TIMP-1 via TWIST1 Induces EMT Phenotypes in Human Breast Epithelial Cells

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Abstract

Tissue inhibitor of metalloproteinase-1 (TIMP-1) regulates intracellular signaling networks for inhibition of apoptosis. Tetranspanin (CD63), a cell surface binding partner for TIMP-1, was previously shown to regulate integrin-mediated survival pathways in the human breast epithelial cell line MCF10A. In the current study, we show that TIMP-1 expression induces phenotypic changes in cell morphology, cell adhesion, cytoskeletal remodeling, and motility, indicative of an epithelial–mesenchymal transition (EMT). This is evidenced by loss of the epithelial cell adhesion molecule E-cadherin with an increase in the mesenchymal markers vimentin, N-cadherin, and fibronectin. Signaling through TIMP-1, but not TIMP-2, induces the expression of TWIST1, an important EMT transcription factor known to suppress E-cadherin transcription, in a CD63-dependent manner. RNAi-mediated knockdown of TWIST1 rescued E-cadherin expression in TIMP-1–overexpressing cells, demonstrating a functional significance of TWIST1 in TIMP-1–mediated EMT. Furthermore, analysis of TIMP-1 structural mutants reveals that TIMP-1 interactions with CD63 that activate cell survival signaling and EMT do not require the matrix metalloproteinase (MMP)–inhibitory domain of TIMP-1. Taken together, these data demonstrate that TIMP-1 binding to CD63 activates intracellular signal transduction pathways, resulting in EMT-like changes in breast epithelial cells, independent of its MMP-inhibitory function.

Implications: TIMP-1’s function as an endogenous inhibitor of MMP or as a “cytokine-like” signaling molecule may be a critical determinant for tumor cell behavior. Mol Cancer Res; 12(9): 1324–33. ©2014 AACR.

Introduction

The tissue inhibitors of metalloproteinases (TIMP1-4) regulate the activities of the matrix metalloproteinases (MMP) critical for the precise turnover of the extracellular matrix (ECM) during morphogenesis and tissue remodeling in normal physiologic conditions (1). Accordingly, deregulation of these enzymatic activities leads to the development or progression of many human diseases including cancers. Increased expression and/or activity of MMPs are thought to play a particular role for tumor cell invasion. Likewise, in vitro and animal studies have established a function of TIMPs in the inhibition of tumor cell invasion and metastasis. However, clinical studies revealed that TIMPs are often upregulated in many cancers. Especially, TIMP-1 overexpression correlates with a poor prognosis in certain malignancies, including metastatic breast cancer (2–4). Although a prognostic value of TIMP-1 is now well established, it is unclear whether increased TIMP-1 expression contributes to tumor progression. Because many transcription factors shown to upregulate TIMP-1 expression can also induce expression of MMPs, it was postulated that increased TIMP-1 expression may be a reflection of stromal responses to the increased MMPs expression in cancer cells. However, increasing evidence suggests the existence of signaling pathways that lead to TIMP-1 upregulation without MMP induction (5).

Importantly, we and others have demonstrated a role for TIMP-1 in the regulation of cell growth and apoptosis inhibition in many different cell types. TIMP-1 regulation of cell survival seems to occur through two coexisting pathways: an MMP-dependent pathway (6–8) or MMP-independent mechanism (9–15). Our discovery of CD63, a member of the tetraspanin family of proteins, as a TIMP-1–interacting protein provided molecular insight into the action of TIMP-1 as a signaling molecule distinct from their MMP-inhibitory activity (16). We demonstrated that...
TIMP-1 binds to CD63 in a complex with integrin β1, a main tetraspanin interacting integrin, on the cell surface and activates cell survival signaling pathways in a CD63-dependent manner (16). In agreement with our results, Egea and colleagues recently highlighted that TIMP-1 downregulates β-catenin signaling via a decrease of let-7f miRNA in a MMP-independent manner and the binding of CD63 to TIMP-1 on the surface is necessary for the TIMP-1–mediated signaling in human mesenchymal stem cells (17). Although these studies may have provided an explanation, at least in part, for a potential tumor-promoting activity of TIMP-1, the molecular actions of TIMP-1 aside from its MMP-inhibitory function are still largely unknown. In this study, we made a novel finding that TIMP-1 induces phenotypic conversion of epithelial cells to a mesenchymal phenotype termed an epithelial–mesenchymal transition (EMT). EMT is a morphologic conversion process initiated by master transcription factors, such as Slug, Snail, the E1f protein SIP1, and TWIST1 (18, 19), that drastically alter gene expression profiles, including the repression of E-cadherin transcription and the induction of N-cadherin, vimentin, and fibronectin. Through an EMT process, epithelial cells undergo drastic remodeling of the cytoskeleton, lose their polarity and cell–cell contact, and acquire a migratory phenotype. Importantly, increasing evidence supports a hypothesis that many of these molecular and cellular changes associated with normal developmental EMT are recapitulated during the progression of human carcinomas, possibly as a transient, dynamic process utilizing the EMT transcription factors (reviewed in refs. 18, 20, 21). Here, we report that TIMP-1 interaction with CD63 activates intracellular signal transduction pathways leading to upregulation of the EMT master transcription factor TWIST1, resulting in a decrease in the epithelial markers and an increase in the mesenchymal markers, along with phenotypic changes such as the transition to a fibroblast-like cell shape and increased cell scattering and cell motility. Importantly, a TIMP-1 mutant devoid of its MMP-inhibitory domain was sufficient to interact with CD63 and to induce phenotypic changes of EMT in human breast epithelial MCF10A cells. These results unveiled an unexpected, novel function of TIMP-1 as an extracellular signal inducer that mediates an EMT-like phenotypic conversion in human breast epithelial cells by a mechanism independent of its MMP-inhibitory activity.

Materials and Methods
Reagents and antibodies
Anti–TIMP-1 mAb was purchased from NeoMarkers, Inc. Anti–CD63, anti-integrin 66 antibody, anti-laminin α5 mAb, and the PAK100 staining kit were purchased from Chemicon. Anti–β-cell, anti-vimentin, anti-mouse IgG peroxidase conjugate, and anti-Rabbit IgG peroxidase conjugate antibodies were purchased from Sigma. Anti–E-cadherin and anti–β-catenin mAbs were purchased from BD transduction laboratories. Anti-cow cytokeratin wide spectrum screening polyclonal antibody (pAb) was purchased from Dako. FITC-conjugated and Texas Red–conjugated rabbit or mouse IgG antibodies and normal donkey serum were purchased from Jackson ImmunoResearch Laboratories. Anti-TWIST1 pAb was generously donated by Carlotta Glackin (Beckman Institute of the City of Hope), and anti-TIMP-1 mAb was purchased from Abcam. Growth factor–reduced Matrigel was purchased from BD Biosciences.

Overexpression of TIMP-1 and its mutants in MCF10A cells
Generation of TIMP-1–overexpressing (TIMP-1) MCF10A clones was previously described (12, 13). For the mutant study, full-length TIMP-1 (amino acids 1–184; T1) or the partial N-terminal without MMP-inhibitory domain and C-terminal domain (amino acids 66–184) of TIMP-1 (T1D) was amplified by PCR using a vector carrying hTIMP-1 cDNA from Open Biosystems, and the various restriction enzyme sites were introduced respectively according to the target vector. The signal peptide was included in the construct for protein to be secreted. The final PCR products containing the signal sequences were fused to p3XFLAG-CMV-14 expression vector at a BamH I site. The correct orientation and in-frame fusion were confirmed by DNA sequencing. The partial N-terminal and C-terminal TIMP-1–FLAG (T1D), pcDNA3.1-TIMP-1, and pcDNA3.1 control vectors were transfected into MCF10A cells using Lipofectamine 2000 from Invitrogen according to the manufacturer’s protocol. Establishment of FLAG-tagged wild-type TIMP-1–transfected MCF10A clone (WT TIMP-1/FLAG #9, referred to as T1#9 in this article) was described previously (13).

RT-PCR analysis of mesenchymal and epithelial markers
Total RNA was isolated using the Qiagen RNA Easy Miniprep Kit (Qiagen) according to the manufacturer’s protocol, and cDNA was synthesized from 1 μg of total RNA using the SuperScript First-Strand Synthesis System for RT-PCR. The thermal cycle profile consisted of an initial denaturation at 94°C for 5 minutes, followed by 25 cycles consisting of a 30-second denaturation at 94°C, a 1-minute annealing of primers at 55°C, and a 1-minute extension at 74°C, followed by a final 7-minute extension at 74°C. The PCR products were then electrophoresed through 1% agarose gels. The primers used are listed in Supplementary Table S1.

Establishment of stable CD63 knockdown cell lines
Plasmids carrying shRNA targeted to CD63 were constructed following Ambion’s Web-based protocol. TIMP-1 MCF10A cells were transfected with either target 2 or control vector using Lipofectamine 2000 from Invitrogen as described previously (16).

Real-time PCR analysis
Real-time PCR reactions were carried out using Brilliant SYBR Green QPCR Master Mix (Stratagene), and thermal cycling was carried out on the Mx4000 multiplex quantitative PCR system (Stratagene) using optimized PCR conditions in triplicate. The median cycle threshold value was
used for analysis, and all cycle threshold values were normalized to the expression of the housekeeping gene GAPDH. The mRNA level in Neo cells was arbitrarily given as 1, and the relative mRNA levels in TIMP-1, TIMP-1 shCont-P, and TIMP-1 shCD63-2 MCF10A cells compared with the levels in the Neo control cells were determined as recommended by the manufacturer (Stratagene).

**Immunoblot analysis**

Cell lysates were obtained by lysing the cell monolayer with SDS lysis buffer [2% SDS, 125 mmol/L Tris-HCl (pH 6.8), and 20% glycerol], and immunoblot analysis was performed according to the protocol described previously (16).

**Immunofluorescent cell staining**

For immunofluorescence analysis, cells were plated on chamber slides at 50% confluence overnight and immunostained with antibodies according to the previously published protocol (22).

**MCF10A morphogenesis assay in three-dimensional cultures**

Three-dimensional (3D) culture of MCF10A cells and immunostaining were conducted according to the published protocol (16). Confocal immunofluorescence microscopic analysis was performed using a Zeiss LSM 510 confocal microscopy system equipped with a C-Apochromat [numerical aperture (NA) = 1.2] 63× Korr objective lenses (Carl Zeiss MicroImaging, Inc.) or (NA = 1.3) 40× lens. Images for figures were colored and resized with Adobe Photoshop 5.5 software.

**Knockdown of TWIST1 using siRNA**

TIMP-1 MCF10A cells were plated onto chamber slides and the next day transiently transfected with control or TWIST1 siRNA oligos (Invitrogen) for 72 hours according to the Lipofectamine protocol (Invitrogen). The cells were fixed, and immunostaining of TWIST1 or E-cadherin and DAPI (nuclear) staining were conducted according to the protocol above. The TWIST1 siRNA and control oligos are listed in Supplementary Table S2.

**Low and high confluent MCF10A cell experiment**

MCF-10A cells were grown at the indicated cell densities [10% to 30% confluence (low confluence) or 80% to 90% confluence (high confluence)] according to the following protocol in a humidified 5% CO2 atmosphere at 37°C (23).

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![Figure 1](image-url) **Figure 1.** TIMP-1 regulates EMT-like phenotypic changes and gene expression. A, phase contrast microscopy of control (Neo) and TIMP-1–overexpressing MCF10A (TIMP-1) cells at 20× magnification. Immunostaining of phalloidin (red) and nuclear DAPI (blue) staining in cross sections through the middle of developing acini of Neo and TIMP-1–overexpressing cells cultured in growth factor reduced Matrigel for 8 to 14 days. B, RT-PCR analysis of the mesenchymal markers vimentin, N-cadherin, and fibronectin (fibro) as well as the epithelial markers E-cadherin, α-catenin, β-catenin, and γ-catenin. GAPDH was used as a loading control (left). Immunoblot analysis of E-cadherin and vimentin in control (Neo) and TIMP-1 MCF10A cells. β-actin was used as a loading control (right). C, immunostaining of E-cadherin (green) and DAPI (blue) staining in cross sections through the middle of developing acini of Neo and TIMP-1–overexpressing cells (top). Immunostaining of cytokeratin (green), vimentin (red), and DAPI (blue) staining in cross sections of the middle of developing acini cultured in growth factor reduced Matrigel (bottom). D, immunostaining of E-cadherin or β-catenin (red) and DAPI (blue) staining in Neo and TIMP-1 MCF10A cells plated on chamber slides. Scale bars, 50 μm.
Production of recombinant TIMP-1/TIMP-2 and treatment of MCF10A cells

Human recombinant TIMP-1 and TIMP-2 were expressed in HeLa cells using a vaccinia expression system and purified to homogeneity as described previously (12). MCF10A cells were treated with rTIMP-1 or rTIMP-2 for 4 days in DMEM/F-12 medium supplemented with 2% horse serum, and cell lysate was collected with 2% SDS lysis buffer and RNA was collected using the Quiagen RNA Kit. Fresh media with rTIMP-1 or rTIMP-2 were supplemented every day of the experiment. Western blot of E-cadherin and TWIST1 was performed with a β-actin loading control. RT-PCR was done to detect the expression of E-cadherin in these samples over time.

Cell migration assay

All cells were grown in complete medium and pretreated with mitomycin C (25 μg/mL) for 30 minutes. Transwell units with polycarbonate filters (Corning Costar) were used for all cell migration assays according to the published protocol (15). Cells on the filters were fixed and stained using the Diff-Quik Stain Set (American Scientific Products), following the direction of the manufacturer.

Results

TIMP-1 overexpression results in EMT-like changes in cell morphology and gene expression

During the course of our study of TIMP-1’s activity in human breast epithelial cell signaling, we observed a remarkable change in the morphology of MCF10A cells upon TIMP-1 overexpression. Although control (Neo) MCF10A cells display cobblestone-like, typical epithelial cell morphology, TIMP-1-overexpressing MCF10A cells show spindle-shaped, fibroblast-like morphology with loss of cell–cell contacts and increased cell scattering (Fig. 1A, top), which may be associated with an EMT-like conversion. Changes in the cytoskeletal structure in a 3D Matrigel culture, as shown by phalloidin staining of F-actin (Fig. 1A, bottom), also suggested TIMP-1–mediated phenotypic changes. A semiquantitative RT-PCR analysis detected changes in gene expression at the mRNA levels, showing a decrease in the epithelial cell–specific markers, E-cadherin, β-catenin, and γ-catenin and an increase in the mesenchymal markers, vimentin, N-cadherin, and fibronectin (Fig. 1B, left). Consistently, TIMP-1 overexpression resulted in a loss of E-cadherin and a gain of vimentin protein levels in MCF10A cells as detected by immunoblot analysis (Fig. 1B, right). Immunostaining in a 3D Matrigel culture also revealed that TIMP-1 mediated a drastic decrease in E-cadherin and a marked induction of vimentin expression, whereas cytokeratin expression was detected in both control and TIMP-1–overexpressing MCF10A cells (Fig. 1C). It is thought that the cytoplasmic domain of E-cadherin forms a complex with the catenin protein family, linking E-cadherin to the actin filament network for the maintenance of the architectural integrity of the epithelium and intercellular adhesion (24). Studies show that loss of E-cadherin often coincides with...
the accumulation of β-catenin in the nucleus for activation of its target genes (25). Next, we examined the effects of TIMP-1 overexpression on the expression level and subcellular localization of the β-catenin protein. As shown in Fig. 1D, β-catenin was found throughout the cells in TIMP-1-overexpressing cells with a loss of E-cadherin expression, whereas it was localized to the cytoplasmic membrane in control MCF10A cells, which also show E-cadherin expression. Immunoblot analysis confirmed a decreased expression of β-catenin in TIMP-1-overexpressing cells (data not shown).

CD63 is essential for TIMP-1 regulation of EMT-like conversion and the EMT master transcription factor TWIST1

EMT, a vital process for morphogenesis during embryonic development, is initiated by master regulators, such as Slug, Snail, the Eฝ1 protein SIP1, and TWIST1 (18, 26), that drastically alter gene expression profiles, including the repression of E-cadherin transcription and the induction of N-cadherin, vimentin, and fibronectin. Interestingly, many of these molecular and cellular changes associated with normal developmental EMT are often characteristics of invasive and metastatic carcinoma cells (18). Therefore, we examined whether TIMP-1-mediated EMT-like changes were associated with induction of transcription factors known to regulate EMT. We found that TWIST1 mRNA level was significantly induced by TIMP-1 expression (3.3-fold) as determined by real-time RT-PCR (Fig. 2A), whereas the levels of Snail and Slug were unaffected (data not shown). TWIST1 is a basic helix-loop-helix (bHLH) transcription factor originally discovered as an essential factor for EMT during mesoderm formation in Drosophila (27). Consistent with a notion that TWIST1 regulates epithelial and mesenchymal markers at the transcriptional level, decreased mRNA expression of the epithelial markers, E-cadherin, β-catenin, and γ-catenin and increased mRNA expression of N-cadherin, vimentin, and fibronectin were found in TIMP-1-overexpressing MCF10A cells (Fig. 1B).

Previously, we demonstrated that TIMP-1 binding to CD63 on the cell surface regulates cell survival signaling pathways involving the tetraspanin/integrin complex. TIMP-1-mediated constitutive activation of CD63/integrin signaling was shown to disrupt MCF10A cell polarization and formation of acini-like structures in a 3D Matrigel culture (16). Here, we asked whether CD63 is required for TIMP-1 induction of TWIST1 and/or downregulation of E-cadherin, a hallmark of EMT. To this end, we examined TWIST1 expression in MCF10A cells overexpressing TIMP-1 with or without CD63 knockdown using shRNA. CD63 knockdown significantly reduced TWIST1 expression in TIMP-1-overexpressing MCF10A cells as determined by real-time RT-PCR analysis (Fig. 2A) and immunostaining (Fig. 2B). CD63 knockdown also partially reversed the TIMP-1-mediated downregulation of E-cadherin expression (Fig. 2C).

The above results suggest that TIMP-1/CD63 signaling upregulates TWIST1 expression leading to EMT-like conversion in MCF10A cells. To evaluate the functional significance of TWIST1 in TIMP-1-mediated EMT, we downregulated TWIST1 expression in TIMP-1-overexpressing MCF10A cells using an siRNA approach. Whereas

Figure 3. TIMP-1 mediates EMT-like conversion independent of cell confluency. Neo, TIMP-1 MCF10A cells, TIMP-1 shCont-P, and TIMP-1 shCD63-2 cells were plated at about 30% (low) and 90% (high) confluency. A, phase contrast microscope images are shown for each cell line. B, immunoblot for E-cadherin and vimentin with β-actin as a loading control. C, RT-PCR for E-cadherin and N-cadherin with GAPDH as a loading control. D, immunostaining of N-cadherin (green, top row), vimentin (green), and cytokeratin (red, second row) along with DAPI nuclear staining (blue).
E-cadherin expression was barely detected in TIMP-1–overexpressing MCF10A with TWIST1 expression. E-cadherin was readily detected in TIMP-1–overexpressing cells upon TWIST1 knockdown (Fig. 2D). Taken together, these results indicate that TIMP-1/CD63 signaling upregulates TWIST1 expression, resulting in EMT-like conversion in human breast epithelial MCF10A cells.

**TIMP-1 induction of EMT is independent of cell confluence**

Although increasing evidence has suggested a critical role for the EMT master transcription factors in cancer progression and metastasis, it has been debated whether "oncogene"-mediated EMT-like changes observed in vitro reflect in vivo tumor biology or it is just a phenomenon only associated with a particular tissue culture condition. Interestingly, Sarrio and colleagues discovered that MCF10A cells undergo spontaneous morphologic and phenotypic EMT-like changes in response to different cell confluences (23). EMT was induced at sparse confluence (15–30%), and the EMT expression pattern was lost when the cells were highly confluent. This study led us to examine if TIMP-mediated EMT is dependent on the confluence of the cell culture.

The control MCF10A cells at subconfluence or confluence displayed a cobblestone-like epithelial cell phenotype. In contrast, TIMP-1–overexpressing cells show a spindle-shaped, fibroblast-like morphology regardless of the cell density (Fig. 3A). Interestingly, TIMP-1–overexpressing cells reached confluence at lower cell density compared with the control MCF10A cells. Immunoblot and RT-PCR analyses confirmed that TIMP-1 overexpression downregulates the epithelial cell marker E-cadherin and induces the mesenchymal makers N-cadherin and vimentin regardless of cell density (Fig. 3B, left and 3C).

In addition, immunostaining of the mesenchymal markers N-cadherin and vimentin shows that in the Neo control cells N-cadherin and vimentin expression is much lower than in the TIMP-1–overexpressing cells, whereas these cells had a higher expression of cytokeratin (Fig. 3D, red staining). With TIMP-1 overexpression, we see expression of N-cadherin and vimentin at both low and high confluence (Fig. 3D, green staining). With TIMP-1 overexpression, the TIMP-1–overexpressing cells are more rounded in shape (Fig. 3A) and E-cadherin expression was increased to some degree, although not completely, in both conditions (Fig. 3B, right).

**Exogenous treatment of MCF10A cells with rTIMP-1, but not rTIMP-2, induces EMT-like changes in gene expression**

To further establish the role for TIMP-1 in EMT, we asked whether exogenously added TIMP-1 proteins can upregulate the EMT master transcription factor TWIST1 and/or downregulate E-cadherin expression. To this end, MCF10A cells were treated daily with 500 ng/mL of rTIMP-1 for 4 days and subjected to daily collection of RNAs and cell lysates. After 3 days of rTIMP-1 treatment, there was a significant increase in TWIST1 expression followed by a reduction in E-cadherin expression (Fig. 4A). Interestingly, rTIMP-2, a closely related MMP inhibitor, had little effect on the expression of E-cadherin, suggesting that the regulation of cell signaling leading to the EMT-like conversion in MCF10A cells overexpressing TIMP-1 was specific to TIMP-1 (Fig. 4B).

**TIMP-1–mediated EMT does not require the N-terminal MMP-inhibitory domain**

TIMP-1 has a two-domain structure with its N- and C-terminal regions each containing six conserved cysteine residues forming three disulfide bonds. The N-terminal domain contains residues that interact with the Zn$^{2+}$-binding pocket of active MMPs. The C-terminal domain of TIMPs is thought to be critical for protein–protein interactions (reviewed in ref. 4). We previously showed that the C-terminal domain of TIMP-1 interacts with CD63, suggesting that TIMP-1's signaling activity is independent of its MMP-inhibitory domain. To establish the structural/functional basis for TIMP-1 regulation of EMT via its interactions with CD63, we generated MCF10A cell clones expressing a full-length TIMP-1 (T1) or a deletion mutant of TIMP-1 lacking the MMP-inhibitory domain (encoding aa
66–184, referred to as T1D) as depicted in Fig. 5A (left). When we examined the cell morphology, T1D-expressing cells (T1D #66 and T1D #69) displayed elongated and fibroblast-like cell shapes similarly to that of the full-length TIMP-1–expressing cells (T1 #9; Fig. 5A, right). T1D expression upregulated mRNA expression of the EMT transcription factor TWIST1 as well as the mesenchymal markers vimentin and N-cadherin, and this was accompanied with a decrease in the epithelial marker E-cadherin as seen in wild-type TIMP-1–expressing cells (Fig. 5B). Consistently, T1D expression resulted in a decrease in the expression of the epithelial cell markers, E-cadherin and β-catenin, and an increase in the mesenchymal marker vimentin as effectively as wild-type TIMP-1 expression as shown by immunoblot analysis (Fig. 5C). Immunostaining of control MCF10A cells showed expression of E-cadherin and β-catenin on the cell–cell junctions (Fig. 5D). Upon TIMP-1 or T1D expression, E-cadherin and β-catenin were barely detectable on the cell surface. Instead, there was an induction of the mesenchymal markers N-cadherin and vimentin (Fig. 5D) compared with the control MCF10A cells. These results clearly demonstrate the TIMP-1–mediated EMT-like transition does not involve its MMP-inhibitory domain.

**TIMP-1 and T1D induce MCF10A cell migration**

The ability of the cells to acquire a migratory phenotype is a functional hallmark of EMT. Thus, we assessed the effects of TIMP-1 expression on cell motility using a transwell chamber assay. TIMP-1 overexpression resulted in approximately 5-fold increased cell migration (Fig. 6A). Upon CD63 knockdown, TIMP-1’s ability to induce cell motility was significantly reduced to less than 2-fold, demonstrating a critical role of CD63 for the TIMP-1–mediated cell motility (Fig. 6B). Importantly, the cell motility rates were comparable between MCF10A cells overexpressing wild-type TIMP-1 (T1#9) and the T1D mutants (T1D #66 and #69; Fig. 6C). These data clearly indicate that TIMP-1 regulates EMT gene expression, cellular phenotypes, and cell motility in a CD63-dependent manner, independent of TIMP-1’s MMP-inhibitory domain.

**Discussion**

The present study unveiled a novel function of TIMP-1 in mediating EMT-derived phenotypic conversion in human breast epithelial cells. Although EMT has not been conclusively confirmed in human cancers by pathologists, increasing evidence supports a notion that subprograms of developmental EMTs are involved during the progression of human carcinoma, possibly as a transient, dynamic process occurring in a small subpopulation of tumor cells at any given time (reviewed in 18, 20, 21). Accordingly, loss or downregulation of E-cadherin expression is frequently found in carcinoma cells, and is often inversely correlated with malignancy (28). Although promoter hypermethylation and inactivating mutations account for loss of E-cadherin expression/function in some tumors, epigenetic mechanisms also play a critical role in downregulation of E-cadherin expression during...
carcinoma progression (29, 30). Interestingly, the PI3K/Akt axis has emerged as a key signaling pathway for induction of EMT-mediating transcription factors that downregulate E-cadherin (26, 31, 32). Importantly, this study found that TIMP-1 signaling induces TWIST1 expression, a bHLH transcription factor known to suppress E-cadherin gene transcription and upregulate mesenchymal markers, providing molecular insight into TIMP-1's role in EMT. The prominent phenotype of TWIST1 knockout mice was a failure of the cranial neural folds to fuse and differentiation of the cranial neural crest cells (33, 34). However, a critical role for TWIST1 has been proposed in the metastatic process, linking the developmental EMT process with tumor metastasis (35). A study showed that 70% of invasive lobular breast carcinomas have increased expression of TWIST1 and decreased E-cadherin, whereas most normal breast tissue samples expressed TWIST1 at a very low level (35). TWIST1 has also been shown to play a role in the regulation of apoptosis. TWIST1 haploinsufficiency in Saethre–Chotzen syndrome, a human autosomal dominant disorder characterized by premature fusion of cranial sutures, alters osteoblast apoptosis (36). Consistently, downregulation of TWIST1 by DNazymes, a group of catalytic nucleic acids designed to cleave target mRNA molecules, resulted in an increase of cellular apoptosis (37). In cancer cells, increased TWIST1 expression was shown to inhibit oncogene- and p53-dependent cell death (38) and result in resistance to certain chemotherapeutic drugs, angiogenesis, and chromosomal instability (39, 40). A study showed that TWIST1 transcriptionally upregulates Akt2 by binding to the E-box elements on the promoter of Akt2 in breast carcinoma cells and that silencing Akt2 reverses TWIST1-mediated EMT, cell migration, invasion, and paclitaxel resistance (41), suggesting a positive feedback signaling loop between the Akt and TWIST1 for the regulation of EMT and cell survival. Importantly, we have previously showed that TIMP-1 activates CD63/integrin survival signaling pathways involving the PI3K/Akt pathways for the regulation of apoptosis. In fact, our recent report also showed TIMP-1 induces an EMT-like process independent of the MMP-inhibitory domain in Madin–Darby canine kidney (MDCK) cells (15), although we could not investigate the involvement of CD63 in the TIMP-1–mediated EMT process in this model because of the limitation of the unidentified canine CD63. However, the MDCK study demonstrated similar results to the TIMP-1 interaction with CD63 and subsequent activation of signal transduction pathways we have found previously in breast epithelial cells. Thus, it seems that TIMP-1 binding to the CD63/integrin signaling complex activates both cell survival and EMT pathways and subsequently undergoes signal amplification by cross-talk between these pathways.

A functional hallmark of EMT is acquisition of migratory characteristics, a critical component of tumor cell invasion during the metastatic process, which requires degradation and remodeling of ECM components. The

Figure 6. TIMP-1 increases MCF10A cell migration in a CD63-dependent manner and is independent of TIMP-1's MMP-inhibitory domain. Transwell migration assay with Neo, TIMP-1 (A) and TIMP-1 shCont-P and TIMP-1 shCD63-2 cells (B). C, Transwell migration assay with control MCF10A, T1 #9, T1D #66, and T1D #69 cells. For all of these experiments, cells were pretreated with mitomycin C (25 μg/mL) for 30 minutes. The total number of cells that migrated to the lower side of the filter was counted using microscopy at 20×. The experiment was done in triplicates, and the data are representative of two or more independent experiments. Each bar, mean ± SD. *, P < 0.05 using a paired t test, considered significant; ***, P < 0.001 using a paired t test, considered extremely significant.
involvement of MMPs including MMP-2, MMP-3, MMP-9, MMP-28, and MMP-14 (MT1-MMP) was suggested in EMT-derived phenotypic conversion in cancer cells (42–44). In addition to MMPs’ ability to remodel ECM, MMP modulation of EMT signaling has been demonstrated in cancer cells. Exposure of mammary epithelial cells to stromelysin (MMP-3) resulted in EMT involving increased expression of an alternative splicing variant of Rac1, resulting in an increase in cellular reactive oxygen species leading to upregulation of the EMT transcription factor Snail (45). Epilysin (MMP-28) was shown to induce EMT through proteolytic processing of latent TGFβ complexes into the active form, resulting in TGFβ-mediated EMT signaling in lung carcinoma cells (46). These MMP-mediated EMT processes can be prevented by chemical inhibitor of MMPs such as BB3103 or GM6001 (46, 47). Considering a well-established role for TIMP-1 in inhibition of MMPs and therefore inhibition of tumor cell invasion, our finding of a novel function of TIMP-1 in mediating EMT is completely unexpected. Importantly, we demonstrated that TIMP-1-mediated EMT is independent of its MMP-inhibitory domain, establishing TIMP-1 as a critical signaling molecule for the regulation of breast epithelial cell morphology and migratory phenotype. Similarly to our study, an association of TIMP-1 with the EMT process was suggested in MDCK cells (48). In these cells, MAPK activation, which regulates gene expression including MMP-13 and TIMP-1, was necessary to induce the first stage of tubulogenesis, the partial epithelial to mesenchymal transition (p-EMT), whereas MMPs were necessary for the second redifferentiation stage. If MMP-mediated proteolytic events were responsible for EMT, TIMP-1 knockdown was expected to enhance or not to affect the EMT processes. TIMP-1 knockdown, however, was shown to result in partial inhibition of EMT in MDCK cells (48), indirectly suggesting a TIMP-1 role in EMT without its inhibition of MMPs, consistent with the present study.

In summary, we report a novel activity of TIMP-1 as a signaling molecule for induction of EMT-derived phenotype in human breast epithelial cells. We propose that TIMP-1 has dual, rather paradoxical, functions during cancer progression: When the cell surface binding partner for TIMP-1 such as CD63 is readily available, TIMP-1 interaction with its cell surface signaling partner activates intracellular signaling pathways leading to EMT-derived phenotype including cell migratory characteristics, exerting a potential oncogenic activity. In a microenvironment where TIMP-1 predominantly interacts with MMPs, TIMP-1 is likely to inhibit tumor cell metastasis via its inhibition of MMPs. Thus, TIMP-1’s function as an endogenous inhibitor of MMP or as a “cytokine-like” signaling molecule may be a critical determinant for tumor cell behavior, and the molecular actions of TIMP-1 may be determined by the localization (soluble vs. pericellular) and the levels of its cell surface partner and MMPs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: R.C. D’Angelo, R. Fridman, H.-R.C. Kim
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References
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