

## Original Article

# Restoration of murine femoral segmental defect using CTGF-overexpressing MC3T3-E1 cells

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**Abstract:** Connective tissue growth factor (CTGF) is a member of the CCN super family and is reported to widely participate in bone development and regeneration. This study aimed to restore murine femoral segmental defect using CTGF-overexpressing MC3T3-E1 cells. MC3T3-E1 cells were transfected by lenti-CTGF (LvCTGF) and lenti-negative control (LvNC) virus to obtain stably transfected cells. Real-time PCR, Western blot, alkaline phosphatase activity assay, and alizarin red staining demonstrated that the overexpression of CTGF enhanced osteogenesis in vitro. Cell migration assay results showed that LvCTGF cells expressed higher migration ability than LvNC cells, while CCK-8 assay revealed no significant difference in cell proliferation. The LvCTGF and LvNC cells were then seeded into a chitosan/ $\beta$ -TCP scaffold and were used to restore a murine femoral segmental defect. Samples were harvested by the end of 2 and 5 weeks respectively. Micro-CT analysis and Masson's trichrome staining results showed that the LvCTGF-scaffold group expressed better bone healing compared with the LvNC-scaffold and scaffold-only groups. CTGF-overexpressed cells serve as an efficient source of seeding cells for bone regeneration.

**Keywords:** Transinfection, osteogenesis, CTGF overexpression, bone segmental defect, bone healing

## Introduction

Connective tissue growth factor (CTGF) is a member of the CCN super family, and its structure consists of four modules: 1) insulin-like growth factor-binding protein-like (IGFBP); 2) von Willebrand factor type C (vWC); 3) thrombospondin type 1 repeat (TSP1); and 4) carboxyl-terminal cysteine knot (CT) [1, 2]. These CTGF sub-structures contribute to the diversity of its bioactivity.

CTGF has been reported to widely participate in bone development and regeneration [1-3]. It plays important roles in cartilage development and maturation [4], and in all procedures of endochondral ossification [3]. Nevertheless, the expression pattern of CTGF during distraction osteogenesis suggested that it may also play roles during intramembranous ossification [5]. Besides, CTGF is capable of inducing osteogenic differentiation and mineralization in various types of cells, such as mesenchymal stem

cells [6, 7], osteoblastic cells [8, 9], vascular smooth muscle cells [10], human dental pulp cells [11], and human periodontal ligament stem cells [12]. Moreover, CTGF reportedly occurred in other osteogenic processes including vascular calcification, which was similar to the procedure of osteogenesis [13, 14]. When introduced into bone regeneration, CTGF also provided favorable outcome [15, 16]. These findings proved that CTGF can serve as a promising candidate for bone tissue regeneration. However, application of CTGF overexpressed cells as seeding cells for bone tissue regeneration has not occurred yet.

Chitosan and  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) have been widely utilized as materials for bone tissue engineering because of their good biocompatibility and osteoinductive activities [17, 18]. The combination of chitosan and  $\beta$ -TCP forms scaffolds that are characterized with interconnected open-pore microstructure and macropore structure, which allows cell inocula-

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tion [19]. In addition, the spongy scaffold was compressible and easy to shape [18]. Our previous studies have already demonstrated the availability of the chitosan/ $\beta$ -TCP (CS/ $\beta$ -TCP) scaffold [20, 21]. Although this scaffold was not ideal for controlled-release of biofactors because of high porosity, it was sufficient for our study because the seeding cells themselves secreted CTGF. We used the hybrid of CTGF-overexpressed cells and CS/ $\beta$ -TCP scaffold to restore a murine femoral defect model.

### Material and methods

#### *Cell culture and lentivirus infection*

MC3T3-E1 cell line (Subclone 14) was purchased from China Center for Type Culture Collection (Wuhan, China). Cells were routinely cultured in  $\alpha$ -MEM (Hyclone, Logan, Utah) supplemented with 10% FBS (Gibco, NY) and 1% Penicillin and Streptomycin (Hyclone). The culture medium was refreshed thrice a week. For cell transinfection, cells were seeded in 24-well plates and separately infected with a GFP (green fluorescent protein)-tagged recombinant mouse CTGF lentivirus (Genepharma Corp., Shanghai, China) as the experimental group (LvCTGF), and a GFP-tagged empty lentivirus as the negative control (LvNC). The transinfected cells were then cultured as in routine medium and amplified for further experiments. For osteogenic differentiation, the transinfected cells were cultured in osteogenesis-inducing medium that consists of  $\alpha$ -MEM, 10% FBS, 1% penicillin and streptomycin, 50 mg/ml ascorbic acid and 10 mM  $\beta$ -glycerophosphate.

#### *Total RNA extraction and quantitative real-time PCR analysis*

For quantitative real-time PCR, cells were seeded in 12-well plates (Corning, Albany, NY). Samples were washed with PBS for 3 times, total RNA was extracted using EZNA total RNA kit (Omega, Norcross, GA) according to the manufacturer's protocol, the RNA templates (1  $\mu$ g) were then reverse-transcribed into cDNA using First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). Quantitative RT-PCR was performed on Bio-Rad CFX 96 real-time PCR machine using the All-in-One™ qPCR Mix (GeneCopoeia, Rockville, MD) following the manufacturer's instructions. Primers were also purchased from GeneCopoeia. Expression of

osteogenic differentiation markers were examined, including alkaline phosphatase (ALP), Osterix (Osx), Osteopontin (OPN) and Runx-related transcription factor 2 (Runx2). All values were normalized to GAPDH and analyzed using  $\Delta\Delta$ Ct method.

#### *Total protein extraction and western blot*

For Western blot, cells were seeded in 3.5 cm flask (Corning) and grew to confluence. Total cellular protein was prepared by lysing cells with RIPA (Thermo Fisher Scientific) supplemented with 1% PMSF (Roche, Basel, Switzerland). The concentration of the total protein was assessed using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Then 20  $\mu$ g protein samples were separated by SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane (Roche) by electro blotting. After 1 hour blocking in 5% degreased milk powder-TBST solution at room temperature, membranes were incubated overnight on a shaker at 4°C with one of the following antibodies: Anti-ALP (Abcam, Cambridge, MA), anti-Runx2 (Abcam), anti-Osterix (Abcam), and anti-Osteopontin (Abcam). The membranes were then incubated with a secondary antibody, including HRP-conjugated goat-anti-rabbit IgG (Abbkine, Redlands, California) or goat-anti-mouse IgG (Abbkine).  $\beta$ -actin was used as the internal reference.

#### *ALP activity*

For ALP activity assay, cells were seeded in 6-well plates (Corning) and treated after reaching sub-confluence. Total protein was then isolated on Day 7. ALP activity was measured using an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. Optic density at 520 nm ( $OD_{520nm}$ ) was used to represent the ALP activity, and  $OD_{520nm}$  normalized to total protein amount was compared among groups.

#### *Alizarin red assay*

Cells were seeded into 12-well plates and cultured to sub confluence in complete medium which was then replaced by osteogenic medium. After 21 days culture with the medium refreshed thrice a week, cells were fixed with 95% ethanol, washed twice with tri-distilled water, and incubated with 1% Alizarin Red S/

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**Table 1.** Grouping protocol

ID	Side	
	Left	Right
1	S	S
2	S	S-N
3	S	S-C
4	S-N	S
5	S-N	S-N
6	S-N	S-C
7	S-C	S
8	S-C	S-N
9	S-C	S-C

S, Scaffold alone; S-N, Scaffold loaded with LvNC cells; S-C, Scaffold loaded with LvCTGF cells; There are 9 animals for each group.

Tris-HCl solution at pH 4.2 for 30 min to stain the mineralized nodules. After that the solution was discarded, cells were gently washed twice with tri-distilled water and air-dried. Microscopic images were obtained using the Nikon inverted microscope. The pigmented mineralized nodules were subsequently dissolved with Cetylpyridinium Chloride (Sigma-Aldrich) separately and optical density at 562 nm (OD 562 nm) was measured.

### Cell proliferation assay

For the measurement of cell proliferation, the cells were cultured in 96-well plates. After the cells reached sub-confluence, FBS concentration was reduced to 1%. Cell numbers were measured using a CCK-8 kit (Dojindo, Mashikimachi, Japan) following the manufacturer's instructions on Days 1, 2, and 3.

### Cell migration assay

For the measurement of cell migration, the cells were seeded into the upper chamber of the Costar Transwell inserts (#3422, pore size, 8  $\mu$ m) (Corning) at a density of  $1.0 \times 10^4$  cells/well. After incubation for 12 h at 37°C, the cells in the upper chamber were carefully removed using cotton swab. The cells went through the permeable supports and were fixed in 4% paraformaldehyde (PFA) and stained with DAPI. Then, cells were photographed with a fluorescence microscope (Leica, Wetzlar, Germany) and quantified using Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MD).

### Hybrid of cells and scaffold

The chitosan/beta-tricalcium phosphate (CS/ $\beta$ -TCP) scaffold with the porosity of 87.5% was fabricated as previously described [19, 20]. The scaffold was shaped into 2 mm\*1 mm\* 1 mm sized cuboid sticks and disinfected in 75% alcohol for 24 h, and then washed thoroughly with sterilized PBS and air-dried under UV light sanitization. Transfected cells were cultured in 100 mm petri dish to confluence and harvested, and then centrifuged into a pellet. After resuspension, the concentration of cells was measured and adjusted to  $1 \times 10^7$ /ml. Then, cells were inoculated into the scaffold by centrifugal force following a previously described protocol [22]. The cell-scaffold hybrids were then incubated for 4 h in an incubator and subsequently used for bone defect restoration.

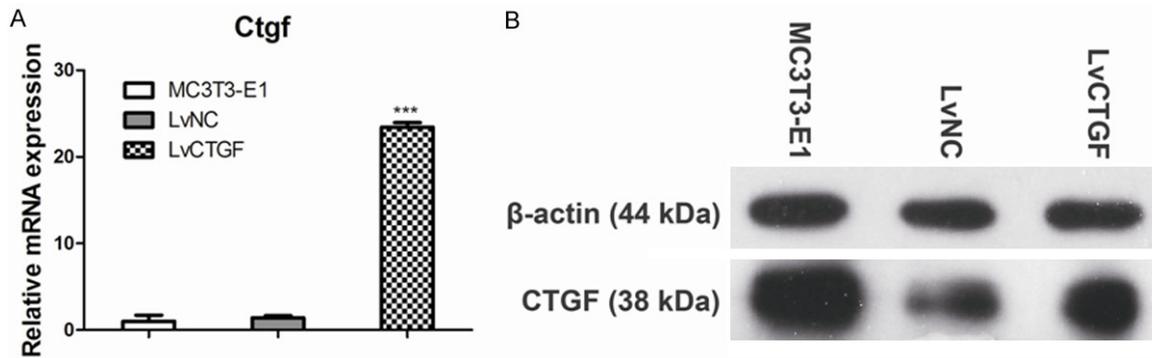
### Animal models and operational treatments

All animals in this study were treated according to internationally recognized guidelines on animal welfare and with the approval of the Medical Ethics Committee of Hospital of Stomatology, Wuhan University. Twenty-three mice were purchased from Hubei Provincial Laboratory Animal Center and bred at the SPF Animal Lab of Wuhan University Stomatology School. One mouse died during anesthetic treatment, 22 underwent bilateral surgery and 4 of the ones that underwent surgery died post-operatively. Mouse femoral segmental defects (1.2 mm) were established according to our previously published protocols [23]. Then, the defects were separately filled with CS/ $\beta$ -TCP scaffold (S group), CS/ $\beta$ -TCP scaffold loaded with LvNC cells (S-N group) and CS/ $\beta$ -TCP scaffold loaded with LvCTGF cells (S-C group), according to the grouping protocol listed in **Table 1**. After 2 and 5 postoperative weeks, the mice were euthanized, and femora were dissected in full length as samples and fixed in 4% PFA for 48 h. Samples were further treated for micro-CT scanning and histological analysis.

### Micro-computed tomography ( $\mu$ -CT) analysis

Femora were flushed using running water for 24 h after 4% PFA fixation, then embedded in paraffin. The fixation devices were carefully removed. The paraffin-embedded samples were scanned using a  $\mu$ -CT 50 imaging system (Scanco Medical AG, Bassersdorf, Switzerland).

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**Figure 1.** CTGF was overexpressed via lentivirus transfection: A. Realtime PCR of *Ctgf* mRNA expression in LvCTGF cells compared with MC3T3-E1 and LvNC cells (\*\* $P < 0.001$ ; One way ANOVA with GraphPad Prism5.0). B. Western blot of CTGF expression in LvCTGF cells compared with MC3T3-E1 and LvNC cells.

The X-ray tube was set at 70 kV, 85 mA with the resolution was 20  $\mu\text{m}$  (Integration time = 400 ms) paralleling the axis of the femora. Analysis and three-dimensional (3D) reconstruction were performed using the software provided by the manufacturer.

### Histology

Following fixation, the samples underwent decalcification, dehydration, waxing and embedding. The fixation devices were then carefully removed from the paraffin-embedded samples, and the voids left were resealed with paraffin. Samples were sliced into 5  $\mu\text{m}$ -thick longitudinal serial sections and stained with Masson's trichrome.

### Data analysis

For all quantitative data, analysis of variance (ANOVA) was employed to evaluate the effect of one variable on two or more independent groups. In the event of a significant group effect, individual pairs of means were compared using the Bonferroni post-hoc test. Data were calculated as mean  $\pm$  standard deviation, and in some cases, converted to percent of control. A value of  $p < 0.05$  was used to determine whether differences were statistically significant.

## Results

### Lenti-virus mediated overexpression of CTGF in MC3T3-E1 cells

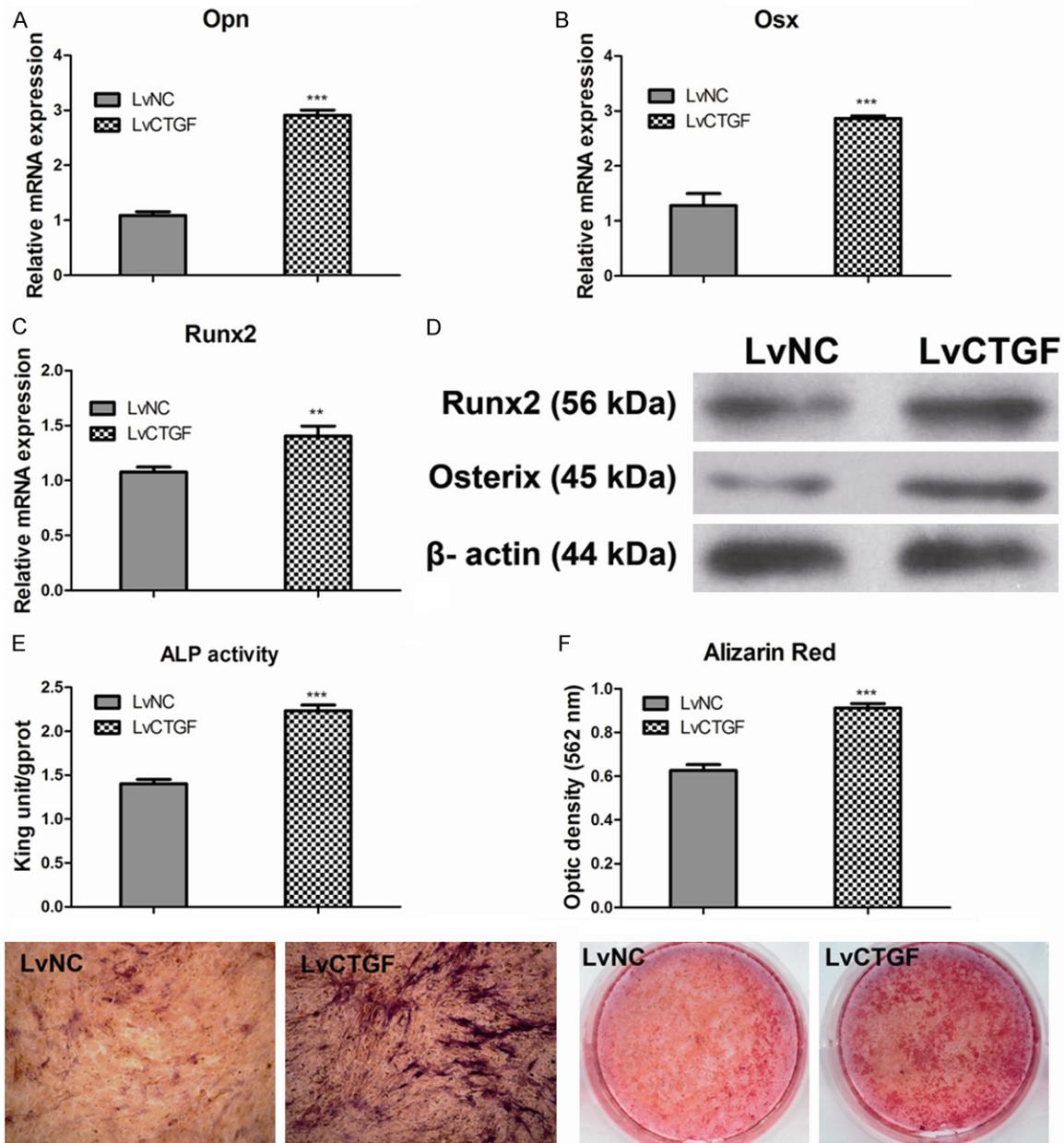
We transfected MC3T3-E1 cells with lenti-NC and lenti-CTGF viruses separately to obtain sta-

bly transfected cell lines. Then the cells were routinely cultured in full medium, and total RNA and protein were extracted after 3 days. When compared with the LvNC group, the LvCTGF group showed more than 7-fold higher expression of CTGF mRNA, as shown by quantitative realtime PCR (**Figure 1A**), and 4.3-fold higher expression of CTGF protein, as shown by Western blot (**Figure 1B**). These results confirmed that the LvCTGF cells expressed higher levels of *Ctgf* mRNA and CTGF protein than LvNC cells.

### CTGF overexpression enhanced osteogenic differentiation of MC3T3-E1 cells in vitro

To determine the osteogenic effect of CTGF overexpression, LvNC and LvCTGF cells were cultured in osteogenesis-inducing medium. Medium was replaced every 3 days. For realtime PCR, cells were cultured for 3 days and total mRNA was extracted. For Western blot and ALP activity quantitative assay, cells were cultured for 7 days, and total protein was extracted. For ALP activity qualitative staining, cells were cultured for 14 days and fixed with 4% PFA. For alizarin red, cells were cultured for 21 days and fixed with 4% PFA. Realtime PCR and western blot results showed that LvCTGF cells expressed significantly higher osteogenic markers, including *OPN*, *Runx2*, and *Osterix*, than LvNC cells (**Figure 2A-D**). ALP activity quantitative assay showed that ALP activity of LvCTGF cells was as 1.59-fold high as that of LvNC cells, in accordance with the ALP activity qualitative staining (**Figure 2E**). Alizarin red assay showed that more mineralized nodules formed in LvCTGF cells than in LvNC cells (**Figure 2F**).

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**Figure 2.** CTGF overexpression enhanced osteogenic differentiation in MC3T3-E1 cells in vitro. A-C. Realtime PCR of Opn, Osx, and Runx2 mRNA expression in LvCTGF cells versus LvNC cells (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; Two-tailed t test with GraphPad Prism5.0). D. Western blot of OPN, Osx, and Runx2 expression in LvCTGF cells versus LvNC cells. E. ALP activity of LvCTGF cells versus LvNC cells (\*\*\* $P < 0.001$ ; Two-tailed t test). F. Alizarin red assay of LvCTGF cells versus LvNC cells (\*\*\* $P < 0.001$ ; Two-tailed t test).

### CTGF overexpression promoted the migration of MC3T3-E1 cells but not proliferation

To assess the influences of CTGF overexpression on migration of MC3T3-E1 cells, transwell assay was performed. As shown in **Figure 3A** and **3B**, LvCTGF cells expressed higher migration behavior than LvNC cells, indicating that CTGF overexpression promoted the migration of MC3T3-E1 cells. However, CCK-8 assay showed that the number of LvCTGF cell

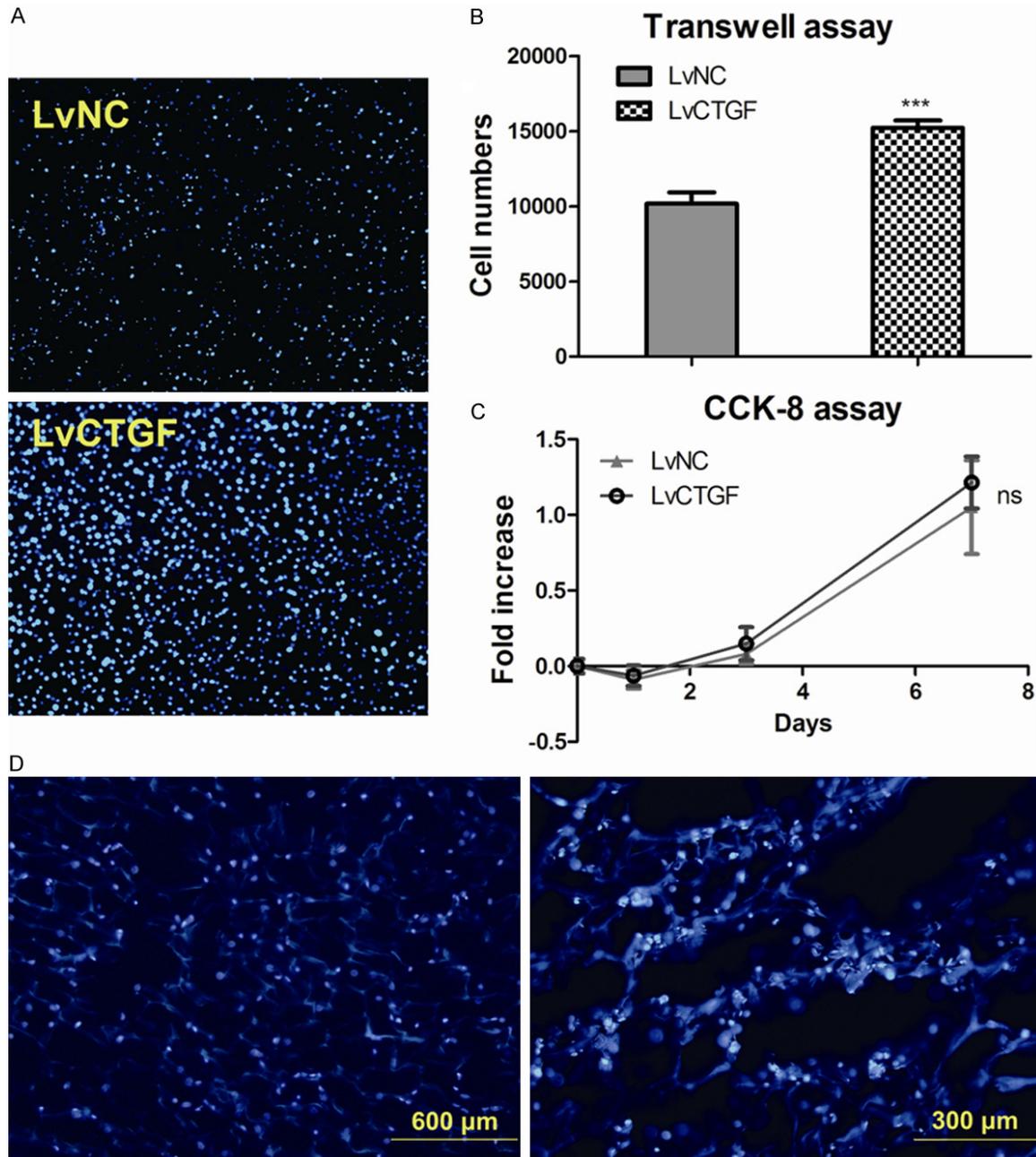
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was not significantly altered when compared with LvNC cells on Day 0, 1, 3 and 7 (**Figure 3C**).

### Hybrid of transfected cells and TCP/Chitosan scaffold

We produced cell-scaffold hybrids as described in the Material and Methods. Some of the hybrids were fixed in 4% PFA for overnight, and then went through the procedures of frozen section. The frozen section slices were then

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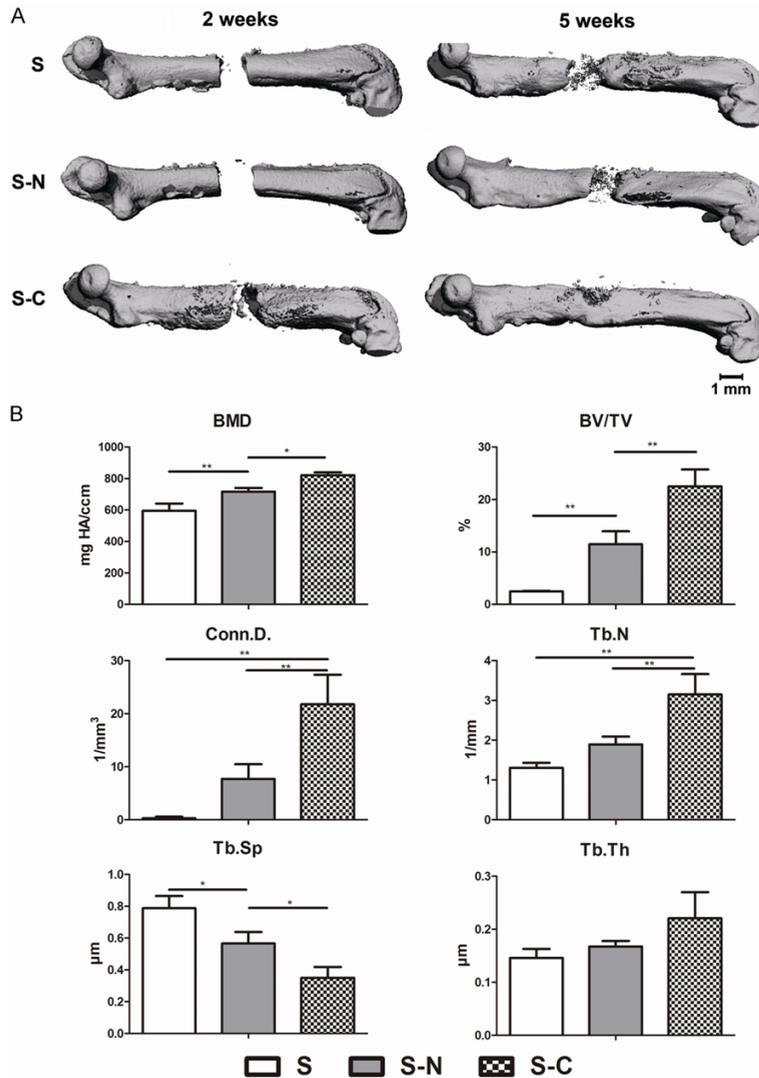
**Figure 3.** CTGF overexpression promoted the migration but did not alter the proliferation of MC3T3-E1 cells. Cells integrated to the scaffold. A. Fluorescent images (Magnification 200 X) of DAPI stained transwell upper chamber, LvNC cells versus LvCTGF cells. Bright blue spots showed the nuclei. B. Quantification data of transwell assay images, nuclei were counted using Image Pro Plus6.0 software (\*\*\*) $P < 0.001$ ; Two-tailed t test with GraphPad Prism5.0). C. CCK-8 assay on Day 0, 1, 3, and 7. Data points showed the relative fold increase to values of Day 0. (ns,  $P > 0.001$ ; two-tailed t test). D. Fluorescent images of DAPI stained frozen section of cell-scaffold hybrid, bright blue spots showed the nuclei, and dark blue structure showed the scaffold, scale bars were stamped.

stained with DAPI and observed using a Leica fluorescence microscope. Images were taken by a Nikon camera. Fluorescent images showed that the cells were well permeated into the scaffold, and most of cells were located on the wall of pores in the scaffold (**Figure 3D**).

*CTGF overexpressed cells promoted healing of murine femoral segmental defect*

Micro-CT analysis and histological staining were performed to evaluate the healing progresses of defects at 2 and 5 weeks, respec-

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**Figure 4.** Micro-CT images and analysis showed the defect healing of group S-C was better than other groups. A. Micro-CT images of defect healing femurs of group S, S-N, and S-C at 2 and 5 weeks. B. Micro-CT quantification data of the defect area of group S, S-N, and S-C at 5 weeks (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; One way ANOVA with GraphPad Prism5.0).

tively. The 3D reconstruction images of  $\mu$ -CT showed that the healing of S-C group was dramatically better than those of the other two groups at 5 weeks postoperatively. However, no great difference was observed at 2 weeks (Figure 4A). Setting the defect area as area of interest, quantitative data of  $\mu$ -CT showed that group S-C was significantly higher than the other two groups in bone mineral density (BMD), bone volume/tissue volume (BV/TV), trabecula number (Tb.N), and connectivity density (Conn.D), but lower in trabecula separation (Tb.Sp) at 5 weeks (Figure 4B). Higher BMD, BV/TV, Tb.N and Conn.D, as well as lower Tb.Sp,

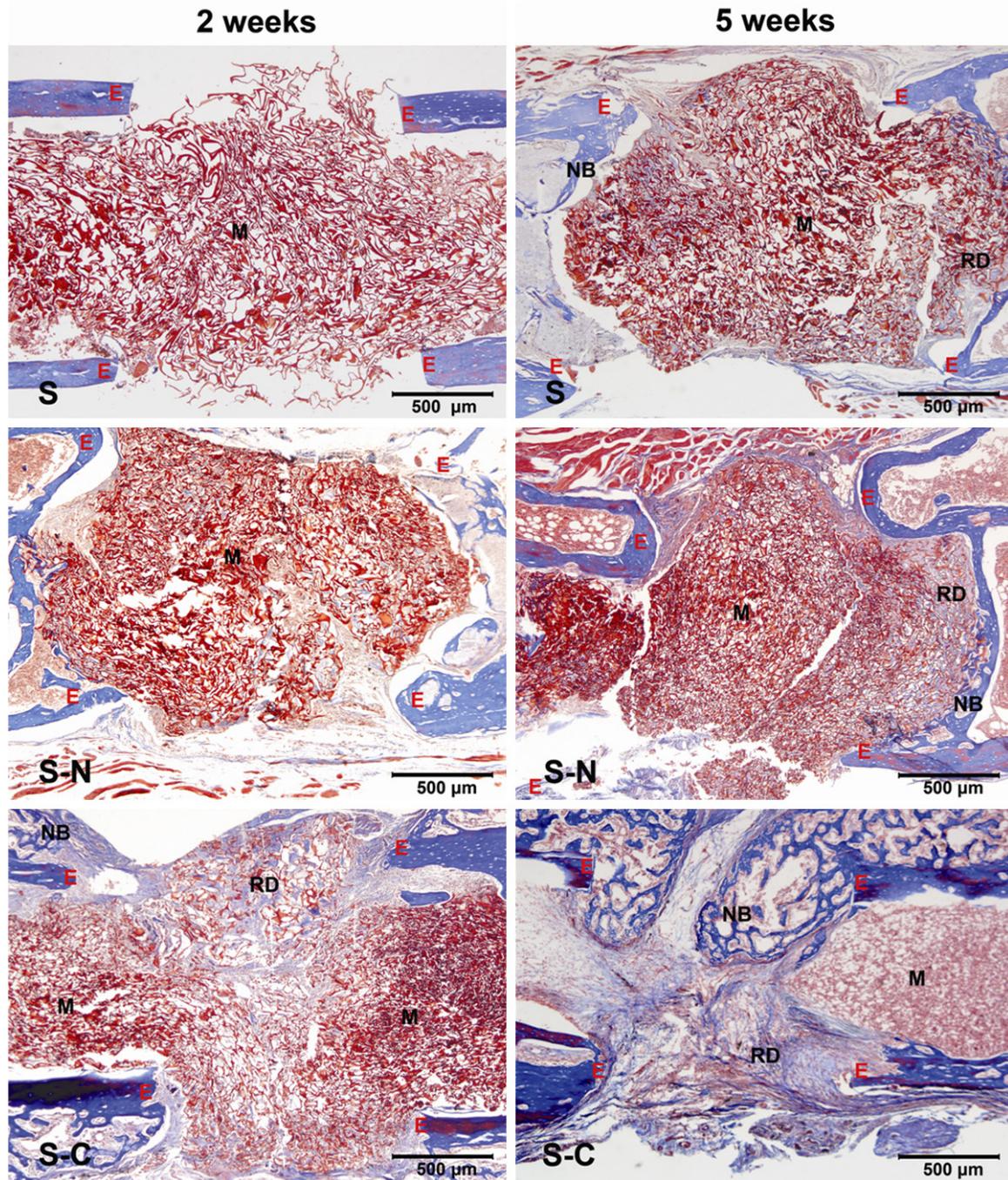
represented better bone healing. Meanwhile, both S-N and S-C group showed the same tendency as S-C group versus S-N group in BMD, BV/TV, Tb.N, Conn.D, and Tb.Sp compared to S group at 5 weeks, thereby indicating that cell-loaded scaffold had better restoration effects than non-cell scaffold. All the quantitative data approximated null, and showed no significant difference at 2 weeks (data not shown). However, trabecula thickness (Tb.Th) showed no significant difference among all groups.

Masson's trichrome staining images showed that at 2 weeks, osteoid and bone remodeling occurred in the defect of S-C group, whereas in the S-N group cells accumulated in scaffold. In the S group, only scaffold structure was present (Figure 5). At 5 weeks all groups formed osteoid in defect area, in group S-C one side of the defect was sufficiently close to be bridged by newly formed bone, whereas in group S-N small amount of osteoid formed and in group S the defect remained unhealed.

### Discussion

Many studies demonstrated that CTGF was capable of inducing osteogenesis in vitro and in vivo [9, 10]. CTGF was proved to be related to several important signaling pathways that regulated osteogenesis including Wnt signaling, BMP signaling and ERK signaling [7, 10, 24]. Contrarily, there were also some studies reported that in some occasions the overexpression of CTGF impaired osteogenesis, these results may be explained by the ability of CTGF to bind to many bio-factors such as BMP2, IGF-1 and subsequently antagonized their effects [25, 26]. In our study we successfully overexpressed CTGF in MC3T3-E1 cells by lentivirus transin-

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**Figure 5.** Masson's trichrome staining images of defect healing femurs of group S, S-N, and S-C at 2 and 5 weeks. Red "E" showed the cortical bone edge of defect. Black "M" stood for scaffold material. "RD" stood for bone remodeling area. "NB" stood for newly formed bone. Scale bars were stamped at lower right corners.

fection, and results of realtime PCR and Western blot demonstrated CTGF overexpression upregulated the osteogenic markers including OPN, Osterix, Runx2 and ALP, in accordance with increased mineralized nodules in the alizarin red assay. Overexpression of CTGF enhanced osteogenesis in vitro.

Cell migration is considered important in bone healing, in some in vitro studies the migration assay was equivalent to wound healing assay [27] or fracture healing assay [28], and the promotion of cell migration was proven to facilitate bone healing [29]. Previous studies also demonstrated that CTGF participated in regulating

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cell migration, the addition of CTGF promotes cell migration [30-32] whereas knockdown of CTGF suppressed cell migration [33]. We performed transwell assay to assess the cell migration ability. CTGF overexpression promoted the migration behavior of MC3T3-E1 cells, representing better "wound healing" capability *in vitro*.

Effect of CTGF on cell proliferation remains ambiguous. Some studies showed that CTGF stimulates the proliferation of some types of cells [4, 34, 35], whereas others showed the contrary results or insignificant effect [36, 37]. The proliferation of MC3T3-E1 was not altered statistically significantly by overexpression of CTGF. The cell numbers of all groups were slightly reduced on Day 1, and then increased normally until Day 7. This slight reduction of cell number on Day 1 may be related to the influence of cell passage procedures and culture condition changes. However, further experiments are needed to clarify the mechanism.

CS/ $\beta$ -TCP composites have been widely used as scaffold material in bone tissue engineering, with good biocompatibility, low cell toxicity and favorable osteoinductivity [18, 19]. CS/ $\beta$ -TCP spongy scaffold was characterized with good plasticity and high porosity and suitable for cell delivery [20, 21]. In our study, after cell inoculation and 4 hours of incubation, fluorescent images of frozen section demonstrated that the seeding cells permeated into the CS/ $\beta$ -TCP scaffold, homogeneously distributed, and adhered onto the wall of pores, thereby indicating that the cells integrated into the scaffold and formed cell-scaffold hybrid.

Besides scaffold, seeding cells and bio-factors were considered indispensable for bone regeneration [38]. By using genetic modified cells, researchers could combine the two elements together. The transinfected seeding cells may affect the osteogenic differentiation of endogenous osteoprogenitor cells orchestrating bone repair and regeneration. In our study, micro-CT showed higher BMD, BV/TV, Tb.N, Conn.D, and lower Tb.Sp in S-C group compared with other groups, suggesting that bone healing of S-C group was superior to other groups. However, there was no significant difference in Tb.Th, showing no observable change in bone microstructure. Therefore, micro-CT and histological results demonstrated that bone healing of

LvCTGF cell-scaffold hybrid was better than LvNC cell-scaffold hybrid and scaffold alone at 2 and 5 postoperative weeks, thereby proving that CTGF overexpression cells facilitate cell accumulation and bone regeneration.

In conclusion, we demonstrated that the overexpression of CTGF enhanced osteogenesis *in vitro*. We also showed that, CTGF overexpression cells serve as efficient seeding cell source for bone regeneration.

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