Twist1 Is a Key Regulator of Cancer-Associated Fibroblasts

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Abstract

Cancer-associated fibroblasts (CAF) are key contributors to malignant progression, but their critical regulators remain largely unknown. In this study, we examined the role of Twist1, a central regulator of epithelial–mesenchymal transition in carcinoma cells, in the transdifferentiation of normal quiescent fibroblasts to CAF and we defined its upstream controls and downstream effectors. Primary human gastric fibroblast and CAF cultures were established from gastrectomy specimens and validated as non-tumor cells by somatic mutation analyses. In these cultures, exposure to the proinflammatory cytokine IL6 commonly expressed in tumors was sufficient to induce Twist1 expression in normal fibroblasts and transdifferentiate them into CAFs via STAT3 phosphorylation. In xenograft models, tumor infiltration of Twist1-expressing CAFs was enhanced strongly by ectopic IL6 expression in gastric or breast cancer cells. We found that Twist1 expression was necessary and sufficient for CAF transdifferentiation. Enforced expression of Twist1 in normal fibroblasts was also sufficient to drive CAF marker expression and malignant character in gastric cancer cells both in vitro and in vivo. Conversely, silencing the expression of Twist1 in CAFs abrogated their tumor-promoting properties. Downstream of Twist1, we defined the chemokine CXCL12 as a transcriptional target. Clinically, CXCL12 and Twist1 expression were correlated in CAFs present in gastric tumor specimens. Finally, ectopic expression of Twist1 in normal fibroblasts suppressed premature senescence, whereas Twist1 attenuation accelerated senescence in CAFs. Our findings define Twist1 as a compelling target to deprioritize the tumor-supporting features of the cancer microenvironment.

Introduction

Tumor growth and progression are markedly influenced by the tumor microenvironment, which comprises the extracellular matrix (ECM) and a variety of stromal cells, such as fibroblasts, monocytes, and endothelial cells (1, 2). Fibroblasts are the most common cells in connective tissue, and their main function is to maintain the ECM and regulate epithelial differentiation by stromal–epithelial cross-talk (3, 4). Fibroblasts display a wide spectrum of activation states: normal fibroblasts show thin, wavy, small spindle-cell morphology, whereas activated fibroblasts are large, plump, spindle-shaped mesenchymal cells with stress fibers (1, 3). Among activated fibroblasts, those found in association with cancer cells are known as cancer-associated fibroblasts (CAF; ref. 3).

CAFs promote cancer progression through remodeling of the ECM, induction of angiogenesis, and recruitment of inflammatory cells and directly stimulate cancer cell proliferation via the secretion of growth factors and mesenchymal–epithelial cell interactions (5). Although the biological and clinical significance of CAFs in tumor microenvironment has been widely recognized, the mechanisms by which normal fibroblasts are transformed into CAFs remain vastly unknown.

In our previous study, we showed that Twist1 is expressed in stromal fibroblasts in gastric cancer tissues and that Twist1-expressing fibroblasts possess CAF-like properties (6). Twist1 is a basic helix–loop–helix transcription factor that is essential for mouse embryogenesis, Twist1 is expressed in various mesodermal tissues including neural crest-derived mesenchyme, branchial arch, muscle, bone, and fibroblasts (9, 10). After birth, Twist1 expression is barely detectable in normal mesenchymal cells of adult tissues and limited to mesenchymal stem cells (11, 12) and mesenchymal cells in human white adipocytes (13). On the other hand, Twist1 is known to be an important inducer of epithelial–mesenchymal transition (EMT) and Twist1 overexpression has been reported in a variety of epithelial cancer cells with clinical correlation with poor prognosis (14–16). However, despite the abundance of Twist1-expressing stromal fibroblasts within cancer tissue, its clinical significance and regulating mechanism remain almost completely unknown.

In the current study, we investigated molecular mechanisms by which Twist1 is induced in CAFs and the role of Twist1 in driving the transdifferentiation of quiescent fibroblasts into CAFs. In addition, we showed that Twist1 expression is necessary and sufficient for CAF transdifferentiation.

Materials and Methods

Cell lines

The gastric normal fibroblast cell line Hs738 was obtained from the ATCC. Hs738 and the stomach cancer cell lines MKN1, MKN28, MKN74, NCI-N87, SNU638, and SNU668, and the
breast cancer cell line MCF7 were used. Cell lines were purchased from the Korean Cell Line Bank that performs cell line characterizations using DNA fingerprinting analysis and passed in our laboratory for fewer than 6 months. Maintenance procedures are described in Supplementary Materials and Methods.

Isolation and culture of fibroblasts

Human stomach tumor specimens were obtained from patients undergoing surgery at Samsung Medical Center of Sung-KyunKwan University of Medicine (Seoul, Korea). An experienced pathologist grossly examined and obtained representative samples of the tumor tissues (CAF, human gastric CAF) and distal normal tissues (NF, human gastric normal fibroblast). Detailed procedures are described in Supplementary Materials and Methods.

Incubation of fibroblasts with conditioned media from gastric cancer cells

Gastric cancer cells were seeded in 10 cm dishes, and after overnight attachment and growth, cells were washed twice with PBS and grown in serum-free D/F12 media. Conditioned medium (CM) was collected after 24 hours (cells reached 70%–80% confluence) and centrifuged at 1,300 rpm for 5 minutes to remove pellet debris. Cells were incubated with CM for 2 days (48 hours). Conditioned media were freshly changed each day. NF cells were treated with recombinant IL6 (Cat#CTP0063, Invitrogen, Life Technologies) for 2 days (48 hours). For inhibition of STAT3 activity, cells were treated with STAT3 inhibitor (cucurbitacin I, Cucumis sativus L., Cat#238590; Calbiochem) for 24 hours.

Tissue specimens and construction of tissue microarrays

A total of 332 formalin-fixed, paraffin-embedded gastric tissue samples were included in this study. All patients provided written informed consent according to institutional guidelines. Detailed procedures are described in Supplementary Materials and Methods.

Immunohistochemical analysis

Detailed methods and list of antibodies used are described in Supplementary Materials and Methods.

Senescence-associated β-galactosidase activity assay

Detailed procedures are described in Supplementary Materials and Methods.

ELISA for CXCL12/SDF1α

The concentration of CXCL12/SDF1α was estimated in CM from NF#14 (GFP/Twist1), NF#32 (GFP/Twist1), CaF#14 (ShNS/ShTwist1), and CaF#32 (ShNS/ShTwist1). Detailed procedures are described in Supplementary Materials and Methods.

Cell viability/proliferation assay

Stomach cancer cells were cultured with CM from normal and CAFs for 2 days. Cells were detached, seeded in 96 well plates, and cultured for 24 hours. Detailed procedures are described in Supplementary Materials and Methods.

Xenograft model

Stomach cancer cells subcutaneously implanted in BALB/c-nude mice as the tumor model. To examine the effect of Twist1-positive fibroblasts on xenograft tumor growth, 5 × 10^5 stomach cancer cells were coinfected with 1.5 × 10^6 Twist1-positive fibroblasts [Hs738(Twist1)]. As controls, the same number of cancer cells were injected alone or with Twist1-negative fibroblast [Hs738(GFP)]. To investigate the effect of IL6 secreted by cancer cells on surrounding CAFs—in vivo, SNU638+GFP and MCF7+GFP cells were injected with IL6-expressing lentivirus. Tumor cells (3 × 10^6; SNU638 and 2 × 10^6; MCF7) were implanted subcutaneously in the right flank in 0.1 ml of serum-free medium and growth factor–reduced Matrigel (1:1). Tumor volume was determined every 2 weeks.

Lentivirus transduction

Detailed procedures are described in Supplementary Materials and Methods.

RNA extraction and quantitative real-time reverse transcription PCR

Total RNA was extracted using the RNeasy Kit (#74104, Qiagen). The cDNA was synthesized from 1 μg of total RNA using High Capacity cDNA Reverse Transcription Kits (#4368813, Applied Biosystems), according to the manufacturer’s protocol. Real-time reverse transcription (RT)-PCR was performed using the ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems). GAPDH was used as an internal loading control. PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Thermal cycling conditions were as follows: 10 minutes at 95°C followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Each measurement was performed in duplicate. PCR product quality was monitored using post-PCR melt-curve analysis. The cycle threshold (Ct), the fractional cycle number at which the amount of amplified target reaches a fixed threshold, was determined. Primer sequences are described in Supplementary Materials and Methods.

SDS-PAGE

Detailed procedures are described in Supplementary Materials and Methods.

Invasion/migration assay

Detailed procedures are described in Supplementary Materials and Methods.

Chromatin immunoprecipitation assay

CAF#14 cells were grown to 70% to 80% confluence and fixed with 1% formaldehyde. Nuclear extracts were sonicated to shear the DNA into fragments smaller than 500 bp. Twist1–DNA complexes were immunoprecipitated using Protein G Dynabeads (Cat. 10003D, Invitrogen) conjugated with anti-Twist1 IgG or control mouse IgG. After the precipitated DNA was eluted, cross-links were reversed by 0.3 mol/L NaCl and purified with the QIAquick PCR Purification Kit (28104, Qiagen). PCR was performed on the purified DNA using primer sets as described in Supplementary Materials and Methods.

Luciferase assay

The pGL3.CXCL12 promoter (−1,442 to +28) luciferase vector was generated by inserting cxcl12 promoter sequence. The 293T cells (5 × 10^4 cells/well) were plated into 24-well plates and incubated for 24 hours. Promoter vectors were cotransfected with either pSG5.hTwist1HA or pSG5.HA vector into cells using Lipofectamine 2000 (Invitrogen). Luciferase reporter gene assays were performed using the Luciferase Assay
The mechanism by which Twist1 is activated in CAFs remains unknown. To address this issue, two primary normal fibroblast cultures (NF#19 and NF#32) were established from gastric nontumor tissues. When we cultured these normal fibroblasts with CM from four gastric cancer cell lines (MKN28, 74, NCI-N87, and SNU668), only CM from SNU668 significantly upregulated mRNA levels of CAF markers, including FAPα, FSP1, PDGFRβ, and Twist1 (Fig. 1A and B). Next, we searched for cytokines selectively secreted by SNU668 because cytokines are important soluble factors in CM for CAF activation (17). The mRNA levels of cytokine genes obtained from the BioCarta data set for gastric cancer cell lines were validated using gene expression data from TCGA project. The results showed that IL6 mRNA levels were distinctly high in SNU668 cells compared with those of other cell lines (Fig. 1C). IL6 protein levels were also markedly increased in the CM of SNU668 (Fig. 1D), suggesting that IL6 is a major inducer of CAF transdifferentiation. To test this hypothesis, the effect of IL6 blocking on CAF transdifferentiation was evaluated by adding IL6-blocking antibody to CM from SNU668 cells, which abolished the enhanced expression of Twist1 and CAF markers induced by CM of SNU668 (Fig. 1E). Furthermore, acquired CAF phenotype in fibroblast by IL6 was maintained even after removal of IL6 (Supplementary Fig. S1).

Next, gastric normal fibroblasts were treated with IL6 at 50 ng/mL for 2 days and its impact on the expression of CAF markers was assessed by Western blot analysis. Twist1 and CAF markers such as FAPα, PDGFRβ, PDGFRβ, α-SMA, and FSP1 were significantly upregulated by IL6 in normal fibroblasts (Fig. 1F and Supplementary Fig. S2A). This IL6-induced upregulation of CAF markers was repressed by silencing of Twist1 expression using shRNA (Fig. 1G and Supplementary Fig. S2B). Because IL6 signaling is mediated by STAT3 phosphorylation in other types of cells (18), we examined whether STAT3 is phosphorylated in gastric fibroblasts in response to IL6 signaling. The results was that IL6 increased pSTAT3 levels as well as Twist1 in two gastric fibroblast lines in a dose-dependent manner, and IL6-induced upregulation of Twist1 was abrogated by a STAT3 inhibitor (JSI-124; Fig. 1H and Supplementary Fig. S2C). These results indicated that Twist1 expression is activated in fibroblast by the IL6/STAT3 axis.

The effect of IL6 on generation of Twist1-expressing CAFs was further investigated in vitro models. Ectopic IL6-expressing SNU638-GFP cancer cells (SNU638-GFP/IL6) and MCF7-GFP cancer cells (MCF7-GFP/IL6), which were originally negative for IL6 (Fig. 2A and B) were generated and injected subcutaneously into immuno-compromised nude mice (Fig. 2C). Tumorigenicity of ectopic IL6-expressing SNU638 and MCF7 was remarkably superior to respective controls (Fig. 2C). Furthermore, Twist1-expressing stromal cells were much more frequent in xenograft tumors formed by these IL6-expressing cancer cells compared with controls (P < 0.001; Fig. 2D and E). These Twist1-expressing stromal cells were negative for GFP but positive for Tenascin-C (Tn-C), indicating that these cells were CAFs not cancer cells (Fig. 2D). This experiment was repeated using another gastric cancer cell, MKN74, and identical result was obtained (Supplementary Fig. S3).

The expression of IL6, pSTAT3, and Twist1 in gastric cancer tissues were studied using immunohistochemistry and IL6 expression was prevalent in the cancer stroma in all cases (n = 46). pSTAT3 expression was detected in both cancer cells and stromal fibroblasts, whereas Twist1 expression was mainly limited to CAFs. There was a significant correlation between pSTAT3 and Twist1 expression in CAFs (P < 0.001; Fig. 2F).
Twist1 expression is sufficient for transdifferentiation of quiescent fibroblasts to CAFs

To determine whether Twist1 alone confers normal fibroblast with CAF-like properties, gastric normal fibroblasts (Hs738, NF#19, and NF#32) were induced to express Twist1 using lentivirus, and we found that the protein levels of CAF markers, such as FAPα, PDGFRα, PDGFRβ, α-SMA, and FSP1, were enhanced by Twist1 in these normal fibroblasts (Fig. 3A and Supplementary Fig. S2D). Next, we collected CM from control fibroblasts (NF#14GFP and NF#32GFP), ectopic Twist1-expressing fibroblasts (NF#14Twist1, NF#32Twist1), and patient matched CAFs (CAF#14, #32), then cultured gastric cancer cells with these CM (Fig. 3B).
CM from Twist1-expressing NF#14Twist1 and NF#32Twist1 significantly enhanced the proliferation of MKN28 and MKN74 cells to the same extent as CM from CAF#14, 32 did (Fig. 3B and Supplementary Fig. S4), and remarkably increased the migratory and invasive abilities of gastric cancer cells (Fig. 3C). Finally, the effect of Twist1-expressing fibroblasts on tumorigenicity of gastric cancer cells was examined in vivo using a xenograft model. Specifically, human SNU638 and SNU668 gastric cancer cells (5 × 10^5 cells each) were co-injected subcutaneously into nude mice in a suspension with either Twist1-expressing fibroblast or control fibroblasts (1.5 × 10^6 cells each). Xenografts containing Twist1-expressing CAFs grew significantly larger and faster than both of xenografts infused with Twist1-negative fibroblasts and xenografts by cancer cells alone (no fibroblasts; P < 0.001; Fig. 3D). Microvessel density and cancer cells' proliferation rate within xenograft tumor were also significantly increased when Twist1-expressing fibroblasts were added (Fig. 3E and F). These data indicated that ectopic Twist1-expressing normal fibroblasts acquired both the functional and expressional attributes of CAFs, strongly suggesting that Twist1 is a sufficient condition for CAF transdifferentiation.
Twist1 expression in CAFs is essential for the maintenance of the CAF phenotype.

To investigate Twist1’s role in CAF, primary cultures of CAFs (CAF#14 and 32) were established from stomach cancer patients’ tissues and both CAFs expressed Twist1. Then, both CAFs’ somatic mutational profiles were compared with those of matched cancer cells using high-throughput sequencing technology to exclude contamination by cancer cells. The results were that cancer cells and Twist1 expression in CAFs is essential for the maintenance of the CAF phenotype.

Twist1 is sufficient for transition of normal fibroblast to CAF. A, the expression of CAF markers in response to Twist1 overexpression in gastric NFs (Hs738, NF#19, NF#32) was evaluated by Western blot analysis. Proliferation (B) and migration/invasion (C) of stomach cancer cells, MKN28, MKN74, and SNU668 after incubation with CM derived from GFP (control)/Twist1-expressing normal fibroblasts (NF#14,32) and CAFs (CAF#14,32). D, SNU638 (left) and SNU668 (right), were coinjected with GFP or Twist1-overexpressing gastric NFs (Hs738) into the flanks of nude mice. Xenograft tumors containing Twist1-expressing Hs738 grew much faster and larger than controls did. **, *P < 0.01. E, microvessel density determined by counting number of microvessel per high-power field in the section stained with an antibody to CD34. F, Proliferative indices of gastric cancer cells in xenograft tumors. The number of Ki67 positive cells per 1,000 total cells was plotted for each tumor. **, *P < 0.05; ***, *P < 0.001.
CAFs had distinctive mutational patterns in both patients, with cancer cells showing frequent mutations, whereas no mutations, except for one synonymous mutation, were identified in CAFs (Fig. 4A). The detailed somatic mutation profiles of the isolated cancer cells and CAFs in the two patients were exported to an Excel table. The different somatic mutation patterns in CAFs and cancer cells suggested that these CAFs were neither contaminated cancer cells nor CAFs originated from cancer cells. Silencing Twist1 expression in both gastric CAFs, CAF#14 and CAF#32, using shRNA markedly reduced the expression of E-cadherin, PDGFRα, PDGFRβ, α-SMA, and β-catenin (Fig. 4B and Supplementary Fig. S2E). Furthermore, the enhanced proliferation of MKN28 and MKN74 gastric cancer cells induced by CM derived from endogenously Twist1-expressing CAF#14 and CAF#32 was not observed when these cancer cells were treated with CM from Twist1-depleted CAF#14 and CAF#32 cells (Fig. 4C). The ability of CAFs to facilitate the migration and invasion of gastric cancer cells was also remarkably reduced by knockdown of Twist1 in gastric CAFs (Fig. 4D). These results indicate that Twist1 is necessary for CAF transdifferentiation and essential for the maintenance of the CAF phenotype.

Genome-wide analyses of the effect of Twist1 on mRNA expression changes in fibroblasts identified Twist1 phenotype-related target genes

To elucidate the functional mechanism of Twist1 in CAFs, changes in mRNA expression profiles induced by Twist1 were analyzed using mRNA expression microarrays. To improve specificity of this analysis, we designed two experimental conditions: (i) gain-of-function effect of twist1 in normal fibroblasts (NF#14 and NF#32) and (ii) loss-of-function effect of twist1 in patient-matched CAFs (CAF#14 and CAF#32). These experiments were repeated twice. Genes upregulated in the two Twist1-induced fibroblast lines (NF#14 and #32) and those downregulated in the two Twist1-deleted CAFs (CAF#14 and #32) were shown using a volcano plot (Fig. 4E). Among approximately 60,000 probes annotating 20,000 genes, 221 genes were significantly upregulated in Twist1-induced fibroblasts and 889 genes were significantly downregulated in Twist1-deleted CAF (cut-off point: fold change > 1.5 and P < 0.05). Among them, 23 genes whose expression was increased in Twist1-induced fibroblasts and simultaneously reduced in Twist1-deleted CAFs were considered candidate target genes upregulated by Twist1. As shown in Fig. 4F, these genes include many CAF phenotype-related genes involved in remodeling of cancer microenvironment, such as SULF2, lysyl oxidase (LOX), CXCL12, Tn-C (TNC), delta-sarcoglycan (SCGCD), and HAPLN1.

CXCL12, a potent regulator of the CAF phenotype, is directly upregulated by Twist1 in fibroblasts with clinical relevance

On the basis of the results of our gene-chip study and functional significance of CXCL12 as a strong tumor-promoting chemokine, we suspect that CXCL12 is a key target of Twist1 in CAFs (19, 20). First, we validated our mRNA microarray results in both mRNA and protein levels. We confirmed that the secretion of CXCL12 was markedly enhanced by Twist1 alone in normal fibroblast (NF#32) and Twist1 is also essential for CXCL12 secretion in CAF (CAF#32) using ELISA assay (Fig. 5A). Besides, Twist1 overexpression increased the mRNA level of CXCL12 in normal fibroblasts (NF#14 and NF#32), whereas knockdown of Twist1 decreased the mRNA level of CXCL12 in CAFs (CAF#14 and CAF#32; Fig. 5B). The association between Twist1 and CXCL12 was analyzed in other types of cancer using public mRNA expression data from TCGA, which showed a significant correlation between Twist1 and CXCL12 expression in various cancers, such as breast, colorectal, ovarian, endometrial, lung, thyroid, and renal cancers, and glioblastoma (Supplementary Fig. S3). These results suggest that Twist1 regulates CXCL12 expression at the transcriptional level.

To further examine the regulation of CXCL12 by Twist1, we performed luciferase reporter assays using the pGL3.CXCL12 vector carrying the CXCL12 promoter region (Fig. 5C). The pGL3.CXCL12 and pSG5.hTwist1.HA vectors were transiently cotransfected into NIH3T3 cells. As shown in Fig. 5D, the CXCL12 promoter-reporter activity was significantly affected when Twist1 was introduced into the cells. Two E-box sequences located at 509 bp and at 1,037 bp upstream of the transcription start site were identified in the promoter region of CXCL12. To confirm the role of these E-boxes, mutations were introduced [CAGGTG (−509) to TTGGTG and CATTCTG (−1,037) to TCTCTG] and the mutant vectors were transfected into NIH3T3 cells. Mutation of the proximal E-box (−509) abrogated the effect of Twist1 on CXCL12 promoter activity, whereas mutation of the distal E-box (−1,037) made no difference, indicating that the proximal E-box is crucial for Twist1 function. Next, we performed chromatin immunoprecipitation (ChIP) assays in endogenous Twist1-expressing CAFs (CAF#14). Specific primer sets for the known Twist1-binding E-box CACATG (PDGFRα −1,839 to −1,834 bp) in the PDGFRα promoter were used as a positive control (17). Twist1 bound specifically to the positive control (PDGFRα −1,839 to −1,834 bp) and the proximal E-box CACATG (Fig. 5E), whereas no amplification was detected in the distal E-box (E-box2) or in the NC site containing no E-box sequence (Fig. S5E). These results indicate that Twist1 binds directly to the endogenous CXCL12 promoter.

In gastric cancer tissues, CXCL12 is expressed in both cancer cells and stromal cells and there is a significant correlation in stromal expressions between CXCL12 and Twist1 (P < 0.001; Fig. 5F). In addition, stromal expression of CXCL12 was associated with an unfavorable clinical outcome (P = 0.0232; Fig. 5G). Patients’ group with both Twist1- and CXCL12-expressing CAFs had the worst prognosis, whereas the other group in which CAFs were negative for both Twist1 and CXCL12 had the best prognosis, and the difference was statistically significant (P < 0.001; Fig. 5G). Additional results for clinical relevance of CXCL12 in gastric cancer were described in Supplementary Results and Tables S1–S4 and Supplementary Fig. S6.

Furthermore, knockdown of CXCL12 in fibroblasts (NF#14, 32) significantly impaired tumor-promoting ability conferred by Twist1 as shown Fig. S1 and Supplementary Fig. S7. CM from CXCL12-deleted fibroblasts (NF#14, 32) failed to enhance migration and invasion of gastric cancer cells even after enforced Twist1 overexpression.

Twist1 contributes to perpetual CAF activation by repressing the senescence of fibroblasts and CAFs

One of the CAF-specific properties that distinguishes them from normal activated fibroblasts such as myofibroblasts is their constitutive activation (3). In contrast to CAFs, myofibroblasts activated during conventional inflammation regress to a normal state through senescence or apoptosis after the source of inflammation is removed (3). Because Twist1 suppresses cellular senescence in various types of epithelial cancer cells (21), we hypothesized that Twist1 induced the constitutive activation of CAFs.
Knockdown of Twist1-inhibited CAF features. 

A, somatic mutation profiles of CAFs (CAF#14, 32) and matched cancer cells using high-throughput sequencing technology. B, the reduced expression of CAF markers in response to Twist1 knockdown by shRNA. Proliferation (C) and migration/invasion (D) of stomach cancer cells, MKN28, MKN74, and SNU668 after incubation with various CM for 48 hours. CM were prepared from either Twist1-expressing or Twist1 knockdown CAFs (CAF#14, 32). E, volcano plots of mRNA expression microarray data demonstrated upregulated genes in Twist1 overexpressing normal fibroblast (NF#14, 32) and downregulated genes in Twist1 knockdown CAFs (CAF#14, 32). F, the gene list whose expression was increased in Twist1-induced fibroblasts and simultaneously reduced in Twist1 deleted CAFs (FC > 1.5, P < 0.05).
through repression of senescence. To validate this hypothesis, the effect of Twist1 on cellular senescence was examined in both normal fibroblasts and CAFs. Twist1 overexpression significantly reduced SA staining and downregulated the expressions of CDKN2B (p15) and CDKN2A (p16), representative inducers of senescence, in gastric normal fibroblasts (NF#5 and NF#15; Fig. 6A). On the other hand, silencing Twist1 expression by shRNA accelerated senescence in CAFs, as detected by increased SA staining in CAF#13 and CAF#36 (Fig. 6B), and upregulated p15 and p16 expressions in all three gastric CAFs (CAF#13, CAF#13, P < 0.05; **, P < 0.01; ***, P < 0.001.)
CAF#14, and CAF#32). Our results strongly suggest that Twist1 contributes to the generation of CAFs by repressing cellular senescence. Together, our findings support a model in which Twist1 is a key regulator of CAFs (Fig. 6C).

Discussion

In our previous study, we showed that Twist1 is frequently overexpressed in stromal fibroblasts surrounding gastric cancer cells (6). In additional, these Twist1-expressing stromal fibroblasts expressed CAF markers such as FSP1 and PDGFRα with association with poor prognosis (6). Despite these results, there was no direct evidence for functional role of Twist1 as a key regulator of CAFs. Therefore, in the current study, we established fibroblasts and CAFs models from gastrectomy specimens and confirmed that Twist1 expression is necessary and sufficient for CAF transdifferentiation. In addition, IL6/STAT3 axis was discovered to be a key upstream

Figure 6. Twist1 repressed cellular senescence of CAFs. A, β-galactosidase staining showed that the number of senescent cells (arrowhead) was decreased in Twist1-overexpressing NFs (#5, #15, and #32; bottom) compared with the GFP-expressing controls (top; magnification, ×100). The mRNA level of the senescence markers, p15 and p16, assayed by qRT-PCR. B, the number of β-galactosidase–stained cells was increased by silencing Twist1 expression (bottom) compared with nontargeting shRNA controls (top). The mRNA levels of p15 and p16 were elevated in shTwist1 cells compared with ShNS controls. C, schematic illustration showing the role of the IL6/STAT3/Twist1 axis in the CAFs.

*, P < 0.05; **, P < 0.01.
control of Twist1. Microarray analysis of the effect of Twist1 on mRNA expression in fibroblasts identified CXCL12 as a key Twist1’s target in CAFs. Moreover, Twist1 was revealed to suppress cellular senescence of normal fibroblasts and CAFs.

As Yang and colleagues first reported that Twist1 is a critical player in cancer metastasis (16), most studies focused on Twist1’s role in cancer cells (22–24), with little attention paid to its possible role in the tumor microenvironment, including CAFs. In contrast to rare expression of Twist1 in adult normal fibroblasts, Twist1 is frequently and strongly expressed in embryonic fibroblasts (9, 11, 25) and pathologic fibroblasts in idiopathic pulmonary fibrosis (26, 27) and desmoid tumor (28). Bridges and colleagues (26) reported that Twist1 enhanced survival and accumulation of fibroblasts in fibrotic lung disease, which is consistent with results of our current study. Bacac and colleagues (28) reported that Twist1 is highly expressed in desmoid tumors (neoplastic myofibroblastic lesions) compared with nodular fasciitis (nonmalignant proliferations of fibroblasts). Furthermore, Spazeh and colleagues reported that CD44-induced acquisition of CAF phenotype in mesenchymal stem cell is mediated by Twist activation. This result is highly consistent with our result and also highlights the importance of Twist1 in CAFs (29).

The role of Twist1 as a major EMT inducer implicates that certain Twist1-expressing CAFs may be derived from malignant epithelial cells. However, EMT of cancer cells probably accounts for only a fraction of CAFs (3, 30). In this current study, CAFs isolated from tumors showed no mutation in contrast to their matched cancer cells displaying more than 9 mutations (Fig. 4A). Despite a few previous studies claiming that CAFs are genetically abnormal, recent evidences showed CAFs to be genetically normal (31, 32). No somatic mutations were detected in CAF cultures generated from primary pancreatic adenocarcinomas (32, 33). In addition, we also found that Twist1-expressing stromal cells in xenograft formed by ectopic GFP/IL6-expressing cancer cells were negative for GFP (Fig. 2). Taken together, our results indicate that Twist1-expressing CAFs are more likely to be derived from noncancer cells.

Recent studies showed that IL6 facilitates CAF transdifferentiation in several types of cancers, including prostate cancer (34–37). The enhancement of actomyosin contractility by the IL6/STAT3 pathway was recently suggested as a mechanism of IL6-induced CAF transdifferentiation (35). However, transcription factors mediating this IL6-induced CAF transdifferentiation remain unknown. Meanwhile, Twist1 was suggested as a downstream transcription factor of the IL6/STAT3 pathway in some types of epithelial cancer cells (38, 39). Twist1 is transcriptionally induced by STAT3 in breast epithelial cancer cells (38) and IL6 stabilizes Twist1 and enhances cancer cell motility in head and neck epithelial cancer cells (39). These results raise the possibility that Twist1 is also a downstream effector of the IL6/STAT3 pathway in fibroblasts and CAFs. Indeed, Twist1 was revealed to be induced by the IL6/STAT3 axis in gastric normal fibroblasts and was indispensable for IL6-induced CAF transdifferentiation (Fig. 1). Furthermore, IL6 was abundant in the cancer stroma and a significant positive correlation between pSTAT3 and Twist1 was observed in cancer tissues (Fig. 2F).

In the current study, we searched for Twist1 target genes in CAFs using mRNA microarray and most of identified candidate genes, such as CXCL12 secreted by CAFs promoted angiogenesis and recruitment of additional inflammatory cells (20). Kojima and colleagues reported that CXCL12 plays a role in the maintenance of the CAF phenotype through formation of an autostimulatory loop (19). Furthermore, CXCL12 has recently emerged as a therapeutic target in various cancers (41–43). However, the transcription factor that regulates CXCL12 remains unknown. In the current study, we showed that CXCL12 was transcriptionally regulated by Twist1 and the expressions of CXCL12 and Twist1 were significantly correlated to each other in cancer tissues with significant clinical relevance. In our previous study, we examined the genome-wide transcriptional regulation by Twist1 in synovial sarcoma combining both ChIP-seq and mRNA array (23). In that study, we found that Twist1 upregulated expression of CXCL12 in synovial sarcoma and bound to a similar sequence in the proximal E-box site (−509 bp), which strongly supports the results of the present study (23).

Although the identity of CAFs remains obscure, one of the characteristics that distinguish CAFs from conventional activated fibroblasts is their constitutive activation. During normal inflammation, once a wound is repaired, the number of activated fibroblasts decreases and resting phenotype is restored (3). Unlike wound healing, CAFs at tumor site remain perpetually activated (3). In the current study, we showed that Twist1 suppressed cellular senescence in fibroblasts. In fact, the suppression of senescence by Twist1 was previously reported in various types of cells. Maestro and colleagues reported that Twist1 inhibits apoptosis and bypasses p53-induced growth arrest by direct and indirect modulation of the ARF/MDM2/p53 pathway in mouse embryonic fibroblasts (25). Ansieau and colleagues showed that Twist1 represses premature senescence by abrogating regulators of p53- and Rb-dependent pathways in breast cancer cells (21). Our results are highly consistent with those of previous studies and suggest that Twist1 is likely to promote CAF transdifferentiation through the suppression of senescence.

Fibroblast is a highly heterogeneous entity. Chang and colleagues showed that fibroblasts from different anatomical regions display characteristic different phenotypes (44). In addition to the differences between fibroblasts from different anatomical sites, fibroblasts separated from a single tissue are not composed of a homogeneous population (45). Likewise, the expression profiles of CAFs may show considerable heterogeneity depending on the anatomical region. Therefore, fibroblasts and CAFs used in study should be derived from the same organ to minimize unexpected bias (3). In the current study, we established primary fibroblast and CAF cultures from gastric cancer tissues to study the interaction between gastric cancer cells and CAFs.

In conclusion, we identified Twist1 as a novel and key transcription factor that regulates CAFs. On the basis of our results, Twist1 is suggested as a potent therapeutic target to reverse unfavorable reprogramming of cancer microenvironment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Authors’ Contributions

Conception and design: K.-W. Lee, S.-H. Kim
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.-W. Lee, S.-Y. Yeo, C.O. Sung, C.O. Sung, S.-H. Kim
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.O. Sung
Writing, review, and/or revision of the manuscript: K.-W. Lee, S.-Y. Yeo, C.O. Sung, S.-H. Kim

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Grant Support

This study was supported by Samsung Biomedical Research Institute grant G1B180411. And this work was also supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST, No. 2013R1A1A0263277). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 5, 2014; revised October 20, 2014; accepted October 20, 2014; published OnlineFirst November 3, 2014.


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