IJC
International Journal of Cancer

The intracellular juxtamembrane domain of discoidin domain receptor 2 (DDR2) is essential for receptor activation and DDR2-mediated cancer progression

Daehwan Kim, Panseon Ko, Eunae You and Sangmyung Rhee

Department of Life Science, Chung-Ang University, Seoul, Korea

Discoidin domain receptors (DDRs) are unusual receptor tyrosine kinases (RTKs) that are activated by fibrillar collagens instead of soluble growth factors. DDRs play an important role in various cellular functions and disease processes, including malignant progression. Compared to other RTKs, DDRs have relatively long juxtamembrane domains, which are believed to contribute to receptor function. Despite this possibility, the function and mechanism of the juxtamembrane domain of DDRs have not yet been fully elucidated. In this study, we found that the cytoplasmic juxtamembrane 2 (JM2) region of DDR2 contributed to receptor dimerization, which is critical for receptor activation in response to collagen stimulation. A collagen-binding assay showed that JM2 was required for efficient binding of collagen to the discoidin (DS) domain. Immunohistochemical analysis of DDR2 expression using a tissue microarray demonstrated that DDR2 was overexpressed in several carcinoma tissues, including bladder, testis, lung, kidney, prostate and stomach. In H1299 cells, inhibition of DDR2 activity by overexpressing the juxtamembrane domain containing JM2 suppressed collagen-induced colony formation, cell proliferation and invasion *via* the inhibition of matrix metalloproteinase-2 and matrix metalloproteinase-9. Taken together, our results suggest that JM2-mediated dimerization is likely to be essential for DDR2 activation and cancer progression. Thus, inhibition of DDR2 function using a JM2-containing peptide might be a useful strategy for the treatment of DDR2-positive cancers.

Cell surface receptors sense and respond to diverse extracellular signals, which in turn initiate appropriate cellular responses. Receptor tyrosine kinases (RTKs) constitute a major class of cell surface receptors with high affinities for various polypeptide growth factors, cytokines and hormones. Hence, RTKs have been implicated in normal physiology, and altered or abnormal RTK regulation has been implicated in pathology, including cancer progression.[1–3]

In the classical model of RTK activation, receptors exist as monomers in the absence of a ligand and dimerize on ligand binding.[4] This dimerization brings the cytoplasmic domains into close proximity, leading to transphosphorylation.[5] However, several studies have shown that receptors for cytokines and growth factors exist in preformed dimeric or trimeric states in the absence of a ligand through their transmembrane (TM) domains or through extracellular juxtamembrane (EJM) and intracellular juxtamembrane (IJM) regions, and these receptors undergo conformational changes in the catalytic domain in response to ligand stimulation.[6–8] It was reported that the IJM region of several RTKs contained phosphorylated Tyr residues, which are used as binding sites for proteins involved in downstream signaling, indicating that IJM has a functional role in RTK activation.[9] For example, the IJM of epidermal growth factor receptor (EGFR) has an autoactivating role.[10] However, the precise molecular mechanism has not been established and may differ among individual RTKs.

Discoidin domain receptors (DDRs), DDR1 and DDR2, bind collagen instead of soluble ligands.[11] Similar to other RTKs, DDRs undergo receptor autophosphorylation on ligand binding; however, this process is unusually slow and sustained.[11] Structurally, DDRs consist of an extracellular region containing the ligand-binding discoidin (DS) domain, a DS-like region, a single TM region connected to a cytoplasmic domain that includes an IJM region and a catalytic domain with a short C-terminal tail. Interestingly, the IJMs of DDRs are substantially longer than those of other RTKs, indicating that they are essential for the functioning of DDRs, as with the IJM of other RTKs.[12] Several signaling proteins, including Src/Shc and Nck1/2, interact with the IJM of DDR1, thus activating many downstream signaling

Key words: discoidin domain receptor 2, dimerization, intracellular juxtamembrane region, cancer cell proliferation, colony formation, invasion, matrix metalloproteinases

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Ministry of Education, Science and Technology [Basic Science Research Program through the National Research Foundation of Korea (NRF)]; **Grant number:** 2010-0024096
DOI: 10.1002/ijc.28901
History: Received 12 Dec 2013; Accepted 2 Apr 2014; Online 17 Apr 2014

Correspondence to: Sangmyung Rhee, Department of Life Science, Chung-Ang University, Seoul 156-756, Korea, Tel.: +82-2-820-5818, Fax: +82-2-825-5206, E-mail: sangmyung.rhee@cau.ac.kr

What's new?
Discoidin domain receptors (DDRs) are activated by collagen, and appear to play an important role in malignant progression. In this study, the authors found that DDR2 is overexpressed in several types of carcinoma, and that its cytoplasmic, "juxtamembrane" region (JM2) is critical for activation of the receptor. They also found that blocking DDR2 activation with excess JM2 reduced proliferation and invasiveness of cancer cells in vitro. Thus, inhibition of DDR2 function with a JM2-containing peptide might be a useful therapeutic strategy for DDR2-positive cancers.

cascades. In contrast, few interacting proteins have been identified for the IJM of DDR2.[13,14] Furthermore, a recent study proposed that DDR1 forms a ligand-independent dimer through multiple interactions with protein modules, including the TM domain and IJM, indicating that the IJMs of DDRs play important roles not only in protein–protein interactions but also in regulating receptor activation.[15]

Owing to their ability to regulate the expression and activity of matrix metalloproteinases (MMPs),[13,16] DDRs regulate cell adhesion, proliferation, migration and matrix remodeling. Recently, it was shown that increased collagen in tumor stroma led to a stiffened microenvironment, which allowed for more efficient tumor cell invasion and metastasis.[17,18] As such, other collagen receptors such as integrins have been extensively studied in cancer progression; in contrast, the functional and structural roles of DDRs in cancer invasion and metastasis remained to be more elucidated.[19] Nevertheless, mutations and abnormal expression of DDR1 and DDR2 have been implicated in cancer progression and a poor prognosis.[17,20,21]

In this study, we found that the juxtamembrane 2 (JM2) region of the cytoplasmic domain of DDR2 is important for receptor dimerization together with the TM domain. Deletion or competitive binding of the JM2 region in this cytoplasmic domain showed that JM2 is necessary for collagen binding to the DS domain and for receptor activation. We also found that disrupting the formation of homodimers by forced expression of a cytoplasmic domain containing JM2 in H1299 nonsmall cell lung carcinoma cells substantially decreased cell proliferation, colony formation and invasion of cells cultured in Type I collagen- or Matrigel-coated Transwells *via* inhibition of collagen-induced MMPs activities. Thus, our results indicate that JM2 is necessary for DDR2 activation, and inhibition of DDR2 function by JM2 overexpression may be a useful therapeutic modality in preventing DDR2-mediated cancer progression.

MATERIAL AND METHODS

Reagents

TransFectin Lipid Reagent was obtained from Bio-Rad (Hercules, CA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). The antibodies used in our study were as follows: mouse anti-myc (sc-40; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-HA (sc-805; Santa Cruz Biotechnology), rabbit anti-DDR1 (sc-532; Santa Cruz Biotechnology), goat anti-DDR2 (sc-7555; Santa Cruz Biotechnology), anti-human DDR2 (AF2538; R&D Systems, Minneapolis, MN), anti-phosphotyrosine (clone 4G10; Upstate Biotechnology, Lake Placid, NY) and peroxidase- and fluorescein-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Protein A agarose beads were obtained from GenDEPOT (Barker, TX). Rat-tail Type I collagen (10.6 mg/mL) was purchased from BD Bioscience (Bedford, MA). Bis(sulfosuccinimidyl)suberate sodium salt (BS^3) and gelatin were purchased from Sigma-Aldrich (St. Louis, MO). Formalin-fixed paraffin-embedded tissue microarray slides containing 30 normal and 29 cancer tissues were purchased from Super Biochips (Seoul, Korea).

Cell culture

HEK293T cells were obtained from the American Type Culture Collection (Manassas, VA). HeLa and H1299 cells were purchased from Korea Cell Line Bank (Seoul, Korea). H1299 cells were cultured in RPMI 1640, and the other cell lines were cultured in DMEM supplemented with 10% FBS. Cell culture and incubations were performed at 37°C in a 5% CO_2 incubator.

Plasmid construction and transfection

The constructs were prepared by polymerase chain reaction. cDNA fragments were amplified by polymerase chain reaction and then introduced into the pcDNA 6.0/Myc-His-HA (Invitrogen, Carlsbad, CA) expression vector. All constructs were myc-tagged at the C-terminus, and TM-JM1-JM2 was additionally cloned with an HA-tag at the N-terminus for coimmunoprecipitation analysis. All clone sequences were verified by DNA sequencing. Transient transfection was performed using TransFectin Lipid Reagent according to the manufacturer's instructions.

Chemical crosslinking

Chemical crosslinking was performed as described previously[15,22,23] with minor modifications. Briefly, 0.1 mM of BS^3 was added directly to cell lysates, and then the samples were incubated for 60 min at room temperature. The reactions were terminated by the addition of 2% SDS.

Coimmunoprecipitation and Western blotting

Immunoprecipitation analysis of protein extracts from HEK293T and H1299 cells transfected with the indicated

10970215, 2014, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ijc.28901 by Chung-Ang University, Wiley Online Library on [10/04/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

plasmids was performed as described previously.[24] Prepared samples were subjected to SDS-PAGE and then transferred to a PVDF membrane (Millipore, Bedford, MA). The membranes were blocked, washed and incubated with primary and secondary antibodies. The signal was developed with ECL reagent. Band density was measured by Quantity One (Bio-Rad).

Immunofluorescence microscopy

The proper orientation of the C-terminal myc-tagged full-length DDR2 and TM-JM1-JM2 protein within transfected HEK293T cells was confirmed by immunofluorescence staining by using nonpermeabilization and permeabilization methods.[25] Microscopic images were collected using a fluorescence microscope (Eclipse 80i; Nikon, Tokyo, Japan) using Plan Fluor 10×/0.30 infinity-corrected objectives. Images were acquired using a digital camera (digital sight DS-Qi1Mc; Nikon) and NIS elements image analysis (Nikon). Image processing was carried out using Photoshop 11.0 (Adobe).

Separation of plasma membrane and cytosolic fractions

Plasma membrane and cytosolic fractionation was performed as previously reported[26] with minor modifications. Briefly, cells were washed and then suspended in ice-cold Buffer A. After rapid freezing in liquid nitrogen and thawing in a water bath, cells were centrifuged at 16,000*g* for 10 min at 4°C. The supernatant was transferred and used as the cytosolic fraction. After washing the pellet with Buffer A, it was lysed with Buffer A containing 1% Triton X-100 and centrifuged at 16,000*g* for 10 min at 4°C. The supernatant was transferred and used as the plasma membrane fraction. Both cytosolic and plasma membrane fractions were separated by SDS-PAGE and analyzed by Western blotting.

Receptor–ligand binding assay

Receptor–ligand binding assays were performed as described previously[27] with minor modifications. Briefly, 96-well plates (SPL, Seoul, Korea) were coated with 50 μg/mL of Type I collagen. HEK293T cells transfected with various DDR2 mutants were lysed and were normalized for DDR2 expression by Western blotting prior to the assay. Samples were added to the wells and then incubated overnight at room temperature. After washing, an anti-myc antibody was added to the wells followed by incubation with the secondary antibody. Bound proteins were detected using *o*-phenylenediamine dihydrochloride (Sigma-Aldrich). The reaction was terminated with the addition of 3 M sulfuric acid. Plates were analyzed at 490 nm using a microplate reader (ASYS UVM 340; Biochrom, Austria).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue microarray slides were deparaffinized, rehydrated and blocked. Antigen retrieval in tissue samples was performed by heating followed by incubation with the anti-DDR2 antibody. Next, the samples were incubated with a biotinylated anti-goat secondary antibody (Vectastain Laboratory, Burlingame, CA) and streptavidin-horseradish peroxidase (Zymed Laboratories, San Francisco, CA). 3,3′-Diaminobenzidine (DAB; Vectastain Laboratory) was used as a chromogen, and eosin was used for counterstaining. Tissue sections were visualized by microscopy (Eclipse 80i), and images were acquired using a digital camera (Digital Sight DS-Qi1Mc) and NIS elements image analysis software (Nikon). The level of DDR2 expression was assessed by measuring the staining intensity using Quantity One software (Bio-Rad).

Anchorage-independent growth assay

Cells in RPMI 1640 containing 0.35% agar (Lonza, Walkersville, MD) were incubated in 12-well plates overlaid with a gel base of 0.5% agar. After 4 weeks, the gels were stained with 0.005% crystal violet to assess the number and projected area of the colonies.

Cell proliferation assay

Cells were seeded on a 35-mm dish and cultured for 7 days. At the indicated time points, cells were harvested and counted using a hemocytometer. For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a WST-1 cell proliferation assay system (Takara, Tokyo, Japan) was used according to the manufacturer's instructions.

Cell invasion through Transwells

Transwell inserts with an 8-μm pore size were coated with 20 μg Type I collagen or 10 μg Matrigel (BD Bioscience) at 37°C overnight. Cells were placed in the upper chamber. RPMI 1640 supplemented with 10% FBS was added to the lower chamber as the chemoattractant. In some experiments, 5 μM GM6001 (Calbiochem, La Jolla, CA) was added to both chambers to prevent MMP-dependent invasion. Nonmigrating or noninvading cells were removed with a cotton swab. Cells were fixed and stained with 0.1% crystal violet and counted in all areas.

Gelatin zymography assay

Cells were seeded in a 35-mm dish and incubated in RPMI 1640 supplemented with 10% FBS for 24 hr. Then, the cells were washed, and the medium was changed to serum-free RPMI 1640. After incubation for 24 or 48 hr, the conditioned medium was centrifuged to remove cellular debris and was normalized to the cell number prior to electrophoresis in 10% polyacrylamide gels with 1 mg/mL gelatin. After SDS-PAGE, the gels were washed four times for 60 min with washing buffer (10 mM Tris-HCl [pH 8.0] and 2.5% Triton X-100), rinsed with distilled water twice for 10 min and then incubated for 20 hr in the developing buffer (50 mM Tris-HCl [pH 7.5], 5 mM $CaCl_2$ and 0.02% NaN_3). Gels were stained with 0.5% Coomassie blue R-250 for 10 min and destained until the gelatinolytic bands were visualized.

10970215, 2014, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ijc.28901 by Chung-Ang University, Wiley Online Library on [10/04/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

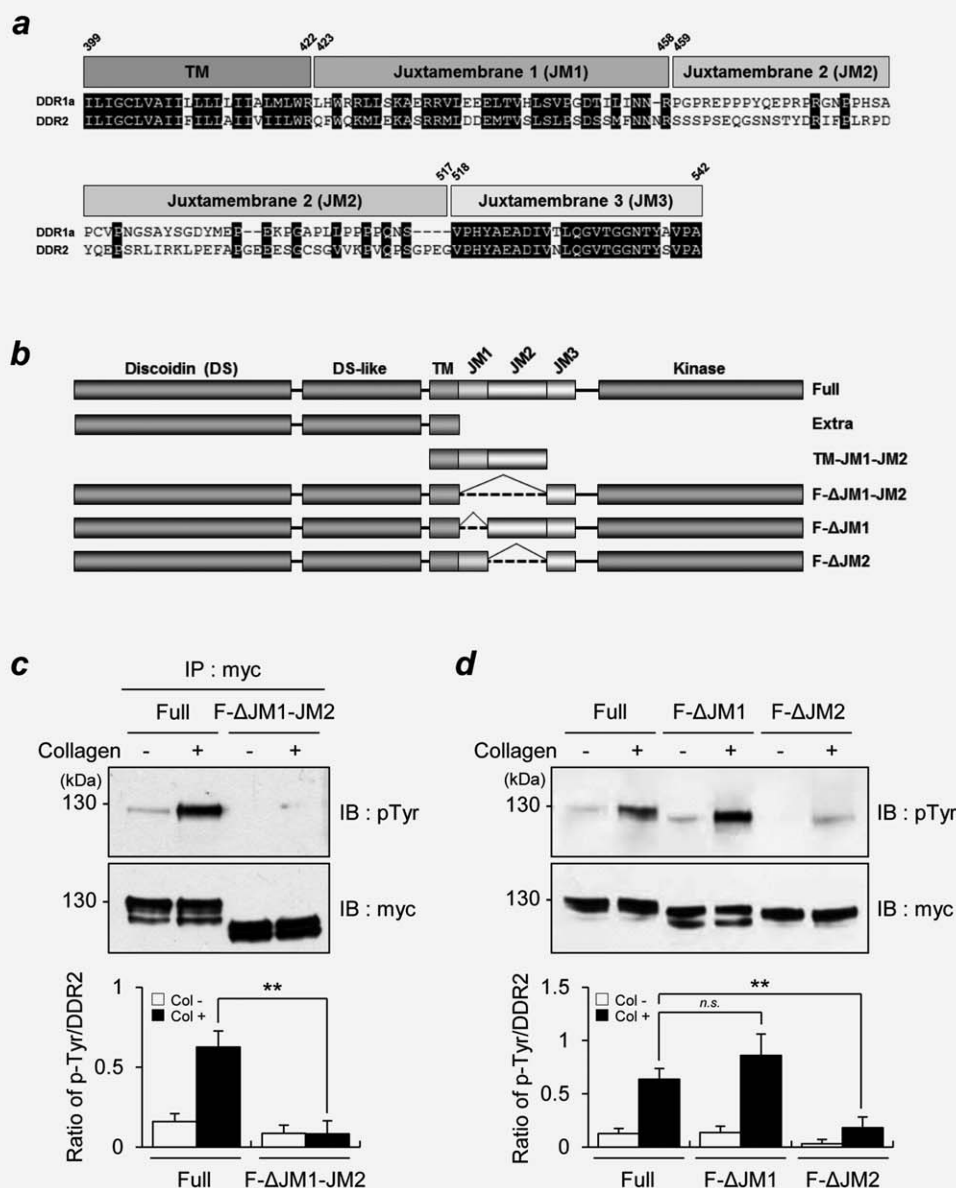

Figure 1. The IJM region is necessary for collagen-induced DDR2 activation. (*a*) Overall topology and alignment of the transmembrane (TM) domain and the intracellular juxtamembrane region (IJM) of DDR1a and DDR2. The IJM was divided into three regions: JM1, JM2 and JM3. (*b*) Schematic diagram of various DDR2 constructs used in our study. (*c*) HEK293T cells transiently transfected with plasmids encoding the full-length DDR2 and F-ΔJM1-JM2 mutant were stimulated by Type I collagen for 60 min. Tyrosine phosphorylation of the F-ΔJM1-JM2 mutant was inhibited compared to that of the full-length DDR2. (*d*) HEK293T cells were transfected with plasmids encoding full-length DDR2, F-ΔJM1 and F-ΔJM2 and were stimulated by collagen. The F-ΔJM2 mutant showed a significant decrease in tyrosine phosphorylation. $^{**}p < 0.01$, Student's *t*-test.

RESULTS

IJM region is necessary for collagen-induced DDR2 activation

To characterize the IJM of DDR2, we compared the 143 amino acid sequence of the IJM in DDR2 to that of DDR1a. Amino acids 423–458, 459–517 and 518–542 of DDR2 shared 68%, 16% and 92% homology with the corresponding regions of DDR1a, which are referred to as juxtamembrane 1 (JM1), juxtamembrane 2 (JM2) and juxtamembrane 3 (JM3), respectively (Fig. 1*a*). Based on the alignment results, a series of constructs was prepared for JM1 and JM2 mutants either in the isolated cytoplasmic region containing the TM domain or in full-length DDR2 (Fig. 1*b*).

To investigate the role of IJM in collagen-induced DDR2 activation, we overexpressed myc-tagged full-length or F-ΔJM1-JM2 in HEK293T cells and performed immunoprecipitation using an anti-myc antibody after collagen stimulation for 1 hr followed by Western blotting with either an anti-myc or anti-phosphotyrosine antibody (4G10). Immunoprecipitation results showed that full-length DDR2 was

10970215, 2014, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ijc.28901 by Chung-Ang University, Wiley Online Library on [10/04/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

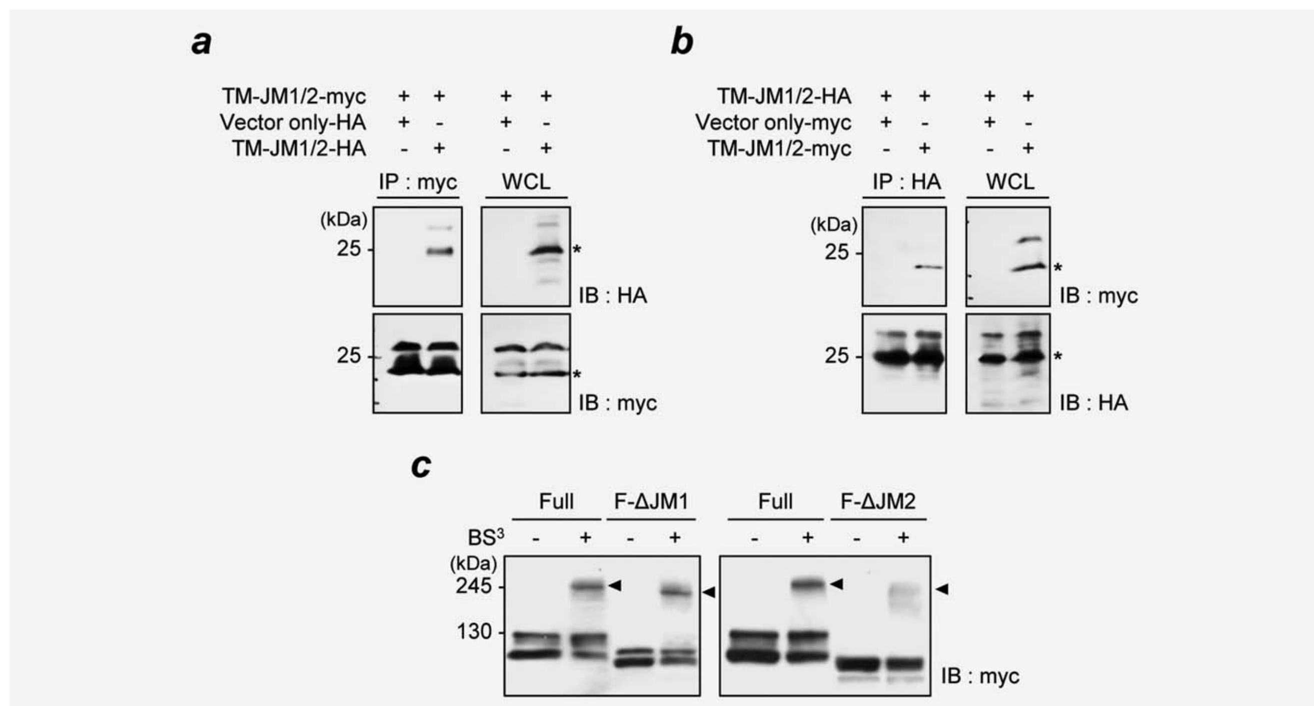

Figure 2. DDR2 dimerizes *via* the JM2 of the IJM region. (*a* and *b*) HEK293T cells were transiently cotransfected with plasmids encoding TM-JM1-JM2-myc and TM-JM1-JM2-HA. Immunoprecipitation and Western blot analysis showed that the cytoplasmic domains of DDR2 bind to each other *via* the intact TM-JM1-JM2 domain and form homodimers. Asterisks indicate the expected size of TM-JM1-JM2. (*c*) HEK293T cells were transfected with plasmids encoding F-ΔJM1 and F-ΔJM2 mutants. A crosslinking assay showed that dimers of F-ΔJM1 were not changed compared to full-length DDR2, whereas dimers were significantly decreased for F-ΔJM2.

extensively phosphorylated by collagen stimulation, whereas F-ΔJM1-JM2 had reduced phosphorylation despite no significant change in the levels of precipitated proteins in the presence or absence of collagen (Fig. 1*c*). To further identify the IJM regions required for DDR2 activation, we examined the phosphorylation of F-ΔJM1 and F-ΔJM2 constructs in response to collagen stimulation. The level of tyrosine phosphorylation in the F-ΔJM1 construct was similar or even slightly increased compared to the full-length DDR2 construct under collagen treatment conditions, whereas tyrosine phosphorylation was significantly decreased in cells transfected with the F-ΔJM2 mutant in response to collagen stimulation (Fig. 1*d*). These results indicate that JM2 has a critical role in DDR2 activation.

JM2 of IJM region mediates DDR2 dimerization

It is known that proper localization and/or dimerization of the IJM is critical for activation of RTKs such as EGFR[10]; therefore, we investigated whether the IJM of DDR2 is involved in receptor dimerization. The sequence homology of the TM domains between DDR1 and DDR2 is up to 87%, and the TM of DDR2 has a leucine-zipper-like motif similar to that of DDR1, suggesting that the TM domain of DDR2 could self-associate similar to DDR1.[15] A coimmunoprecipitation assay showed that HA-tagged TM-JM1-JM2 interacted with myc-tagged TM-JM1-JM2 and *vice versa* (Figs. 2*a* and 2*b*). Thus, the IJM of DDR2 likely participates in receptor homodimerization.

To test whether JM2 is specifically involved in dimerization, we analyzed the extent of DDR2 dimerization with JM1- and JM2-deletion mutants by using the homobifunctional cross-linker BS^3. Dimerization of F-ΔJM1 was similar to that of full-length DDR2, whereas F-ΔJM2 exhibited significantly decreased dimerization (Fig. 2*c*), suggesting that JM2, not JM1, of the cytoplasmic domain is necessary for DDR2 dimerization. Taken together with the results shown in Figure 1*c*, we conclude that dimerization through JM2 is essential for DDR2 activation in response to collagen stimulation.

Inhibition of receptor dimerization via JM2 prevents collagen-induced DDR2 activation

On the basis of the above results, we hypothesized that overexpression of the JM2-containing cytoplasmic domain would lead to heterodimerization with endogenous DDR2, which could have a dominant-negative effect on collagen-induced DDR2 activation. To test this hypothesis, we examined whether TM-JM1-JM2 interacts with full-length DDR2, leading to the formation of heterodimers. HA-tagged TM-JM1-JM2 interacted with myc-tagged full-length DDR2 and *vice versa* (Figs. 3*a* and 3*b*). To test whether the heterodimeric structure of full-length DDR2 and TM-JM1-JM2 proteins were in the correct conformation within the plasma

10970215, 2014, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ijc.28901 by Chung-Ang University, Wiley Online Library on [10/04/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

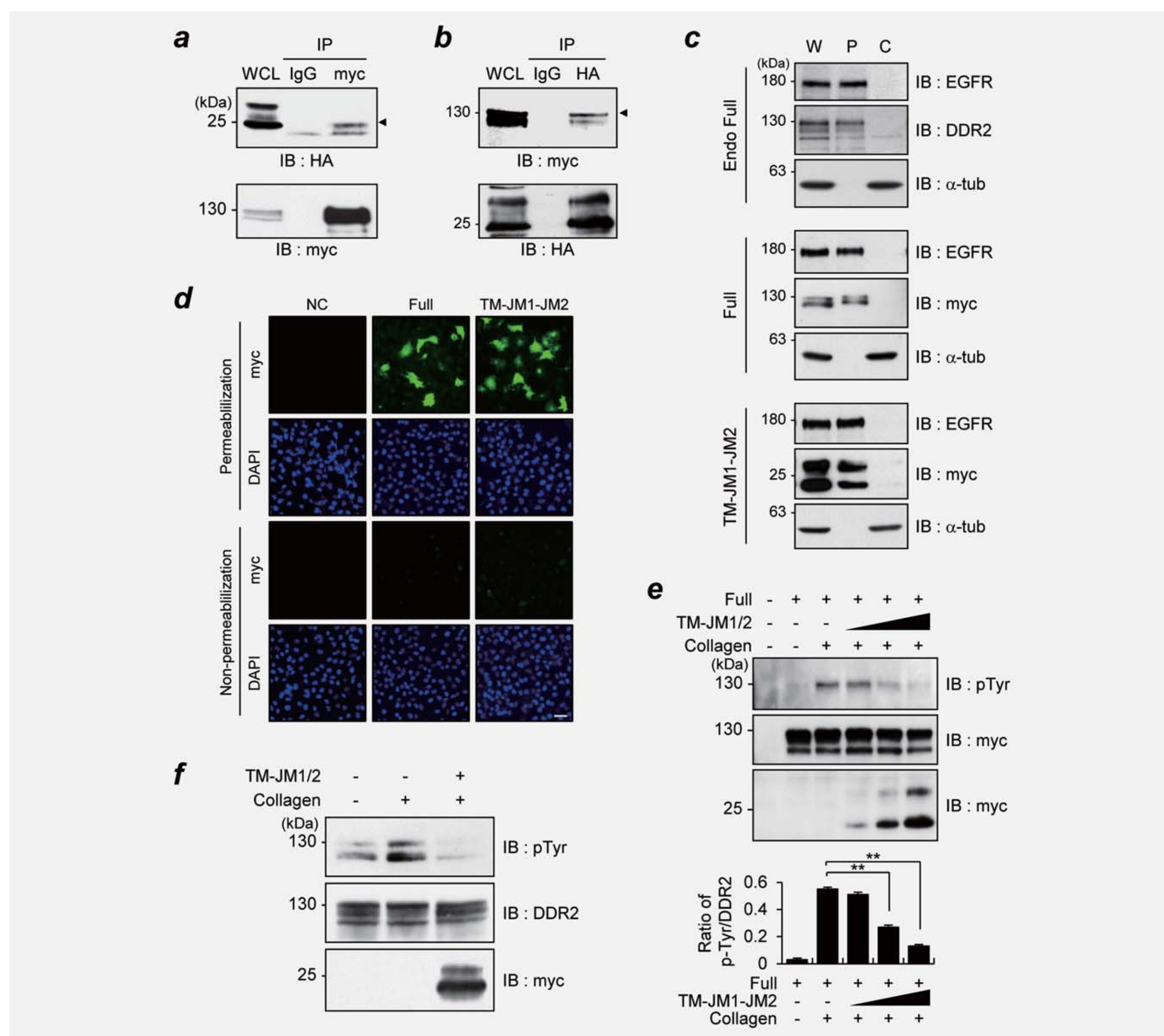

Figure 3. JM2 has a dominant-negative effect on DDR2 activation. (*a* and *b*) HEK293T cells were cotransfected with plasmids encoding full-length DDR2-myc and TM-JM1-JM2-HA. Immunoprecipitation and Western blotting showed that the full-length DDR2 and TM-JM1-JM2 bind to each other to form heterodimers. The immunoprecipitates obtained with anti-IgG antibodies were used as a negative control. (*c*) H1299 cells transfected with plasmids encoding full-length DDR2 and TM-JM1-JM2 and HeLa cells were lysed, and the whole cell lysates (W) were separated into the plasma membrane (P) and cytosol (C) fractions. EGFR and α-tubulin were used as positive controls for the plasma membrane and cytosol fractions, respectively. Endogenous full-length DDR2 (HeLa cells), forced-expressed full-length DDR2 and TM-JM1-JM2 proteins were appropriately localized in the plasma membrane. (*d*) HEK293T cells were transfected with plasmids encoding a C-terminally myc-tagged full-length DDR2 and TM-JM1-JM2. Only under the permeabilized condition, full-length DDR2-myc and TM-JM1-JM2-myc were visualized, indicating that the C-termini of these proteins were located in the cytosol and not extracellular space. Full-length DDR2 was used as a positive control. Bar, 50 μm. (*e*) HEK293T cells were cotransfected with plasmids encoding full-length DDR2 (500 ng) and an increasing amount of TM-JM1-JM2 (100, 300 and 500 ng) as indicated and then stimulated with collagen. Tyrosine phosphorylation gradually decreased with an increasing amount of TM-JM1-JM2. (*f*) HeLa cells were transiently transfected with a plasmid encoding TM-JM1-JM2 and were then stimulated. Tyrosine phosphorylation was significantly decreased in endogenous DDR2. $^{**}p < 0.01$, Student's *t*-test. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

membrane, we performed membrane fractionation assays and immunocytochemistry. As shown in Figure 3*c*, overexpressed full-length DDR2 (middle panel) and TM-JM1-JM2 (lower panel) were both localized in the plasma membrane fraction at levels similar to those of endogenous full-length DDR2 (upper panel). Specifically, JM1-JM2 (without the TM domain) was localized in the cytosol and not in the plasma membrane, suggesting that the TM domain plays a critical role in the proper transportation to the plasma membrane (Supporting Information Fig. S1). In addition, the myc tags

10970215, 2014, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ijc.28901 by Chung-Ang University, Wiley Online Library on [10/04/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

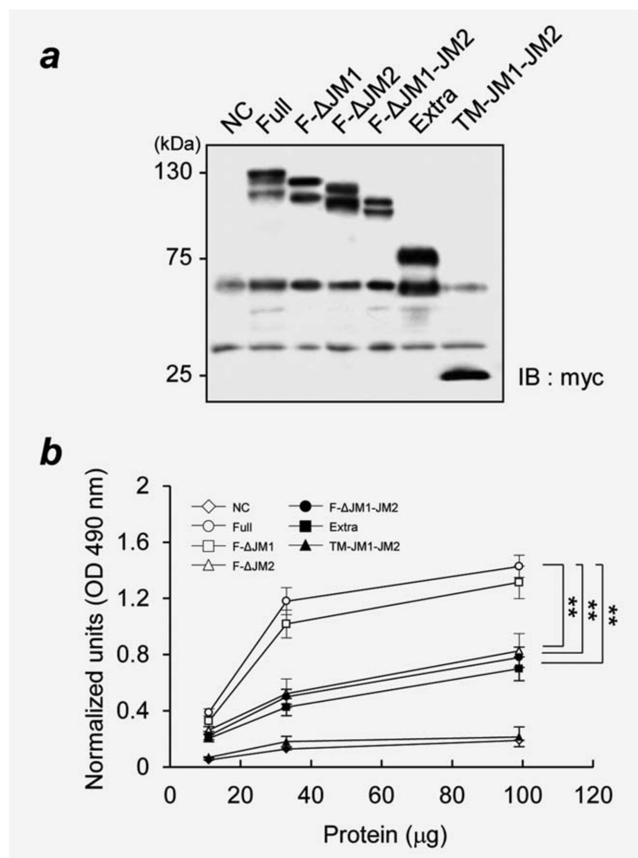

Figure 4. JM2 regulates the collagen-binding affinity of DDR2. (*a* and *b*) HEK293T cells transiently transfected with various DDR2 constructs were harvested, and protein expression was verified by Western blotting (*a*). Collagen-binding affinities were reduced in F-ΔJM2, F-ΔJM1-JM2 and extra mutants but not the F-ΔJM1 mutant in a dose-dependent manner (*b*). Nontransfected (NC) and TM-JM1-JM2 samples were used as negative controls. $^{**}p < 0.01$, Student's *t*-test.

at the C-termini of full-length DDR2 and TM-JM1-JM2 were visualized in permeabilized samples, whereas they were not visualized in nonpermeabilized samples (Fig. 3*d*), indicating that the C-termini of full-length DDR2 and TM-JM1-JM2 are located in the cytosol, not in the extracellular space, and that these proteins are correctly positioned within the plasma membrane. Fluorescence microscopy showed that overexpressed TM-JM1-JM2 was localized in the plasma membrane at sites with abundance of endogenous DDR2 (Supporting Information Fig. S2). These results suggest that TM-JM1-JM2 is suitable for acting as a dominant-negative mutant. Therefore, we verified whether overexpression of TM-JM1-JM2 affected DDR2 activation. Forced expression of TM-JM1-JM2 decreased DDR2 phosphorylation following collagen stimulation in a TM-JM1-JM2 concentration-dependent manner, although no significant change in total DDR2 expression was observed (Fig. 3*e*). We next analyzed whether overexpression of TM-JM1-JM2 inhibited endogenous DDR2 activation. HeLa cells expressing endogenous DDR2 but not DDR1 were transiently transfected with the TM-JM1-JM2 construct, and then collagen-induced DDR2 phosphorylation was analyzed by Western blotting. TM-JM1-JM2 overexpression resulted in a complete loss of collagen-induced endogenous DDR2 phosphorylation (Fig. 3*f*). These results indicate that JM2, not JM1, is essential for DDR2 activation.

JM2 is required for collagen interaction with the DS domain of DDR2

The IJMs of several receptors, including integrin, are known to regulate ligand–receptor association.[28,29] We also showed that deletion of JM2 in the full-length DDR2 substantially decreased receptor activation on collagen stimulation; therefore, it is likely that the JM2 deletion mutant would decrease the ligand affinity of the DS domain. To test this possibility, we performed a ligand-binding assay with various DDR2 mutants. There was a significant decrease in the collagen-binding affinity of the JM1-JM2 and JM2 deletion mutants, which was similar to that of the extracellular domain only, whereas the binding affinity of JM1 deletion mutant was not substantially changed compared to that of full-length DDR2 (Figs. 4*a* and 4*b*). These results suggest that the JM2 is critical for high-affinity collagen binding to the DS domain in the extracellular domain.

Inhibition of dimerization mediated by JM2 prevents cancer cell proliferation and invasion

DDR1 is implicated in cancer progression, but recent studies have reported that DDR2 is also involved in the progression of several tumor types.[13,17,30] We compared DDR2 expression in various cancer and normal tissues by immunohistochemical analysis using a tissue microarray chip that contained 30 normal and 29 cancer tissue samples. Among the tested samples, DDR2 expression was significantly upregulated in lung, prostate, stomach, bladder, testis and kidney cancers compared to normal tissues (Figs. 5*a* and 5*b*).

Because results from our microarray experiments and results from other studies have indicated that DDR2 expression is prominent in lung cancer,[13,17,31] we examined whether inhibition of DDR2 activity by JM2 overexpression could alter features associated with cancer progression, such as anchorage-independent proliferation and invasion, in a lung cancer cell line. We established a stable cell line expressing TM-JM1-JM2 by using the H1299 nonsmall cell lung carcinoma line. Because H1299 cells express both DDR1 and DDR2,[32] we performed an immunoprecipitation analysis of stable TM-JM1-JM2–expressing H1299 cells by using antibodies against DDR1 and DDR2 to test whether JM2 overexpression specifically inhibits DDR2, but not DDR1, following collagen stimulation. The amount of DDR2 precipitated was greater than that of DDR1, which was significantly activated on collagen stimulation in H1299 cells (Fig. 5*c*). Moreover, TM-JM1-JM2 was coprecipitated with DDR2 but not with DDR1, and DDR2 activation was specifically inhibited by TM-JM1-JM2, confirming that JM2 mediates DDR2 activation in response to collagen stimulation. To test whether overexpression of JM2-containing proteins could reduce the

10970215, 2014, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ijc.28901 by Chung-Ang University, Wiley Online Library on [10/04/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

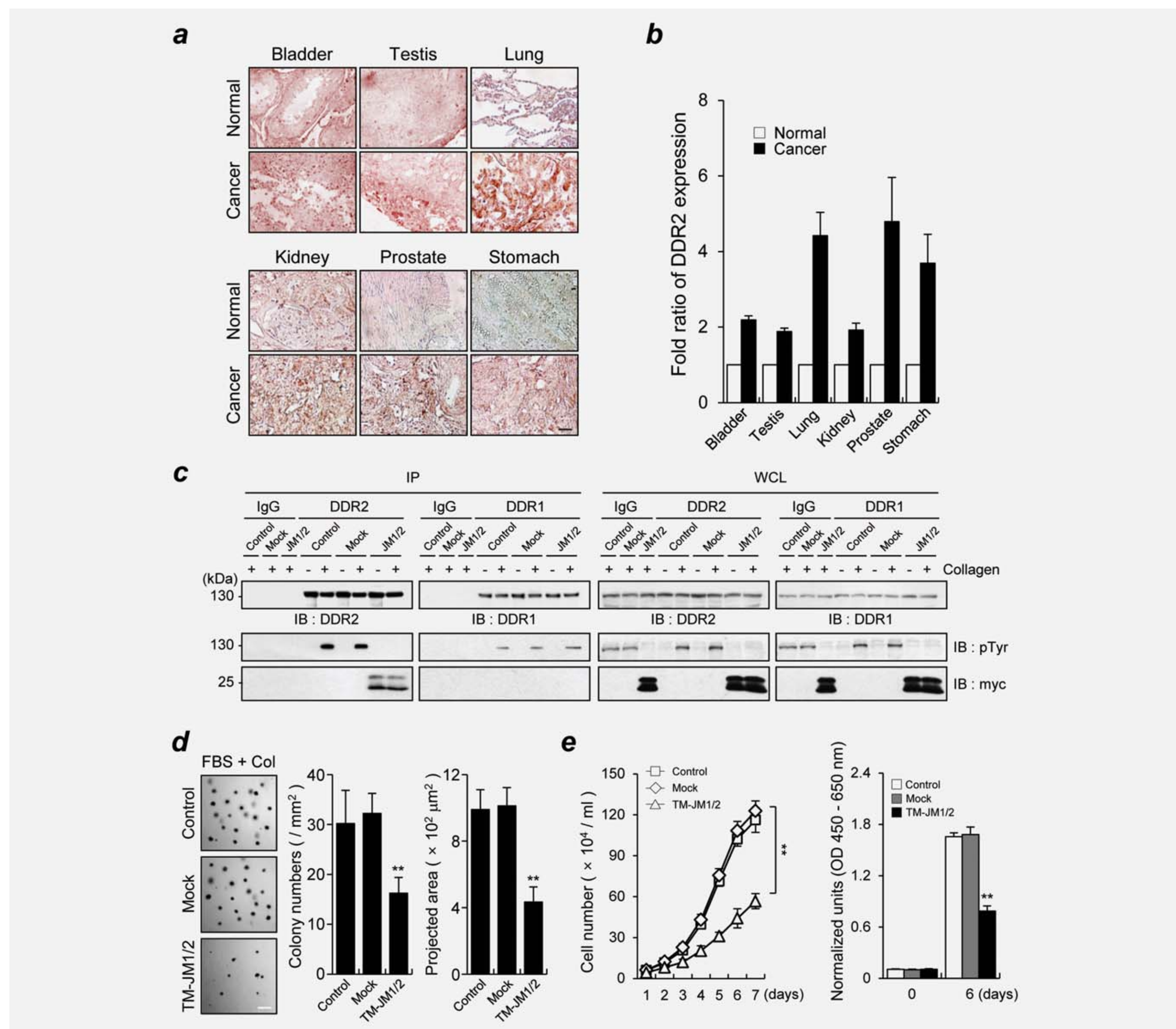

Figure 5. Colony formation and proliferation of tumor cells are suppressed by overexpression of JM2. (*a* and *b*) Formalin-fixed tissue microarray slides were used in immunohistochemistry experiments. DDR2 was overexpressed in bladder, testis, lung, kidney, prostate and stomach cancers. Bar, 50 μm. (*c*) Stable TM-JM1-JM2–expressing H1299 cells were stimulated by collagen and then harvested. Immunoprecipitation and Western blot analysis showed that tyrosine phosphorylation of DDR2 was decreased by TM-JM1-JM2 overexpression (labeled JM1/2), but phosphorylation of DDR1 was unaffected. (*d*) A colony-forming assay of H1299 cells showed that the number and projected area of colonies were decreased by TM-JM1-JM2 overexpression. Bar, 100 μm. (*e*) Proliferation of H1299 cells was assessed by cell counting (left) and an MTT assay (right). Cell proliferation was inhibited by TM-JM1-JM2 overexpression. $^{**}p < 0.01$, Student's *t*-test; control, nontransfected cells; Mock, empty vector stably transfected cells; JM1/2, TM-JM1-JM2 stably transfected cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

tumorigenicity of H1299 cells, we analyzed the colony-forming ability of H1299 cells in response to collagen stimulation on soft agar plates. Compared to Mock-transfected cells, TM-JM1-JM2–overexpressing cells showed a significant suppression of collagen-stimulated colony formation as determined by the number and projected area of colonies (Fig. 5*d*). Overexpression of TM-JM1-JM2 inhibited collagen-stimulated H1299 cell proliferation, as measured by cell counting and the MTT assay (Fig. 5*e*). This finding suggested that the impairment of anchorage-independent growth by TM-JM1-JM2 overexpression is likely due to the inhibition of cell proliferation.

We next investigated whether JM2 overexpression could suppress the invasive capacity of H1299 cells by examining cell migration through a Matrigel- or Type I collagen-coated Transwell insert. Invasion of TM-JM1-JM2–expressing H1299 cells was significantly lower on both Type I collagen- and Matrigel-coated membranes than that of Mock-transfected cells (Fig. 6*a*). The invasion of Mock-transfected cells treated

10970215, 2014, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ijc.28901 by Chung-Ang University, Wiley Online Library on [10/04/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

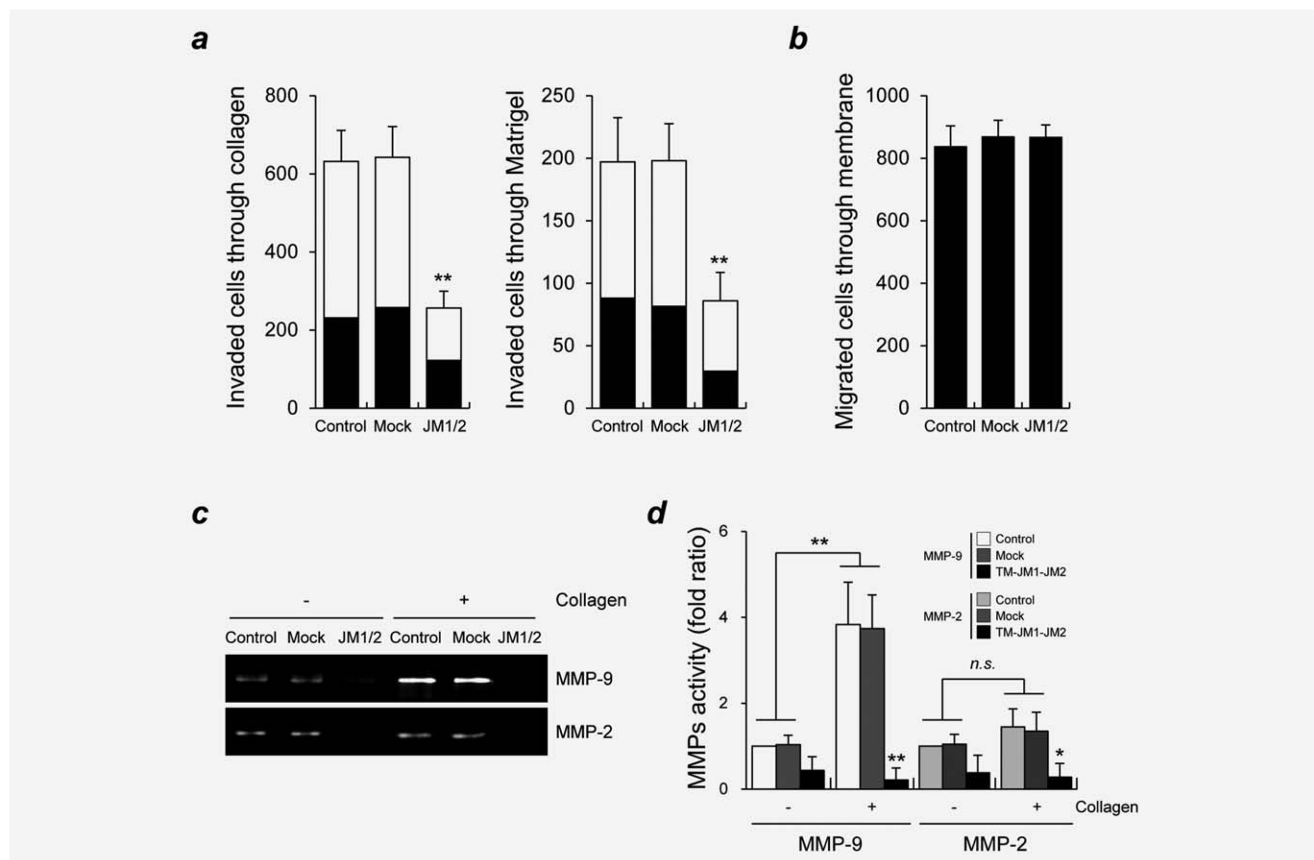

Figure 6. Inhibition of JM2-mediated dimerization decreases tumor cell invasion. (*a*) Transwell assays were performed with stable TM-JM1-JM2–expressing H1299 cells. Invasion was suppressed by TM-JM1-JM2 overexpression in both collagen- (left) and Matrigel-coated inserts (right). Filled bars indicate invaded cells in the presence of 5 μM GM6001. (*b*) H1299 cells were allowed to migrate through uncoated inserts for 4 hr. The number of migrated cells was not significantly changed by TM-JM1-JM2 overexpression. (*c* and *d*) Conditioned media from cells were normalized to the cell number prior to performing gelatin zymography. Densitometric intensities were measured for MMP-2 and MMP-9. Increased activities of MMP-2 and MMP-9 after collagen treatment were decreased by TM-JM1-JM2 overexpression. $*p < 0.05$, $**p < 0.01$, Student's *t*-test; n.s., not significant; control, nontransfected cells; Mock, empty vector stably transfected cells; JM1/2, TM-JM1-JM2 stably transfected cells.

with GM6001, a general MMP inhibitor, was similar to that of untreated TM-JM1-JM2–expressing cells on Type I collagen- or Matrigel-coated membranes (Fig. 6*a*), suggesting that the decrease in the invasive ability of TM-JM1-JM2–expressing H1299 cells was MMP-dependent. However, no significant differences in cell invasion were observed when the cells were applied to an uncoated insert, which indicates that TM-JM1-JM2 overexpression did not alter cell motility (Fig. 6*b*). Because MMP activation significantly contributes to cancer invasion,[30] and DDR2 controls MMP-2 expression and activity,[33,34] we investigated whether TM-JM1-JM2 overexpression could inhibit collagen-induced MMP activity (Figs. 6*c* and 6*d*). Using gelatin zymography, we found that H1299 cells secreted not only MMP-2 but also MMP-9, and the activity of MMP-9 was significantly increased by collagen treatment, whereas MMP-2 activity was nonsignificantly increased. TM-JM1-JM2 overexpression significantly decreased collagen-induced MMP-2 and MMP-9 activity. These results suggest that MMP-2 and MMP-9 activities depend on DDR2 activation and that JM2 overexpression is sufficient to decrease these activities.

DISCUSSION

In this study, we demonstrated that the IJM contributed to DDR2 activation on collagen stimulation. In addition, we showed that the JM2 region of the IJM mediates receptor dimerization along with the TM domain. Disruption of DDR2 dimerization by overexpression of the JM2-containing cytoplasmic region in a nonsmall cell lung cancer cell line, H1299, inhibited typical characteristics of malignancy, such as anchorage-independent growth and invasiveness. Therefore, our results suggest that the JM2 domain of the IJM plays an important role in DDR2 activation.

The DDR family is thought to exist as preformed dimers through multiple interactions between the extracellular, TM and cytoplasmic domains in a ligand-independent manner; among these regions, the TM domain is believed to be the most important for DDR dimerization.[15] Accordingly, our

10970215, 2014, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ijc.28901 by Chung-Ang University, Wiley Online Library on [10/04/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

10970215, 2014, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ijc.28901 by Chung-Ang University, Wiley Online Library on [10/04/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

results also showed that constructs possessing the TM domain coimmunoprecipitate with the IJM (Figs. 2*a* and 2*b*). However, TM-JM1 failed to associate with TM-JM2, although both constructs contain the TM domain. Similarly, the TM-domain-deleted JM1-JM2 was not immunoprecipitated with TM-JM1-JM2 (data not shown). These data indicate that the TM domain itself is insufficient for cytoplasmic domain association. Furthermore, a crosslinking assay using BS^3 showed that the JM2 domain is critical for DDR2 dimerization (Fig. 2*c*). Thus, these results indicate that DDR2 dimerization requires both TM and IJM, especially the JM2 domain.

Because DDRs possess a relatively long IJM, it has been suggested that the IJM is involved in receptor activation.[13] The cytoplasmic domain of the IJM in most RTKs serves a positive or negative role in catalytic activity.[9] Our JM2-domain-deletion and dominant-negative mutants also suggested that the IJM, especially JM2, of DDR2 plays a positive role in DDR2 activation (Fig. 1*d*). Supporting this conclusion, forced expression of the JM2-containing cytoplasmic domain enabled heterodimer formation with full-length DDR2 (Figs. 3*a* and 3*b*), which may hinder homodimerization of DDR2. Moreover, TM-JM1-JM2 overexpression significantly decreased collagen-induced activation of endogenous DDR2 (Fig. 3*e*). The IJM region of several RTKs that form ligand-independent dimers, including EGFR and EpoR, is thought to be involved in the proper positioning of the catalytic domain to allow maximal activation on ligand stimulation.[35] Thus, the intact association with the IJM through the TM and JM2 domains may allow for the precise interaction of the catalytic domain for receptor activation after collagen-induced conformational changes.[19]

In addition to receptor dimerization, the JM2 domain controlled collagen binding to the DS domain of DDR2 (Fig. 4). There are several examples in which signaling proteins bind to the IJM, leading to a conformational change of the extracellular domain to increase the receptor–ligand interaction.[28,29] In the case of integrin, ligand affinity is mediated by talin binding to the β-cytoplasmic tail, which leads to a conformational change of the ectodomain and regulates ligand-binding affinity.[28] In addition, the intracellular domain of EGFR can regulate the binding affinity for EGF.[29] Few proteins, including Shc, SHP-2 and Src, have been identified as interacting proteins with the IJM of DDR2. Of these proteins, Shc and SHP-2 did not seem to be involved in DDR2 activation. Furthermore, an interaction between Src and DDR2 may not be mediated by the JM2 domain, although Src kinase activity is required for maximal activation of DDR2.[33,36,37] Interestingly, a recent report identified three phosphorylated sites, including a previously undescribed serine phosphorylation site, in the JM2 domain of DDR2, and some were phosphorylated even in the inactive state.[36] This suggests that additional proteins are recruited to the JM2 domain. Thus, future studies should identify the proteins that bind to the JM2 region and investigate their relevance to DDR2 activation.

Tyrosine kinase inhibitors (TKIs) have been used to disrupt the intracellular kinase activities of RTKs, including DDRs, as targeted cancer therapy.[3,38] Some agents such as dasatinib and imatinib inhibit collagen-induced DDR1 and DDR2 activation by blocking kinase activity.[39,40] Treatment with TKIs simultaneously inhibits several RTKs, including DDRs. For example, one TKI, LCB 03–0110, suppresses DDR1 and DDR2 activation and may effectively inhibit other RTKs.[41] These multitarget TKIs may be more convenient for use in patients but are associated with toxicity, such as serosal inflammation and cardiotoxicity, due to off-target kinase inhibition.[42,43] Therefore, the development of more specific agents targeting specific RTKs is required for improved cancer treatments. Based on our results, JM2 overexpression specifically inhibits DDR2 activity without affecting DDR1 under collagen treatment conditions (Fig. 5*c*), indicating that JM2 is highly specific for DDR2. This finding suggests that anticancer agents such as polypeptides or chemicals containing the JM2 sequence could be candidates for the treatment of DDR2-positive cancers. However, in terms of cellular delivery efficiency, the possibility and significance of the use of TM-JM1-JM2 peptide in cancer treatment is limited. Thus, the identification of small chemical inhibitors that are involved in the blockage of JM2-mediated dimerization might be useful against DDR2-mediated cancer progression.

Acknowledgement

This research was also supported by the Chung-Ang University Research Scholarship Grants in 2012.

References

1. Bennasroune A, Gardin A, Aunis D, et al. Tyrosine kinase receptors as attractive targets of cancer therapy. *Crit Rev Oncol Hematol* 2004;50:23–38.
2. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. *Nature* 2001;411:355–65.
3. Gschwind A, Fischer OM, Ullrich A. The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat Rev Cancer* 2004;4:361–70.
4. Lemmon MA, Schlessinger J. Regulation of signal transduction and signal diversity by receptor oligomerization. *Trends Biochem Sci* 1994;19:459–63.
5. Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* 2000;103:211–25.
6. Cammett TJ, Jun SJ, Cohen EB, et al. Construction and genetic selection of small transmembrane proteins that activate the human erythropoietin receptor. *Proc Natl Acad Sci USA* 2010;107: 3447–52.
7. Schuster B, Meinert W, Rose-John S, et al. The human interleukin-6 (IL-6) receptor exists as a preformed dimer in the plasma membrane. *FEBS Lett* 2003;538:113–16.
8. Yu X, Sharma KD, Takahashi T, et al. Ligand-independent dimer formation of epidermal growth factor receptor (EGFR) is a step separable from ligand-induced EGFR signaling. *Mol Biol Cell* 2002;13:2547–57.
9. Hubbard SR. Juxtamembrane autoinhibition in receptor tyrosine kinases. *Nat Rev Mol Cell Biol* 2004;5:464–71.
10. Red Brewer M, Choi SH, Alvarado D, et al. The juxtamembrane region of the EGF receptor functions as an activation domain. *Mol Cell* 2009;34:641–51.
11. Vogel W, Gish GD, Alves F, et al. The discoidin domain receptor tyrosine kinases are activated by collagen. *Mol Cell* 1997;1:13–23.
12. Carafoli F, Hohenester E. Collagen recognition and transmembrane signalling by discoidin

domain receptors. *Biochim Biophys Acta* 2012; 1834:2187–94.
13. Vogel WF, Abdulhussein R, Ford CE. Sensing extracellular matrix: an update on discoidin domain receptor function. *Cell Signal* 2006;18: 1108–16.
14. Fu HL, Valiathan RR, Arkwright R, et al. Discoidin domain receptors: unique receptor tyrosine kinases in collagen-mediated signaling. *J Biol Chem* 2013;288:7430–7.
15. Noordeen NA, Carafoli F, Hohenester E, et al. A transmembrane leucine zipper is required for activation of the dimeric receptor tyrosine kinase DDR1. *J Biol Chem* 2006;281: 22744–51.
16. Yoshimura T, Matsuyama W, Kamohara H. Discoidin domain receptor 1: a new class of receptor regulating leukocyte-collagen interaction. *Immunol Res* 2005;31:219–30.
17. Hammerman PS, Sos ML, Ramos AH, et al. Mutations in the DDR2 kinase gene identify a novel therapeutic target in squamous cell lung cancer. *Cancer Discov* 2011;1:78–89.
18. Provenzano PP, Inman DR, Eliceiri KW, et al. Collagen density promotes mammary tumor initiation and progression. *BMC Med* 2008;6:11.
19. Valiathan RR, Marco M, Leitinger B, et al. Discoidin domain receptor tyrosine kinases: new players in cancer progression. *Cancer Metastasis Rev* 2012;31:295–321.
20. Ford CE, Lau SK, Zhu CQ, et al. Expression and mutation analysis of the discoidin domain receptors 1 and 2 in non-small cell lung carcinoma. *Br J Cancer* 2007;96:808–14.
21. Yang SH, Baek HA, Lee HJ, et al. Discoidin domain receptor 1 is associated with poor prognosis of non-small cell lung carcinomas. *Oncol Rep* 2010;24:311–19.
22. Leitinger B. Molecular analysis of collagen binding by the human discoidin domain receptors, DDR1 and DDR2. Identification of collagen binding sites in DDR2. *J Biol Chem* 2003;278: 16761–9.
23. Li N, Zhou J, Weng D, et al. Adjuvant adenovirus-mediated delivery of herpes simplex virus thymidine kinase administration improves outcome of liver transplantation in patients with advanced hepatocellular carcinoma. *Clin Cancer Res* 2007;13:5847–54.
24. Kim JW, Jang SM, Kim CH, et al. New molecular bridge between RelA/p65 and NF-κB target genes via histone acetyltransferase TIP60 cofactor. *J Biol Chem* 2012;287:7780–91.
25. Bai XY, Chen X, Sun AQ, et al. Membrane topology structure of human high-affinity, sodium-dependent dicarboxylate transporter. *FASEB J* 2007;21:2409–17.
26. Hiramoto-Yamaki N, Takeuchi S, Ueda S, et al. Ephexin4 and EphA2 mediate cell migration through a RhoG-dependent mechanism. *J Cell Biol* 2010;190:461–77.
27. Abdulhussein R, McFadden C, Fuentes-Prior P, et al. Exploring the collagen-binding site of the DDR1 tyrosine kinase receptor. *J Biol Chem* 2004;279:31462–70.
28. Wegener KL, Campbell ID. Transmembrane and cytoplasmic domains in integrin activation and protein–protein interactions (review). *Mol Membr Biol* 2008;25:376–87.
29. Macdonald-Obermann JL, Pike LJ. The intracellular juxtamembrane domain of the epidermal growth factor (EGF) receptor is responsible for the allosteric regulation of EGF binding. *J Biol Chem* 2009;284:13570–6.
30. Labrador JP, Azcoitia V, Tuckermann J, et al. The collagen receptor DDR2 regulates proliferation and its elimination leads to dwarfism. *EMBO Rep* 2001;2:446–52.
31. Rikova K, Guo A, Zeng Q, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell* 2007;131: 1190–203.
32. Valencia K, Ormazabal C, Zandueta C, et al. Inhibition of collagen receptor discoidin domain receptor-1 (DDR1) reduces cell survival, homing, and colonization in lung cancer bone metastasis. *Clin Cancer Res* 2012;18:969–80.
33. Ikeda K, Wang LH, Torres R, et al. Discoidin domain receptor 2 interacts with Src and Shc following its activation by type I collagen. *J Biol Chem* 2002;277:19206–12.
34. Hung WC, Tseng WL, Shiea J, et al. Skp2 overexpression increases the expression of MMP-2 and MMP-9 and invasion of lung cancer cells. *Cancer Lett* 2010;288:156–61.
35. Thiel KW, Carpenter G. Epidermal growth factor receptor juxtamembrane region regulates allosteric tyrosine kinase activation. *Proc Natl Acad Sci USA* 2007;104:19238–43.
36. Iwai LK, Payne LS, Luczynski MT, et al. Phosphoproteomics of collagen receptor networks reveals SHP-2 phosphorylation downstream of wild-type DDR2 and its lung cancer mutants. *Biochem J* 2013;454:501–13.
37. Yang K, Kim JH, Kim HJ, et al. Tyrosine 740 phosphorylation of discoidin domain receptor 2 by Src stimulates intramolecular autophosphorylation and Shc signaling complex formation. *J Biol Chem* 2005;280:39058–66.
38. Su J, Yu J, Ren T, et al. Discoidin domain receptor 2 is associated with the increased expression of matrix metalloproteinase-13 in synovial fibroblasts of rheumatoid arthritis. *Mol Cell Biochem* 2009;330:141–52.
39. Rix U, Hantschel O, Durnberger G, et al. Chemical proteomic profiles of the BCR-ABL inhibitors imatinib, nilotinib, and dasatinib reveal novel kinase and nonkinase targets. *Blood* 2007;110: 4055–63.
40. Day E, Waters B, Spiegel K, et al. Inhibition of collagen-induced discoidin domain receptor 1 and 2 activation by imatinib, nilotinib and dasatinib. *Eur J Pharmacol* 2008;599: 44–53.
41. Sun X, Phan TN, Jung SH, et al. LCB 03-0110, a novel pan-discoidin domain receptor/c-Src family tyrosine kinase inhibitor, suppresses scar formation by inhibiting fibroblast and macrophage activation. *J Pharmacol Exp Ther* 2011;340:510–19.
42. Kelly K, Swords R, Mahalingam D, et al. Serosal inflammation (pleural and pericardial effusions) related to tyrosine kinase inhibitors. *Target Oncol* 2009;4:99–105.
43. Cheng H, Force T. Molecular mechanisms of cardiovascular toxicity of targeted cancer therapeutics. *Circ Res* 2010;106:21–34.

10970215, 2014, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ijc.28901 by Chung-Ang University, Wiley Online Library on [10/04/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License