Molecular Biology of the Cell
Vol. 15, 3580–3590, August 2004

NG2 Proteoglycan Promotes Endothelial Cell Motility and Angiogenesis via Engagement of Galectin-3 and $\alpha 3\beta 1$ Integrin

Jun-ichi Fukushi,* Irwan T. Makagiansar, and William B. Stallcup

The Burnham Institute, La Jolla, California 92037

Submitted March 19, 2004; Revised May 22, 2004; Accepted May 27, 2004
Monitoring Editor: Martin A. Schwartz

The NG2 proteoglycan is expressed by microvascular pericytes in newly formed blood vessels. We have used in vitro and in vivo models to investigate the role of NG2 in cross-talk between pericytes and endothelial cells (EC). Binding of soluble NG2 to the EC surface induces cell motility and multicellular network formation in vitro and stimulates corneal angiogenesis in vivo. Biochemical data demonstrate the involvement of both galectin-3 and $\alpha 3\beta 1$ integrin in the EC response to NG2 and show that NG2, galectin-3, and $\alpha 3\beta 1$ form a complex on the cell surface. Transmembrane signaling via $\alpha 3\beta 1$ is responsible for EC motility and morphogenesis in this system. Galectin-3–dependent oligomerization may potentiate NG2-mediated activation of $\alpha 3\beta 1$. In conjunction with recent studies demonstrating the early involvement of pericytes in angiogenesis, these data suggest that pericyte-derived NG2 is an important factor in promoting EC migration and morphogenesis during the early stages of neovascularization.

INTRODUCTION

Angiogenesis, the formation of new blood vessels by sprouting from existing vessels, plays a key role in a large number of physiological processes, including development of the embryo, formation of the corpus luteum, endochondral ossification, and wound healing as well as pathological processes such as the enlargement of solid tumors, progression of ocular diseases, psoriasis, and rheumatoid arthritis (Folkman, 1995). The regulation of neovascular development is heavily dependent on cross-talk between the two microvascular cell types: endothelial cells (EC) and pericytes (Beck and D'Amore, 1997). This cell-cell communication is mediated by both soluble growth factors and cell adhesion molecules (Gerhardt and Betsholtz, 2003).

The critical functional role of pericytes in microvascular morphogenesis is vividly illustrated by the pathological phenotypes of mice in which pericyte development is interrupted or delayed by ablation of genes for platelet-derived growth factor (PDGF)-B and PDGF β-receptor (Lindahl *et al.*, 1997). A growing body of evidence suggests that pericytes are present at very early stages of angiogenesis, placing them in a position to help stimulate EC recruitment and guide the formation of capillary tubes via either cell-cell interaction or the release of soluble factors (Schlingemann *et al.*, 1990; Nehls *et al.*, 1992; Wesseling *et al.*, 1995; Redmer *et al.*, 2001; Ozerdem *et al.*, 2001, 2002; Gerhardt and Betsholtz, 2003; Ozerdem and Stallcup, 2003). Elucidation of the early contribution of pericytes to angiogenesis has been facilitated by the use of markers such as PDGF β-receptor, aminopeptidase A, and NG2 that characterize "activated" pericytes at early points in their development (Lindahl *et al.*, 1997; Schlingemann *et al.*, 1996; Ozerdem *et al.*, 2001). NG2 is a transmembrane chondroitin sulfate proteoglycan that is widely expressed in newly formed blood vessels. During normal development, NG2 is detected in both microvessels and large vessels like the embryonic aorta (Ozerdem *et al.*, 2001). In mature animals NG2 expression is associated with vasculature formed during tumor development and wound healing (Schlingemann *et al.*, 1990; Ozerdem and Stallcup, 2003).

In addition to establishing its value as a marker, our observations concerning NG2 have suggested several possible means by which the proteoglycan may positively regulate angiogenesis. NG2 has been shown to bind directly to basic FGF (bFGF) and PDGF-AA (Goretzki *et al.*, 1999), and may enhance interaction of these growth factors with cell surface receptors (Grako *et al.*, 1999). NG2 also binds to and modulates the activities of kringle domain-containing proteins such as plasminogen and angiostatin (Goretzki *et al.*, 2000). In addition, expression of NG2 by tumor cells has been shown to promote tumor growth and metastasis, possibly by increasing tumor angiogenesis (Burg *et al.*, 1998; Chekenya *et al.*, 2002).

An additional possibility is that NG2 directly mediates communication between pericytes and vascular ECs. The appeal of this idea is enhanced by the observation that the extracellular domain of NG2 can be proteolytically released (shed) from the cell surface both in vitro and in vivo (Nishiyama *et al.*, 1995; Jones *et al.*, 2002). Thus, soluble NG2 released from pericyte cell surfaces might stimulate recruitment of ECs to sites of neovascularization. To investigate this aspect of the proteoglycan's function during angiogenesis, we have studied the ability of the NG2 ectodomain to interact with ECs and affect their behavior. In this report we demonstrate that soluble recombinant NG2 binds to ECs and

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E04–03–0236. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E04–03–0236.

* Corresponding author. E-mail address: fukushi@burnham.org.

Abbreviations used: bFGF, basic fibroblast growth factor; EC, endothelial cell(s); HMVEC, human microvascular endothelial cells; MAEC, mouse aortic endothelial cells; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; NG2/EC, extracellular domain of NG2; PDGF, platelet-derived growth factor.

promotes both their migration and the formation of cellular networks in vitro. When implanted in the cornea, NG2 induces in vivo angiogenesis in this normally avascular tissue. In addition, we identify the galactose-binding protein galectin-3 and the $\alpha3\beta1$ integrin as two putative NG2 receptors on the surface of ECs. NG2 binding appears to activate transmembrane signaling via $\alpha3\beta1$. Galectin-3 oligomerization may be important for clustering NG2 to permit more effective activation of $\alpha3\beta1$. These findings suggest that NG2 expressed by pericytes may help regulate angiogenesis by stimulating the motility and morphogenesis of neighboring ECs.

MATERIALS AND METHODS

Cell Culture

Human dermal microvascular ECs were obtained from Cambrex Bioscience Walkersville, Inc. (Walkersville, MD) and maintained in Cambrex EC growth medium EGM-MV. The BALB/c mouse aortic EC (MAEC) line 22106 (Bastaki *et al.*, 1997) was provided by Dr. Yu Yamaguchi (The Burnham Institute, La Jolla, CA). Cells were maintained in DMEM containing 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin sulfate. Neither of these EC lines express cell surface NG2 as determined by immunofluoresence microscopy. A375, MDA-MB-435, and MDCK cells were obtained from ATCC (Manassas, VA). SV7tert cells (Arbiser *et al.*, 2001) were a gift from Dr. Jack Arbiser, Emory University School of Medicine.

Reagents

Type I collagen (Vitrogen 100) was obtained from Cohesion (Palo Alto, CA). Growth factor reduced Matrigel was purchased from Becton Dickinson (Bedford, MA). Bovine plasma fibronectin was from Sigma (St. Louis, MO). The rat anti-mouse galectin-3 mAb (M3/38)-producing hybridoma TIB-166 was obtained from ATCC. mAbs FB12 (anti-$\alpha1$), P1B5 (anti-$\alpha3$ blocking antibody), P4C2 (anti-$\alpha4$), and polyclonal antibodies against the cytoplasmic tails of $\alpha3$, $\alpha5$, and $\beta1$ integrin chains are products of Chemicon International, Inc. (Temecula, CA). Polyclonal antibodies against the cytoplasmic tails of the $\alpha2$ and $\alpha6$ integrin subunits were provided by Dr. Kristina Vuori (The Burnham Institute, La Jolla, CA). PharMingen (La Jolla, CA) was the source of mAbs IM7 (anti-CD44), MEC 13.3 (anti-CD31), 9EG7 and HUTS-21 (antiactivated $\beta1$ integrin subunit). Mouse monoclonal (Eric-1) antibody against NCAM was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal and mouse monoclonal (N11.4) antibodies against NG2 have been described previously (Nishiyama *et al.*, 1991; Ozerdem *et al.*, 2001).

Purified Proteins

A 753-base pair fragment of human galectin-3 DNA corresponding to the coding region of nucleotides 19–771 (GenBank accession no. NM002306) was generated by RT-PCR and subcloned into pGEX-2T (Amersham Biosciences, Piscataway, NJ). GST-galectin-3 fusion protein was expressed in *Escherichia coli* BL21 (DE3) (Stratagene, San Diego, CA). Induction with IPTG and purification of fusion proteins were carried out as previously described (Barritt *et al.*, 2000) using glutathione-agarose beads (Sigma). Two recombinant NG2 species were used in these experiments: NG2/EC$^-$ comprising the whole extracellular domain (residues 1–2223) without glycosaminoglycan chains, and NG2/EC$^+$ with glycosaminoglycan chains (Tillet *et al.*, 1997). These were purified by DEAE Sepharose chromatography from tissue culture supernatant of 293 cells transfected with cDNA coding for the ectodomain of rat NG2. Tissue culture supernatant of 293 cells transfected with vector alone was subjected to parallel DEAE Sepharose fractionation. High salt fractions corresponding to those containing the NG2/EC$^+$ species were designated as "mock purified" material and used as a control in several experiments.

Flow Cytometry and Immunofluorescence

For flow cytometry, cells were dissociated using enzyme-free cell dissociation buffer (Invitrogen, Carlsbad, CA), resuspended in PBS containing 1% BSA, and incubated with antigalectin-3 antibody or control IgG for 30 min on ice. After several washes with PBS containing 1% BSA, cells were incubated with fluorescein-coupled goat secondary antibody against rat IgG (Biosource, Camarillo, CA) for 20 min. After final washing, cells were examined using a FACSort instrument (Becton Dickinson, Oxford, CA).

Expression of the activated $\beta1$ integrin epitope was evaluated in living cells by immunofluorescence staining with mAbs 9EG7 and HUTS-21. Cells were cultured on glass coverslips, serum-starved overnight, and incubated with 50 μg/ml NG2/EC$^+$ or phosphate-buffered saline (PBS) for 30 min. After washing with PBS, cells were incubated for 1 h on ice with the primary antibodies. After several washes with DMEM containing 2% fetal calf serum (FCS), cells were incubated with fluorescein-coupled goat secondary antibody against rat (for 9EG7) or mouse (for HUTS-21) IgG for 30 min. After final washing and fixation with 95% ethanol, specimens were cover-slipped in Vectashield with DAPI (H-1200, Vector Laboratories, Burlingame, CA) and examined using a Nikon Optiphot microscope attached to a Coolsnap procamera with a 1392 × 1040 resolution (Media Cybernetics, Silver Spring, MD). Images were captured with a PlanApo 40× objective using Image-ProExpress software (Media Cybernetics). Postimaging processing was performed in Adobe Photoshop 5.0 (San Jose, CA). For galectin-3 detection, cells were cultured overnight on glass coverslips, and a similar staining protocol was followed (omitting the incubation with NG2/EC$^+$).

Coexpression of NG2, $\alpha3$ integrin and CD31 was examined in the neovasculature of healing wound tissue. C57Bl/6 mice aged 3 months were anesthetized with Avertin, and paired 6-mm-diameter, full-thickness, excisional wounds were made through the skin on the back. Wound tissue was harvested at 7 days after wounding and snap-frozen in liquid nitrogen before embedding in TissueTek OCT compound (Sakura Finetek USA, Torrance, CA). Cryostat sections were fixed with ice-cold acetone, washed and blocked with PBS containing 1% FCS, and incubated with the primary antibodies overnight at 4°C. After two washes with PBS, sections were incubated with fluorescein- or rhodamine-coupled secondary antibodies for 2 h, and examined as described above.

NG2 Binding Assay

MAEC were washed three times with PBS and incubated in PBS + 1% BSA with or without 50 μg/ml NG2/EC$^+$ at 4°C for 5 h. After washing with PBS, cell-bound NG2 was detected by immunofluorescence using a rabbit antibody against the proteoglycan.

Cell Attachment Assay

Ninety-six-well plates (Becton Dickinson, Franklin Lakes, NJ) were coated overnight at 4°C with various concentrations of NG2/EC or ECM proteins and then blocked with PBS containing 1% BSA for 2 h. Serum-starved ECs were harvested using enzyme-free cell dissociation buffer and plated in DMEM + 1% BSA at a density of 12,500 cells/well. Attachment was allowed to proceed for 1 h at 37°C. Nonadherent cells were removed by floatation on a 1.10 g/ml Percoll solution (Sigma), and the remaining adherent cells were fixed with glutaraldehyde and stained for 30 min with 0.1% crystal violet in water. After extensive washes, adherent cells were counted under the microscope. Four random 200× fields were counted for each well, and each assay point was determined in triplicate.

Cell Spreading Assay

Wells were coated overnight at 4°C with either NG2/EC (10 μg/ml) or type I collagen (30 μg/ml) and blocked for 2 h with a solution of 1% BSA in PBS. Cells were harvested with cell dissociation buffer, plated on the cover slips, and allowed to attach for 1 h at 37°C. Nonadherent cells were removed by several washings with PBS. Adherent cells were fixed with 2.5% paraformaldehyde (PFA) in PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and incubated for 45 min with rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR). Specimens were examined as described in "Immunofluorescence."

Cell Migration Assay

EC migration was assayed using 6.5-mm Transwell chambers (Corning Costar, Cambridge, MA). The undersides of 8-μm pore size chambers were coated overnight at 4°C with various concentrations of NG2/EC or extracellular matrix proteins. The chambers were then blocked for 2 h with 1% BSA in PBS and put in a 24-well cluster plate filled with medium containing 1% FCS. In some experiments, rabbit polyclonal anti-NG2 antibodies or control IgG were added to the cluster plate and incubated for 1 h before cells were plated. ECs were released with enzyme-free cell dissociation buffer and resuspended in medium containing 1% FCS. Cells were added into the Transwells at 4×10^5 cells/well, and migration to the underside of the precoated filter was examined after 2-h (MAEC) or 5-h (human microvascular ECs [HMVEC]) incubation at 37°C. The membranes were fixed and stained with Diff-Quick (Dade Diagnostics, Aguada, PR). In some experiments, cells were preincubated with mAbs (anti-$\alpha3$ P1B5, antigalectin-3 M3/38, anti-CD44, or control IgG) for 15 min before being added to the chambers.

Assay for EC Network Formation

Type I collagen gels (2.4 mg/ml, 100 μl/well) containing various doses (2–50 μg/ml) of NG2/EC were prepared in 96-well plates (Fukushi *et al.*, 2000). MAEC were released with enzyme-free cell dissociation buffer, resuspended in DMEM containing 1% FCS, and plated onto the gel at 2×10^5 cells/well. After 48-h incubation at 37°C, gels were fixed with 4% PFA. Morphogenesis within the gel was examined using a Nikon Optiphot as described above. The images were captured using Image-ProExpress and the total length of the cord-like structures was determined using NIH-Image software. Four random fields were measured for each dish.

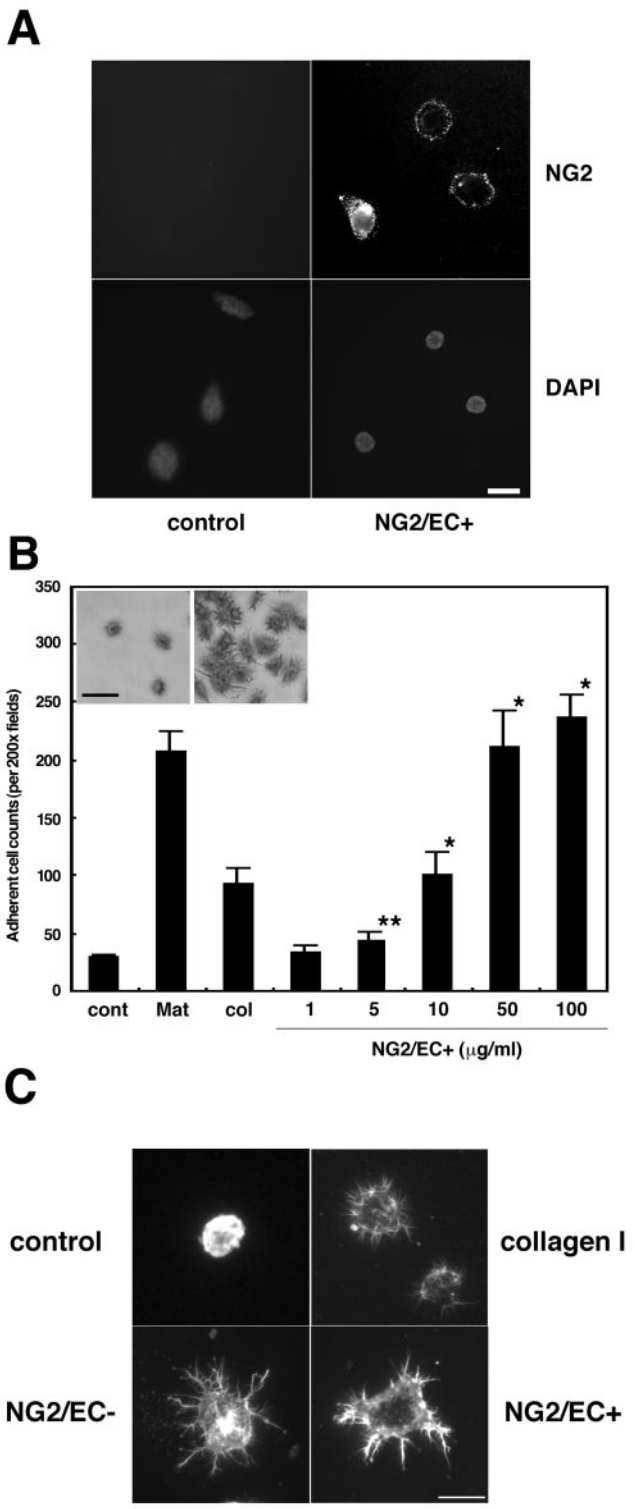


Figure 1. Attachment and spreading of ECs on NG2. (A) NG2 binding to the EC surface. NG2 immunoreactivity is seen on the surfaces of MAEC incubated with soluble NG2/EC$^+$, but not on untreated cells (control). DAPI was used as a nuclear counterstain. Scale bar, 20 μm. (B) EC attachment to NG2-coated surfaces. MAEC were tested for their ability to adhere to wells coated with Matrigel (30 μg/ml), type I collagen (30 μg/ml), and various concentrations of NG2/EC$^+$ (1–100 μg/ml). Three high-power microscope fields were counted in each replicate well, and results were expressed as cells per field. Each bar represents the mean ± SD (n = 3). Statistically significant differences compared with the control are indicated (*$p < 0.005$; **$p < 0.05$). Insets show the stained cells in control (left) and NG2-coated (100 μg/ml) wells (right). Adherent cells in the right-hand panel represent roughly 90% of the input cells. Scale bar, 50 μm. (C) Spreading of ECs on NG2-coated surfaces. Staining with rhodamine-phalloidin was used to visualize F-actin organization in MAEC after adherence for 1 h to wells coated with BSA (control), type I collagen (30 μg/ml), NG2/EC$^+$ (10 μg/ml), or NG2/EC$^-$ (10 μg/ml). Scale bar, 20 μm.

Corneal Pocket Assay

Hydron pellets (0.5 μl for rat and 0.1 μl for mice; IFN Sciences, New Brunswick, NJ) containing NG2/EC$^+$ (500 ng for rats and 200 ng for mice) or bFGF (100 ng for rats and 50 ng for mice) were prepared and implanted in the corneas of 8-week-old male Sprague Dawley rats, C57BL/6 mice, or BALB/c mice (Fukushi *et al.*, 2000). In some cases mAb against NG2 (N11.4, 2 μg/pellet) or isotype matched control IgG was added directly to the NG2/EC/Hydron preparation. After 5 days, the animals were sacrificed by CO_2 inhalation, and the corneal vessels were photographed using a Leica MZ125 stereo-microscope (Deerfield, IL) equipped with a Canon PowerShot S45 digital camera (Lake Success, NY). The images were captured with a 1.0× PLAN objective and processed in Adobe Photoshop 5.0. The corneas were scored as "positive" when any blood vessels were observed growing toward the implanted pellet from the preexisting vasculature of the limbus. These studies were approved by the authors' institutional review board and were performed in accordance with NIH guidelines.

Cell Lysis, Immunoprecipitation, and Immunoblotting

For immunoprecipitation, A375 cells or MAEC were lysed for 30 min in ice-cold lysis buffer containing 25 mM octylglucopyranoside, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 2 mM Na_3VO_4, 1 mM PMSF, and protease inhibitor cocktail. After centrifugation, cleared supernatants were incubated for 2 h at 4°C with continuous mixing with rabbit anti-NG2, anti-β1 antibodies, or IgG coupled to protein A-Sepharose (Sigma). Immunoprecipitates were washed five times with lysis buffer.

For immunoblotting, samples were separated using SDS-PAGE, transferred to nitrocellulose, blocked with 5% BSA in Tris-buffered saline, and probed with primary antibodies. Antibody binding was detected using peroxidase-labeled second antibody and ECL kit (Amersham Pharmacia, Buckinghamshire, United Kingdom).

NG2 Affinity Matrix and Binding Experiments

To prepare an affinity matrix (NG2 beads), NG2/EC$^+$ was coupled to cyanogen bromide-activated Sepharose CL-4B (Amersham Biosciences). MAEC were lysed for 30 min in ice-cold lysis buffer containing 1% NP40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and protease inhibitor cocktail. After centrifugation, cleared supernatants were collected and incubated with NG2 beads or control Sepharose CL-4B for 1 h at 4°C. Beads were washed three times with lysis buffer, and bound proteins were separated using SDS-PAGE followed by silver staining or immunoblotting. After silver staining, gel pieces containing individual proteins were excised and subjected to in-gel trypsin digestion. Proteins were identified by determining the masses of tryptic peptides using MALDI-TOF mass spectrometry.

Pull-down Assays

Glutathione *S*-transferase (GST)-galectin-3 fusion protein was incubated with NG2 beads or CL-4B Sepharose beads for 1 h at 4°C with constant agitation. Similarly, NG2/EC was incubated with GST-galectin-3 fusion protein-coated beads or GST-coated beads. Beads were then washed three times with PBS containing 0.1% NP40, and bound proteins were eluted with 100 mM lactose. The samples were separated using SDS-PAGE and then subjected to immunoblotting using polyclonal antibody against NG2 or mAb against galectin-3 as described above.

Statistical Analyses

Data were analyzed using the Student's *t*-test (two-tailed).

RESULTS

Attachment and Spreading of ECs on NG2-coated Surfaces

To investigate the interaction of NG2 with ECs, we incubated MAEC with the purified extracellular domain of the proteoglycan (NG2/EC$^+$). Indirect immunohistochemistry detected NG2 associated with MAEC surfaces, whereas no NG2 signal was seen on MAEC in the absence of exogenous

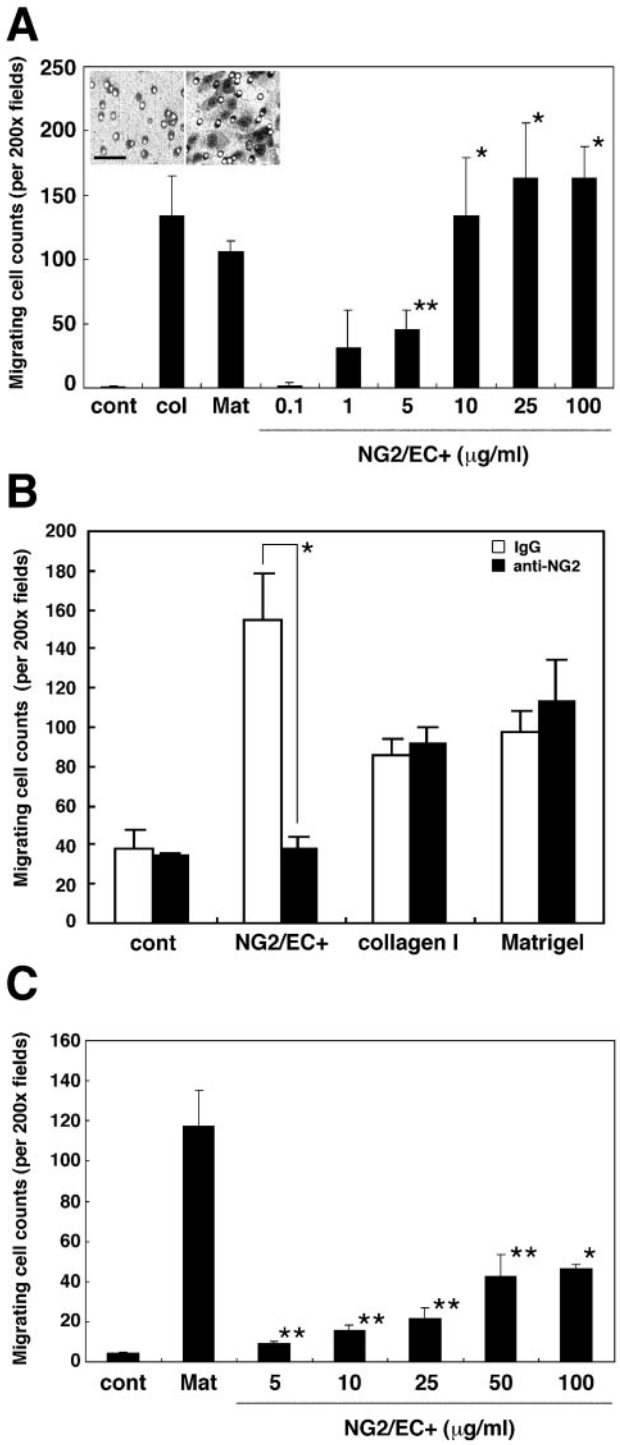


Figure 2. NG2-stimulated EC migration. (A) MAEC were tested for migration through Transwell membranes coated on the underside with type I collagen (30 μg/ml), Matrigel (30 μg/ml), or various concentrations of NG2/EC$^+$ (0.1–100 μg/ml). Three high-power microscope fields were counted in each replicate well, and results were expressed as cells per field. Each bar represents the mean ± SD (n = 3). Statistically significant differences compared with the control are indicated (*$p < 0.001$; **$p < 0.01$). Insets show representative micrographs of stained cells on the underside of control (left) and NG2-coated (25 μg/ml) wells (right). Scale bar, 50 μm. (B) Transwells were coated with type I collagen (30 μg/ml), Matrigel (30 μg/ml), or NG2/EC+ (10 μg/ml), and preincubated with NG2 antibody or control IgG (20 μg/ml) for 1 h before MAEC were added. Cell migration was quantified as described above. Each bar represents the mean ± SD (n = 3). A statistically significant difference was obtained for anti-NG2 compared with the value obtained in the presence of control IgG (*$p < 0.001$). (C) HMVEC migration was quantified in Transwells coated with Matrigel (30 μg/ml) or various concentrations of NG2/EC+ (5–100 μg/ml). Each bar represents the mean ± SD (n = 3). Statistically significant differences compared with the control are indicated (*$p < 0.0001$; **$p < 0.01$).

proteoglycan (Figure 1A). As an additional demonstration of NG2 interaction with the EC surface, we tested MAEC adherence to various types of substrata. As shown in Figure 1B, MAEC adhere to type I collagen- and Matrigel-coated surfaces. MAEC also adhere to NG2/EC-coated surfaces in a dose-dependent manner, independent of the presence (NG2/EC$^+$; Figure 1B) or absence of the chondroitin sulfate chain (NG2/EC$^-$; unpublished data). Wells coated with "mock purified" material from NG2-negative 293 cell supernatants (see MATERIALS AND METHODS) did not promote EC adherence (unpublished data). To determine whether interaction of ECs with NG2-coated surfaces is accompanied by reorganization of the actin cytoskeleton, we used rhodamine-labeled phalloidin to examine the cytoskeletal architecture of adherent MAEC. As shown in Figure 1C, cell spreading was observed on type I collagen-coated surfaces, but not on BSA-treated control surfaces. On NG2/EC-coated surfaces, cells were extensively spread with well-developed filopodia.

NG2 Stimulates Migration of ECs

In light of the dynamic effects of NG2 on EC morphology, we examined the effect of the proteoglycan on EC proliferation and migration, two critical aspects of angiogenesis. Consistent with our previous report (Goretzki *et al.*, 2000), addition of soluble NG2 had no obvious effect on EC proliferation under several different conditions (unpublished data). We then investigated whether ECs migrate through polycarbonate filters coated with NG2/EC. As shown in Figure 2A, MAEC migration through Transwells was stimulated in a dose-dependent manner by coating the undersides of the filters with NG2/EC$^+$. A similar stimulatory effect was observed with NG2/EC$^-$ coated filters (unpublished data). This migration induced by NG2/EC$^+$ was almost completely inhibited in the presence of rabbit polyclonal anti-NG2 antibody (20 μg/ml; Figure 2B). The lack of effect of the NG2 antibody on EC migration in response to collagen I and Matrigel emphasizes the specificity of this inhibition. The stimulatory effect of NG2/EC$^+$ on migration was also observed for HMVEC derived from skin (Figure 2C). EC migration was not stimulated by the "mock purified" preparation (unpublished data), further demonstrating that migration depends on the presence of NG2 rather than on contaminants in the purified material.

NG2 Promotes Formation of Multicellular Endothelial Networks

Extending the studies on EC morphogenesis and motility, we determined whether NG2 can stimulate the formation of EC networks in three-dimensional type I collagen gels. As shown in Figure 3A, MAEC plated on the surface of a type I collagen gel can invade the matrix and assume spindle-shaped morphologies during a 48-h incubation period (left panel). In the presence of NG2/EC$^+$, increased numbers of cells developed spindle-shaped morphologies and assembled into multicellular cord-like networks (right panel).

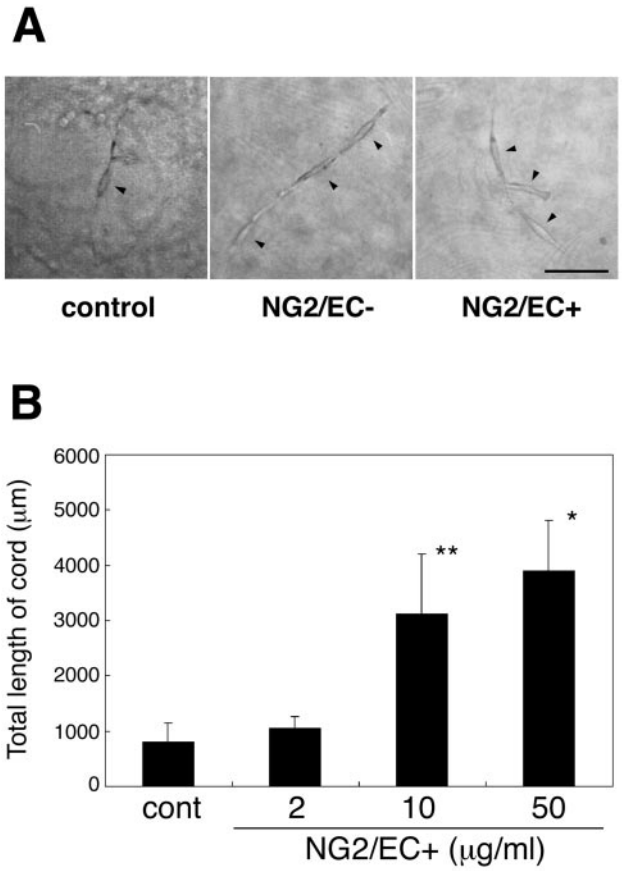
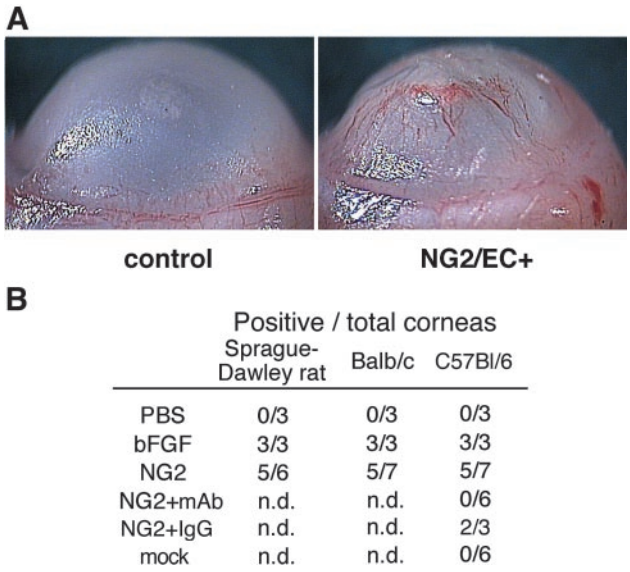


Figure 3. EC network formation in three-dimensional collagen gels. (A) Formation of cord-like endothelial networks. MAEC were plated onto type I collagen gels in the absence of NG2 (control) or in the presence of either NG2/EC$^+$ or NG2/EC$^-$ (50 μg/ml). Morphogenesis was analyzed after a 48-h incubation. Cell nuclei are indicated by arrowheads. Scale bar, 100 μm. (B) Quantitative analysis of morphogenesis. The total length of cord-like networks in each gel was measured in four random fields, and the total length per field was calculated. Each bar represents the mean ± SD (n = 3). Statistically significant differences compared with the control are indicated (*$p < 0.01$; **$p < 0.05$).



	Sprague-Dawley rat	Balb/c	C57Bl/6
PBS	0/3	0/3	0/3
bFGF	3/3	3/3	3/3
NG2	5/6	5/7	5/7
NG2+mAb	n.d.	n.d.	0/6
NG2+IgG	n.d.	n.d.	2/3
mock	n.d.	n.d.	0/6

Figure 4. Corneal angiogenesis. (A) Hydron pellets containing PBS (control) or NG2/EC$^+$ (500 ng) were implanted into Sprague-Dawley rat corneas. Five days later, vessels growing toward the pellet implant from the limbus were photographed. (B) Rat and mouse corneal angiogenesis in summary. Pellets containing 200 ng of NG2/EC$^+$ or 50 ng of bFGF were implanted in the case of BALB/c and C57Bl/6 mice, and 500 or 100 ng, respectively, were used for Sprague-Dawley rats. For antibody inhibition, anti-NG2 mAb or isotype-matched control IgG (2 μg/pellet) was directly added to the pellet before implantation into corneas of C57Bl/6 mice. "Mock purified" material was also tested in C57Bl/6. n.d., not determined.

NG2/EC$^-$ produced qualitatively similar effects on EC morphogenesis (middle panel). Quantitative analysis of the total length of these cord-like structures within the gel revealed that the presence of NG2/EC$^+$ increased network assembly three- to fourfold over control levels (Figure 3, A and B).

NG2 Induces Corneal Angiogenesis

We next examined whether NG2 can induce angiogenesis in vivo. We implanted Hydron pellets impregnated with bFGF (as a positive control) or with NG2/EC$^+$ into the corneas of rats and mice. bFGF invariably induced angiogenesis in this system (Figure 4B). Administration of 500 ng of NG2/EC$^+$ induced angiogenesis in five of six rat corneas (Figure 4, A and B). Two hundred nanograms of NG2/EC$^+$ induced a positive angiogenic response in five of seven mouse corneas, regardless of the mouse strain, whereas pellets containing PBS or "mock purified" material exhibited no stimulatory effect on corneal angiogenesis (Figure 4, A and B). Coadministration of mouse monoclonal anti-NG2 antibody N11.4, which can inhibit NG2-induced endothelial migration in vitro (unpublished data), completely inhibited the angiogenic response induced by NG2/EC$^+$ in all six corneas tested (zero of six C57Bl/6 corneas were positive; Figure 4B).

Identification of Galectin-3 as an Endothelial Receptor for NG2

Because binding of NG2 to the EC surface appears to trigger the observed increases in cell motility and the formation of multicellular networks, we screened ECs for potential NG2 receptors. An affinity matrix was prepared by coupling NG2/EC$^+$ to CL-4B Sepharose beads (hereafter referred to as NG2 beads). Detergent lysates of MAEC were incubated with NG2 beads, and bound proteins were separated by SDS-PAGE and visualized by silver staining. As shown in Figure 5A, several specifically bound proteins were identified in lane 3 (arrow and arrowheads) when compared with CL-4B control matrix (lane 2) or NG2 beads themselves (lane 1). Using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, the 30-kDa protein band (Figure 5A, arrow) was identified as galectin-3 (Figure 5B). This 30-kDa band was also confirmed as galectin-3 by immunoblotting with antigalectin-3 antibody (Figure 5C).

Galectin-3 has been shown to bind in a galactose-dependent manner to a number of protein ligands, including laminin, fibronectin, and tenascin C (Hughes, 2001). Because NG2 can bind to laminin and tenascin C (Burg *et al.*, 1996), it is possible that the NG2/galectin-3 interaction is an indirect one. We determined whether NG2 directly binds to galectin-3 by attaching a GST-galectin-3 fusion protein to glutathione-Sepharose beads and performing a pull-down assay with NG2/EC. As shown in Figure 5D, NG2/EC bound to the GST-galectin-3 fusion protein and was eluted from the

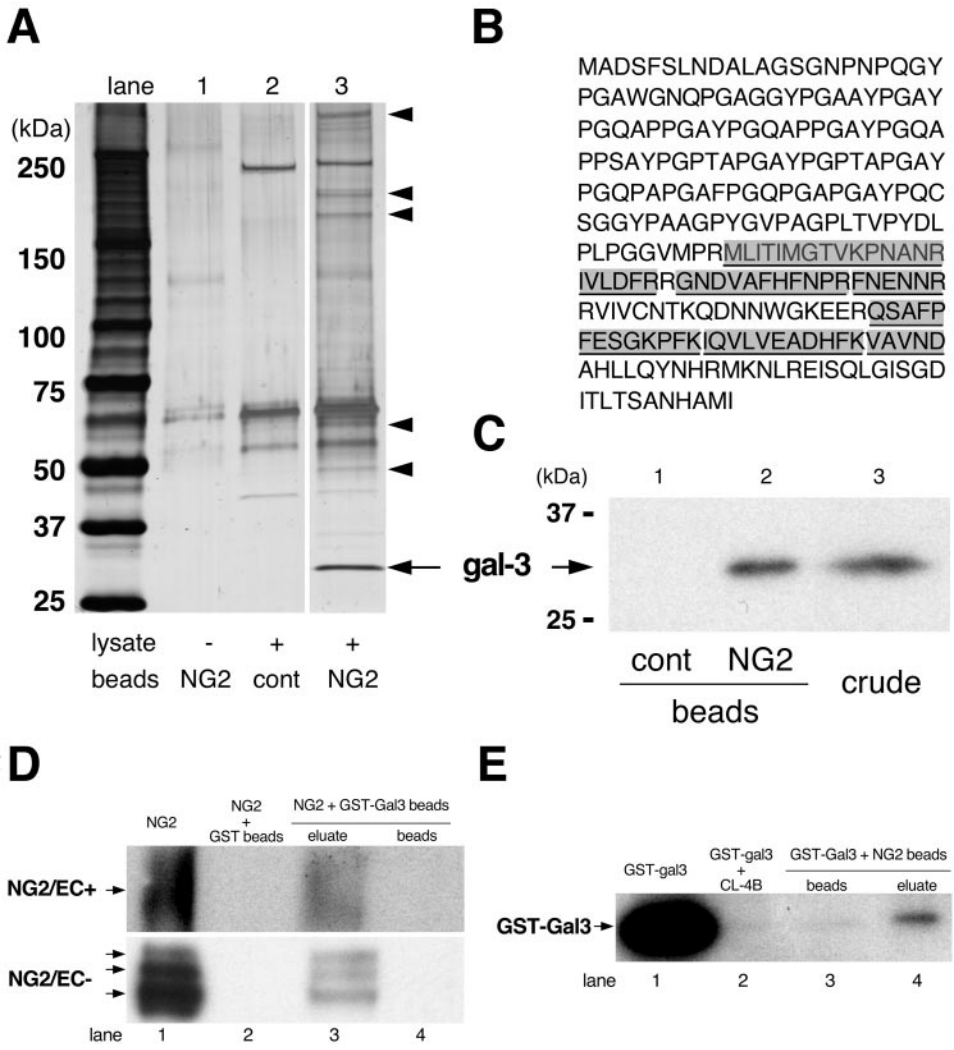


Figure 5. Identification of galectin-3 as an NG2-binding protein. (A) Identification of NG2-interacting proteins. Extracts from MAEC were incubated with either NG2 beads (lane 3) or CL-4B Sepharose beads as a control (lane 2). Bound proteins were visualized on 4–12% Tris-glycine gels by silver staining. NG2 beads alone were also loaded as another control (lane 1). MAEC proteins bound specifically to NG2 are labeled with arrowheads. The 30-kDa protein band (arrow) was analyzed by MALDI-TOF mass spectrometry. (B) Amino acid sequence of galectin-3. Shaded sequences indicate tryptic peptides derived from the 30-kDa band, as determined by mass spectrometry. (C) MAEC proteins bound to NG2 beads (lane 2) or CL-4B Sepharose beads (lane 1) were analyzed by immunoblotting with antigalectin-3 antibody. A crude lysate of MAEC was also loaded (lane 3). (D) NG2/EC$^+$ or NG2/EC$^-$ were incubated with glutathione-agarose beads carrying either GST-galectin-3 fusion protein (lane 3 and 4) or GST (lane 2) as a control. Bound proteins were eluted from GST-galectin-3 fusion protein beads with 100 mM lactose (lane 3), and the stripped beads were boiled in SDS-PAGE loading buffer (lane 4). Samples were separated by SDS-PAGE, and immunoblotted using anti-NG2 antibody. (lane 1) NG2/EC was loaded as a positive control. NG2/EC$^+$ migrates as a broad smear because of the presence of the chondroitin sulfate chain. NG2/EC$^-$ appears as three distinct bands because of proteolytic degradation during its purification. (E) GST-galectin-3 fusion protein was incubated with either NG2 beads (lane 3 and 4) or control CL-4B beads (lane 2). After washing, bound proteins were eluted from NG2 beads with lactose (lane 4). Stripped beads were also analyzed (lane 3). Samples were treated as described above and immunoblotted using antigalectin-3 antibody. (lane 1) GST-galectin-3 fusion protein was loaded as a positive control.

fusion protein beads by 100 mM lactose (lane 3), independent of the presence or absence of the chondroitin sulfate side chain. In contrast, NG2/EC did not bind to control GST beads (lane 2). We also used NG2 beads as an affinity matrix for a pull-down assay with GST-galectin-3 fusion protein. GST-galectin-3 fusion protein bound to NG2 beads and was eluted by 100 mM lactose (Figure 5E, lane 4). Thus, NG2/EC directly binds to galectin-3 in a carbohydrate-dependent manner.

Although galectin-3 lacks a signal sequence, it is secreted from cells and can then bind to glycosylated proteins on the cell surface (Hughes, 2001). To evaluate the availability of galectin-3 as an NG2 receptor, we examined the surface expression of galectin-3 on living, unpermeabilized ECs. As shown by immunostaining and flow cytometry (Figure 6A), both MAEC and HMVEC display galectin-3 on their cell surfaces. mAb against galectin-3 was further evaluated for its ability to inhibit NG2-induced motility of MAEC. This mAb reduced NG2/EC-induced migration of MAEC by 70% (Figure 6B), demonstrating the importance of galectin-3 in the EC response to NG2. As in the case of the NG2 antibody (Figure 2), Matrigel- or type I collagen–induced migration was not affected by the antigalectin-3 mAb. Although fibronectin-induced migration was reduced by 20% in this experiment, this effect could not be shown to be statistically significant. Antibody against CD44, another cell surface molecule expressed on MAEC, had no significant effect on NG2/EC-induced migration (Figure 6B).

Identification of α3β1 Integrin as a Mediator of NG2 Signaling

Although the foregoing experiments demonstrate a clear role for galectin-3 as an endothelial receptor for NG2, the nonmembrane spanning nature of this protein does not suggest an obvious mechanism for NG2-mediated signaling. Because both NG2 and galectin-3 have been shown to interact with β1 integrins (Burg *et al.*, 1998; Ochieng *et al.*, 1998; Hughes, 2001), we wondered whether β1 integrin signaling could be involved in the EC response to NG2. Accordingly, living ECs were immunostained with mAbs (9EG7 for MAEC and HUTS-21 for HMVEC) that recognize an activated epitope in the β1 integrin subunit (Lenter *et al.*, 1993;

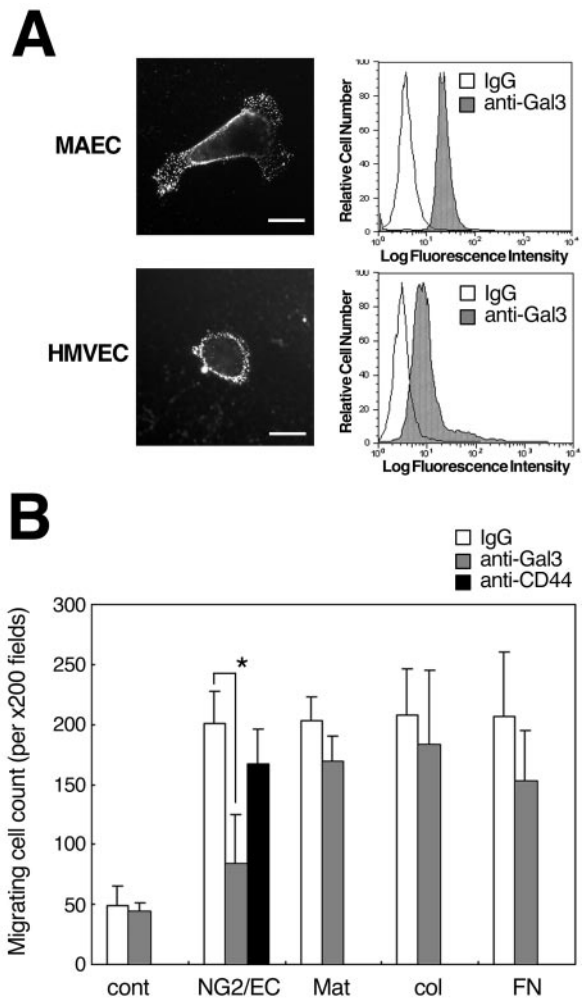


Figure 6. Inhibition of EC migration by antigalectin-3 antibody. (A) Detection of galectin-3 on the surface of ECs. Immunofluorescence (left panels) and flow cytometry (right panels) were performed on unpermeabilized MAEC (top panels) and HMVEC (bottom panels) using antigalectin-3 antibody. Scale bar, 20 μm. (B) MAEC were preincubated with either nonimmune rat IgG, anti-CD44, or antigalectin-3 antibody (15 μg/ml) for 15 min and assayed for cell motility in Transwells coated with Matrigel (30 μg/ml), type I collagen (30 μg/ml), fibronectin (10 μg/ml), or NG2/EC$^+$ (10 μg/ml). Each bar represents the mean $\pm$ SD (n = 3). A statistically significant difference was obtained for antigalectin-3 on NG2/EC-coated Transwells compared with the value obtained in the presence of control IgG (*$p < 0.02$).

Luque *et al.*, 1996). Although the 9EG7 or HUTS-21 epitope was barely detectable on serum-starved EC (Figure 7, A and B, top left), a more intense signal was observed after a 30-min incubation with NG2/EC$^+$ (Figure 7, A and B, top right). A similar increase in 9EG7 and HUTS-21 binding was observed in the presence of Mn^{2+}, a known activator of integrins (unpublished data).

We used immunoblotting to identify integrin subunits expressed by MAEC. As shown in Figure 7C, in addition to the β1 subunit, MAEC express the α3 and α6 subunits, suggesting that $\alpha3\beta1$ and $\alpha6\beta1$ are the predominant integrin heterodimers assembled by these cells. To investigate the NG2 binding capability of these integrin species, we used detergent extracts of MAEC to perform coimmunoprecipitation experiments with NG2/EC$^+$. Proteins coimmunoprecipitated along with NG2/EC$^+$ were separated by SDS-PAGE, and immunoblotting was performed using antibodies against the α3, α6, and β1 integrin subunits. These tests revealed that the α3 and β1 subunits, but not α6, were coimmunoprecipitated with NG2/EC$^+$ (Figure 7D). $\alpha3\beta1$ therefore appears to be the primary NG2-binding integrin on MAEC. Significantly, the NG2/EC immunoprecipitate prepared from MAEC lysate contains not only $\alpha3\beta1$ integrin, but also galectin-3 (Figure 7D). This observation demonstrates that addition of exogenous NG2 to MAEC results in the formation of a multimolecular complex containing the proteoglycan, $\alpha3\beta1$ integrin, and galectin-3. These molecules were also coimmunoprecipitated from HMVEC lysate along with NG2/EC (unpublished data).

Because well-characterized antibodies are available against human integrins, we tested whether NG2/EC-induced HMVEC migration could be inhibited by blocking antibodies against the α3 integrin subunit. As shown in Figure 8A, NG2/EC-induced migration of HMVEC was reduced by 35% in the presence of anti-α3 antibody. This same degree of inhibition was produced by the antigalectin-3 antibody. Moreover, the combination of the α3 integrin and galectin-3 antibodies produced a greater effect than either alone, reducing NG2/EC-dependent migration by 55%. In parallel experiments, this antibody combination produced no inhibition of cell motility in response to Matrigel (Figure 8A), emphasizing that the inhibition is restricted to NG2-dependent motility. These results indicate that the $\alpha3\beta1$ integrin and galectin-3 not only form a complex with NG2, but also that both molecules are important for NG2-dependent stimulation of EC migration.

Because β1 integrin binding has been demonstrated for galectin-3 (Ochieng *et al.*, 1998), we examined whether the $\alpha3\beta1$ integrin could bind to galectin-3 in ECs. Detergent extracts of MAEC were used for immunoprecipitations with anti-β1 antibody. As shown in Figure 8B, both the α3 subunit and galectin-3 were coimmunoprecipitated along with the β1 subunit, suggesting that binding to $\alpha3\beta1$ integrin may provide a means of anchoring galectin-3 on the EC surface. As an additional demonstration that $\alpha3\beta1$ integrin, galectin-3, and NG2 can form a multimolecular complex, we performed immunoprecipitation experiments with detergent extracts of A375 melanoma cells, which express all three components on their cell surface. As shown in Figure 8C, incubation with affinity-purified NG2 or β1 integrin antibody resulted in coimmunoprecipitation of both $\alpha3\beta1$ integrin and galectin-3 along with NG2. This result demonstrates that NG2 forms a complex with $\alpha3\beta1$ integrin and galectin-3 not only when the proteoglycan is added exogenously, but also when it is expressed as an endogenous cell surface component. The absence of NCAM from the immunoprecipitates demonstrates that the complex has a relatively restricted composition.

We examined the spatial relationship between NG2 and $\alpha3\beta1$ integrin in newly formed blood vessels in vivo. In cross section of capillaries in healing wound tissue, NG2-positive pericytes are seen surrounding CD31-positive ECs (Figure 9, bottom panels). Similarly, α3 immunoreactivity was detected in ECs surrounded by NG2-positive pericytes in newly formed blood vessels (top panels), demonstrating the feasibility of an NG2-$\alpha3\beta1$ integrin interaction at the pericyte-EC interface.

DISCUSSION

Unlike angiogenic growth factors that induce proliferation of ECs, molecules such as soluble VCAM-1, soluble E-selec-

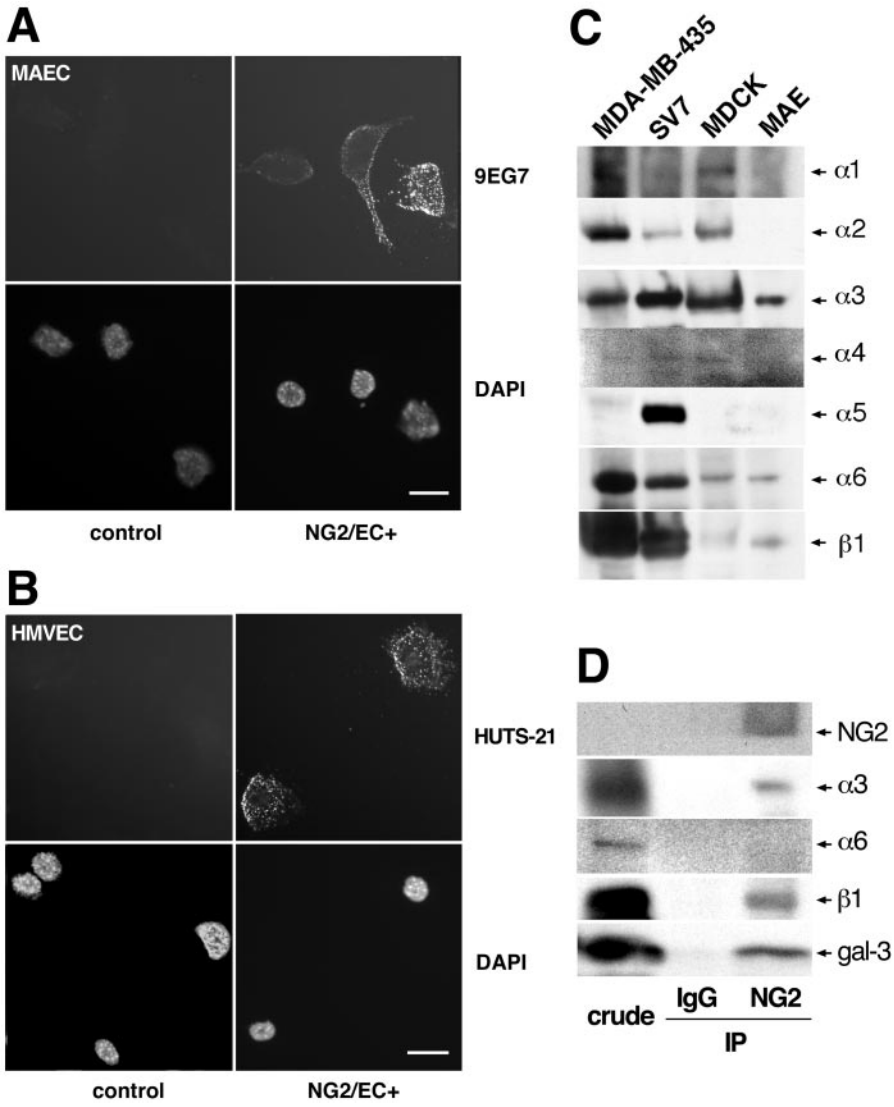


Figure 7. Identification of $\alpha 3\beta 1$ integrin as an NG2 receptor. (A and B) Serum-starved MAEC (A) or HMVEC (B) were incubated with or without NG2/EC$^+$ (25 μg/ml) for 30 min and then immunostained with mAb 9EG7 (A) or HUTS-21 (B) that recognizes activated $\beta 1$ integrins. DAPI was used as a nuclear counterstain. Scale bar, 20 μm. (C) Detergent extracts of the indicated cells were fractionated by SDS-PAGE and immunoblotted with antibodies against various integrin subunits. (D) Identification of NG2-binding integrin. Extracts from MAEC were incubated with NG2/EC$^+$ and immunoprecipitated with anti-NG2 antibody or control IgG. Immunoprecipitates were fractionated by SDS-PAGE and immunoblotted with anti-NG2, anti-$\alpha 3$, anti-$\alpha 6$, anti-$\beta 1$, and anti-galectin-3 antibodies. Crude MAEC lysate was also loaded as a positive control.

tin (Koch *et al.*, 1995), soluble ICAM-1 (Gho *et al.*, 1999), and osteopontin (Leali *et al.*, 2003) have been shown to exert potent proangiogenic activities in vivo despite their inability to induce mitosis of ECs in vitro. Similarly, soluble forms of the NG2 proteoglycan are nonmitogenic, but can induce EC migration, promote the assembly of multicellular EC networks, and stimulate corneal angiogenesis.

NG2 appears to accomplish these functions by binding to cell surface receptors on ECs. Using an NG2 affinity matrix and immunoprecipitation, we identified two EC binding partners for soluble NG2: namely galectin-3 and the $\alpha 3\beta 1$ integrin. Intriguingly, NG2, galectin-3, and $\alpha 3\beta 1$ integrin are able to form a multimolecular complex, as demonstrated by coimmunoprecipitation of the three components either from extracts of MAEC incubated with exogenous NG2/EC$^+$ or from extracts of A375 cells that endogenously express all three proteins. The existence of this complex suggests a potential model for understanding why both galectin-3 and $\alpha 3\beta 1$ integrin appear to be important for NG2-mediated enhancement of EC motility.

In contrast to galectin-3, whose involvement in signal transduction remains poorly understood because of its lack of a membrane-spanning sequence, integrins are well-established as mediators of transmembrane communication. On NG2 stimulation, increased levels of activated $\beta 1$ integrin are observed in ECs, as judged by binding of the activation-dependent mAbs 9EG7 (MAEC) and HUTS-21 (HMVEC; Lenter *et al.*, 1993; Luque *et al.*, 1996). NG2-dependent EC migration is partially inhibited in the presence of antibody against the $\alpha 3$ integrin subunit. Although we cannot exclude the possible involvement of $\alpha v\beta 3$ or other $\beta 1$ integrins (such as $\alpha 5\beta 1$) that may be expressed at low levels in the EC lines, these observations establish $\alpha 3\beta 1$ as a transducer of the NG2 signal. The $\alpha 3\beta 1$ integrin has been previously implicated in regulating EC migration, tube formation, and angiogenesis (Yanez-Mo *et al.*, 1998; Chandrasekaran *et al.*, 2000; Dominguez-Jimenez *et al.*, 2001). Integrin activation via binding to extracellular ligands is known to stimulate FAK phosphorylation, an important trigger for many cytoplasmic signaling pathways (Schlaepfer *et al.*, 1999). Among these is the ERK signaling pathway (Chung *et al.*, 2003), which is critical for angiogenesis (Eliceiri *et al.*, 1998). We have observed the phosphorylation of both FAK and ERK1/2 in ECs upon NG2-stimulation (unpublished data). Further studies will be required to elucidate the detailed signaling mechanisms induced by NG2/$\alpha 3\beta 1$ integrin engagement.

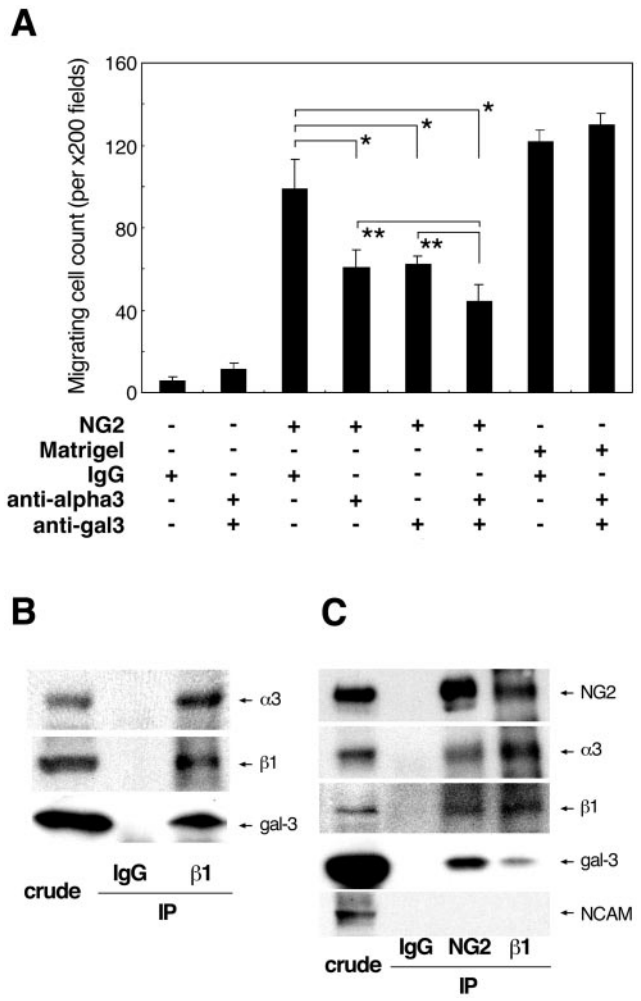


Figure 8. EC response to NG2 involves both galectin-3 and $\alpha 3\beta 1$ integrin. (A) HMVEC preincubated for 15 min with either nonimmune IgG, anti-$\alpha 3$, or antigalectin-3 antibody (15 μg/ml) were assayed for motility in Transwells coated with Matrigel (30 μg/ml) or NG2/EC$^+$ (50 μg/ml). Each bar represents the mean ± SD (n = 3). *$p < 0.02$ indicates statistically significant differences from the value obtained in the presence of control IgG, and **$p < 0.05$ from the value obtained in the presence of either $\alpha 3$ or galectin-3 antibody alone. (B) Detergent extracts of MAEC were immunoprecipitated with anti-$\beta 1$ integrin antibody or control IgG. Immunoprecipitates were fractionated by SDS-PAGE and immunoblotted with antibodies against $\alpha 3$ integrin, $\beta 1$ integrin, and galectin-3. Crude MAEC lysate was also loaded as a positive control. (C) Detergent extracts of A375 cells were immunoprecipitated with anti-NG2 antibody, anti-$\beta 1$ integrin antibody or control IgG. Immunoprecipitates were fractionated by SDS-PAGE, and immunoblotted with antibodies against NG2, $\alpha 3$ integrin, $\beta 1$ integrin, and galectin-3. NCAM was immunoblotted as a negative control to show the specificity of the immunoprecipitations.

Antibody against galectin-3 is also effective in inhibiting NG2-induced EC motility. The combined inhibition produced by antigalectin-3 and anti-$\alpha 3$ is greater than that produced by either antibody alone, demonstrating the dual role of these proteins in mediating the effects of soluble NG2. Galectin-3 has been previously shown to be involved in EC morphogenesis in vitro (Nangia-Makker *et al.*, 2002). Here we demonstrate a novel interaction between NG2 and galectin-3. Pull-down assays reveal that these two proteins

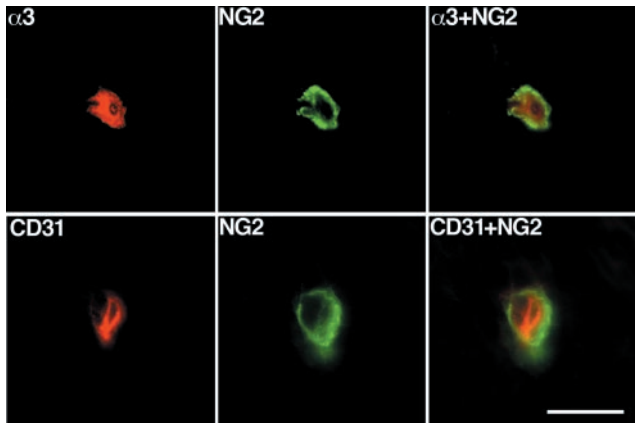


Figure 9. Coexpression of NG2 and $\alpha 3\beta 1$ integrin at the pericyte-EC interface. Sections from healing wound tissue at 7 days after surgery were immunostained for NG2, $\alpha 3$ integrin subunit, and CD31. Cross section of a newly formed capillary reveals the investment of CD31-positive EC by NG2-positive pericytes (bottom panels). $\alpha 3$ integrin is also expressed in EC surrounded by NG2-expressing pericytes (top panels). Galectin-3 staining is also found in these blood vessels, but is not restricted to the vasculature (unpublished data). Scale bar, 20 μm.

directly interact with each other and that the interaction is disrupted by lactose, suggesting a dependence on beta-galactosides. NG2 has a number of potential N-glycosylation sites, at least some of which are utilized (Nishiyama *et al.*, 1991).

Galectin-3 is found in the cytoplasm of various cells, but can be secreted and bound on the cell surface by a variety of glycoconjugate ligands, including L1, NCAM, CD98, NCA160, and $\alpha 1\beta 1$ integrin (Hughes, 2001). Our results suggest that the $\alpha 3\beta 1$ integrin may serve as an anchor for galectin-3 on the EC surface. Once localized to the cell surface, galectin-3 is capable of oligomerization. The resultant cross-linking of surface glycoproteins into multimolecular complexes can provide focal points for promoting cell adhesion. For example, galectin-3 on the EC surface is reported to mediate the adhesion of tumor cells to the vascular endothelium (Glinsky *et al.*, 2001). It seems possible in our system that galectin-3–mediated clustering of NG2 and $\alpha 3\beta 1$ integrin may lead to enhanced integrin-dependent signaling.

The angiogenic or antiangiogenic activities of heparan sulfate proteoglycans are dependent on both the heparan sulfate chains and core proteins (Iozzo and San Antonio, 2001). In contrast, the similarity in behavior between the NG2/EC$^+$ and NG2/EC$^-$ species provides no evidence for the involvement of chondroitin sulfate chains in NG2-dependent EC migration and network formation. The interaction between NG2 and galectin-3 is also independent of the presence of chondroitin sulfate chains. Similarly, we have previously reported the ability of NG2/EC$^-$ to interact with angiostatin and neutralize its antagonistic effect on EC proliferation (Goretzki *et al.*, 2000). Taken together, these observations emphasize the importance of the NG2 core protein for promoting angiogenesis.

NG2 is widely expressed by newly formed blood vessels. Immunohistochemical studies have established that NG2 expression in neovasculature is limited to the microvascular pericytes (Schlingemann *et al.*, 1990; Ozerdem and Stallcup, 2003). In contrast, both $\alpha 3\beta 1$ integrin and galectin-3 have been shown to be expressed by vascular ECs in newly

formed blood vessels (Lotan *et al.*, 1994; Gonzalez *et al.*, 2002). Thus, $\alpha 3\beta 1$ and galectin-3 on ECs and NG2 on pericytes are localized in complementary manner in newly formed blood vessels, demonstrating the feasibility of NG2 interaction with $\alpha 3\beta 1$ and galectin-3 during angiogenesis.

Pericytes are intimately associated with ECs in developing microvasculature and are thought to affect the growth and maturation of vascular tubes. Traditional opinion holds that pericyte interaction with ECs occurs at a relatively late time point in vascular tube formation and that pericytes serve primarily to stabilize the neovasculature (Beck and D'Amore, 1997; Benjamin *et al.*, 1998). However, more recent reports using new markers for nascent pericytes are demonstrating the presence of these cells at very early stages of angiogenesis. Pericytes may therefore be in position to stimulate and guide capillary tube formation (Schlingemann *et al.*, 1990; Nehls *et al.*, 1992; Wesseling *et al.*, 1995; Redmer *et al.*, 2001; Ozerdem *et al.*, 2001, 2002; Ozerdem and Stallcup, 2003; Gerhardt and Betsholtz, 2003). For example, in both corneal and retinal angiogenesis, NG2-positive pericytes are found in association with ECs at the tips of nascent capillary sprouts (Ozerdem *et al.*, 2002; Ozerdem and Stallcup, 2003). NG2-positive pericytes are also present during the earliest phases of tumor vascularization (Ozerdem and Stallcup, 2003).

The findings in the present study are consistent with the possibility of an early role for pericytes in vascular tube formation. Specifically, NG2 expressed by pericytes may be an important stimulus for recruitment of ECs. Studies of angiogenesis in the corpus luteum provide an example that seems especially relevant to our current results. Pericytes are the first vascular cells to invade this tissue, preceding the appearance of ECs (Redmer *et al.*, 2001). It is postulated that pericyte-derived factors, including VEGF, are important for stimulating EC invasion. In the current work we have utilized the soluble NG2 ectodomain to stimulate EC motility, morphogenesis, and angiogenesis. It remains to be determined whether soluble NG2 shed from the pericyte surface is responsible for promoting normal angiogenesis in vivo or whether this response is mediated by NG2 anchored on the pericyte surface. It is possible that both mechanisms may be operative. Further work will be also be required to understand the detailed mechanisms of interaction between NG2, $\alpha 3\beta 1$ integrin, and galectin-3 during pericyte/EC cross-talk. In addition, the coexpression of NG2, $\alpha 3\beta 1$ integrin, and galectin-3 by some types of neoplasms (e.g., A375 melanoma cells used in Figure 8C) suggests that investigation of this complex may yield valuable information about the behavior of tumor cells.

ACKNOWLEDGMENTS

We thank Dr. Kuniko Kadoya (The Burnham Institute) for providing wound tissue samples and Drs. Yu Yamaguchi (The Burnham Institute) and Fu-Tong Liu (University of California Davis School of Medicine) for providing helpful discussions and valuable reagents. We are indebted to Tristan Williams for technical help with the MALDI-TOF analysis, and Yoav Altman for help with the FACSort instrument. This work was supported by National Institutes of Health Grants RO1 CA95287 and PO1 HD25938 (W.S.B.).

REFERENCES

Arbiser, J.L. *et al.* (2001). The generation and characterization of a cell line derived from a sporadic renal angiomyolipoma: use of telomerase to obtain stable populations of cells from benign neoplasms. Am. J. Pathol. *159*, 483–491.

Barritt, D.S., Pearn, M.T., Zisch, A.H., Lee, S.S., Javier, R.T., Pasquale, E.B., and Stallcup, W.B. (2000). The multi-PDZ domain protein MUPP1 is a cytoplasmic ligand for the membrane-spanning proteoglycan NG2. J. Cell. Biochem. *79*, 213–224.

Bastaki, M. *et al.* (1997). Basic fibroblast growth factor-induced angiogenic phenotype in mouse endothelium. A study of aortic and microvascular endothelial cell lines. Arterioscler. Thromb. Vasc. Biol. *17*, 454–464.

Beck, L., Jr., and D'Amore, P.A. (1997). Vascular development: cellular and molecular regulation. FASEB J. *11*, 365–373.

Benjamin, L.E., Hemo, I., and Keshet, E. (1998). A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development. *125*, 1591–1598.

Burg, M.A., Tillet, E., Timpl, R., and Stallcup, W.B. (1996). Binding of the NG2 proteoglycan to type VI collagen and other extracellular matrix molecules. J. Biol. Chem. *271*, 26110–26116.

Burg, M.A., Grako, K.A., and Stallcup, W.B. (1998). Expression of the NG2 proteoglycan enhances the growth and metastatic properties of melanoma cells. J. Cell. Physiol. *177*, 299–312.

Chandrasekaran, L., He, C.Z., Al-Barazi, H., Krutzsch, H.C., Iruela-Arispe, M.L., and Roberts, D.D. (2000). Cell contact-dependent activation of alpha3beta1 integrin modulates endothelial cell responses to thrombospondin-1. Mol. Biol. Cell *11*, 2885–2900.

Chekenya, M. *et al.* (2002). NG2 proteoglycan promotes angiogenesis-dependent tumor growth in CNS by sequestering angiostatin. FASEB J. *16*, 586–588.

Chung, C.H., Wu, W.B., and Huang, T.F. (2003). Aggretin, a snake venom-derived endothelial integrin alpha2beta1 agonist, induces angiogenesis via expression of vascular endothelial growth factor. Blood *103*, 2105–2113.

Dominguez-Jimenez, C., Yanez-Mo, M., Carreira, A., Tejedor, R., Gonzalez-Amaro, R., Alvarez, V., and Sanchez-Madrid, F. (2001). Involvement of alpha3 integrin/tetraspanin complexes in the angiogenic response induced by angiotensin II. FASEB J. *15*, 1457–1459.

Eliceiri, B.P., Klemke, R., Stromblad, S., and Cheresh, D.A. (1998). Integrin alphavbeta3 requirement for sustained mitogen-activated protein kinase activity during angiogenesis. J. Cell Biol. *140*, 1255–1263.

Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med. *1*, 27–31.

Fukushi, J., Ono, M., Morikawa, W., Iwamoto, Y., and Kuwano, M. (2000). The activity of soluble VCAM-1 in angiogenesis stimulated by IL-4 and IL-13. J. Immunol. *165*, 2818–2823.

Gerhardt, H., and Betsholtz, C. (2003). Endothelial-pericyte interactions in angiogenesis. Cell Tissue Res. *314*, 15–23.

Gho, Y.S., Kleinman, H.K., and Sosne, G. (1999). Angiogenic activity of human soluble intercellular adhesion molecule-1. Cancer Res. *59*, 5128–5132.

Glinsky, V.V., Glinsky, G.V., Rittenhouse-Olson, K., Huflejt, M.E., Glinskii, O.V., Deutscher, S.L., and Quinn, T.P. (2001). The role of Thomsen-Friedenreich antigen in adhesion of human breast and prostate cancer cells to the endothelium. Cancer Res. *61*, 4851–4857.

Gonzalez, A.M., Gonzales, M., Herron, G.S., Nagavarapu, U., Hopkinson, S.B., Tsuruta, D., and Jones, J.C. (2002). Complex interactions between the laminin alpha 4 subunit and integrins regulate endothelial cell behavior in vitro and angiogenesis in vivo. Proc. Natl. Acad. Sci. USA *99*, 16075–16080.

Goretzki, L., Burg, M.A., Grako, K.A., and Stallcup, W.B. (1999). High-affinity binding of basic fibroblast growth factor and platelet-derived growth factor-AA to the core protein of the NG2 proteoglycan. J. Biol. Chem. *274*, 16831–16837.

Goretzki, L., Lombardo, C.R., and Stallcup, W.B. (2000). Binding of the NG2 proteoglycan to kringle domains modulates the functional properties of angiostatin and plasmin(ogen). J. Biol. Chem. *275*, 28625–28633.

Grako, K.A., Ochiya, T., Barritt, D., Nishiyama, A., and Stallcup, W.B. (1999). PDGF (alpha)-receptor is unresponsive to PDGF-AA in aortic smooth muscle cells from the NG2 knockout mouse. J. Cell Sci. *112*, 905–915.

Hughes, R.C. (2001). Galectins as modulators of cell adhesion. Biochimie *83*, 667–676.

Iozzo, R.V., and San Antonio, J.D. (2001). Heparan sulfate proteoglycans: heavy hitters in the angiogenesis arena. J. Clin. Invest. *108*, 349–355.

Jones, L.L., Yamaguchi, Y., Stallcup, W.B., and Tuszynski, M.H. (2002). NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors. J. Neurosci. *22*, 2792–2803.

Koch, A.E., Halloran, M.M., Haskell, C.J., Shah, M.R., and Polverini, P.J. (1995). Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1. Nature *376*, 517–519.

Leali, D. *et al.* (2003). Osteopontin (Eta-1) and fibroblast growth factor-2 cross-talk in angiogenesis. J. Immunol. *171*, 1085–1093.

Lenter, M., Uhlig, H., Hamann, A., Jeno, P., Imhof, B., and Vestweber, D. (1993). A monoclonal antibody against an activation epitope on mouse integrin chain beta 1 blocks adhesion of lymphocytes to the endothelial integrin alpha 6 beta 1. Proc. Natl. Acad. Sci. USA *90*, 9051–9055.

Lindahl, P., Johansson, B.R., Leveen, P., and Betsholtz, C. (1997). Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science *277*, 242–245.

Lotan, R., Belloni, P.N., Tressler, R.J., Lotan, D., Xu, X.C., and Nicolson, G.L. (1994). Expression of galectins on microvessel endothelial cells and their involvement in tumour cell adhesion. Glycoconj. J. *11*, 462–468.

Luque, A., Gomez, M., Puzon, W., Takada, Y., Sanchez-Madrid, F., and Cabanas, C. (1996). Activated conformations of very late activation integrins detected by a group of antibodies (HUTS) specific for a novel regulatory region (355–425) of the common beta 1 chain. J. Biol. Chem. *271*, 11067–11075.

Nangia-Makker, P., Hogan, V., Honjo, Y., Baccarini, S., Tait, L., Bresalier, R., and Raz, A. (2002). Inhibition of human cancer cell growth and metastasis in nude mice by oral intake of modified citrus pectin. J. Natl. Cancer Inst. *94*, 1854–1862.

Nehls, V., Denzer, K., and Drenckhahn, D. (1992). Pericyte involvement in capillary sprouting during angiogenesis in situ. Cell Tissue Res. *270*, 469–474.

Nishiyama, A., Dahlin, K.J., Prince, J.T., Johnstone, S.R., and Stallcup, W.B. (1991). The primary structure of NG2, a novel membrane-spanning proteoglycan. J. Cell Biol. *114*, 359–371.

Nishiyama, A., Lin, X.H., and Stallcup, W.B. (1995). Generation of truncated forms of the NG2 proteoglycan by cell surface proteolysis. Mol. Biol. Cell *6*, 1819–1832.

Ochieng, J., Leite-Browning, M.L., and Warfield, P. (1998). Regulation of cellular adhesion to extracellular matrix proteins by galectin-3. Biochem. Biophys. Res. Commun. *246*, 788–791.

Ozerdem, U., Grako, K.A., Dahlin-Huppe, K., Monosov, E., and Stallcup, W.B. (2001). NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis. Dev. Dyn. *222*, 218–227.

Ozerdem, U., Monosov, E., and Stallcup, W.B. (2002). NG2 proteoglycan expression by pericytes in pathological microvasculature. Microvasc. Res. *63*, 129–134.

Ozerdem, U., and Stallcup, W.B. (2003). Early contribution of pericytes to angiogenic sprouting and tube formation. Angiogenesis *6*, 241–249.

Redmer, D.A., Doraiswamy, V., Bortnem, B.J., Fisher, K., Jablonka-Shariff, A., Grazul-Bilska, A.T., and Reynolds, L.P. (2001). Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum. Biol. Reprod. *65*, 879–889.

Schlaepfer, D.D., Hauck, C.R., and Sieg, D.J. (1999). Signaling through focal adhesion kinase. Prog. Biophys. Mol. Biol. *71*, 435–478.

Schlingemann, R.O., Rietveld, F.J., de Waal, R.M., Ferrone, S., and Ruiter, D.J. (1990). Expression of the high molecular weight melanoma-associated antigen by pericytes during angiogenesis in tumors and in healing wounds. Am. J. Pathol. *136*, 1393–1405.

Schlingemann, R.O., Oosterwijk, E., Wesseling, P., Rietveld, F.J., and Ruiter, D.J. (1996). Aminopeptidase a is a constituent of activated pericytes in angiogenesis. J. Pathol. *179*, 436–442.

Tillet, E., Ruggiero, F., Nishiyama, A., and Stallcup, W.B. (1997). The membrane-spanning proteoglycan NG2 binds to collagens V and VI through the central nonglobular domain of its core protein. J. Biol. Chem. *272*, 10769–10776.

Wesseling, P., Schlingemann, R.O., Rietveld, F.J., Link, M., Burger, P.C., and Ruiter, D.J. (1995). Early and extensive contribution of pericytes/vascular smooth muscle cells to microvascular proliferation in glioblastoma multiforme: an immuno-light and immuno-electron microscopic study. J. Neuropathol. Exp. Neurol. *54*, 304–310.

Yanez-Mo, M., Alfranca, A., Cabanas, C., Marazuela, M., Tejedor, R., Ursa, M.A., Ashman, L.K., de Landazuri, M.O., and Sanchez-Madrid, F. (1998). Regulation of endothelial cell motility by complexes of tetraspan molecules C.D81/TAPA-1 and C.D151/PETA-3 with alpha3 beta1 integrin localized at endothelial lateral junctions. J. Cell Biol. *141*, 791–804.