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DDR2 plays a role in fibroblast migration independent of adhesion ligand and collagen activated DDR2 tyrosine kinase

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ABSTRACT

Discoidin domain receptor-2 (DDR2) is a cell surface tyrosine kinase receptor that can be activated by soluble collagen and has been implicated in diverse physiological functions including organism growth and wound repair. In the current studies, we used fibronectin and collagen-coated 2D surfaces and collagen matrices in combination with siRNA technology to investigate the role of DDR2 in a range of fibroblast motile activities. Silencing DDR2 with siRNA inhibited cell spreading and migration, and similar inhibition occurred regardless whether cells were interacting with fibronectin or collagen surfaces. Under the assay conditions used, DDR2 tyrosine kinase activation was not observed unless soluble collagen was added to the incubation medium. Finally silencing DDR2 also inhibited human fibroblast migration in 3D collagen matrices but had no effect on 3D collagen matrix remodeling and contraction. Taken together, our findings suggest that DDR2 is required for normal fibroblast spreading and migration independent of adhesion ligand and collagen activation of DDR2 tyrosine kinase.

1. Introduction

Interactions between tissue cells and extracellular matrix (EM) play a central role in normal physiological processes. Changes in EM interactions contribute to tissue fibrosis and malignant transformation. Integrins and syndecans represent well known families of receptors that mediate cell–matrix interactions in synergistic manner. The extracellular domains of these receptors bind to protein molecules of the EM such as collagens, fibronectin and laminin and to intracellular elements conforming the cell cytoskeleton elements as well as to other cytoplasmic signaling molecules. The interplay between EM with cell membrane adhesion molecules results in the physical and mechanical integration of cells into tissues in addition to a coupled with functional of the cell processes such as regulation of gene expression, cell proliferation, and cell motile activity [1–3].

DDR1 and DDR2 are transmembrane adhesion molecules that bind native collagens and can function as direct signaling molecules [4,5] through an intracellular tyrosine kinase domain that is activated by collagen binding. DDR activation depends on the triple helical binding domain present in individual collagen molecules and does not require supramolecular collagen organization [6].

DDR receptor effector mechanisms are not well understood. Most descriptions of DDR function have focused on downstream signaling pathways activated by collagen that regulate cell proliferation and transcriptional activation [7]. Natural occurring or induced DDR2−/− genotypes in mice induce dwarfism, reduce fibroblasts have proliferation [8,9], delayed cutaneous wound repair, as well as decreased expression of syndecan 1 and the collagen crosslinking enzymes lysyl oxidase and lysyl hydroxylase [10]. Conversely, functional mutations in DDR2 contribute to development of squamous cell lung cancer [11]. However, DDR functions involving collagen interactions, e.g., DDR1−/− smooth muscle cell adhesion to collagen [12] and DDR control of collagen are independent of tyrosine kinase activation [13,14]. In addition, DDR1 is required for cell chemotaxis towards soluble collagen [15–17]: yet not all aspects of DDR-dependent cell motile activity depend on DDR-collagen interactions. DDR1 is required for collective cancer cell migration in organotypic collagen culture models. Fibroblast migration through matrigel, which is inhibited in DDR2−/− cells, occurs with serum rather than collagen as the chemotactic stimulus [18].

In the current studies, we expected possible differences between the cellular behavior of the two types of adhesion ligands since absence of collagen-binding DDR2 receptors potentially would have different effects depending on whether the cells were interacting with collagen or another adhesion ligand. We used fibronectin and collagen-coated adhesion surfaces and collagen matrices in combination with siRNA technology to investigate the role of DDR2 in a range of fibroblast motile activities. Our findings suggest that DDR2 is required for normal fibroblast spreading and migration independent of adhesion ligand and collagen activation of DDR2 tyrosine kinase.

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2. Materials and methods

2.1. Materials

DMEM and 0.25% trypsin/EDTA and oligofectamin solution from Invitrogen (Gaithersburg, MD). Opti-MEM (Carlsbad, CA). Fetal bovine serum (FBS) from Atlanta Biologicals (Atlanta, GA). Platelet-derived growth factor (PDGF) from Upstate Biotechnology (Lake Placid, NY). Fatty acid free bovine serum albumin (BSA), Alexa Fluor 488 phalloidin, Alexa Fluor 594 phalloidin and propidium iodide (PI) from Molecular Probes (Eugene, OR). RNase (DNase free) from Roche (Indianapolis, IN). Fluoromount G from Southern Biotechnology Associates (Birmingham, AL). Type I bovine collagen PureCol (3 mg/ml) from Inamed Biomaterials (Fremont, CA). Primary antibodies used were goat anti-human DDR2 (polyclonal) antibody from R&D Systems (Minneapolis, MN); mouse anti-β-actin from Sigma–Aldrich (St. Louis, MO); mouse anti-FAK from BD Biosciences (Bedford, MA); rabbit (polyclonal) anti-pFAK (Y397) from Invitrogen (Gaithersburg, MD); and mouse anti-phosphotyrosine (pTyr) clone 4G10 from Millipore Corp. (Bedford, MA). Secondary antibodies were rabbit anti-goat (affinity purified) from Jackson Immuno Research (Baltimore, MD); horseradish peroxidase (HRP) conjugated goat anti-rabbit Ig from Dako (Ely, UK), and HRP-conjugated goat anti-mouse IgG (H+L) from MP Biomedical Life Sciences (Solon, OH).

2.2. Cell culture

BR5 cells are hTERT-immortalized, early passage human foreskin fibroblasts [19]. Use of de-identified human foreskin fibroblasts was approved by the University Institutional Review Board (Exemption #4). Cells were cultured in DMEM with 10% FBS. Cell culture and experimental incubations were carried out at 37 °C in a 5% CO_2 incubator.

2.3. DDR2 silencing by siRNA

SiRNA oligonucleotides for human DDR2 (siGenome SMARTpool and four individual siRNAs) were designed and provided by Dharmacon (Chicago, IL). Cell cultures (60–70% confluent) were rinsed with antibiotic-free DMEM and treated with trypsin–EDTA for 1 min to elicit cell rounding but not detachment. Antibiotic-free DMEM/10% FBS was added to quench the trypsin. Cells were rinsed with antibiotic-free DMEM, they were incubated overnight at 37 °C with 1 ml Opti-MEM and 0.2 mM oligofectamine containing 100 nM siRNA. Transfected cells were cultured for an additional 72 h. Mock transfected cells were treated with the transfection reagent but no siRNA at 37 °C for 72 h or cells treated with siRNA oligonucleotides (siIntegrin α5 oligonucleotides from mouse), they does not have effect in DDR2 expression.

2.4. Immunoblotting and immunoprecipitation

Cells were washed with PBS (3 mM KCl, 1 mM KH_2PO_4, 150 mM NaCl, and 6 mM Na_2HPO_4, pH 7.2.) containing 1 mM sodium vanadate and lysed with 0.5% NP-40 buffer in PBS containing protease and phosphatase inhibitors (1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 mM AEBSF, 1 mM sodium orthovanadate, 50 mM NaF and 1 mM ammonium molybdate). The lysates were cleared and subjected to SDS–PAGE followed by Western blot. Primary antibodies used were anti-DDR2, anti-actin, anti-pTyr, anti-pFak, anti-Fak). Secondary antibodies were HRP-conjugated goat-anti-rabbit or anti-mouse and their signals ware developed with ECL reagent (GE Healthcare, Buckinghamshire, UK).

For immunoprecipitation, cells were washed with PBS and lysed in modified RIPA (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM EGTA, 10% glycerol, 1% Triton X-100, 10 μg/ml aprotinin, 10 mM NaF, 1 mM PMSF and 1 mM sodium orthovanadate). After clearing by centrifugation, cell lysates (500 μg) were mixed with anti-DDR2 antibodies (2 μg in 0.2 ml PBS) overnight at 4 °C. Protein A/G PLUS agarose (20 μg; Santa Cruz Biotechnology) were added to the mixture and incubated at 4 °C for 2 h. The beads were washed three times with modified RIPA buffer. Samples were extracted by addition of 4× sample buffer, boiled for 3 min, and subjected to SDS–PAGE and immunoblotting.

2.5. Immunofluorescence microscopy

Cell preparations were paraformaldehyde-fixed, blocked with glycine and permeabilized with 0.5% Triton X-100 in PBS. Primary antibody used was anti-DDR2. For actin staining, samples were fixed and incubated with Alexa Fluor 594-conjugated phalloidin or Alexa Fluor 564-conjugated phalloidin. For PI stained (8 μg/ml) PI in the presence of 20 μg/ml RNAse (DNAse-free) (Roche, Mannheim, Germany). Samples were mounted with Fluoromount G and images were collected with a Nikon Elipse 400 fluorescent microscope using 10×/0.45, 20×/0.75, and 40×/0.75 Plan Fluor infinity corrected objectives, a Photometrics SenSys camera, and MetaView acquisition software (Universal Imaging Corp.).

2.6. Matrix contraction and cell migration

Methods for measuring floating collagen matrix contraction (FMC), stressed-released matrix contraction (SMC), and cell migration in nested collagen matrices have been described previously [20,21]. FMC and are shown in Fig. S1. Samples were paraformaldehyde-fixed. The appearance and matrix diameter of collagen matrices paraformaldelhyde-fixed was recorded using an Epson 4870 photo scanner, and matrix diameter measured. Extent of matrix contraction was calculated by subtracting final matrix diameter from the starting diameter (12 mm).

The analysis of cell migration was performed using cell containing nested floating collagen matrices pre-contracted for 6 h in DMEM/10% FBS. These cell-containing pre-contracted matrices (dermal equivalents) were re-embedded in 200 μl cell-free outer collagen matrices and then incubated for 16 h in DMEM/BSA + 50 ng/ml PDGF. Samples were fixed and stained with Alexa Fluor-conjugated phalloidin to visualize actin and with PI to stain cell nuclei. Cell migration index was calculated by counting the average number of cell nuclei that had migrated out of dermal equivalents in four 10× microscopic fields selected arbitrarily. Each field included the border of the dermal equivalent and the farthest migrated cells (PI).

To analyze 2D cell migration, agarose barrier culture surfaces were prepared in 24 well culture dishes. Culture dish surfaces were covered 15 min at 22 °C with 1.5 ml agarose (3% in DMEM). Polymerized agarose was removed from half the culture surfaces, which were then coated for 15 min at 37 °C with 50 μg/ml collagen or 20 μg/ml fibronectin in DMEM.

Subsequently, the dishes were incubated 6-8 h with cells (4×10^4) in 0.5 ml DMEM 10% FBS, after which the barrier was removed. Samples were re-treated with collagen or fibronectin, rinsed, and then cultured overnight in DMEM/BSA plus 50 ng/ml PDGF. Samples were fixed and stained to visualize actin or cell nuclei as above. Cell migration index was calculated as above by counting in five 10× microscopic fields the number of cell nuclei in the space previously occupied by the barrier in five experiments.

2.7. Data analysis

Cell spreading, matrix contraction and cell migration data are presented as averages ± standard deviations for duplicate or triplicate samples. All experiments were carried out three or more times. Statistical significance between two groups was determined by one way ANOVA. $P \leqslant 0.05$ was considered significant.

3. Results

3.1. Dependence of human fibroblast spreading and migration on DDR2

We used siRNA technology to silence DDR2 expression in human fibroblasts. By immunoblotting, DDR2 exhibits double or triple bands representing different degrees of glycosylation [22].

Fig. 1A shows representative gel the extent of silencing after 72 h treatment with DDR2-specific ribo-oligonucleotides. Once silenced, DDR2 levels remained low for several days. Fig. 1B shows that even after seven days, DDR2 expression had not returned to control levels. Fig. 1C shows representaive images of the distribution of actin and Mock control and siRNA-silenced (72 h) cells after the cells were cultured overnight on collagen-coated surfaces. DDR2 staining occurred along the cell surface in control cells but was absent from siRNA-silenced cells indicating the DDR2 levels had decreased in most cells in the preparations.

Fig. 1C shows that DDR2-silenced cells appeared to be less spread with fewer actin stress fibers than control cells. Spreading was visualized by fluorescence actin imaging and morphometric studies were carried out to quantify differences in cell spreading, measurements of area with Photometrics SenSys camera, and MetaView acquisition software. We compared cell spreading over 24 h on collagen and fibronectin-coated surfaces. We expected possible differences between the cellular behaviors of the two types of adhesion ligands since absence of collagen-binding DDR2 receptors potentially would have different effects depending on whether the cells were interacting with collagen or another adhesion ligand.

Morphometric measurements presented in Fig. 2A and B shows representative images of the extent of cell spreading (area) of control and DDR2-silenced cells. By 4 h control cells spread more than DDR2-silenced cells on collagen ($4.327069 \pm 1.103887 \times 10^3\ \mu m^2$ vs $2.495937 \pm 0.654092 \times 10^3\ \mu m^2$) and fibronectin-coated surfaces ($4.168429 \pm 0.405292 \times 10^3\ \mu m^2$ vs $3.26734 \pm 1.174817 \times 10^3\ \mu m^2$). The difference became even more pronounced by 24 h (on coll $6.706534 \pm 1.064165 \times 10^3\ \mu m^2$ vs $3.899571 \pm 0.471334 \times 10^3\ \mu m^2$; on fibronectin $8.975644 \pm 1.152123 \times 10^3\ \mu m^2$ vs $4.969903 \pm 1.476136 \times 10^3\ \mu m^2$). In addition to extent of cell spreading, the shape of spread cells were different by 24 h. On both fibronectin and collagen-coated surfaces, silenced cells were less polarized than control cells and had fewer stress fibers (Fig. 2B).

Experiments were carried out to test the effects of DDR2 silencing on fibroblasts in 3D collagen matrices. Fibroblasts embedded in 3D collagen matrices cause extensive matrix remodeling. Floating matrix contraction (FMC) occurs in the absence of cell tension (i.e., without formation of actin stress fibers); stressed matrix contraction (SMC) occurs when contraction is initiated after cells develop tension. Serum and PDGF stimulate FMC, whereas serum but not PDGF stimulates SMC [23]. Fig. S1 demonstrate that DDR2 silencing (with two different siRNA oligonucleotides had no effect on FMC or SMC) in three independent experiments.

We also tested fibroblast migration in nested 3D collagen matrices. Cells migrate from an inner cell-containing matrix to an outer cell free matrix. In contrast to FMC and SMC, cell migration in nested collagen matrices is stimulated by PDGF but not by serum [23]. Fig. 3A and B shows the overall morphology of cells migrating out of the inner cell matrix. In nested collagen matrices, DDR2-silencing inhibited cell migration compared to control cells (33 ± 5.86435 vs 202 ± 10.858257 outer cells free matrix/field).

Inhibition of cell migration by DDR2-silencing was not specific to nested collagen matrices. Fig. 3C also shows overall cell morphology and cell nuclei for fibroblast migration on 2D surfaces that had been coated with collagen (COLcs) or fibronectin (FNcs). The cell migration index (Fig. 3D) of DDR2-silenced cells was decreased compared to control cells, on collagen (99.4 ± 10.3653 vs 15.6 ± 2.57682 cells) on fibronectin (137.4 ± 10.53755 vs 32.4 ± 3.720215 cells).

3.2. DDR2 activation by soluble collagen

DDR2 has a cytoplasmic tyrosine kinase domain that becomes activated (tyrosine phosphorylated) in response to soluble, native collagen. Fig. 4A shows that if human fibroblasts were attached to collagen or fibronectin-coated surfaces and incubated with

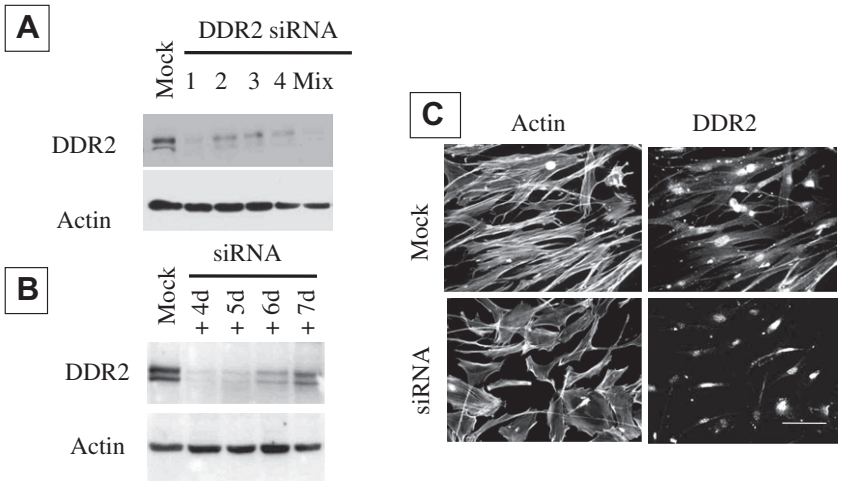

Fig. 1. siRNA silencing of DDR2 expression in BR5. (A) Transfection of cells with four different siRNA oligonucleotides (100 nM, 72 h) resulted in DDR2-silencing as analyzed by immunoblotting. (B) DDR2-silenced cells were cultured for up to 1 week. DDR2 levels analyzed by immunoblotting remained low. (C) Control (mock) and DDR2-silenced cells were harvested and cultured 24 h on culture dishes in DMEM/10% FBS. Actin and DDR2 distribution was detected by immunofluorescence. DDR2 in control cells was evident along the cell surface but undetectable in siRNA-treated cells. DDR2 silenced cells appeared to have fewer actin stress fibers and were less polarized than control cells. The figure represents a typical result from five independent experiments. Scale bar = 50 μm.

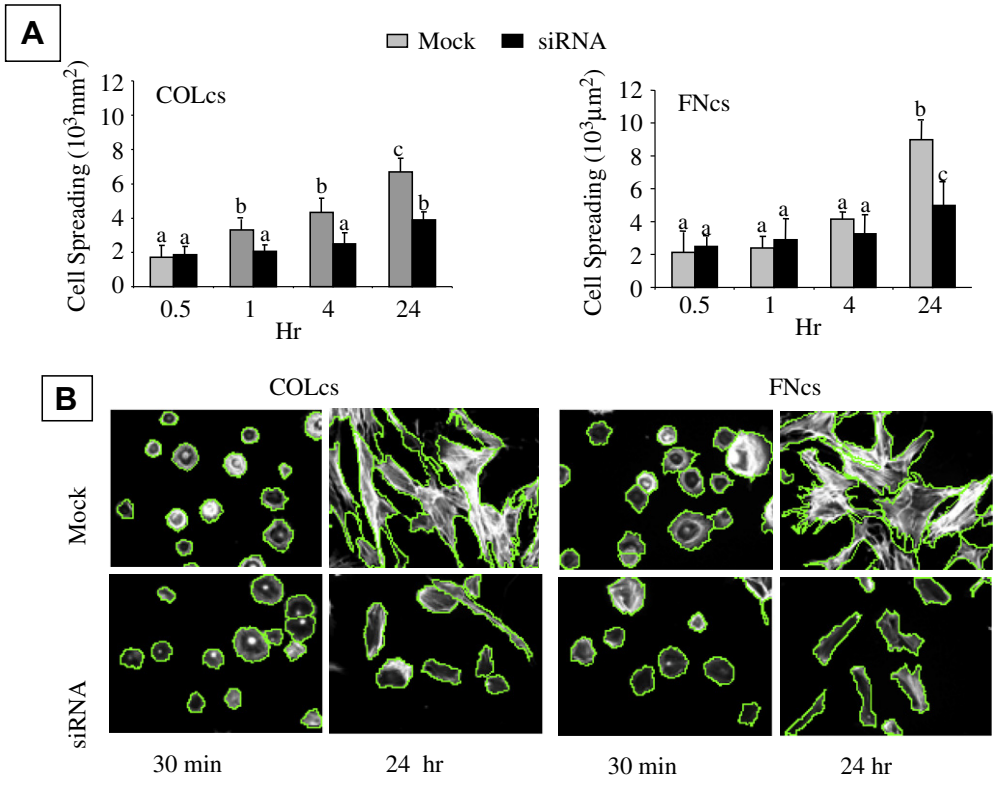

Fig. 2. DDR2-silenced BR5 cells showed decreased spreading on collagen and fibronectin coated surfaces. (A, B) Control and DDR2-silenced cells were cultured in DMEM/10% FBS for the time periods indicated on substrata (50 µg/ml collagen coated COLcs) or 20 µg/ml fibronectin (FNcs). As early as 4 h, DDR2-silenced cells appeared to spread less well than control cells on either COLcs or FNcs. Cell spreading was quantified by morphometric analysis (area). Scale bar = 50 µm. Cell spreading data presented are averages ± SD of five independent experiments. Lowercase letters denote significant differences ($P \leqslant 0.05$).

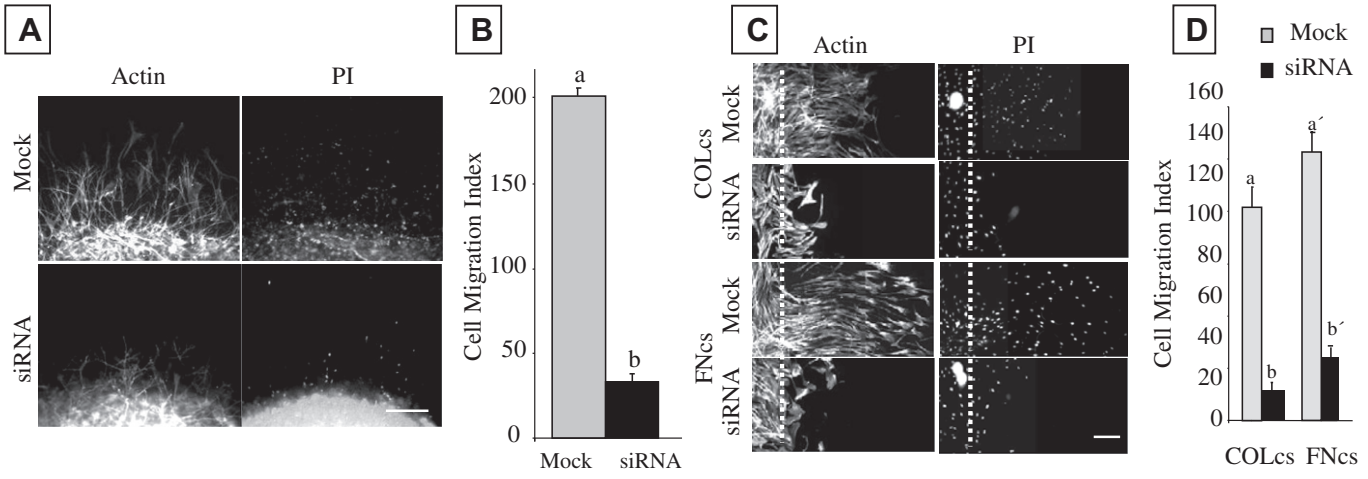

Fig. 3. DDR2-silenced BR5 cells showed reduced cell migration. (A) Nested collagen matrices were prepared with dermal equivalents precontracted in serum-containing medium and then incubated 16 h in DMEM/BSA + 50 ng/PDGF. Actin distribution and cell nuclei were detected by immunofluorescence. (C) Cells were incubated 6–24 h on agarose barrier culture surfaces coated with collagen (COLcs) or fibronectin (FNcs) after which the agarose barrier was removed and cells incubated 24 h in DMEM/BSA + 50 ng/ml PDGF. Actin distribution and cell nuclei were detected by immunofluorescence. (B, D) Cell migration index data presented are averages ± SD of five independent experiments. Lowercase letters denote significant differences ($P \leqslant 0.05$). Scale bar = 100 µm.

soluble collagen, then new DDR2 ("a") and pTyr ("c") bands were detected by SDS–PAGE analysis. In the absence of added collagen, these new bands were not observed regardless whether the cells were incubated on collagen or fibronectin-coated surfaces and regardless whether or not PDGF was added to the incubations. The new pTyr band ("b") that appeared in the presence of PDGF corresponds in size to the phosphorylated PDGF receptor. DDR2 and pTyr bands also were not observed if the cells had been DDR2 silenced by siRNA treatment (Fig. S3). The immunoblotting findings were confirmed by immunoprecipitation experiments. Fig. 4B shows that DDR2 IPs prepared from cells activated by incubation on collagen with collagen in the medium contained three DDR2 bands ("a") and a corresponding pTyr band ("c").

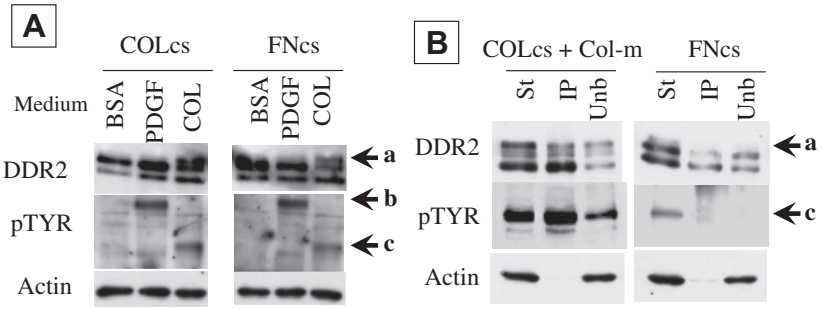

Fig. 4. DDR2 activation by soluble collagen. (A) Cells were incubated 4 h in DMEM/BSA or DMEM/BSA with 50 ng/ml PDGF or 20 μg/ml collagen added to the medium as indicated on 50 μg/ml COLcs or 20 μg/ml FNcs surfaces. Cell extracts were immunoblotted for DDR2 and pTYR using actin as a loading control. (B) Cells were incubated 4 h in DMEM/BSA on 50 μg/ml collagen-coated surfaces with 20 μg/ml collagen added to the medium. Cell extracts were subjected to immunoprecipitation with anti-DDR2 antibodies. Equal protein loads of the starting, IP, and unbound fractions were immunoblotted for DDR2 and pTYR with actin as a specificity control. The figure represents a typical result from six independent experiments.

Cell adhesion to fibronectin and collagen-coated surfaces results in integrin-stimulated activation (pTyr) of focal adhesion kinase (FAK) [24,25]. Since FAK and DDR2 migrate in the same region on SDS gels, activation of one might be mistaken for activation of the other. Moreover, potential signaling interactions between DDR, integrins and FAK have been reported [26–28]. Therefore we compared DDR2 and FAK activation on collagen and fibronectin-coated surfaces with and without collagen in the medium. Fig. S2 shows that the patterns of DDR2 and FAK were not distinct. However, pFAK activation was affected by addition of soluble collagen.

DDR2 silencing on fibroblasts show decrease of pFak anf pTyr on collagen coated with collagen in the medium (Fig. S3).

4. Discussion

In the current studies, using fibronectin and collagen-coated 2D surfaces and collagen matrices in combination with siRNA technology to investigate the role of DDR2 in a range of fibroblast motile activities. As will be discussed, our findings suggest that DDR2 is required for normal fibroblast spreading and migration independent of adhesion ligand and collagen activation of DDR2 tyrosine kinase.

DDR2 played a role in cell motile behavior, but the relationship between motility and collagen activation of DDR2 tyrosine kinase was unclear. Studies with smooth muscle cells from DDR1−/− mice demonstrated a defect in cell attachment to collagen and a loss of cell chemotaxis towards soluble collagen, but only the latter required a functional DDR-kinase domain [15]. Investigators reported that DDR1 was a negative regulator of cell spreading [16], which could be attributed to DDR1 activation of myosin [29] or inhibition of α2β1 function [28]. Yet others reported that DDR2 was required for fibroblast migration through matrigel, but the chemotactic stimulus for migration was serum not soluble collagen [18]. Finally, rather than interacting with collagen, the role of DDR1 in cell–cell interaction [30] and collective cell migration in organotypic collagen culture models [17] was found to depend on DDR1 interaction with E-cadherin.

We found that silencing DDR2 inhibited BR5 cells spreading and migration regardless whether cells were interacting with fibronectin or collagen-coated 2D surfaces. However, cells attached to either surface did not show DDR2 tyrosine kinase activation unless soluble collagen was added to the medium. The requirement for DDR2 in spreading and migration appeared to be independent of adhesion ligand and collagen-stimulated DDR2 activation but dependent of activation of pFak. Inhibition of cell migration by silencing DDR2 also was observed for human fibroblasts migrating in nested collagen matrices, but collagen matrix contraction by these cells was not inhibited indicating the specificity of the DDR2 silencing effect. Our new observations support previous work comparing cell migration and collagen matrix contraction, which showed that different growth factors and regulatory mechanisms control migration and contraction in 3D collagen matrices [23].

Decreased spreading and formation of actin stress fibers by DDR2 silenced cells could reflect a link between DDR2 activation and of focal adhesion kinase. Other studies have suggested that DDR1 signaling affects myosin function activation [29] or inhibition [17,29] depending on cell type and experimental assay under investigation.

The current findings should be distinguished from work on DDR function in cell behavior is analyzed with and without soluble collagen in the medium. While it is well established that soluble collagen can activate DDR tyrosine kinase [4,5] and that activation depends on a triple helical binding domain within individual collagen molecules [6]. Indeed, in fibrous connective tissue, it seems unlikely that significant soluble collagen would be present in the normal tissue environment [31]. Regulation of collagen fibrillogenesis does not require the DDR tyrosine kinase domain [13,14]. The relative ability of collagen fragments and fibrillar structures as activators of DDRs and downstream signally pathways have yet to be studied, will be critical to understanding DDR2 functions depend on collagen-stimulated tyrosine kinase signaling.

In conclusion, our findings suggest that DDR2 is required for normal fibroblast spreading and migration independent of adhesion ligand and collagen activation of DDR2 tyrosine kinase. The diversity of physiological functions in which DDRs participates likely reflects the ability of this cell surface receptor to participate in both collagen dependent and independent regulatory mechanisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.103.

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