



Original Article

Connective tissue growth factor enhances TGF- β 1-induced osteogenic differentiation via activation of p38 MAPK in mesenchymal stem cells

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ABSTRACT

Objectives: Cellular differentiation is based on the effects of various growth factors. Transforming growth factor (TGF)- β 1 plays a pivotal role in inducing osteogenic differentiation of mesenchymal stem cells (MSCs). In this study, we investigated the influence of connective tissue growth factor (CTGF), known to function synergistically with TGF- β 1, on osteogenic differentiation in MSCs.

Methods: UET-13 cells were treated with TGF- β 1 and/or CTGF. Subsequently, protein levels of intracellular signaling pathway molecules were determined through western blot analysis. The mRNA expression levels of osteogenic differentiation markers were investigated using reverse transcription-quantitative polymerase chain reaction. Bone matrix mineralization was evaluated through alizarin red staining.

Results: Co-treatment with TGF- β 1 and CTGF resulted in the suppression of TGF- β 1-induced phosphorylation of extracellular signal-regulated kinase 1/2, an intracellular signaling pathway molecule in MSCs, while significantly enhancing the phosphorylation of p38 mitogen-activated protein kinase (MAPK). In MSCs, co-treatment with CTGF and TGF- β 1 led to increased expression levels of alkaline phosphatase and type I collagen, markers of osteogenic differentiation induced by TGF- β 1. Osteopontin expression was observed only after TGF- β 1 and CTGF co-treatment. Notably, bone sialoprotein and osteocalcin were significantly upregulated by treatment with CTGF alone. Furthermore, CTGF enhanced the TGF- β 1-induced mineralization in MSCs, with complete suppression observed after treatment with a p38 MAPK inhibitor.

Conclusions: CTGF enhances TGF- β 1-induced osteogenic differentiation and subsequent mineralization in MSCs by predominantly activating the p38 MAPK-dependent pathway.

1. Introduction

Mesenchymal stem cells (MSCs) are adult stem cells isolated from various tissues, such as bone marrow, adipose tissue, and umbilical cord blood [1,2]. MSCs differentiate into various cells that constitute mesodermal, ectodermal, and endodermal lineages including bone cells, adipose cells, chondrocytes, muscle cells, neurons, islet cells, and hepatocytes [3,4]. Osteogenesis occurs throughout development and adult life. The formation of new bone involves a complex cascade of cell proliferation, osteogenic cell differentiation, extracellular matrix maturation, and bone matrix mineralization. MSCs differentiate into

osteoblasts and then synthesize and secrete bone matrix proteins, which subsequently form the mineralized tissue.

Connective tissue growth factor [CTGF, also known as cellular communication network 2 (CCN2)], a member of the CCN protein family, is a multifunctional secretory protein present in various fibrous tissues, and it is involved in embryonic development, tumorigenesis, and osteoarthritis [5]. CTGF regulates proliferation, migration, adhesion, survival, and differentiation of cells along with synthesis of extracellular matrix proteins in various cells [6–9]. CTGF has been found to play a role in osteogenesis when its overexpression was detected in osteopetrotic bones [10]. Further studies showed that CTGF plays a role in

Abbreviations: BMP, Bone morphogenetic protein; CTGF, Connective tissue growth factor; ERK, Extracellular signal-regulated kinase; FBS, Fetal bovine serum; IPF, Idiopathic pulmonary fibrosis; MAPK, Mitogen-activated protein kinase; MSC, Mesenchymal stem cell; PDGF, Platelet-derived growth factor; SD, Standard deviation; SDS, Sodium dodecyl sulfate.

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palate development and craniofacial bone formation [11–13]. Parada et al. reported that CTGF regulates cell proliferation as a mediator of the Smad-dependent canonical pathway of transforming growth factor (TGF)- β signaling during palatogenesis [14]. Notably, TGF- β can induce CTGF expression in diverse cell types predominantly in fibrotic disorders [15–18], and CTGF contains a TGF- β response element in its promoter [19]. CTGF has a molecular weight of 38 kDa and contains: insulin-like growth factor-binding protein domain, von Willebrand factor C-type repeat domain (VWC), thrombospondin type-1 repeat domain, and cysteine knot-containing C-terminal domain [8]. Recent studies have suggested that the presence or absence of other growth factors may influence the biological response of target cells, and individual domains within the molecule may regulate various cellular functions [20–22]. The VWC domain in CTGF has been reported to function as bone morphogenetic protein (BMP)- and TGF- β -binding domains in certain cases [23–26]. CTGF can antagonize BMP activity by preventing its binding to BMP receptors; however, it has the opposite effect on TGF- β 1 where it enhances receptor binding [27,28].

CTGF was originally described as a secretory platelet-derived growth factor (PDGF)-like factor that competed with PDGF for binding to PDGF receptors present on fibroblasts. The primary amino acid sequences of CTGF and PDGF have a sequence similarity of 40% [29]. We previously demonstrated that TGF- β 1-induced osteogenic differentiation of human bone marrow-derived MSCs is markedly enhanced by PDGF, although PDGF alone does not induce differentiation [30]. In addition, the enhancement of TGF- β 1-induced osteogenic differentiation by PDGF-induced phosphoinositide 3-kinase (PI3K)/Akt-mediated signaling depends on TGF- β 1-induced mitogen-activated protein kinase (MEK) activity. In the present study, we investigated the osteogenic differentiation of human MSCs following stimulation with exogenous TGF- β 1 and CTGF. We also investigated the mechanisms via which intracellular signals induced by TGF- β 1 and/or CTGF control the osteogenic differentiation of MSCs.

2. Materials and methods

2.1. Reagents

Recombinant human TGF- β 1 and CTGF were purchased from Pepro Tech (Cranbury, NJ, USA). The p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 was obtained from Selleck chemicals (Houston, TX, USA). The MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor U0126 was purchased from Calbiochem (La Jolla, CA, USA). Rabbit polyclonal antibodies against p38 MAPK (#9212S), phospho-p38 MAPK (pp38 MAPK, T180/Y182; #9211S), p44/42 MAPK (ERK1/2, #9102S), phospho-p44/42 MAPK (pERK1/2, T202/Y204; #9101S), stress-activated protein kinase (SAPK)/Jun amino-terminal kinase (JNK) (JNK, #9252S), phospho-SAPK/JNK (pJNK, T183/Y185; #9251S), Akt (#9272), phospho-Akt (Ser473) (pAkt, S473; #9271), phospho-Smad2/3 (pSmad2/3; #8828), were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibody against Smad2/3 (#610842) was purchased from BD Transduction Laboratories (Franklin Lakes, NJ, USA). Mouse polyclonal antibody against β -actin (#H0522) was purchased from Cosmo Bio (Tokyo, Japan).

2.2. Cell culture

The human bone marrow-derived MSCs UE7T-13 cells, the lifespan of which was prolonged via infection with a retrovirus encoding human papillomavirus E7 and human telomerase reverse transcriptase [30–33], were purchased from the Health Science Research Resources Bank (JCRB No.1154; Japan Health Sciences Foundation, Tokyo, Japan). UE7T-13 cells were cultured in growth medium comprising Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified

incubator with an atmosphere of 5% CO₂. The medium was changed every 2–3 d.

2.3. RNA isolation and reverse transcription-quantitative polymerase chain reaction

UE7T-13 cells (1×10^5 cells/well) were seeded in 24-well plastic plates containing growth medium. After 48 h of incubation, the medium was replaced with growth medium containing 5.0 ng/ml TGF- β 1 and/or 100.0 ng/ml CTGF and incubated for 1 week. Total RNA was isolated using ISOGEN I reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. cDNA was synthesized from the extracted total RNA using the PrimeScript RT reagent Kit (Takara-Bio, Shiga, Japan). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was subsequently performed on a Thermal Cycler Dice Real Time System (Takara Bio) using SYBR Premix Ex Taq II (Takara Bio); the sequences of the primers used are listed in Table 1. The mRNA expression levels of RUNX family transcription factor 2 (*RUNX2*), alkaline phosphatase, biomineralization associated (*ALPL*), collagen type I alpha 1 chain (*COL*), secreted phosphoprotein 1 (osteopontin, *OPN*), integrin-binding sialoprotein (bone sialoprotein, *BSP*), bone gamma-carboxyglutamate protein (osteocalcin, *OSC*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were measured. The gene expression levels were normalized to that of *GAPDH* and expressed in terms of fold-change relative to that of the control sample.

2.4. Alkaline phosphatase staining

UE7T-13 cells (1×10^5 cells/well) were seeded in 24-well plastic plates containing growth medium. After 48 h of incubation, the medium was replaced with growth medium containing 5.0 ng/ml TGF- β 1 and/or 100.0 ng/ml CTGF and incubated for 1 week. The cells were then stained with Alkaline phosphatase using the TRAP/ALP staining kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's instructions.

Table 1
Primer sequences used in this study.

Gene name	symbol	primer sequence (5'–3')
Runx-related transcription factor 2	<i>RUNX2</i>	forward CACTGGCTGCAACAAGA
		reverse CATTCCGGAGCTCAGCAGAATAA
Alkaline phosphatase, liver/bone/kidney	<i>ALPL</i>	forward GGACCATTCCTCCAGCTCTTCAC
		reverse CCTTGTAGCCAGGCCATTG
Collagen, type I, alpha 1	<i>COL</i>	forward TCTAGACATGTTACGCTTGTGGAC
		reverse TCTGTACGCAGGTGATTGGTG
Secreted phosphoprotein 1, osteopontin	<i>OPN</i>	forward ACACATATGATGGCCGAGGTGA
		reverse TGTGAGGTGATGTCCTCGTCTGTAG
Integrin-binding sialoprotein, bone sialoprotein	<i>BSP</i>	forward GGCCACGATATTATCTTTACAAGCA
		reverse TCAGCCTCAGAGTCTTCATCTTCA
Bone gamma-carboxyglutamate (gla) protein, osteocalcin	<i>OSC</i>	forward AGGTGCAGCCTTTGTGTCCA
		reverse GGCTCCAGCCATTGATACAG
Glyceraldehyde-3-phosphate dehydrogenase	<i>GAPDH</i>	forward GCACCGTCAAGGCTGAGAAC
		reverse ATGGTGGTGAAGACGCCAGT

2.5. Alizarin red staining

UE7T-13 cells (5×10^4 cells) were seeded in 24-well plates containing growth medium. After 48 h of incubation, the growth medium was replaced with basal osteogenic differentiation medium comprising Minimum Essential Medium alpha (Sigma-Aldrich) supplemented with 100 nM dexamethasone (Sigma-Aldrich), 50 μ g/ml ascorbic acid (Nacalai Tesque, Kyoto, Japan), 10 mM β -glycerophosphate (Sigma-Aldrich), and 10% FBS (PAA Laboratories, Seattle, WA, USA) and treated with 5.0 ng/ml TGF- β 1 and/or 100.0 ng/ml CTGF following culturing for 4 weeks. Half of the medium in each dish was changed every 2–3 d. After 4 weeks, bone matrix mineralization was evaluated via staining with Alizarin red S (Sigma-Aldrich). Alizarin red was extracted by adding calcified nodule lysate (PG Research, Tokyo, Japan), and the absorbance at 450 nm was measured using an MPR-A4i microplate reader (Tosoh, Tokyo, Japan).

2.6. Western blot analysis

UE7T-13 cells (5×10^5 cells) were seeded in 6-cm culture dishes containing growth medium. After 24 h of incubation, the growth medium was replaced with FBS-free starvation medium, and the cells were cultured for 24 h. The cells were then treated with 10.0 ng/ml TGF- β 1 and/or 100.0 ng/ml CTGF for 10, 30, 60, and 120 min. Subsequently, the cells were washed twice with phosphate-buffered saline and solubilized using radioimmunoprecipitation acid lysis buffer [50 mM tris-HCl (pH 7.2), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). The protein content in cell extracts was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of extracted proteins were

separated via 10–20% SDS-polyacrylamide gel electrophoresis (ATTO CORPRATION, Tokyo, Japan) and transferred to a polyvinylidene fluoride membrane (Merck KGaA, Darmstadt, Germany). After blocking the membrane with 5% skim milk in Tris-buffered saline containing Tween-20 (pH 7.6; 50 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20), the membrane was incubated with primary antibodies for 24 h at 4 °C. The membrane was incubated with secondary antibodies for 1 h at room temperature and the blots were detected using the BCIP/NBT Phosphatase Substrate Kit (SeraCare, Milford, MA, USA). Densitometry of the bands was performed using Image-J software (version 1.53e; National Institutes of Health, Bethesda, MD, USA).

2.7. Statistical analysis

Data are presented as the mean \pm standard deviation (SD), and values of $p < 0.05$ statistically analyzed via Tukey’s multiple comparison test using SPSS software (IBM, Armonk, NY, USA) were considered significant. The results are representative of at least three separate experiments.

3. Results

3.1. CTGF enhanced the activation of p38 MAPK in MSCs

To identify the signaling pathways activated by CTGF during the TGF- β 1-induced osteogenic differentiation of UE7T-13 cells, we evaluated the phosphorylation status of the Smad2/3-, PI3K/Akt-, and MAPK-mediated pathways via western blot analysis. The phosphorylation status of ERK1/2 was enhanced by treatment with TGF- β 1 alone but was attenuated via co-treatment with TGF- β 1 and CTGF (Figs. 1 and 2). Therefore, TGF- β 1-induced activation of ERK1/2 may be suppressed in

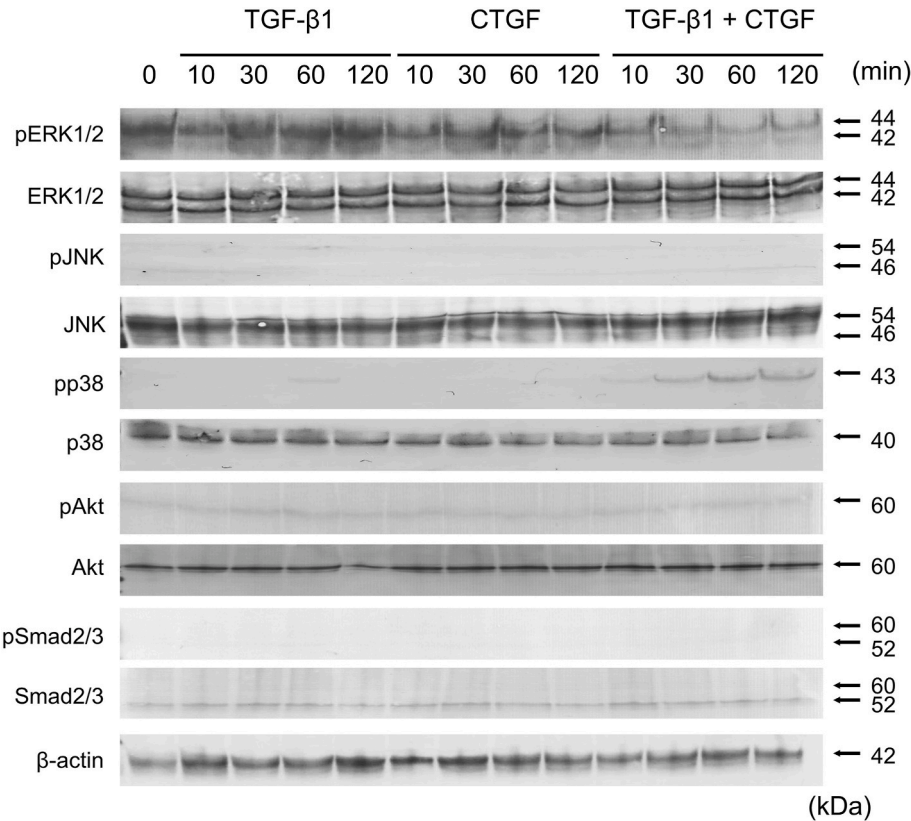


Fig. 1. TGF- β 1 activates ERK1/2 but not p38 MAPK in MSCs. CTGF activates p38 MAPK but not ERK1/2. UE7T-13 cells cultured in 6-cm culture dishes containing growth medium were serum-starved overnight and then stimulated with 10.0 ng/ml TGF- β and/or 100.0 ng/ml CTGF for 10, 30, 60, and 120 min. Phosphorylation status was analyzed via western blot analysis using specific phosphorylated antibodies. TGF- β 1, transforming growth factor beta 1; CTGF, connective tissue growth factor; ERK1/2, extracellular signal-regulated kinase 1/2; MAPK, mitogen-activated protein kinase.

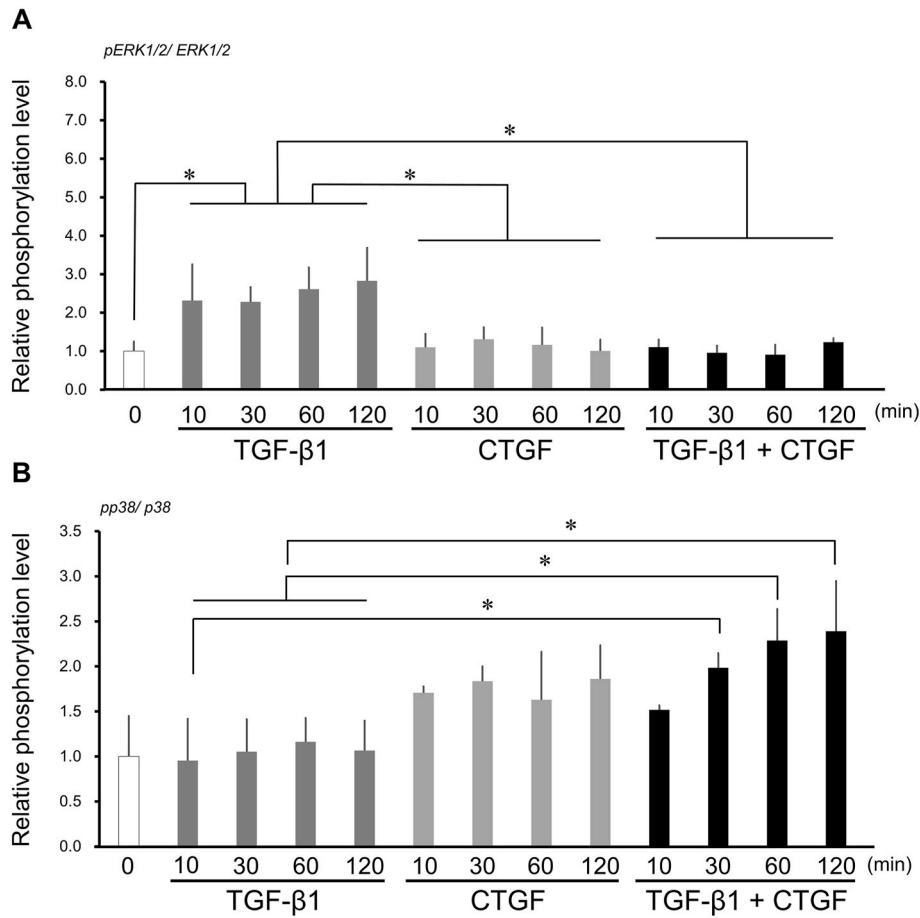


Fig. 2. CTGF promotes phosphorylation of p38 MAPK and attenuates TGF-β1-induced phosphorylation of extracellular ERK1/2 in MSCs. Densitometry was performed with Image-J software. Data are expressed as the ratio of the phosphorylated to total molecular bands illustrated in Fig. 2 each (A) ERK1/2 and (B) p38 MAPK. Data are presented as the mean \pm SD ($n = 3$). * $p < 0.05$ indicate statistical significance. TGF-β1, transforming growth factor beta 1; CTGF, connective tissue growth factor; ERK1/2, extracellular signal-regulated kinase 1/2; MAPK, mitogen-activated protein kinase.

the presence of CTGF. In addition, co-treatment of cells with TGF-β1 and CTGF significantly enhanced p38 MAPK phosphorylation 1 h after treatment. In contrast, TGF-β1 and/or CTGF were not involved in phosphorylation of JNK, Akt, and Smad2/3 in UE7T-13 cells.

3.2. Co-treatment of TGF-β1 and CTGF induced mRNA expression of osteogenic differentiation marker genes in MSCs

UE7T-13 cells were treated with TGF-β1 alone or in combination with CTGF, and then the mRNA expression levels of osteogenic differentiation marker genes were determined via RT-qPCR. The expression levels of *ALPL*, *COL*, and *OPN* were significantly increased after co-treatment with TGF-β1 and CTGF (Fig. 3B–D). Notably, the mRNA expression levels of *BSP* and *OSC* were increased not only by co-treatment with TGF-β1 and CTGF but also by treatment with CTGF alone (Fig. 3E and F). In contrast, treatment with TGF-β1 and/or CTGF did not affect the mRNA expression level of *RUNX2* in UE7T-13 cells (Fig. 3A). In addition, alkaline phosphatase staining was only enhanced by co-treatment with TGF-β1 and CTGF (Fig. 4). Increased expression levels of osteogenic differentiation marker genes and enhancement of alkaline phosphatase staining were suppressed by treatment with SB203580, an inhibitor of p38 MAPK, which is activated by co-treatment with TGF-β1 and CTGF (Fig. 3B–F and 4). These results suggest that CTGF increases the expression levels of osteogenic differentiation marker genes in MSCs, and this effect is enhanced in the presence of TGF-β1 via activation of p38 MAPK.

3.3. CTGF markedly enhanced the TGF-β1-induced extracellular matrix mineralization in MSCs

In general, MSCs that differentiate into osteoblasts induce extracellular matrix mineralization [34–36]. We investigated the osteogenic differentiation in UE7T-13 cells treated with TGF-β1 and/or CTGF using alizarin red staining to assess extracellular matrix mineralization. TGF-β1 treatment induced matrix mineralization in the UE7T-13 cells (Fig. 4). We then examined the synergistic effects of TGF-β1 and CTGF on osteogenic differentiation. Alizarin red staining of TGF-β1- and CTGF- treated cells revealed that the TGF-β1-induced matrix mineralization was enhanced by CTGF co-treatment, whereas CTGF treatment alone did not induce mineralization. Furthermore, the enhancement of mineralization by co-treatment with TGF-β1 and CTGF in UE7T-13 cells was reduced to the level of mineralization obtained via TGF-β1 treatment alone upon treatment with the p38 MAPK inhibitor SB203580. Therefore, CTGF synergistically promotes TGF-β1-induced osteogenic differentiation in MSCs by activating p38 MAPK. Our previous study demonstrated that TGF-β1-induced osteogenic differentiation in MSCs depends on TGF-β1-induced ERK1/2 activity [30]. Interestingly, TGF-β1-induced mineralization enhanced by CTGF in UE7T13 cells was significantly reduced by the MEK inhibitor U0126.

4. Discussion

In this study, CTGF increased the mRNA expression levels of several osteogenic differentiation marker genes such as *ALP*, *COL*, and *OPN* after

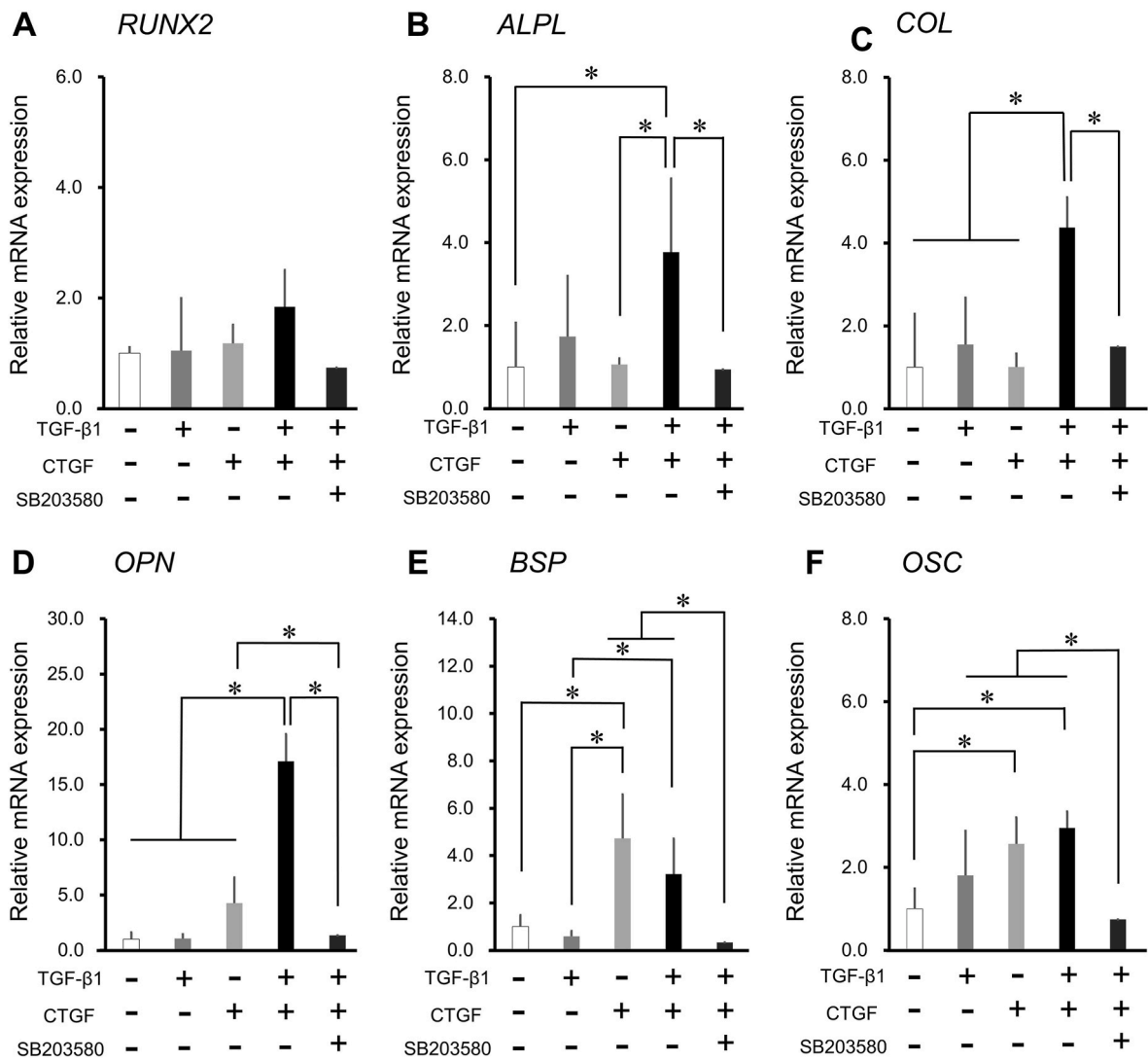


Fig. 3. Co-treatment of UE7T-13 cells with TGF-β1 and CTGF induces the mRNA expression of osteogenic differentiation marker genes in MSCs. UE7T-13 cells were cultured in 24-well plates containing growth medium and treated with 5.0 ng/ml TGF-β1 and/or 100.0 ng/ml CTGF. After 1 week of culture, reverse transcription-quantitative polymerase chain reaction was performed with specific oligonucleotide primers (Table 1). mRNA expression levels of (A) *RUNX2*, (B) *ALPL*, (C) *COL*, (D) *OPN*, (E) *BSP*, and (F) *OSC* were normalized to that of *GAPDH*, and the results are expressed as the fold-change relative to the respective control. Data are presented as the mean ± SD (n = 5). **p* < 0.05 indicates significance. TGF-β1, transforming growth factor beta 1; CTGF, connective tissue growth factor; *RUNX2*, runt-related transcription factor 2; *ALPL*, alkaline phosphatase, liver/bone/kidney; *COL*, collagen, type I, alpha 1; *OPN*, secreted phosphoprotein 1 (osteopontin); *BSP*, integrin-binding sialoprotein (bone sialoprotein); *OSC*, bone gamma-carboxyglutamate protein (osteocalcin); *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

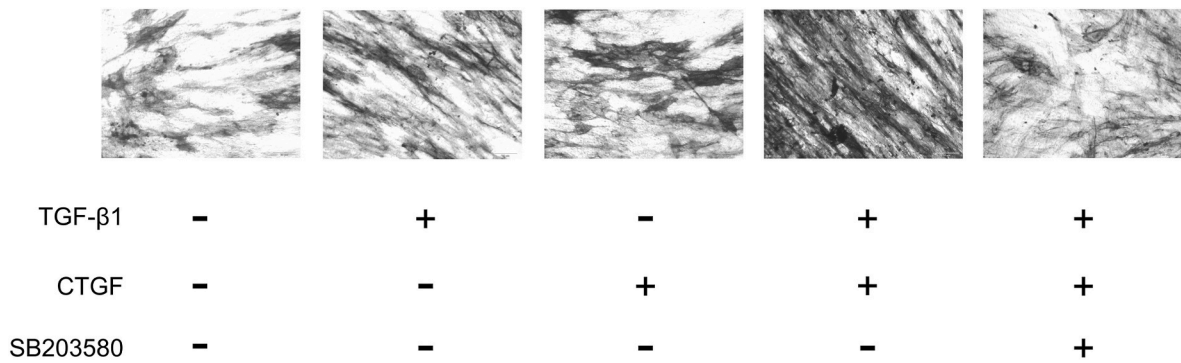


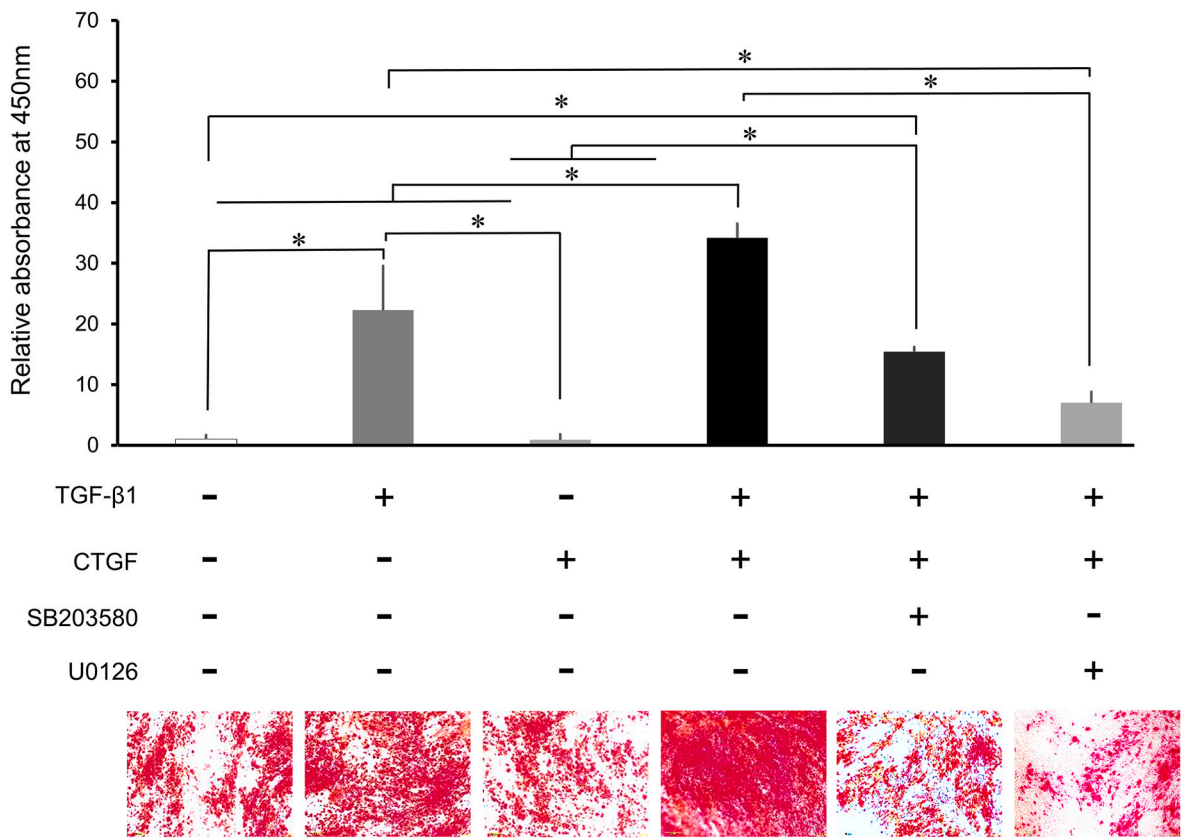
Fig. 4. CTGF enhances TGF-β1-induced alkaline phosphatase staining in MSCs. UE7T-13 cells were cultured in 24-well plates containing growth medium and treated with 5.0 ng/ml TGF-β1 and/or 100.0 ng/ml CTGF. After 1 week of culture, staining for alkaline phosphatase activity was performed. TGF-β1, transforming growth factor beta 1; CTGF, connective tissue growth factor.

co-treatment with TGF-β1 in UE7T-13 cells. However, *BSP* and *OSC* were upregulated after treatment with CTGF alone (Fig. 3). We previously found that the mRNA expression levels of *ALP* and *BSP* are significantly increased in UE7T-13 cells co-treated with TGF-β1 and PDGF; the presence of PDGF is not associated with the expression of *COL*, *OPN*, and *OSC* [30]. The effect of CTGF on TGF-induced osteogenic differentiation in this experiment was characterized by the enhanced expression of bone matrix proteins such as *COL*, *OPN*, and *OSC* in the same cells. CTGF enhances TGF-β1-induced intracellular signal pathway activation by binding to TGF-β1 via its VWC domain [27]. The treatment of primary osteoblasts or osteoblastic cell lines (SaOS-2 or MC3T3-E1) with exogenous recombinant CTGF upregulates *ALPL*, *COL*, *OPN*, and *OSC* [37, 38]. Therefore, it was suggested that the expression of osteogenic differentiation markers in MSCs is enhanced by TGF-β1 and/or CTGF. The osteogenic differentiation proceeds sequentially with the appearance of specific osteogenic marker mRNAs. Usually, *ALP* and *COL* mRNAs are expressed first, followed by *OPN* and *BSP* mRNAs, while *OSC* mRNA emerges last [39]. It was suggested that CTGF not only enhances the expression of *ALP* but also the production of extracellular matrix, whose expression is promoted in the middle to late stages. One of the characteristics of the CCN protein family is that no specific receptor has been identified [40]. The induction of *BSP* and *OSC* expression upon treatment with CTGF alone in this study is predicted to be caused by molecules other than TGF-β1 that interact with each domain of CTGF. The four domains of CTGF interact with factors that mediate cell adhesion, such as fibronectin and integrins [20]. Therefore, CTGF alone may induce the expression of *BSP* and *OSC* through these effects.

Treatment with TGF-β1 enhanced the phosphorylation of ERK1/2 in

UE7T-13 cells, but no change was observed in cells co-treated with CTGF (Figs. 1 and 2). In contrast, pp38 MAPK expression was enhanced only when cells were co-treated with TGF-β1 and CTGF. No phosphorylation of Smad2/3 was detected even though it represents one of the major pathways of TGF-β stimulation in these cells [30]. TGF-β1 activates intracellular effectors such as MAPKs [41,42]. At least three distinct groups of MAPKs have been identified previously: ERK1/2, JNK, and p38 MAPK. Notably, TGF-β1-induced intracellular signaling affects MSC differentiation [30,43–46]. Our previous study demonstrated that the enhancement of TGF-β1-induced osteogenic differentiation in MSCs by PDGF-induced PI3K/Akt-mediated signaling depends on TGF-β1-induced ERK1/2 activity [30]. In the present study, p38 MAPK phosphorylation was detected only after co-treatment with TGF-β1 and CTGF. Since CTGF can enhance receptor binding to TGF-β1 [27], it was suggested that in the presence of CTGF, the effect of TGF-β1 on MSCs is amplified and p38 MAPK is activated.

Alizarin red staining revealed that UE7T-13 cells undergo TGF-β1-induced osteogenic differentiation and significant mineralization (Fig. 5); the mineralization was further enhanced upon co-treatment with TGF-β1 and CTGF. The enhancement of TGF-β1-induced mineralization by CTGF was reduced upon treatment with SB203580, an inhibitor of p38 MAPK, to the mineralization level obtained using TGF-β1 alone. CTGF-null (KO) mice exhibit multiple skeletal abnormalities owing to impaired bone formation/mineralization, growth plate chondrogenesis, and angiogenesis [11]. The temporal pattern of CTGF expression in osteoblasts increases to maximum levels during matrix production and maturation and remains at high levels during mineralization [37]. Several studies have similarly demonstrated that CTGF



stimulates osteoblast proliferation, extracellular matrix production, and differentiation [10,11,38,47,48]. *Ex vivo* studies have shown that primary osteoblasts purified from CTGF-KO mice contain reduced COL, ALP, and OSC expression levels as well as reduced alkaline phosphatase activity and osteoblast proliferation; these defects can be rescued by adding recombinant CTGF [49].

Our previous study showed that ERK1/2 phosphorylation is induced by stimulation with TGF- β 1 alone, whereas the combination of TGF- β 1 and PDGF partially reduces ERK1/2 phosphorylation [30]. In those experiments, unexpectedly, MEK upstream of ERK1/2; treatment with inhibitor U0126 completely inhibits TGF- β 1-induced mineralization in UE7T-13 cells. The PDGF-mediated synergistic promotion of TGF- β 1-induced mineralization is also completely inhibited by U0126 treatment, indicating that PDGF enhances TGF- β 1-induced osteogenic differentiation in a MEK-dependent manner. In the present study, CTGF enhanced the TGF- β 1-induced mineralization in MSCs. In addition, the enhancement was completely suppressed by inhibition of p38 MAPK. Therefore, it was suggested that CTGF enhances TGF- β 1-induced osteogenic differentiation and subsequent mineralization in MSCs by activating the p38 MAPK-dependent pathway. In contrast, CTGF partially inhibited the TGF- β 1-induced phosphorylation of ERK1/2. In this study, the synergistic promotion of TGF- β 1-induced mineralization through CTGF was also completely inhibited by U0126, indicating that CTGF enhances TGF- β 1-induced osteogenic differentiation in a MEK-dependent manner. Taken together, our results suggest that TGF- β 1-induced osteogenic differentiation in MSCs depends on the MAPK pathways including MEK/ERK and p38 MAPK.

5. Conclusions

This study demonstrated the effect of CTGF, which has a functional effect in synergy with TGF- β 1, on osteogenic differentiation in MSCs. Our findings suggest that CTGF enhances TGF- β 1-induced osteogenic differentiation and subsequent mineralization in MSCs by predominantly activating the p38 MAPK-dependent pathway.

Ethical approval

All authors declare that ethical approval is not required for this original article.

CRediT authorship contribution statement

Hironori Yoshida: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Seiji Yokota:** Methodology, Validation, Writing – review & editing. **Kazuro Satoh:** Conceptualization, Validation. **Akira Ishisaki:** Conceptualization, Supervision, Validation, Writing – review & editing. **Naoyuki Chosa:** Conceptualization, Methodology, Data curation, Investigation, Supervision, Validation, Writing – original draft, Writing – review & editing. All the above authors have read and agreed to the submission of the manuscript.

Declaration of competing interest

The authors declare no potential conflicts of interest with respect to this article's authorship and publication.

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