Downregulation of connective tissue growth factor reduces migration and invasiveness of osteosarcoma cells

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Abstract. As one of the most serious types of primary bone tumor, osteosarcoma (OSA) features metastatic lesions, and resistance to chemotherapy is common. The underlying mechanisms of these characteristics may account for the failure of treatments and the poor prognosis of patients with OSA. It has been reported that inhibition of Cyr61 suppresses OSA cell proliferation as it represents a target of statins. In addition to cystein-rich protein 61 (Cyr61) **and nephroblastoma overex-pression**, connective tissue growth factor (CTGF) is a member of the CCN family and may therefore exhibit effects on human OSA cells similar to those of Cyr61. In the current study, acridine orange/ethidium bromide staining were used to determine the rate of apoptosis. The present study demonstrated that small interfering RNA-mediated silencing of CTGF promoted cell death and suppressed OSA cell migration and invasion, as indicated by wound healing and Transwell assays, while lentivirus-mediated overexpression of CTGF reversed these effects. Furthermore, a colorimetric caspase assay demonstrated that CTGF knockdown enhanced the efficacy of chemotherapeutic drugs. The results of the present study provided a novel molecular target which may be utilized for the treatment of metastatic OSA.

Introduction

Osteosarcoma (OSA) is globally one of the most common types of primary bone tumor and is predominantly observed in children and adolescents (<20 years old) (1). Patients with localized disease have a five-year recurrence-free survival rate of 80%; however, the prognosis of OSA is poor in metastatic osteosarcoma. In spite of OSA occurring in any type of bone in the body, the metaphyseal (actively growing) regions of the distal femur, proximal tibia and proximal humerus are the most frequent origins of the primary tumor and the sites with the highest probability of metastasis are the lungs and distant bones (2).

It has been reported that several genes are able to regulate cell proliferation and differentiation; these genes carry numerous mutations associated with significant neoplasmic abnormalities in OSA (3-9). Of note, mutations in tumor suppressor genes, including p53, MDM2 and riboblastoma protein have been reported to have major roles in the tumorigenesis of OSA (3,4). OSA is also associated with the aberrant expression of certain transcription factors expressed in bones, including c-fos, whose overexpression has been shown to result in OSA in the bones of mice (5), as well as osteoblast differentiation factor osterix (6,7). In OSA cell lines, Runx2 was found to be downregulated or dysfunctional (8), and in high-grade pediatric OSA, genomic aberrations in the Twist have been reported (9).

Resistance to conventional chemotherapy is one of the characteristics of metastatic OSA and represents a considerable obstacle for its clinical treatment (10). However, only a small number of genes, including HES1 (11-13) and Ezrin (10) have been implicated in the progression and metastasis of OSA.

It has been reported that statins exert anti-tumoral effects on OSA cells (13-15). Cystein-rich protein 61 (Cyr61), a member of the Cyr61/connective tissue growth factor (CTGF)/nephroblastoma overexpressed (NOV) (CCN) family of secreted proteins, was among the factors downregulated by statins. This CCN protein family comprises Cyr61, CTGF, NOV and Wnt-induced secreted proteins (WISP)1, -2 and -3 (16). As a member of the CCN family, CTGF was

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hypothesized have effects on osteocarcinoma similar to those of statins. The present study therefore assessed the effects of CTGF knockdown or lentivirus-mediated overexpression of CTGF as well as statin treatment on the biological properties of OSA cells.

Materials and methods

Cell lines and culture. The SaOS2, U2OS, MG63, OHS4 and CAL72 cell lines (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal calf serum (FCS; Thermo Fisher Scientific) at 37°C in a humidified atmosphere containing 5% CO_2 in air.

RNA extraction and reverse-transcription quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent (Invitrogen; Thermo Fisher Scientific) was used to isolate RNA according to the manufacturer's instructions, which was stored at -20°C. cDNA was synthesized using 3 μg RNA, which was denaturated and reverse-transcribed by using 300 U Moloney murine leukemia virus reverse transcriptase, 15 mg oligo dT primers and 1 mM deoxynucleoside triphosphate (dNTP) (Promega, Madison, WI, USA) in a total volume of 30 μl. SYBR Green Master Mix kit (ABGen, Courtaboeuf, France) was used for qPCR. A total of 0.5 mM of each primer (Invitrogen; Thermo Fisher Scientific) was used with sequences as follows: Human CTGF, forward 5'-CAGGCTAGAGAAGCAGAG CC-3' and reverse 5'-GTAATGGCAGGCACAGGTCT-3'; β-actin, forward 5'-CTCCATCCTGGCCTCGCTGT-3' and reverse 5'-GCTGTCACCTTCACCGTTCC-3'. Thermocycling was conducted using the ABI 7500 (Applied Biosystems; Thermo Fisher Scientific) and the cycling conditions were as follows: Initial denaturation at 95°C for 15 min, followed by 40 cycles of 20 sec at 95°C, 15 sec at 58°C and 15 sec at 72°C, and final extension at 72°C for 7 min. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative quantities of RNA.

Plasmid transduction. In order to investigate the role of CTGF in OSA, cell lines were transduced with lentiviral vectors (LV) encoding either the full-length sequence (LV-CTGF) or a specific short hairpin (sh)RNA (sh-CTGF). The full-length CTGF ORF (1047 base pairs; GenBank accession number, CR541759.1) was amplified from the pFLAG-CMV2-CTGF plasmid (Invitrogen; Thermo Fisher Scientific). The primer sequences were as follows: Forward, 5'-TACTGGCGGCGGTATACCCG-3' and reverse, 5'-TGCCATGTCTCCGTACAT-3'. The PCR product was inserted into the expression vector pcDNA3.1/myc-His(-)B-3X FLAG-IRES-hrGFP, derived from pcDNATM3.1/myc-His(-)B (Invitrogen; Thermo Fisher Scientific). Cell transduction was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific) according to the manufacturer's instructions.

Proliferation assay. A bromodeoxyuridine (BrdU) incorporation assay was used to quantify cell replication. A previously described procedure was used in the present study (17). In brief, cells were cultured for 24 h in the presence of increasing concentrations of bisphosphonates (10^{-9}-10^{-4} M) and labeled with BrdU for the last 6 h (kit purchased from GE Healthcare Life Sciences, Roosendaal, The Netherlands).

Detection of apoptosis and necrosis. Double staining with ethidium bromide and acridine orange was performed to visualize and quantify the number of viable cells (green nuclei), apoptotic cells (nuclei condensed and colored orange), and necrotic cells (red nuclei). In briefly, 2 μl dye mixture (100 μg/ml acridine orange and 100 μg/ml ethidium bromide) was added to 20 μl cell suspension and immediately examined with the 40X oil immersion objective using a Leitz DMRB fluorescence microscope (green/red filter; 100 W lamp; Leica Microsystems GmbH, Wetzlar, Germany) equipped with a photometrics CCD camera and the Logikon image analysis system (Numeris Benelux SA, Ath, Belgium). Several fields, randomly chosen, were digitized and 600-800 nuclei for each sample were counted and scored. Results were expressed as the relative percentages of viable, apoptotic and necrotic cells to the total number of cells scored.

Caspase activity. Effector caspase activity was performed as previously described (14,15). In brief, cells were treated with 10 mM atorvastatin (Adooq BioScience LLC, Irvine, CA, USA) or the solvent for 24 h then the caspase activity was determined. Cellular extracts (50 μg) were incubated with 0.2 mM acetyl-Asp-Glu-Val-Asp-p-nitroanilide (caspases-3, -6 and -7; Enzo Life Sciences, Inc., Farmingdale, NY, USA), Ac-LEHD-pNA (caspase-9; Enzo Life Sciences, Inc.) or Ac-IETD-pNA (caspase-8; Enzo Life Sciences, Inc.) as the substrates for the previously reported times (14,15) at 37°C in the presence or the absence of the specific caspase inhibitors Ac-DEVD-CHO, Ac-LEHD-CHO and Ac-IETD-CHO (10 μM). The specific activity (nmol of pNA/min/mg protein) was expressed as treated over control ratios.

Migration and invasion assays. A wound-healing assay was performed following the manufacturer's instructions (ibidi GmbH, Martinsried, Germany). A Transwell migration and invasion assay as performed as described previously (14). In brief, the cells (50,000 cells/insert) were incubated 2 h with or without statin and/or z-VAD-fmk prior to seeding into the inserts and incubation for a further 22 h. The cells that did not migrate through the filter were removed from the upper surface of the membrane using cotton-tipped swabs. The cells migrated to the underside were fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C and stained with crystal violet (Amresco, Solon, OH, USA). The membranes were then cut from the insert and observed under a microscope (Axioplan 2 Imaging Mot Microscope System; Zeiss, Oberkochen, Germany). Five fields were randomly selected and counted and each assay was performed in duplicate.

Western blot analysis. A protocol of a previous study was used for the preparation of cell lysates (14) In brief, the proteins (30 μg) were resolved using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Protogel, Atlanta, GA, USA) and transferred onto polyvinylidene difluoride nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). The filters were incubated at room temperature for 2 h in 50 mm Tris-HCl (pH 7.4), 150 mm NaCl, 0.1% (v/v)
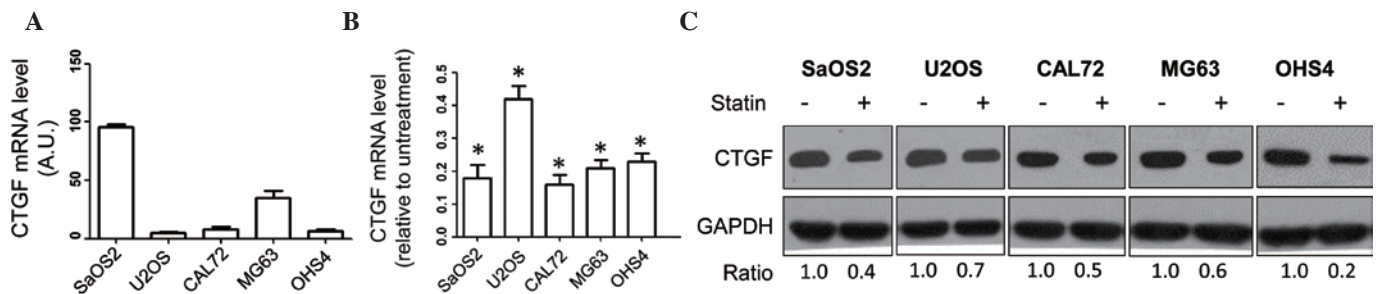
Figure 1. CTGF expression levels in osteosarcoma cell lines are reduced by atorvastatin. (A) RT-qPCR was performed to evaluate CTGF mRNA expression in several human OSA cell lines. Expression was normalized to β-actin. (B) Human OSA cell lines were treated with 10 mM atorvastatin for 24 h and RT-qPCR was used to evaluate mRNA levels of CTGF. Relative expression levels compared with those in untreated cells are shown. Values are expressed as the mean ± standard deviation (n=3). *P<0.05 vs. A. (C) Immunoblot analysis was used to assess protein levels of CTGF in human OSA cell lines treated with 10 mM atorvastatin for 24 h. Ratios of protein expression compared with those of untreated cells are presented, as an average of three blots. Statin, atorvastatin; CTGF, connective tissue growth factor; A.U., arbitrary units; RT-qPCR reverse-transcription quantitative polymerase chain reaction.

Tween 20, 0.5% (w/v) bovine serum albumin (TBST/BSA) and then overnight at 4°C on a shaker with the rabbit monoclonal anti-GAPDH (ab181602) and anti-CTGF (ab6992) antibodies (1:1,000 in TBST/BSA; Abcam, Cambridge, UK). The membranes were washed twice with TBST and incubated for 2 h with the horseradish peroxidase-conjugated secondary antibody (1:20,000 in TBST/BSA). Following the final washes, the signals were visualized with Enhanced Chemiluminescence Western Blotting Detection Reagent (GE Healthcare Life Sciences) and autoradiographic film (X-Omat AR; Kodak, Rochester, NY, USA). Densitometric analysis using ImageQuant software was performed following digital scanning (Odyssey® Fc; Agfa-Gevaert, Mortsel, Belgium).

Immunoblot analysis. A protocol of a previous study was used for the preparation of cell lysates (14,15). Incubation with rabbit monoclonal anti-GAPDH (ab181602; 1:200) and rabbit polyclonal anti-CTGF (ab6992; 1:200) antibodies was conducted at 4°C overnight. Cell extracts were collected in 2X loading lysis buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% 2-mercaptoethanol, 10% glycerol and protease inhibitor cocktail; Sigma-Aldrich, St. Louis, MO, USA]. The total cellular proteins were separated using 8% SDS–PAGE and trans- ferred to Hybond-C nitrocellulose membranes (GE Healthcare Life Sciences, Chalfont, UK). Subsequent to blocking with PBS containing 5% BSA or nonfat milk, the membranes were incubated with the primary antibodies, followed by incubation with IRDye 800CW or 680RD secondary antibodies (1:10,000; LI-COR Biosciences, Lincoln, NE, USA). The protein bands were detected using the Odyssey Infrared Imaging System (Li-COR Biosciences).

Statistical analysis. Values are expressed as the mean ± standard deviation. Two-factor analysis of variance was used to compare values between groups, using SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference between values.

Results

CTGF expression is reduced by atorvastatin (statin) in OSA cells. RT-qPCR analysis of CTGF was performed in the

SaOS2, U2OS, CAL72, MG63 and OHS4 human OSA cell lines, revealing that CTGF mRNA was expressed in all cell lines, particularly in SaOS2 cells (Fig. 1A). Furthermore, the effect of statin treatment on the expression of CTGF was assessed in the OSA cell lines. CTGF mRNA expression in the panel of OSA cell lines was markedly decreased following treatment with statin (10 mM) (P<0.05 vs. untreated) (Fig. 1B). In addition, the effect of statin (10 mM) on the protein levels of CTGF in the panel of cell lines was assessed by immunoblot analysis, revealing that the protein levels of CTGF were decreased following statin (Fig. 1C). Collectively, these results indicated that statin treatment led to the downregulation of CTGF in human OSA cells. As the SaOS2 and U2OS cell lines expressed the highest and lowest levels of CTGF, respectively, they were selected to be used in the subsequent experiments.

CTGF expression does not affect OSA cell proliferation. A BrdU incorporation assay were used to determine the proliferative rates of transduced and parental cells, revealing that these were not affected by plasmid transduction (Fig. 2A). The results therefore indicated that CTGF had no significant effects on OSA-cell proliferation in human cell lines.

Evasion of apoptosis by OSA cells is dependent on CTGF expression. The present study investigated the effects of CTGF on OSA cell death. As shown in Fig. 2B, apoptotic and necrotic indices of sh-CTGF-transduced cells were higher than those of parental cells. By contrast, LV-CTGF-transduced cells displayed lower apoptotic and necrotic indices compared with those of parental cells. Furthermore, it was revealed that sh-CTGF-transduced cells exhibited increased caspase activity and an elevated Bax/Bcl2 ratio compared with those of parental cells. By contrast, caspase activity and the Bax/Bcl2 ratio were reduced in CTGF-overexpressing OSA cells compared with those in parental cells (Fig. 2C and D). These results indicated that CTGF expression was associated with the evasion of apoptosis by OSA cells.

The dose-dependent cytotoxic effects of doxorubicin, cisplatin and methotrexate on OSA cell viability are utilized for the chemotherapeutic treatment of OSA (14). The present study revealed that CTGF silencing significantly enhanced the caspase activity in SaOS2 cells following treatment with doxorubicin, cisplatin or methotrexate, whereas LV-CTGF
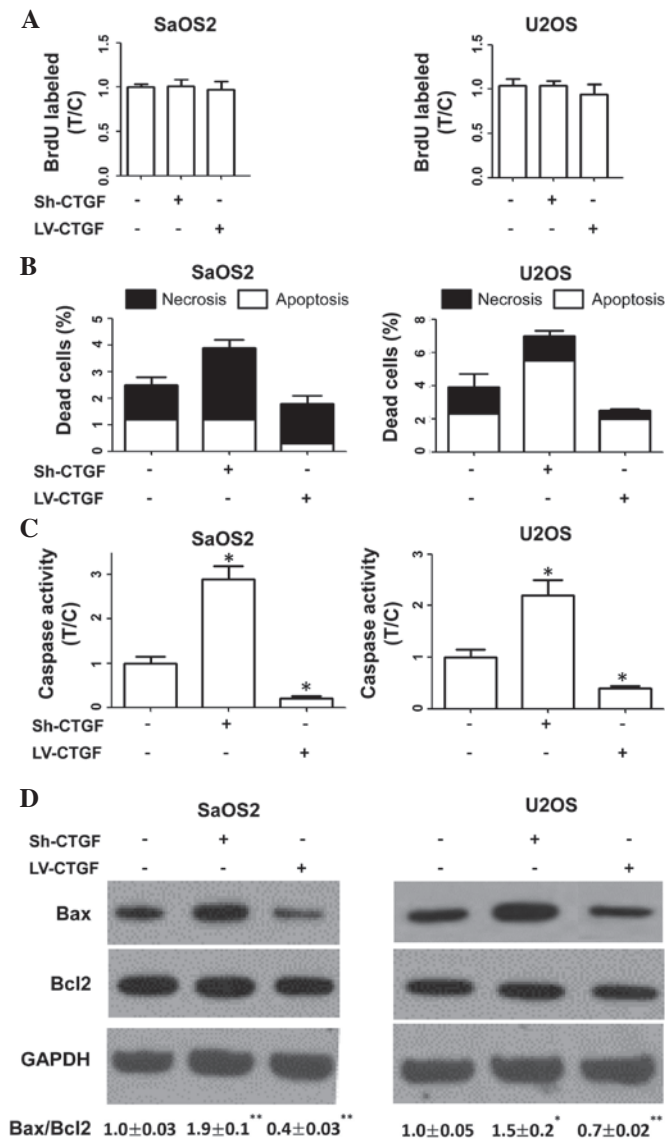
Figure 2. Osteosarcoma cell viability is modulated by CTGF expression. (A) Following 48 h of BrdU incorporation, cell proliferation was evaluated. (B) Acridine orange/ethidium bromide staining were performed and staining intensity was scored to determine the numbers of viable, apoptotic and necrotic cells. Necrotic and apoptotic cells are expressed as a percentage of the total cell population. (C) A colorimetric assay was used to evaluate caspase activity. (D) Immunoblot analysis was used to assess levels of apoptosis-associated proteins Bcl-2 and Bax in cell lysates. Blots were quantified to determine the Bax/Bcl2 ratio. *P<0.05 versus parental cells. Values are expressed as the mean ± standard deviation (n=3). Bcl2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; LV, lentivirus; Sh, short-hairpin RNA; BrdU, bromodeoxyuridine; CTGF, connective tissue growth factor; T/C, treatment/control.
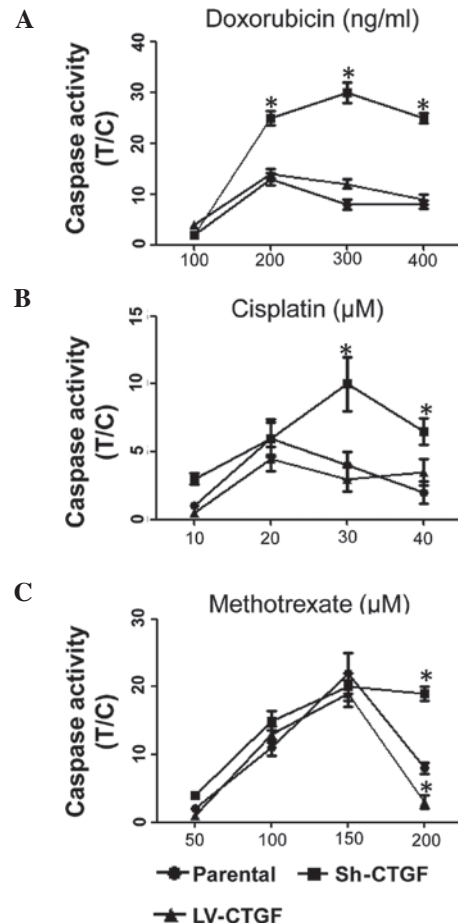

Figure 3. Efficacy of chemotherapeutics in OSA cells is enhanced by CTGF knockdown. Transduced SaOS2 cells were incubated with various doses of (A) doxorubicin, (B) cisplatin or (C) methotrexate. A colorimetric assay was used to evaluate caspase activity. *P<0.05 vs control. Values are expressed as the mean ± standard deviation (n=3). LV, lentivirus; Sh, short-hairpin RNA; CTGF, connective tissue growth factor; T/C, treatment/control.

slighly decreased caspase levels compared with those in native SaOS2 cells treated with the chemotherapeutics (Fig. 3A-C). It is therefore concluded that silencing of CTGF enhanced the efficacy of chemotherapeutic drugs against OSA.

Cell migration and invasion are dependent on CTGF expression in vitro. The present study further investigated the invasiveness and migratory potential of transduced OSA cell lines, which represent the main characteristics of OSA progression and the development of metastasis. The results showed that CTGF silencing inhibited wound healing in sh-CTGF-transduced

cells compared with that in parental cells, while CTGF overexpression enhanced wound healing (Fig. 4A). In addition, CTGF overexpression enhanced the migratory potential in a Transwell assay (Fig. 4B). The observed inhibition of the migratory potential by statin was not able to be rescued by overexpression of CTGF (Fig. 4C). Furthermore, a Transwell assay using Matrigel-coated inserts revealed that silencing of CTGF inhibited the invasive capacity of OSA cells, while cell invasiveness was promoted by CTGF overexpression (Fig. 4D). All of these results implied that CTGF had positive effects on cell migration and invasiveness *in vitro*, whereas invasion and migration were reduced in CTGF-silenced OSA cells. It can be concluded that CTGF expression is associated with the aggressiveness and metastatic potential of OSA cells.

Discussion

Conserved cysteine residues covalently bound to isoprenoids can be post-translationlly modified by prenylation, which is essential for the pro-tumorigenic activity of certain guanosine triphosphatases, including Ras and Rho-like proteins (18,19). Synthetic bisphosphonates with inhibitory activities on geranylgeranyltransferase type and farnesyltransferase can be utilized
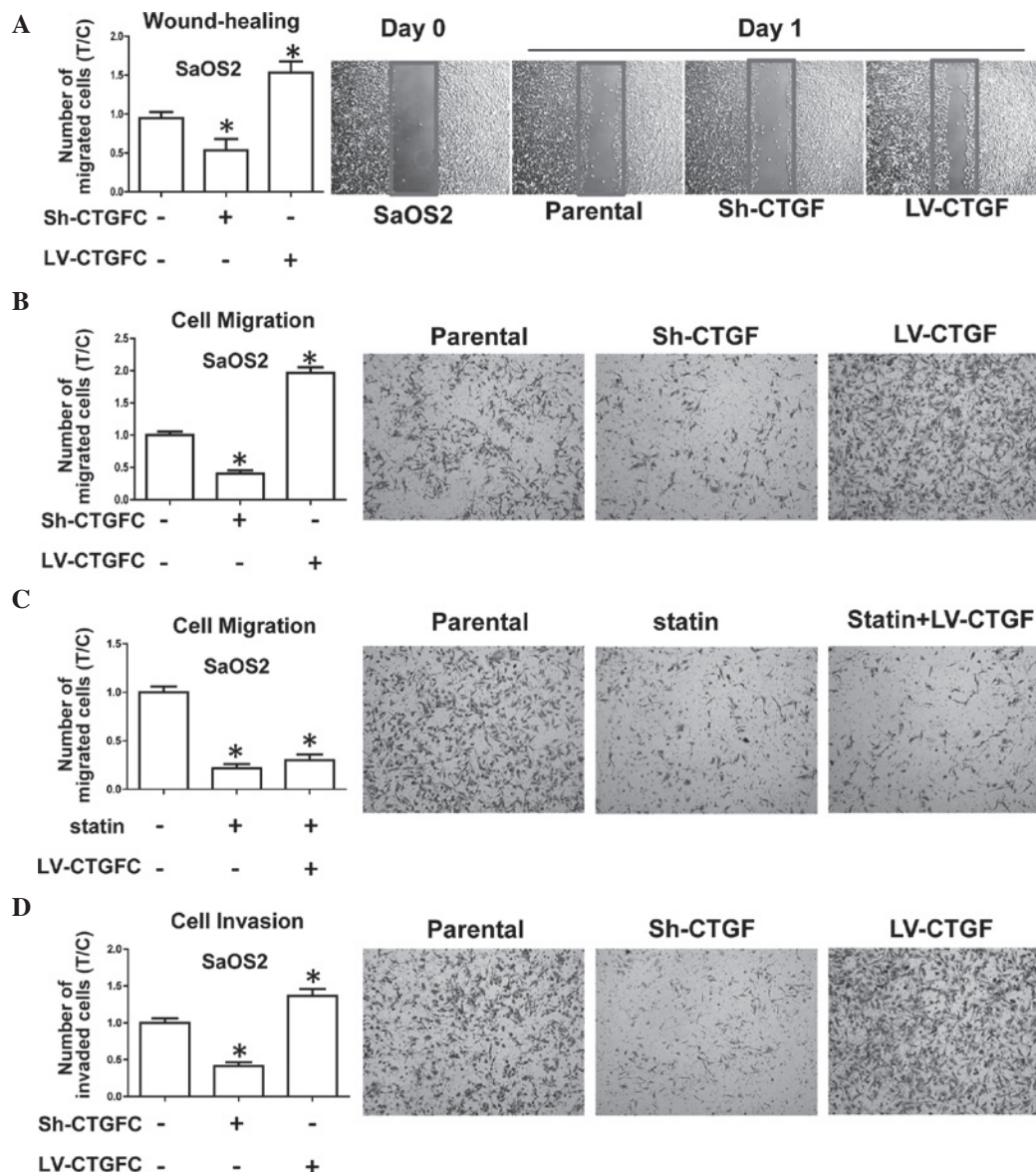
Figure 4. OSA cell migration and invasion are affected by CTGF expression. SaOS2 cells were subjected to CTGF knockdown, plasmid-mediated overexpression of GTGF and/or statin treatment. (A) A wound healing assay was used to assess the migratory potential of SaOS2 cells was assessed at one day one after wounding. Phase-contrast microscopy images were captured on days 0 and 1 after wounding. (B and C) A 24-h Transwell assay was used to assess the migratory capacity of SaOS2 cells. (D) The invasive capacity of SaOS2 cells was assessed using a 24-h Transwell assay using Matrigel-coated inserts. Values are expressed as the mean ± standard deviation determined from eight fields of view. Magnification, x40; stained with crystal violet. *P<0.05 vs. parental cells. Statin, atorvastatin; LV, lentivirus; Sh, short-hairpin RNA; CTGF, connective tissue growth factor; T/C, treatment/control.

as anti-cancer drugs which partly block prenylation through inhibition of farnesyl pyrophosphate (FPP) synthase activity; this approach is a novel therapeutic strategy for several cancer types, including OSA and bone metastases (20-25). Statins act as hypocholesterolemic agents with inhibitory effects on the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (26) and represent another class of drug which acts through depleting downstream isoprenoid residues, including such as geranylgeranylpyrophosphate or FPP. Previous studies on OSA reported that statins not only induced apoptosis but also reduced cell migration and invasion, and potentiated the effects of chemotherapeutic agents (13-15). However, the anti-cancer efficacy of statins *in vivo* remains to be clarified. Previous clinical studies indicated that statins, apart from exhibiting anti-cancer effects, may also be associated with an increased risk for the development of cancer *de novo* (27-29).

These conflicting results indicate that the understanding of the mechanisms of action of statins is required to be expanded and refined, and that novel targets for cancer therapy require to be discovered.

Previous studies reported that Cyr61, which encodes a secreted protein known to modulate tumor development and progression, was downregulated by statins (30-32) and that CTGF is also among the molecular targets of statins (33,34). CTGF is a matricellular protein of the CCN family of extracellular matrix-associated heparin-binding proteins, which comprises Cyr61, CTGF, NOV and WISP1-3 (35-37). CTGF has important roles in numerous biological processes, including cell adhesion, migration, proliferation, angiogenesis, skeletal development and tissue wound repair, and is critically involved in fibrotic disease and several types of cancer (33,34,38). Members of the CCN protein family have similar domains,

indicating that CTGF may have the similar roles in OSA cells to those of Cyr61.

The present study enhanced or silenced the expression of CTGF in human OSA cells to determine the role of CTGF in OSA development and progression. A BrdU incorporation assay did not reveal any significant effects of CTGF on the proliferation of human OSA cell lines. By contrast, CTGF silencing slightly increased OSA cell death and enhanced the anti-neoplasic and pro-apoptotic effects of the chemotherapeutics doxorubicin, cisplatin and methotrexate, which may represent a novel strategy to enhance the efficacy of OSA treatments. A positive combinatory effect of statins with chemotherapeutic drugs in OSA or other cancer types has been indicated by previous studies (13,39-42). The present study focused on CTGF expression in OSA cells, independent of the presence of statins. As silencing of CTGF enhanced the anti-tumoral effects chemotherapeutic drugs, it was indicated that CTGF knockdown may reduce the resistance of OSA cells to chemotherapy.

OSA bears the characteristics of rapid and frequent development of metastatic lesions. *In vitro* experiments performed in the present study demonstrated that the migratory and invasive capacities of human OSA cells were reduced by CTGF silencing, whereas CTGF overexpression led to an increase in cell migration and invasion. By contrast, previous studies reported that silencing or inhibition of CTGF reduced the motility and invasiveness of breast and prostate cancer cells (43,44). Due to this discrepancy, the roles of CCN family proteins, particularly CTGF, in OSA require further study. In OSA cell lines, Nov was reported to be expressed at variable levels (45) and may be associated with poor prognosis and an increased risk of developing metastases (46). The predictive value of CTGF expression levels with regard to the outcome and progression of human OSA requires to be investigated in future studies analyzing CTGF expression in primary and metastatic tumors.

In conclusion, the results of the present study revealed that OSA cell invasion and migration was regulated by CTGF *in vitro*. CTGF was indicated to have a critical role in the genesis and progression of human OSA, and to be involved in the evasion of apoptosis, aggressiveness and metastasis formation of OSA. Targeting of CTGF may be a strategy to enhance the efficacy of chemotherapeutics in the treatment of OSA as well as to reduce the aggressiveness of OSA cells.

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