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Type I collagen promotes epithelial-mesenchymal transition through ILK-dependent activation of NF-κB and LEF-1

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Abstract

Collagen I has been shown to promote epithelial-mesenchymal transition (EMT), a critical process of embryonic development and disease progression. However, little is known about the signaling mechanisms by which collagen I induces this cellular transformation. Here we show that collagen I causes ILK-dependent phosphorylation of IκB and subsequent nuclear translocation of active NF-κB, which in turn promotes increased expression of the Snail and LEF-1 transcription factors. ILK also causes inhibitory phosphorylation of GSK-3β, a kinase that prevents functional activation of both Snail and LEF-1. These transcription factors alter expression of epithelial and mesenchymal markers to initiate EMT and stimulate cell migration. These data provide a foundation for understanding the mechanisms by which collagen I stimulates EMT and identify potential therapeutic targets for suppressing this transition in pathological conditions.

Keywords

EMT; collagen I; ILK; NF-κB; LEF-1; epithelial-mesenchymal transition

Introduction

Epithelial-mesenchymal transition is a fundamental mechanism of embryogenesis (Hay, 1995) that is normally dormant in the adult organism, but can be triggered to induce pathological conditions such as organ fibrosis (Kalluri and Neilson, 2003) and tumor metastasis (Thiery, 2002). While commonly initiated by growth factors such as TGF-β (Zavadil and Bottinger, 2005), extracellular matrix molecules have also been implicated in causing this phenomenon. The most prominent of these is collagen I, which has been linked to transformation of embryonic epithelium to mesenchyme (Hay and Zuk, 1995), as well as induction of EMT in lung (Shintani et al., 2007), breast (Gilles et al., 1997), and pancreatic (Menke et al., 2001; Koenig et al., 2006; Imamichi et al., 2007) carcinoma cells. Furthermore, in vivo EMT is perturbed in *COL1A1* knockout mice, particularly during craniofacial

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development (Lavrin et al., 2001). Also, collagen I is highly expressed in metastatic tumors (van Kempen et al., 2003), providing an in vivo basis for its function in stimulating pathologic EMT. It has been established that this cellular transformation occurs through interaction of collagen I fibrils with α2β1 integrin (Valles et al., 1996), yet little is known about the downstream signaling mechanisms by which this interaction causes EMT.

Activation of transcription factors that suppress cell-cell adhesion is essential for initiating EMT (Thiery, 2003). Members of the Snail family of transcriptional repressors have been shown to directly bind and suppress expression of genes encoding adherens junction and tight junction proteins such as E-cadherin, claudins, occludin, etc. (Peinado, et al., 2004; Medici et al., 2006). LEF-1, a member of the T-cell factor (TCF) family of transcriptional regulators, has also been shown to directly inhibit expression of the *E-cadherin* gene (Jamora et al., 2003) and promote EMT (Kim et al., 2002; Nawshad and Hay, 2003; Nawshad et al., 2007; Medici et al., 2008). It has recently been found that both Snail and LEF-1 have unique and essential roles in disrupting cell-cell adhesion (Medici et al., 2006).

Here we identify a signaling mechanism by which these factors cooperate to induce EMT upon cellular stimulation with collagen I. Our data suggest that this signaling is mediated by integrin linked kinase (ILK), a substrate for β1 integrin that regulates EMT (Hannigan et al., 2005), which increases phosphorylation of Akt, GSK- 3β, and IκB. These phosphorylations cause functional activation of the NF-κB, Snail, and LEF-1 transcription factors, which work in unison to induce EMT. NF-κB mediates EMT signaling by increasing expression of both Snail and LEF-1, which in turn reduces E-cadherin expression and promotes synthesis of the mesenchymal marker vimentin, thus stimulating EMT and cell migration.

Results and discussion

To investigate the role of ILK in collagen I-dependent EMT, we assessed phosphorylation levels of potential primary targets for ILK activity in human pancreatic carcinoma (PANC-1), colon carcinoma (DLD1), and normal kidney proximal tubule epithelial (HK-2) cells. Immunoblotting showed increased activating phosphorylation of Akt, and inhibitory phosphorylation of IκB and GSK-3β in cells grown on type I collagen compared those grown on laminin (negative control). These increases were prevented by the presence of ILK siRNA (Fig. 1). Inhibitory phosphorylation of IκB should permit NF-κB function, so we conducted immunocytochemistry showing that cells under control conditions lacked nuclear NF-κB, but those grown on type I collagen had strong nuclear localization of NF-κB and a distinct change in cell morphology (Fig. 2A). pGL3-NF-κB-Lux (containing NF-κB binding sites) reporter assays showed that NF-κB transcriptional activity was significantly higher in cells grown on collagen I than in control cells. These increases in NF-κB activity were inhibited with ILK siRNA (Fig. 2B).

Immunoblotting showed that expression of the Snail and LEF-1 transcription factors was greatly increased in cells grown on type I collagen, compared to control cells. These up-regulations are NF-κB-dependent, as NF-κB siRNA prevented collagen I-induced increases in Snail and LEF-1 expression (Fig. 3A). To measure β-catenin–LEF-1 transcriptional activity we performed pTOPFLASH-Lux (containing LEF-1 binding sites) reporter assays. We found that cells grown on collagen I had significantly higher β-catenin–LEF-1 activity than control cells. These levels were reduced to background upon exposure to ILK siRNA or NF-κB siRNA (Fig. 3B).

To assess EMT we observed gene expression levels of the epithelial marker *E-cadherin* and the mesenchymal marker *vimentin* by real-time quantitative PCR. We found that *E-cadherin* was greatly reduced, while *vimentin* was increased in cells grow on type I collagen. These

changes were prevented in these cells upon exposure to Snail siRNA or LEF-1 siRNA. Interestingly, while LEF-1 siRNA had no effect on *Snail* expression, Snail siRNA prevented collagen I-induced up-regulation of *LEF-1* (Fig. 4A). These data are supported by recent evidence suggesting a regulatory role for Snail in mediating *LEF-1* expression (Medici et al., 2008). To detect post-EMT activity, we performed transwell migration assays showing that cells seeded on type I collagen had higher levels of migration than control cells seeded on laminin. The presence of Snail siRNA or LEF-1 siRNA prevented collagen I-induced migration (Fig. 4B).

These data provide a foundation for understanding the signaling mechanisms that control collagen I-induced EMT. Collagen I fibrils interact with α2β1 Integrin (Valles et al., 1996) causing ILK-induced phosphorylation of Akt, IκB, and GSK-3β. The inhibitory phosphorylation of IκB causes release of functional NF-κB, which enters the nucleus to aid in transcription of target genes such as *Snail* and *LEF-1* . Inhibitory phosphorylation of GSK-3β (by Akt and ILK) is essential for functional activation of NF-κB, Snail, and LEF-1 (Lustig and Behrens, 2003; Zhou et al., 2004; Bachelder et al., 2005). Snail is known to repress *E-cadherin* transcription (Peinado et al., 2004), a process that reduces the membrane substrate for β-catenin, a binding activator for LEF-1. While LEF-1 can also reduce *E-cadherin* levels (Jamora et al., 2003), it promotes transcription of EMT target genes such as *vimentin* .

Our results provide the basis for understanding how collagen I promotes the invasive, metastatic phenotype in cancer cells and fibrogenesis in normal epithelial cells. This mechanism unifies the function of several key signaling molecules and transcription factors in causing EMT. Knowledge of these pathways may provide therapeutic targets for treatment of pathologic conditions and insight into understanding EMT in other systems.

Experimental procedures

Cell culture

PANC-1, DLD1, and HK-2 cells were acquired from the American Tissue Culture Collection (ATCC). Characterization of the physiology and ability of these cells to undergo EMT have been previously documented (Menke et al., 2001; Docherty et al., 2005; Medici et al., 2008). Cells were grown in culture with DMEM (for PANC-1), RPMI 1640 (for DLD1) or K-SFM + bovine pituitary extract + EGF (for HK-2) [Gibco] + 10% FBS + 1% Penicillin/Streptomycin. FBS and supplemental growth factors were removed 24 hours prior to all experimental conditions. Cells were grown on 250 ng/ml type I collagen (BD Biosciences) for all relative experiments. Cells grown on 250 ng/ml standard basement membrane laminin (Sigma) were used as negative controls. Protein phosphorylation was assessed 15 minutes after cell adherence on the culture dish (1 hour after addition of cells) was observed. Snail and LEF-1 expression were measured 24 hours post attachment, while EMT and migration were observed 48 hours after adherence. All experiments for this study were performed in triplicate.

RNA interference

siRNA gene expression knockdown studies were performed using the TriFECTa RNAi kit (IDT) and corresponding protocol. Each 27mer RNAi duplex was transfected into cells using X-tremeGene siRNA transfection reagent (Roche) following the manufacturer's guidelines. siRNA was synthesized (IDT) using the following sequences: **ILK:** 5′-ACGGCUAUUGAGUGCAUGUCGUGGGAU-3′; **NF-κB:** 5′-CUAAUUUCUGCGCCAGAGUAGCCCA-3′; **Snail:** 5′-CCACAGAAAUGGCCAUGGGAAGGCCUC-3′; **LEF-1:** 5′-CCGGGAUUUGCGCGCGGAGAACGCCGG-3. For all relative control experiments, cells

were exposed to a scrambled non-specific siRNA duplex with the following sequence: 5′-CACAAGGGAGAGAAAGAGAGGAAGGA-3′.

Immunoblotting and immunocytochemistry

Immunofluorescence and Western blotting were performed using the following antibodies at concentrations and using protocols recommended by the respective manufacturers: ILK, Phospho-IκB, IκB, NF-κB (p50), SNAI1, LEF-1, E-cadherin (Santa Cruz Biotechnology), Phospho-Akt, Akt, Phospho-GSK-3β, GSK-3β (Cell Signaling Technology), β-actin, vimentin (Sigma). HRP-conjugated secondary antibodies (Calbiochem) were used at a dilution of 1:5000. Rhodamine-conjugated secondary antibodies (Roche) were used at a dilution of 1:250. Images were acquired using a Nikon 80i fluorescence microscope.

Reporter gene assays

Luciferase reporter gene assays were conducted using the Luciferase Assay System (Promega) and its corresponding protocol. All plasmids (500 ng) were transfected into cells using Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer's guidelines. Light units were measured with a Luminometer TD-20/20 (Turner Designs). Assays were normalized for transfection efficiency by cotransfecting cells with a β-galactosidase (β-gal) control plasmid and were detected with the Luminescent β-gal control assay kit (Clontech). Experimental (luciferase) results were divided by the β-gal results to provide normalized data. The pGL3-NF-κB-Lux plasmid was provided by A. Rao (Harvard Medical School, Boston, MA, USA). The pTOPFLASH-Lux reporter construct was provided by H. Clevers (Hubrecht Laboratory, Utrecht, The Netherlands). Control cells were transfected with a pGL3 empty vector plasmid (Promega).

Transwell migration assays

Migration was assessed using the Innocyte Cell Migration Assay Kit (EMD Biosciences). Cells migrated towards 10% serum into the lower chambers. Migrated cells in the lower chamber were stained with a Calcein-AM fluorescent dye. Excitation max (485 nm)/emission max (520 nm) was assessed using a fluorescent plate reader (BD FACSArray bioanalyzer).

Real-time quantitative PCR

RNA extractions were performed using the RNeasy Mini kit (Qiagen) and protocol. Samples were submitted to a core facility (Biopolymers Facility: Harvard Medical School: Department of Genetics) where real-time PCR experiments were conducted using the Syber Green PCR system (ABI) on an ABI 7500 cycler, with 40 cycles per sample. Cycling temperatures were as follows: denaturing 95°C; annealing and extension, 60°C. The following primers were used: ***Snail***: Forward: 5′-ACCACTATGCCGCGCTCTT-3′; Reverse: 5′-GGTCGTAGGGCTGCTGGAA-3′; ***LEF-1***: Forward: 5′-CCGAAGAGGAAGGCGATTTAGC-3′; Reverse: 5′-GGTCCCTTGTTGTAGAGGCC-3′; ***vimentin***: Forward: 5′- TCTACGAGGAGGAGATGCGG-3′; Reverse: 5′-GGTCAAGACGTGCCAGAGAC-3′; ***E-cadherin***: Forward: 5′-GTCAGTTCAGACTCCAGCCC-3′; Reverse: 5′-AAATTCACTCTGCCCAGGACG-3′; ***GAPDH***: Forward: 5′-ACCACAGTCCATGCCATCAC-3′; Reverse: 5′-TCCACCCTGTTGCTGTA-3′.

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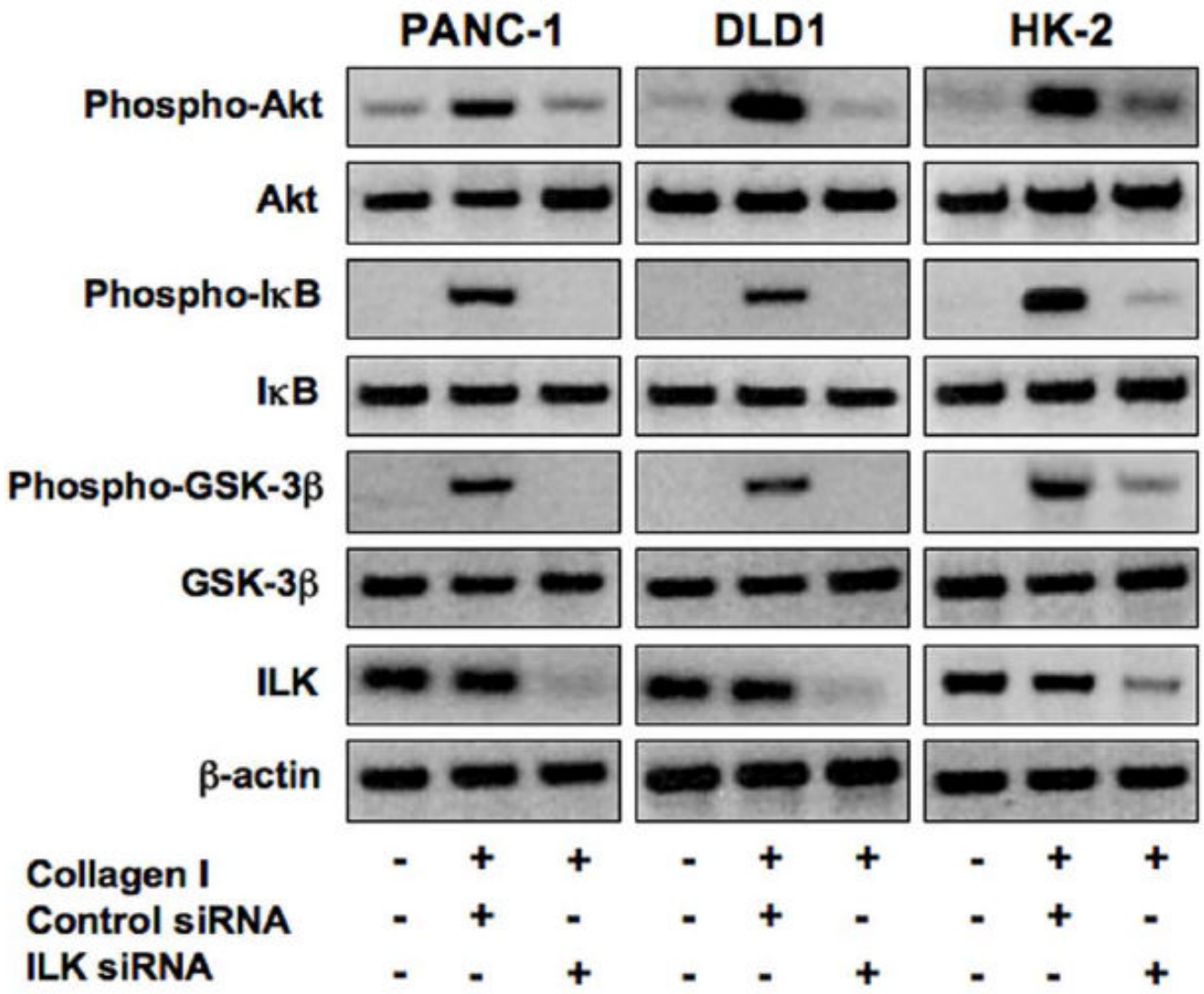

Fig. 1.

Signaling events induced by type I collagen. Immunoblotting showing that collagen I increases phosphorylation of Akt, IκB, and GSK-3β. These phosphorylations are inhibited by exposure to ILK siRNA. β-actin was used as an internal control.

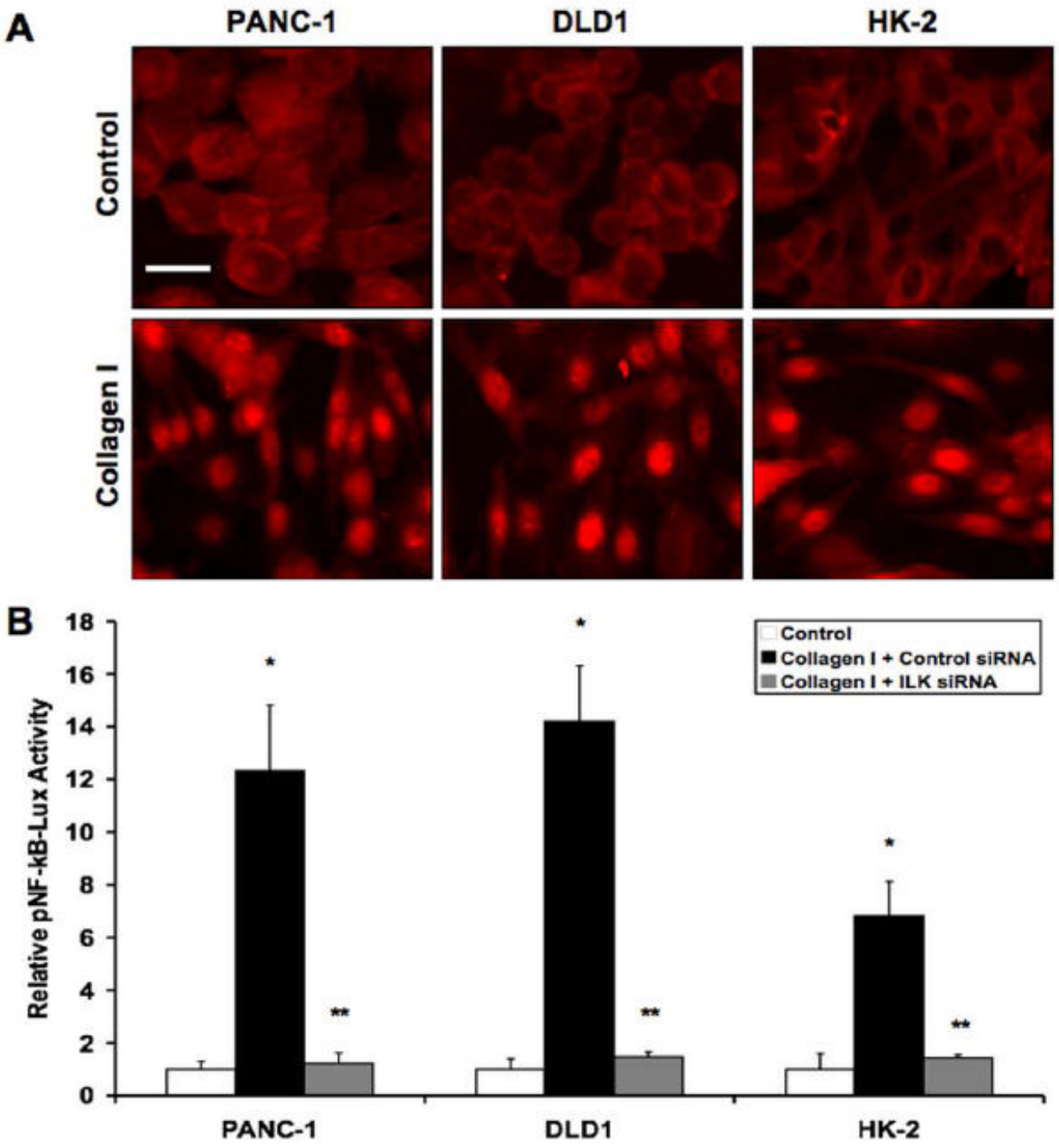

Fig. 2.

Collagen I causes ILK-dependent activation of NF-κB. A) Immunocytochemistry showing collagen I-induced changes in cell morphology and nuclear localization of NF- κB. Bar, 10μm. B) pGL3-NF-κB-Lux reporter gene assay demonstrating increased activity of the NF-κB transcription factor by seeding cells on collagen I. ILK siRNA inhibits this activity. Data represent mean (n=3) ± S.D., **P*<0.001 compared to control; ***P*<0.01 compared to collagen I + control siRNA.

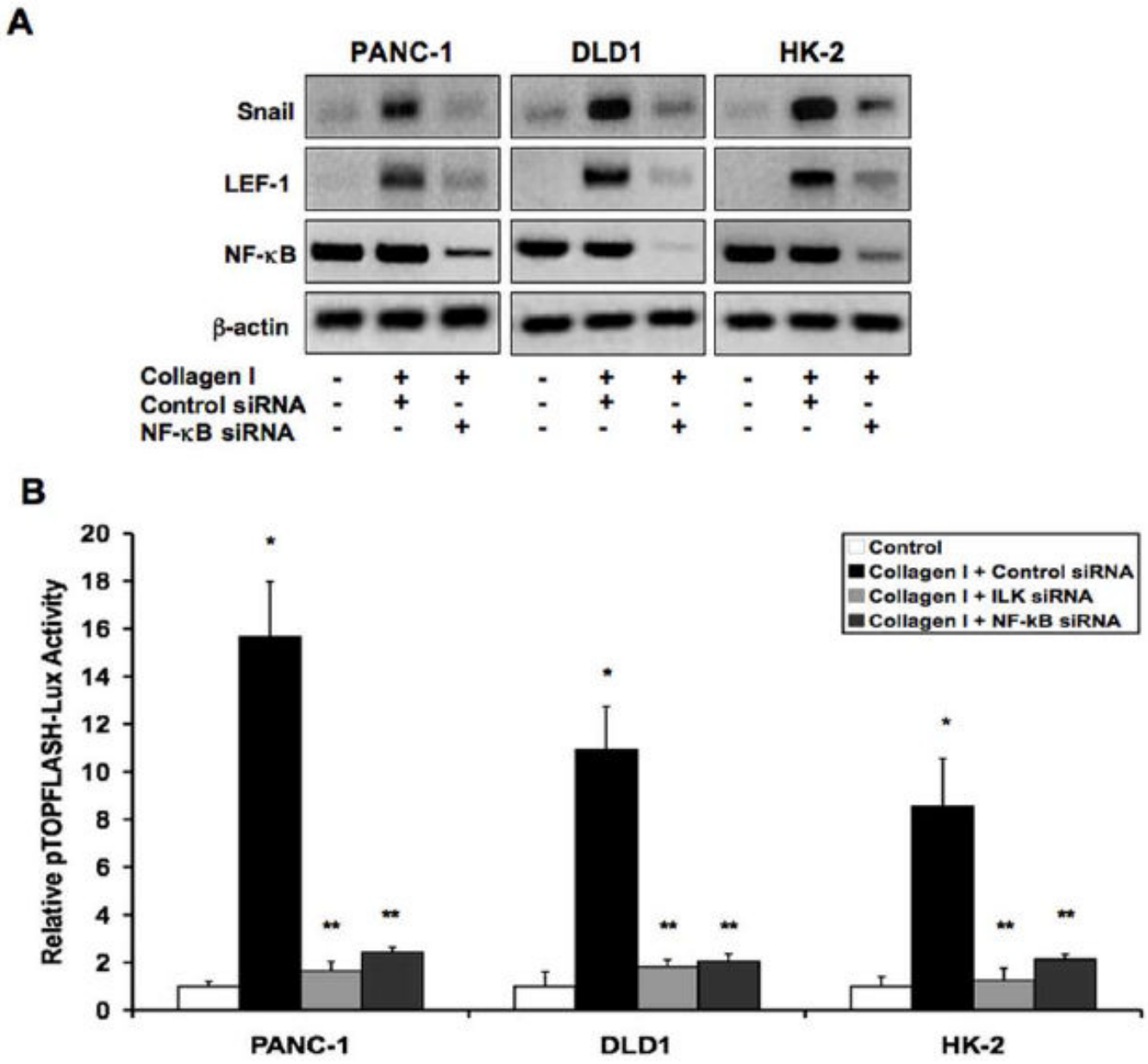

Fig. 3.

NF-κB increases expression of Snail and LEF-1 transcription factors. A) Immunoblotting showing that collagen I-induced up-regulation of Snail and LEF-1 is NF- κB-dependent. β-actin was used as a loading control. B) pTOPFLASH-Lux reporter gene analysis demonstrating that β-catenin-LEF-1 transcriptional activity is increased by seeding cells on collagen I. Inhibition of NF-κB prevents these increases. Data represent mean (n=3) ± S.D., **P*<0.001 compared to control; ***P*<0.001 compared to collagen I + control siRNA.

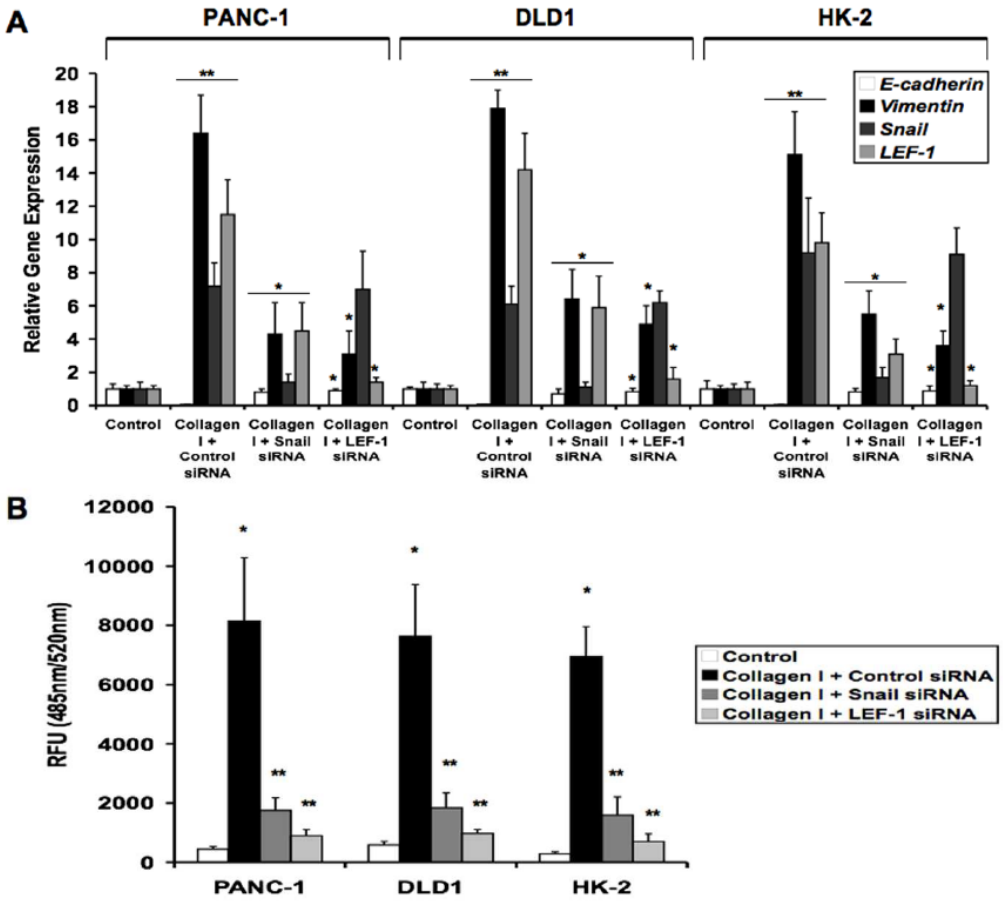

Fig. 4.

Snail and LEF-1 are essential for collagen I-induced EMT. A) Real-time quantitative PCR showing collagen I-induced EMT and that loss of *E-cadherin* and gain of *vimentin* expression is Snail and LEF-1-dependent. Data represent mean (n=3) ± S.D., **P* <0.001 compared to collagen I + control siRNA; ***P* <0.01 compared to control. Horizontal lines denote all underlying graphic data as statistically significant. B) Cell migration assays confirming that collagen I-induced increases in cell migration are Snail- and LEF-1-dependent. Data represent mean (n=3) ± S.D., **P* <0.001 compared to control; ** *P* <0.01 compared to collagen I + control siRNA.