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Discoidin domain receptor 2 is a critical regulator of epithelial-mesenchymal transition

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Abstract

Discoidin domain receptor 2 (DDR2) is a collagen receptor that is expressed during epithelial-mesenchymal transition (EMT), a cellular transformation that mediates many stages of embryonic development and disease. However, the functional significance of this receptor in EMT is unknown. Here we show that Transforming Growth Factor-beta1 (TGF- β 1), a common stimulator of EMT, promotes increased expression of type I collagen and DDR2. Inhibiting expression of COL1A1 or DDR2 with siRNA is sufficient to perturb activity of the NF- β B and LEF-1 transcription factors and to inhibit EMT and cell migration induced by TGF- β 1. Furthermore, knockdown of DDR2 expression with siRNA inhibits EMT directly induced by type I collagen. These data establish a critical role for type I collagen-dependent DDR2 signaling in the regulation of EMT.

Keywords

EMT; DDR2; collagen; TGF-beta; LEF-1; epithelial-mesenchymal transition

Introduction

Epithelial-mesenchymal transition is a cellular transformation that controls several stages of embryonic development including gastrulation, neural crest and somite dissociation, and craniofacial tissue fusion (Hay, 1995). It is also a primary mechanism of tissue repair and disease progression, mediating such processes as wound healing, organ fibrosis, and cancer metastasis (Thiery, 2003; Kalluri and Neilson, 2003). EMT is characterized by changes in cell polarity and expression of biochemical markers. Expression of epithelial markers such as E-cadherin and cytokeratins decreases while mesenchymal markers such as vimentin, FSP1, and N-cadherin increase (Kalluri and Weinberg, 2009). Cells that undergo EMT are highly invasive and migratory (Thiery, 2002). EMT is usually initiated by growth factors such as TGF- β 1 (Akhurst and Derynck, 2001; Zavadil and Bottinger, 2005).

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Type I collagen has been shown to be essential for embryonic EMT (Hay and Zuk, 1995). Craniofacial EMT is severely inhibited in *COL1A1* knockout mice (Lavrin et al., 2001). Type I collagen also directly stimulates EMT of 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, type I collagen is highly expressed in metastatic tumors (van Kempen et al., 2003), and EMT is the primary mechanism of metastasis (Thiery, 2002). We previously reported that type I collagen stimulates EMT by increasing NF- κ B transcription factor activity to increase expression of the transcription factor LEF-1 (Medici and Nawshad, 2010), which acts to inhibit cell-cell adhesion and promote EMT (Jamora et al., 2003; Medici et al., 2006; Nawshad et al., 2007; Medici et al., 2008).

DDR2 is a receptor tyrosine kinase that binds to type I, type II, type III, and type X collagen (Leitinger and Kwan, 2006; Carafoli et al., 2009). The downstream signaling pathways regulated by DDR2 are unclear, but DDR2 is known to increase expression of matrix metalloproteinases and may have an essential role in causing osteoarthritis (Xu et al., 2010). Previous studies have shown that expression of DDR2 increases during epithelial-mesenchymal transition (Maeyama et al., 2008; Goldsmith et al., 2010). A similar collagen receptor termed discoidin domain receptor 1 (DDR1) has been determined to have an essential role in type I collagen-induced EMT (Shintani et al., 2008). However, nothing is known regarding potential function of DDR2 in promoting EMT.

Since type I collagen stimulates EMT (Menke et al., 2001; Medici and Nawshad, 2010) and DDR2 is a receptor for type I collagen (Carafoli et al., 2009) that is up-regulated during EMT (Maeyama et al., 2008; Goldsmith et al., 2010), we hypothesize that DDR2 may have a critical role in the signaling mechanisms that mediate this process.

Results and discussion

To assess the effects of TGF- β 1 on type I collagen and DDR2 expression we treated human renal proximal tubule epithelial cells (HK-2) with recombinant TGF- β 1 for 8 hours. We then performed real-time quantitative PCR using RNA extracted from the cells. TGF- β 1-treated cells showed an increase in mRNA levels of *COL1A1* and *DDR2* compared to vehicle treated cells (Fig. 1A). Immunoblotting using lysates from our cultures showed increased protein expression of *COL1A1* and *DDR2* induced by TGF- β 1 (Fig. 1B).

To determine the role of type I collagen in TGF- β 1-induced EMT, we transfected cells with *COL1A1* siRNA or a scrambled non-specific siRNA duplex as a negative control. We then treated the cells with recombinant TGF- β 1 for 8 hours and assessed mRNA levels of *COL1A1* by real-time PCR. *COL1A1* siRNA dramatically reduced expression of *COL1A1* in both vehicle treated cells and TGF- β 1 treated cells (Fig. 2A).

To assess the activity of the transcription factors NF- κ B and LEF-1 that we previously determined to be essential for type I collagen-mediated EMT (Medici and Nawshad, 2010), we transfected cells with pGL3-NF- κ B-Lux (containing NF- κ B binding sites) or pTOPFLASH-Lux (containing LEF-1 binding sites) reporter plasmids. We repeated siRNA transfections then treated the cells with TGF- β 1 for 48 hours to induce EMT. Luciferase expression from both reporter plasmids was dramatically increased by treating the cells with exogenous TGF- β 1. Furthermore, *COL1A1* siRNA significantly inhibited these increases in NF- κ B and LEF-1 activity driven by TGF- β 1 (Fig. 2B).

siRNA transfections were repeated and cells were treated with TGF- β 1 for 48 hours to induce EMT. We collected cell lysates and performed immunoblotting to acquire biochemical confirmation of EMT. TGF- β 1 promoted expression loss of the epithelial marker E-cadherin and gain of the mesenchymal marker vimentin. *COL1A1* siRNA

inhibited these changes (Fig. 2C). We then assessed the post-EMT activity of cell migration with transwell migration assays. TGF- β 1 caused increased migration of cells, which was significantly inhibited by COL1A1 siRNA (Fig. 2D).

Next, we attempted to determine whether there is any potential role for DDR2 in mediating EMT signaling. We transfected cells with control siRNA or DDR2 siRNA followed by treatment with TGF- β 1 for 8 hours to assess *DDR2* gene expression. RNA was extracted from these cultures and real-time PCR was performed to detect mRNA levels of DDR2. DDR2 siRNA greatly reduced levels of DDR2 mRNA in either vehicle or TGF- β 1 treated cells (Fig. 3A). Cells containing pGL3-NF- κ B-Lux or pTOPFLASH-Lux reporter plasmids were transfected with control siRNA or DDR2 siRNA, then exposed to recombinant TGF- β 1 for 48 hours. DDR2 siRNA significantly reduced NF- κ B and LEF-1 activity induced by TGF- β 1 (Fig. 3B).

Lysates from cells transfected with control siRNA or DDR2 siRNA, then treated with recombinant TGF- β 1 for 48 hours were used for immunoblotting experiments. We observed that DDR2 mediates TGF- β 1-induced EMT by inhibiting changes in E-cadherin and vimentin expression (Fig. 3C). DDR2 siRNA was also sufficient to reduce post-EMT cell migration stimulated by TGF- β 1 in transwell migration assays (Fig. 3D).

Similar results for DDR2 knockdown inhibiting TGF- β 1-induced EMT were observed in A549 lung carcinoma cells (Fig. S1). DDR1 expression knockdown with siRNA also showed similar effects as observed with DDR2 knockdown (Fig. S2), suggesting that both discoidin domain receptors play a significant role in mediating TGF- β 1-induced EMT.

We next sought to determine whether DDR2 has an essential role in regulating EMT induced directly by type I collagen. Cells were cultured on matrices of either standard epithelial basement membrane laminin (negative control) or type I collagen. Activation of DDR2 was determined by immunoprecipitation with antibodies specific for DDR2. Immunoblotting was performed with these precipitates using antibodies specific for phospho-tyrosine (P-Y). We observed that type I collagen promoted phosphorylation of the DDR2 receptor, but laminin did not (Fig. 4A). Cells containing pGL3-NF- κ B-Lux or pTOPFLASH-Lux reporter plasmids were transfected with control siRNA or DDR2 siRNA, then grown on laminin or type I collagen for 48 hours. Cells grown on type I collagen showed much higher reporter activity for both plasmids than those grown on laminin. DDR2 siRNA significantly reduced elevated NF- κ B and LEF-1 activity induced by type I collagen (Fig. 4B). To assess EMT, immunoblotting was performed using lysates from cells transfected with control siRNA or DDR2 siRNA then grown on either laminin or type I collagen for 48 hours. We found that type I collagen reduced expression of E-cadherin and increased expression of vimentin. These expression changes were inhibited by the presence of DDR2 siRNA (Fig. 4C). Transwell migration assays showed that type I collagen increased cell migration, which was inhibited by DDR2 siRNA (Fig. 4D).

Similar results were observed for DDR2 knockdown in inhibiting type I collagen-induced EMT in A549 lung carcinoma cells (Fig. S3). DDR1 siRNA also had similar effects to those observed with DDR2 siRNA (Fig. S4), suggesting that DDR1 also has a significant role in mediating type I collagen-induced EMT.

These data identify DDR2 as an essential mediator of TGF- β 1/type I collagen-induced EMT. Both DDR2 and type I collagen play a significant role in TGF- β 1-induced EMT. Also, DDR2 signaling clearly regulates activity of transcription factors that promote this transformation, yet the specific signaling kinases involved remain elusive. Future studies are necessary to dissect the web of signaling downstream of DDR2 that initiates EMT.

It is unclear why such dramatic and opposing expression changes in DDR1 and DDR2 occur during EMT. Both receptors appear to be necessary for type I collagen-mediated signaling that activates EMT-inducing transcription factors, yet DDR1 levels decrease and DDR2 levels increase. DDR1 has been shown to interact with integrin receptors and activate the Pyk2/Rap1/MLK3/MKK7/JNK1/c-Jun signaling pathway, which promotes expression of the mesenchymal marker N-cadherin (Shintani et al., 2008). Specific kinase activities associated with DDR2 are unknown. Furthermore, some studies have demonstrated that DDR2 is not up-regulated during EMT (Camara and Jarai, 2010), suggesting that increased levels of DDR2 may not be critical for its function in regulating EMT.

DDR2 has already been implicated in disease as a stimulator of osteoarthritis (Xu et al., 2010), but DDR2-dependent regulation of other pathological processes has yet to be described. Since EMT is the primary mechanism of several diseases including fibrosis and tumor metastasis (Kalluri and Weinberg, 2009), inhibiting DDR2 in vivo may prove beneficial for anti-fibrotic and/or anti-cancer therapeutics.

Experimental procedures

Cell culture

HK-2 and A549 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 (Docherty et al., 2005; Kasai et al., 2005). HK-2 cells were grown in culture with K-SFM + bovine pituitary extract + EGF (Gibco) + 10% FBS + 1% penicillin/streptomycin. A549 cells were grown in F12K medium (ATCC) + 10% FBS + 1% penicillin/streptomycin. FBS and supplemental growth factors were removed 24 hours prior to all experimental conditions. Recombinant TGF- β 1 (R&D Systems) was added to the serum free culture medium at a concentration of 10ng/mL to treat cells grown on tissue culture plastic. In other experiments, cells were grown on 250ng/ml type I collagen (BD Biosciences) or on 250ng/ml standard basement membrane laminin (Sigma). Cells were treated with TGF- β 1 or grown on matrix proteins for 8 hours to assess effects of signaling on gene expression or for 48 hours to assess EMT and cell migration, processes that occur at much later time points beyond initial signaling. All experiments for this study were performed in triplicate.

Real-time quantitative PCR

RNA extractions were performed using the RNeasy Mini kit (Qiagen) and protocol. 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: **COL1A1**: Forward: 5'-CAGGTCTCGGTCATGGTACCT-3'; Reverse: 5'-GTCGAGGGCCAAGACGAA-3'; **DDR1**: Forward: 5'-ATGGAGCAACCACAGCTTCTC-3'; Reverse: 5'-CTCAGCCGGTCAAACCTCAAACCT-3'; **DDR2**: Forward: 5'-AGTCAGTGGTCAGAGTCCACAGC-3'; Reverse: 5'-CAGGGCACCAGGCTCCATC-3'; **GAPDH**: Forward: 5'-ACCACAGTCCATGCCATCAC-3'; Reverse: 5'-TCCACCCTGTTGCTGTA-3'.

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 (Integrated DNA Technologies) using the following sequences: **COL1A1**: 5'-CGAUGUUUCUGCUUUGUCGUGGCCCUU-3'; **DDR1**: 5'-

UCAAGCUAGGUCCAUAUAUUAGUGUAA-3'; **DDR2**: 5'-UCAACUUUAAGCAUUGGAUCAUAGGUU-3'; **negative control**: 5'-UCACAAGGGAGAGAAAGAGAGGAAGGA-3'.

Reporter gene assays

Luciferase reporter gene assays were conducted using the Luciferase Assay System (Promega) and its corresponding protocol. All plasmids (500ng) 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- κ B-Lux plasmid was provided by A. Rao (Harvard Medical School, Boston, MA, USA). The pTOPFLASH-Lux reporter construct was provided by H. Clevers (Hubrecht Institute, Utrecht, The Netherlands). Negative control cells were transfected with a pGL3 empty vector plasmid (Promega).

Immunoprecipitation and immunoblotting

Western blotting and/or immunoprecipitation were performed using the following antibodies at concentrations and using protocols recommended by the respective manufacturers: P-Y, COL1A1, DDR1, DDR2, E-cadherin (Santa Cruz Biotechnology), vimentin, β -actin (Sigma-Aldrich). HRP-conjugated IgG TrueBlot reagents (eBioscience) were used at a dilution of 1:1000. TrueBlot IgG beads (eBioscience) were used for immunoprecipitation experiments.

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 (485nm)/emission max (520nm) was assessed using a fluorescent plate reader (BD FACSArray bioanalyzer).

Statistical Analyses

One-way analysis of variance (ANOVA) was performed and confirmed with two-tailed paired student's t test using GraphPad Prism 4 software. P values less than 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. TGF- β 1 increases expression of type I collagen and DDR2. A) Real-time quantitative PCR analysis showing increased gene expression of *COL1A1* and *DDR2* in HK-2 cells treated with TGF- β 1. B) Immunoblotting showing increased protein expression of COL1A1 and DDR2 in HK-2 cells treated with TGF- β 1. Data represent mean (n=3) $\pm$ S.D., * P <0.05 compared to vehicle.

Fig. 2.

Type I collagen mediates TGF-β1-induced EMT. A) Real-time PCR analysis confirming expression knockdown of *COL1A1* in HK-2 cells with COL1A1 siRNA. B) Reporter gene assays showing TGF-β1-induced activity of the NF-κB and LEF-1 transcription factors, which is reduced by COL1A1 siRNA. C) Immunoblotting demonstrating that TGF-β1-induced loss of E-cadherin and gain of vimentin expression is inhibited by COL1A1 siRNA. D) Transwell migration assays showing increased cell migration induced by TGF-β1, which is perturbed by COL1A1 siRNA. Data represent mean (n=3) ± S.D., * $P < 0.01$ compared to vehicle + control siRNA; ** $P < 0.05$ compared to TGF-β1 + control siRNA.

Fig. 3. DDR2 mediates TGF-β1-induced EMT. A) Real-time PCR analysis of *DDR2* gene expression confirming the knockdown effects of DDR2 siRNA in HK-2 cells. B) Luciferase reporter gene assays demonstrating that DDR2 siRNA inhibits TGF-β1-induced activation of the NF-κB and LEF-1 transcription factors. C) Immunoblotting showing that DDR2 siRNA inhibits expression changes of E-cadherin and vimentin induced by TGF-β1. D) Transwell migration assays showing that DDR2 siRNA inhibits cell migration promoted by TGF-β1. Data represent mean (n=3) ± S.D., * $P < 0.01$ compared to vehicle + control siRNA; ** $P < 0.05$ compared to TGF-β1 + control siRNA.

Fig. 4.

Inhibition of DDR2 prevents type I collagen-induced EMT. A) Immunoprecipitation showing that type I collagen stimulates tyrosine phosphorylation (P-Y) of DDR2, in HK-2 cells but laminin does not. B) Luciferase reporter gene assays showing that DDR2 siRNA inhibits NF-κB and LEF-1 transcriptional activity induced by type I collagen. C) Immunoblotting demonstrating that expression knockdown of DDR2 with siRNA prevents type I collagen-dependent changes in E-cadherin and vimentin protein expression. D) Transwell migration analysis showing that increased cell migration induced by type I collagen is inhibited by the presence of DDR2 siRNA. Data represent mean (n=3) ± S.D., * $P < 0.01$ compared to laminin + control siRNA; ** $P < 0.01$ compared to collagen I + control siRNA.