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A novel anti-CCN1 monoclonal antibody suppresses Rac-dependent cytoskeletal reorganization and migratory activities in breast cancer cells

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ABSTRACT

CCN1, a secreted matrix-associated molecule, is involved in multiple cellular processes. Accumulating evidence supports that CCN1 plays an important role in tumorigenesis and progression of breast cancer. In this study, we have developed a novel CCN1 function-blocking monoclonal antibody (mAb), designated YM1B. YM1B binds to human CCN1 with high specificity, recognizing the native CCN1 structure with undisturbed disulfide linkages. Our analyses have mapped the YM1B recognition region to domain IV of CCN1, likely in proximity to the DM site. In breast cancer cells, CCN1 can induce actin reorganization, formation of lamellipodia, and cell migration/invasion through the αV integrins/Rac1/ERK signaling axis; these CCN1-dependent activities can be effectively suppressed by YM1B. Our results also suggest that YM1B may exert its CCN1-blocking effect by perturbing the interaction of CCN1 with vitronectin and fibronectin, which are ligands of αV integrins and instrumental for integrin activation. This CCN1-specific mAb may open a new potential avenue for therapeutic intervention of breast cancer progression.

1. Introduction

CCN1 (original name CYR61), a secreted extracellular matrix (ECM)-associated molecule, belongs to the matricellular CCN family. The CCN proteins, CCN1–CCN6, are composed of four structural domains: an insulin-like growth factor-binding protein-like domain I, a von Willebrand factor type C repeat domain II, a thrombospondin type I repeat domain III, and a cysteine knot-containing domain IV [1–3]. As a matricellular protein, CCN1 plays a minimal structural role in matrix integrity, but serves an important function in regulating cellular activities such as substratum attachment, migration, survival, growth, and gene expression [4]. The plethora of CCN1 activities can be attributed to its interaction with multiple cell surface integrin receptors and proteoglycans [4,5]. By connecting to integrin αV-mediated signaling, CCN1 promotes angiogenesis [4]. In fibroblasts, CCN1 is capable of inducing integrin α6-dependent actin reorganization, which leads to the formation of lamellipodia and filopodia [6]. CCN1 also triggers the activation of signaling molecules in focal complexes and regulates a spectrum of gene expression [6,7].

Dysregulation of CCN1 expression associates with various types of tumors. Overexpression of CCN1 has been found in tissues of breast, pancreas, oral, and prostate cancers [8–11]. It has been shown that forced expression of CCN1 in breast cancer cells enhances the tumorigenic activity [12,13]. Recent studies suggest that upregulation of CCN1 contributes to drug resistance in breast cancer and is a signature feature of cancer microenvironment [14,15]. Silencing of endogenous CCN1 reduces cancer outgrowth [16,17]. Thus, CCN1 is considered as a candidate therapeutic target for cancer treatments [1].

In this study, we set up a functional screening to isolate monoclonal antibodies (mAbs) targeting the CCN1 activity. Using the breast cancer cell model, we examined a panel of mAbs raised against CCN1 for inhibitory effects on the CCN1-induced Rac1-dependent change of cell morphology. We have developed a mAb, designated YM1B, which reduces CCN1-mediated formation of lamellipodia and activation of signaling molecules in focal complexes. YM1B represents a novel function-blocking mAb that effectively inhibits CCN1- and Rac1-dependent invasiveness of cancer cells and may provide an alternative therapeutic agent in breast cancer treatment.

2. Materials and methods

2.1. Reagents, chemicals, and antibodies

Rabbit antibodies against total- and phosphorylated-paxillin, FAK, and Erk1/2 were from Cell Signaling Technology (Boston,

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MA). StrepTactin-HRP conjugate and the anti-StrepII monoclonal antibody were from IBA GmbH (Germany). The Rac1 activation detection kit was from Pierce (Rockford, IL). The function-blocking anti-integrin mAbs, including anti-α6 (GoH3), anti-αV (17E6), and anti-αVβ3 (LM609), were from Millipore (Billerica, MA). CCN6 and other protein reagents were from PeproTech (Rocky Hill, NJ) or BD Biosciences (San Jose, CA).

2.2. Cell culture

MCF7 and MDA-MB-231 human breast cancer cells were cultured in DMEM medium (Life Technologies, NY) supplemented with 10% fetal bovine serum at 37 °C in a humidified incubator containing 5% CO_2.

2.3. Preparation of recombinant native and mutant CCN1 proteins

The Bac-to-Bac baculovirus expression system (Life Technologies, NY) was used for recombinant CCN1 protein production. Secreted CCN1 was collected from the serum-free media and further purified as described previously [18,19]. The structural mutants of human CCN1, previously reported as D125A, SM, DM, and TM [4,19–21], were prepared by substituting the corresponding residues in CCN1 with alanine using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). CCN1 proteins were fused at the C-terminus with the StrepII peptide tag (WSHPQFEK) and purified by StrepTactin affinity chromatography (IBA GmbH, Germany). For CCN1 truncated fragments, the Fc region of IgG was fused to the C-terminus of CCN1 to increase the solubility of the expressed proteins and facilitate purification by Protein A (Millipore, MA). Purified proteins were concentrated by an ultrafiltration column (GE Healthcare, NJ) into a buffer (50 mM phosphate containing 500 mM NaCl, pH 7.0), and sterilized by filtration through a 0.22-μm membrane.

2.4. Production of monoclonal antibodies against CCN1

BALB/c mice were immunized with purified human CCN1 protein; hybridoma clones were established following standard hybridoma protocols using SP2/0 myeloma cells, and screened by indirect ELISA for the production of mAbs reacting with immobilized CCN1. Two CCN1-specific mAbs, YM1B and YM1E, were purified using the Protein G-based affinity chromatography kit (Millipore, MA) for further studies. Isotying analysis indicated that both mAbs belong to the murine IgG1 (κ) class.

2.5. ELISA

CCN1 and other test proteins (10 μg/ml) were coated onto 96-well plates at 4 °C overnight. The plates were washed with PBS, and incubated with a blocking buffer (1% BSA and 0.05% Tween 20 in PBS) for 2 h. After washing, the mAb (0.1–1 μg/ml) was added to each well and allowed to react with the test protein for 1 h. The amount of bound mAb was measured by absorbance at 450 nm after incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody and detection reagents in an ELISA kit (Millipore, MA). For detecting the interaction between CCN1 and specific ECM components, StrepII-tagged CCN1 and HRP-conjugated StrepTactin were used.

2.6. Western blotting

Purified proteins (5 ng/lane) or freshly prepared cell lysates (40 μg/lane) were separated by 10% SDS–PAGE. For detecting interaction with YM1B, dithiothreitol was omitted during sample preparation for non-reducing electrophoresis. Proteins were electro-blotted onto a PVDF membrane (Millipore, MA) and probed with specific antibodies. An ECL kit (Millipore) was used for detection.

2.7. Fluorescence cell staining

Cells were seeded at 30% confluency on coverslips and incubated for 24 h. After fixation in 4% formaldehyde and permeabilization in 0.4% Triton X-100, cells were washed with PBS and incubated with rhodamine-phalloidin for F-actin, or specific primary antibodies followed by FITC-conjugated secondary antibodies (Millipore, MA). Cell nuclei were counterstained by DAPI. Images were captured under the Olympus FV1000 laser scanning confocal microscope.

2.8. Transwell assays

Cell migration was assayed using transwell inserts with 8-μm pores (BD Bioscience, San Jose, CA). Cells were harvested by mild trypsinization (0.02%), resuspended at 5×10^5/300 μl in a medium with or without the addition of test proteins, loaded onto the upper chamber, and allowed to migrate for 24 h. Cells that migrated were counted in at least 10 random fields under a microscope (at 200× magnification); the average cell number per field was presented. Cell invasion was assayed as the above except that the transwell filter was precoated with Matrigel.

2.9. Data analysis

All experiments were independently performed for at least three times. Results were expressed as mean ± SD and statistical significance was determined by the Student's *t* test.

3. Results

3.1. CCN1 induces cytoskeletal reorganization and enhances migration of breast cancer cells through αV integrins/Rac/ERK signaling

CCN1 has been shown to induce focal complexes along with persistent formation of filopodia and lamellipodia in fibroblasts [6]. We investigated whether CCN1 induces cytoskeletal rearrangements in MCF7 breast carcinoma cells which express low levels of endogenous CCN1. Direct addition of the CCN1 protein induced lamellipodia formation and concomitant recruitment of paxillin to tips of lamellipodia (Fig. 1A). Analysis of several molecular components involved in lamellipodia formation showed that CCN1 increased the abundance of GTP-bound Rac1 and the phosphorylation of paxillin and Erk1/2. These CCN1 effects were diminished by the simultaneous presence of a Rac1 inhibitor (Fig. 1B). CCN1 induced the migration of MCF7 cells, which could be abrogated by an inhibitor for either Rac1 or ERK (Fig. 1C). These results indicate that CCN1 is capable of inducing cytoskeletal reorganization and cell migration via Rac1 and Erk1/2-dependent mechanisms in MCF7 cells. It has been established that CCN1 employs distinct integrin receptors to mediate multiple cellular functions [4]. We investigated the involvement of integrins in the above-mentioned CCN1 actions using function-blocking mAbs against specific integrins. We found that anti-αV and anti-αVβ3, but not anti-α6, mAbs inhibited CCN1 effects (Fig. 1D and E), suggesting that CCN1-mediated activation of Rac1 and downstream signaling is largely mediated by αV integrins but not α6 integrins in breast cancer cells.

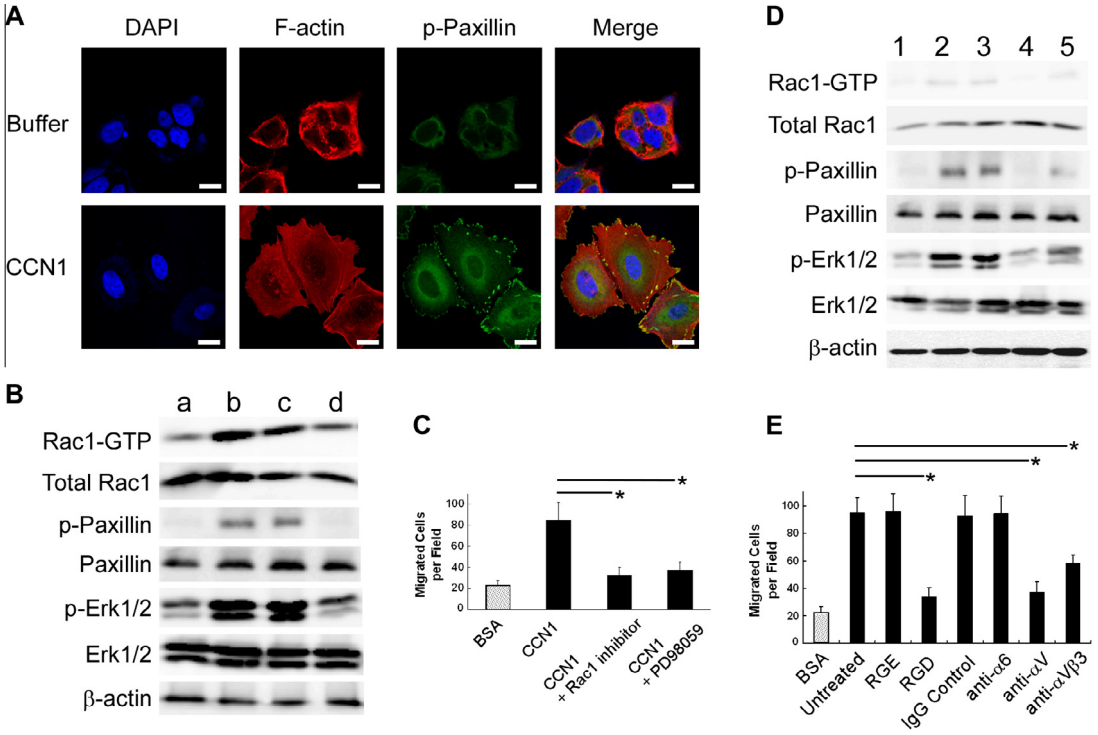

Fig. 1. CCN1 induces morphological changes with formation of prominent lamellipodia and promotes cell migration through activating a signaling axis involving αV integrins, Rac1, and focal complex-associated effectors. (A) CCN1 induces actin cytoskeleton reorganization along with formation of prominent lamellipodia. MCF7 cells were cultured on glass coverslips, treated with CCN1 protein (4 μg/ml) overnight, and stained with rhodamine-conjugated phalloidin for F-actin, or undergone immunofluorescence staining to detect phospho-paxillin. Cell nuclei were visualized with DAPI staining. Scale bar, 20 μm. (B) CCN1 induces the activation of Rac1, paxillin, and Erk1/2. Lysates were prepared from cells under indicated treatments and subjected to Rac activation assays or Western analysis using specific antibodies for active phosphorylated forms of paxillin (p-paxillin) and p42/44 MAP kinase (p-Erk1/2). Treatments used: lane a, none; b, recombinant CCN1; c, CCN1 and vehicle buffer; d, CCN1 and Rac1 inhibitor. (C) Exogenous CCN1 stimulates cell migration in a Rac1/MAP kinase dependent manner. Cells undergone indicated treatments were subjected to transwell assays for migration. BSA, bovine serum albumin; PD98059, MAP kinase inhibitor. (D) CCN1-induced activation of Rac1 and downstream signaling is mainly mediated by αV integrins. Lysates were prepared from cells under indicated treatments and subjected to Western analysis. Treatments used: lane 1, buffer; 2, recombinant CCN1 alone; 3, CCN1 and anti-α6 mAb; 4, CCN1 and anti-αV mAb; 5, CCN1 and anti-αVβ3 mAb. (E) CCN1-indued cell migration is inhibited by addition of the RGD peptide or anti-αV mAbs. The results presented are the average numbers of migrated cells counted per microscopic field. Data (mean ± SD) shown in bars are from three independent experiments. $^{*}P < 0.05$.

3.2. Generation of a novel CCN1-specific function-blocking monoclonal antibody

We next set out to generate CCN1-specific mAbs and screen for those that could inhibit CCN1-induced activities in breast cancer cells. We found a novel mAb, designated YM1B, able to inhibit the CCN1-induced lamellipodia formation (Fig. 2A). YM1B was purified by Protein G-based affinity chromatography to apparent homogeneity as shown by SDS–PAGE (Fig. S1). ELISA showed that YM1B bound specifically to human CCN1, but not to other members of the CCN family or a panel of matrix proteins (Fig. 2B). The binding between YM1B and the immobilized CCN1 was dose-dependent, with an EC50 (~0.175 nM) comparable to that between the commercial anti-StrepII mAb and StrepII-tagged CCN1 (Fig. 2C). Western analysis on proteins separated under non-reducing conditions showed that YM1B reacted with both purified CCN1 and endogenous CCN1 in lysates from either the CCN1-overexpressing MCF7 or the highly invasive MDA-MB-231 cells (Fig. 2D). No signals could be detected when Western blotting was performed on proteins separated under reducing conditions (not shown). Moreover, YM1B failed to detect CCN1 that was pretreated with disulfide reducing or oxidizing agents, or PDI (an enzyme that catalyzes disulfide rearrangement) (Fig. 2E). Together, these results suggest that YM1B is a CCN1-specific function-blocking mAb that recognizes native CCN1 with intact disulfide bonds.

3.3. YM1B inhibits CCN1-dependent Rac1 activation and intracellular signaling in MCF7 cells and MDA-MB-231 breast cancer cells

We further characterized the effects of YM1B on CCN1 functions in breast cancer cells. Immunofluorescence staining of MCF7 cells showed that YM1B reduced the CCN1-induced formation of lamellipodia and phosphorylation of paxillin (Fig. 3A). YM1B inhibited the CCN1-stimulated activation of Rac1, as demonstrated by the lowered levels of GTP-bound Rac1 (Fig. 3B). Western blotting for phosphorylated active forms of signaling molecules showed that YM1B abrogated the CCN1-induced activation of two focal complex proteins, paxillin and FAK, as well as Erk1/2 (Fig. 3C). CCN1-stimulated cell migration was also decreased by YM1B (Fig. 3D).

We next tested whether YM1B can inhibit the cellular activities of the highly invasive MDA-MB-231 breast cancer cells, which express high levels of endogenous CCN1. Silencing of endogenous CCN1 in MDA-MB-231 cells suppressed cell spreading and reduced Rac1 activity (not shown). Consistent with the notion that YM1B is a CCN1 function-blocking mAb, addition of YM1B reduced cell spreading and the formation of lamellipodia (Fig. S2A), lowered the abundance of GTP-bound Rac1 (Fig. S2B), and downregulated the levels of phosphorylated paxillin, FAK, and Erk1/2 (Fig. S2C). In addition, results from scratch wound healing assays (Fig. S2D) and transwell invasion assays (Fig. S2E) demonstrated that YM1B inhibited the migration and invasion of MDA-MB-231 cells, respectively. Taken together, our findings indicate that YM1B hinders the

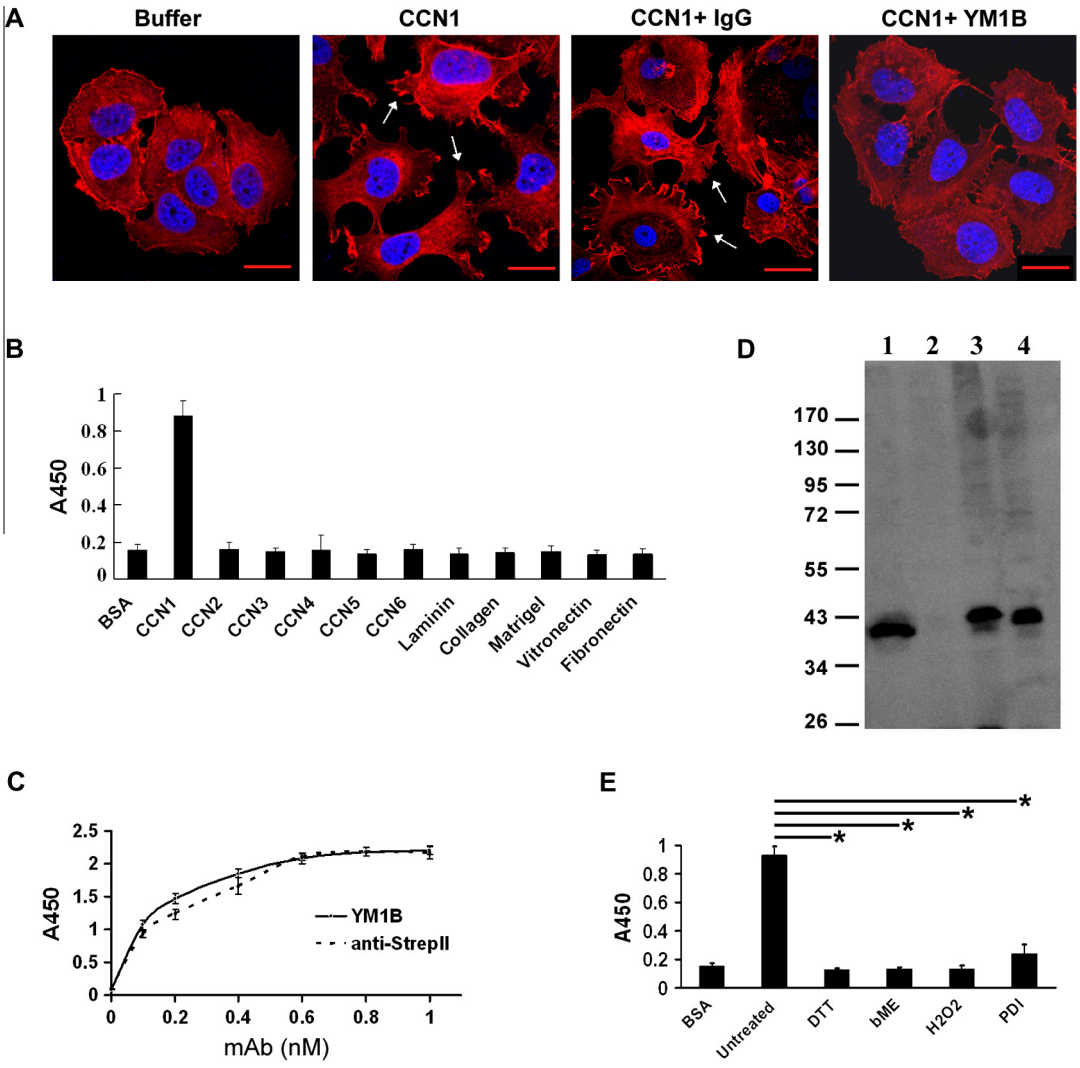

Fig. 2. Characterization of the CCN1-specific YM1B mAb. (A) YM1B suppresses the CCN1-induced formation of lamellipodia. MCF7 cells were cultured on coverslips, treated with buffer or CCN1 (4 μg/ml) in the presence of control murine IgG or YM1B (20 μg/ml) for 16 h, and stained with phalloidin for F-actin. Arrows, CCN1-induced lamellipodia. Scale bar, 20 μm. (B) YM1B binds specifically to CCN1. YM1B was added onto the 96-well plate pre-coated with indicated ECM molecules for 1 h, and ELISA was performed to evaluate the binding of YM1B to the test protein. The absorbance at 450 nm is used as a measurement of binding. (C) YM1B binds to StrepII-tagged CCN1. The StrepII-tagged CCN1 protein (5 μg/ml) was immobilized on ELISA plates. Various doses of YM1B were applied into microtiter wells. Anti-StrepII mAb was assayed for comparison. (D) YM1B recognizes native CCN1. Samples were subjected to non-reducing SDS–PAGE and Western blotting using YM1B (1 μg/ml) as the primary antibody. Lanes: 1, purified recombinant CCN1 (5 ng); 2, MCF7 cell lysates; 3, CCN1-overexpressing MCF7 cell lysates; 4, MDA-MB-231 cell lysates (40 μg). (E) YM1B recognizes CCN1 with intact disulfide bonds. The microtiter wells were coated with CCN1 (10 μg/ml), and incubated with various reagents for 60 min. After washing, binding of YM1B was detected as in C. DTT, dithiothreitol (1 mM); bME, beta-mercaptoethanol (5 mM); H_2O_2, hydrogen peroxide (0.1 M); PDI, protein disulfide isomerase (10 μM). Shown are data (mean ± SD) obtained from three independent experiments. *$P < 0.05$ compared to the control group.

CCN1-dependent activation of effectors involved in cytoskeletal rearrangement and invasiveness of breast cancer cells.

3.4. YM1B recognizes an epitope near the DM region in domain IV of CCN1

We next mapped the YM1B-binding site in CCN1. A series of CCN1 variants carrying truncation or point mutations were generated; these CCN1 mutants were tagged with the immunoglobulin Fc fragment or the StrepII peptide (Fig. 4A). ELISA results suggested that domain IV of CCN1 was necessary and sufficient for YM1B binding, as a mutant form devoid of domain IV (D123-Fc) was unable while domain IV alone (D4-Fc) was able to bind to YM1B (Fig. 4B). CCN1-DM and TM, two structural mutants harboring amino acid mutations in domain IV and known to be inactive for receptor binding [4], were incapable of interacting with YM1B. By contrast, CCN1-D125A and SM, which bear mutated residues in domain II and III, respectively, could still interact with YM1B. Western analysis demonstrated that YM1B could not react with CCN1-DM, -TM, or D123-Fc (Fig. 4C), while another mAb specific to domain IV of CCN1, i.e., YM1E, could recognize all the tested CCN1 mutants except for the domain IV deletion mutant, D123-Fc (Fig. S3). Together, these results mapped the YM1B-binding region to domain IV of CCN1, and suggested an epitope recognized by YM1B near the DM region.

CCN1 has been shown to interact with vitronectin [22], but the physiological significance of this interaction is unclear. We found that CCN1 could bind to vitronectin and fibronectin, which are two ECM molecules interacting with αV integrins; in comparison, interaction of CCN1 with laminin, which binds mainly to α6 integrins, was much weaker (Fig. 4D). Results also suggest that domain IV of CCN1 is important for ECM interaction, as D4-Fc but not D123-Fc was able to bind to ECM molecules (Fig. 4D). YM1B inhibited the binding of CCN1 to vitronectin or fibronectin, but

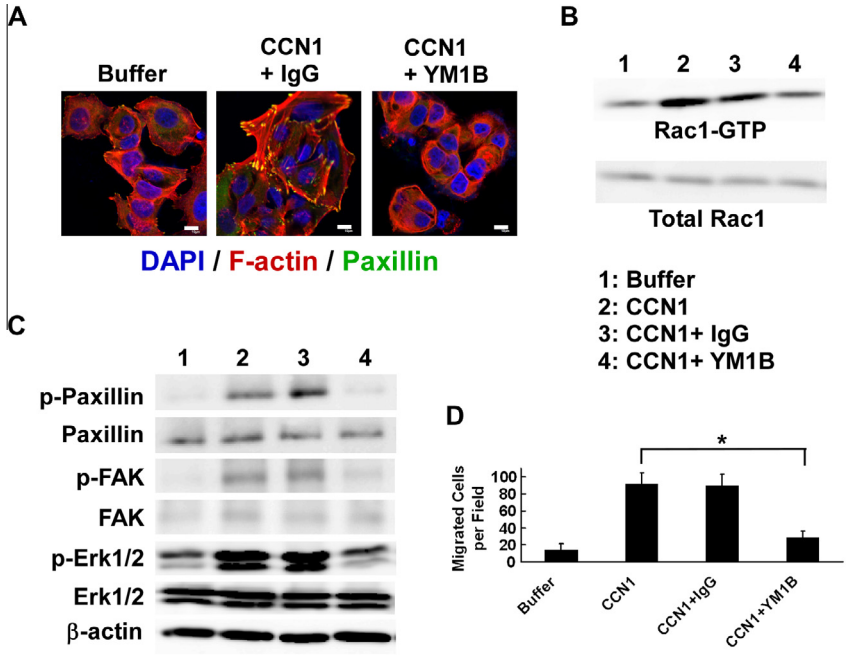

Fig. 3. YM1B blocks CCN1-induced intracellular signaling and cell migration in MCF7 cells. (A) YM1B inhibits CCN1-induced cytoskeletal changes. Cells were treated with CCN1 (4 μg/ml) and YM1B (20 μg/ml) or as indicated, and subsequently processed and stained for nuclei (DAPI), F-actin, and phospho-paxillin. (B) CCN1-induced increase of Rac1 activity can be abolished by YM1B. Immunoblot analysis was performed on lysates prepared from cells after indicated treatments. (C) YM1B suppresses the CCN1-induced activation of signaling molecules relevant to formation of focal complexes. Cells were treated as indicated in B. and lysates prepared from these cells were subjected to Western analysis for phosphorylated paxillin (p-paxillin), focal adhesion kinase (p-FAK), and ERK1/2 (p-ERK1/2). (D) YM1B suppresses CCN1-induced cell migration. Cells treated as indicated were assessed for migration activity using the transwell migration assay. Results are the average cell numbers per microscopic field. Bars represented data (mean ± SD) from three independent experiments. $^{*}P < 0.05$.

had no significant effects on the weak CCN1-laminin interaction (Fig. 4E). We also observed that CCN1-overexpressing breast cancer cells displayed enhanced cell spreading and mobility on fibronectin- or vitronectin- but not laminin-coated coverslips, and these CCN1/ECM-induced phenotypes were abrogated by YM1B (Fig. S4A–C). Together, our findings suggest that YM1B blocks CCN1-induced αV integrins-dependent downstream signaling in breast cancer cells by disrupting the interaction between CCN1 and ECM ligands of αV integrins.

4. Discussion

CCN1 has been reported to induce cytoskeletal reorganization in fibroblast cells via a Rac GTPase-dependent mechanism [6]. Activation of Rac is also shown to be involved in breast tumorigenesis and metastasis [23,24]. We have now demonstrated that CCN1 induces the activation of Rac1 and phosphorylation of focal complex-associated signaling molecules in breast cancer cells to promote migration and invasion. We also provide evidence indicating that these CCN1 effects are mediated mainly via αV but not α6 integrins. Our results have defined a CCN1/αV integrins/Rac1/ERK signaling axis for mediating invasiveness of breast cancer cells.

To our best knowledge, the novel mAb YM1B we developed in this study represents the first reported CCN1 function-blocking mAb that targets CCN1/αV integrins/Rac1/ERK signaling. Our analyses mapped domain IV of CCN1 as the necessary and sufficient interacting region for YM1B. We synthesized peptides containing overlapping sequences encompassing domain IV, and none of these peptides were capable of YM1B binding (not shown), suggesting that the epitope recognized by YM1B in domain IV is conformational and might not be defined by consecutive amino acids. A previous report has also pointed out the importance of domain IV in CCN1-mediated spreading and migration of breast cancer cells [25]; however, the underlying mechanisms have not been elucidated. Here we show that domain IV mediates the interaction of CCN1 with fibronectin and vitronectin, i.e. two ECM molecules which are ligands of αV integrins; the domain IV-targeting YM1B specifically inhibits CCN1 binding to vitronectin and fibronectin, but does not affect binding to the α6 integrin ligand laminin. Taking into consideration the above-mentioned conclusion that CCN1 effects in breast cancer cells are dependent on αV integrins, we speculate that CCN1 facilitates vitronectin/fibronectin-dependent activation of αV integrins. In this scenario, YM1B disrupts the interaction between CCN1 and vitronectin/fibronectin, and therefore perturbs the activation of αV integrins and downstream signaling, resulting in the suppression of CCN1-induced Rac1-dependent activation of signaling molecules involved in cytoskeletal reorganization and cell migration/invasion.

Function-blocking mAbs affecting the interaction of CCN1 with specific receptor(s) and binding partners likely have specific effects on selective CCN1-mediated activities. Consistent with this notion, another CCN1-specific mAb which recognizes an epitope in domain II of CCN1 appears to affect a mechanism distinct from the signaling axis perturbed by YM1B; it has been shown that this domain II-targeting mAb inhibits malignant phenotypes of breast cancer cells through downregulating the phosphorylation of Akt and ERKs, as well as upregulating the MMP1 inhibitors, TIMP1 and TIMP2 [26]. In addition, as domain IV of CCN1 mediates other cellular activities such as apoptosis and cellular senescence [4], it is of interest to further investigate whether YM1B affects these domain IV-linked CCN1 functions as well. In this respect, YM1B and other CCN1 function-blocking mAbs may serve as useful reagents for structure–function studies on the CCN1 protein.

Breast cancer is among the leading causes of cancer-related mortality worldwide [27], and novel therapeutic agents are in demand to improve its treatment. ECM molecules are important components of the tumor microenvironment and play significant roles in regulating cancer cell survival and growth [28–30]. Expression of the matricellular protein CCN1 is induced by hypoxia and tu-

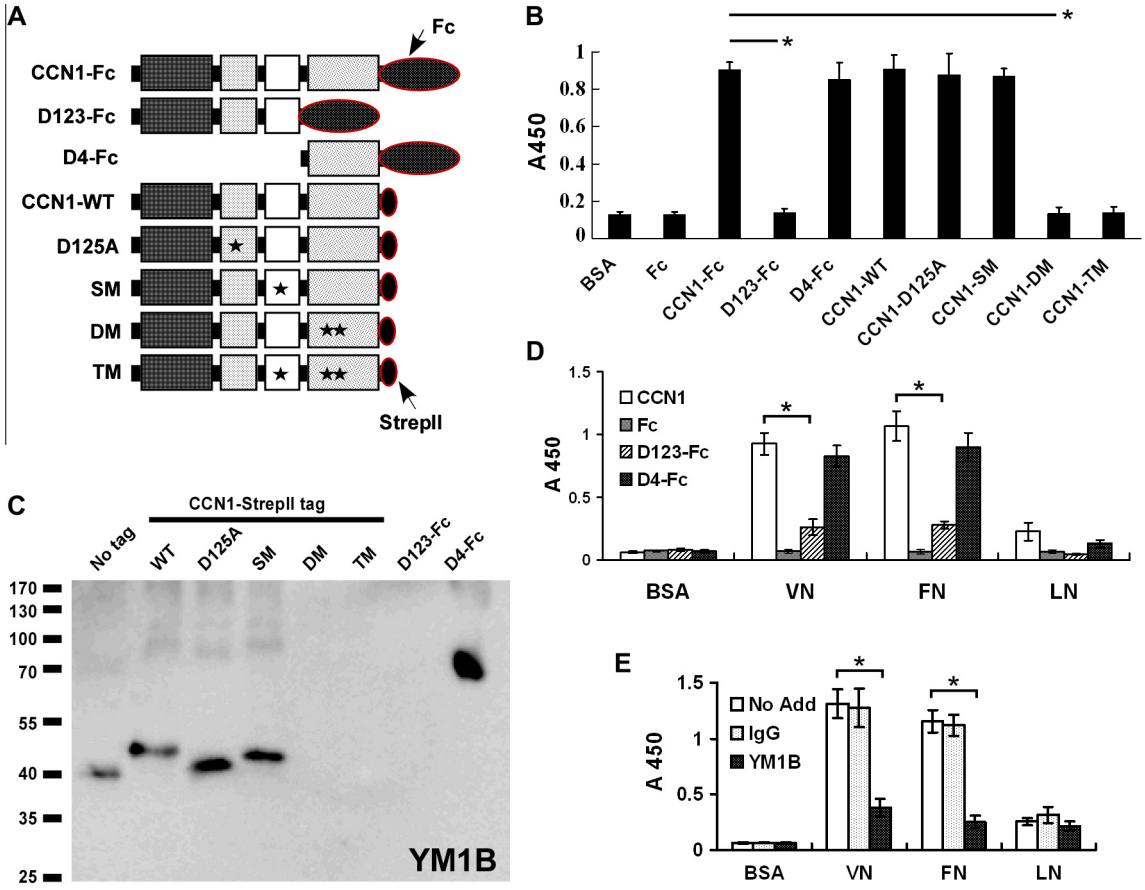

Fig. 4. Characterization of the YM1B–CCN1 interaction. (A) A schematic diagram of the four-domain structure of CCN1, showing the truncation/mutation constructs used in this study. Asterisks indicate the positions of mutated amino acids. Truncation mutants were fused with the Fc fragment while point mutants were fused with a StrepII peptide. (B) Domain IV of CCN1 is required for YM1B interaction. CCN1 variants were immobilized in microtiter wells and binding of YM1B was analyzed by ELISA. (C) YM1B binds to native CCN1. Western blot analysis was performed on proteins separated under non-reducing conditions; each lane contained 5 ng of purified proteins. No tag, CCN1 protein without a peptide tag. (D) Interaction between CCN1 and ECM proteins. CCN1 (1 μg/ml) was incubated with immobilized ECM proteins, and the binding was measured using ELISA. VN, vitronectin; FN, fibronectin; LN, laminin. (E) YM1B inhibits the binding of CCN1 to specific ECM components. The binding of CCN1 to immobilized test protein was analyzed as in D in the absence or presence of YM1B or control murine IgG (10 μg/ml). Results are mean ± SD from three independent experiments. $^{*}P < 0.05$.

mor-associated macrophages, and the protein is enriched in the secretome of breast cancer cells [31–33]. Indeed, CCN1 has been implicated in breast cancer tumorigenesis and progression [1,4]. Our finding that addition of YM1B inhibits the migration/invasion of CCN1-overexpressing breast cancer cells suggests a possible application of YM1B in repressing CCN1-mediated malignant behaviors of cancer cells. We are currently testing whether YM1B is effective against the progression of breast cancers *in vivo* using animal models. In conclusion, the development and characterization of the CCN1 function-blocking mAb YM1B in this study highlights the possibility of targeting CCN1/integrins/Rac1/ERK signaling as a novel strategy for the therapeutic intervention of breast cancers.

Acknowledgments

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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.2013.04.045.

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