Supporting Information

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SI Materials and Methods

Animals. ICR mice and Wister and Lewis rats were purchased from Nippon-Clea and Sankyo Labo Service, respectively. All animal experiments and procedures used in the present study were approved by the Animal Care and Use Committees of Aichi Medical University and Osaka University.

Materials. Mouse laminin-111 and porcine type I collagen were obtained from Iwaki Glass. Types II (chicken), V (bovine), and VI (bovine) collagens were purchased from Chemicon International. Rat type III collagen was obtained from Biogenesis. Mouse type IV collagen and human laminin-211/221 were purchased from BD Biosciences. Gelatin was obtained from Sigma. Fibronectin was purified from bovine serum. Laminin-511/521 was purified as described in ref. 1 or was purchased from Sigma. Anti-GFP mouse mAb was obtained from Santa Cruz Biotechnology. Anti-myc and anti-hexahistidine (his6) mAbs were purchased from Invitrogen. Horseradish peroxidase-conjugated sheep anti-mouse antibodies were obtained from GE Healthcare. Anti-heparan sulfate stub (F69-3G10) and anti-chondroitin sulfate stub (1B5, 2B6, and 3B3) mAbs and GAG-cleaving enzymes (heparitinase, heparinase, and chondroitinase ABC) were purchased from Seikagaku. Hyaluronidase was obtained from Sigma.

Cell Culture. 293T, MG63 (Health Science Research Resources Bank, Osaka, Japan), C2C12 (Dainippon Sumitomo Pharma), and MEF cells were maintained in DMEM supplemented with 10% FBS, penicillin, and streptomycin at 37°C under 5% CO_2. COS cells were maintained in DMEM supplemented with 10% FBS, penicillin, streptomycin, and nonessential amino acids. 293F cells (Invitrogen) were maintained in serum- and protein-free FreeStyle 293 expression medium (Invitrogen) in 125-ml Erlenmeyer flasks at 37°C under 8% CO_2 with rotation at 120 rpm. When 293T or COS cells were transfected, the complete medium was replaced with OptiMEM (Invitrogen) after the transfection and the cells were cultured for the indicated periods of time.

Construction of Mammalian Expression Plasmids. Individual candidate cDNAs encoding complete ORFs, including a Kozak sequence but not a stop codon, were amplified by PCR with KOD-Plus DNA polymerase (Toyobo) by using forward and reverse primers tagged with restriction enzyme recognition sequences. For cDNAs that did not have a typical Kozak sequence upstream of the ATG initiation codon, we introduced mutations to create a consensus Kozak sequence. The PCR products were incubated with restriction enzymes, purified on MultiScreen-PCR96 plates (Millipore), and directionally introduced into the multicloning site of an EGFP-expression vector pEGFP-N3 (BD Biosciences) or a modified version of pCIkanaEGFP that included tandem triple repeats of a myc tag at the C terminus. The resulting plasmids were transformed into OneShot competent cells (Invitrogen) and purified by using Montage Plasmid Miniprep96 kits (Millipore). For some clones, the PCR-amplified cDNAs were introduced into the multicloning site of the pSecTag2A mammalian expression vector (Invitrogen) to produce full-length proteins tagged with myc and his6 epitopes at their C termini. A plasmid coding for a secreted form of EGFP (sGFP) was constructed by inserting cDNA encoding a protein C inhibitor signal sequence adjacent to the initiation codon of EGFP.

Screening for Protein Secretion. 293T cells were plated in 96-well tissue culture plates, transfected with the GFP-fusion plasmids by using FuGENE6 (Roche) or Lipofectamine 2000 (Invitrogen), and cultured in serum-free OptiMEM medium. 293F cells cultured in 96-deep-well plates in FreeStyle 293 expression medium were transfected with the plasmids by using 293fectin (Invitrogen). At 3–4 days posttransfection, the conditioned media were collected, centrifuged, and passed through 0.2-μm filters to remove any cell debris. The samples were then analyzed by Western blot analysis with anti-GFP mAb. Proteins were detected by using an enhanced chemiluminescence detection kit (GE Healthcare).

Detection of Neoepitopes Generated by Cleavage of GAG Chains. To confirm GAG attachment to the core protein, C-terminally his6-tagged full-length proteins were expressed in 293F or COS cells and precipitated with Ni-resin (Qiagen). The precipitated proteins were then treated with the GAG-cleaving enzymes and subjected to Western blot analysis with anti-stub mAbs (3G10 for heparan sulfate; 1B5, 2B6, and 3B3 for chondroitin sulfate; Seikagaku) that recognize neoepitopes produced after treatment with GAG-degrading enzymes. Proteins were detected by using an enhanced chemiluminescence detection kit (GE Healthcare).

Antibody Production. Polyclonal antibodies against the candidate proteins were raised in rabbits by immunization with recombinant proteins or synthetic peptides. We usually produced more than two nonoverlapping antigenic fragments for each candidate protein to obtain at least two independent antibodies both working in immunohistochemistry for validation of the specificities of the immunostaining. The antigenic fragments used in this study are listed in Table S6. Synthetic peptides were conjugated to keyhole limpet hemocyanin via a cysteine residue added to the N-terminal end of each peptide. For the purification of the recombinant proteins, cDNA fragments encoding antigenic fragments were PCR amplified and introduced into the multicloning site of pGEX-4T1 (GE Healthcare), pET42a (Novagen Merck), or pIVEX-GST (Roche) for expression in bacteria or pSecTag2A, pFLAG-CMV-5a (Sigma), or pCIneo-myc-his for expression in 293F cells (Table S6). To construct pCIneo-myc-his, a cDNA fragment encoding a myc-his6 epitope tag was PCR amplified by using pSecTag2A as a template and introduced between the XbaI and NotI sites of pCIneo (Promega). The GST-fusion proteins expressed in BL21 or JM109 bacteria (Invitrogen) were affinity purified by using glutathione-conjugated columns (GE Healthcare) according to the manufacturer's instructions. When pET42a was used as an expression vector, the GST-his6-fusion proteins were purified on glutathione-Sepharose columns, followed by Ni-resin chromatography. Recombinant proteins expressed and secreted by 293F cells were affinity purified by using Ni-resin (for his6-tagged proteins) or anti-FLAG (Sigma) affinity chromatography. The antisera were produced by Medical & Biological Laboratories. The resulting antibodies were purified by affinity chromatography by using the corresponding immunogenic recombinant proteins or synthetic peptides. The antibodies raised after immunization with GST-fusion proteins were passed through GST-Sepharose columns to remove any antibodies directed against GST. The antibodies raised after immunization with synthetic peptides conjugated to keyhole limpet hemocyanin were affinity purified with antigenic peptides coupled to thioester-activated Sepharose (GE Healthcare).

mAbs against the subunits of mouse laminin were produced as described in ref. 1. In brief, 50–100 μg of antigen emulsified with Freund's complete adjuvant was injected into the hind footpads of 6- to 9-month-old female rats (WKY/NCrj or LEW/SsN Slc). Lymph node cells were subsequently fused with Sp2/0-Ag14 mouse myeloma cells. In some cases, a booster injection of antigen was given 3 days before cell fusion. After screening with ELISAs for the antigens, positive clones were further screened by immunohistochemical staining of mouse tissues. mAbs against type IV and XVIII collagen subunits were produced as described (2).

The specificities of the antibodies were confirmed by using ELISA and Western blot analysis with immunogenic recombinant fragments and full-length recombinant proteins expressed in mammalian cells as GFP-fusion proteins.

1. Kikkawa Y, Sanzen N, Sekiguchi K (1998) Isolation and characterization of laminin-10/11 secreted by human lung carcinoma cells: laminin-10/11 mediates cell adhesion through integrin $\alpha 3\beta 1$. *J Biol Chem* 273:15854–15859.

2. Sado Y, *et al.* (1995) Establishment by the rat lymph node method of epitope-defined monoclonal antibodies recognizing the six different α chains of human type IV collagen. *Histochem Cell Biol* 104:267–275.

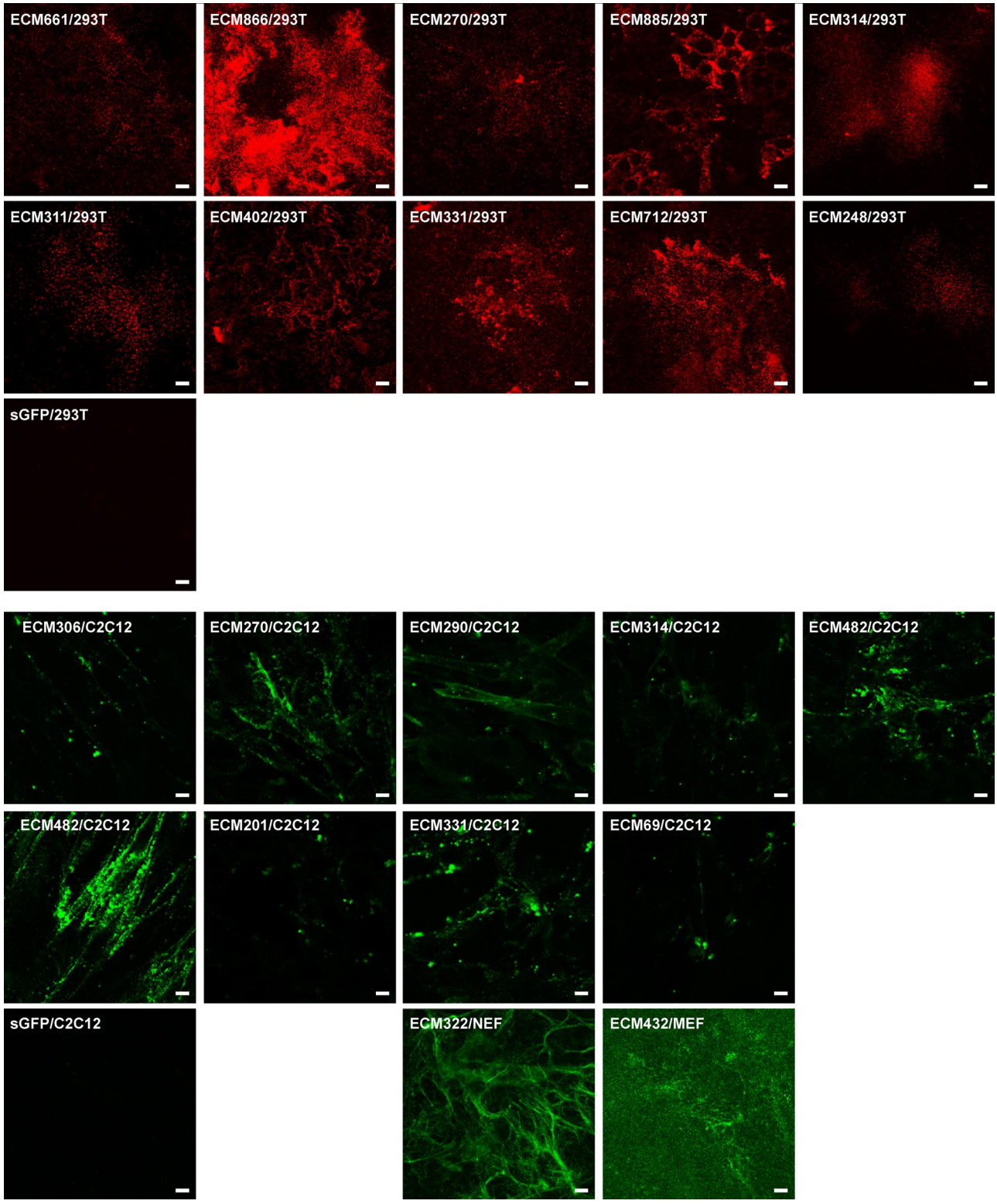

Fig. S1. Candidate proteins with matrix assembly activities. Protein immobilizations into the ECM of 293T cells were detected by immunocytochemical staining with an anti-GFP mAb followed by visualization with a rhodamine-conjugated secondary antibody. Deposition into the ECM of C2C12 or MEF cultures was visually screened by observation of GFP fluorescence. A signal peptide-tagged GFP (sGFP) was used as a negative control. (Scale bars, 10 μm.)

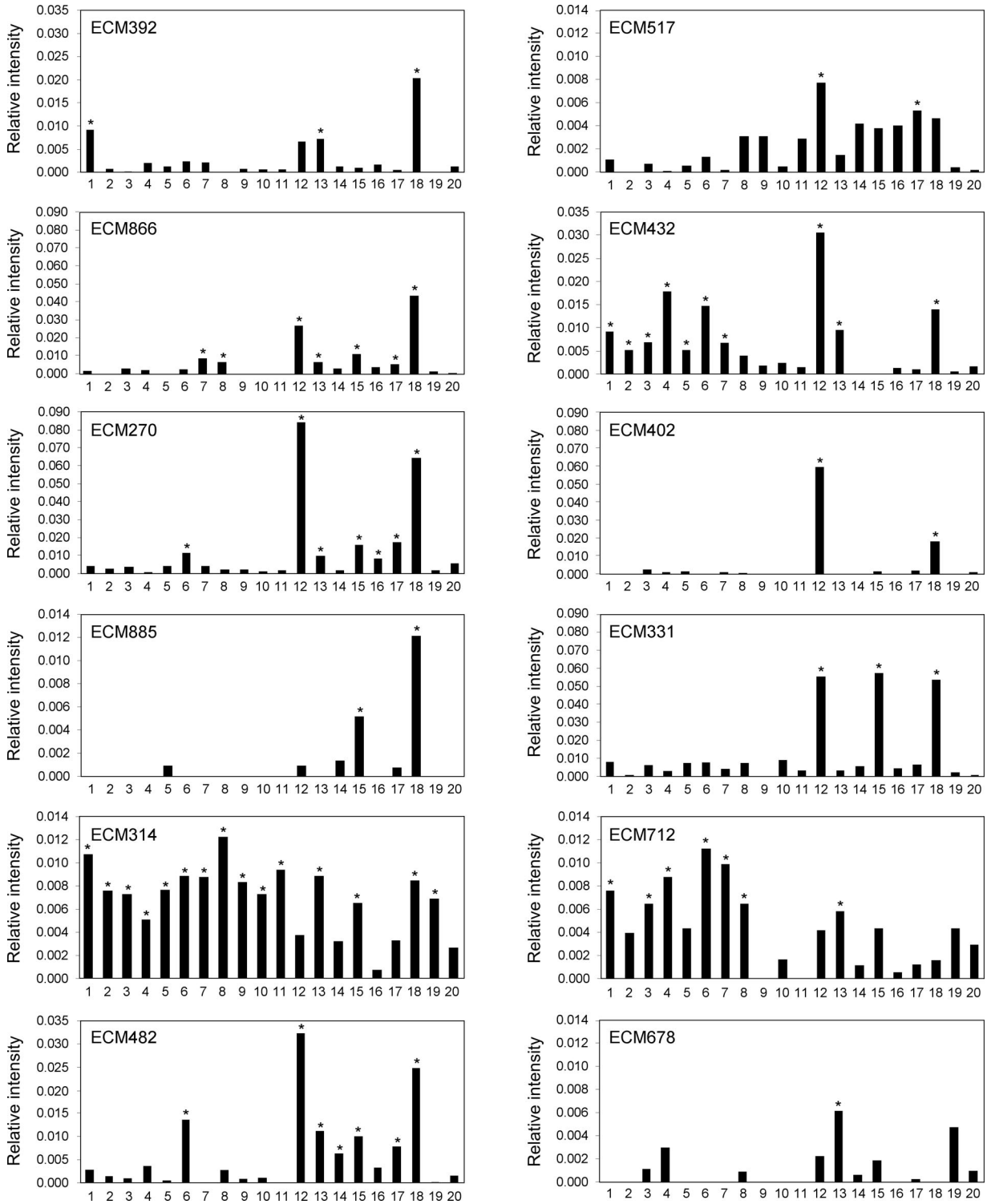

Fig. S2. Candidate proteins capable of binding to known ECM molecules. The ECM molecules used in the solid-phase binding assays were: 1, collagen type I; 2, collagen type II; 3, collagen type III; 4, collagen type IV; 5, collagen type V; 6, collagen type VI; 7, gelatin; 8, fibronectin; 9, laminin-111; 10, laminin-211/221; 11, laminin-511/521; 12, heparin; 13, heparan sulfate; 14, chondroitin sulfate A; 15, dermatan sulfate; 16, chondroitin sulfate C; 17, chondroitin sulfate D; 18, chondroitin sulfate E; 19, hyaluronic acid. BSA (20) was used as a negative control. The amounts of bound GFP-fusion proteins were determined by measuring the GFP intensities. The intensity of bound sGFP was subtracted from the individual readouts as the background signal. Data with the intensity over the threshold value are marked with asterisks.

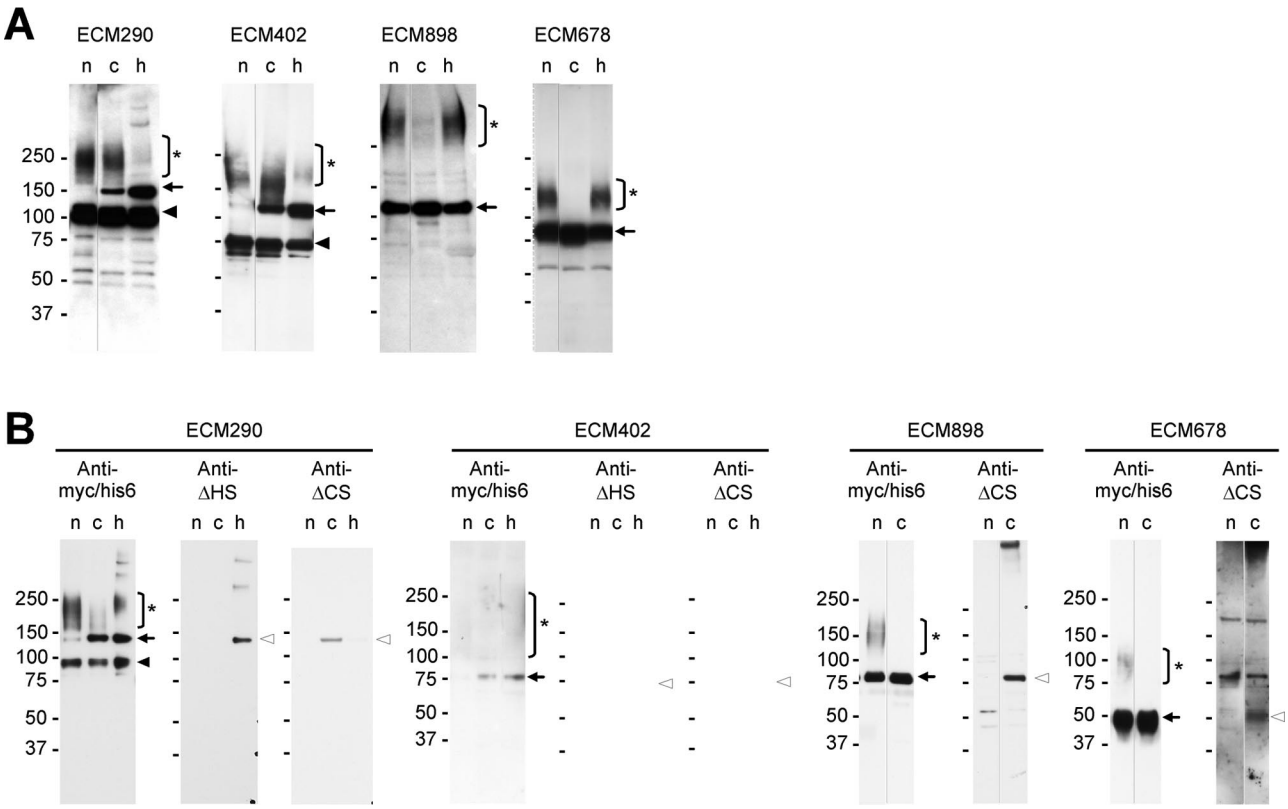

Fig. S3. Candidate proteins modified by GAG chains. (*A*) Attachment of GAG chains was screened by incubating GFP-fusion proteins in conditioned media of transfected 293F (ECM290/nectican, ECM402/epidermacan, and ECM898/mamcan) or COS (ECM678/MCP-11) cells with heparinase and heparitinase (h), chondroitinase ABC (c), or buffer alone (n), followed by Western blot analyses with an anti-GFP mAb to detect mobility shifts of the candidate proteins. After incubation with the GAG-degrading enzymes, the smeared bands (asterisks) disappear (or diminish) with the concomitant appearance (or increase) of faster migrating bands (arrows). Putative proteolytic fragments of ECM290/nectican and ECM402/epidermacan fusion proteins are indicated by arrowheads. (*B*) Covalent attachment of GAG chains to the proteins was verified by using anti-stub mAbs. Candidate proteins fused with myc-his6 tags were partially purified from the conditioned media of transfected COS cells by using Ni-resin and then treated with heparinase and heparitinase (h), chondroitinase ABC (c), or buffer alone (n). Proteins treated with the GAG-degrading enzymes were subjected to SDS/PAGE, followed Western blot analysis with a mixture of anti-myc and anti-his6 mAbs (anti-myc/his6) to detect mobility shifts of the corresponding bands. To verify the covalent attachment of GAG chains to the proteins, neoepitopes generated on cleavage of heparan sulfate or chondroitin sulfate chains were detected by Western blotting by using anti-stub mAbs (anti-ΔHS and anti-ΔCS mAbs, respectively). The bands detected with the anti-stub mAbs are indicated with open arrowheads.

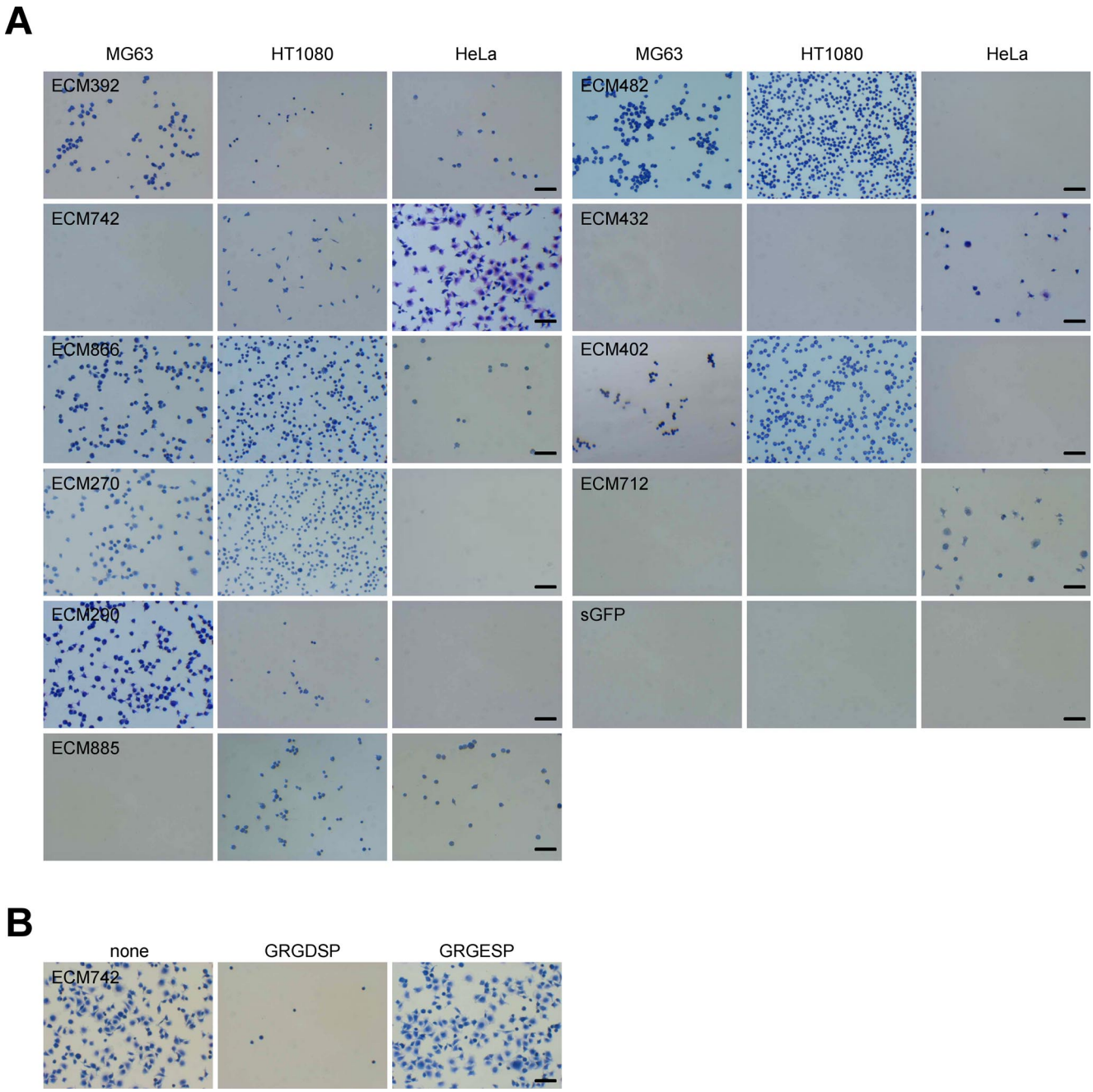

Fig. S4. Candidate proteins with cell adhesion-promoting activities. (*A*) The cell adhesion-promoting abilities were screened by incubating MG63, HT1080, and HeLa cells in 96-well plates containing immobilized GFP-tagged candidate proteins. Cells adhering to the substrates were visualized by staining with Diff-Quick (Wako). (*B*) HeLa cells were plated on ECM742/cradin-coated substrates in the absence (none) or presence of 1 mM peptide containing either an RGD (GRGDSP) or RGE (GRGESP) sequence. The RGD-containing peptide, but not the RGE-containing peptide, inhibits ECM742/cradin-promoted cell adhesion. (Scale bars, 100 μm.)

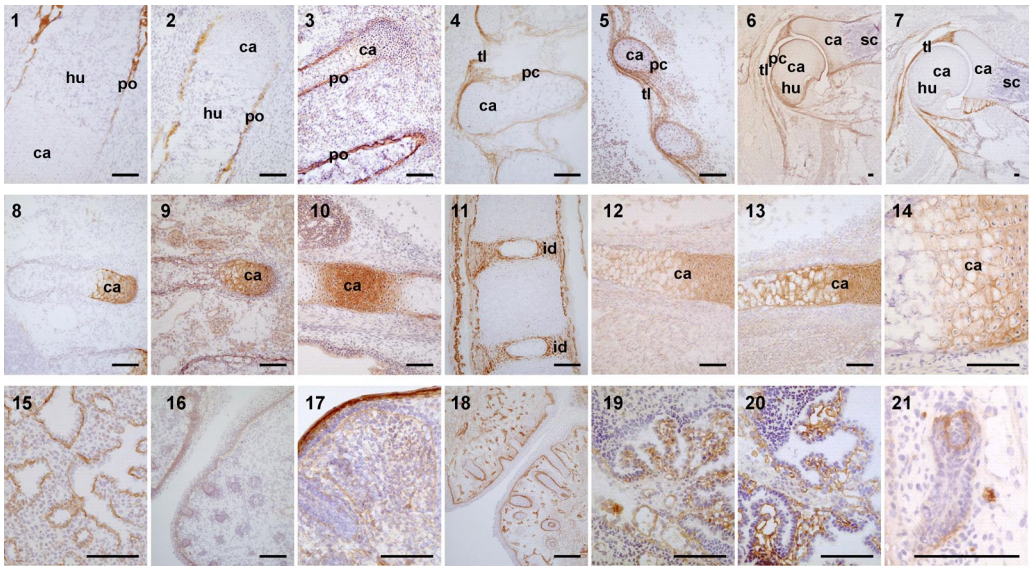

Fig. S5. Immunohistochemical analyses of the ECM localizations of the candidate proteins. Sagittal sections of E16.5 mouse embryos (sections 1–5 and 8–21) or newborn mice (sections 6 and 7) were immunohistochemically stained with affinity-purified antibodies. The following representative localization data are shown: ECM311/IGFBP-rP10 (1, periosteum of the humerus); ECM201/periolin (2, periosteum of the humerus); ECM432/tenonectin (3, periosteum of ribs; 6, tendons connecting the scapula and humerus to muscles); ECM322/ADAMTSL-4 (4, ligaments and associated perichondrium surrounding the spinal cartilage); ECM517/RAINB2 (5, ligaments and associated perichondrium surrounding the spinal cartilage); ECM482/eratin (7, perichondrium surrounding the humerus and tendons connecting the scapula and humerus to muscles); ECM314/emprin (8, rib cartilage); ECM898/mamcan (9, rib cartilage); ECM885/vitrin (10, skull base cartilage); ECM306/WARP (11, intervertebral disks in the spinal cord; 18, lip epithelium); ECM866/URB [12, rib cartilage; 19, choroid plexus epithelium (arrows) and associated blood vessels (arrowheads)]; ECM270/SMOC-2 (13, rib cartilage; 20, choroid plexus epithelium and associated blood vessels); ECM290/nectican (14, rib cartilage; 21, hair follicle); ECM661/MAEG (15, lung epithelium); ECM392/ependolin (16, lip epithelium); and ECM742/cradin (17, lip epithelium and capillaries). ca, cartilage; pc, perichondrium; po, periosteum; tl, tendon or ligament; id, intervertebral disk; hu, humerus; sc, scapula. (Scale bars, 100 μm.)

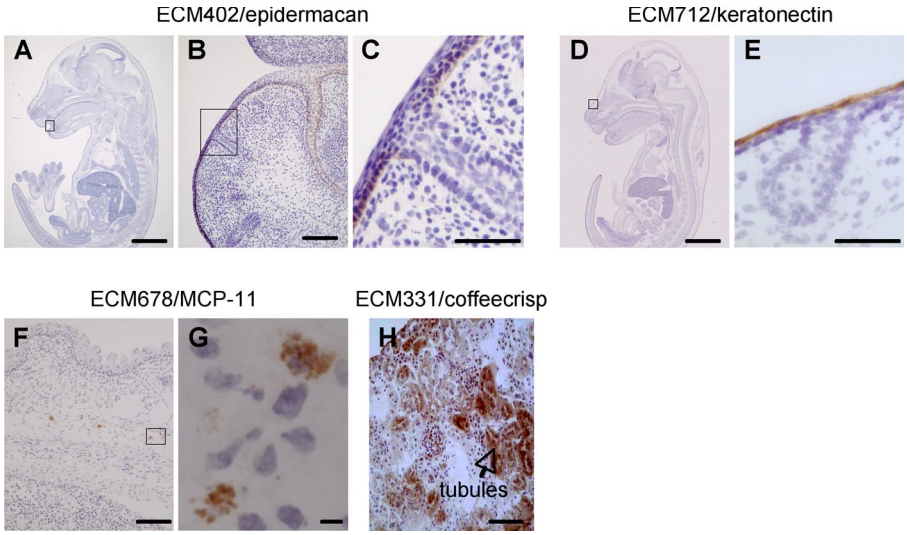

Fig. S6. Tissue localizations of candidate proteins displaying non-ECM staining patterns. Sagittal sections of E16.5 mouse embryos (*A*–*G*) and adult kidneys (*H*) were immunohistochemically stained with affinity-purified antibodies. (*Right*) High-magnification views of the boxed areas. An antibody against ECM402/epidermacan specifically stains interfollicular basal cells in the epidermis of an E16.5 mouse embryo (*A*–*C*). ECM712/keratonectin is a specific component of suprabasal keratinocytes. Its restricted localization pattern is consistent with previous reports of the mRNA expression profiles of these proteins [Moffatt P, *et al.* (2004) Identification of a conserved cluster of skin-specific genes encoding secreted proteins. *Gene* 334:123–131; Matsui T, *et al.* (2004) Identification of novel keratinocyte-secreted peptides dermokine-α/-β and a new stratified epithelium-secreted protein gene complex on human chromosome 19q13.1. *Genomics* 84:384–397.] (*D* and *E*). An antibody against ECM678/MCP-11 stains some cells (probably mast cells) in the dermis of an E16.5 embryo (*F* and *G*). ECM331/coffeecrisp is detected in renal tubules in the adult kidney (*H*). The immunohistochemical data for ECM343/photomedin-1 and ECM501/photomedin-2 have already been published [Furutani Y, *et al.* (2005) Identification and characterization of photomedins: Novel olfactomedin-domain-containing proteins with chondroitin sulfate-E-binding activity. *Biochem J* 389:675–684.] (Scale bars, *A* and *D*, 2.5 mm; *B* and *F*, 100 μm; 10 μm in G; and 50 μm in C, E and H.

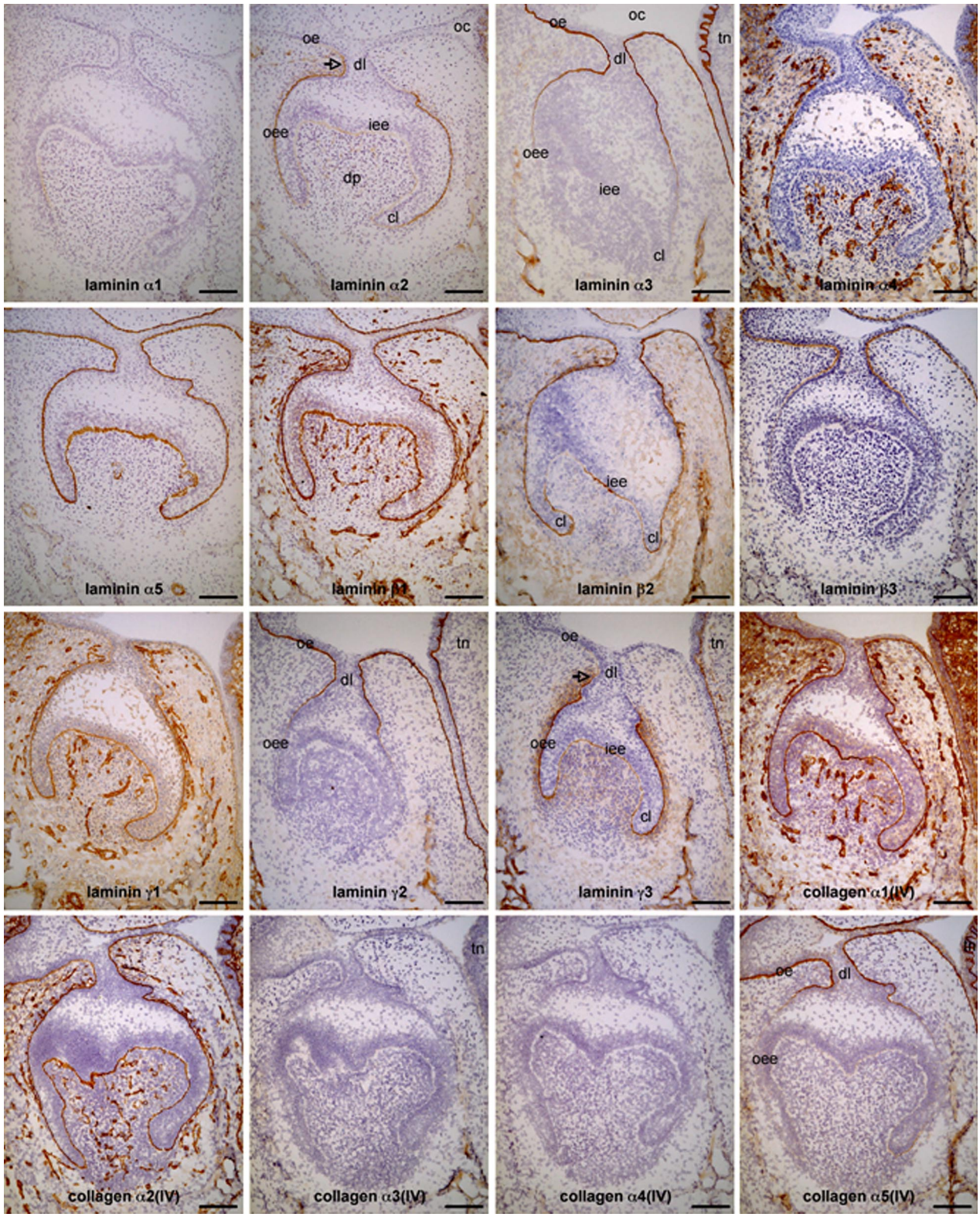

Fig. S7. Localizations of BM proteins in the E16.5 mouse first mandibular molar. Frontal sections of E16.5 mouse heads were stained with 38 antibodies against BM proteins. The arrows indicate asymmetries in the staining intensities found in individual epithelial BMs. oe, oral epithelium; oee, outer enamel epithelium; iee, inner enamel epithelium; dl, dental lamina; cl, cervical loop; tn, tongue; oc, oral cavity; df, dental follicle; dp, dental papilla. (Scale bars, 100 μm.)

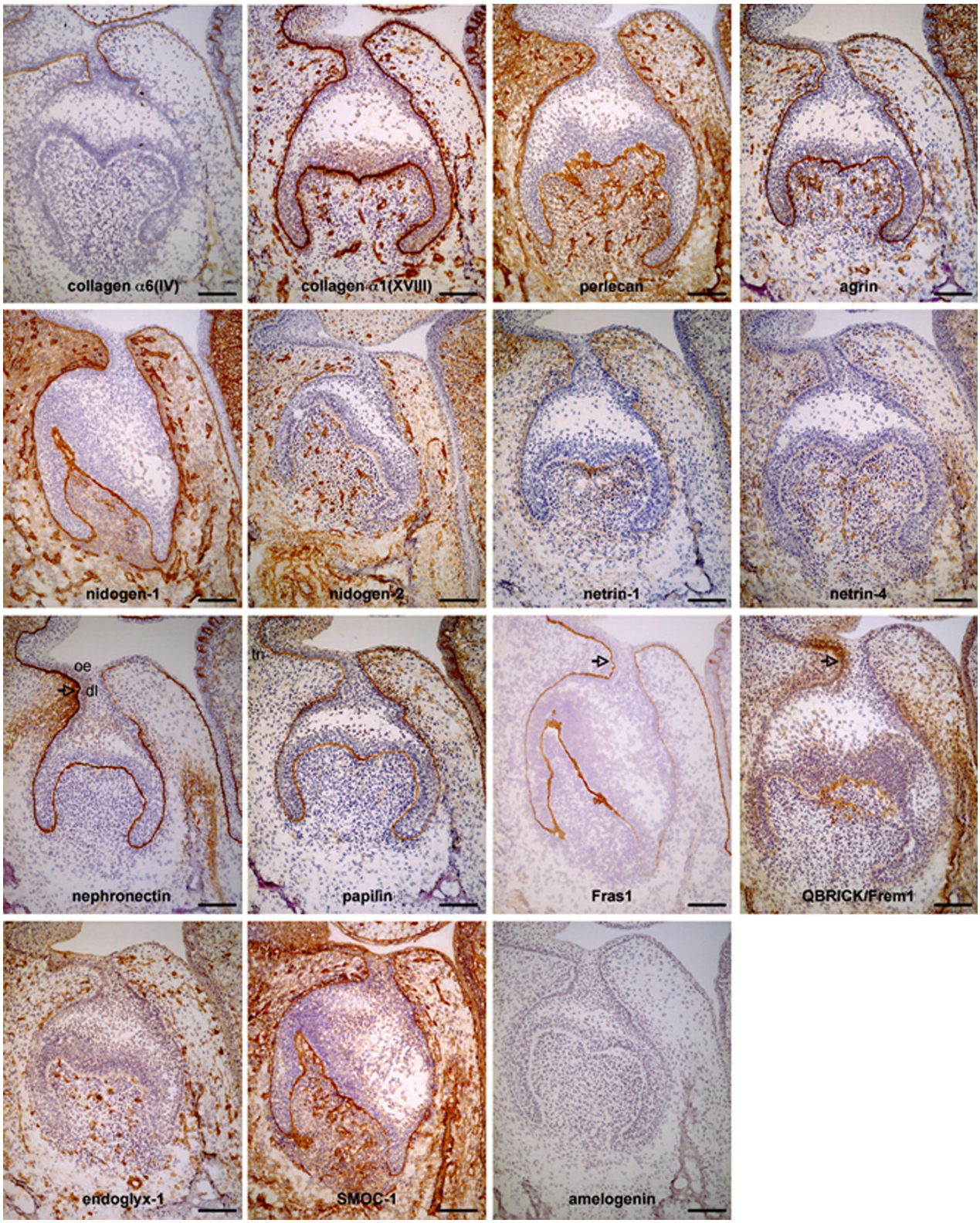

Fig. S7. (*Continued*).

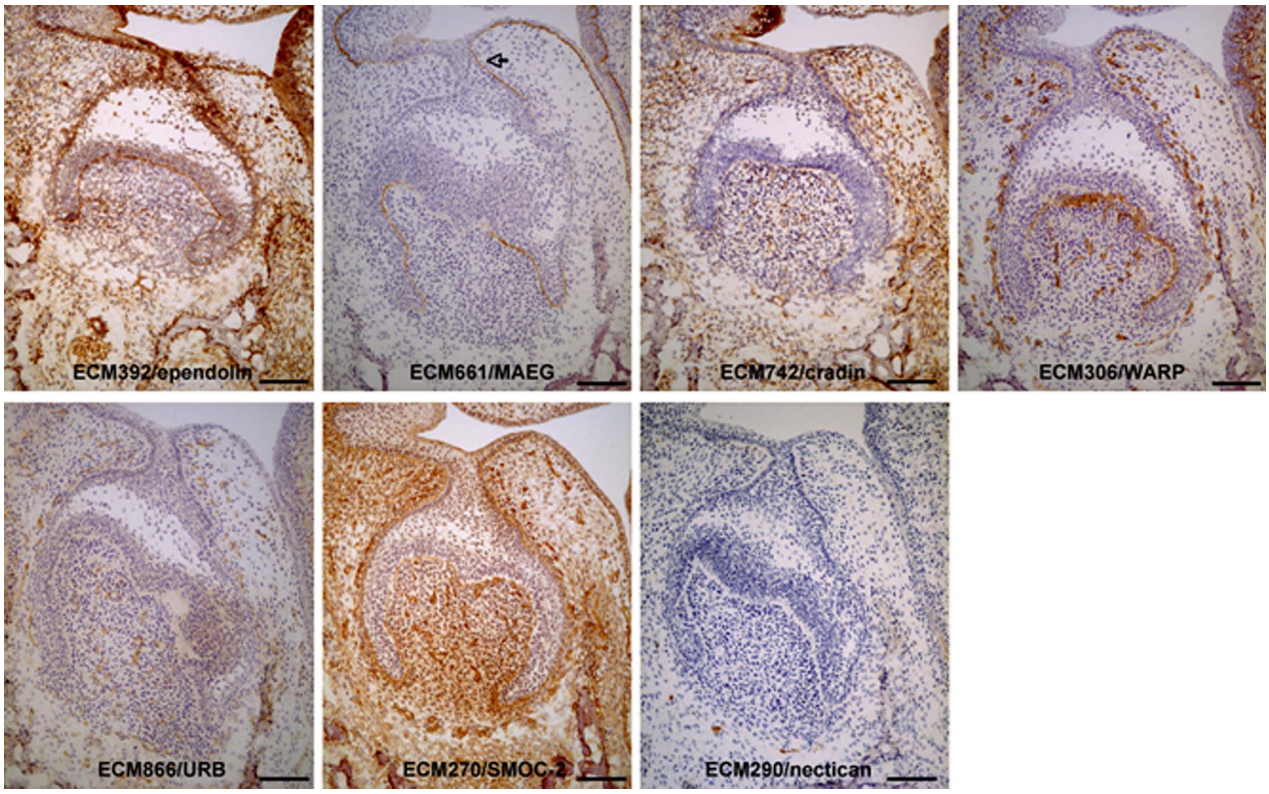
ECM392/ependolin
ECM661/MAEG
4
ECM742/cradin
ECM306/WARP
ECM866/URB
ECM270/SMOC-2
ECM290/nectican

Table S1. Designation of proteins

Clone name	Protein name*	Designation of protein
ECM392	ependolin	Protein localizing in basement membrane zones of the epidermis and vascular endothelium.
ECM661	MAEG	According to the published literature (1).
ECM742	cradin	CR (chordin-like cysteine-rich) domain-containing adhesive protein.
ECM306	WARP	According to the published literature (2).
ECM866	URB	According to the published literature (3).
ECM270	SMOC-2	According to the published literature (4).
ECM290	nectican	Proteoglycan with RGD-dependent cell adhesion-promoting activity.
ECM898	mamcan	MAM domain-containing proteoglycan.
ECM885	vitrin	Derived from an identical mouse EST clone (GenBank accession no. AF454755).
ECM314	emprin	Extracellular matrix protein with prion homology.
ECM482	eratin	Extracellular matrix protein isolated in our ERATO project.
ECM322	ADAMTSL-4	Protein structurally similar to ADAMTSL proteins.
ECM517	RAINB2	Protein structurally similar to human RAINB1 (5).
ECM432	tenonectin	Protein expressed in tendinous structures that shows RGD-dependent cell adhesion-promoting activity. Likely to be a mouse ortholog of human ECM2 (6).
ECM311	IGFBP-rP10/BONO1	According to the published literature (7, 8).
ECM201	periolin†	Periosteum-specific leucine-rich repeat-containing protein.
ECM402	epidermacan	Epidermis-specific proteoglycan.
ECM343	photomedin-1	Olfactomedin domain-containing protein expressed in the retinal photoreceptor layer (9).
ECM501	photomedin-2	Olfactomedin domain-containing protein expressed in the retinal photoreceptor layer (9).
ECM331	coffeecrisp	Protein structurally similar to the human EST clone CocoaCrisp (GenBank accession no. AF329197). Seems to be a mouse ortholog of rat LGL1 (10).
ECM712	keratonectin	Secreted protein specifically expressed in suprabasal keratinocytes that shows cell adhesion-promoting activity. Likely to be an isoform of SK89 (11) and dermokine-β (12).
ECM678	MCP-11	According to the published literature (13).
ECM69	gfod2	According to an EST clone named glucose-fructose oxidoreductase domain containing 2 (GenBank accession no. NP_081745).
ECM248	serac1	According to the published literature (14).

*Proteins that were given new names in this study are underlined.
†This protein is identical to the recently identified protein nephrocan (15).

1. Buchner G, *et al* (2000) Identification of a new EGF-repeat-containing gene from human Xp22: a candidate for developmental disorders. *Genomics* 65:16–23.
2. Fitzgerald J, Tay Ting S, Bateman J-F (2002) WARP is a new member of the von Willebrand factor A-domain superfamily of extracellular matrix proteins. *FEBS Lett* 517:61–66.
3. Aoki K, *et al.* (2002) Cloning, expression, and mapping of a gene that is upregulated in adipose tissue of mice deficient in bombesin receptor subtype-3. *Biochem Biophys Res Commun* 290:1282–1288.
4. Vannahme C, *et al.* (2003) Characterization of SMOC-2, a modular extracellular calcium-binding protein. *Biochem J* 373:805–814.
5. Merrill R-A, Plum L-A, Kaiser M-E, Clagett-Dame M (2002) A mammalian homolog of unc-53 is regulated by all-trans retinoic acid in neuroblastoma cells and embryos. *Proc Natl Acad Sci USA* 99:3422–3427.
6. Nishiu J, Tanaka T, Nakamura Y (1998) Identification of a novel gene (ECM2) encoding a putative extracellular matrix protein expressed predominantly in adipose and female-specific tissues and its chromosomal localization to 9q22.3. *Genomics* 52:378–381.
7. Shibata Y, *et al.* (2004) Role of a new member of IGFBP superfamily, IGFBP-rP10, in proliferation and differentiation of osteoblastic cells. *Biochem Biophys Res Commun* 325:1194–1200.
8. James M-J, Jarvinen E, Thesleff I (2004) Bono1: A gene associated with regions of deposition of bone and dentine. *Gene Expression Patterns* 4:595–599.
9. Furutani Y, *et al.* (2005) Identification and characterization of photomedins: novel olfactomedin-domain-containing proteins with chondroitin sulphate-E-binding activity. *Biochem J* 389:675–684.
10. Kaplan F, *et al.* (1999) A novel developmentally regulated gene in lung mesenchyme: homology to a tumor-derived trypsin inhibitor. *Am J Physiol* 276:L1027–L1036.
11. Moffatt P *et al* (2004) Identification of a conserved cluster of skin-specific genes encoding secreted proteins. *Gene* 334:123–131.
12. Matsui T *et al* (2004) Identification of novel keratinocyte-secreted peptides dermokine-α/-β and a new stratified epithelium-secreted protein gene complex on human chromosome 19q13.1. *Genomics* 84:384–397.
13. Wong G-W, *et al.* (2004) Mouse chromosome 17A3.3 contains 13 genes that encode functional tryptic-like serine proteases with distinct tissue and cell expression patterns. *J Biol Chem* 279:2438–2452.
14. Schimenti J-C, Reynolds J-L, Planchart A (2005) Mutations in Serac1 or Synj2 cause proximal *t* haplotype-mediated male mouse sterility but not transmission ratio distortion. *Proc Natl Acad Sci* USA 102:3342–3347.
15. Mochida Y, *et al.* (2006) Nephrocan, a novel member of the small leucine-rich repeat protein family, is an inhibitor of transforming growth factor-β signaling. *J Biol Chem* 281:36044–36051.

Table S2. *In vitro* functional assays for known ECM proteins

	Protein name	PSORTII PSG	PSORTII GvH	Matrix assembly	ECM molecule binding	GAG attachment	Cell adhesiveness
1	vitronectin	7.06	0.70				
2	thrombospondin-1	5.80	-2.19				
3	elastin	-4.40	3.23				
4	DEL1	5.38	2.57				
5	SPARC	7.53	5.57				
6	SMOC-1	3.49	3.57				
7	nephronectin	5.54	-1.62				
8	emilin	5.18	-1.10				
9	EMU-2	4.54	4.05				
10	collagen (α1) VIII	7.32	-2.90				
11	matrilin-1	7.30	3.56				
12	osteoprotegerin	5.75	-3.46				
13	testican-2	5.85	1.95				
14	testican-3	4.50	1.93				
15	biglycan	5.99	7.48				
16	decorin	6.95	2.08				
17	DMP-1	7.26	3.79				
18	nidogen-1	-4.40	1.70				
19	fibulin-1	4.08	2.21				
20	fibulin-2	-4.40	0.15				
21	fibulin-3	6.41	-0.59				
22	fibulin-5	5.43	0.92				
23	bone sialoprotein	5.85	0.68				
24	β ig-H3	5.21	1.70				
25	cyr-61	5.01	-2.17				
26	NOV	6.90	0.83				

To examine the feasibility of the strategy for *in vitro* functional screenings, 26 known ECM proteins were subjected to individual functional assays for their ECM assembly, binding to other ECM molecules, GAG attachment, and cell-adhesive activities. Items in yellow and grey indicate positive and negative results obtained in each experiment. The PSG and GvH scores derived from PSORTII searches are also shown.

Table S3. Clones selected by *in silico* screening but eliminated after *in vitro* functional assays

DDBJ accession no.	Amino acids	PSORTII		Pfam domain
		PSG	GvH	
AK037507	527	7.36	−4.72	Protein of unknown function(DUF563)
AK046675	437	4.28	0.14	PF00530; SRCR
AK015620	399	5.90	0.87	None
AK046350	841	4.49	1.96	EGF-like domain; EF hand; Kazal-type serine protease inhibitor domain; Immunoglobulin domain x2
AK014340	613	6.10	2.97	DOMON domain; Copper typeII ascorbate-dependent monooxygenase N-terminal domain; Copper type II ascorbate-dependent monooxygenase C-terminal domain
AK028377	408	−13.40	0.74	EGF-like domain; collagen triple helix repeat x20
AK028866	563	−4.40	0.91	None
AK014680	452	6.62	3.80	Serine carboxypeptidase
AK016685	438	6.95	3.92	20G-Fe(II) oxygenase superfamily
AK017087	434	6.90	−3.59	Serine carboxypeptidase
AK030604	685	4.69	0.07	Acyltransferase family
AK031608	392	6.73	7.13	None
AK018432	459	5.22	−1.38	EGF-like domain
AK033488	363	−5.55	−1.38	None
AK033642	1038	3.24	−0.81	None
AK035674	470	5.11	−0.79	None
AK050223	546	7.26	−2.76	None
AK054356	1182	6.59	−6.44	von Wille brand factor type A domain x6
AK005465	439	7.78	9.51	Sushi domain x2; EGF-like domain x2
AK013028	343	4.60	−0.22	Carbohydolate kinase
AK014608	426	5.75	−7.11	None
AK028580	464	−7.03	−1.60	None
AK029377	555	5.54	−3.16	None
AK033983	596	−4.40	1.08	None
AK034323	623	4.60	0.33	SAM domain (sterile alpha motif)
AK034617	410	5.44	−6.31	None
AK035259	504	4.71	−1.34	Galactosyltransferase
AK039202	359	1.51	2.94	None
AK043957	417	4.51	−3.89	None
AK044739	462	5.34	−7.93	None
AK046065	677	6.53	1.21	None
AK047738	493	4.50	−4.77	None
AK028081	358	5.57	2.81	None
AK036568	593	4.07	−1.03	None
AK012720	601	5.89	2.49	None
AK031254	359	−4.40	0.85	EGF-like domain x2; CUB domain, SUSHI domain
AK013520	329	5.01	−0.05	EF-hand
AK046301	837	8.25	1.11	Leucine-rich repeat
AK044704	389	6.27	−2.50	EGF-like domain x2; laminin G
AK030132	570	−4.40	−1.78	None
AK045363	408	5.54	−0.08	None
AK029104	541	−4.40	−1.22	Protein of unknown function (DUF1171)
AK077120	483	−0.13	1.16	None
AK078812	483	−0.81	2.04	None
AK084987	597	−1.92	0.72	None
AK142898	454	4.24	0.23	EGF-like domain x3; NTR/C345C module
AK076022	308	−2.45	1.35	Domain of unknown function (DUF323)
AK086819	496	3.29	2.47	Olfactomedin-like domain
AK076682	329	−4.62	−0.18	None
AK087856	448	2.49	1.80	None
AK079886	392	7.13	1.09	None
AK086298	311	4.80	1.31	EGF-like domain; SUSHI domain
AK076222	643	1.07	2.23	Glycosyltransferase family 25
AK076281	409	0.98	1.45	None
AK145082	489	2.94	2.00	None
AK132143	318	7.28	1.68	EF-hand
AK139382	410	−6.31	0.92	None
AK133986	347	−2.30	2.56	NHL repeat
AK157301	1045	−0.10	0.84	Cysteine-rich repeat; NF-X1 type Zinc-finger
AK160156	396	1.25	1.93	Glycosyl hydrolases family 18

DDBJ accession no.	Amino acids	PSORTII		Pfam domain
		PSG	GvH	
AK132922	320	−6.80	1.79	None
AK143522	517	−4.28	0.73	None
AK148185	356	−1.63	0.95	None
AK152969	467	2.09	2.51	Phospholipase D. Active site motif
AK146068	472	−2.45	0.93	Immunoglobulin domain x4
AK133536	505	4.09	1.38	Olfactomedin-like domain
AK135322	532	4.90	3.38	None
AK140169	402	−0.58	1.00	Domain of unknown function (DUF323)
AK140848	680	0.17	1.30	None

Table S4. BM proteins known to date

	Protein name*
1	laminin α1
2	laminin α2
3	laminin α3
4	laminin α4
5	laminin α5
6	laminin β1
7	laminin β2
8	laminin β3
9	laminin γ1
10	laminin γ2
11	laminin γ3
12	collagen α1(IV)
13	collagen α2(IV)
14	collagen α3(IV)
15	collagen α4(IV)
16	collagen α5(IV)
17	collagen α6(IV)
18	collagen α1(VII)
19	collagen α1(XV)
20	collagen α1(XVIII)
21	perlecan
22	agrin
23	nidogen-1
24	nidogen-2
25	netrin-1
26	netrin-4
27	nephronectin
28	papilin
29	Fras1
30	QBRICK/Frem1
31	Frem2
32	Frem3
33	endoglyx-1
34	SMOC-1
35	amelogenin
36	ECM392/ependolin
37	ECM661/MAEG
38	ECM742/cradin
39	ECM306/WARP
40	ECM866/URB
41	ECM270/SMOC-2
42	ECM290/nectican
43	usherin
44	TIN-ag
45	AMACO
46	amelotin

BM proteins are defined by their predominant localizations in BM zones on immunohistochemistry. Some interstitial ECM proteins, e.g., fibronectin, fibrillins, collagens, and fibulins, have also been shown to localize at BM zones, but are not classified as BM proteins here because of their predominant localizations in connective tissue matrices adjacent to the BM zones.
*BM proteins whose tissue localizations were determined by immunohistochemistry in the present study are underlined.

Table S5. Summary of BM protein composition in developing oral and tooth germ epithelia

BM protein	Epithelial BMs					
	Oral cavity adjacent to tooth germ	Tooth germ				
		Dental lamina	Outer enamel epithelium	Inner enamel epithelium		
				Apical zone	Middle zone	Basal zone
laminin α5						
laminin β1						
collagen α1(IV)						
collagen α2(IV)						
collagen α1(XVIII)						
nidogen1						
papilin						
perlecan						
agrin						
SMOC-1						
ECM270/SMOC-2						
nephronectin	*	*				
netrin-4						
laminin γ1						
nidogen2						
Fras1	*	*	*			
QBRICK	*	*	*			
ECM306/WARP						
netrin-1						
laminin β2						
ECM661/MAEG	**	**				
ECM392/ependolin						
ECM742/cradin						
laminin α2		*				
laminin γ3		*				
collagen α5(IV)						
collagen α6(IV)						
laminin α3						
laminin β3						
laminin γ2						
laminin α1						
laminin α4						
collagen α3(IV)						
collagen α4(IV)						
endoglyx-1						
amelogenin						
ECM866/URB						
ECM290/nectican						
Number of proteins detected	28	30	24	27	25	25

The expression levels of 38 BM proteins, including 7 newly identified proteins, in different regions of the E16.5 mouse first mandibular molar and oral mucosa are graded into three categories, namely strong (orange), moderate/slight (yellow), and undetectable (grey), based on their immunostaining intensities. The inner enamel epithelium is divided into three zones designated the apical (including the cervical loop), middle, and basal (including cusp and intercusp) zones. The numbers of BM proteins detected in the individual zones are indicated at the bottom.

*Stronger staining on the buccal side than on the lingual side.

**Stronger staining on the lingual side than on the buccal side.

Table S6. Antibodies used in the immunohistochemical analyses

Basement membrane protein	Antibody			Antigen*				GenBank or RIKEN FANTOM Accession no.	Pretreatments**
				Antigen 1		Antigen 2			
	Type	Clone name	Cat no./Vendor	Polypeptide	Expression vector	Polypeptide	Expression vector		
laminin α1	mAb	5B7-H1		C749-P1156	pSecTag2A			J04064	formaldehyde, acid, and enzymes
laminin α2	mAb	4H8-2	L0663/SIGMA Aldrich						formaldehyde, acid, and enzymes
laminin α3	mAb	M35-N3-B9		L231-Q639	pSecTag2A			X84013	acetone, acid, and enzymes
laminin α4	mAb	M49-N7-F3		R1032-I1228	pSecTag2A			NM_010681	formaldehyde, acid, and enzymes
laminin α5	mAb	M5N8-C8		G1643-E2167	pSecTag2A			NM_001081171	formaldehyde, acid, and enzymes
laminin β1	mAb	1B5-D12		C821-P1226	pSecTag2A			M15525	formaldehyde, acid, and enzymes
laminin β2	mAb	B24-N8-D6		R523-A831	pSecTag2A			NM_008483	acetone, acid, and enzymes
laminin β3	mAb	B31-N8-G8		Q18-C575	pSecTag2A			U43298	formaldehyde, acid, and enzymes
laminin γ1	mAb	A5	MAB1914/Chemicon						formaldehyde and acid
laminin γ2	mAb	C21-N1-C9		H197-C602	pSecTag2A			U43327	acetone, acid, and enzymes
laminin γ3	mAb	C38-N4-F4		C767-S927	pSecTag2A			NM_011836	acetone, acid, and enzymes
collagen α1(IV)	mAb	H11		(1)					acetone and acid
collagen α2(IV)	mAb	H22		(1)					acetone and acid
collagen α3(IV)	mAb	H31		(1)					acetone and acid
collagen α4(IV)	mAb	RH43		(2)					acetone and acid
collagen α5(IV)	mAb	H53		(2)					acetone and acid
collagen α6(IV)	mAb	B66		(2)					acetone
collagen α1(XVIII)	mAb	CM186		A1101-D1136	(3)			NM_009929	acetone and enzymes
perlecan	mAb	A7L6	MAB1948/Chemicon						formaldehyde and enzymes
agrin	pAb			E778-D958	pET42a			BC059259	formaldehyde and enzymes
nidogen-1	mAb	ELM1	RT797/NeoMarkers						formaldehyde, acid, and enzymes
nidogen-2	pAb			T953-K1403	pSecTag2A	F275-T510	pET42a	AK140832	formaldehyde and enzymes
netrin-1	pAb	Ab-2	PC364/Oncogene Science						formaldehyde and enzymes
netrin-4	pAb			M1-V628	pSecTag2A			AF268066	formaldehyde and enzymes
nephronectin	pAb			M1-C561	pFLAG-CMV-5a			AK050484	formaldehyde and enzymes
papilin	pAb			M1-W309	pSecTag2A	R801-L1063	pET42a	AK086767	formaldehyde and enzymes
Fras1	pAb			(4)		C27-C86	pSecTag2A	CAD33519.1	formaldehyde and enzymes
QBRICK/Frem1	pAb			(5)					ethanol, acid, and enzymes
endoglyx-1	pAb			T38-N56	KLH	G921-P939	KLH	AK077884	formaldehyde, acid, and enzymes
SMOC-1	pAb			M1-V463	pCImychis			AK040931	acetone, acid, and enzymes
amelogenin	pAb		PC-062/Kamiya Biomedical Co.						formaldehyde and enzymes
ECM392/ependolin	pAb			E175-M303	pET42a	K42-E160	pET42a	A830089I08	no pretreatment
ECM661/MAEG	pAb			(6)				6820436K20	formaldehyde and enzymes
ECM742/cradin	pAb			G153-M324	pSecTag2A	S30-D148	pET42a	A930041G11	no pretreatment
ECM306/WARP	pAb			G207-P415	pGEX4T1	G33-Q217	pGEX4T1	4932416A11	formaldehyde and enzymes
ECM866/URB	pAb			K850-F863	KLH	S45-G59	KLH	3321404F21	formaldehyde and enzymes
ECM270/SMOC-2	pAb			M1-G446	pSecTag2A	M1-T284	pSecTag2A	5830469N15	formaldehyde, acid, and enzymes
ECM290/nectican	pAb			N232-S346	pGEX4T1			A030011G10	formaldehyde and enzymes
ECM898/mamcan	pAb			G24-E169	pET42a	A661-Y686	KLH	9530067G07	ethanol and enzymes
ECM885/vitrin	pAb			A136-N261	pET42a	D467-R635	pIVEX	F930032K14	formaldehyde and enzymes
ECM314/emprin	pAb			P70-E159	pET42a	G247-P349	pET42a	9930102M17	ethanol and enzymes
ECM482/eratin	pAb			Q22-S107	pET42a	G110-S215	pET42a	D130017E17	ethanol and enzymes
ECM322/ADAMTSL-4	pAb			R95-D258	pGEX4T1			B230114P05	formaldehyde and enzymes
ECM517/RAINB2	pAb			Q93-S232	pGEX4T1	D402-R576	pGEX4T1	A430070G12	formaldehyde and enzymes
ECM432/tenonectin	pAb			S158-G261	pGEX4T1			A530057I08	ethanol and enzymes
ECM311/IGFBP-rP10	pAb			E176-Y313	pGEX4T1	Q32-G120	pGEX4T1	5430425E24	formaldehyde, acid, and enzymes
ECM201/periolin	pAb			K441-D512	pGEX4T1			5730451H22	acetone, acid, and enzymes
ECM402/epidermacan	pAb			G294-S380	pGEX4T1			4831422J22	formaldehyde
ECM343/photomedin-1	pAb			(7)				9530014B09	formaldehyde
ECM501/photomedin-2	pAb			(7)				4832415H08	formaldehyde
ECM331/coffeecrisp	pAb			E417-Q495	pGEX4T1	P26-G208	pGEX4T1	9330102O19	formaldehyde
ECM712/keratoadhesin	pAb			N380-F393	KLH			C130083I19	acetone and acid
ECM678/MCP-11	pAb			G236-S318	pET42a			C130091P12	formaldehyde and enzymes

*In most of the immunohistochemical analyses for newly identified ECM proteins, two nonoverlapping antigens (antigens 1 and 2) were used for the production of antibodies. Antibodies raised against antigen 1 were used for representation of the immunohistochemical data. The amino acid numbers refer to the polypeptides used as antigens. The expression vectors used for production of the antigens are also shown. KLH (keyhole limpet hemocyanin) indicates that synthetic peptides conjugated to KLH were used for the antibody production. Numbers in parentheses are references.

**To optimize the staining with antibodies, the types of fixatives and conditions for pretreatments with acid (0.1 M KCl-HCl, pH 1.5) and/or enzymes (hyaluronidase and chondroitinase ABC) were determined for individual antibodies.

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Other Supporting Information Files

SI Appendix (pdf)