Online Supplemental Material for

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Supplemental Materials and Methods

Cloning of the Synovial Cadherin Gene. To determine the identity of synovial cadherins, we performed PCR using degenerate oligonucleotides based on the highly conserved cytoplasmic domains in human cadherins (1). Specifically, there are four regions of human E-, P-, and N-cadherin with near-identical amino acid sequences, corresponding to human E-cadherin residues 753–762 (EEGGGEEDQD), residues 840–847 (SLSSL[N/T]SS), residues 853–859 (QDYDYLN), and residues 865–875 (FKKLADMYGGG). Messenger RNA was extracted from a pure population of rheumatoid FLS as described previously (2), reverse transcribed, and used as a template for PCR amplification using Taq polymerase and degenerate oligonucleotides spanning the region between residues 753–875 (sense, 5′-GCGGGATCCGAIGARGGIGGNGGNGA-3′, and antisense, 5′-GGGGAGCTCTCIGCIARYTTYTTRAA-3′; where N5A,T,G,C; I5 Inosine; Y5C,T and R5 A,G), at 95°C for 30 s, 60°C for 1 min, 72°C for 1 min for 30 cycles. PCR products of the expected 385-bp size were obtained from FLS cDNA that were absent in the negative control where no template was included. These products were cloned into the pCRII plasmid using the TA cloning system (Invitrogen), subsequently analyzed by automated sequencing, and identified as cadherin-11 fragments when compared with the GenBank database. Six out of eight clones obtained matched the canonical cadherin-11 sequence. A full-length cadherin-11 clone was obtained by PCR with PFU polymerase (Stratagene) using cDNA generated from FLS and the following primers XV14 (5′-CCAAAAATGAAGGAGAACTACT-3′) and XV15 (5′-ATTGTTAAGAATCGTCATCAAA-3′) encompassing the entire coding region of cadherin-11. The product of 2.4 kb was cloned into pBluescript SK (Stratagene) and canonical sequence was confirmed. Two previous papers reported immunohistochemical staining for E-cadherin in RA synovium (3, 4), although not in normal synovium (4). However, we found no evidence for the presence of E-cadherin in this location by degenerate PCR cloning, Northern analysis, flow cytometry of cultured FLS, or by immunohistochemical staining of RA synovium (unpublished data).

Generation of L Cell–Cadherin-11 Stable Transfectants. The cadherin-11 cDNA was inserted into the HindIII–BamHI sites of the expression plasmid pCEP4 (Invitrogen). L cells, known to contain the catenins but no endogenous cadherins, were transfected with pCEP4/cadherin-11 or with the pCEP4 vector alone as described before. Transfected cells were selected by culture in 0.8 mg/ml hygromycin B, and cadherin-11 expression was confirmed by flow cytometry.

Production of Cadherin-11–Fc Fusion Protein. To evaluate whether cadherin-11 mediates cell adhesion, we constructed a fusion protein corresponding to the predicted extracellular domain of cadherin-11 and the Fc region of human IgG1 as shown in Fig. S1 A and as reported previously for E-cadherin–Fc (5). A double stranded DNA adaptor containing a 59 MscI site and the final five codons of the human cadherin-11 extracellular region, and a 39 XhoI site was produced by PCR from the human cadherin-11 cDNA using the oligonucleotides XVCad11A (5′-GCTGGCACCGTGGTTGGGAGAGT-3′) and XVCad11E (5′-GGGGGGCTCGAGGTAGGCCTCTGCGTTGCAGG-3′) and PFU polymerase according to the manufacturer's recommendations. This MscI–XhoI–digested adaptor was ligated to the MscI site at the 3′ end of the extracellular region of human cadherin-11. The resulting cadherin-11 extracellular domain fragment was introduced upstream of the hinge and Fc region of human IgG1 in a derivative of pCDM8 (pCDM8Fc; reference 6). The sequence of the junctional region is shown in Fig. S1 A. The construct was sequenced to confirm its integrity at this region. Finally, the cadherin-11-Fc cDNA was excised from pCDM8 and inserted into the Hind III and Not I sites of the expression vector pCEP4 (Invitrogen).

HEK293 cells were transfected with pCEP4/cadherin-11-Fc using the Mammalian transfection kit (Stratagene). Stably transfected clones expressing cadherin-11 Fc were identified after culture in selective media containing 0.2 mg/ml hygromycin B by ELISA. Cadherin-11–Fc protein was produced and purified as described previously for E-cadherin–Fc (5) and the concentration was determined by Bradford assay using BSA as the standard (BioRad Laboratories, Inc.). SDS-PAGE of the purified material revealed the presence of a protein of the expected size of ~120 kD under reducing conditions (Fig. S1 B). Under nonreducing conditions, the fusion protein migrated at ~240 kD as expected for dimeric fusion proteins linked through disulfide bonds in the Fc region.

Production and Characterization of Cadherin-11 Monoclonal Antibodies. To produce mAb that specifically recognized the extracellular region of human cadherin-11, we immunized BALB/c mice with intraperitoneal injections of 20 mg of purified cadherin-11–Fc, first in CFA and subsequently in IFA at 2-wk intervals, followed by a final boost of 30 mg administered i.v. 3 d later, splenocytes were isolated and fused with NS1 murine myeloma cells in the presence of PEG, m.w. 1450, as described previously (7). Hybridomas were selected with aminopterin-containing medium, and hybridoma supernatants were screened by differential ELISA in plates coated with either cadherin-11–Fc, E-cadherin–Fc (5), or human IgG1 (Calbiochem). These clones were further screened by flow cytometry for staining of cadherin-11–expressing cells but not E-cadherin–expressing cells. The hybridomas selected were subcloned thrice by limiting dilution and three were further charac-

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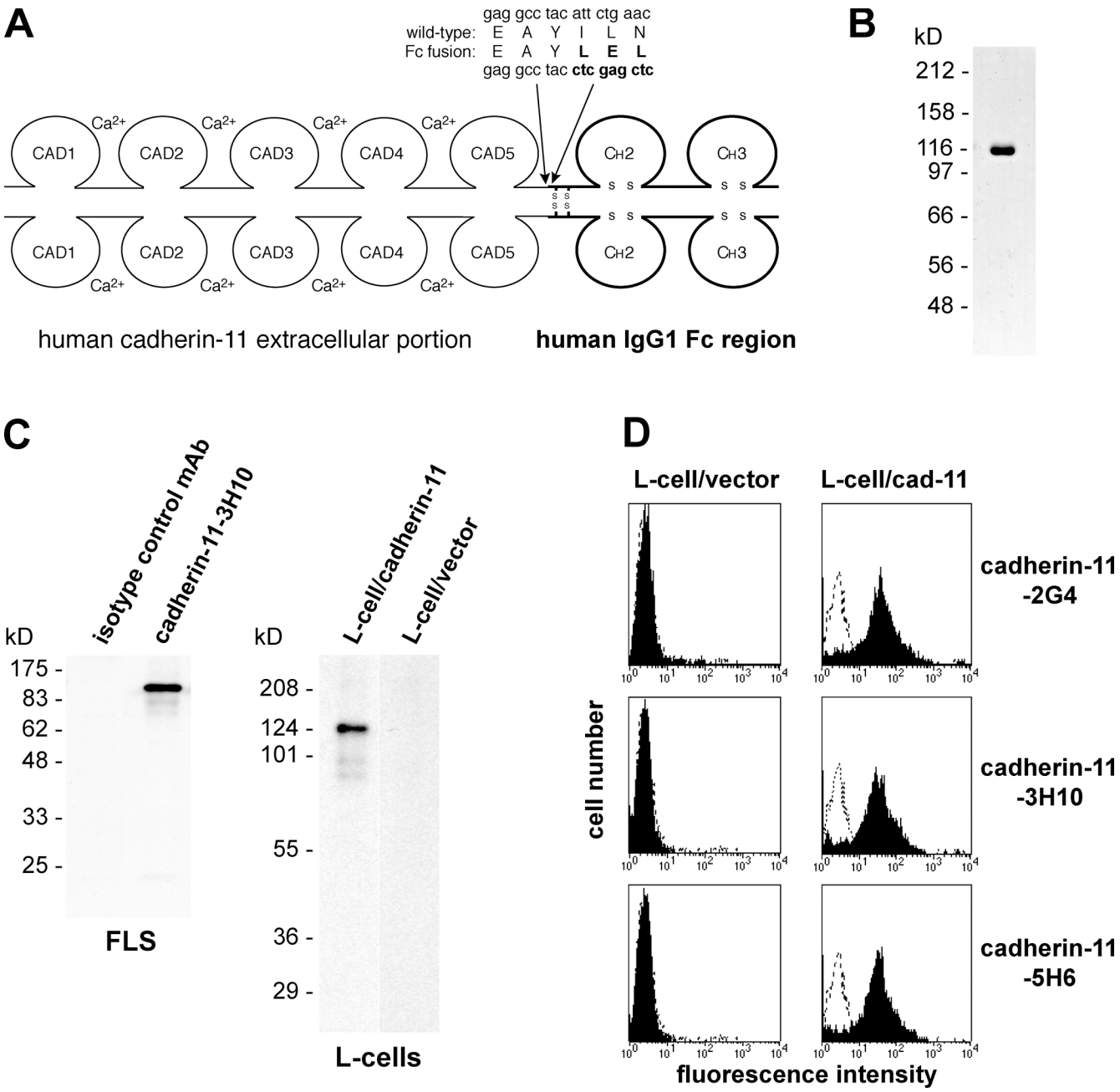

Figure S1. Cadherin-11 fusion protein, transfectants, and monoclonal antibodies. (A) Schematic structure of the human cadherin-11–Fc fusion protein. The five immunoglobulin-like extracellular domains (CAD1-5) and Fc domains of human IgG1 are depicted. The sequence of the extracellular juxtamembrane region of wild-type cadherin-11 and the alterations resulting from fusion with the human Fc region are shown at the joint. Regions corresponding to the Fc portion are shown in bold. (B) SDS-PAGE (7.5%, reducing conditions) of purified human cadherin-11–Fc protein stained with Coomassie blue. (C) Immunoprecipitation of cadherin-11. (left) Immunoprecipitation with anti–cadherin-11-3H10 or anti-paxillin isotype control antibody from FLS analyzed by 10% nonreducing SDS-PAGE. (right) Immunoprecipitation of cadherin-11 from L-cell/cadherin-11 but not L ell/vector lysates using cadherin-11–3H10 mAb, analyzed by 11% nonreducing SDS-PAGE. (D) Flow cytometric analysis of L cells transfected with cadherin-11 cDNA. Vector-transfected L cells (left column, L/vector) or L cells transfected with cadherin-11 (right column, L/cad-11) were stained with control mAb P3 (unshaded) or with the anti–human cadherin-11 mAbs (cadherin-11-2G4, cadherin-11-3H10, cadherin-11-5H6) generated in this work (shaded).

terized: mAb cadherin-11–2G4, cadherin-11–3H10, and cadherin-11–5H6. All mAb were of the IgG1 isotype as determined by ELISA using murine isotype-specific mAb (Jackson Immunoresearch Laboratories). To examine the specificity of the mAbs, we conducted immunoprecipitation from L cell transfectants and cultured FLS lysed in 1% Brij 96 in HBS with 2 mM CaCl2 and 5 mM MgCl2 essentially as described previously (8). Each mAb immunoprecipitated a protein of ~120 kD from 125I or biotin surface-labeled cultured FLS (Fig. S1 C and not depicted). Western blotting confirmed

that cadherin-11–3H10 immunoprecipitated cadherin-11 from L-cell/cadherin-11 but not L-cell/vector transfectant lysates (Fig. S1 C), and flow cytometry showed that all three anti–cadherin-11 mAb stained L-cell/cadherin-11 transfectants, but not L-cell/vector transfectants (Fig. S1 D). These data confirmed the specificity of these mAb for cadherin-11.

Other Antibodies. Mouse mAbs used were as follows. Anti–human E-cadherin (E4.6, IgG1) was raised previously in this laboratory (9). Anti-CD68 (EMB11, IgG1) was from DakoCytomation, anti–E-cadherin (HECD1, IgG1), and antipaxillin (Z035, IgG1) from Zymed Laboratories; anti-CD55 (Clone 67, IgG1), anti-CD45 (H130, IgG1), MOPC-21-biotin, and –FITC (control IgG1) from Caltag; P3 (control IgG1), anti–mouse MHC class I (H-2Kk; 36–7-5, IgG2a), and anti–VCAM-1 (51-10C9, IgG1) from BD Biosciences.

Cell Lines. The murine fibroblast cell line L-M (ATCC CCL1.3; L cells) was grown in DMEM, 10% FBS, 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM nonessential amino acids (GIBCO BRL), 100 U/ml penicillin, 100 mg/ml streptomycin sulfate, and 50 mM 2-mercaptoethanol at 10% CO2. L cell transfectants were grown in the aforementioned medium with 0.8 mg/ml hygromycin B (Invitrogen). Human embryonic kidney HEK293 cells (American Type Culture Collection) were maintained in 10% FBS, DMEM at 37°C at 10% CO_2. Human breast epithelial 16E6.A5 cells were maintained as described previously (9).

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