Runx2/Smad3 Complex Negatively Regulates TGF-β-Induced Connective Tissue Growth Factor gene Expression in Vascular Smooth Muscle Cells

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Aim: Connective tissue growth factor (CTGF), a direct target gene of transforming growth factor-β (TGF-β) signaling, plays an important role in the development of atherosclerosis. We previously showed that Runx2, a key transcription factor in osteoblast differentiation, regulates osteogenic conversion and dedifferentiation of vascular smooth muscle cells (VSMCs). In this study, we investigated the hypothesis that Runx2 modulates CTGF gene expression via the regulation of TGF-β signaling.

Methods and Results: Expression of the Runx2 gene was decreased, and CTGF mRNA levels were reciprocally increased by TGF-β in a time-dependent manner in cultured human aortic smooth muscle cells (HASMCs) and C3H10T1/2 cells. Forced expression of Runx2 decreased and the reduction of Runx2 expression by small interfering RNA enhanced both basal and TGF-β-stimulated CTGF gene expression in HASMCs. Site-directed mutation analysis of the CTGF promoter indicated that transcriptional repression by Runx2 was mediated by the Smad-binding element (SBE) under basal and TGF-β-stimulated conditions. Data obtained from immunoblots of Runx2-, Smad3- or Smad4-transfected cells and chromatin immunoprecipitation analysis indicated that Runx2 interacts with Smad3 at the SBE. Immunohistochemistry revealed that the expression of Runx2 and CTGF was distinct and almost mutually exclusive in human atherosclerotic plaque.

Conclusions: These results for the first time demonstrate that Runx2/Smad3 complex negatively regulates endogenous and TGF-β-induced CTGF gene expression in VSMCs. Thus, the induction of Runx2 expression contributes to the phenotypic modulation of VSMCs, in which the TGF-β/Smad pathway plays a major role.


Key words: Atherosclerosis, Smooth muscle cell, Differentiation, Signaling

Introduction

Transforming growth factor-β (TGF-β) plays a critical role in determining the phenotype of vascular smooth muscle cells (VSMCs) by regulating a diverse set of cellular responses, including cell proliferation, migration, and the production of extracellular matrix (ECM). TGF-β and receptors are abundantly expressed in VSMCs in atherosclerotic lesions and stimulate proteoglycan production and a collagen-rich fibrous cap. Stable lesions in atherosclerotic plaque express greater amounts of TGF-β than unstable lesions. Among a variety of genes whose expression is directly regulated by the TGF-β/Smad pathway is the gene for connective tissue growth factor (CTGF), a cysteine-rich secreted peptide that is a key regulator of extracellular matrix (ECM). CTGF plays an important role in atherosclerosis and restenosis by multiple processes, including the stimulation of ECM produc-
tion, monocyte migration into lesions, and intimal angiogenesis\(^4,5\). It is generally believed that modulation of TGF-\(\beta\) signaling affects plaque stability and CTGF may be a therapeutic target of atherosclerosis.

TGF-\(\beta\) acts through binding to a transmembrane receptor, which in turn phosphorylates R-Smads, such as Smad2 and Smad3\(^6-8\). These activated R-Smads then translocate to the nucleus with binding partner Smad4. R-Smad and Smad4 complex regulates a wide range of target genes that are relevant to phenotypic changes of VSMCs through cis-regulatory elements. Smad proteins consist of two conserved globular domains, known as the MH1 (Mad homology 1) and MH2 domains, coupled by a linker region. The MH1 domain recognizes the Smad-binding element (SBE) in the target gene promoter, whereas the MH2 domain binds to transcriptional coactivators such as p300 and CREB-binding protein (CBP)\(^9\). A ubiquitously expressed homeodomain protein of the TALE class, TGIF, represses TGF-\(\beta\)-induced transcription by deacetylase recruitment via a Smad-TGIF complex\(^10\). C-Ski\(^1,12\) and its related protein SnoN (Ski-related novel gene) are also Smad-binding transcriptional co-repressors\(^13\). Thus, various proteins are recruited to Smads and regulate TGF-\(\beta\) signaling.

The transcription factor Runx2, also known as Cbfa1/Os2/AML3/PEBP2aA, is one of the three mammalian members of the Runt-related transcription family\(^14\). Runx2 directly regulates osteoblast-specific genes, including osteocalcin, osteopontin, and type I collagen, through OSE (osteooblast-specific cis-acting element)\(^15\). Forced expression of the Runx2 gene in non-osteoblastic cells induces osteoblast differentiation in vitro\(^10\). Mice deficient in Runx2 do not form mineralized bone in any part of their skeleton\(^14,15,17\), and inherited mutations of the Runx2 gene in human cause cleidocranial dysplasia, characterized by severely impaired osteogenesis\(^18\). From these results, Runx2 is an essential transcription factor for both intramembranous and endochondral ossification. We have previously reported that Runx2 represses SMC gene expression and promotes osteogenic gene expression in human aortic smooth muscle cells (HASMCs)\(^19\). Runx2 physically interacts with SRF, disrupts the SRF/myocardin ternary complex, and inhibits myocardin-induced SMC gene expression; however, the role of Runx2 in the regulation of TGF-\(\beta\) signaling remains to be determined.

We undertook the present study to determine whether Runx2 exerts its direct effect on TGF-\(\beta\) signaling pathways in VSMCs. We found that Runx2 inhibits endogenous and TGF-\(\beta\)-induced CTGF gene expression. The inhibition occurs through a complex formation of Runx2 with Smad3 within the CTGF promoter. To our knowledge, this is the first demonstration of the repression of TGF-\(\beta\) signaling by Runx2, which acts as a Smad corepressor.

### Materials and Methods

#### Plasmids

FLAG-Runx2 was described previously\(^{19}\). Myc-Smad3 and Myc-Smad4 were gifts from K. Miyazono\(^20\) (Tokyo University, Japan). pGL(SBE)-Luc was kindly provided by S. Itoh\(^21\) (Showa Pharmaceutical University, Japan). Various deletion mutants of CTGF-Luc (\(-745, \ -201, \ -57\) and \(+1/+203\) from the translation start site) were constructed by subcloning polymerase chain reaction (PCR) -amplified fragments of the human CTGF genomic sequence into pGL4 vector (Promega).

#### Cell Culture, Transfection, and Luciferase Assays

TGF-\(\beta\) was purchased from Roche. C3H10T1/2 cells and COS7 cells were obtained from the American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS). HASMCs were obtained from Kurabo and cultured in Humedia supplemented with 5% FBS, 2 ng/mL human fibroblast growth factor 2, 0.5 ng/mL human epidermal growth factor, and 5 \(\mu\)g/mL insulin. C3H10T1/2 cells were transiently transfected with plasmid DNA using a modified calcium phosphate precipitation method as described previously\(^{12}\). The total amount of DNA per well was kept constant by adding the corresponding amount of expression vector without a cDNA insert. Seventy-two hours later, cells were harvested and used for subsequent experiments, including RNA analyses and luciferase assays (Promega). Reporter gene luciferase activity was normalized to the amount of total protein in lysates. These assays were performed in triplicate and repeated at least four times, and results from a representative experiment are shown with standard deviations.

#### RNA Analysis and Small Interfering RNA (siRNA)

Total RNA was isolated from cells with TRIzol reagent (Invitrogen) according to the manufacturer’s directions. Single-strand cDNAs were synthesized from 1 \(\mu\)g total RNA, and semiquantitative reverse transcription-PCR (RT-PCR) was performed using an RT-PCR kit (Takara) and Advantage 2 polymerase mix (Clontech). All experiments were repeated at least three times. The primers used for RT-PCR are listed in Table 1.
The Runx2-specific siRNA (siRunx2) and GFP siRNA (siGFP) were introduced into HASMCs using Lipofectamine2000 (Invitrogen). The details are described elsewhere\(^1\)\(^9\).

### Adenovirus

Adenoviruses encoding FLAG-Runx2 (Ad-Runx2) and LacZ (Ad-LacZ) were produced using the Gateway system (Invitrogen). Protein expression was confirmed by Western blotting (data not shown).

### Immunoprecipitation and Western Blots

COS7 cells were transiently transfected with FLAG-Runx2, Myc-Smad3 and Myc-Smad4, as indicated in the figure legends, by the calcium phosphate precipitation method. Cells were harvested in phosphate-buffered saline (PBS) at 72 h after transfection and were lysed in NETN buffer (20 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM EDTA and 0.5% NP-40) supplemented with freshly prepared protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 2 µg/mL pepstatin A and aprotinin). Following brief sonication and the removal of cellular debris by centrifugation, epitope-tagged proteins were precipitated with the indicated antibodies and protein A/G-Sepharose beads (GE Healthcare). The bound proteins were washed four times in NETN buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. Membranes were immunoblotted with antibodies as indicated, and proteins were visualized with a chemiluminescence detection system (Millipore).

### Chromatin Immunoprecipitation (ChIP)

ChIP assay was performed as described previously\(^2\)^\(^9\). Cultured cells were cross-linked with 1% formaldehyde for 10 min at room temperature. After the cells had been collected in PBS, the pellets were resuspended in SDS lysis buffer (1% SDS, 5 mM EDTA, 50 mM Tris-HCl [pH 8] and protease inhibitors). Cross-linked chromatin was sonicated to shear genomic DNA. Cross-linked DNA/protein extracts were diluted with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl [pH 8] and protease inhibitors) and immunoprecipitated with 5 µg normal rabbit immunoglobulin G (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-FLAG or anti-Myc antibodies overnight at 4°C. Protein A-Sepharose beads were added to the supernatant, and the mixture was incubated for 1 h. The beads were washed sequentially with TSE \(_1\) buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 0.5% NP-40), TSE \(_2\) buffer (0.1% SDS, 1% Triton X-100, 1 mM EDTA, 500 mM NaCl and 20 mM Tris-HCl [pH 8]), TSE \(_3\) buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA and 10 mM Tris-HCl [pH 8]), and TE buffer (10 mM Tris-HCl [pH 8] and 1 mM EDTA) twice. The precipitates were eluted with 150 µL elution buffer (1% SDS and 0.1 M NaHCO\(_3\)), and incubated with 6 µL of 5 M NaCl at 65°C for 4 h to reverse cross-linking. After treatment with proteinase K (Roche) and RNase A (Sigma), DNA fragments were purified with a PCR purification kit (Qiagen). Input DNA and DNA isolated from precipitated chromatin were subjected to conventional PCR. The primers used for ChIP assays are listed in Table 1.

### Immunohistochemical Analysis

The atherosclerosis specimen was obtained at Gunma University Hospital by directional coronary atherectomy (DCA) from a patient with ischemic heart disease, with informed consent from the patient.

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**Table 1.** Primer sequences used for RT-PCR analyses and ChIP assays

<table>
<thead>
<tr>
<th>Test</th>
<th>Gene</th>
<th>Primer sequence</th>
<th>Primer sequence</th>
</tr>
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<tr>
<td>Mouse semiquantitative PCR</td>
<td>Runx2</td>
<td>5'-GAGGGCAACAGTTATCTCG-3'</td>
<td>5'-CGCTCCGGCCCAAAATC-3'</td>
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<td></td>
<td>CTGF</td>
<td>5'-AGCACTGGAGAAGTTTG-3'</td>
<td>5'-GCTGTTTGAAGCTAC-3'</td>
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<tr>
<td></td>
<td>GAPDH</td>
<td>5'-ACCACGAGGCAATC-3'</td>
<td>5'-TCCACCCGCTTGTGTA3'</td>
</tr>
<tr>
<td>Human semiquantitative PCR(^a)</td>
<td>Runx2</td>
<td>5'-CGCATCTCCTACCCAGTA-3'</td>
<td>5'-GACTGGCCGGGTGTAGTA3'</td>
</tr>
<tr>
<td></td>
<td>CTGF</td>
<td>5'-TTCCAGAGCGCTGCAAAT-3'</td>
<td>5'-TGGAGATTGGAGGTTACG-3'</td>
</tr>
<tr>
<td>ChIP assays(^a)</td>
<td>CTGF-SBE</td>
<td>5'-TGCGAAGAGTAGGAAA-3'</td>
<td>5'-TACGCGGAGAGTTGGTA3'</td>
</tr>
<tr>
<td>Subcloning</td>
<td>CTGF-SBE mutant</td>
<td>5'-CAGCTTTTGAGCTCGAGAATCT-3'</td>
<td>5'-AGCATCTCTGATCCAAAAGCTG-3'</td>
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</table>

\(^a\)The primers for GAPDH are the same as the mouse GAPDH sequences used in semiquantitative PCR.
Human carotid artery tissues were obtained at autopsy at Gunma University Hospital from a patient with ischemic heart disease, with informed consent from his family. These protocols were approved by the Institutional Review Board at Gunma University Hospital. Tissue samples were fixed in 10% buffered formalin, embedded in paraffin, and serial sections were prepared. After deparaffinization and blocking, samples were incubated with antibodies against CTGF (L-20) and Runx2 (PEBP2aA,M-70), SM α-actin (Enzo; CGA7) overnight at 4°C, and for double-immunofluorescence staining, samples were incubated with Cy3- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Sigma) for 1 h at room temperature. Nuclear staining was performed with 4′,6-diamidino-2-phenylindole (DAPI). Immunohistochemical staining of sections was performed using the CSA kit (DAKO) according to the manufacturer's protocol. Serial sections were also stained using hematoxylin-eosin (HE).

Results

Runx2 Inhibits CTGF gene Expression in HASMCs

To examine whether Runx2 regulates CTGF gene expression in VSMCs, Runx2 was adenovirally overexpressed in HASMCs. RT-PCR analyses of RNA prepared from HASMCs transduced with Ad-Runx2 and Ad-LacZ showed that overexpression of Runx2 significantly repressed the basal expression of the CTGF gene in HASMCs (Fig. 1A). We found that Runx2 is constitutively expressed at minimal but detectable levels in VSMC under unstimulated conditions, and knockdown of Runx2 expression in HASMCs by siRNA increased CTGF mRNA levels (Fig. 1B). Together, these results suggest that Runx2 inhibits endogenous CTGF gene expression in HASMCs.

To gain further insight into the mechanisms by which Runx2 inhibits CTGF expression, we performed transient transfection assays using CTGF promoter constructs that contain various lengths of the 5′ flanking region of the human CTGF gene. We employed mouse mesenchymal fibroblast C3H10T1/2 cells, which are capable of undergoing differentiation into SMCs or osteoblasts. As shown in Fig. 1C, the luciferase activity of CTGF-745Luc and CTGF-201Luc was significantly decreased in the presence of Runx2 in C3H10T1/2 cells. Deletion of the DNA sequence from -745 to -201 increased luciferase activity, suggesting the presence of a negative regulatory sequence. Further deletion of the 5′-flanking DNA sequence to -57 substantially decreased luciferase activity, and overexpression of Runx2 only minimally inhibited the luciferase activity of CTGF-57Luc. These results suggest that the DNA sequence between -201 and -57 contains the cis-regulatory element responsible for the repression of CTGF expression in response to Runx2.

We aimed to identify the sequences that are critical for the inhibition of CTGF expression by Runx2. No sequences identical or similar to OSE, a canonical binding sequence for Runx2, were found; however, we identified the consensus Smad-binding element (SBE), AGAC24, at -180 of the human CTGF promoter (Fig. 1D). Because Smad proteins are the primary TGF-β receptor substrates that translocate to the nucleus and regulate gene expression in concert with other transcription factors, we hypothesize that Runx2 inhibits CTGF expression directly or indirectly through interference with the function of Smads.

TGF-β Inhibits Runx2 Expression and Induces CTGF Transcripts in HASMCs and C3H10T1/2 Cells

To determine the role of Runx2 in the regulation of TGF-β-induced CTGF expression, we examined the expression of Runx2 and CTGF in response to TGF-β stimulation in HASMCs and C3H10T1/2 cells. Consistent with previous reports, CTGF mRNA levels of HASMCs increased in a time-dependent manner by TGF-β stimulation (Fig. 2A). In contrast, Runx2 transcripts decreased in response to TGF-β and the propensity was an inverted mirror image of CTGF expression. A similar tendency was observed in C3H10T1/2 cells (Fig. 2B). These results prompted us to investigate whether Runx2 negatively regulates TGF-β-induced CTGF expression.

In order to elucidate the mechanism by which TGF-β inhibits Runx2 expression, we examined whether TGF-β inhibits Runx2 mRNA via the induction of de novo protein synthesis. As shown in Fig. 2C, regardless of the presence of cycloheximide (CHX), an inhibitor of protein synthesis, TGF-β decreased Runx2 mRNA in HASMCs. These results suggest that repression of Runx2 mRNA expression does not require de novo protein synthesis. Our result is consistent with the previous paper in which Smad3 repressed Runx2 expression in osteoblasts.

Runx2 Represses TGF-β-Induced CTGF Expression in HASMCs through a Sequence Containing SBE

Previous reports described that TGF-β stimulation strongly induces CTGF gene expression. We next examined the effects of Runx2 on TGF-β-induced CTGF expression. Runx2 overexpression significantly attenuated the TGF-β-mediated induction of CTGF
Runx2 Acts as a Smad Repressor in VSMCs

expression in HASMCs (Fig. 3A). Conversely, knockdown of Runx2 by siRNA enhanced the induction of CTGF expression in response to TGF-β (Fig. 3B). These results suggest that Runx2 inhibits TGF-β-induced CTGF expression in HASMCs.

To determine the mechanisms by which Runx2 represses TGF-β-induced CTGF expression, a luciferase assay was performed. As expected, the results showed that SBE mutation of the 5'-flanking region of the CTGF gene completely abolished basal and TGF-β-induced CTGF gene promoter activity. Runx2 repressed basal and TGF-β-stimulated CTGF promoter activity in an SBE-dependent manner (Fig. 3C). These results suggest that Runx2 represses TGF-β-induced CTGF expression at the transcriptional level through the sequence of SBE.
Runx2 Represses TGF-β Signaling by Interacting with Smad3

To examine the role of Smads in Runx2-mediated repression of TGF-β-induced CTGF expression, we utilized a reporter plasmid, pGL(SBE)-Luc, in which luciferase expression is controlled by four tandem copies of the SBE. In C3H10T1/2 cells, this promoter is transcribed efficiently by TGF-β or by overexpression of Smad3 (data not shown). As in the case with CTGF-Luc construct, TGF-β-induced luciferase activity derived from pGL(SBE)-Luc was markedly inhibited by Runx2 (Fig. 4A and 4B). These data suggest that the transcriptional repression function by Runx2 was dependent on Smad proteins; to test this possibility, we cotransfected COS7 cells with Myc-Smad3, Myc-Smad4 and FLAG-Runx2 and performed coimmunoprecipitation studies. The results showed that FLAG-Runx2 was immunoprecipitated with Myc-Smad3 but not with Myc-Smad4 (Fig. 4C).

Next, to confirm the interaction between Runx2 and Smad proteins within the native chromatin context, we performed the ChIP assay. Chromatin fragments from lysates of COS7 cells cotransfected with Myc-Smad3, Myc-Smad4 and FLAG-Runx2 were precipitated with anti-FLAG antibody or control IgG and subjected to PCR analysis using primers specific to the SBE of the CTGF gene promoter. As shown in Fig. 4D, anti-FALG antibody immunoprecipitated the SBE sequence in the presence of FLAG-Runx2, Myc-Smad3 and Myc-Smad4, whereas the SBE signal was barely detected in the absence of FLAG-Runx2 or in negative control IgG immunoprecipitates. These results indicate that Runx2 interacts with the Smad3/Smad4 complex at SBE (Fig. 4D).

Expression of Runx2 and CTGF in Human Atherosclerotic Plaques

We performed immunohistochemistry of the atherosclerotic plaque obtained from the patient with coronary artery disease by DCA (Fig. 5A) or autopsy (Fig. 5B). Fluorescent photomicrographs revealed that Runx2 and CTGF were abundantly expressed in the plaque and the cells expressing Runx2 and CTGF appear to be distinct (Fig. 5A). The few double-positive cells supported our hypothesis that Runx2 potently inhibits CTGF expression in VSMCs. In addition, we found Runx2- and CTGF-positive cells in the intima of human calcifying carotid artery where SMα-actin is abundantly expressed, suggesting that Runx2- and CTGF-expressing cells are derived from VSMCs (Fig. 5B).

Discussion

In this study, we demonstrated that Runx2 inhibited basal and TGF-β-induced CTGF expression in HASMCs by interfering with the Smad3-mediated signaling pathway. This conclusion is supported by several lines of experimental evidence. First, overexpression of Runx2 inhibits TGF-β-induced CTGF gene expression in an SBE-dependent manner. Second, siRNA for Runx2 enhanced TGF-β-induced CTGF expression. Third, Runx2 does not bind SBE, but interacts with Smad3 at SBE within CTGF promoter. To the best of our knowledge, this is the first investigation to demonstrate the Runx2/Smad3 complex as a corepressor of TGF-β-induced gene expression in VSMCs.

Runx2 works as a Corepressor without DNA Binding

Previous analyses demonstrated that Runx2 con-
Runx2 Acts as a Smad Repressor in VSMCs

A, HASMCs were infected with an MOI of 10 of either Ad-Runx2 or Ad-LacZ. B, HASMCs were transfected with either siRunx2 or siGFP. After 2 days, cells were serum starved for 24 h and subsequently treated with TGF-β for 12 h. Expression of CTGF and Runx2 transcripts was detected by RT-PCR analyses. GAPDH was used as an internal control for RNA loading. C, C3H10T1/2 cells were transiently transfected with the indicated CTGF reporter plasmid and FLAG-Runx2 or pcDNA3.1. After 24 h, cells were serum starved for 24 h and subsequently treated with TGF-β for 12 h. Values are presented as relative luciferase activity compared with the pcDNA3.1 vector alone.

**Fig. 3.** Runx2 represses TGF-β-induced CTGF expression in HASMCs.

So far, several studies have described the interaction between Runx2 and Smad proteins. Bone morphogenetic protein 2 (BMP2) promotes osteogenic differentiation through Smad1 and Runx2 interactions. This Smad-mediated gene expression is dependent on the Smad interacting domain (SMID) of Runx2. Smad3 interacts with Runx2 and confers functional TGF-β stimulation of collagenase-3 expression in a human breast cancer cell line. In contrast, TGF-β inhibits osteoblast differentiation by the Smad3-mediated interference of Runx2 function. Further studies to understand the molecular mechanism underlying the repression of Smad-mediated signaling by Runx2 are warranted.

Contains transcriptional repression domains that inhibit both basal and activated transcription. Given the emerging role of histone deacetylases (HDACs) in regulating Runx2, it is plausible that the Runx2/Smad3 complex recruits such corepressors. In view of the fact that DNA binding has been considered to be fundamentally required for Runx2-dependent gene repression, it is worth stressing that we found no cis-regulatory DNA sequences for Runx2 within the CTGF gene promoter. A previous report showed that Runt, the *Drosophila* homologue of Runx proteins, represses the *Drosophila* melanogaster segmentation gene *engrailed* (en) by a mechanism that does not involve DNA binding. In that study, the possibility that Runt tethers to the DNA by interacting with the zinc-finger DNA-binding protein Tramtrack (Ttk) has been proposed. Further studies to understand the molecular mechanism underlying the repression of Smad-mediated signaling by Runx2 are warranted.
Fig. 4. Runx2-mediated repression is dependent on SBE and Smad3 interaction. 

A, C3H10T1/2 cells were transiently transfected with pGL(SBE)4-Luc and FLAG-Runx2 or pcDNA3.1. After 2 days, cells were serum starved for 24 h and subsequently treated with TGF-β for 12 h. B, pGL(SBE)4-Luc and Myc-Smad3 or pcDNA3.1 were transiently transfected with or without FLAG-Runx2 in C3H10T1/2 cells. After 2 days, cells were harvested and the luciferase assay was performed. Values are presented as relative luciferase activity compared with the pcDNA3.1 vector alone. C, COS7 cells were transiently transfected with FLAG-Runx2 and Myc-Smad3 or Myc-Smad4. Cell lysates were incubated with anti-Flag antibody or normal mouse IgG, and immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-Flag or anti-Myc antibodies as indicated. Whole cell extracts were loaded for input. D, COS7 cells were transiently transfected with FLAG-Runx2 and/or Myc-Smad3 and Myc-Smad4. ChIP assay was performed as described in Materials and Methods. The cross-linked chromatin was immunoprecipitated with normal mouse IgG or anti-Flag or anti-Myc antibodies, followed by PCR amplification using primers containing the SBE of human CTGF. Input represents the amount of DNA prior to immunoprecipitation in each sample.
As shown in Fig. 4C, we detected Runx2 and Smad3 interaction in COS7 cells; however, Runx2 and Smad3 proteins synthesized by the in vitro transcription and translation system did not form a complex (data not shown). We assume that Runx2 does not directly interact with Smad3 but rather recruits p300/CBP or histone deacetylase (HDAC) that physically associate with both Runx2 and Smad3. Consistent with this assumption, we found no interaction between Runx2 and Smad3 in 293T cells in which adenovirus type 5 E1A and SV40 large T antigen are abundantly expressed, and p300/CBP or HDAC are sequestered from the transcription factor complex (data not shown).

Runx2 Inhibits Endogenous CTGF gene Expression in VSMCs

Runx2 has been proved to be a key regulator of both chondrocyte hypertrophy and osteoblast differentiation, and CTGF is considered to be a major factor that promotes endochondral ossification. In fact, in situ hybridization studies demonstrated that CTGF expression is correlated with Runx2 expression during embryonic skeletal development; no CTGF mRNA was detected in Runx2 null mice in chondrocytes.\(^{(38)}\) In this regard, it is interesting to note that, unlike in chondrocytes, Runx2 represses CTGF gene expression in VSMCs, and Runx2-positive cells scarcely express CTGF in atherosclerotic plaque. The most likely explanation for the discrepant effect of Runx2 on the CTGF gene between chondrocytes and VSMCs is that Runx2 exerts differential effects on gene expression depending on the cell type, stage of cell development and experimental conditions, a so-called ‘context dependency’.

It is noteworthy that CTGF expression is repressed by Runx2 in the absence of TGF-\(\beta\) (Fig. 1A, 1C). Given that Smad3 and Smad4 form a complex in the nucleus even in the absence of TGF-\(\beta\)\(^{(39, 40)}\), it is likely that Runx2 acts as corepressor of the Smad3/Smad4 complex to inhibit basal activity of the CTGF promoter under basal conditions. Consistent with this assumption, previous studies showed that CTGF expression is reduced in cells derived from Smad3-null mice\(^{(41, 42)}\), suggesting that the Smad3/Smad4 complex supports basal CTGF gene expression and is subjected to Runx2-mediated repression, irrespective of TGF-\(\beta\)
stimulation.

**Runx2 Inhibits TGF-β-Induced CTGF Expression by Interacting with Smad3**

We employed the *CTGF* promoter to analyze the role of Runx2 in TGF-β/Smad signaling because it has been established that TGF-β stimulates *CTGF* production, at least partly through Smad-dependent pathways, and functional SBE has been identified within the *CTGF* promoter. Previously, several ligands and molecules were reported to inhibit TGF-β-induced *CTGF* gene expression. Suppression of TNF-α on TGF-β-induced *CTGF* expression is due to the competition for p300 by p65 and Smad4. Interleukin-1α and -1β inhibit TGF-β-stimulated Smad3 phosphorylation by increasing Smad7 expression and by TGF-activated kinase1 (TAK1). Interfering with Smad3, PPARγ and its ligands suppress CTGF expression in response to TGF-β. Our study demonstrates that overexpression of Runx2 blunted the TGF-β induction of *CTGF* expression. Knockdown of Runx2 expression by siRNA augmented the induction of *CTGF* expression by TGF-β. Furthermore, transient transfection assays of the *CTGF* promoter and SBE-derived luciferase gene revealed that SBE is responsible for the repression by Runx2. These results all point to the possibility that by associating with TGF-β-activated Smads, Runx2 forms a Smad-dependent transcriptional repressor complex.

**Implications for Vascular Disease**

Given the crucial role of TGF-β in cellular differentiation, the synthesis of ECM, and the proliferation/apoptosis of VSMCs, our finding of the ability of Runx2 to repress TGF-β signaling implies that Runx2 plays an important role in the phenotypic change of VSMCs and thus in the development of vascular disease. The TGF-β/Smad signaling pathway increases the transactivating function of the myocardin/SRF complex by possibly recruiting Smad coactivators. In our previous study, we showed that Runx2 inhibits SMC-specific gene expression by interfering with the function of myocardin/SRF. Runx2 disrupts the complex formation of myocardin/SRF. We also found that Runx2 inhibits TGF-β-induced SMC-specific gene expression (data not shown).

Increasing evidence demonstrates that vascular calcification, which is often encountered in advanced atherosclerotic lesions and is a common consequence of aging, shares features with normal embryonic bone formation. Runx2 and other osteoblast-specific genes are expressed in atherosclerotic and Moenckeberg types of calcification. Furthermore, Runx2 plays a role in the osteoblastic differentiation of VSMCs cultured in media containing high β-glycerophosphate or inorganic phosphate levels. We have recently shown that Runx2 transactivates the expression of osteopontin and osteocalcin genes in VSMCs in response to fibroblast growth factor-2 (FGF-2), and raised the possibility that Runx2 mediates phenotypic transition from VSMCs to an osteoblastic phenotype in atherosclerotic lesions where FGF-2 and its receptor (FGFR1) are abundantly expressed. The results of the present study provide additional evidence suggesting that Runx2 augments osteoblastic changes of VSMCs by repressing TGF-β-activated Smad signaling, which plays a major role in the determination of SMC differentiation. In addition, it is interesting to note that TGF-β inhibits Runx2 expression, as shown in Fig. 2A and 2B. These data are consistent with a report showing that TGF-β inhibits osteoblast differentiation through repression of the function of the Runx2/Smad3 complex in osteoblasts. In contrast, TGF-β has been reported to activate Runx2-mediated osteoblastic gene expression in cooperation with BMP-2 in C2C12 cells. These apparently conflicting reports may be reconciled by genomic modification controlled in a cell-intrinsic manner.

**TGF-β-Induced CTGF Expression as a Therapeutic Target?**

It remains unclear whether Runx2 inhibition of TGF-β/Smads signaling retards plaque formation and progression toward an unstable phenotype. CTGF is overexpressed predominantly in areas with ECM accumulation, and especially along with the shoulder of fibrous caps in advanced atherosclerotic plaques. In vitro studies showed that overexpression of CTGF induces capase-3 activity and hence induces apoptosis of VSMCs. In addition, CTGF promotes monocyte migration into lesions and induces intimal angiogenesis. In contrast, CTGF may contribute to plaque stabilization by increasing ECM accumulation. Although the precise role of CTGF in plaque stability in atherosclerosis awaits further studies, the expression levels of CTGF, the location of CTGF expression, and the environmental cue of VSMCs in plaque may be important, as in the case of TGF-β.

In summary, we show for the first time that Runx2 represses TGF-β-induced CTGF expression by interfering with the Smad3 signaling pathway. Together with our recent data, this study suggests that Runx2 regulates plaque stability not only by inducing osteoblastic conversion of VSMCs but also by repress-
ing CTGF production, which represents a downstream target for TGF-β. Runx2 may therefore be a therapeutic target of atherosclerosis.

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