035±1.582	0.0745±0.004	6.5056±0.014	0.0792±0.005	1.2839±0.077 <sup>a</sup>
CCN1 over- expression	0.4541±0.03383 <sup>b</sup>	25.5417±1.19068	0.0784±0.00367	5.4492±0.36592	0.0921±0.0078	1.7539±0.16949 <sup>b,c</sup>

<sup>a</sup>Compared with controls, P=0.014, P=0.003. <sup>b</sup>Compared with controls, P=0.031; P=0.001. <sup>c</sup>Compared with zoledronic acid, P=0.012.

*Runx2 and  $\beta$ -catenin upregulation in OBs after CCN1 stimulation.* The mRNA expressions of Runx2,  $\beta$ -catenin and BMP2 were analyzed to observe OB differentiation. These expressions were critical transcription factors of early OB differentiation (22). The median mRNA of Runx2 in CCN1 group (30 ng/l) was 0.9965 (range, 0.3751-9.7473), which was significantly higher than that in the blank group (0.5421, 0.0938-1.8725) (P=0.012). The mRNA of  $\beta$ -catenin had a similar result. The median mRNA of  $\beta$ -catenin after CCN1 stimulating increased significantly from 0.6538 (0.2836-1.8751) to 0.7540 (0.3893-4.7142) (P=0.011). However, the mRNA of BMP2 had no significant difference before and after CCN1 stimulation. The median mRNA of BMP2 in the blank and CCN1 group were 1.2414 (0.0488-1.167) and 1.5390 (0.2001-1.3756) (Fig. 1F-H).

*Bone disease of MM animal model.* In order to evaluate the myeloma animal model, we investigated the serum human monoclonal Ig $\lambda$  level by ELISA at 3, 5 and 7 weeks after myeloma cells injection. Compared with 3 weeks, there was a corresponding increase in Ig $\lambda$  level. The serum human Ig $\lambda$  level increased from 164.6 to 611.93 (5 weeks) and 1308.9  $\mu$ g/ml (7 weeks) (Fig. 2A).

Myeloma cells in mouse bone marrow were observed by H&E staining and immunohistochemistry (CD138) at 7 weeks. The results showed myeloma cells in tibiae (Fig. 2B) by morphology and positive CD138 expression (Fig. 2C), which confirmed that human myeloma cells were injected directly into the bone marrow of the model mice.

Furthermore, we observed bone disease by microCT at 3, 5 and 7 weeks and TRAP staining in BM at 7 weeks. TRAP

staining, which revealed increased number of osteoclasts in tibiae, was stronger in the mouse model (Fig. 2D-b) compared with controls (Fig. 2D-a). We observed the lateral, coronal and sagittal scan of mouse tibiae and analyzed several three-dimensional (3D) parameters to assess the bone disease. The results showed that bone damage existed in images from 5 weeks and became worse at 7 weeks after injection (Fig. 2E-G). Table IV shows the three-dimensional (3D) parameters. The total bone volume/total volume at 5 and 7 weeks significantly increased compared with controls (P=0.041 and 0.017). Also, trabecular bone volume at 5 and 7 weeks were also significantly higher than controls (P=0.027 and 0.004). While other parameters had no significant changes.

*CCN1 effect on bone remodeling in MBD mouse model.*

To evaluate the efficacy of CCN1 on bone remodeling, we established a CCN1 overexpression model of MM, in which transduced RPMI-8226/CYR61 cells were injected directly into the marrow tibial cavity of mice. Moreover, we used RPMI-8226/EV as negative control and zoledronic acid as positive control. Compared with RPMI-8226/EV, the transduced RPMI-8226/CYR61 cells expressed CCN1 by RT-PCR, ELISA and western blotting, but the proliferation of the cells was not affected by transduction (Fig. 3).

Radiographic analysis of the bones showed that bones exhibited decreased resorption in CCN1 overexpression group and zoledronic acid group, while severe resorption in negative control group (Fig. 4A-C). TRAP staining was negative in CCN1 overexpression group and zoledronic acid group, compared with control (Fig. 4D). Table V shows that total



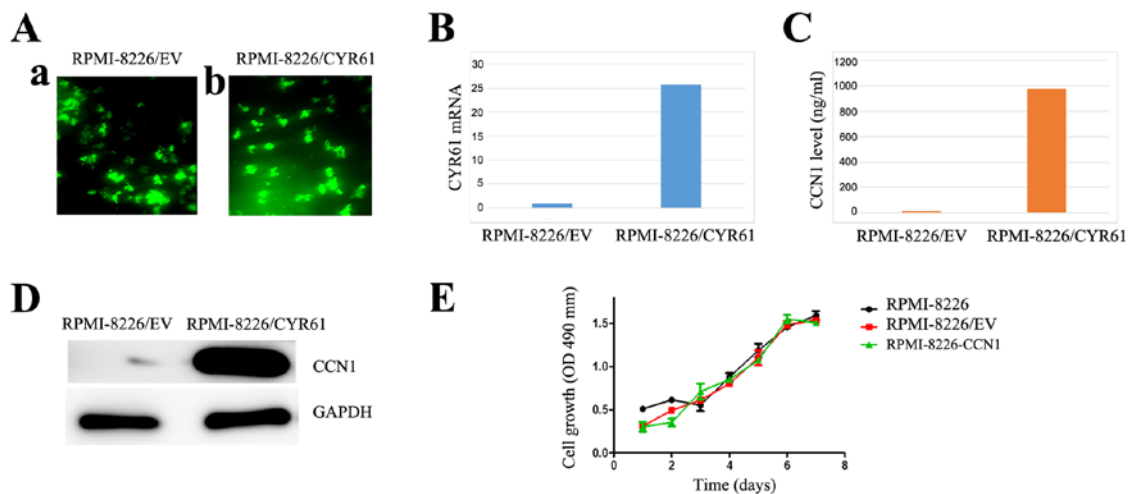


Figure 3. The transduced RPMI-8226/CYR61 cells overexpressed CCN1, while their growth was not affected. (A) RPMI-8226 cells were transduced with lentivirus expressing either CYR61-GFP or GFP only (empty vector, EV). (B) CYR61 mRNA expressed positively in RPMI-8226/CYR61 cells. (C) CCN1 levels in the conditioned medium of RPMI-8226/CYR61 or RPMI-8226/EV transduced cells were measured by ELISA. (D) Transduced RPMI-8226/CYR61 cells overexpressed CCN1 compared to RPMI-8226/EV cells by western blotting. (E) Non-transduced RPMI-8226 and transduced RPMI-8226/CYR61 or RPMI-8226/EV cells were cultured for 7 days and the proliferation of cells was detected by CCK-8.

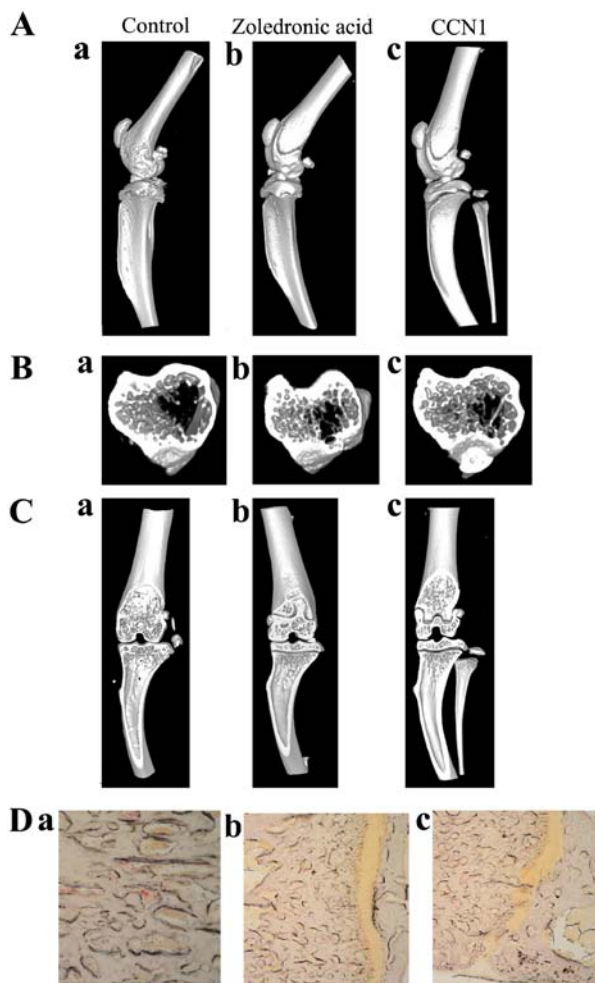


Figure 4. The effect of CCN1 in MBD mouse model. The mice were divided into three groups: control, zoledronic acid group and CCN1 overexpression group. MicroCT showed that the bones exhibited decreased resorption in CCN1 overexpression group (A-c, B-c and C-c) and zoledronic acid group (A-b, B-b and C-b), while severe resorption in control group (A-a, B-a and C-a). TRAP staining was negative in CCN1 overexpression group (D-c) and zoledronic acid group (D-b), compared with control (D-a) (x200).

bone volume/total volume and trabecular bone volume in both CCN1 overexpression group and zoledronic acid group were significantly increased compared with controls. Furthermore, trabecular bone volume in CCN1 overexpression group was ( $1.7539 \pm 0.16949$ ), which was significantly higher than that in zoledronic acid group ( $1.2839 \pm 0.077$ ) ( $P=0.012$ ).

## Discussion

MM is a malignancy of terminally differentiated plasma cells and produces bone disease in 80% of patients (23,24). How to cure the bone disease of MM patients is still a challenge for the researchers and physicians. Recent studies indicated many cytokines in BM microenvironment play a role in the pathogenesis of malignant diseases (25,26). CYR61, which encodes the secreted protein CCN1, plays an important role on bone remodeling in physiological and pathological processes. We found that CCN1 levels in BM supernatant and OBs both increased in the newly diagnosed MM patients, especially in the patients without bone disease, indicating CCN1 maybe play a protective role in MBD.

In order to observe the effect of CCN1, we used two concentrations of CCN1 (10 and 30 ng/ml) to stimulate OBs, the results showed that the quantity and mineralization ability of OBs increased significantly at 30 ng/ml concentration. Furthermore, we detected the mRNA levels of Runx2,  $\beta$ -catenin and BMP2 to evaluate the differentiation of OBs. As known, the canonical Wnt/ $\beta$ -catenin pathway is an important pathway regulating the differentiation of OBs (27,28). BMP2 and Runx2 also take part in the osteogenetic process. Some studies have identified that Runx2 is one of major target gene of BMP2, which is a key regulator of osteoblast differentiation and bone development (29-33). In our study, the mRNA levels of  $\beta$ -catenin and Runx2 increased after the CCN1 stimulation, while the BMP2 had no significant change. Gaur *et al* (34) suggested that canonical Wnt signaling promoted osteogenesis by directly stimulating Runx2 gene expression. Therefore, the

CCN1 stimulated the growth and mineralization of OBs in MBD patients mainly via Wnt signaling pathway.

It is well known that MM is a heterogeneous malignant disease, featured by various subtypes and different outcomes (35). It also determines that the characteristics of myeloma cells in different patients are diverse. Because myeloma cells react with OBs in different ways (36,37), the OBs in different patients maybe exhibit diverse performances. Our results in real-time PCR are presented as median, which perhaps support this hypothesis.

In order to investigate the role of CCN1 in bone remodeling *in vivo*, we tried to build a mouse model of MBD. There are several kinds of MBD models, including 5T2MM model (38), MOPC35.BM model (39) and transgenic mouse models (Myc/Bcl-XL mice) (40). However, these models are made by injection or transgene of mouse myeloma cells, which can not recapitulate the human condition of BM microenvironment and bone disease. Considering the availability, technology and cost, we injected the human myeloma cells (RPMI-8226) to mice, similar to Labrinidis *et al* (19). We detected the myeloma cells in mouse bone marrow by H&E staining and immunohistochemistry staining (CD138), which indicated the human myeloma cells grew in the mice. Furthermore, we observed the bone lesions by microCT and TRAP staining to identify the bone disease.

In this mouse model, overexpressed CCN1 in engrafted RPMI-8226 cells resulted in reduced bone disease, suggesting that CCN1 plays a positive effect on progression of bone lesions. Our results also indicated that the effect of CCN1 may be better than zoledronic acid, a first line treatment of MBD (41).

In conclusion, CCN1 is overexpressed in BM supernatant and OB in newly diagnosed myeloma patients, especially in the patients without bone disease, which could stimulate the proliferation and differentiation of OBs *in vitro* and contribute to bone remodeling *in vivo*. However, we still need to deeply investigate the signaling pathway of CCN1 on OBs to explore its mechanism.

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