

# An RGD Motif Present in Cadherin 17 Induces Integrin Activation and Tumor Growth\*

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Rubén A. Bartolomé<sup>†1</sup>, Alberto Peláez-García<sup>‡2</sup>, Inmaculada Gomez<sup>§</sup>, Sofía Torres<sup>‡3</sup>,  
María Jesús Fernandez-Aceñero<sup>¶</sup>, Beatriz Escudero-Paniagua<sup>‡2</sup>, J. Ignacio Imbaud<sup>§</sup>, and J. Ignacio Casal<sup>†4</sup>

From the <sup>†</sup>Department of Cellular and Molecular Medicine, Centro de Investigaciones Biológicas (CIB-CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain, <sup>§</sup>Protein Alternatives SL, Tres Cantos, Madrid, Spain, and the <sup>¶</sup>Department of Pathology, Fundación Jiménez Díaz, Madrid, Spain

**Background:** The interaction between cadherin 17 and  $\alpha 2\beta 1$  integrin promotes cell adhesion and proliferation.

**Results:** Cadherin 17 contains an RGD motif that constitutes the critical switch for integrin binding and activation.

**Conclusion:** The cadherin RGD motif is a critical ligand for tumor growth and metastasis.

**Significance:** Cadherin 17 is the first known integrin-ligand RGD-cadherin. Other RGD-cadherins might play important roles in cancer metastasis.

Little is known about the mechanism of integrin activation by cadherin 17 (CDH17). Here we observed the presence of a tripeptide motif, RGD, in domain 6 of the human CDH17 sequence and other cadherins such as cadherin 5 and cadherin 6. The use of CDH17 RAD mutants demonstrated a considerable decrease of proliferation and adhesion in RKO and KM12SM colon cancer cells. Furthermore, RGD peptides inhibited the adhesion of both cell lines to recombinant CDH17 domain 6. The RGD motif added exogenously to the cells provoked a change in  $\beta 1$  integrin to an active, high-affinity conformation and an increase in focal adhesion kinase and ERK1/2 activation. *In vivo* experiments with Swiss nude mice demonstrated that cancer cells expressing the CDH17 RAD mutant showed a considerable delay in tumor growth and liver homing. CDH17 RGD effects were also active in pancreatic cancer cells. Our results suggest that  $\alpha 2\beta 1$  integrin interacts with two different ligands, collagen IV and CDH17, using two different binding sites. In summary, the RGD binding motif constitutes a switch for integrin pathway activation and shows a novel capacity of CDH17 as an integrin ligand. This motif could be targeted to avoid metastatic dissemination in tumors overexpressing CDH17 and other RGD-containing cadherins.

Cadherin 17 (CDH17), also known as liver-intestine cadherin, is a non-canonical, 7D-domain cadherin. Its sequence is formed by seven extracellular domains and a very short cytoplasmic domain (1). CDH17 is present in the fetal liver and gastrointestinal tract, exhibiting elevated expression during

embryogenesis (2). The gene is silenced in the adult healthy liver and gut. However, CDH17 is expressed again in gastric cancer, esophagus carcinoma, pancreatic cancer (3), and hepatocarcinoma (4). In primary colon cancer tumors, poorly differentiated tumors, and lymph nodes, CDH17 is expressed at low levels (5). However, CDH17 expression is increased in patients with metastasis and is correlated with a poor prognosis, suggesting an association between CDH17 expression and final hepatic colonization during late stages of metastasis (6, 7).

CDH17 expression was increased in highly metastatic KM12SM colon cancer cells (8). An exhaustive proteomic analysis of cell membrane proteins in these cells detected only five integrin subunits:  $\alpha 2$ ,  $\alpha 6$ ,  $\alpha v$ ,  $\beta 1$ , and  $\beta 4$  (8). No expression of other integrins in epithelial colon cancer cells has been described, except for some  $\beta 6$  integrin constructs. CDH17 was part of a large protein complex containing, among other proteins,  $\alpha 2\beta 1$  and  $\alpha 6\beta 4$  integrins in colorectal cancer cells (7). After immunoprecipitation of CDH17, only  $\alpha 2$ ,  $\alpha 6$ ,  $\beta 1$ , and  $\beta 4$  were found, but not  $\alpha v$ . Although  $\alpha 6\beta 4$  integrin was present in the complex, only the interaction with  $\alpha 2\beta 1$  triggered the integrin signaling pathway and caused the activation of the focal adhesion kinase (FAK),<sup>5</sup> Ras, ERK1/2, and cyclin D1 to increase cell adhesion and proliferation. In agreement with our data, it has been described that  $\alpha 2$  integrin mediates collagen type IV-dependent activation of FAK and mediates selective liver metastasis (9). We also noticed strong binding of KM12SM cells to collagen IV but not to collagen type I (7).

Integrins are a well known family of cell adhesion molecules that serve as receptors for extracellular matrix proteins (10). The tripeptide RGD motif is a major recognition site for integrins within some ligands, such as fibronectin or fibrinogen (11, 12). However, although  $\alpha 2\beta 1$  integrin contains an RGD-binding site, their natural ligands, collagens type I and type IV, used a different binding sequence (GFOGER) (13) and are classified as RGD-independent ligands (14, 15). Therefore, the reasons

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<sup>2</sup> Recipient of an FPI fellowship.

<sup>3</sup> Recipient of a Juan de la Cierva program grant.

<sup>4</sup> To whom correspondence should be addressed: Dept. of Cellular and Molecular Medicine, Centro de Investigaciones Biológicas (CIB-CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain. Tel.: 34-91-8373112; Fax: 34-91-5360432; E-mail: icasal@cib.csic.es.

<sup>5</sup> The abbreviations used are: FAK, focal adhesion kinase; Ecd, ectodomain; DOM6, domain 6; RAD, Arg-Ala-Asp; BCECF-AM, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein tetrakis(acetoxymethyl) ester.

## The CDH17-RGD Motif Is Critical for Integrin Activation

for the presence of the RGD-binding site in  $\alpha 2\beta 1$  integrin and its role in activation were unclear. Moreover, the capacity of cadherins to act as integrin ligands in cancer cells was completely unknown.

Here we investigated the presence of integrin-binding sites in CDH17 and discovered the presence of an RGD motif. We decided to evaluate the role of this motif in the activation of integrin signaling by CDH17. To this end, we used RAD mutants of CDH17, recombinant fragments, and synthetic peptides. We provide evidence that CDH17 modulates integrin activity through this motif, provoking a conformational change in the  $\beta 1$  subunit of the integrin that increased its affinity. Experiments with nude mice demonstrated a major role of the RGD motif in tumor growth and dissemination. This report describes, for the first time, the capacity of CDH17 to act as an integrin ligand through an RGD motif to promote integrin activation that results in enhanced tumor proliferation.

### EXPERIMENTAL PROCEDURES

**Cell Lines, Antibodies, and Peptides**—KM12SM human colon cancer cells were obtained directly from the laboratory of Dr. Fidler (MD Anderson Cancer Center). RKO human colon cancer cells and the pancreatic cancer cell lines BxPc3, Capan-1, and PANC1 were purchased from the ATCC. The 786-O kidney clear cell carcinoma cell line was provided by M. J. Calzada (Hospital de la Princesa, Madrid, Spain). As a control we also used the MCF7 breast carcinoma cell line and the SK-MEL-103 and A375 melanoma cell lines. All cell lines were used within 6 months of purchase and cultured in DMEM (Invitrogen) containing 10% FCS (Invitrogen) and antibiotics at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere.

The antibodies anti-CDH17 (H-167), anti-CDH6, anti-CDH5, anti-FAK, anti-RhoGDI, anti- $\alpha 2$ , anti- $\beta 1$ , anti- $\alpha 6$ , anti- $\beta 4$ , and anti- $\alpha v$  integrin were purchased from Santa Cruz Biotechnology. Anti-pFAK, anti-ERK1/2, anti-pERK1/2, and blocking anti- $\beta 1$  integrin (Lia 1/2.1) were from Cell Signaling Technology. Anti- $\beta 1$  integrin specific for high-affinity conformation (HUTS-21) was from BD Biosciences. The synthetic peptide RGDS was purchased from Sigma. The peptides RADS, SILRGDYQD (CDH5), RAIRRGDTEG (CDH16), and VSLRGDTRG (CDH17) were synthesized using solid phase chemistry with a Focus XC instrument (AAPPtec).

**Cloning and Mutagenesis of CDH17, Protein Purification, and Transfections**—CDH17 mRNA from the human colorectal cancer cell line Caco2 was reverse-transcribed by Superscript III first strand synthesis kit (Invitrogen), and the cDNA was RT-PCR-amplified using the primers 5'-AGCTCGAGGATCTGAGTTGATCAATCTGCTTAGTG-3' and 5'-CGGGTACCATGAGATGGTTGTTGCTGAAATAG-3' with Advantage 2 polymerase (Clontech). The PCR product was digested with XhoI and KpnI and cloned into pcDNA3.1. Mutagenesis of CDH17 to change motif <sup>603</sup>RGD<sup>605</sup> into RAD was performed with the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies), using the primers 5'-GGACATAAGCTATTTACTGAGGGCAGACACAAGAGTTGG-3' and 5'-CCAACCTCTTGTGTCTGCCCTCAGTGAATAGCTTATGTCC-3'. The presence of the mutation was confirmed by DNA sequencing. Cells were transiently transfected with

CDH17 WT or CDH17 RAD in pcDNA3.1 using JetPrime (Polyplus Transfection). After 48 h, a fraction of the transfectants was lysed and analyzed by Western blotting to assess the expression of CDH17. Control siRNA and siRNA against CDH17 (SASI\_Hs01\_00166354) were from Sigma-Aldrich.

The recombinant ectodomains of CDH17 (WT and RAD mutant) were expressed with the baculovirus system in insect cells according to standard procedures. Recombinant domains 6 (WT and mutant) were produced in *Escherichia coli* and purified according to standard procedures (16).

**Reverse Transcription PCR**—For CDH6 and CDH20 amplification, cells were lysed in TRIzol reagent (Ambion). RNA was extracted and reverse-transcribed using MoMLV reverse transcriptase (Promega). Amplification of CDH6 was done by PCR using primers 5'-GTCATCACCGACCAGGAAAC-3' and 5'-TGCAGGGTCTGAATCAACTG-3'. For CDH20, the primers were 5'-AGAGGAGCTGGGTTTGGAA-3' and 5'-GCATCTGTGGCTGCTACTTG-3'. The PCR profile was 33 cycles of 30 s at 94 °C, 30 s at 56 °C, and 45 s at 72 °C with TaqDNA polymerase (Invitrogen).

**Cell Adhesion and Soluble Binding Assays**—For cell adhesion, 96-well plates were coated with Matrigel (4  $\mu$ l/ml) (BD Biosciences) or type IV collagen (5  $\mu$ g/ml) (Sigma-Aldrich) in coating buffer (0.1 M NaHCO<sub>3</sub> (pH 8.8)) for 20 h and incubated with adhesion medium (0.4% BSA in serum-free DMEM) for 2 h to block unspecific binding. Cells were starved for 5 h without serum, labeled with BCECF-AM (Invitrogen), detached with 2 mM EDTA in PBS, and resuspended in serum-free DMEM, and then 7  $\times 10^4$  cells in 100  $\mu$ l were added to plates in triplicate and incubated for 25 min. Non-adherent cells were removed by three washes with DMEM. Bound cells were quantified using a fluorescence analyzer (POLARstar Galaxy).

For soluble binding assays, cells were detached, incubated for 40 min with CDH17 ectodomain (10  $\mu$ g/ml) at 37 °C in Ca<sup>2+</sup>-, Mg<sup>2+</sup>-, and Mn<sup>2+</sup>-free Hanks' balanced salt solution medium (Invitrogen), washed, incubated with anti-CDH17 antibodies at 4 °C, washed again, incubated with secondary antibodies, and analyzed by flow cytometry. For cell adhesions to CDH17 domain 6 (2–10  $\mu$ g/ml), adhesion assays in 96-well microtiter plates were performed as above but in medium containing 1 mM MnCl<sub>2</sub>, and cells were washed gently using a multi-channel pipette. For blocking assays, cells were preincubated with anti- $\beta 1$  integrin (5  $\mu$ g/ml) for 10 min before adhesion.

**Affinity Chromatography for  $\alpha 2\beta 1$  Integrin**—1 mg of purified CDH17 domain 6 WT was coupled to a HiTrap NHS 1 ml column (GE Healthcare). KM12SM cell extracts (20 mg) were loaded into the column, incubated for 10 min, and washed with 10 ml of lysis buffer at a flow rate of 0.4 ml/min using an ÄKTA system. Elution was performed with 5 ml of 1.5 mM RGDS peptide.

**Cell Separation**—Magnetic beads coated with protein G (Invitrogen) were incubated with 10  $\mu$ g of anti- $\alpha 2$  integrin for 1 h at 4 °C. 4  $\times 10^5$  RKO cells, knocked down or not knocked down for  $\alpha 2$  integrin, were resuspended in 0.5% BSA in PBS and incubated for 40 min at 4 °C with the coated beads. Cells were separated magnetically, and each population was subjected to Western blot analysis using anti- $\alpha 2$  integrin antibodies to assess the efficacy of isolation.

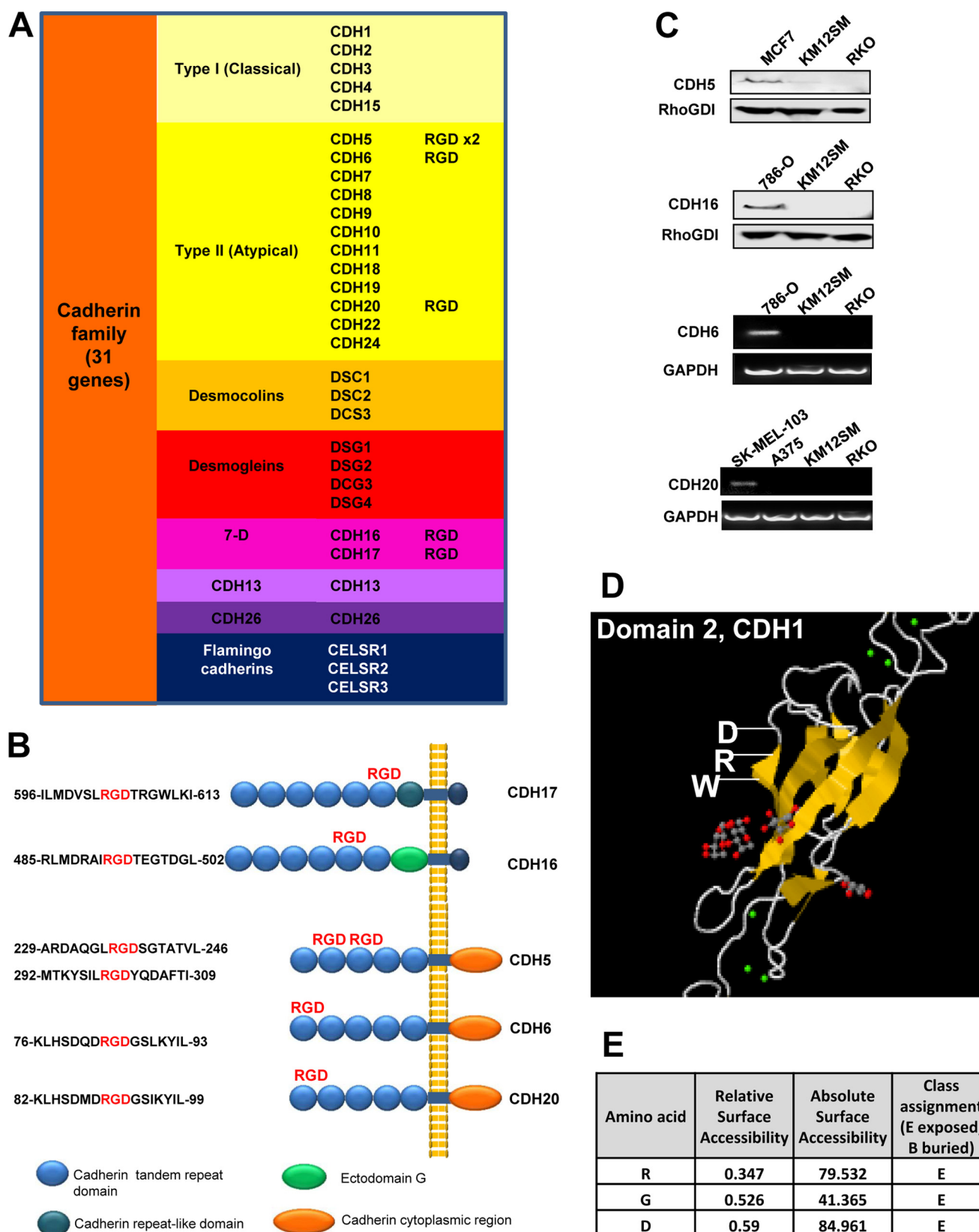
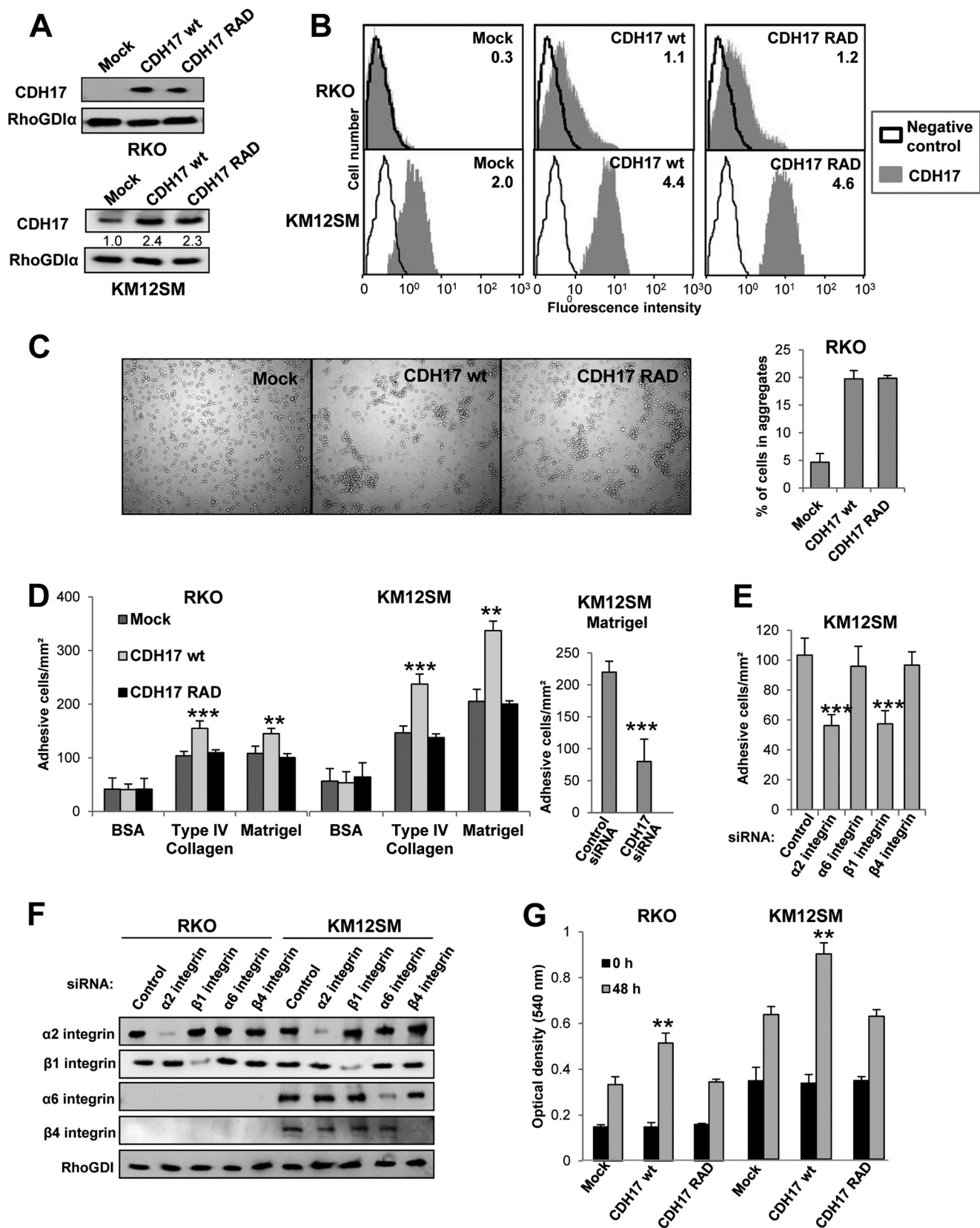


FIGURE 1. **Sequence analysis of the cadherin protein family reveals a RGD motif in several cadherins.** *A*, the different genes of human cadherin families. The presence of RGD motifs is indicated. *B*, structure of five cadherins containing RGD motifs (*right*) and the flanking sequences of such motifs (*left*). *C*, CDH16, CDH6, and CDH20 were not detected, and CDH5 was barely detected in KM125M and RKO cells by Western blot analysis (CDH5 and CDH16) or PCR amplification assays (CDH6 and CDH20). As positive controls we used breast cancer (MCF7), kidney clear cell carcinoma (786-O), and SK-MEL-103 and A375 melanoma cell lines. *D*, according to Jpred3, domain 6 of CDH17 matched most significantly (Blast E-value =  $7 \times 10^{-10}$ ) with domain 2 of CDH1 (PDB code 3Q2V) in PDB modeling. Alignment of the domains showed that the RGD motif of CDH17 matched with exposed WRD residues (372–374) in CDH1. *E*, according to NetSurfP protein surface accessibility and secondary structure predictions software, the three amino acids of the RGD motif in CDH17 were exposed.

## The CDH17-RGD Motif Is Critical for Integrin Activation

**Flow cytometry**—Cells were detached with 2 mM EDTA in PBS, incubated at 4 °C with primary antibodies (10 μg/ml) for 30 min, washed, and incubated with Alexa Fluor 488-labeled

secondary antibodies (anti-mouse IgG or anti-rabbit IgG, Dako). Fluorescence was analyzed in a Coulter Epics XL cytofluorometer. Mean fluorescence intensities for the indicated



antibodies are shown in each figure. As a reference, irrelevant control antibodies (anti-cadherin-11, Santa Cruz Biotechnologies) gave a mean fluorescence intensity of 0.3.

**Cell Aggregation Assays**— $10^5$  Cells were detached with 2 mM EDTA in PBS, resuspended in 100  $\mu$ l of DMEM, and allowed to aggregate for 30 min at 37 °C with constant shaking at 30 rpm. Total cells and cells forming aggregates were counted under a microscope in five different fields.

**Proliferation Assays**—Cells were seeded at  $1 \times 10^4$  cells/well on 96-well plates and incubated for 24–48 h at 37 °C in DMEM with 0.5% serum, followed by 1 h incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.6 mg/ml, Sigma-Aldrich). Cell proliferation was determined by absorbance at 560 nm and compared with control cells collected at time 0.

**Western Blot Analysis and Immunoprecipitation**—Cells were starved for 4 h and allowed to bind to domain 6 of CDH17-coated plates for 45 min. Then, cells were detached, washed, and lysed with 1% Igepal, 100 mM NaCl, 2 mM MgCl<sub>2</sub>, and 10% glycerol in 50 mM Tris-HCl containing proteases and phosphatases inhibitors. Protein extracts were separated in SDS-PAGE gels, transferred to nitrocellulose membranes, and incubated with primary antibodies (1  $\mu$ g/ml), followed by incubation with either HRP anti-mouse IgG (Thermo Scientific) or HRP anti-rabbit IgG (Sigma-Aldrich). Reactive proteins were visualized with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). Densitometric analyses were carried out using Quantity One (Bio-Rad). For immunoprecipitation, cells were lysed, and 500  $\mu$ g of cell lysate was incubated with the indicated antibodies (5  $\mu$ g/ml). The immunocomplexes were captured by adding 50  $\mu$ l of protein G-Sepharose beads (Sigma-Aldrich). After washing, samples were resuspended in loading buffer, boiled for 5 min, centrifuged, and subsequently loaded on 10% SDS-PAGE gels for Western blot analysis.

**Immunohistochemistry**—A total of 48 patients diagnosed and treated for pancreatic cancer in Fundación Jiménez Díaz (Madrid, Spain) between 2003 and 2013 were used for the study. Informed written consent was obtained from all participants, as required and approved by the Research Ethics Committee of the Hospital Fundación Jiménez Díaz (Madrid, Spain). Samples were fixed and stained as described previously (7).

**In Vivo Assays**—*In vivo* assays were performed using Swiss nude mice (Charles River Laboratories). The Ethical Committee of the Consejo Superior de Investigaciones Científicas (Madrid, Spain) approved the protocols used for experimental work with mice. Liver homing assays were performed as described previously (17). Briefly,  $10^6$  cells, KM12SM or RKO, transfected with vectors encoding for CDH17 WT, RAD, or empty vectors (mock) were inoculated intrasplenically into

mice ( $n = 3$ ), which were euthanized after 24 h. RNA was isolated from mouse livers using TRIzol and retrotranscribed. The cDNA was subjected to 30 cycles of PCR with TaqDNA polymerase to amplify human GAPDH, a housekeeping gene. As a loading control, amplification of murine  $\beta$ -actin was performed. For xenografts, tumors were induced by subcutaneous injection of  $5 \times 10^6$  cells, KM12SM or RKO, in PBS with 0.1% glucose into nude mice ( $n = 3$ ). After 10 days, mice were euthanized, and tumors were excised and weighed.

**Statistical Analyses**—Data were analyzed by one-way analysis of variance followed by Tukey-Kramer multiple comparison test. In both analyses, the minimum acceptable level of significance was  $p < 0.05$ .

## RESULTS

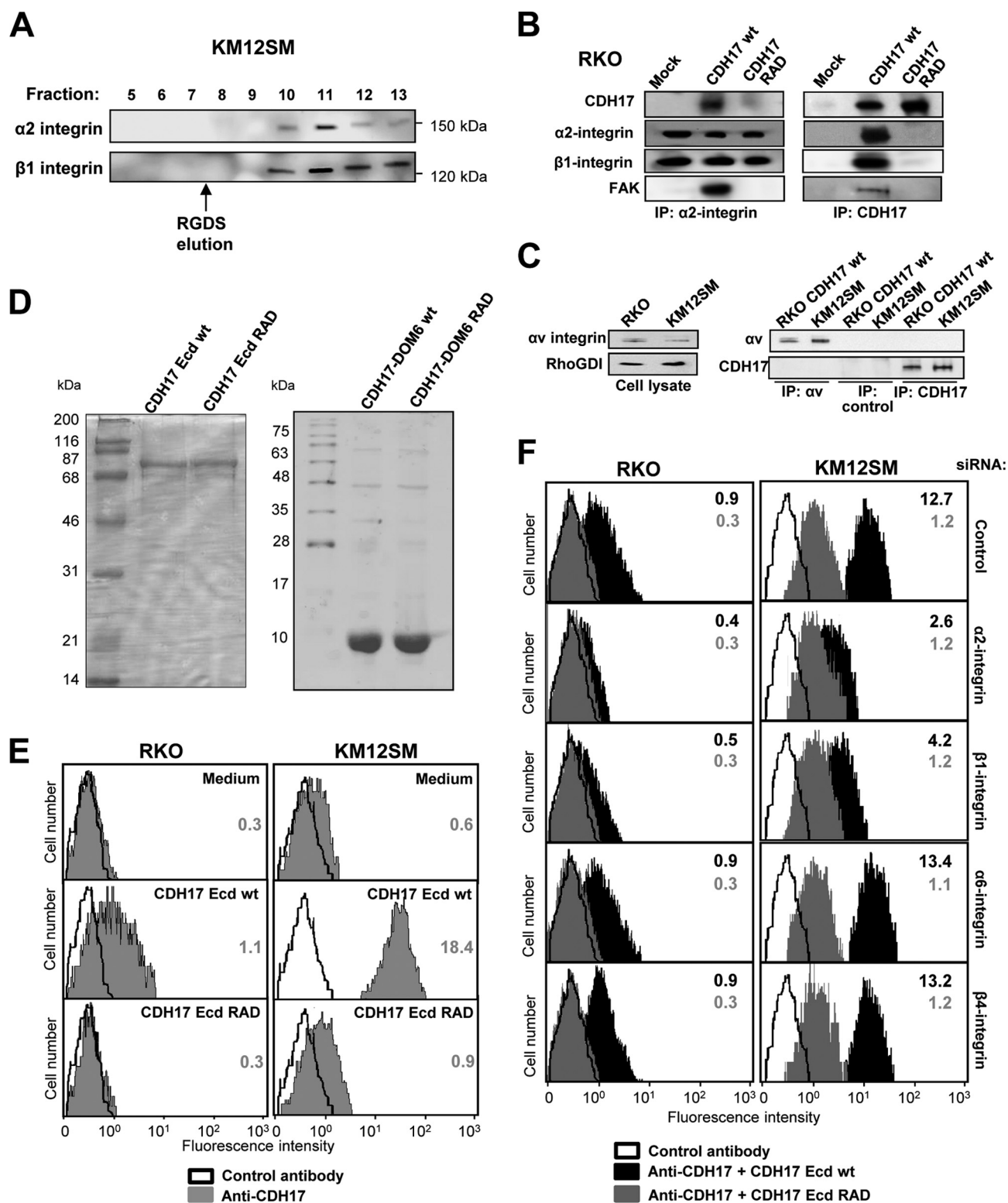
**The Human CDH17 Sequence Contains an RGD Motif**—After revising the sequence of 31 human cadherin genes (Fig. 1A), an RGD motif was found in an extracellular domain of CDH17 and CDH16. Other cadherins containing RGD motifs were VE-cadherin (CDH5), fetal kidney K-cadherin (CDH6), and CDH20. All of them are classified as type II atypical cadherins (Fig. 1A). The RGD motif was present in domain 6 of CDH17 and domain 5 of CDH16 (Fig. 1B). Analysis of CDH17 orthologs showed that the RGD motif in CDH17 was exclusively present in some mammals (data not shown). For instance, mouse CDH17 does not contain an RGD motif. The flanking sequences of RGD (Fig. 1B) are different from other reported consensus sequences for integrin ligands, like fibronectin (18). To discard additional cadherins in our colon cancer cells, we tested the expression of other RGD cadherins either by Western blot analysis or PCR. RKO and KM12SM cells did not express CDH6, CDH16, or CDH20. CDH5 was barely detected in KM12SM cells but not in RKO cells (Fig. 1C). Breast, kidney, and melanoma cancer cells were used as positive controls.

The structure of CDH17 is unknown, but the RGD motif is predicted to be exposed using two different bioinformatic approaches. According to Jpred 3 (19), CDH17 domain 6 matched most significantly with the known structure of CDH1 domain 2. The alignment of both domains showed that the RGD motif matched exposed WRD residues (372–374) in CDH1 (Fig. 1D). Accordingly, NetSurfP software (20), which calculates the surface accessibility of protein residues, predicted an exposition of the RGD motif in CDH17 (Fig. 1E). These predictions suggest the accessibility of the RGD motif for protein-protein interactions.

**Mutation of RGD in CDH17 Reduces Adhesion and Proliferation in Colorectal Cancer Cells but Does Not Affect Aggregation**—To examine the effect of the RGD motif on colorectal cancer cells, vectors encoding for wild-type CDH17 (CDH17-WT) or a

**FIGURE 2. The presence of CDH17 RGD is required for the increase in cell adhesion and proliferation.** A and B, RKO and KM12SM cells were transfected with vectors encoding for CDH17 WT, a mutant form (CDH17 RAD), or empty vectors (Mock). Transfectants were analyzed by Western blotting (A) or flow cytometry (B) to assess the expression of CDH17 in whole lysates and cell membranes, respectively. C, RKO transfectants were subjected to cell aggregation assays. Representative pictures and a quantification of cell forming aggregates are shown. D, transfectants were subjected to cell adhesion assays on collagen type IV or Matrigel. Adhesion was enhanced significantly by overexpression of CDH17 wild-type or decreased by silencing of endogenous CDH17. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . E, RKO and KM12SM were transfected with siRNAs for the indicated integrin subunits or with a control siRNA. After 48 h, transfectants were lysed, and the extracts were analyzed by immunoblotting to assess the interference in the expression. Anti-RhoGDI was used as a loading control. F, KM12SM transfectants were subjected to cell adhesion assays on Matrigel. Cell adhesion was inhibited significantly by silencing of the indicated proteins. \*\*\*,  $p < 0.001$ . G, transfectants were incubated in 0.5% serum for 48 h and subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Cell proliferation was increased significantly by overexpression of CDH17 WT or decreased by silencing of endogenous CDH17. \*\*,  $p < 0.01$ .

# The CDH17-RGD Motif Is Critical for Integrin Activation



**FIGURE 3. The CDH17 RGD motif is a ligand of  $\alpha 2\beta 1$  integrin.** *A*, binding of  $\alpha 2\beta 1$  integrin to immobilized CDH17 DOM6 WT. KM12SM cells were lysed and loaded onto a 1-ml column of CDH17 domain 6 WT coupled to agarose. After extensive washing, the column was eluted with RGDS peptide. The fractions (1 ml) were precipitated and subjected to Western blot analysis using anti- $\alpha 2$  and anti- $\beta 1$  integrin antibodies. RGDS elution started at fraction number 7. *B*, RKO and KM12SM cells transfected with vectors encoding for CDH17 WT, CDH17 RAD, or empty vectors (*Mock*) were lysed, subjected to immunoprecipitation (*IP*) with anti- $\alpha 2$  integrin or anti-CDH17 antibodies, and analyzed by Western blotting with the indicated antibodies. *C*, expression of  $\alpha v$  integrin in RKO and KM12SM cells, detected by Western blot analysis (*left panel*) and immunoprecipitation assays with anti- $\alpha v$  integrin, anti-CDH17, or control antibodies showing the lack of association between this integrin subunit and CDH17 (*right panel*). *D*, polyacrylamide gels stained with Coomassie Blue showing the expression of purified Ecd (*left panel*) or of purified domain 6 (*DOM6*, *right panel*) of CDH17, both the WT or the mutant lacking the RGD motif (*RAD*). *E*, soluble binding assays using the CDH17 ectodomain as a ligand. Flow cytometry showed that, after incubation with the wild-type ectodomain, this protein fragment was bound to the cell surface. Mean fluorescence intensity is indicated. *F*, soluble binding assays using the CDH17 ectodomain as ligand in cells silenced for the indicated integrin subunits. Mean fluorescence intensity is indicated for both CDH17 ectodomain WT and RAD.

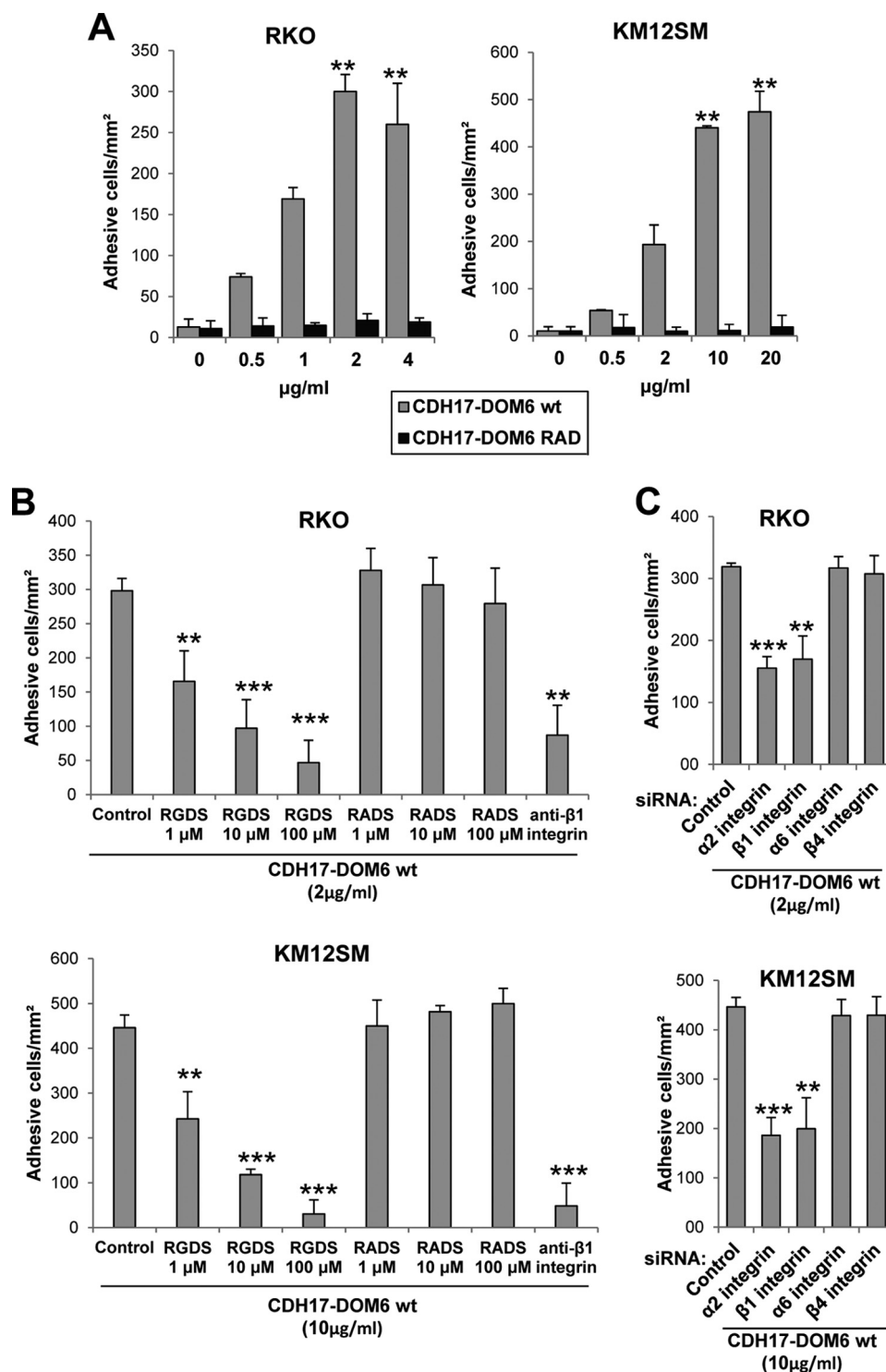


FIGURE 4. **The CDH17 RGD motif is able to mediate cell adhesion.** A, RKO and KM12SM cells were subjected to cell adhesion assays in plates coated with different concentrations of CDH17-DOM6 WT or CDH17-domain 6 RAD in the presence of 1 mM MnCl<sub>2</sub>. Adhesion was increased significantly in plates coated with CDH17-DOM6 WT compared with plates not coated. \*\*,  $p < 0.01$ . B, cell adhesion assays for CDH17 domain 6 WT were done in the presence of the RGDS and RADS peptides or anti-β1 integrin-blocking antibodies. Adhesion was inhibited significantly by the addition of peptides or antibodies. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . C, cell adhesion assays for CDH17 domain 6 WT with cells silenced for the indicated integrin subunits. Adhesion was inhibited significantly by the silencing of the indicated integrin subunits. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

RAD mutant (CDH17-RAD) were used to transfect KM12SM and RKO human colorectal cancer cells. Poorly differentiated RKO cells are non-metastatic cells that do not express CDH17, whereas KM12SM cells are highly metastatic colon cancer cells expressing CDH17 (7). After transfection, wild-type and RAD

mutant CDH17 were overexpressed in both cell lines at similar levels, as detected by Western blot analysis and flow cytometry (Fig. 2, A and B). First, we tested the aggregation of cells expressing CDH17. RKO CDH17 transfectants showed a significant increase in cell aggregation in respect to mock cells after

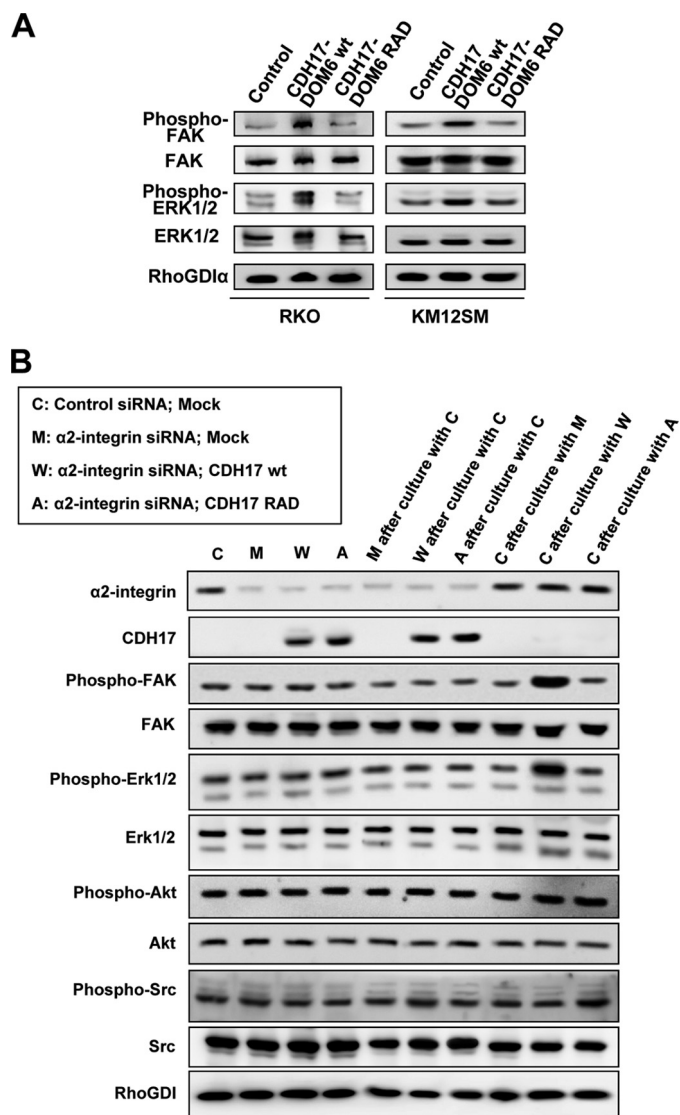
## The CDH17-RGD Motif Is Critical for Integrin Activation

detachment (Fig. 2C). This increase was RGD-independent because CDH17 RAD transfectants showed the same increase in aggregation. This result suggests a homotypic aggregation capacity for CDH17, as described for other cadherins (21).

Then, we assessed the capacity of the transfectants for cell adhesion to Matrigel or collagen type IV. Overexpression of CDH17-WT in both cell lines increased the adhesion to both types of extracellular matrix (Fig. 2D). In contrast, CDH17-RAD transfectants showed a basal adhesion, as mock transfectants, suggesting that the RGD motif was necessary to increase cell adhesion. Also, CDH17 silencing significantly reduced the adhesion of KM12SM cells (Fig. 2D). Cell adhesion required  $\alpha 2\beta 1$  integrin but not  $\alpha 6\beta 4$  integrin, as demonstrated by using siRNAs against each integrin subunit in adhesion assays with KM12SM cells (Fig. 2, E and F). Finally, cells expressing CDH17-WT showed a significant increase in proliferation, whereas the CDH17-RAD mutant was unable to increase cell proliferation (Fig. 2G). Collectively, these data indicate that the presence of the RGD motif increased cell adhesion and proliferation in colon cancer cells through  $\alpha 2\beta 1$  integrin but does not affect homotypic aggregation.

*The CDH17 RGD Binding Motif Is a Ligand for  $\alpha 2\beta 1$  Integrin*—Previously, we showed that CDH17 and  $\alpha 2\beta 1$  integrin coimmunoprecipitated together. To prove a direct interaction, we decided to purify  $\alpha 2\beta 1$  integrin using affinity chromatography on the basis of the coupling of CDH17 domain 6 WT to Sepharose following a similar strategy as Pytela *et al.* (22). KM12SM lysates were loaded on the affinity column and washed extensively, and then the column was eluted with an RGDS peptide. Both  $\alpha 2\beta 1$  integrin subunits were detected by Western blot analysis in the eluted fractions, confirming the integrin binding to CDH17 and the role of the RGD motif in that binding (Fig. 3A). To confirm that this association was RGD-dependent, CDH17 was coimmunoprecipitated with  $\alpha 2\beta 1$  integrin in RKO cells after transfection with CDH17-WT. In contrast, transfected CDH17-RAD was not detected after immunoprecipitation with  $\alpha 2$  integrin and vice versa in RKO (Fig. 3B). The  $\alpha 2\beta 1$  integrin was associated with FAK when CDH17-RGD WT was present but not after RAD mutation. Therefore, to start signaling, the binding of the RGD motif was critical for the integrin-FAK association. To discard  $\alpha \nu$  integrin, we carried out further coimmunoprecipitations. We did not detect  $\alpha \nu$  integrin in CDH17 coimmunoprecipitates and vice versa (Fig. 3C).

To evaluate whether exogenous CDH17 could be a ligand of integrins, we used the purified recombinant CDH17 ectodomain expressed in baculovirus and the recombinant CDH17 domain 6 (571–665) expressed in *E. coli*. In addition, we prepared RAD mutants of the ectodomain and domain 6 (Fig. 3D). After incubation, the CDH17 ectodomain bound to the cell surface (Fig. 3E). However, the RAD mutant form of the CDH17 ectodomain was unable to bind to cells. To confirm that this binding was specific for  $\alpha 2\beta 1$  integrin, we silenced the expression of the  $\alpha 2\beta 1$  and  $\alpha 6\beta 4$  integrins using siRNAs (Fig. 2D). In both types of cells, RKO and KM12SM, the silencing of  $\alpha 2\beta 1$  integrin subunits inhibited the binding of the CDH17-RGD ectodomain. However, the silencing of  $\alpha 6\beta 4$  integrin subunits did



**FIGURE 5.  $\beta 1$  integrin signaling after exposition to CDH17 RGD.** A, RKO and KM12SM cells were allowed to adhere to plates coated or not coated with CDH17 domain 6 WT or RAD or without protein (*control*) for 45 min. Cells were lysed, and the extracts were analyzed by Western blotting with antibodies against FAK and ERK1/2 and their phosphorylated forms. B, RKO cells were transfected with vectors encoding for CDH17 WT, RAD mutant, and empty vectors (*mock*) and with siRNAs targeting  $\alpha 2$  integrin or the control. The combinations were as follows:  $\alpha 2$  integrin-expressing transfectants (C) were cocultured with CDH17-expressing transfectants (M, W, and A) for 45 min. Then,  $\alpha 2$  integrin-expressing transfectants were isolated using anti- $\alpha 2$  integrin antibodies coupled to magnetic beads and analyzed separately. Extracts from transfectants not subjected to cocultures were also analyzed by Western blotting.

not affect the ability of the CDH17 ectodomain to bind the cell surface (Fig. 3F).

Then, we tested recombinant domain 6 as a ligand in cell adhesion assays. We found that 2 and 10  $\mu\text{g/ml}$  were the optimal doses in RKO and KM12SM cells, respectively, for promoting cell adhesion to plates coated with domain 6 (Fig. 4A). Moreover, there was a clear dose-dependent effect of the RGDS peptide to inhibit cell adhesion to CDH17 domain 6 WT in both cell lines. The competition started at 1  $\mu\text{M}$  and was similar to that obtained with a blocking antibody, anti- $\beta 1$  integrin (Fig. 4B). In contrast, the RADS peptide did not inhibit cell adhesion



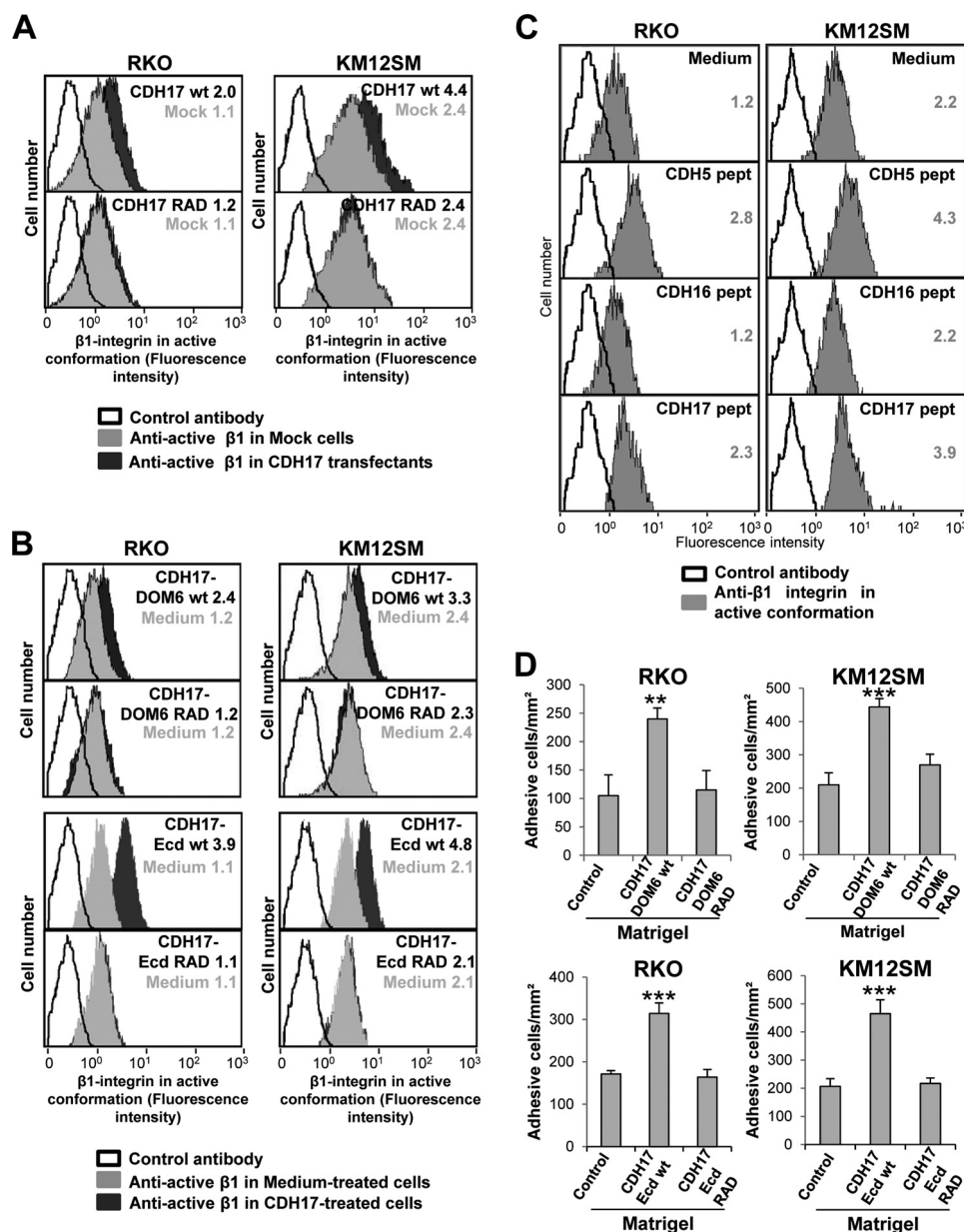


FIGURE 6. RGD enhances  $\beta 1$  integrin activation and cell adhesion to Matrigel. *A*, RKO and KM12SM cells were transfected with vectors coding to CDH17 WT or RAD or with empty vectors (*mock*) for 45 min and subjected to flow cytometry assays with HUTS21 antibody, which recognizes  $\beta 1$  integrin in the high-affinity conformation, or with a control antibody. Mean fluorescence intensity is indicated. *B*, RKO and KM12SM cells were exposed to CDH17 DOM6 (2  $\mu\text{g/ml}$ ) or Ecd (10  $\mu\text{g/ml}$ ) (WT or RAD) or medium for 45 min and subjected to flow cytometry assays with HUTS21 antibody or a control antibody as in *A*. *C*, RKO and KM12SM cells were exposed to nine amino acid peptides (0.5  $\mu\text{g/ml}$ ), whose sequences include the RGD motif and flanking amino acids belonging to CDH5, CDH16, and CDH17, for 45 min and subjected to flow cytometry as in *A*. *D*, after incubation with CDH17 DOM6 or Ecd (WT or RAD), cells were collected and subjected to cell adhesion assays on Matrigel. Adhesion was enhanced significantly by incubation with CDH17 DOM6 WT or Ecd WT. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

(Fig. 4*B*). This result suggests that CDH17 domain 6 mediates cell adhesion in an RGD-dependent manner. Again, to confirm that the binding was integrin-dependent, we silenced the expression of the four integrin subunits. Only silencing of  $\alpha 2\beta 1$  integrin significantly inhibited cell adhesion to CDH17 domain 6 (Fig. 4*C*).

*Role of the RGD Motif in Integrin Signaling*—To assess the role of the RGD motif in integrin activation, we treated both cell lines with CDH17 domain 6 WT and the RAD mutant. Incubation with CDH17 domain 6 WT increased phospho-FAK and phospho-ERK1/2 activity, whereas CDH17 domain 6 RAD did not (Fig. 5*A*). To investigate whether signaling pathways could

be directly activated through CDH17, we prepared four types of RKO transfectants: control siRNA (C),  $\alpha 2$  integrin silenced (M), expressing CDH17 with  $\alpha 2$  integrin silenced (W), and expressing CDH17 RAD with  $\alpha 2$  integrin silenced (A). Control siRNA transfectants were allowed to interact with the other transfectants for 45 min. Then, control transfectants were separated using anti- $\alpha 2$  integrin magnetic beads. All transfectants were lysed separately and subjected to Western blotting to analyze the activation of several signaling proteins, such as FAK, ERK1/2, Akt, and Src. FAK and ERK1/2 were activated only in control transfectants (expressing  $\alpha 2$  integrin) cocultured with transfectants expressing CDH17 with  $\alpha 2$  integrin silenced

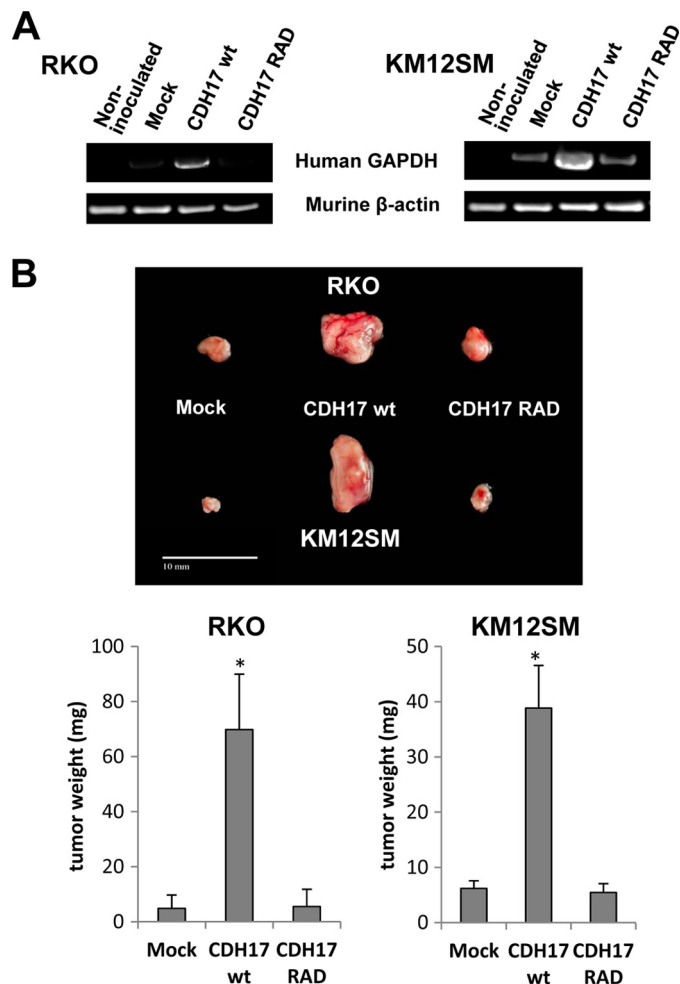
## The CDH17-RGD Motif Is Critical for Integrin Activation

(Fig. 5B). In contrast, transfectants expressing CDH17, with  $\alpha 2$  integrin silenced cocultured with control transfectants, did not show activation of any of these proteins (Fig. 5B). Therefore, signaling activation was triggered exclusively through the integrin, whereas CDH17 was unable to signal into the cell. Collectively, these data confirmed that the RGD motif was critical for the activation of the integrin-triggered proliferation cascade.

**Role of the RGD Motif in  $\beta 1$  Integrin Activation**—To assess the role of the RGD motif in integrin activation, we transfected both cell lines with vectors containing CDH17 WT and the RAD mutant. Expression of  $\alpha 2\beta 1$  integrin was not affected by altering CDH17 expression levels in colorectal cancer cells (7). However, CDH17-WT increased the high-affinity conformation of  $\beta 1$  integrin in RKO and KM12SM cells, as shown by using the HUTS21 antibody, which specifically recognizes this conformation (Fig. 6A). In contrast, cells transfected with CDH17-RAD showed similar amounts of high-affinity conformation  $\beta 1$  integrin as mock cells (Fig. 6A). Therefore, the RGD motif in CDH17 provoked the change to the high-affinity conformation required for  $\beta 1$  integrin activation. In the same way, preincubation with the CDH17 ectodomain or domain 6 WT enhanced the amount of  $\beta 1$  integrin in the high-affinity conformation, whereas incubation with the RAD mutants had no effect (Fig. 6B). In addition, we tested the effect of the RGD flanking sequences on integrin activation. Besides the CDH17 peptide, we tested the CDH16 and CDH5 RGD flanking sequences. Exposition to the CDH17 peptide increased high-affinity conformation  $\beta 1$  integrin in both cell lines (Fig. 6C). A similar effect was observed when cells were exposed to the CDH5 RGD peptide (Fig. 6C). In contrast, CDH16 RGD had no effect on  $\beta 1$  integrin activation (Fig. 6C). These results confirm the relevance of the RGD flanking sequences on  $\beta 1$  integrin activation.

Finally, we assessed whether the change to the high-affinity conformation of  $\beta 1$  integrin increased the adhesive capacity of the cells. The CDH17 ectodomain and domain 6 WT caused a significant increase in cell adhesion to Matrigel, whereas the RAD mutant hardly increased the basal levels (Fig. 6D).

**The CDH17 RGD Motif Is Critical for Tumor Growth and Metastasis**—We carried out subcutaneous and intrasplenic inoculations of Swiss nude mice with RKO and KM12 cells containing CDH17 WT or mutant RAD in triplicate. Mice were sacrificed 24 h after intrasplenic injection of cells, and livers were collected for DNA extraction and PCR analysis (Fig. 7A). Liver DNA from mice inoculated with CDH17-RGD cells was positive by PCR using human GAPDH primers. In contrast, mice inoculated with CDH17-RAD mutant cells showed negligible DNA amplification in the liver. After subcutaneous inoculation, CDH17 WT cells developed considerable tumors. In contrast, cells containing RAD mutants showed very small tumors, similar to the control (Fig. 7B). Tumor weight corroborated these differences between CDH17-RGD and RAD cells (Fig. 7B). These results support a critical role for the RGD motif in the tumor growth and metastatic dissemination in colon cancer.



**FIGURE 7. The RGD motif is critical for tumor growth and metastatic dissemination.** A, Swiss nude mice were inoculated intrasplenically with RKO or KM12SM cells transfected with vectors encoding for CDH17 WT, CDH17 RAD, or empty vectors (Mock). Human GAPDH was RT-PCR-amplified from RNA isolated from the livers 24 h after inoculation. Amplification of murine  $\beta$ -actin was used as a control. B, the same transfectants were inoculated subcutaneously. Top panel, representative picture of tumors developed after 10 days. Bottom panel, tumor weight after 10 days was increased significantly in cells expressing CDH17 WT. \*,  $p < 0.05$ .

**The CDH17 RGD Motif Is Relevant in pancreatic Cancer Cells**—CDH17 overexpression has been reported in some pancreatic tumors (3). Here, in a sample set of 48 patients with pancreatic cancer, we detected CDH17 expression in 60.4% of tumors by immunohistochemistry. Among CDH17-positive tumors, 62.1% showed intense staining, indicating overexpression of CDH17 (Fig. 8A). To learn whether our findings could be extended to pancreatic cancer, we used the BxPC3, PANC-1, and CAPAN pancreatic cancer cell lines. PANC-1 and CAPAN cells showed expression of CDH17, but BxPC3 did not (Fig. 8B). After transfection with CDH17-WT and CDH17-RAD mutant vectors, we observed clear differences in adhesion capacity. Although CDH17-WT caused a clear increase in the three cell lines, the RAD mutant failed to increase adhesion above basal levels (Fig. 8C). Moreover, cell proliferation increased in CDH17-RGD transfectants but not in cells transfected with CDH17-RAD (Fig. 8D). These results support an extension of our findings to other cancers expressing CDH17, like pancreatic cancer.

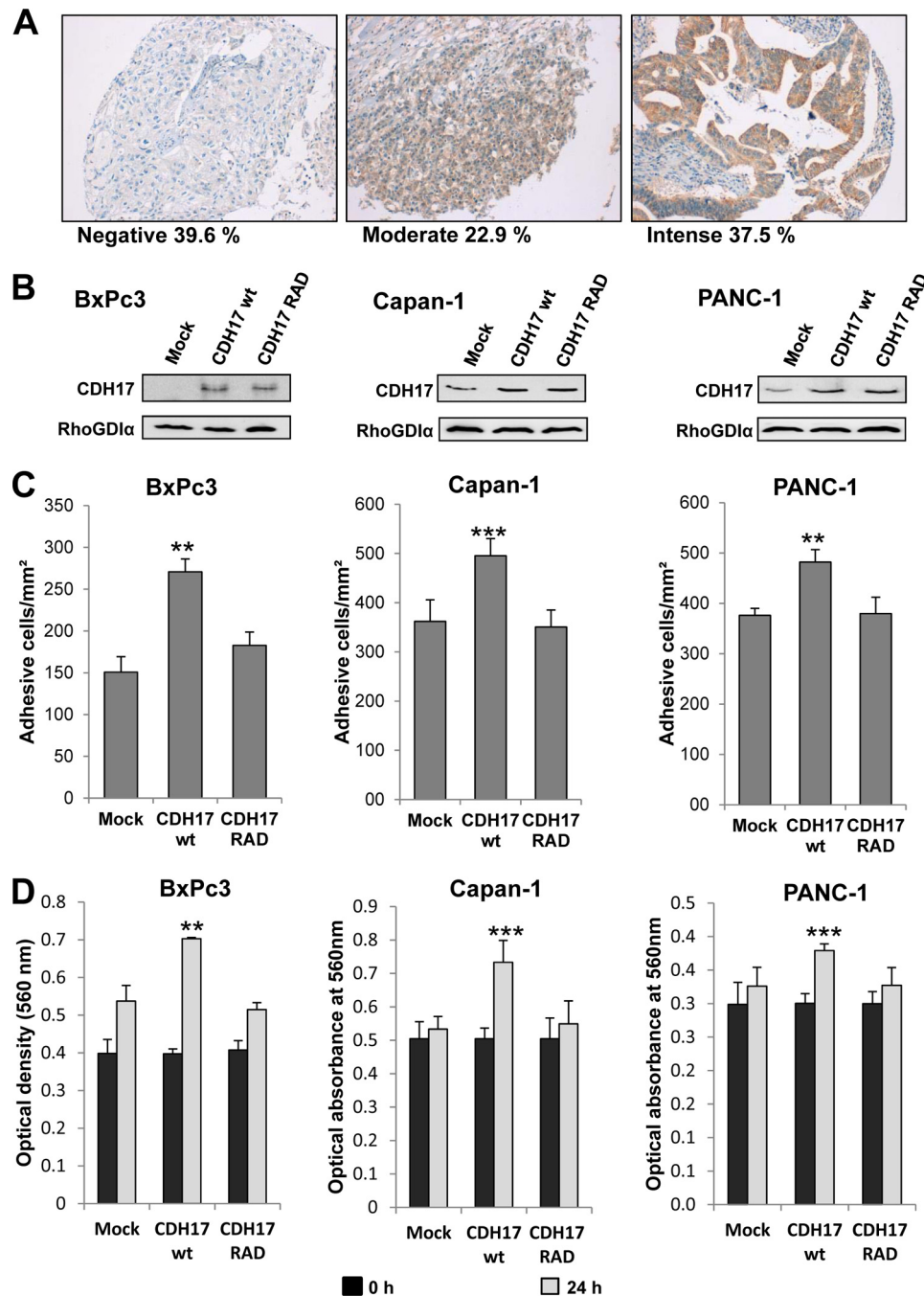


FIGURE 8. CDH17 expression promotes cell adhesion and proliferation in pancreatic cancer cells. *A*, immunohistochemistry analysis of CDH17 expression in human pancreatic cancer samples ( $n = 48$ ) showing representative images of strong, moderate, or negative staining and the percentage in each classification. *B*, BxPc3, Capan-1, and PANC-1 cells were transfected with vectors encoding for CDH17 WT, CDH17 RAD, or empty vectors (*Mock*). Transfectants were lysed, and the extracts were subjected to Western blot analysis to confirm the overexpression of CDH17. *C*, transfectants were subjected to cell adhesion assays on Matrigel. Adhesion was enhanced significantly by overexpression of CDH17 WT. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . *D*, transfectants were incubated in 0.5% serum for 24 h and subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Cell proliferation was increased significantly by overexpression of CDH17 WT. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . As a control, a fraction of the cells was subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays at time 0.

## DISCUSSION

We found that the human 7D-cadherin, CDH17, contains an RGD site with the capacity to act as a new ligand for integrin binding. This conclusion was obtained from the following observations. Interaction of CDH17 with  $\alpha 2\beta 1$  integrin required the presence of the RGD binding site; the capacity of the RGD motif to specifically bind  $\alpha 2\beta 1$  integrin in colon cancer cells was supported

by different binding and cell adhesion assays, including siRNA experiments; the CDH17-RGD ectodomain was able to bind colon cancer cells and activate  $\beta 1$  integrin when added exogenously; and, after *in vivo* inoculation, tumor cells expressing mutant CDH17 RAD showed a considerable delay in tumor growth and liver colonization. In summary, RGD works as a switch that regulates the integrin activation in colon cancer metastatic cells.

## The CDH17-RGD Motif Is Critical for Integrin Activation

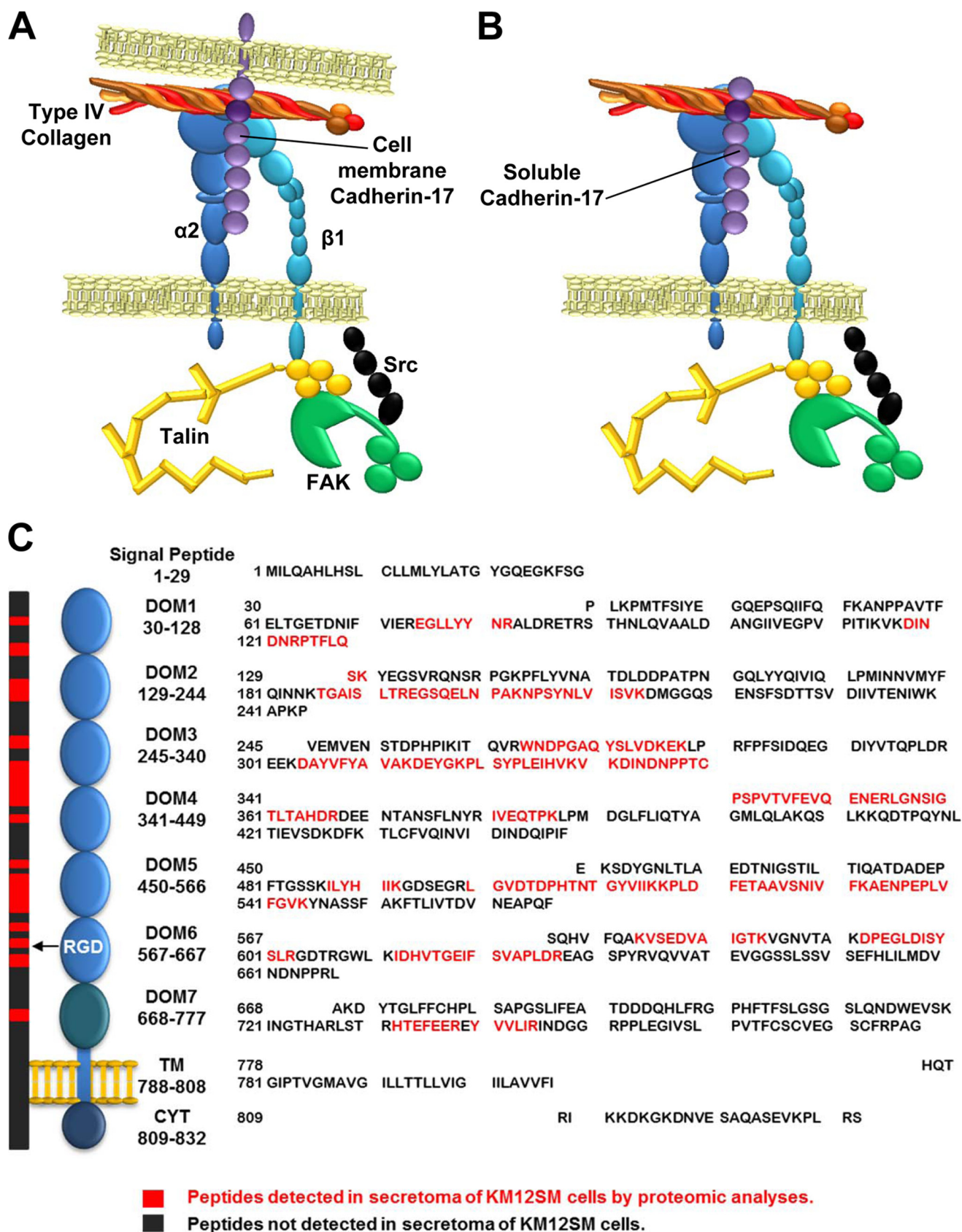


FIGURE 9. **Proposed models for the interaction between CDH17 and  $\alpha 2\beta 1$  integrin.** *A* and *B*, either CDH17 of a contiguous cell (*A*) or the soluble CDH17 ectodomain (*B*) can modulate the binding of  $\alpha 2\beta 1$  integrin to collagen type IV. *C*, medium conditioned for 24 h from KM12SM was collected, concentrated, resolved by SDS-PAGE, and in-gel-digested with trypsin. Mass spectra were acquired on an LTQ-Orbitrap Velos mass spectrometer, and the files were searched against the SwissProt database using the MASCOT search engine. Peptides assigned to CDH17 are marked in red in the sequence of CDH17 (*right*). All detected peptides belong to the ectodomain (domains 1–7) of CDH17 (*left*). 32% of the ectodomain was detected by the proteomics analysis.

CDH17, as other cadherins, can mediate cell-cell interactions by homotypic binding (21). In fact, RKO transfectants showed an increase in cell aggregation after cell detachment. The aggregation was not RGD-dependent, suggesting a CDH17 homotypic interaction different from integrin-cadherin interaction. Binding assays in the absence of divalent cations indicated that the RGD-containing CDH17 ectodomain was sufficient to mediate binding to the integrin. Moreover, coimmunoprecipitation assays showed that CDH17- $\alpha 2\beta 1$  integrin interaction was quite stable in the presence of different detergents (1% of digitonin, Igepal, or Triton X-100). Only cell adhesion to CDH17 DOM6 RGD-coated plates required the presence of manganese, indicating that the interaction was relatively weak when this domain was used as a substrate in coated plates. In any case, integrin activation, including activation of FAK and ERK proteins, in cells bound to the wild-type CDH17 domain 6 (in the absence of divalent cations) was comparable with the activation in cells adhered to collagen. Therefore, the CDH17- $\alpha 2\beta 1$  integrin binding works as a functional interaction between a ligand and its receptor, which triggers the integrin signaling pathway.

Other integrins, different from  $\alpha 2\beta 1$  integrin, have been classified as RGD receptors (23); for instance, combinations of  $\beta 1$  with  $\alpha 5$ ,  $\alpha v$ , or  $\alpha 8$  or combinations of  $\alpha v$  with  $\beta 3$ ,  $\beta 5$ ,  $\beta 6$ , or  $\beta 8$  (24). However, apart from  $\beta 1$ , from these possible combinations, only  $\alpha v$  integrin was detected in KM12SM cells after proteomic analyses, and we proved that was not associated with CDH17 (Fig. 3B). Only  $\alpha 2\beta 1$  integrin was required for binding to CDH17 in KM12SM and RKO cells, but not  $\alpha 6\beta 4$  integrin.  $\alpha 6\beta 4$  integrin is a receptor for laminins, and the binding motif in laminins is still unknown, but it seems to be RGD-independent. Therefore, although we do not discard the hypothesis that CDH17 might bind other integrins (*i.e.*  $\alpha v\beta 6$ ) in other cell lines, we provide strong evidence that  $\alpha 2\beta 1$  integrin is the only binder of CDH17 in KM12SM and RKO colon cancer cells.

On the basis of our results,  $\alpha 2\beta 1$  integrin should work simultaneously as a receptor RGD-dependent for CDH17 and RGD-independent for collagen IV, suggesting a simultaneous binding cooperative effect of both cadherin and collagen in the fine-tuning of  $\alpha 2\beta 1$  integrin activation (Fig. 9, A and B). In recent reviews (Ref. 23 and later), integrins have been divided in several groups, those that work as collagen receptors (GFOGER), *i.e.*  $\alpha 2\beta 1$  integrin, or those that work as RGD receptors (fibronectin, etc.), *i.e.*  $\alpha v\beta 3$  or  $\alpha 5\beta 1$ . In light of our results with  $\alpha 2\beta 1$  integrin and RGD cadherins, this classification should be reevaluated, and these two groups should probably be combined into one because both groups work as RGD receptors but with different ligands. In fact,  $\alpha 2\beta 1$ -mediated adhesion to collagen can be inhibited by an RGD-containing cyclic peptide (23).

Another relevant question is whether the cadherin/integrin interaction takes place in *cis* or in *trans*. The *trans* model would require the presence of CDH17 on the surface of a contiguous cell (Fig. 9A) or the presence of soluble ectodomains of CDH17 (Fig. 9B) to facilitate the contact in the interface of  $\alpha 2$  and  $\beta 1$  subunits, which is the ligand recognition site. Our data suggest that an interaction in *trans* is more probable because incubation with the soluble recombinant ectodomain, followed by

integrin activation, would mimic the interaction with the soluble form of CDH17 after shedding of this molecule from the cell surface membrane. Furthermore, the coculture of cells expressing only CDH17 with cells expressing only  $\alpha 2$  integrin reflects a *trans* interaction between two cells. Still, an interaction in *cis* cannot be totally ruled out.

Other cadherins, like CDH5 (which contains two RGD motifs), CDH6, or CDH20, also contain RGD-binding sites, and they could play important roles in cancer metastasis in other cancer types, where they are overexpressed. Here we provide evidence that CDH5 RGD, but not CDH16, also activates  $\alpha 2\beta 1$  integrin. Therefore, the flanking sequences of the cadherin RGD motif strongly influence the binding capacity to integrins and confer specificity to the interaction. In agreement with this hypothesis, recent studies point out a role for these cadherins in cancer and vascular damage. CDH5 and CDH6 seem to promote cancer progression (25), thrombus formation, and vascular injury because of the induction of platelet aggregation (26). Some studies have reported an up-regulation of CDH5 in invasive human breast tumors and in a breast cancer model (25), and CDH5 induction was responsible for vasculogenic mimicry in aggressive melanomas (27). In platelets, a previous study revealed a role for CDH6 as a novel ligand for  $\alpha_{IIb}\beta_3$  integrin, this binding being responsible for platelet aggregation and thrombus formation (26). This interaction might play a role in the signs of thrombosis occurring in patients at the metastatic stage and the contribution of platelets to tumor metastasis (28). Further experiments are required to clarify the integrin-binding capacity as a general mechanism of RGD-containing cadherins for the promotion of metastasis.

CDH17, as other cadherins, is a target for ectodomain shedding because of the presence of elevated protease activity in the tumor microenvironment. Previous observations confirmed the shedding of CDH17 in the conditioned medium of KM12SM cells (29). The secreted soluble form of CDH17 contained the RGD domain. This shedding in metastatic cells makes CDH17 a candidate biomarker for detection in biofluids (serum or plasma) of colon cancer patients. Therefore, CDH17 could be useful for patient stratification and targeted therapy.

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## **An RGD Motif Present in Cadherin 17 Induces Integrin Activation and Tumor Growth**

Rubén A. Bartolomé, Alberto Peláez-García, Inmaculada Gomez, Sofía Torres, María Jesús Fernandez-Aceñero, Beatriz Escudero-Paniagua, J. Ignacio Imbaud and J. Ignacio Casal

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