Smad3 and Extracellular Signal-Regulated Kinase 1/2 Coordinately Mediate Transforming Growth Factor-β-Induced Expression of Connective Tissue Growth Factor in Human Fibroblasts

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Connective tissue growth factor (CTGF) is secreted by fibroblasts stimulated with transforming growth factor-β (TGF-β). CTGF is a potent enhancer of fibroblast proliferation, chemotaxis, and extracellular matrix deposition, and it is thought to mediate some of the fibrogenic effects of TGF-β. Here, we have elucidated signaling pathways involved in regulating the TGF-β-induced production of CTGF in primary fibroblasts. TGF-β induced the expression of CTGF messenger RNA and protein in human gingival fibroblasts after 2 h of treatment. Adenoviral overexpression of Smad3 enhanced the TGF-β-elicited expression of CTGF, whereas Smad7 and dominant-negative Smad3 suppressed the effects of TGF-β on CTGF and Cyr61 expression. Pre-treatment of cells with PD98059, an inhibitor for extracellular signal-regulated kinase (ERK)1/2-activator mitogen-activated protein kinase (MAPK)/ERK kinase (MEK)1, potently inhibited the TGF-β-induced expression of CTGF. Furthermore, co-expression of Smad3 with constitutively active MEK1 resulted in potent induction of CTGF production without exogenous TGF-β stimulation. Together, these results demonstrate that Smad3 and ERK1/2 coordinately mediate TGF-β-induced release of CTGF by fibroblasts. It is conceivable that the crosstalk between Smad3 and ERK1/2 signaling cascades plays an important role in regulating CTGF expression, e.g., in wound repair and tissue fibrosis and could be exploited in therapeutic targeting of fibrotic conditions.

Key words: connective tissue growth factor/cyr61/mitogen-activated protein kinase/Smad/TGF-β
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Connective tissue growth factor (CTGF) is a 36–38 kD cysteine-rich heparin-binding protein originally discovered in the conditioned medium of human umbilical vein endothelial cells. CTGF belongs to the CCN (CTGF, Cyr61, and nephroblastoma overexpressed (Nov)) family of growth factors, the additional members of which include Elm-1/Wnt1-inducible signaling pathway protein (WISP-1), Cop1/WISP-2, and WISP-3. CTGF exhibits diverse cellular functions depending on the cell type, which include matrix production, cell migration, angiogenesis, and cell adhesion. Transforming growth factor-β (TGF-β) induces the expression of CTGF in connective tissue cells, but not in epithelial cells (Grotendorst, 1997; Holmes *et al*, 2001; Chen *et al*, 2002; Leask *et al*, 2003). Thus, CTGF is thought to mediate the stimulatory effects of TGF-β on proliferation and matrix production by fibroblastic cells. In addition, CTGF stimulates the synthesis of type I collagen and fibronectin (Frazier *et al*, 1996), and appears to account for the profibrogenic effects attributed to TGF-β.

The cellular effects of TGF-β are mediated via Smad and mitogen-activated protein kinase (MAPK) signaling pathways (Massague, 2000). TGF-β-activated Smad transription factors are divided into three groups according to their function: receptor-activated Smad (Smad2 and Smad3), common-mediator Smad (Smad4), and inhibitory Smad (Smad7). Receptor-activated Smad2 and Smad3 are phosphorylated by activated TGF-β receptor complex, and following phosphorylation these Smads associate with Smad4. Subsequently, this hetero-oligomeric complex translocates into the nucleus, where Smads bind to DNA or associate with other transcription co-activators or co-repressors, and regulate the transcription of various TGF-β-responsive genes (Massague and Wotton, 2000). Smad7 is an inhibitory Smad, the expression of which is induced by TGF-β, and that is capable of inhibiting phosphorylation of Smad2 and Smad3 by competitively interacting with the TGF-β receptor complex (Nakao *et al*, 1997).

In addition to Smad, TGF-β signaling is mediated via MAPK pathways. TGF-β can activate distinct MAPKs in-

Abbreviations: CCN, Cyr61, CTGF, Nov family; CTGF, connective tissue growth factor; Cyr61, cysteine-rich 61; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MKK, MAPK kinase; MMP, matrix metalloproteinase; MOI, multiplicity of infection; Nov, nephroblastoma overexpressed; PAI, plasminogen activator inhibitor; TGF-β, transforming growth factor-β; TIMP, tissue inhibitor of metalloproteinases; WISP, Wnt-induced secreted protein

cluding extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK), and p38 in various types of cells. In human gingival fibroblasts, TGF-β stimulation results in the activation of ERK1/2 and p38 MAPK pathways (Ravanti *et al*, 1999). There is evidence for crosstalk between distinct cell signaling cascades activated by TGF-β, e.g., MAPK and Smad pathways. ERK1/2, JNK, and p38 MAPK are capable of activating or inhibiting Smad pathway by phosphorylating Smad2 or Smad3 (de Caestecker *et al*, 1998; Brown *et al*, 1999; Dennler *et al*, 2000; Leivonen *et al*, 2002; Hayashida *et al*, 2003). In addition, delayed phosphorylation of p38 MAPK by TGF-β has recently been shown to be mediated by the Smad pathway (Takekawa *et al*, 2002; Ungefroren *et al*, 2003).

The promoter region of CTGF contains a TGF-β response element, which is mainly responsible for the basal expression of CTGF (Grotendorst, 1997; Holmes *et al*, 2001). In addition, a functional Smad binding element has been identified from the CTGF promoter, and it is essential for TGF-β induction of CTGF promoter activity in lung and skin fibroblasts, and in mesangial cells (Holmes *et al*, 2001; Chen *et al*, 2002). There is also recent evidence that activation of Ras/MAPK/ERK kinase (MEK)1/2/ERK1/2 pathway is required for activation of CTGF promoter by TGF-β in fibroblasts (Leask *et al*, 2003). Here, we have elucidated the role of Smad and MAPK signaling in the regulation of endogenous CTGF in human gingival fibroblasts. Our results show, that TGF-β potently induces secretion of soluble CTGF in fibroblasts, and that this is mediated by cooperation between ERK1/2 and Smad3 signaling pathways. It is conceivable that this interplay may play an important role in regulating CTGF expression, e.g., in wound repair and tissue fibrosis and could be exploited in therapeutic targeting of CTGF expression in fibrotic conditions.

Results

TGF-β induces the expression of CTGF in human gingival fibroblasts TGF-β induces the expression of CTGF in human dermal fibroblasts (Igarashi *et al*, 1993). To also examine whether human gingival fibroblasts express CTGF in response to TGF-β, we treated human gingival fibroblasts obtained from three different donors with TGF-β (5 ng per mL) for 24 h, and analyzed the expression of CTGF by northern and western blots. CTGF is a secreted protein that can exist as a soluble 36–38 kDa glycosylated form, or alternatively, it can be associated with the cell layer (Steffen *et al*, 1998). As shown in Fig 1*A*, a 24-h TGF-β stimulation led to potent induction of CTGF production both in to the conditioned media and the cell layers of all gingival fibroblast strains studied. Cyr61 protein was detected in the cell layers of gingival fibroblasts, and its expression was also potently upregulated by TGF-β. In comparison, the levels of tissue inhibitor of metalloproteinases-1 (TIMP-1) in the conditioned media of fibroblasts remained unaltered in response to TGF-β (Fig 1*A*).

We also studied the kinetics of TGF-β induction of CTGF expression by treating the cultures with TGF-β for different periods of time, and analyzing the expression of CTGF messenger RNA (mRNA) and protein by northern and western blots. As shown in Fig 1*B*, CTGF mRNA was potently induced 2 h after TGF-β addition, and the levels remained elevated until 24 h. In addition, plasminogen activator inhibitor (PAI)-1 mRNA were detected 2 h after TGF-β stimulation, whereas biglycan mRNA were potently upregulated after 24-h TGF-β treatment (Fig 1*B*). Western blot analyses showed that CTGF protein appeared in the cell layer of TGF-β-treated fibroblasts even after 2 h, whereas soluble CTGF was detected in the conditioned media after 18 h of treatment (Fig 1*C*). The levels of soluble CTGF were elevated up to 48 h, indicating that the production of CTGF protein is rapidly induced after TGF-β addition, but it accumulates in the conditioned media relatively slowly.

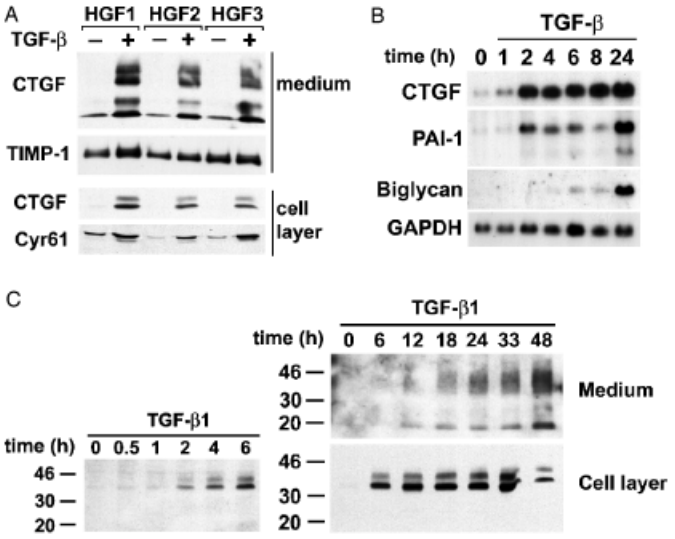

Figure 1
Expression of connective tissue growth factor (CTGF) is induced by transforming growth factor-β (TGF-β) in human gingival fibroblasts. (*A*) Human gingival fibroblasts (HGF1–3) established from three different donors were serum starved for 18 h, and subsequently treated with TGF-β1 (5 ng per mL) for 24 h, as indicated. The conditioned media and cell layers were harvested and analyzed by western blotting for the production of CTGF, tissue inhibitor of metalloproteinases (TIMP)-1, and Cyr61 with specific antibodies. (*B,C*) HGF were serum starved for 18 h, and subsequently treated with TGF-β1 (5 ng per mL) for different periods of time, as indicated. The cell layers were harvested for RNA extraction and analyzed for the expression of CTGF, plasminogen activator inhibitor (PAI)-1, biglycan, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA by northern blotting (*B*). Conditioned media were collected and analyzed for the production of soluble CTGF, and the cell layers were analyzed for the production of CTGF by western blotting (*C*).

TGF-β-induced expression of CTGF is mediated by Smad3 In order to elucidate the role of Smad signaling pathway in TGF-β-induced expression of endogenous CTGF gene in human gingival fibroblasts, we utilized adenoviral gene delivery of Smad7 (RAdSmad7) and dominant-negative Smad3 (RAdSmad3DN). Human gingival fibroblasts were infected with the corresponding recombinant adenoviruses and with empty control virus RAdpCA3, treated with TGF-β for 24 h, after which the cells were harvested and analyzed for the expression of CTGF by western blot hybridization. TGF-β potently induced the accumulation of CTGF in the cell layer of control virus RAdpCA3-infected cells, as compared with untreated control (Fig 2*A*). Adenoviral overexpression of Smad7 potently (by 73%) inhibited

the effect of TGF-β on CTGF production, indicating that Smad signaling pathway is essential for TGF-β-induced CTGF expression. In addition, expression of dominant-negative Smad3 inhibited TGF-β-induced expression of CTGF, indicating that Smad3 specifically mediates the upregulatory effect of TGF-β on CTGF gene expression. Similarly, basal and TGF-β-induced Cyr61 expression was suppressed by adenoviral expression of Smad7 and Smad3DN (Fig 2*A*).

To further study the role of Smad signaling in the induction of endogenous CTGF production by TGF-β in human gingival fibroblasts, we utilized adenoviral gene delivery of wild-type Smad2 and Smad3, and dominant-negative Smad3, and analyzed CTGF expression by northern and western blots. As expected, a 24-h TGF-β treatment of empty control virus RAd66 infected cells resulted in potent induction of the expression of CTGF mRNA and protein, as compared with untreated cells (Fig 2*B* and *C*). Interestingly, the TGF-β-induced expression of CTGF mRNA was clearly (by 3-fold) enhanced by Smad3 overexpression, as compared with RAd66-infected cells treated with TGF-β (Fig 2*B*), resulting in a marked increase in secretion of soluble CTGF in the conditioned media of fibroblasts (Fig 2*C*). In accordance with the above results, expression of dominant-negative Smad3 potently inhibited the TGF-β-induced expression of CTGF mRNA by 85%, indicating that Smad3 is essential for the induction of CTGF production by TGF-β (Fig 2*B* and *C*). In contrast, overexpression of Smad2 had no effect on the induction of CTGF expression by TGF-β (Fig 2*B* and *C*). The production of TIMP-1 was not affected by Smad2 or Smad3 overexpression in the absence or presence of TGF-β (Fig 2*C*).

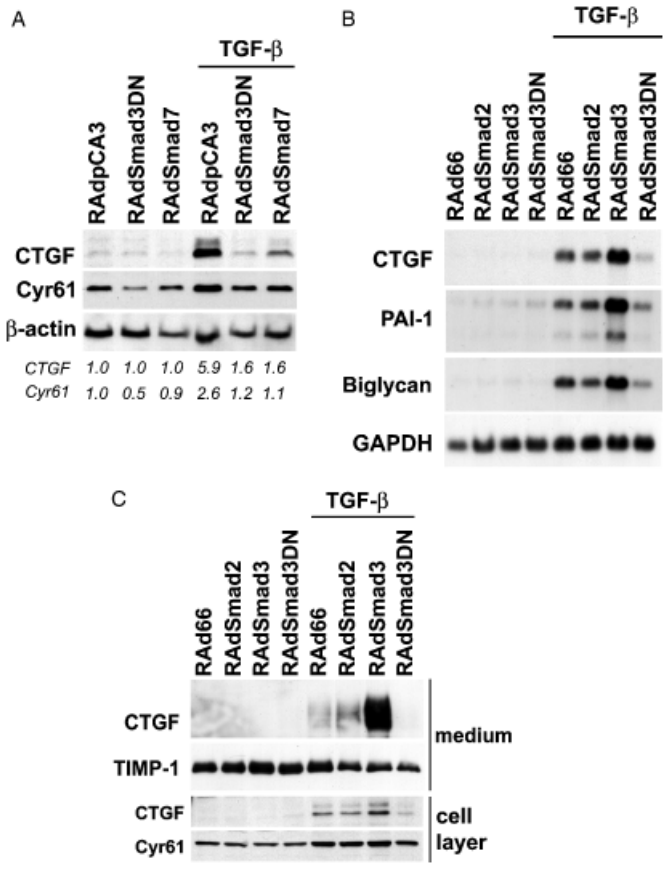

Figure 2
Smad3 mediates transforming growth factor-β- (TGF-β-) induced expression of connective tissue growth factor (CTGF) in human gingival fibroblasts. (*A*) Human gingival fibroblasts were infected with recombinant adenoviruses for dominant-negative Smad3 (RAdSmad3DN), and wild-type inhibitory Smad7 (RAdSmad7), and with empty control virus RAdpCA3 at multiplicity of infection (MOI) 500. After infection, the cells were incubated for 24 h, and treated with TGF-β1 (5 ng per mL) for 24 h, as indicated. The cell layers were harvested and analyzed for the levels of CTGF and Cyr61 by western blotting using corresponding antibodies. Equal loading was confirmed by probing the same filter with specific antibody against β-actin. The levels of CTGF and Cyr61 quantitated by densitometric scanning and normalized to β-actin levels are shown below the blot relative to the levels in RAdpCA3-infected control cells (1.0). (*B,C*) Human gingival fibroblasts were infected with recombinant adenoviruses for Smad2 and Smad3 (RAdSmad2 and RAdSmad3, respectively), and RAdSmad3DN, and with control virus RAd66 at MOI 500, and treated with TGF-β1 (5 ng per mL) for 24 h, as indicated. Total cellular RNA were analyzed for the expression of CTGF, plasminogen activator inhibitor (PAI)-1, biglycan, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA by northern blotting (*B*). Conditioned media were analyzed for the levels of CTGF and tissue inhibitor of metalloproteinases (TIMP)-1, and the cell layers for the expression of CTGF and Cyr61 with western blotting (*C*).

Biglycan expression in human gingival fibroblasts is potently induced by TGF-β (Kähäri *et al*, 1991), and recent studies have identified biglycan as a Smad-responsive gene in pancreatic cells (Chen *et al*, 2002; Ungefroren *et al*, 2003). Therefore, we examined the role of Smad signaling in regulating biglycan gene expression in gingival fibroblasts. TGF-β potently induced the expression of biglycan mRNA, and the overexpression of Smad3 enhanced the effect of TGF-β by 2-fold (Fig 2*B*). In addition, inhibition of the Smad pathway by dominant-negative Smad3 suppressed the expression of biglycan mRNA by 75%. These results indicate that biglycan is a Smad3-responsive gene in human gingival fibroblasts.

ERK1/2 mediates TGF-β-induced expression of CTGF in human gingival fibroblasts TGF-β activates ERK1/2 and p38 MAPK pathways in human gingival fibroblasts (Ravanti *et al*, 1999). To examine the role of ERK1/2 and p38 MAPK pathways in mediating the effect of TGF-β on CTGF gene expression, we used PD98059 (30 μM), an inhibitor for MEK1, the upstream activator of ERK1/2, and SB203580 (10 μM), a specific chemical inhibitor for p38 MAPK. Gingival fibroblasts were serum-starved for 18 h, and subsequently treated with the corresponding MAPK inhibitors for 1 h prior to addition of TGF-β. TGF-β-induced CTGF mRNA was detected 3 h after TGF-β treatment, and their levels were further increased after 8 or 16 h of TGF-β treatment (Fig 3*A*). Interestingly, PD98059 potently (by 80%) inhibited the effect of TGF-β on CTGF mRNA abundance, whereas SB203580 slightly (by 30%) inhibited the effect of TGF-β, noted after 16 h incubation. These results indicate that the ERK1/2 pathway mediates the effects of TGF-β on

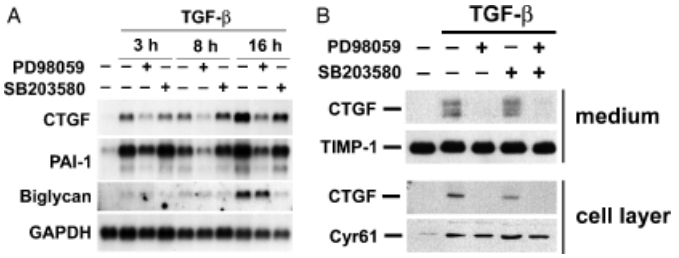

Figure 3
Transforming growth factor-β- (TGF-β-) induced expression of connective tissue growth factor (CTGF) in human gingival fibroblasts is mediated by extracellular signal-regulated kinase (ERK)1/2. (*A*) Human gingival fibroblasts were serum starved for 18 h, and treated for 1 h with PD98059 (30 μM), or SB203580 (10 μM), specific chemical inhibitors for mitogen-activated protein kinase/ERK kinase (MEK)1 or p38, respectively. Subsequently, TGF-β1 (5 ng per mL) was added, and the cultures were incubated for 3, 8, or 16 h, as indicated. Total cellular RNA were harvested and analyzed for the levels of CTGF, plasminogen activator inhibitor (PAI)-1, biglycan, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA by northern blot hybridizations. (*B*) Human gingival fibroblasts were treated with PD98059 or SB203580, and TGF-β1, as in (*A*) and incubated for 24 h. The conditioned media were analyzed for the levels of CTGF and tissue inhibitor of metalloproteinases (TIMP)-1, and the cell layers for the expression of CTGF and Cyr61 with western blotting.

CTGF gene expression. In addition, PD98059 suppressed the TGF-β-induced PAI-1 mRNA levels by 80%–90% (Fig 3*A*), indicating that in gingival fibroblasts ERK1/2 also mediates TGF-β-induced expression of PAI-1. In comparison, PD98059 had no marked effect on the levels of biglycan mRNA detected after 16-h TGF-β treatment (Fig 3*A*). In contrast, SB203580 potently (by 90%) inhibited the effect of TGF-β on biglycan gene expression, providing evidence that the activity of p38 MAPK is essential for TGF-β-induced biglycan expression (Fig 3*A*). This is in accordance with recent observations showing that MKK6/p38 pathway is needed for induction of biglycan gene expression by TGF-β (Ungefroren *et al*, 2003).

To further elucidate the role of ERK1/2 and p38 MAPK in TGF-β-induced expression of CTGF, we treated gingival fibroblasts with the corresponding MAPK inhibitors and TGF-β for 24 h, and analyzed the conditioned medium and cell layers for the production of CTGF by western blotting. In accordance with the above observations, a 24-h TGF-β treatment of cells potently induced the production of CTGF protein both into the cell layer and conditioned media (Fig 3*B*). Consistent with the results shown in Fig 3*A*, inhibition of MEK1 activity by PD98059 totally abrogated TGF-β-induced CTGF accumulation in the cell layer and secretion into conditioned media (Fig 3*B*). In contrast, SB203580 slightly reduced the TGF-β-induced accumulation of CTGF to the cell layer, but this had no marked effect on the production of soluble CTGF (Fig 3*B*). These results indicate that ERK1/2 signaling pathway is essential for the TGF-β-induced expression of CTGF in human gingival fibroblasts. In comparison, neither PD98059 nor SB203580 had any marked effect on Cyr61 or TIMP-1 production (Fig 3*B*).

Co-expression of Smad3 with constitutively active MEK1 results in induction of CTGF production in the absence of TGF-β stimulation Smad signaling is also regulated through crosstalk with other signaling cascades, e.g., MAPK pathways p38, ERK1/2 and JNK, and Cam kinase II (Brown *et al*, 1999; Wicks *et al*, 2000; Blanchette *et al*, 2001; Watanabe *et al*, 2001; Leivonen *et al*, 2002). Furthermore, ERK1/2 has been shown to activate Smad2 or Smad3 by phosphorylation (Funaba *et al*, 2002; Hayashida *et al*, 2003). As TGF-β treatment of gingival fibroblasts leads to rapid phosphorylation of ERK1/2 (Ravanti *et al*, 1999), and the above results show the involvement of Smad3 and ERK1/2 in TGF-β-induced CTGF expression, we examined the possible crosstalk between Smad3 and ERK1/2 signaling pathways in regulating the CTGF gene expression in human gingival fibroblasts. We utilized adenovirus RAdMEK1CA for delivery of constitutively active MEK1, the upstream activator or ERK1/2, and adenovirus for constitutively very active MKK3b (RAdMKK3bE), the upstream activator of p38α, together with Smad3 adenovirus. Gingival fibroblasts were infected with the corresponding adenoviruses, incubated for 24 h, and the conditioned media and cell layers were harvested for western analysis. In order to confirm that ERK1/2 and p38 MAPK were activated by adenovirally expressed MEK1CA and MKK3bE, respectively, we first analyzed the levels of activated ERK1/2 and p38 in infected fibroblasts by western blot. As shown in Fig 4*A*, infection of cells with adenoviruses for constitutively active MKK3b and MEK1 resulted in potent activation of p38 and ERK1/2 MAPK, respectively. Furthermore, overexpression of Smad3 had no effect on the phosphorylation of p38 or ERK1/2. Phosphorylation of Smad3 in its C-terminal SSXS-motif could not be detected in these samples (data not shown).

Next, we analyzed the production of CTGF in the same cells by western blotting. In the absence of TGF-β, activation of ERK1/2 by MEK1CA and simultaneous co-expression of Smad3 resulted in significant induction of CTGF accumulation into the cell layer and secretion into the conditioned media of infected gingival fibroblasts (Fig 4*A*). On the contrary, activation of endogenous p38α by constitutively active MKK3bE and simultaneous co-expression of Smad3 or MEK1CA had no effect on the accumulation of CTGF in the cell layer or on the conditioned media of infected gingival fibroblasts (Fig 4*A* and *B*). Interestingly, activation of ERK1/2 and simultaneous overexpression of Smad3 also resulted in upregulation of Cyr61 production in the cell layer or infected cells (Fig 4*A*). Activation of ERK1/2 by MEK1CA slightly augmented pro-matrix metalloproteinase (MMP)-1 production, and co-expression of Smad3 further induced pro-MMP-1 protein production (Fig 4*B*). In addition, activation of p38 together with activated ERK1/2 resulted in potent induction of pro-MMP-1 protein levels (Fig 4*B*). Activation of ERK1/2 by constitutively active MEK1 also enhanced the production of TIMP-1 in the conditioned media of the same cells (Fig 4*B*).

Discussion

CTGF is a pleiotropic growth factor, the expression of which is rapidly induced by TGF-β in fibroblastic cells. It belongs to the CCN family of growth factors, the other members including Cyr61-, Nov-, and Wnt-induced secreted proteins-1, -2, and -3 (WISP-1, -2, and -3). CCN members ex-

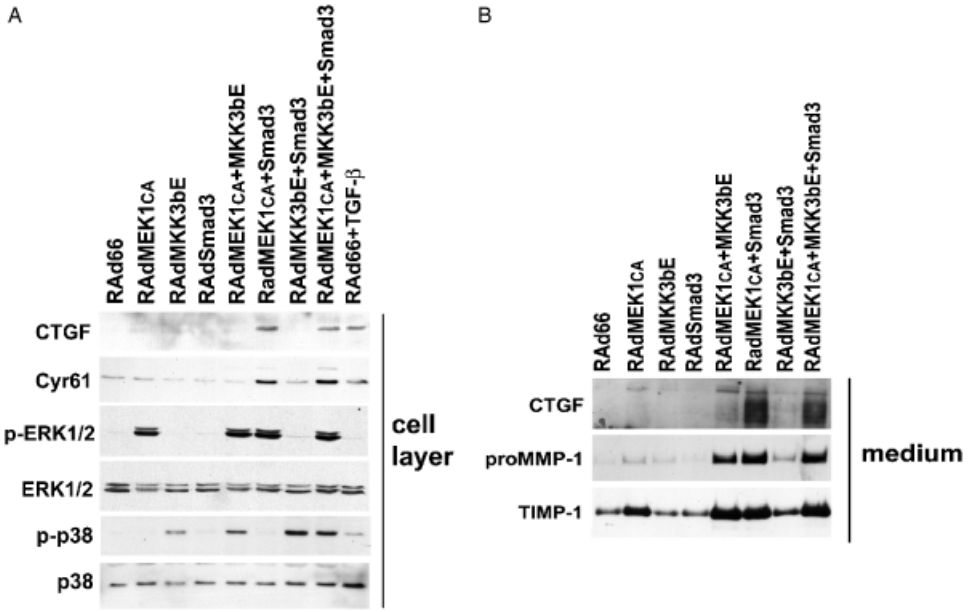

Figure 4
Expression of Smad3 and activation of extracellular signal-regulated kinase (ERK)1/2 results in induction of connective tissue growth factor (CTGF) gene expression in the absence of transforming growth factor-β (TGF-β) stimulation. (*A*, *B*) Human gingival fibroblasts were infected with recombinant adenoviruses for constitutively active mitogen-activated protein kinase/ERK kinase (MEK)1 (RAdMEK1CA), constitutively active MKK3b (RAdMKK3bE), Smad3 (RAdSmad3) and control virus RAd66, as indicated at multiplicity of infection 500. After 24 h incubation, the cell layers were analyzed for the levels of CTGF and Cyr61, and for the levels of activated p38 MAPK (p-p38) and ERK1/2 (p-ERK1/2) and total p38 and ERK1/2 with specific antibodies (*A*). Conditioned media were harvested and analyzed for the production of CTGF, pro-matrix metalloproteinase-1, and tissue inhibitor of metalloproteinases (TIMP-1) (*B*).

hibit a wide range of functions that regulate biological processes such as cell adhesion, angiogenesis, and tissue fibrosis. CTGF is a potent enhancer of fibroblast proliferation, chemotaxis, and extracellular matrix (ECM) deposition, and it is thought to mediate some of the fibrogenic effects of TGF-β (Frazier *et al*, 1996).

In this study, we have elucidated in detail the cellular signaling pathways involved in regulating the TGF-β-induced expression of CTGF in human gingival fibroblasts. Major cell signaling pathways activated by TGF-β include MAPK pathways ERK1/2, JNK, and p38, and the Smad pathway. The Smad pathway is activated when TGF-β type I receptor kinase ALK5 phosphorylates SSXS motif in the C-terminus of receptor-regulated Smad2 and Smad3. TGF-β is a potent stimulator of the production of many ECM components, and Smads are involved in mediating the effects of TGF-β on the expression of e.g., collagenase-3 (MMP-13), biglycan, aggrecan, PAI-1, type I collagen, and type VII collagen (Dennler *et al*, 1998; Watanabe *et al*, 2001; Leivonen *et al*, 2002; Ungefroren *et al*, 2003). Although the role of different cellular signaling pathways in regulating the activity of CTGF promoter has been widely studied (Grotendorst *et al*, 1996; Holmes *et al*, 2001; Chen *et al*, 2002; Leask *et al*, 2003), there are only a few reports studying the regulation of endogenous CTGF gene expression. Therefore, we used our recently constructed recombinant Smad adenoviruses (Leivonen *et al*, 2002) in dissecting the role of Smad signaling pathway in regulating the endogenous CTGF gene expression in human gingival fibroblasts. The results of this study show that Smad3, but not Smad2, mediates the effect of TGF-β on the production of endogenous CTGF. Adenoviral overexpression of Smad3 resulted in a significant enhancement in TGF-β-induced levels of CTGF mRNA and resulted in a potent increase in the secretion of soluble CTGF. In addition, Smad7 and dominant-negative Smad3 potently inhibited the effect of TGF-β on CTGF production. These results are in accordance with a previous report, where Smad3 and Smad4 mediated the activation of CTGF promoter by TGF-β in the human lung and skin fibroblasts (Holmes *et al*, 2001). Interestingly, our results also demonstrate that the basal and TGF-β-induced expression of Cyr61 is Smad-dependent. Inhibition of Smad signaling by adenovirally delivered dominant-negative Smad3 and inhibitory Smad7 potently suppressed Cyr61 production in the cell layer of infected fibroblasts. It seems that the regulation of CCN family members CTGF and Cyr61 differs from the regulation of the third member of the family, Nov, as TGF-β downregulates the expression of Nov in a Smad-independent manner (Lafont *et al*, 2002).

In gingival fibroblasts, TGF-β stimulation leads to activation of ERK1/2 and p38 MAPK pathways (Ravanti *et al*, 1999). Therefore, we elucidated the role of these signaling cascades in mediating the effects of TGF-β on CTGF gene expression. MEK1/2 inhibitor PD98059 abrogated the effects of TGF-β on the induction of CTGF gene expression, whereas p38 inhibitor SB203580 had no marked effect on the production and release of CTGF by fibroblasts. This is in accordance with recent results showing that ERK1/2 is involved in the activation of CTGF promoter by TGF-β in skin fibroblasts (Leask *et al*, 2003). In addition, our results provide evidence for the importance of crosstalk between ERK1/2 and Smad3 signaling in CTGF regulation, as co-expression of Smad3 with constitutively active MEK1 resulted in potent induction of CTGF production without exogenous TGF-β stimulation. There is recent evidence that kinases of MAPK pathways are capable of activating Smad signaling. For example, activated ERK1/2 can phosphorylate Smad2 in its linker region and N-terminal domain, but not in the usual C-terminal SSXS motif, which is phosphorylated by ALK5 (Funaba *et al*, 2002). Furthermore, MEK1/2/ERK1/2 inhibitors PD98059 and UO126 decrease the total serine phosphorylation of Smad2/3, but not phosphorylation of the C-terminal SSXS motif, suggesting that ERK1/2 is capable of phosphorylating Smad in a region other than the C-terminus (Hayashida *et al*, 2003). In our study, phosphorylated Smad3 could not be detected in the cell lysates where Smad3 was co-expressed with constitutively active MEK1, as the phospho-

specific antibody used here detects only Smad3 phosphorylated in its C-terminus (Piek *et al*, 1999; Dooley *et al*, 2001). It is therefore possible that ERK1/2 also induces activation of Smad3 in our model. This could explain why Cyr61 is upregulated when Smad3 is co-expressed with activated ERK1/2. Inhibition of ERK1/2 pathway by PD98059 had no effect on TGF-β-induced Cyr61 expression, suggesting that ERK1/2 is not alone involved in the regulation of Cyr61. On the contrary, our results demonstrated that Smad3 mediated the expression of Cyr61. Together, these results suggest that activation of Smad3 by ERK1/2 is sufficient to induce Cyr61 expression in the absence of TGF-β under the conditions where Smad3 is co-expressed with MEK1-activated ERK1/2.

Although CTGF is a secreted soluble protein, it also associates with the ECM (Steffen *et al*, 1998). In this study, secretion of soluble CTGF in the conditioned media of TGF-β-stimulated gingival fibroblasts was markedly increased when Smad3 was overexpressed. In addition, co-expression of Smad3 with constitutively active MEK1 resulted in potent secretion of soluble CTGF by these cells. Interestingly, the expression of MMP-1 was also potently upregulated, when Smad3 was co-expressed with constitutively active MEK1, i.e., the secretion of CTGF in the conditioned media correlated with the production of MMP-1. MMPs are a large family of zinc-dependent endopeptidases collectively capable of degrading essentially all components of the ECM. Moreover, MMPs can cleave molecules other than ECM components, such as interleukin-1β and Fas-ligand (Ito *et al*, 1996; Mitsiades *et al*, 2001). There is also recent evidence that certain MMPs can cleave CTGF (Hashimoto *et al*, 2002). It is therefore possible, that MMPs are involved in the release of CTGF from the ECM into the conditioned media of fibroblasts.

In conclusion, the results of this study demonstrate that activation of Smad3 and ERK1/2 signaling cooperatively mediates the stimulatory effect of TGF-β on CTGF production in human fibroblasts (Fig 5). It is likely, that TGF-β elicited induction of CTGF expression by an interplay between Smad3 and ERK1/2 plays a role in situations in which CTGF mediate the effects of TGF-β, such as tissue repair and fibrosis. It is conceivable that this interplay can be exploited in therapeutic targeting of CTGF expression, e.g., in fibrotic conditions.

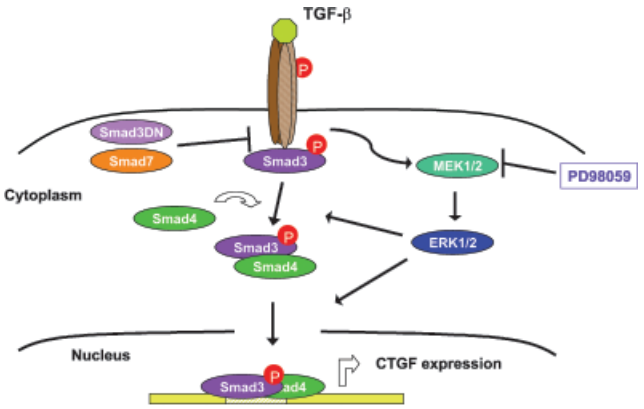

Figure 5
A schematic representation of the transforming growth factor-β (TGF-β) signaling pathways regulating connective tissue growth factor (CTGF) gene expression in human gingival fibroblasts. Stimulation of human gingival fibroblasts with TGF-β results in activation of Smad3 and extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase (MAPK). Smad3 associates with Smad4, and mediates induction of CTGF expression by TGF-β. Smad7 and dominant-negative Smad3 (Smad3DN) inhibit phosphorylation of Smad3 by TGF-β receptor complex. Activated ERK1/2 and Smad3 cooperate in regulating the expression of CTGF. MAPK/ERK kinase (MEK)1/2 activity is inhibited by specific chemical inhibitor, PD98059.

Materials and Methods

Cell cultures and reagents Human gingival fibroblasts were established from three healthy donors (Ravanti *et al*, 1999; Leivonen *et al*, 2002). All studies were approved by the ethical committee of the University of Turku. Participants gave their written informed consent, and the study was conducted according to the declaration of Helsinki. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St Louis, Missouri) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU per mL penicillin-G, and 100 μg per mL streptomycin. Human recombinant TGF-β1 was obtained from Sigma, and p38 MAPK inhibitor SB203580 and MEK1/2 inhibitor PD98059 were from Calbiochem (San Diego, California).

Transduction of human gingival fibroblasts with recombinant adenoviruses The construction of empty control virus RAdpCA3 and recombinant adenoviruses RAdSmad2, RAdSmad3, RAdSmad4 for HA-tagged Smad2, Smad3, and Smad4, respectively, has been described previously (Leivonen *et al*, 2002). Recombinant adenoviruses for Smad7 (RadSmad7) (Fujii *et al*, 1999) and dominant-negative Smad3 (RAdSmad3DN) (Pardali *et al*, 2000) were kindly provided by Dr Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). Adenovirus for constitutively active MEK1 (RAdMEK1CA) (Foschi *et al*, 1997) was kindly provided by Dr Marco Foschi (University of Florence, Italy), and adenovirus for constitutively active MKK3b (RAdMKK3bE) (Wang *et al*, 1998) by Dr Jiahuai Han (Scripps Research Institute, La Jolla, California). Control adenovirus RAd66 (Wilkinson and Akrigg, 1992) was kindly provided by Dr Gavin W.G. Wilkinson (University of Cardiff, Cardiff, UK).

Adenoviral infections of human gingival fibroblasts were performed as previously (Leivonen *et al*, 2002). Briefly, cells were infected in suspension with a multiplicity of infection 500, plated, and incubated for 18 h in DMEM with 1% FCS. Thereafter, the medium was replaced with DMEM without FCS, and the incubations were continued for 24 h. The cultures were treated with TGF-β1 (5 ng per mL) for 24 h, as indicated. Thereafter, the conditioned media were harvested, and analyzed for the production of CTGF, pro-MMP-1, or TIMP-1 by western blotting. The cell layers were harvested either for RNA extraction to detect CTGF, PAI-1, biglycan, or MMP-1 mRNA, or for the determination of CTGF or Cyr61 proteins and activated p38 and ERK1/2 from the cell lysates by western blotting.

Immunoblotting and antibodies Western blottings were performed as described previously (Leivonen *et al*, 2002). Polyclonal rabbit anti-CTGF antibody pAb2 was obtained from Fibrogen (South San Francisco, California). Polyclonal antibody against Cyr61 was from Santa Cruz Biotechnology (Santa Cruz, California), and polyclonal anti-TIMP-1 from Chemicon International (Temecula, California). Polyclonal rabbit antiserum raised against human MMP-1 was a kind gift from Dr Henning Birkedal-Hansen (NIDCR, National Institutes of Health, Bethesda, Maryland). Polyclonal antibodies for phospho-p38, p38, phospho-ERK1/2, and ERK1/2 were from Cell Signaling Technology (Beverly, Massachussetts), and monoclonal antibody for β-actin was from Sigma. Antiserum for phospho-Smad1, which shows cross-reactivity with phosphorylated Smad3, was a kind gift from Dr Aristidis Moustakas (Ludwig Institute for Cancer Research) (Piek *et al*, 1999; Dooley *et al*, 2001). The blots were visualized by the enhanced chemilumi-

nescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK), and quantitated by densitometric scanning.

Northern blot hybridizations Total cellular RNA was extracted with Qiagen's Rapid RNA Purification Kit (Qiagen, Chatsworth, California), and northern blot hybridizations were performed as described previously (Leivonen *et al*, 2002). For hybridizations, a 1.2-kb human CTGF complementary DNA (cDNA), a 2.0-kb human MMP-1 cDNA (Goldberg *et al*, 1986), human PAI-1 cDNA (Keski-Oja *et al*, 1988), a 1.7-kb human biglycan cDNA (Fisher *et al*, 1989), and a 1.3-kb rat glyceraldehyde-3-phosphate dehydrogenase cDNA (Fort *et al*, 1985) were used. The 1.2 kb cDNA for the coding region of human CTGF (Bradham *et al*, 1991) was obtained by RT-PCR of TGF-β-treated gingival fibroblast RNA samples using specific primers (sense 5′-ATC GAT ATC CGC AGT GCC AAC CAT GAC-3′, and antisense 3′-GTA CCG TAC TTC GGT CTC TCC TAG GAC A-5′).

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