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Ectopic expression of Ligand-of-Numb protein X promoted TGF- β induced epithelial to mesenchymal transition of proximal tubular epithelial cells

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Key Words: LNX, TGF- β , Epithelial to mesenchymal transition, ubiquitin ligase, proximal tubular epithelial cell

Abstract

Ligand-of-Numb protein X (LNx) was initially characterized as a RING finger type E3 ubiquitin ligase that targeted the intrinsic cell fate determinant Numb for ubiquitination dependent degradation. However, the physiological function of LNx remains largely unknown. In the present study, we demonstrate that ectopic expression of LNx in human proximal tubular epithelial cells (HK-2 cells) significantly enhanced TGF- β 1 induced epithelial to mesenchymal transition (EMT). The EMT-promoting effect of LNx manifested as strong inhibition of E-cadherin expression, enhanced expression of vimentin, fibronectin or PAI-1, and increased cell migration. This function of LNx was shown to be independent of its ligase activity because ectopic expression of a mutant form of LNx (C48ALNx) that lacks E3 ligase activity had the similar effect as the wild-type LNx. Overexpression of E-cadherin could inhibit LNx augmented EMT. This study suggests a potential role for LNx in promoting EMT in human proximal tubular epithelial cells.

Introduction

The epithelial–mesenchymal transition (EMT) is a fundamental process governing morphogenesis in multicellular organisms. This process is also re-activated in a variety of diseases including fibrosis and cancers [1]. EMT is usually initiated by an extracellular stimulus, which leads to a loss of junctional contacts between neighboring cells, detachment from the basement membrane, remodeling of the actin cytoskeleton, and ultimately, an increase in cell motility. After the initiation of EMT, cells move from the basement membrane to the interstitial matrix where they acquire the characteristics of fibroblasts/myofibroblasts [2]. Of the many factors that can induce EMT, transforming growth factor- β 1 (TGF- β 1) is the most intensively studied. Previous investigations into the molecular mechanism of TGF- β induced EMT in the mammary epithelium led to the identification of a number of mediators of this process that include the Smads proteins, phosphatidylinositol 3-kinase, RhoA, p38, and integrin- β 1, among others [3–8]. Besides alterations in signaling pathways involving these molecules, EMT is also characterized by extensive genetic modifications and by alterations in protein expression and degradation [9, 10]. Ubiquitination of a protein is initiated by an E1 ubiquitin-activating enzyme, facilitated by an E2 ubiquitin-conjugating enzyme, and carried out by an E3 ubiquitin ligase. In the ubiquitin-proteasome pathway, E3 ligases play a critical role in the recognition of target proteins and subsequent protein degradation by 26 S proteasomes. Two types of E3 ubiquitin ligases, namely the RING finger-type and HECT-type ligases, have been

well characterized in mammals [11]. Many proteins containing RING finger domains have been found to function as E3 ligases, some of which are involved in maintaining epithelial cell polarity and in regulating EMT. For example, Hakai, a Cbl-like E3, was found to ubiquitinate and induce endocytosis of E-cadherin, a key component of the adherens junction [12]. Ecto, a RING-type E3, localizes in the nucleus, binds specifically to Smad4, leading to its ubiquitination and proteasome-mediated degradation and thereby blocking TGF- β signaling [13]. Recently, Wrana's group identified that Smurf1, an E3 ubiquitin ligase, functions as a key regulator of tight junction disassembly in TGF- β 1 induced EMT [14].

Murine LNX was originally identified in a search for proteins interacting with the phosphotyrosine-binding domain of the cell fate determinant Numb. Two isoforms of LNX have been identified to date. They are LNXp80 that harbors an N-terminal RING finger domain and LNXp70 that lacks the RING finger due to alternative splicing [15]. We previously reported that LNXp80 functions as a RING finger-type E3 ubiquitin ligase that targets Numb for proteasome degradation [16]. However, the physiological function of LNX remains largely unknown. Recently, Numb was shown to regulate integrin endocytosis for directional cell migration and to interact with the Par3/Par6/aPKC polarity complex [17]. In polarized MDCK cells, endogenous LNXp70 was detected at the tight junction and was found to function as an endocytic scaffold for JAM4 in TGF- β induced cell junctional reorganization[18]. These studies prompted us to address the question whether LNX mediated Numb degradation could affect the progress of EMT. In the present study, we show that ectopic expression of

LNX promotes TGF- β 1-induced EMT in human proximal tubular epithelial cells (HK-2 cells). Overexpression of LNX led to a decrease in E-cadherin suppression, an increase in vimentin and fibronectin expression, and an enhancement in cell migration. Interestingly, a mutant of LNX that lacks E3 ligase activity had the similar effect as wild-type LNX, indicating that the EMT promoting effect of LNX is independent of its E3 ligase activity.

Materials and Methods

Reagents and plasmids constructs

Recombinant human TGF- β 1 was purchased from R&D Systems. TRIzol and reverse transcriptase were from Invitrogen (Carlsbad, CA, USA). Antibody against E-cadherin and fibronectin was from BD Biosciences (San Jose, CA); antibody against vimentin was from Sigma (St. Louis, Missouri, USA), and polyclonal PAI-1 antibody was purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Wild type and mutant LNX constructs have been described previously [16]. Anti-LNX antibody was raised against residues 31-221 of the mouse LNXp80 and was provided by Dr. Jane McGlade (University of Toronto, Canada). pRc/CMV-E-cadherin was provided by Dr. Shawn Li (University of Western Ontario, Canada).

Cell culture and transfection

HK-2 cells were maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum. DNA constructs (1-2 μ g) were transfected into HK-2 cells using

LipofectAMINE (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After transfection, cells were treated with indicated amount of TGF- β 1 for 24h.

Real-time PCR

Real-time PCR was performed with the ABI PRISM 7000 sequence detection system. SYBR green real-time PCR mix (Invitrogen, Carlsbad, CA) was used for PCR reaction, with a primer concentration of 400 nM. Primers for real-time PCR were as follow:

human E-cadherin	sense 5'-CGC CCT ATG ATT CTC TGC TCG-3''
	antisense 5'- TCG TCC TCG CCG CCT CCG TA-3'
human fibronectin	sense 5'-GTG TGT TGG GAA TGG TCG TG-3'
	antisense 5'- GAC GCT TGT GGA ATG TGT CG-3'
human vimentin	sense 5'-GAA TGA CCG CTT CGC CAA CT-3'
	antisense 5'-CCG CAT CTC CTC CTC GTA G-3'
human PAI-1	sense 5'-CAA CTT GCT TGG GAA AGG AG-3'
	antisense 5'-GAC AGT GCT GCC GTC TGA TT-3'
human GAPDH	sense 5'-TGG ACC TGA CCT GCC GTC TA-3'
	antisense 5'-GAG TGG TGT CGC TGT TGAA-3'

Reaction conditions were as follow: 95°C for 2 min for UDG incubation and 95°C for 10 min for UDG inactivation and DNA polymerase activation, then 40 repeats of 95°C for 15 s, 62°C for 30 s and each sample was performed triply in 25 μ l reaction

volume. GAPDH was used as reference gene. Relative quantification of gene expression was performed using the $2^{-\Delta\Delta C_t}$ method based on C_t values for both target and reference genes. In order to calculate the fold increase, results of real-time PCR analysis are given as mean \pm SD.

Western blot analysis

Cells were washed with PBS and scraped into lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_5 , 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin and 20 μ g/ml aprotinin). Protein was quantified by the Bradford assay (BioRad, Hercules, CA) and Samples were heated at 100°C for 10 min before loading. Equal amount of protein were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). After blocking in 5% skim milk for 1h at room temperature, the membranes were incubated with indicated primary antibody at 4°C overnight followed by horseradish peroxidase-conjugated second antibody for 1h at room temperature and detected by chemiluminescence (Amersham Life Science). Quantification of the Western blot data was performed by measuring the intensity of the hybridization signals using NIH Image analysis program.

Immunofluorescence staining

HK-2 cells were seeded on glass coverslips. After transfection, cells were washed

with PBS and fixed with cold methanol for 20 min at 4°C. Following three extensive washings with PBS, cells were permeabilized in PBS containing 0.1% Triton X-100 for 3 min and then blocked with PBS containing 5% BSA for 45 min at 37°C. Cells were incubated overnight at 4°C with indicated primary antibodies diluted in PBS. After extensive washing, either FITC-conjugated and/or Cy3-conjugated second antibody in 5% BSA in PBS were added for 45 min at 37°C. Samples were mounted in fixation medium (Biomedex, Foster City, CA). DAPI was used to stain the nucleus. Images were analyzed and collected with Zeiss LSM 510 Confocal Imaging System (Zeiss, Germany).

Migration assays.

Cell migration was evaluated using a Boyden chamber motility assay with tissue culture-treated Transwell filters (Millipore, Billerica, MA, USA). HK-2 Cells were initially cultured on 35mm dishes. After transfection and TGF- β 1 treatment, cells (1×10^4) were resuspended and seeded onto the filters (8 μ m pore size) in the top compartment of the chamber. After 10h of incubation at 37°C, filters were fixed with methanol for 15 min at room temperature and stained with 1% crystal violet for another 15 min, and the upper surface of the filters was carefully wiped with a cotton-tipped applicator. Cells and cell extensions that passed through the pores were counted in five nonoverlapping $\times 40$ fields and photographed.

Statistics

The results were expressed as mean \pm SD from at least three experiments. Statistical analysis was carried out using SPSS11.0. The difference between wLNX or empty vector transfected cells upon TGF- β 1 treatment were analyzed by one-way ANOVA. The remaining data were analyzed using Student's t-test. A $P < 0.05$ was considered to be significant.

Results

Ectopic expression of LNX was insufficient to cause EMT

We first examined the subcellular distribution of LNX p80 in proximal epithelial cells (HK-2 cells). FLAG-tagged wild-type LNX (wLNX), C48ALNX, a mutant of LNX that lacks E3 ligase activity, or an empty vector was transfected into HK-2 cells, respectively. The localization of LNX was detected by immunofluorescence using the antibody that specifically recognized the N-terminal of LNXp80 as described in materials. As shown in Figure 1A-F, both wLNX and C48ALNX were detected mainly in the cytosol, but also at the plasma membrane. No endogenous LNXp80 was detected in HK-2 cells (Fig. 1G). In addition, no apparent morphological changes were observed in LNX-expressing cells compared to cells transfected with an empty vector. Western blot analysis indicated that LNX over-expressing cells had similar level of the junctional protein E-cadherin as the control cells. Furthermore, no significant change was observed in the expression of mesenchymal marker proteins such as vimentin, fibronectin and PAI-1 when wLNX or C48ALNX was overexpressed (Fig. 1G). These results suggest that ectopic expression of LNX does

not directly cause EMT.

TGF- β 1 induced E-cadherin suppression was enhanced by LNX overexpression

There is increasing evidence that TGF- β 1 is a key factor for initiating and completing the full course of EMT, and a hallmark of an early stage of EMT is the loss of E-cadherin expression [19]. We therefore investigated whether LNX could affect TGF- β 1-dependent suppression of E-cadherin expression in HK-2 cells. FLAG-tagged wLNX or an empty vector was transiently transfected into HK-2 cells. At 24h post-transfection, cells were treated with incremental concentrations of TGF- β 1 ranging from 0.1 to 5ng/ml. Cell lysates were harvested 24h after TGF- β 1 treatment and Western blots were performed to determine E-cadherin protein levels. As expected, the protein level of E-cadherin decreased gradually with increasing concentrations of TGF- β 1 in vector-transfected cells. However, the E-cadherin level in LNX-transfected cells was significantly lower than that of vector transfected cells at all tested concentrations of TGF- β 1 (Fig. 2A). Quantification of the gel bands indicated that the drop in E-cadherin protein level was most pronounced at 0.1ng/ml TGF- β 1 treatment in comparison to the vector control (Fig. 2B). In contrast to the vector-transfected cells in which E-cadherin level was almost the same as that without TGF- β 1 treatment, E-cadherin was reduced to approximately 60% of the resting level in the LNX-expression cells under the same treatment (Fig. 2B). These results suggest that LNX sensitize HK-2 cells to TGF- β 1 treatment. The suppression of E-cadherin expression by LNX was further confirmed by real-time RT-PCR. Consistent with

Western blot analysis, the level of E-cadherin transcript in LNX-transfected cells was significantly lower than that of vector-transfected cells upon 0.1ng/ml TGF- β 1 treatment (Fig. 2C). Collectively these results suggest that LNX may function synergistically with TGF- β 1 in regulating E-cadherin gene expression and protein level during the process of EMT.

Because previous studies suggested a role for the transcription factor Snail in suppressing E-cadherin expression during tumorigenesis of epithelial cells [20, 21], we next investigated the potential involvement of Snail in LNX-dependent E-cadherin suppression. As shown in Figure 2D, real time RT-PCR revealed a dose-dependent increase of Snail expression in control cells after TGF- β 1 treatment. However, the Snail mRNA level in LNX-overexpressing cells was lower than that in vector-transfected cells upon TGF- β 1 treatment. This result suggests that LNX suppress E-cadherin expression by a mechanism independent of Snail transcription factor in tubular epithelial cells.

LNX promoted TGF- β 1- induced vimentin expression

Vimentin is a phenotypic marker of myofibroblasts and its expression is a hallmark of EMT at advanced stages [22]. Compared to control cells in which TGF- β 1 induced a gradual and dose-dependent increase in the vimentin protein level, LNX overexpression resulted in a more robust increase in vimentin at all concentrations of TGF- β 1. At 0.1ng/ml TGF- β 1 treatment, the vimentin level in vector-transfected control cells was similar to that in untreated cells. But the protein level of vimentin in

the LNX-expressing cells was two folds higher than of untreated cells. The difference between LNX-transfected cells and control cells was statistically significant. After 5ng/ml of TGF- β 1 treatment, the vimentin level in the LNX-expressing cells was approximately 3 times of that in vector-transfected cells under the same treatment (Fig. 3A-B). Real-time RT-PCR showed a dose dependent increase of vimentin transcript after TGF- β 1 stimulation in both control and LNX expressing cells. The mRNA level of vimentin increased significantly upon 0.1ng/ml of TGF- β 1 treatment. Interestingly, although the mRNA level of vimentin in LNX-expressing cells was slightly higher than that of control cells, the difference was not statistically significant (Fig. 3C). The discrepancy between the Western blot and Real time RT-PCR results suggest that LNX may stabilize vimentin mRNA or protect its protein from degradation.

LNX expression accentuated TGF- β 1 triggered accumulation of extracellular matrix

One of the cellular consequences of EMT is the extracellular matrix accumulation (ECM) due to increased synthesis of ECM proteins and production of protease inhibitors that block ECM degradation [23, 24]. To explore a potential role of LNX in this process, we examined the effect of LNX on the expression of fibronectin, a component of extracellular matrix. As shown in Figure 4A, fibronectin expression in LNX-transfected cells was slightly higher than that in the empty vector-transfected cells in the absence of TGF- β 1 yet the difference was insignificant. Under the treatment of 0.1ng/ml of TGF- β 1, this difference in fibronectin protein level was

drastically enhanced (Fig. 4B). We confirmed this observation by immunofluorescence and found that fibronectin was present in assembled fibrils at the extracellular compartment of the cells and that this fibril network was significantly more extensive in LNX-overexpressing cells than in normal HK-2 cells at 0.1ng/ml of TGF- β 1 treatment (Fig. 4C-H).

We next investigated whether LNX promoted TGF- β 1 induced expression of plasminogen activator inhibitor-1 (PAI-1), the primary inhibitor of the serine proteases tissue-type and urokinase-type plasminogen activators. As shown in Figure 5, PAI-1 protein in LNX-transfected cells was slightly higher than that of empty vector-transfected cells at 0.1ng/ml TGF- β 1. The difference grew significantly larger at 0.5ng/ml TGF- β 1 (Fig. 5B). Taken together, these data indicate that LNX expression promoted ECM accumulation by increasing synthesis of ECM components and inhibiting ECM degradation.

LNX expression promoted cell migration

We further examined the morphology of cells after TGF- β 1 treatment. As shown in Figure 6A-B, after 0.1ng/ml of TGF- β 1 stimulation for 24h, empty vector-transfected cells still remained the cobblestone morphology similar to the untreated cells. However, LNX-transfected cells exhibited a more elongated, spindle-like shape.

Increased expression of vimentin and morphological changes of LNX-overexpressing cells upon TGF- β 1 treatment implies that LNX might promote cell migration. To test this possibility, cell motility assay using a Boyden chamber system was carried out.

As shown in Figure 6C-D, upon 0.1ng/ml TGF- β 1 treatment for 24h, LNX-transfected cells displayed a greater ability to migrate than the controls. Quantification of the data indicated an approximately 50% increase in the number of cells migrating across the Transwell filters for LNX-overexpressing cells over control cells (Fig. 6E).

The effect of LNX on TGF- β 1 induced EMT is independent of its E3 activity

To interrogate whether the E3 ligase activity of LNX is necessary for its role in EMT, a mutant version of LNX, C48ALNX which lacks E3 activity was transfected into HK-2 cells. Upon 24h treatment with 0.1ng/ml TGF- β 1, cell lysates were collected and Western blot analysis was performed. As shown in Figure 7A, compare to empty vector-transfected cells, both wild type LNX and C48ALNX markedly promoted TGF- β 1 induced E-cadherin suppression, vimentin induction, PAI-1 induction, and fibronectin expression. However, no significant difference in the expression levels of the above proteins was detected between wild type LNX and C48ALNX transfected cells. In addition, Boyden chamber motility assay demonstrated that an essentially equal amount of either wLNX- or C48ALNX-expressing cells passed through the transwell filter upon TGF- β 1 treatment (Fig.7B-D). This result suggests that the effect of LNX on promoting TGF- β 1-induced EMT is not dependent on its E3 ligase activity.

Overexpression of E-cadherin inhibited LNX augmented EMT

Given the dramatic inhibitory effect of LNX on E-cadherin expression both at protein

and mRNA level, we hypothesized that overexpression of E-cadherin would attenuated LNX augmented EMT. To test this hypothesis, we transfected E-cadherin together with wild type LNX into HK-2 cells and then stimulate the cells with 0.1ng/ml TGF- β 1. Upon 24h treatment, cell lysates were collected and Western blot analysis was performed. As shown in Figure 8A, the protein level of E-cadherin in LNX and E-cadherin co-transfected cells was higher than that of LNX transfected cells. Similarly, the protein level of fibronectin, vimentin and PAI-1 in co-transfected cells was significantly lower than that of LNX transfected cells. Moreover, Boyden chamber motility assay demonstrated a significantly decreased number of E-cadherin and LNX co-transfected cells migrated across the transwell filter (Fig. 8B) compared to that of LNX-transfected cells. These data suggest that overexpression of E-cadherin could inhibit LNX-augmented EMT.

Discussion

The principle finding of this study is that overexpression of LNX significantly promoted TGF- β 1 induced EMT in HK-2 cells by modulating the expression of E-cadherin, vimentin, fibronectin and PAI-1. Overexpression of LNX also facilitates cell migration. Interestingly, this novel function of LNX is independent of its E3 ligase activity.

EMT is proposed as an orchestrated process that consists of four key steps: (1) loss of epithelial cell adhesion; (2) *de novo* mesenchymal marker protein expression and actin reorganization; (3) disruption of tubular basement membrane; and (4) enhanced

cell migration and invasion [25]. In this work, we examined the effect of LNX on promoting TGF- β 1-induced EMT at all four steps. The early event of EMT is the disruption of cellular junction characterized by loss of epithelial markers such as E-cadherin. Consistent with previous studies, we found that ectopic expression of LNX significantly enhanced TGF- β 1-induced suppression of E-cadherin expression. The protein level of E-cadherin in LNX transfected cells is about 50% of that in empty vector- transfected cells after 0.1ng/ml TGF- β 1 treatment. Moreover, Real time RT-PCR revealed that LNX expression also significantly decreased E-cadherin transcript after TGF- β 1 treatment. These data indicated that ectopic expression of LNX has a profound effect on inhibiting E-cadherin expression. In agreement with the previous finding that Snail plays a critical role in suppressing E-cadherin expression in tumor epithelial cells [20, 21], we observed TGF- β 1 induced Snail expression in HK-2 cells. However, Snail transcripts level in LNX-transfected cells was lower than that of vector control cells after TGF- β treatment. Therefore, it is likely that suppression of E-cadherin expression by LNX may be independent of Snail in tubular epithelial cells.

Vimentin is a type III intermediate filament protein normally expressed in cells of mesenchymal origin. Using an in vitro wound healing model, it was shown that vimentin mRNA and protein expression were exclusively induced in cells at the wound's edges that were actively migrating towards the center of the lesion [26]. In our study, we found a remarkable increase in vimentin protein level in LNX transfected-cells compared to control cells under TGF- β 1 treatment. The increased

vimentin expression correlated with the observed morphological changes and migratory phenotype of LNX-overexpressing cells after TGF- β 1 treatment. Nevertheless, the difference in vimentin mRNA level, as determined by Real time RT-PCR, was found to be insignificant between LNX-transfected cells and control cells after TGF- β 1 treatment, suggesting that LNX may stabilize vimentin mRNA or protect its protein from degradation. However, we are unable to rule out the possibility that the low level of transient LNX expression in our study might have undermined its cellular effect.

Cellular changes associated with EMT also manifest as an increase in the synthesis of extracellular matrix molecules and the inhibitors of ECM degradation proteins [23, 24]. We found a significant increase in the production of fibronectin, a component of ECM, in LNX-transfected cells following TGF- β 1 treatment, which is consistent with a more fibroblast-like phenotype. The PAI-1 level was also significantly elevated in LNX-transfected cells. Collectively, these results indicate that overexpressing LNX could sensitize HK-2 cells to TGF- β treatment and thus promote TGF- β 1-induced EMT.

One important observation in this study is that a proportion of exogenously expressed LNX is localized at regions of cell-cell contact where adhesion molecules such as E-cadherin are enriched. In line with our finding, endogenous LNXp70 was reported to localize at tight junction in polarized MDCK cells [18]. However, we were not able to identify a cell line that endogenously express LNXp80 protein at levels detectable by Western blot using our antibody, although LNXp80 transcript was detected in a

number of mouse tissues by Northern blot [15]. Moreover, our attempts to generate cell lines that stably express LNX invariably caused cell death. These observations imply that expression of LNX is tightly regulated in cells under normal conditions. We previously reported that LNX functions as an E3 ligase and mediates mammalian Numb protein degradation via ubiquitin-proteasome pathway [16]. Numb is expressed in early embryonic development and adult tissues in a ubiquitous manner [27, 28]. Previous studies revealed that Numb plays a critical role in neurogenesis and in maintaining cell polarity [29]. Recent study demonstrated that Numb functions as a tumor suppressor by preventing p53 degradation [30]. Taken together with the EMT-promoting effect of LNX revealed in present study, it is plausible to postulate that the restricted expression pattern of LNXp80 is crucial for maintaining cell morphology and cell function. Thus, dysregulation of LNXp80 expression could have a detrimental effect on cell polarity and proliferation, which would in turn initiate and/or promoted the pathological progression such as renal fibrosis and tumorigenesis. The mechanism by which LNX promotes TGF- β 1-induced EMT is currently under investigation, and several possibilities can be envisaged: (i) the distribution of LNX at areas of cell-cell contact as well as its dramatic inhibitory effect on E-cadherin expression imply that LNX may have a direct effect on cell adhesion molecules and overexpression of LNX may thus disturb the integrity of cellular junction. Extensive research in tumorigenesis and developmental biology has solidified the concept that intercellular contacts are not merely passive structure, but active regulators of EMT. Accordingly, loss of E-cadherin promotes EMT whereas forced E-cadherin expression

can restore the epithelial phenotype in transformed tumor cells [22, 31-33]. In agreement with this study, we found that overexpression of E-cadherin could inhibit the EMT augmented effect of LNX. The potential effect of LNX on the integrity of cell junction was supported by the finding that LNX interacts with JAM4 at tight junction and facilitates JAM4 endocytosis in MDCK cells [18]. In neuroepithelium, several adherens junction proteins, including E-cadherin, N-cadherin and catenins (α -E-catenin, -catenin) could be co-immunoprecipitated with endogenous Numb. Numb inactivation disrupts adherens junctions and polarity of radial glial cells [29]. It should be noted that Numb is required for all of the above interactions. While it is tempting to propose that LNX may regulate cell polarity and adhesion through Numb, our observation that a ligase-inactive mutant of LNX had the same effect as the wild type LNX in promoting TGF- β 1 induced EMT argues against this scenario, but support conferring this novel function to the PDZ domains of LNX. (ii) PDZ domain containing protein ZO-2 has been shown to localize in the nucleus and interacts directly with the DNA-binding protein SAF-B, which is proposed to be involved in transcriptional regulation [34]. Moreover, ZO-1 could translocate into nuclei of migrating epithelial cells [35]. We also observed the nucleus localization of LNX when it was overexpressed in HEK293 cells (data not shown). Thus, it is possible that LNX itself could act as a transcription regulator. The profound inhibitory effect of LNX on E-cadherin expression lends support to this notion.

In summary, our data provide the first evidence demonstrating that ectopic expression of LNX can promote TGF- β 1-induced EMT in human proximal tubular epithelial

cells manifested by inhibition of E-cadherin expression, induction of vimentin, fibronectin and PAI-1 expression, and enhanced cell migration. The effect of LNX on promoting EMT is independent of its E3 ligase activity. Identification of the region in LNX that is responsible for mediating this effect and its binding partners would help elucidate the molecular basis of LNX's role on EMT.

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Figure legends

Figure 1. (A-F) Subcellular distribution of LNX in HK-2 cells. Normal HK-2 cells (G, H, I) or HK-2 cells transfected with either FLAG-tagged wLNX (A, B, C) or C48ALNX (D, E, F) were co-stained with anti-LNX (green; A, D and G) and anti-E-cadherin (red; B, E and H). Merged images are shown in (C, F and I). Images were obtained by confocal microscopy. Magnification 600x. (J) Ectopic expression of LNX did not affect the protein level of epithelial and mesenchymal marker proteins in HK-2 cells in the absence of TGF- β 1. HK-2 cells transfected with empty pFLAG-CMV₂, FLAG-tagged wLNX or C48ALNX, respectively. 24h after transfection, cell lysates were harvested, quantitated and equivalent amounts of protein were resolved by SDS-PAGE and immunoblotted with indicated antibodies.

Figure 2. Ectopic expression of LNX promoted TGF- β 1 induced E-cadherin suppression. (A) HK-2 cells transfected with either empty pFLAG-CMV₂, or FLAG-tagged wLNX. 24h after transfection, cells were treated with indicated amount of TGF- β 1 for 24h. Cells lysates were blotted with antibody against E-cadherin. Membrane was then stripped and blotted with anti-LNX to verify LNX expression and anti-GAPDH to verify equivalent protein loading. Representative blot of three experiments. (B) Graphical presentation of the relative E-cadherin abundance normalized to GAPDH following TGF- β 1 treatment in either empty vector or wLNX transfected HK-2 cells. Data are means \pm SEM of three experiments. * P <0.05 vs vector transfected cells upon the same concentration of TGF- β 1 treatment. (C) Real

time RT-PCR analysis of E-cadherin expression in either empty vector or wLNX transfected HK-2 cells in the absence or presence of indicated amount of TGF- β 1 treatment for 24h. n=3, each sample assayed in triplicate. * P <0.05 vs TGF- β 1 0ng/ml; # P <0.05 vs empty vector transfected cells. (D) Real time RT-PCR analysis of Snail expression in either empty vector or wLNX transfected cells in the absence or presence of indicated amount of TGF- β 1 treatment for 24h. n=3, each sample assayed in triplicate. * P <0.05 vs TGF- β 1 0ng/ml;

Figure 3. Ectopic expression of LNX promoted TGF- β 1 induced vimentin production.

(A) HK-2 cells transfected with either empty pFLAG-CMV₂, or FLAG-tagged wLNX. 24h after transfection, cells were treated with indicated amount of TGF- β 1 for 24h. Cells lysates were blotted with antibody against vimentin. Membrane was then stripped and blotted with anti-LNX to verify LNX expression and anti-GAPDH to verify equivalent protein loading. Representative blot of three experiments. (B) Graphical presentation of relative vimentin abundance normalized to GAPDH following TGF- β 1 treatment in either empty vector or wLNX transfected HK-2 cells. Data are means \pm SEM of three experiments. * P <0.05 vs vector transfected cells upon the same concentration of TGF- β 1 treatment. (C) Real time RT-PCR analysis of vimentin expression in either empty vector or wLNX transfected HK-2 cells in the absence or presence of indicated amount of TGF- β 1 treatment for 24h. n=3, each sample assayed in triplicate. * P <0.05 vs TGF- β 1 0ng/ml; # P >0.05 vs empty vector

transfected cells.

Figure 4. Ectopic expression of LNX promoted TGF- β 1 induced fibronectin production (A) HK-2 cells transfected with either empty pFLAG-CMV₂, or FLAG-tagged wLNX. 24h after transfection, cells were treated with indicated amount of TGF- β 1 for 24h. Cells lysates were blotted with antibody against fibronectin. Membrane was then stripped and blotted with anti-LNX to verify LNX expression and anti-GAPDH to verify equivalent protein loading. Representative blot of three experiments. (B) Graphical presentation of relative fibronectin abundance normalized to GAPDH following TGF- β 1 treatment in either empty vector or wLNX transfected HK-2 cells. Data are means \pm SEM of three experiments. * P <0.05 vs vector transfected cells upon the same concentration of TGF- β 1 treatment. (C-J) Immunofluorescence evidence of wLNX promoted TGF- β 1 induced fibronectin production. HK-2 cells transfected with either empty pFLAG-CMV₂, or FLAG-tagged wLNX. 24h after transfection, cells were treated with 0.1ng/ml TGF- β 1 for 24h. Cells were co-stained with anti-LNX and goat anti-rabbit antibody conjugated to AlexaFluor488 to detect LNX (green; E and I) and anti-fibronectin was detected by goat anti-mouse antibody conjugated to CY3 (red; C and G). Nuclei were stained with DAPI (blue; D and H). Merged images are shown in (F) and (J). Images were obtained by confocal microscopy. Magnification 600x.

Figure 5. Ectopic expression of LNX promoted TGF- β 1 induced PAI-1 production.

Western blot analysis of PAI-1 expression in either empty vector or wLNX transfected cells in the absence or presence of indicated amount of TGF- β 1 treatment for 24h. Cells lysates were blotted with antibody against PAI-1. Membrane was then stripped and blotted with anti-LNX to verify LNX expression and anti-GAPDH to verify equivalent protein loading. Representative blot of three experiments. (B) Graphical presentation of relative Fibronectin abundance normalized to GAPDH following TGF- β 1 treatment in either empty vector or wLNX transfected HK-2 cells. Data are means \pm SEM of three experiments. * P <0.05 vs vector transfected cells upon the same concentration of TGF- β 1 treatment.

Figure 6. Forced expression of LNX enhances TGF- β 1 induced migration of tubular epithelial cells. HK-2 cells were initially cultured on 35mm dishes and transfected with FLAG-tagged wLNX or vector, and then incubated in the absence or presence of 0.1 ng/ml of TGF- β 1 for 24h. (A-B) showed morphological changes of cells after TGF- β 1 treatment. Photographs were taken using a Nikon microscope (phase contrast). Magnification 200x. A, empty pFLAG-CMV₂ transfected cells; B, FLAG-tagged wLNX transfected cells; For Boyden chamber motility assay, Vector (C) or LNX-overexpressing (D) cells (1×10^4) were resuspended and seeded onto the filters in the top compartment of the chamber and incubated for 10h. Cells and cell extensions that migrated through the pores of Transwell plates were counted and reported (E). * P < 0.05 vs vector transfected cells, n = 3.

Figure 7. LNX promoting TGF- β 1 induced EMT is not dependent on its E3 ligase activity. HK-2 cells were transfected with empty pFLAG-CMV₂, FLAG-tagged wLNX or C48ALNX, respectively. 24h after transfection, cells were further treated with 0.1ng/ml TGF- β 1 for 24h and then cell lysates were harvested for Western blot analysis by using indicated antibodies (A). For Boyden chamber motility assay, wLNX (B) or C48ALNX (C) transfected cells (1×10^4) were resuspended and seeded onto the filters in the top compartment of the chamber and incubated for 10h. Cells and cell extensions that migrated through the pores of Transwell plates were counted and reported (D). * $P > 0.05$ vs wLNX transfected cells, $n = 3$.

Figure 8. Overexpression of E-cadherin inhibited LNX-augmented EMT. (A) HK-2 cells were transfected with empty vector, LNX or LNX together with E-cadherin, respectively. 24h after transfection, cells were further treated with 0.1ng/ml TGF- β 1 for 24h and then cell lysates were harvested for Western blot analysis using indicated antibodies. (B) For Boyden chamber motility assay, LNX-transfected cells or LNX and E-cadherin co-transfected cells (1×10^4) were resuspended and seeded onto the filters in the top compartment of the chamber and incubated for 10h. Cells and cell extensions that migrated through the pores of Transwell plates were counted and reported. * $P < 0.05$ vs LNX-transfected cells, [#] $P > 0.05$ vs vector transfected cells, $n = 3$.

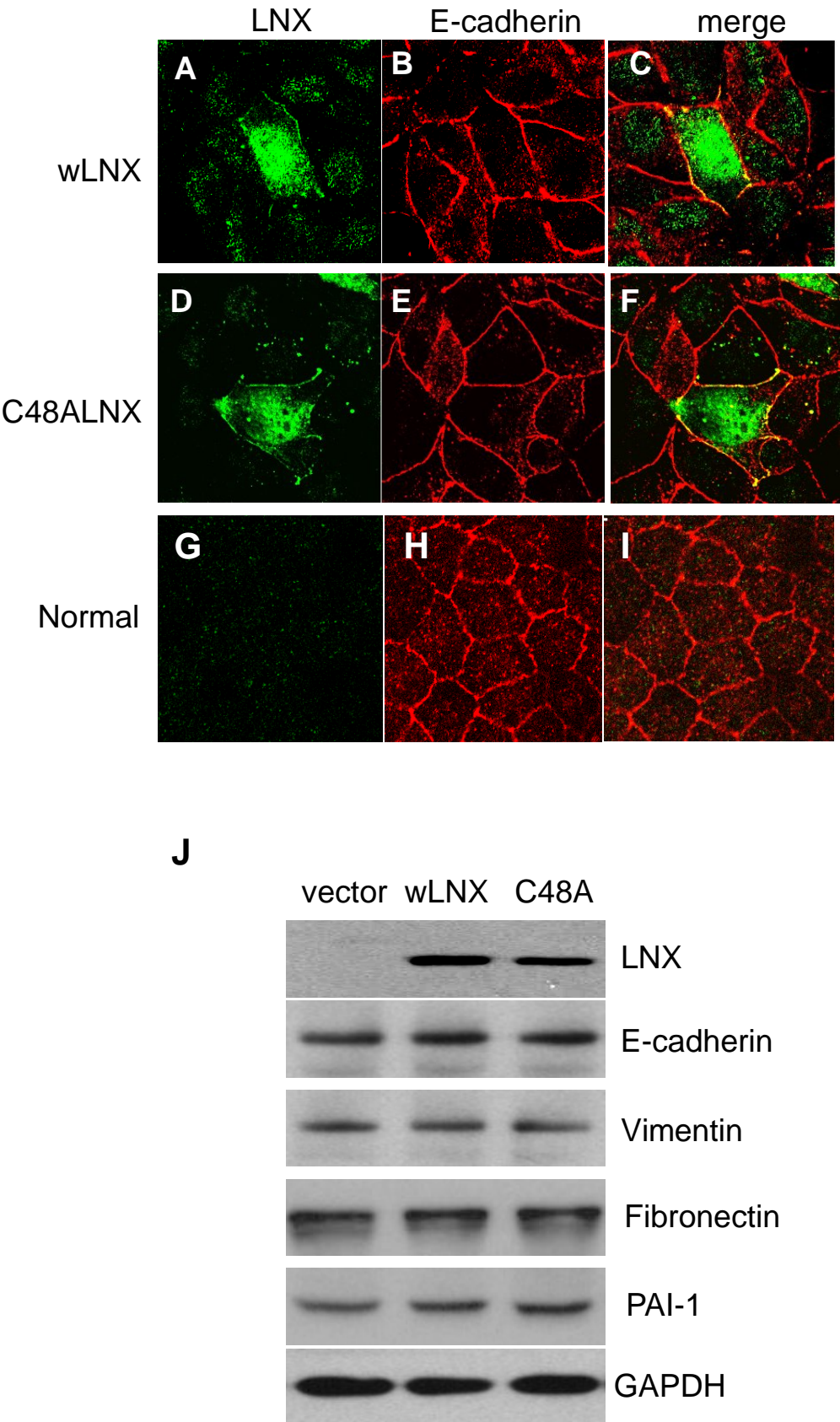
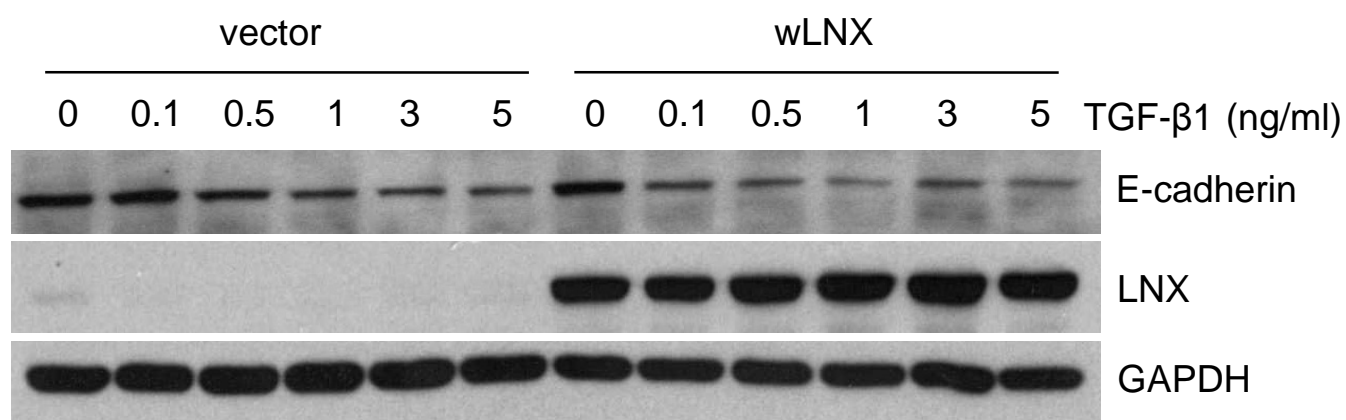
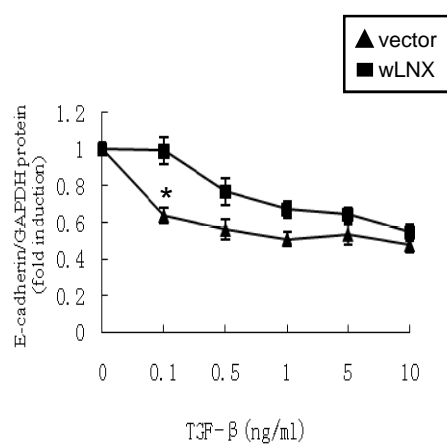


Figure 1

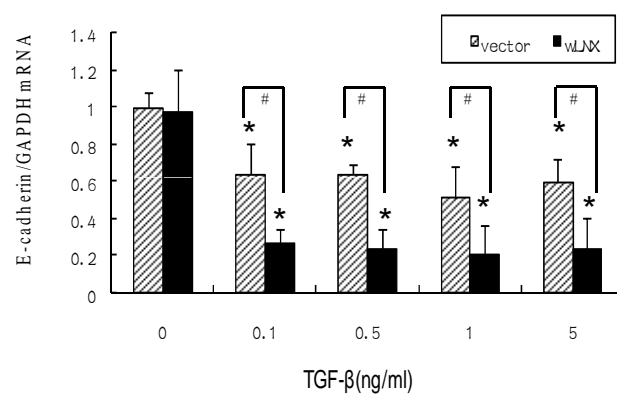
A



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C



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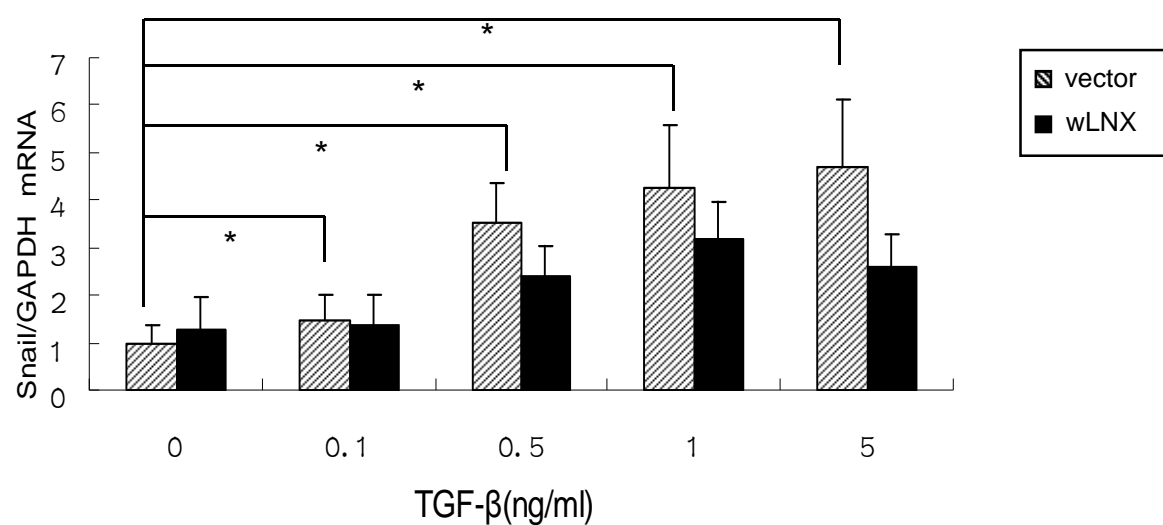
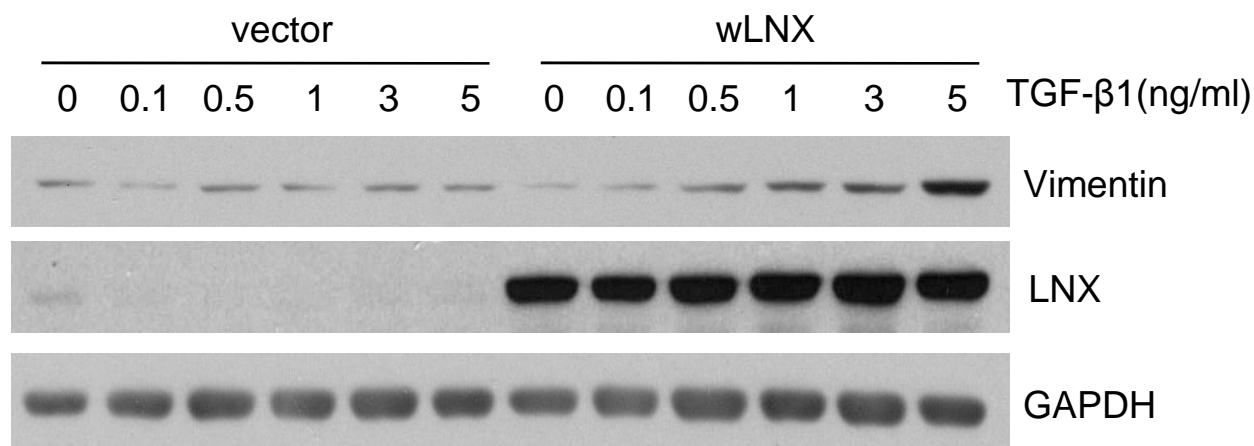
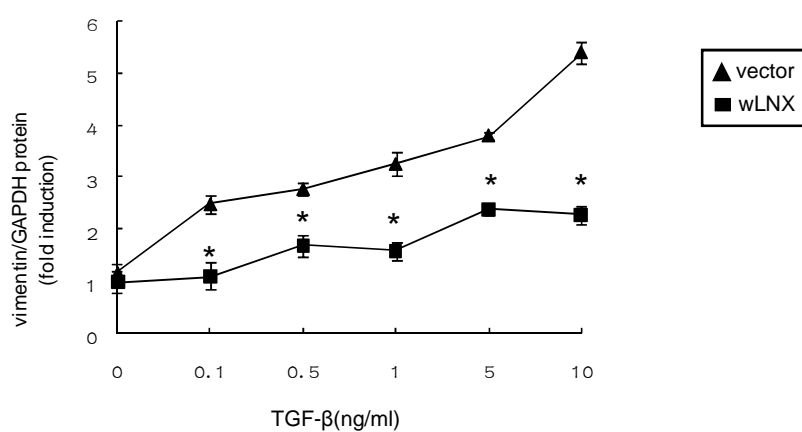


Figure 2

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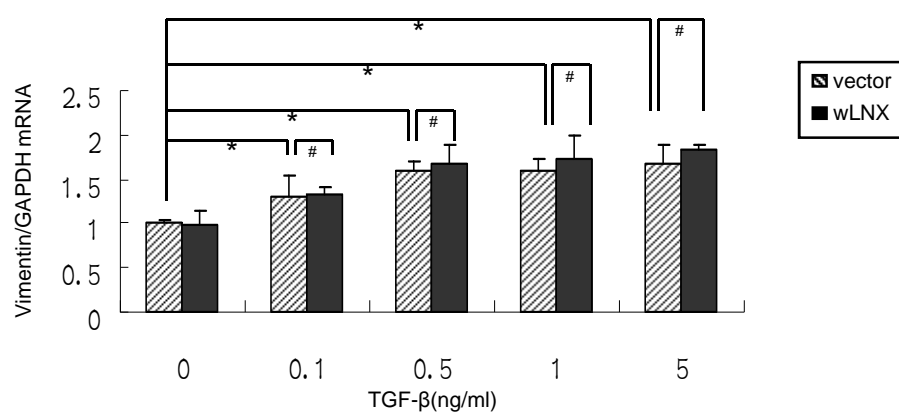


Figure 3

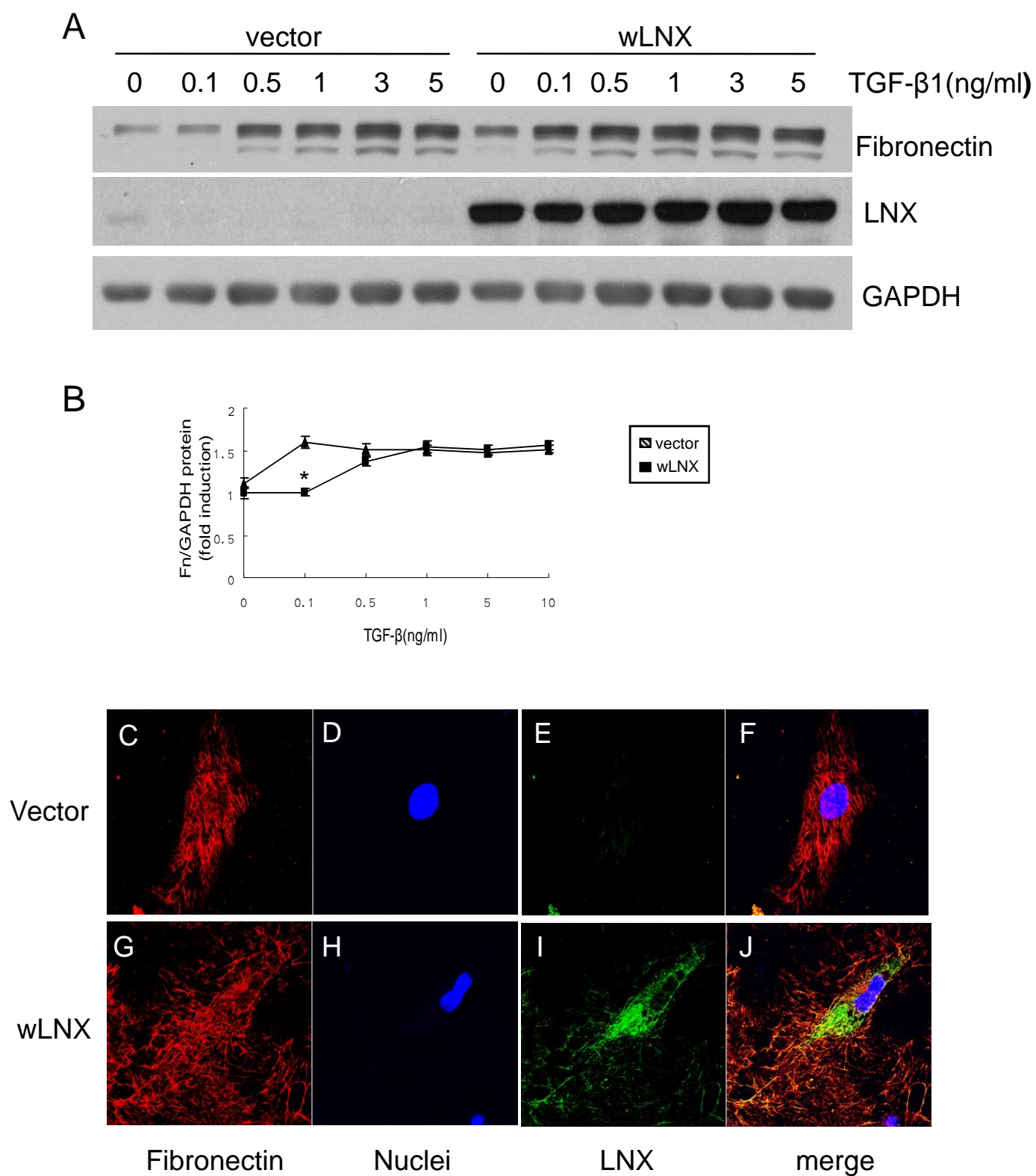
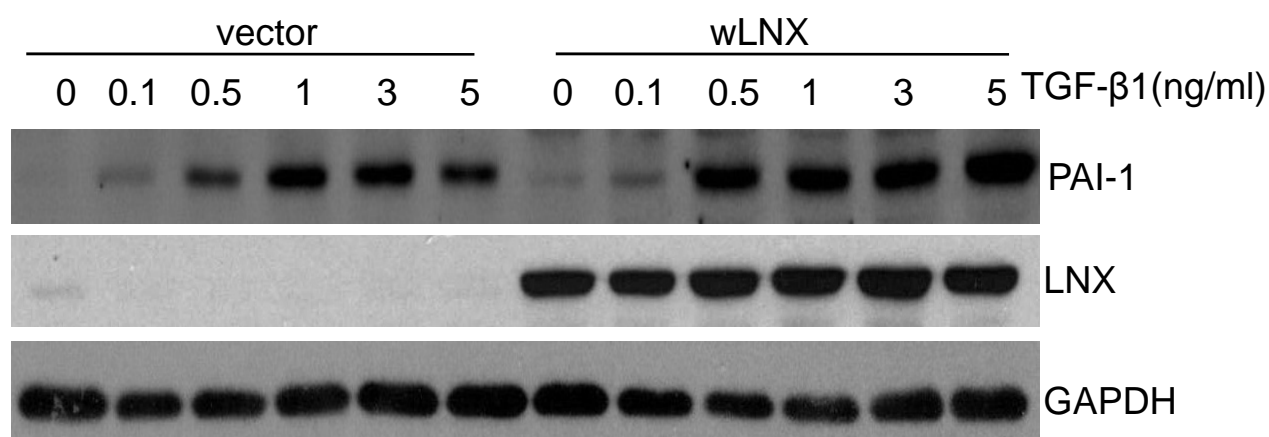


Figure 4

A



B

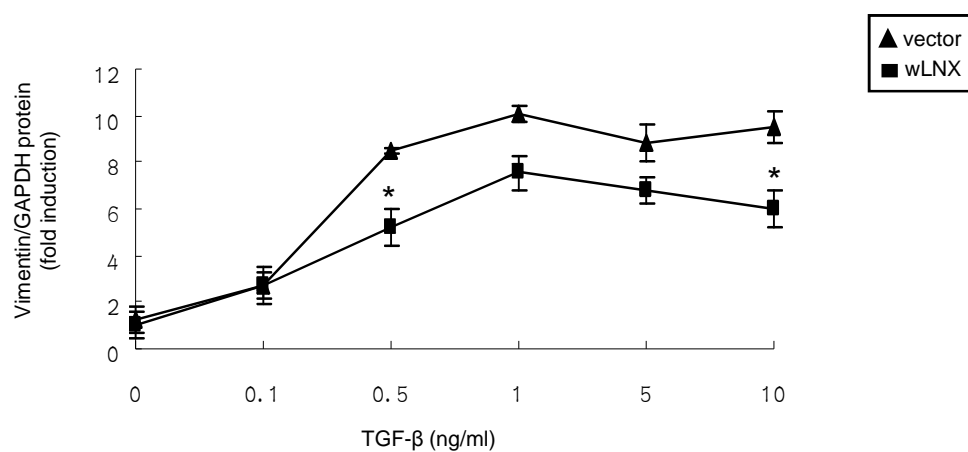


Figure 5

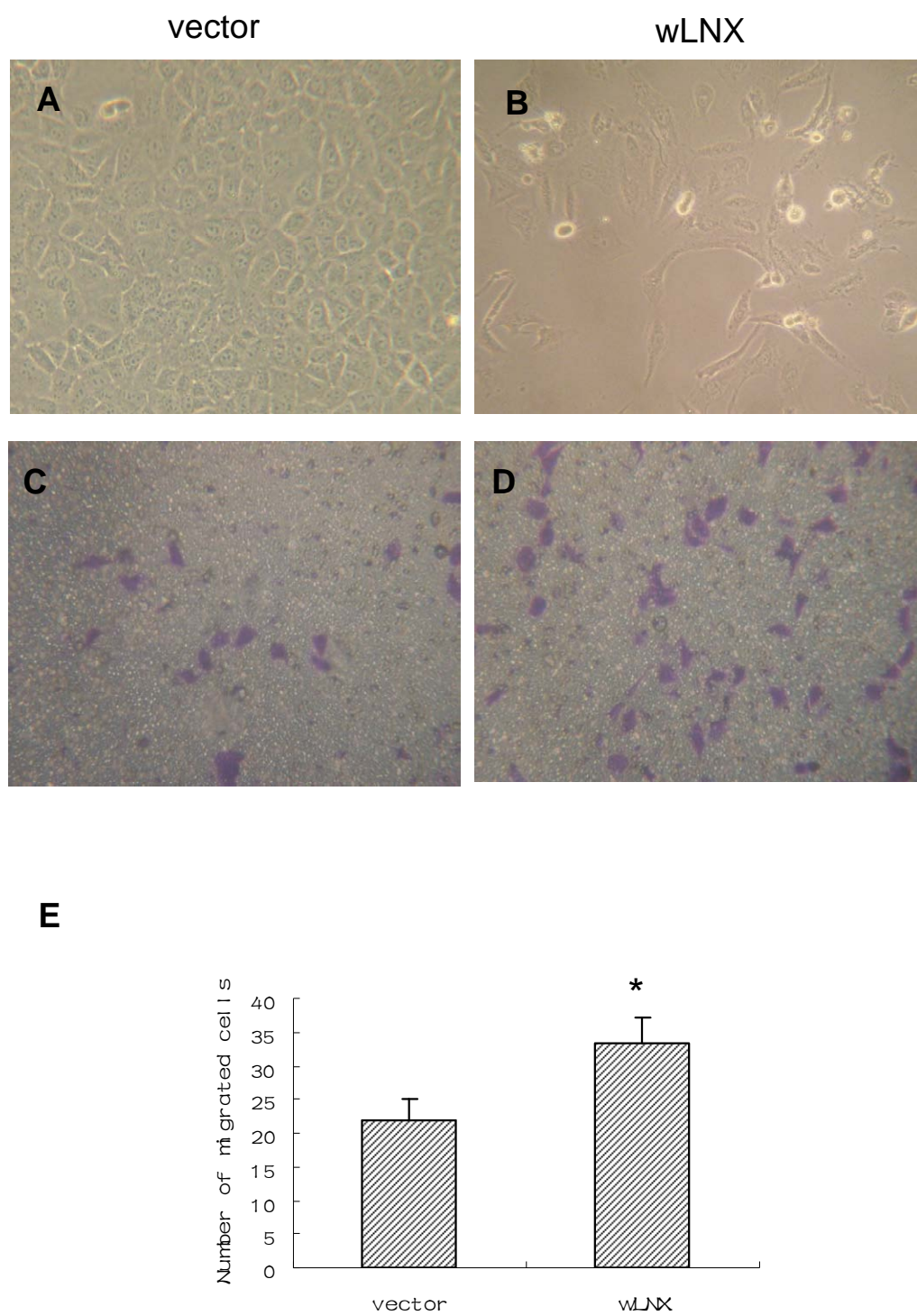


Figure 6

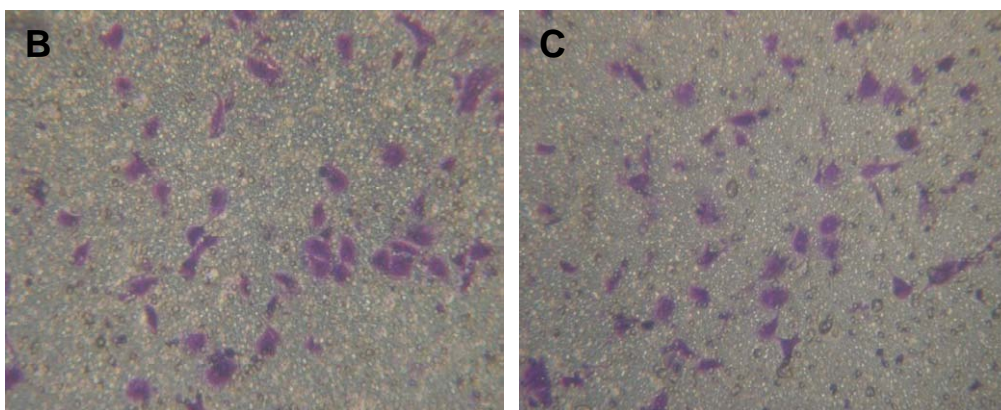
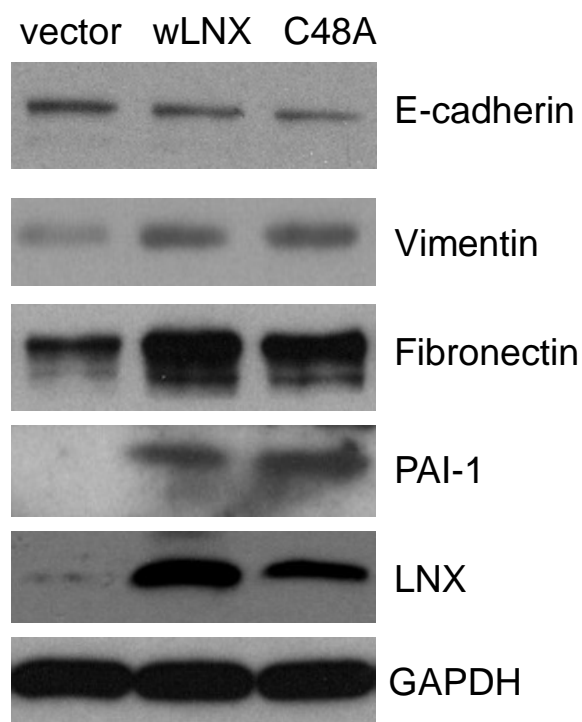
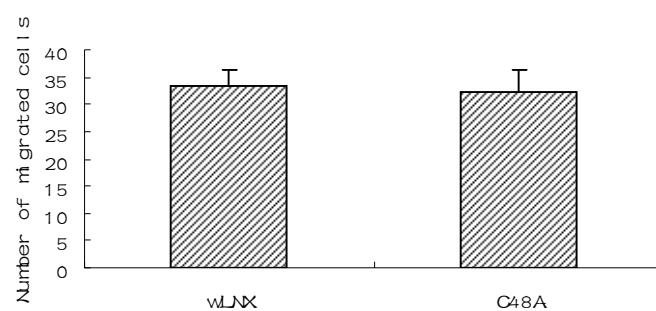
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Figure 7

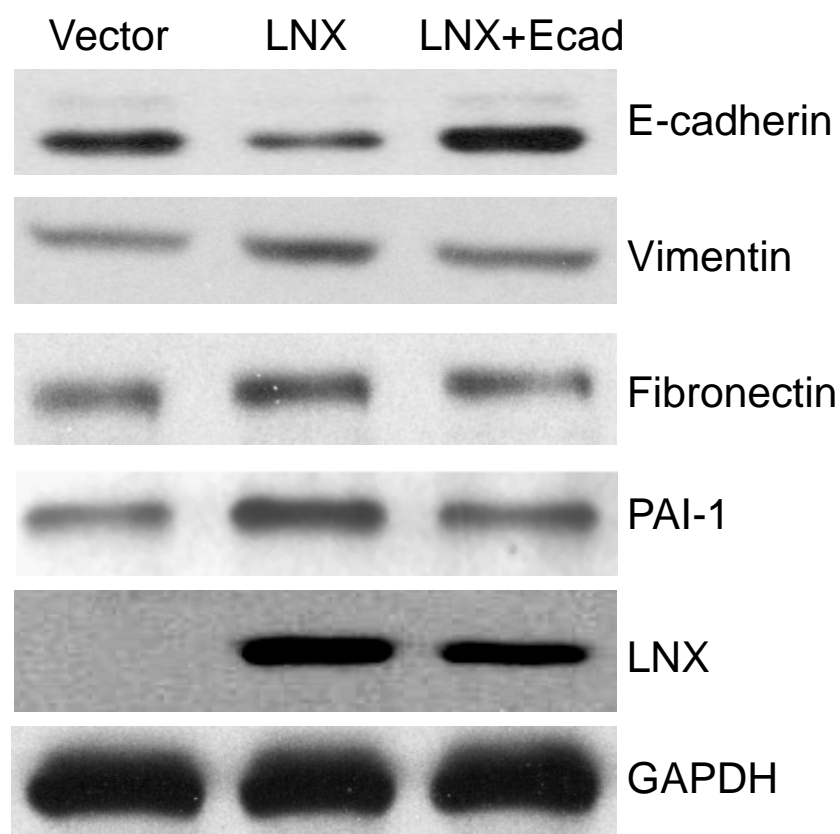
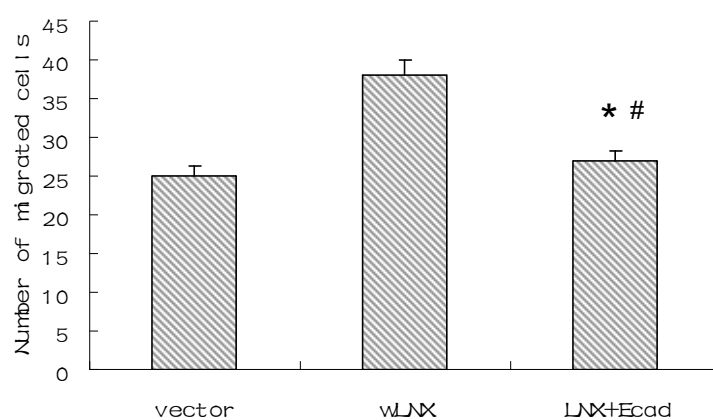
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Figure 8