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Cortactin is involved in transforming growth factor-β1-induced epithelial-mesenchymal transition in AML-12 cells

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Cortactin is an F-actin binding protein, regulating cell movement and adhesive junction assembly. However, the function of cortactin in epithelial-mesenchymal transition (EMT) remains elusive. Here we found that during transforming growth factor-β1 (TGF-β1)-induced EMT in AML-12 murine hepatocytes, cortactin underwent tyrosine dephosphorylation. Inhibition of the dephosphorylation of cortactin by sodium vanadate blocked TGF-β1-induced EMT. Knockdown of cortactin by RNAi led to decrease of intercellular junction proteins E-cadherin and Zonula occludens-1 and induced expression of mesenchymal protein fibronectin. Additionally, knockdown of cortactin further promoted TGF-β1-induced EMT in AML-12 cells, as determined by EMT markers and cell morphological changes. Moreover, migration assay showed that cortactin knockdown promoted the migration of AML-12 cells, and also enhanced TGF-β1-induced migration. Our study showed the involvement of cortactin in the TGF-β1-induced EMT.

Keywords epithelial-mesenchymal transition; cortactin; transforming growth factor-β; migration

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Introduction

Epithelial-mesenchymal transition (EMT) describes a switch in which epithelial cells acquire mesenchymal-like characteristics, including increased motility and invasiveness. EMT was originally defined as the formation of mesenchymal cells from epithelia during different embryonic development stages. It is now known to occur in a variety of diseases including the fibrotic diseases in lung, kidney, and liver [1–4], and malignant tumor progression [5]. Epithelial cells are characterized by the formation of intercellular junctions, which connect and immobilize adjacent cells. During EMT, intercellular junctions of epithelial cells are interrupted or decreased by downregulation of adhesion molecules E-cadherin and tight junction component Zonula occludens-1 (ZO-1). The subsequent disassembly of adhesion junction increases cell motility. Epithelial tight junction participates in the maintenance of epithelial integrity that protects multicellular organisms from the external environment. ZO-1 belongs to a family of membrane-associated guanylate kinase homologues that function as an essential tight junctional plaque protein. During EMT, the loss of tight junctions and, accordingly, the delocalization of their structural components (e.g. ZO-1) from cell–cell contacts were implicated in the depolarization of epithelial cells [6]. EMT is also characterized by morphological changes from epithelial to fibroblast-like, with upregulation of mesenchymal markers, including fibronectin and vimentin.

Cortactin is an F-actin binding protein that stabilizes F-actin networks and stimulates Arp2/3 (Actin related proteins 2/3)-mediated actin polymerization and branched actin assembly [7,8]. Cortactin was identified as one of the major substrates for tyrosine kinases such as Src and Fer. Tyrosine phosphorylation modification of cortactin regulates F-actin cross-link [9] and mediates the actin filament-driven centrosome separation at G2-M transition [10]. Because cortactin distributes mainly in cell motility structures such as lamellipodia and invadopodia, the role of cortactin in cell movement was studied. Overexpression of cortactin has been shown to enhance cell motility in a variety of assays [11,12], mainly due to its roles in actin assembly and promotion of the persistence of lamellipodial protrusion [13]. Cortactin is also reportedly involved in the formation of

adhesion junction after recruitment to cell–cell adhesive contacts [14], suggesting that cortactin has a potential function in maintaining epithelial properties. Transforming growth factor (TGF)-β1 is extensively involved in EMT induction in various epithelial cells [15]. However, whether cortactin participates in the disruption of intercellular junction during TGF-β1-induced EMT still remains unclear.

In this study, we examined the status of phosphorylation and the expression level of cortactin during TGF-β1-induced EMT. We also investigated the role of cortactin in TGF-β1-induced EMT using RNA interference technique in AML-12 murine hepatocyte cell line. The results revealed that TGF-β1 induced a decrease in the levels of tyrosine phosphorylated cortactin during EMT. Knockdown of cortactin led to a disruption of tight junction and adhesion junction, causing an enhancement of TGF-β1-induced EMT and increased the migratory capacity of AML-12 cells.

Materials and Methods

Materials

Cell culture and transfection reagents were purchased from Invitrogen (Carlsbad, USA). AML-12 murine hepatocytes were purchased from the American Type Culture Collection (Manassas, USA). TGF-β1 was from Chemicon/Millipore (Billerica, USA). Rabbit polyclonal antibodies against cortactin, tyrosine phosphorylation (PY99), vimentin, and fibronectin were purchased from Santa Cruz (Santa Cruz, USA). Mouse monoclonal antibody against ZO-1 was purchased from Invitrogen. Rabbit polyclonal antibody against fibronectin was from Sigma (St Louis, USA). Mouse monoclonal antibody against E-cadherin was from BD Biosciences (San Jose, USA). Mouse monoclonal antibody against tyrosine phosphorylation (4G10) was from Upstate/Millipore. pPGK super RNAi vector was a kind gift from Dr Yuan Wang (Institute of Biochemistry and Cell Biology, Shanghai, China). Other reagents were purchased from Sigma unless otherwise indicated.

Cell culture

AML-12 cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 10% fetal calf serum and supplemented with insulin-transferrin-selenium-X, dexamethasone (40 ng/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 until 30–50% confluence was reached.

RNA interference

The target sequence of cortactin is 5′-GGATCGGATGGACAAGAAT-3′, which was inserted into the pPGK super vector at the *Bgl*II and *Hin*dIII sites. An empty vector was used as negative control.

Transfection

Cells (30–50% confluence) in 35-mm plates were transfected with the plasmids as indicated using Lipofectamine™ reagent according to the manufacturer's instructions. For transient transfection, the expression of the indicated plasmids was examined 48 h after transfection. Stable transfection was proceeded with G418 (800 μg/ml) selection after the transient transfection.

Examination of morphological change

The morphological changes of the cells were observed under an inverted phase-contrast microscope (Olympus). The photographs were taken at 200× magnifications by a digital camera.

Migration assays

Cell migration assays were performed using Transwell migration chambers (8 μm pore size; Costar, Conning, USA). After TGF-β1 treatment (5 ng/ml, 48 h), the cells (2×10^4 cells per Transwell) were plated into the insert in medium containing 0.5% FBS and were allowed to migrate from upper compartment to lower compartment toward a 10% FBS gradient for 12 h. The cells that remained on the top of the filter were scrubbed off, and the cells that migrated to the underside of the filter were fixed in methanol, followed by H&E staining. The number of migrated cells was counted manually.

Preparation of cell lysates and immunoblotting

Cells were lysed in 10 mM Tris (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 10 mM $Na_4P_2O_7 \cdot 10H_2O$, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM PMSF. Aliquots (50 μg) of protein lysates were electrophoresed on SDS-polyacrylamide gels (8 or 10%) and transferred to nitrocellulose membranes (Hybond™ ECL). The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and subsequently incubated with primary antibody (1:3000 dilution) overnight at 4°C. After washing with TBS-T for 1 h at room temperature, the membrane was further

incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz) for 2 h, followed by 45 min of washing (with three to five changes of the wash buffer). Protein bands were visualized with the Super Signal Reagents (Pierce/Thermo Fisher Scientific, Rockford, IL, USA).

Statistical analysis

Quantitative data are presented as mean ± SD. Statistical significance was determined by the two-tailed student's *t*-test or one-way ANOVA followed by the LSD *t*-test for multiple comparisons. A *P*-value of <0.05 was considered statistically significant.

Results

TGF-β1 downregulated the levels of tyrosine phosphorylated cortactin during EMT

AML-12 murine hepatocytes have a typical epithelial phenotype with polygonal morphology and tight arrangement. In response to TGF-β1, AML-12 cells acquired a spindle-like mesenchymal morphology, which could be detected after 12 h, and became highly prominent after 48 h [**Fig. 1(A)**] [4,16]. Furthermore, downregulation of E-cadherin and ZO-1, the two well-known EMT markers, was observed during TGF-β1 treatment in time-dependent manner [**Fig. 1(B)**], suggesting a dissolution of adhesion and tight junctions. TGF-β1 treatment also increased fibronectin and vimentin levels in AML-12 cells [**Fig. 1(B)**], indicating that these cells acquired a mesenchymal phenotype. The results demonstrate that AML-12 cells could undergo EMT in response to TGF-β1.

In order to know whether TGF-β1 regulates cortactin, we detected the protein levels of cortactin by immunoblotting. The results showed that the protein levels of cortactin remained unchanged before and after TGF-β1 treatment from 6 to 48 h [**Fig. 1(C)**]. Because the tyrosine phosphorylation of cortactin was reported to modify its activity and function, we also examined the effect of TGF-β1 on the phosphorylated-cortactin levels. As shown in **Fig. 1(D)**, a decrease in the levels of tyrosine-phosphorylated cortactin was detected by immunoblotting with PY99 or 4G10, two general antibodies against phospho-tyrosine, after the immunoprecipitation with antibody against cortactin. TGF-β1-mediated downregulation of tyrosine-phosphorylated cortactin was observed 12 h after TGF-β1 treatment and reached to an almost undetectable level 48 h after TGF-β1 treatment. The kinetics of the alterations in tyrosine phosphorylated cortactin levels highly correlated with that of the EMT processes.

To know if the dephosphorylation of cortactin might be involved in TGF-β1-induced EMT, AML-12 cells were treated with tyrosine phosphatase inhibitor sodium vanadate, which markedly blocked TGF-β1-induced cortactin tyrosine dephosphorylation [**Fig. 2(A)**]. Interestingly, sodium vanadate treatment suppressed TGF-β1-induced cell morphological changes [**Fig. 2(B)**], the downregulation of ZO-1 and E-cadherin, and the upregulation of fibronectin [**Fig. 2(C)**].

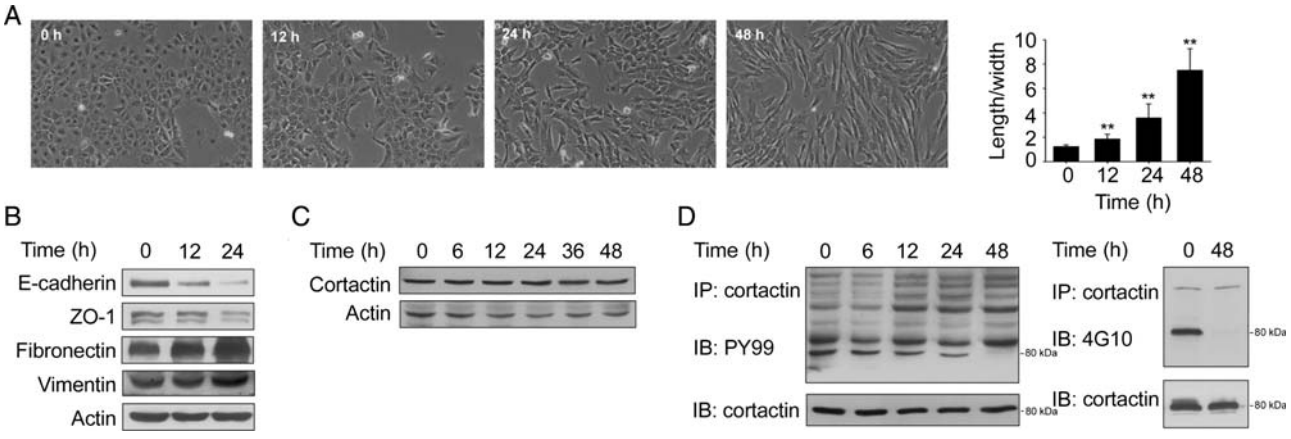

Figure 1 Analysis of protein level and phosphorylation modification of cortactin in response of TGF-β1 (A) AML-12 cells were treated with TGF-β1 (5 ng/ml) for indicated time periods. Cell morphology was examined and quantified by the ratio of cell length vs. width $^{**}P < 0.001$. Magnification, 200×. (B) The expressions of epithelial markers (E-cadherin and ZO-1) and mesenchymal marker (fibronectin and vimentin) were examined by immunoblotting. (C) AML-12 cells were treated with TGF-β1 (5 ng/ml) for indicated time periods. The protein level of cortactin was examined by immunoblotting. (D) AML-12 cells were treated with TGF-β1 (5 ng/ml) for indicated time periods. Cells were lysed and immunoprecipitated with anti-cortactin antibody followed by immunoblotting with anti-phosphotyrosine antibodies (PY99 or 4G10) and anti-cortactin antibody.

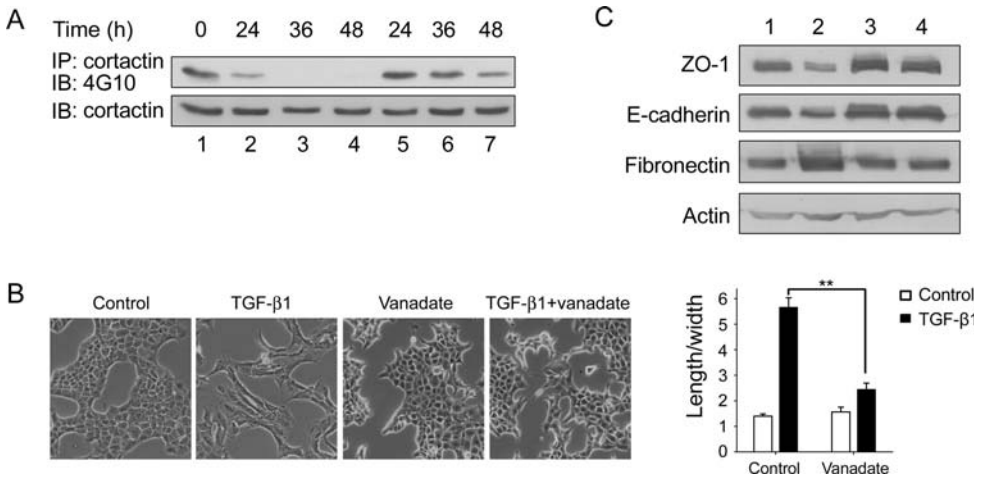

Figure 2 Sodium vanadate inhibits dephosphorylation of cortactin and EMT (A) AML-12 cells were treated with TGF-β1 (5 ng/ml) or/and vanadate (10 μM) for indicated time periods. Cells were lysed and immunoprecipitated with anti-cortactin antibody followed by immunoblotting with anti-phosphotyrosine antibodies (4G10) and anti-cortactin antibody. 1, control; 2–4, TGF-β1; 5–7, TGF-β1+vanadate. (B) AML-12 cells were treated with TGF-β1 (5 ng/ml) or/and vanadate (10 μM) for 48 h. Morphological changes and the average length/width ratio of cells were shown $^{**}P < 0.001$. Magnification, 200×. (C) The protein level of ZO-1, E-cadherin, and fibronectin were analyzed by immunoblotting. Actin levels were used as loading controls. 1, control; 2, TGF-β1; 3, vanadate; 4, TGF-β1+vanadate.

Knockdown of cortactin induced EMT-like changes

The alterations of the phosphorylated cortactin levels during EMT raises the possibility that cortactin may play a role in TGF-β1-induced EMT. To test this possibility, we knocked down cortactin by RNAi in AML-12 cells, and selected two cortactin RNAi clones [**Fig. 3(A)**]. E-cadherin and ZO-1 were dramatically downregulated in cortactin RNAi cells, as compared with control cells. As an important mesenchymal marker, the level of fibronectin was also found elevated in cortactin RNAi cells. Moreover, a change from well-arranged rectangle shape to irregular morphology was observed [**Fig. 3(B)**]. These data suggest that knockdown of cortactin induced molecular and phenotypic changes essential for EMT in AML-12 cells.

Knockdown of cortactin promoted TGF-β1-induced EMT

We next examined the effect of knockdown of cortactin on TGF-β1-induced EMT in AML-12 cells. As shown in [**Fig. 4(A)**], TGF-β1 induced decrease in the levels of E-cadherin and ZO-1 was significantly enhanced in cortactin RNAi cells. Similarly, the increase of vimentin, another EMT marker, was also enhanced in cortactin RNAi cells. In addition, TGF-β1-induced apparent morphological changes were accelerated in cortactin RNAi

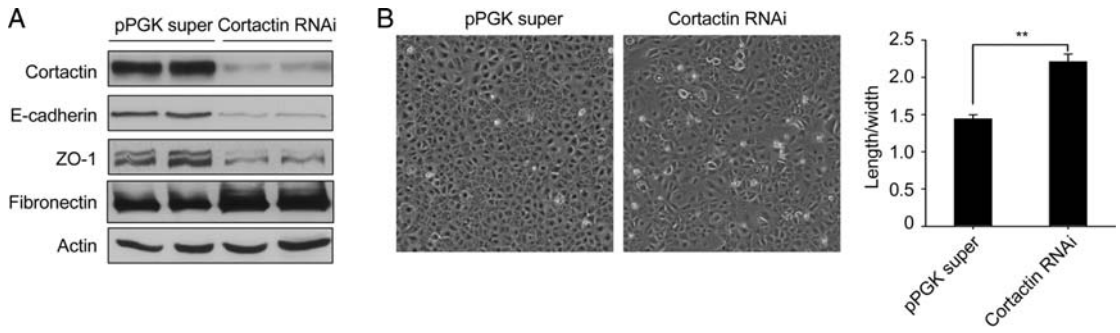

Figure 3 Effect of cortactin RNAi on EMT markers and cell morphology in AML-12 cells (A) Plasmids encoding cortactin siRNA and empty vector (pPGK super) were transfected into AML-12 cells, respectively. Stable clones were selected. The protein level of cortactin, E-cadherin, ZO-1, and fibronectin were analyzed by immunoblotting. Actin levels were used as loading controls. (B) The morphology and the average length/width ratio of pPGK super and cortactin RNAi plasmids transfected cells $^{**}P < 0.001$. Magnification, 200×.

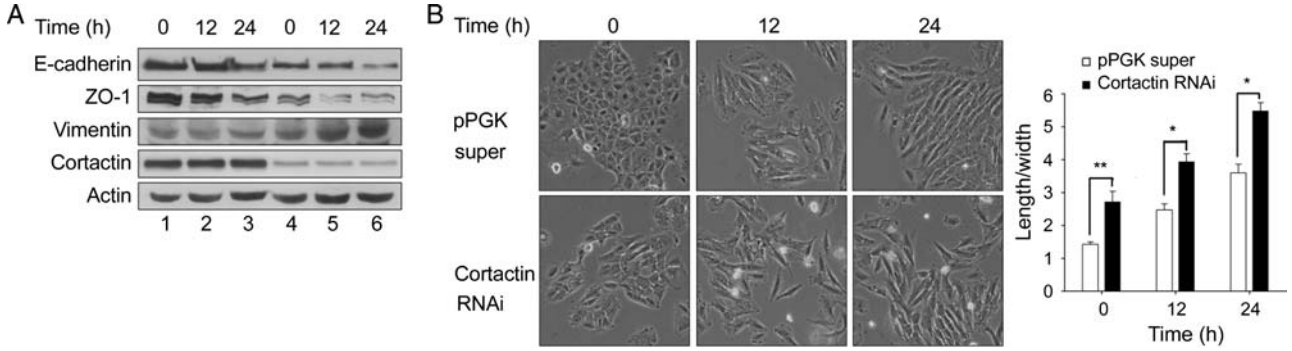

Figure 4 Effect of cortactin RNAi on TGF-β1-induced EMT (A) Empty vector (pPGK super) and cortactin RNAi plasmids transfected cells were treated with TGF-β1 (5 ng/ml) for indicated time periods. The protein levels of E-cadherin, ZO-1, vimentin and cortactin were examined by immunoblotting. 1-3, pPGK super+TGF-β1; 4-6; cortactin RNAi+TGF-β1. (B) Morphological changes and the average length/width ratio of cells were shown $*P < 0.05$, $**P < 0.001$. Magnification, 200×.

cells [**Fig. 4(B)**], indicating that knockdown of cortactin promoted TGF-β1-induced EMT.

Knockdown of cortactin enhances cell migratory capacity

Since EMT can increase the cell motility, we examined whether the downregulation of cortactin by RNAi has any effect on the cell migration. We performed transwell assay to compare the migration of cortactin RNAi cells with control cells. The results showed that cortactin RNAi promoted the migration of AML-12 cells, and enhanced TGF-β1-induced migration (**Fig. 5**).

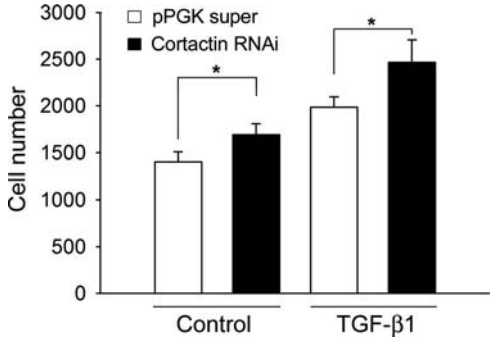

Figure 5 Migration assay of cortactin RNAi cells with or without TGF-β1 treatment Empty vector (pPGK super) cells or cortactin RNAi cells were treated with TGF-β1 (5 ng/ml) for 48 h or left untreated, and then trypsinized and counted. Cells (2×10^4) were plated into the insert in medium containing 0.5% FBS and allowed to migrate from upper compartment to lower compartment toward a 10% FBS gradient for 12 h. The cells that migrated to the underside of the filter were fixed and counted. Results from three independent experiments were calculated. $*P < 0.05$.

Discussion

As an important cytoskeleton regulator protein, cortactin was reported implicated in various biological events such as migration and matrix remodeling [17], mitosis [10], and cell endocytosis [18]. It has been reported that cortactin is required for adhesive contacts formation through interaction with E-cadherin and promoting F-actin accumulation in adhesive complex; inhibition of cortactin activity significantly reduced cadherin adhesive contact zone extension [14]. Using yeast two-hybrid screening, Katsube *et al*. [19] found that cortactin also can bind to tight junction protein ZO-1. Our study showed that after knockdown of cortactin in AML-12 cells, the protein levels of E-cadherin and ZO-1 dramatically decreased, suggesting that cortactin is required for maintaining the levels of these important molecules. Furthermore, as we have shown, inhibition of cortactin enhanced the expression of fibronectin, which is a specific mesenchymal marker, indicating that cortactin is also involved in maintaining the epithelial properties of cells. However, knockdown of cortactin did not induce the full EMT morphology as that induced by TGF-β treatment. These results indicate that the regulation of cortactin by TGF-β1 was mainly involved in the process of dissociation of intercellular junctions during EMT. Our recent studies have shown that PKA, STAT3, labile iron pool/ROS, and other signaling events are involved in the control of EMT [20,21]. Therefore, the data presented also suggest that cortactin only partially involved in the mediation of the maintenance of the epithelial phenotype.

Overexpression of cortactin and amplification of the cortactin gene, *EMS1*, have been found in cancer cells

[22–24], and have been shown to enhance the cell motility and invasion [12,25,26]. However, there was a discrepancy in the published reports. It has been reported that knockdown of cortactin impaired the cell motility [27] or showed no significant effect on the cell motility [28,29]. Jia *et al*. [30] reported that knockdown of cortactin in cells with high level of cortactin tyrosine phosphorylation enhanced cell migration in gastric cancer cells and breast cancer cells MCF7. In the present study, we found that cortactin RNAi in AML-12 cells enhanced the cell migration in the absence or presence of TGF-β1. Our results also suggest that the effect of cortactin knockdown on cell migration is dependent on a disruption of intercellular junctions and is related to EMT process. We concluded that cortactin plays an important role in intracellular junction formation. In tumor cells, cortactin knockdown reportedly impaired the persistence of lamellipodial, leading to a selective defect in motility [13]. The effect of disruption of intercellular junctions was not evident because of the dispersive property of tumor cells. However, in normal epithelial cells such as AML-12 cells, the disruption of intercellular junctions may account for the dominant effect of cortactin knockdown. These observations provide an explanation for the paradoxical effects of cortactin knockdown.

Tyrosine phosphorylation is an important posttranscriptional modification of cortactin. Phosphorylation of cortactin by Src kinase occurs at tyrosine residues 421, 466, and 482 through a progressive manner with initial phosphorylation at tyrosine 421 followed by 466 [31]. The tyrosine phosphorylation has been shown to attenuate its ability to cross-link F-actin *in vitro* [32] and to inhibit its activation on N-WASP [33], an important protein involving in actin polymerization. However, it is not clear whether tyrosine phosphorylation of cortactin is involved in assembly of intercellular junctions. In cortactin over-expressing cells, cortactin has been shown to redistribute from the cytoplasm to contact sites at the margins of cells, concurrent with an increase in its levels of phosphorylation [34]. Tyrosine phosphorylation of cortactin and p130Cas (Crk-associated substrate) have been shown to coincide with the tyrosine phosphorylation of focal adhesion kinase during integrin-mediated cell adhesion to extracellular matrix [35]. Our results demonstrated that TGF-β1 reduced the phosphorylated cortactin levels, which was accompanied by the disruption of adhesion junction and tight junction in AML-12 cells. These results suggested that the tyrosine phosphorylation of cortactin might play a role in cell adhesion and assembly of intercellular junctions.

One of the critical processes of EMT is disruption of adhesive junction and tight junction, which relieves cells from mutual restriction and makes free migration possible. As the main component of adhesive junction, the downregulation of E-cadherin is generally accepted as a hall marker of EMT. The mechanism of downregulation of E-cadherin was intensively studied during TGF-β1-induced EMT. Many key transcription factors such as snail family proteins and zinc finger E-box binding family proteins activated directly or indirectly by Smad2/3 were identified to transcriptionally inhibit the expression of E-cadherin [36]. In fact, the assembly and disruption of adhesive junction is far more complicated than regulation of the level of E-cadherin. The ectodomain of E-cadherin interacts with other E-cadherin in neighbor cells in homotypic manner. The cytoplasmic domain of E-cadherin binds to β-catenin, which interacts with α-catenin and cortactin and anchors to the actin cytoskeleton [14,37]. Considering the important role of cortactin in assembly of adhesion junction complex, regulation of cortactin may be a way involving in disruption of adhesion junction during EMT. Our study showed that during EMT, TGF-β1 indeed decreased the phosphorylated cortactin levels, which can affect the activity of cortactin. Knockdown of cortactin accelerated TGF-β1-induced EMT. This study suggested a new mechanism of regulating of disassembly of adhesive junction.

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