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13. ABSTRACT (Maximum 200 Words)

Pericytes are critical for neovascularization to form and maintain angiogenic sprouts in cancer. In May 2005 annual report we elucidate an extrinsic (pharmacological) targeting method, consisting of loading hydron polymer pellets with anti-NG2 neutralizing antibody to target nascent pericytes in the neovasculature of PC3 and LNCaP tumors. Hydron pellets containing NG2 neutralizing antibody decreased corneal neovascularization induced by PC3 tumor xenografts significantly. Mean corneal neovascularization in the treated eyes and control eyes were 0.5341 mm^2, and 2.789mm^2 respectively (n=20 eyes, $p<0.0001$). Hydron pellets containing NG2 neutralizing antibody decreased corneal neovascularization induced by LNCaP tumor xenografts significantly. Mean corneal neovascularization in the treated eyes and control eyes were 0.3393 mm^2, and 3.443 mm^2 respectively (n=10 eyes, $p=0.0079$). We have also encountered extensive lymphangiogenesis in PC3, LNCAP and TRAMP tumors in addition to angiogenesis. We have grown TRAMP prostate tumors in NG2 knockout and wild type mice to elucidate the anti-neovascular effect of intrinsic (genetic) targeting of NG2 on nascent pericytes. Task 3B of our statement of work is under way: We are in the process of comparing these tumor tissues by using an image analysis software (Volocity) in terms of microvascular density and lymphatic endothelial density.

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PROGRESS REPORT-DAMD 17-03-1-0012

This progress report outlines the outcome of the research April 2004 through May 2005.

INTRODUCTION

Prostate cancer is the most common cancer diagnosed in men and is the second leading cause of cancer-related death among men in the United States (Jemal et al., 2005). American Cancer Society statistics project 232,090 new prostate cancer cases for 2005, and that 30,350 patients will die this year. It is therefore imperative to find new therapies for coping with this major health problem. Anti-angiogenic therapy offers an attractive option for the treatment, since tumor progression depends on angiogenesis.

Microvascular wall is composed of two cell types; endothelium and pericyte. The functional role of pericytes in angiogenesis and their potential as anti-angiogenic targets in prostate cancer have only recently drawn attention of investigators. Overall aim of our research supported by DAMD17-03-1-0012 is to establish pericyte and NG2 proteoglycan located on pericyte as novel anti-angiogenic target for treatment of prostate cancer. This overall aim is organized into 3 sub-divisions (tasks) for experimental testing:

1) Analysis of neovasculature in prostate cancer.
2) Demonstrate the feasibility of pericyte-based targeting by investigating the ability of NG2-neutralizing antibody to inhibit angiogenesis in LNCaP and PC-3 prostate cancers grown in nude mouse cornea.
3) Analysis of transgenic adenocarcinoma of mouse prostate (TRAMP) grown in NG2 knockout and wildtype mice to elucidate the effect of intrinsic (genetic) targeting of NG2 proteoglycan on pericytes.

PROGRESS OF THE RESEARCH IN THE LAST 12 MONTHS IN THE LIGHT OF THE STATEMENT OF WORK

Task 1. Mapping the pericyte/endothelial cell relationship in prostate tumors
Task 1A was accomplished and reported already in last year's progress report.
Task 1B. Analysis of vasculature in prostate tumors: Vasculature in LNCaP and PC-3 tumor tissues along with TRAMP tissues were analyzed in more detail. We encountered lymphatic vessels in addition to blood vessels in prostate tumor tissues and therefore expanded the analysis of neovasculature to include lymphangiogenic vessels in Task 3. Immunohistochemical and confocal microscopic analysis of PC-3 and LNCaP, and TRAMP tumors revealed the following findings:

1) NG2+ and PDGF receptor-β+ pericytes invest the endothelium in neovasculature extensively in all three types of prostate tumors studied.
2) All three types of tumors have extensive lymphangiogenic vessels expressing LYVE-1. Since we encountered extensive lymphangiogenesis in Task 1B, we wish to analyze not only blood vessel microvascular density but lymphatic vessel density in Task 3 in TRAMP tumors grown in NG2 knockout and wild type mice.

Task 2. Anti-angiogenic effects of NG2 antibody on prostate cancer

Establishment of LNCaP and PC-3 prostate tumors in the cornea and Analysis (quantification) of extrinsic targeting of pericyte-NG2 in PC-3 and LNCAP tumor xenografts grown in nude mouse cornea.

The cornea is a thin (400 microns), transparent, avascular tissue in which the growth of all new angiogenic vessels generated in response to implanting tumor xenografts can be quantified in a straightforward manner using a stereomicroscope (Kenyon et al., 1996) (Kenyon et al., 1997). In a modification of four established *in vivo* techniques (Muthukkaruppan and Auerbach, 1979) (Kenyon et al., 1996) (Kenyon et al., 1997) (Fernandez et al., 2001) (Ozerdem, 2005), pellets containing anti-NG2 antibody were tested for their ability to inhibit corneal angiogenesis induced by PC-3 or LNCAP tumor fragments. These tests were performed to reveal the extent to which NG2 blockage can slow the angiogenesis that occurs in response to prostate tumors implanted in six-week-old, male, outbred athymic mice (Crl:nu/nu) (Charles River Laboratories, Wilmington, MA).

The surgical procedures for inducing corneal angiogenesis in the mouse (Muthukkaruppan and Auerbach, 1979) (Kenyon et al., 1996) (Kenyon et al., 1997) (Fernandez et al., 2001) are modified to accommodate tumor xenografts and hydron pellets containing either rabbit anti-NG2, or isotype-matched non-immune globulin (control) by making a wider keratotomy incision and a deeper micropocket. Male nude athymic mice (Crl:nu/nu) were obtained from Charles River Laboratories, (Wilmington, MA). Male nude mice (Crl:nu/nu) were obtained from Charles River Laboratories, (Wilmington, MA). and used for inducing PC-3 and LNCAP prostate tumor xenografts.

Hydron pellets (0.4x0.4x0.2 mm) containing the NG2 antibody or control non-immune globulin, and tumor fragment (0.3x0.3x0.3 mm) were implanted in the corneal pocket in male nude mice (Crl:nu/nu). Tumor fragments were prepared from subcutaneous PC-3 or LNCAP prostate tumor xenografts grown in nude mice (total 4 donor mice). Slow-release polyhydroxyethyl methacrylate (hydron) (Hydro Med Sciences, Cranbury, NJ) pellets are formulated to contain 45μg sucrose aluminum sulfate (sucralfate) (Sigma, St.Louis, MO) plus one of two experimental additives: 0.8μg affinity-purified rabbit anti-NG2 antibody (Ozerdem, 2005) (Ozerdem and Stallcup, 2004) (Ozerdem and Stallcup, 2003), or 0.8 μg isotype-matched non-immune globulin. Six-week-old mice were anesthetized with Avertin (0.015-0.017 ml/g body weight), and under an operating microscope, one pellet and tumor fragment were surgically implanted into the corneal stroma of one eye at a distance of 0.7 mm from the corneo-scleral limbus. Corneal stromal micropockets were created by using a modified von Graefe knife. Treatment pellet and matching control pellet eyes received the tumor fragment from the same donor. Twenty (20) eyes were operated for PC 3 tumor implantation. Ten (10) eyes were operated for LNCAP tumor implantation. On postoperative day 7, angiogenesis was quantified by determining the area of vascularization by using Olympus Stereoscope SZX 12 (Olympus USA, Melville, NY) as described previously (Kenyon et al., 1996) (Kenyon

et al., 1997). Hydron pellets containing NG2 neutralizing antibody decreased corneal neovascularization induced by PC3 or LNCaP fragments xenografted in the cornea. **(Figure 1. and 2)**

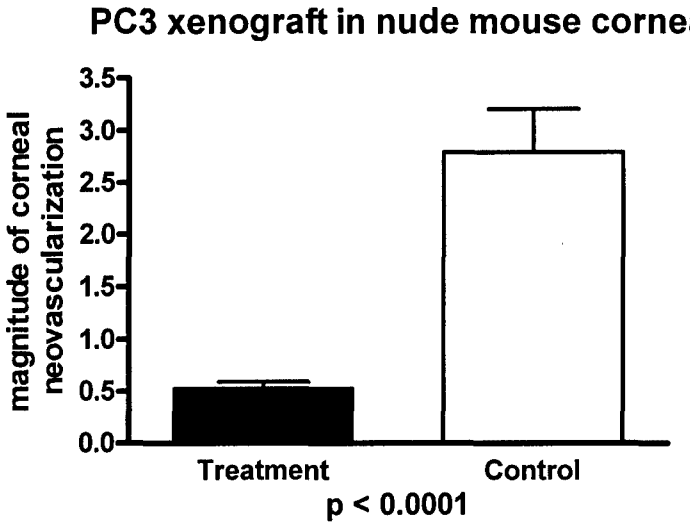

Figure 1. Hydron pellets containing NG2 neutralizing antibody decreased corneal neovascularization induced by PC3 tumor xenografts significantly. Mean corneal neovascularization in the treated eyes and control eyes were 0.5341 mm^2, and 2.789mm^2 respectively (n=20 eyes, p<0.0001 Mann Whitney U test).

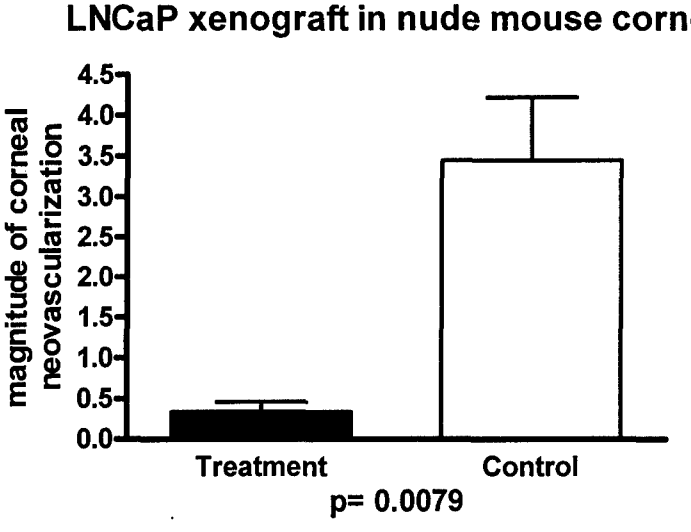

Figure 2. Hydron pellets containing NG2 neutralizing antibody decreased corneal neovascularization induced by LNCaP tumor xenografts significantly. Mean corneal neovascularization in the treated eyes and control eyes were 0.3393 mm^2, and 3.443 mm^2 respectively (n=10 eyes, p=0.0079 Mann Whitney U test).

Task 3.Analysis of the TRAMP model of prostate cancer

Task 3A Establishment of the TRAMP tumors in NG2+/+ and NG2-/-mice

Intrinsic targeting of pericyte-NG2: TRAMP tumor fragment implantation or TRAMP-C1 cell line inoculation in NG2 knockout and wild type mice

We have established a TRAMP (***Tr***ansgenic ***A***denocarcinoma of ***M***ouse ***P***rostate) mouse colony breeding pairs obtained from Mouse Models of Human Cancers Consortium (Frederick, Maryland). NG2 null mice (Grako et al., 1999) (Ozerdem and Stallcup, 2004) were generated via a conventional homologous recombination approach (Mansour et al., 1988) (Capecchi, 1989). The mice were back-crossed onto a C57BL/6 genetic background and NG2+/-heterozygotes were mated to establish separate male NG2 knockout (NG2-/-) and wild type (NG2+/+) colonies. NG2 knockout mouse has shown decreased neovascularization in cornea and retina challenged with angiogenic growth factors and ischemia in a previous study when challenged with angiogenic growth factors and ischemia respectively (Ozerdem and Stallcup, 2004). TRAMP (***Tr***ansgenic ***A***denocarcinoma of ***M***ouse ***P***rostate) (Greenberg et al., 1995) C57BL/6-TgN (TRAMP) 8247Ng mouse breeders were obtained from the National Cancer Institute, Mouse Models of Human Cancers Consortium (MMHCC catalog number 01XC6 Frederick, MD). For genotyping, genomic DNA from tail was isolated, and the transgene was identified by PCR as described previously(Greenberg et al., 1995). The mice had been back-crossed at least 10 generations onto the C57BL/6 background at the time of these experiments. TRAMP prostate tumor fragments and cell lines have previously been verified to be transplantable and tumorigenic when implanted in mouse with C57BL/6 genetic background (Foster et al., 1997) (Ozerdem and Stallcup, 2003). This tumor transplantation paradigm is similar to transplantation of mouse transgenic mammary tumor fragments in C57BL/6 mouse (Maglione et al., 2004) (Namba et al., 2004) (Ozerdem and Stallcup, 2003). Four, male TRAMP mice **(24 week old)** carrying transgene served as donor for tumor implantation in 6-week-old male NG2 knockout and wild type mice. Four NG2 knockout and 4 wild type control mice were used as recipients. Following a 3-mm skin incision in the dorsum, a 2mm x 2mmx 2mm fragment of tumor is implanted subcutaneosuly and the skin incision is closed with LiquiVet tissue adhesive (American Health Service, Libertyville, IL). The mice were followed for 3 weeks and sacrificed for tumor retrieval. Seven NG2 knockout and 7 wild type control mice were used for TRAMP-C1 cell line inoculation. TRAMP-C1 cells (**5x 10^6** cells) were injected subcutaneously in the dorsum. The mice were followed for 3 weeks and sacrificed for tumor retrieval.

Immunohistochemistry, Confocal Microscopic Imaging, and Image Analysis

Tissues were fixed in 4% paraformaldehyde for 6 hours, cryoprotected in 20% sucrose overnight, and frozen in O.C.T. embedding compound (Miles, Inc., Elkhardt, IN). Cryostat sections (40 μm) were air-dried onto Superfrost slides (Fisher Scientific, Pittsburgh, PA). Blood vessel endothelial cells (BEC) were identified using a cocktail of rat antibodies against mouse endoglin (CD105), PECAM-1 (CD31), and VEGF receptor-2 (flk-1) (Pharmingen, San Diego, CA) (Chang et al., 2000) (Ozerdem and Stallcup, 2003) (Ozerdem and Stallcup, 2004) a strategy that was utilized previously to maximize labeling of all blood vessel endothelial cells. Lymphatic endothelium (LEC)

was identified by immunolabeling with rabbit anti-mouse LYVE-1 antibody as described (Witmer et al., 2004) (Petrova et al., 2004). Pericytes were identified by labeling with rabbit PDGF β-receptor antibody, and rabbit antibodies against the NG2 proteoglycan (Ozerdem, 2005) (Ozerdem and Stallcup, 2003) (Ozerdem and Stallcup, 2004). Both NG2 and PDGF β-receptors are regarded as sensitive markers for identifying activated, immature pericytes, i.e. neovascular pericytes(Ozerdem, 2005) (Ozerdem and Stallcup, 2003) (Ozerdem and Stallcup, 2004) (Gerhardt and Betsholtz, 2003) (McDonald and Choyke, 2003) (Abramsson et al., 2002) (Rajantie et al., 2004) (Sundberg et al., 1993; Lindahl et al., 1997) (Schlingemann et al., 1991). Slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA).

Following cryosectioning, 5 sections representing the entire thickness of the tumor tissue were selected from the numbered sections by using systematic random sampling (Dawson and Trapp, 2001). The sampled histological sections (220 sections from TRAMP and TRAMP-C1 grafts) were imaged with a multi-channel laser scanning confocal microscope for microvascular density(MVD) and lymphatic vascular density (LVD). Briefly, optical sections were obtained from the specimens using the Fluoview 1000 laser scanning confocal microscope (Olympus USA, Melville, NY) in the three-channel sequential scanning mode. Serial optical sections (1 μm each) across the entire thickness (40 μm) of the histological specimens were overlaid (Z-stack) to provide reconstructions of entire vessels. This allowed unambiguous identification of the spatial relationship between pericytes and endothelial cells in the vessel wall.

The Volocity image analysis software (Openlab-Improvision Inc, Lexington MA) (serial # 88262001) will be used for quantification of MVD, and LVD in prostate tumors grown in NG2 knockout and wild type mice. The Classifier Module will be set Volocity to automatically identify MVD or LVD following multi-channel scanning to accomplish Task 3B in upcoming months.

KEY RESEARCH ACCOMPLISHMENTS

- Our results arising from finished Task 2-related experiments suggest that as early players in angiogenesis pericytes and NG2 proteoglycan represent an additional target for treatment of prostate cancer. Neovascular pericytes in prostate cancer can thus be targeted extrinsically (pharmacologically) by using hydron pellets loaded with NG2 neutralizing antibodies to inhibit neovascularization.

REPORTABLE OUTCOMES

Extrinsic targeting of pericytes and NG2 proteoglycan resulted in decreased neovascularization in corneal neovascularization model induced by bFGF corroborating the decreased neovascularization in PC 3 and LNCaP-induced neovascularization in cornea by a similar extrinsic targeting strategy. I published this data in Angiogenesis journal in the last 12 months. Since this result supports and complements the rationale behind targeting pericytes in prostate cancer, the U.S. Department of Defense Prostate Cancer Research Program's support was accordingly

acknowledged in the Acknowledgement section of the publication in Angiogenesis Journal.

CONCLUSIONS

Research supported by DAMD17-03-1-0012 showed a significant effect of extrinsic targeting of pericytes and NG2 proteoglycan by using hydron pellets loaded with anti-NG2 neutralizing antibodies to inhibit neovascularization.

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APPENDIX

A copy of the paper published in *Angiogenesis* journal in 2004 complementary to the research supported by DAMD17-03-1-0012 is attached.(Ozerdem U, Stallcup WB. 2004. Pathological angiogenesis is reduced by targeting pericytes via the NG2 proteoglycan. Angiogenesis 7:269-27). the U.S. Department of Defense Prostate Cancer Research Program's support was accordingly acknowledged in the Acknowledgement section of the publication in Angiogenesis Journal.

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Pathological angiogenesis is reduced by targeting pericytes via the NG2 proteoglycan

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Abstract

The NG2 proteoglycan is expressed by nascent pericytes during the early stages of angiogenesis. To investigate the functional role of NG2 in neovascularization, we have compared pathological retinal and corneal angiogenesis in wild type and NG2 null mice. During ischemic retinal neovascularization, ectopic vessels protruding into the vitreous occur twice as frequently in wild type retinas as in NG2 null retinas. In the NG2 knock-out retina, proliferation of both pericytes and endothelial cells is significantly reduced, and the pericyte:endothelial cell ratio falls to 0.24 from the wild type value of 0.86. Similarly, bFGF-induced angiogenesis is reduced more than four-fold in the NG2 null cornea compared to that seen in the wild type retina. Significantly, NG2 antibody is effective in reducing angiogenesis in the wild type cornea, suggesting that the proteoglycan can be an effective target for anti-angiogenic therapy. These experiments therefore demonstrate both the functional importance of NG2 in pericyte development and the feasibility of using pericytes as anti-angiogenic targets.

Abbreviations: BrdU – bromodeoxyuridine; CD31 – PECAM-1; CD105 – endoglin; flk 1 – VEGF receptor-2; NG2 – nerve/glial antigen 2; PBS – phosphate-buffered saline; PDGF β-receptor – platelet-derived growth factor beta receptor

Introduction

Angiogenesis is an essential element of many pathological processes, including tumor growth and metastasis, psoriasis, acne rosacea, rheumatoid arthritis, proliferative diabetic retinopathy, retinopathy of prematurity, and age-related macular degeneration [1, 2–4]. The development of anti-angiogenic therapies for treating these pathologies has therefore become an increasingly important goal of biomedical research. Most of these strategies have focused on targeting endothelial cells, which form the inner lining of the vascular tube and are by far the best understood component of neovasculature. However, the walls of typical angiogenic microvessels have a second cellular component: namely, pericytes (mural cells) which form an outer sheath around the endothelium [2, 5, 6]. Much less is known about these perivascular cells, as evidenced by the 115-fold difference in the number of publications on endothelial cells and pericytes, respectively (revealed by a recent search of the *PUBMED* database). The origin, function, and even reliable identification of pericytes have been elusive [5, 7, 8]. As a result, the benefits of using pericytes as an additional target for anti-angiogenic therapy are just beginning to be explored [9, 10].

The effectiveness of using pericytes as anti-angiogenic targets would be expected to depend heavily on the importance of these cells in the development and function of microvessels: i.e. the more important their function, the greater the impact of targeting them. The functional importance of pericytes during angiogenesis is vividly illustrated by the phenotypes of mice in which pericyte development is disrupted. Ablation of PDGF-B or PDGF β-receptor, critical elements for the recruitment and development of pericytes, gives rise to mice that are pericyte-deficient. Depending on the timing and specificity of the ablations, microvessels in these animals, at the very least, have dramatically altered morphologies [11, 12] and in some cases are subject to lethal microaneurysms [13]. Despite their importance, PDGF β-receptor and PDGF-B do not necessarily represent the only effective means of targeting pericytes. During the process of angiogenesis, extensive cross-talk occurs between pericytes and endothelial cells [2, 14, 15]. Accordingly, other cell surface and soluble components that mediate or modulate this cellular

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cross-talk are likely to be important candidates for targeting. One such pericyte component is the NG2 chondroitin sulfate proteoglycan, which is expressed on the surfaces of vascular mural cells during both normal and pathological angiogenesis [16–20].

The NG2 proteoglycan binds with high affinity to basic fibroblast growth factor (bFGF), platelet-derived growth factor AA (PDGF-AA), and the kringle domains of plasminogen and angiostatin [21, 22]. In addition, NG2 appears to mediate signal transduction events that lead to increased cell spreading and motility [23–27]. This combination of properties, coupled with the high level of NG2 expression on nascent microvascular pericytes during developmental angiogenesis [19], has led us to investigate the functional role of the proteoglycan in neovascularization. Towards this end, we have utilized well-characterized retinal and corneal models to compare the details of pathological angiogenesis in wild type and NG2 null mice. We have previously demonstrated that NG2 expression is restricted to microvascular pericytes, rather than endothelial cells, in pathological ocular angiogenesis [18] and tumor angiogenesis [17]. The genetic ablation of NG2 can therefore be regarded as a specific "intrinsic" targeting of pericytes in pathological microvasculature. We have also used anti-NG2 antibodies for "extrinsic" targeting of pericyte-expressed NG2. Both types of studies demonstrate the functional importance of NG2 during pathological neovascularization, establishing the potential value of the proteoglycan as a pericyte-specific target for anti-angiogenic therapy.

Materials and methods

Experimental animals

NG2 null mice [28] were generated via a conventional homologous recombination approach [29, 30]. The mice were back-crossed onto a C57Bl/6 genetic background for six generations, and NG2+/– heterozygotes were mated to establish separate NG2 knockout (NG2–/–) and wild type (NG2+/+) colonies.

Animal models

All animal studies were performed in accordance with National Institutes of Health Office of Laboratory Animal Welfare (OLAW) guidelines, and were approved by the authors' institutional animal research committees.

Ischemia-induced retinal angiogenesis

Ischemic retinal angiogenesis was induced by withdrawal of neonatal mice from hyperoxia [31]. Litters of postnatal day 7 (P7) NG2 knockout and wild type mice were placed along with their nursing dams in an environmentally controlled chamber (75% oxygen–25% nitrogen atmosphere) for 5 days. At P12, the animals were returned to room air, and at P17 the mice were sacrificed and the eyes enucleated. In total, five mice of each genotype were utilized, allowing comparison of 10 wild type and 10 knockout eyes. The right and left eyes of each mouse were frozen in the same block and sectioned in a plane oriented sagitally to the optic nerve, so that each section represented complete slices of both eyeballs and retinas. Serial sections were cut through the entire thickness of the eyes, yielding between 85 and 132 sections per pair of eyes. Despite this range in the number of sections obtained, the variation was random, with no statistical difference between the number of sections derived from wild type or knockout eyes ($P = 0.0952$, Mann–Whitney test). The total number of cryosections obtained from each group of mice was 1104.

Using systematic random sampling [32], we selected five sections per mouse to provide a representative sampling of both retinas. The sections were stained using the periodic acid-Schiff (PAS) method, with hematoxylin counterstaining as described [31]. This allowed the identification of the so-called neovascular tufts, or clusters of pathological angiogenic vessels protruding beyond the internal limiting membrane of the retina into the vitreous. Quantification of pathological angiogenesis was accomplished by counting the number of vascular cell nuclei in these tufts. We compiled the data according to the number of angiogenic nuclei per section (with each section representing a pair of eyes). We processed five sections per mouse, and therefore compared 25 wild type sections with 25 knockout sections.

In a separate experiment, mice received daily intraperitoneal injections of BrdU (80 μg/g body weight) on postnatal days 14 through 18 after withdrawal from hyperoxia. This allowed subsequent identification of mitotic cells in the pathological vascular tufts present in the P18 retinas. Right and left eyes from each mouse (one wild type and one NG2 null mouse) were frozen in pairs into OCT blocks and sectioned. Two sets of right and left pairs were mounted on each slide. Systematic random sampling was then used to select five slides for each animal. Thus, each animal was represented by 20 sections (10 left eyes and 10 right eyes). Slides were immunostained for PDGF β-receptor and BrdU and counter-stained with hematoxylin. The percentages of mitotic pericytes and endothelial cells were determined after quantifying the number of BrdU-positive nuclei in each of the two immunohistochemically-defined cell types.

Dual hydron pellet corneal angiogenesis

The surgical procedure for inducing corneal angiogenesis in the mouse [33] was modified for this study to incorporate two pellets in the corneal pocket instead of just a single pellet. Slow-release polyhydroxyethyl methacrylate (hydron) (Hydro Med Sciences, Cranbury, New Jersey) pellets ($0.4 \times 0.4 \times 0.2$ mm) were formulated to contain 45 μg sucrose aluminum sulfate (sucralfate) (Sigma, St. Louis, Missouri) plus one of three experimental additives: 90 ng recombinant bFGF (Life Technologies, Carlsbad, California), 0.8 μg affinity-purified rabbit anti-NG2 antibody [17–19], or PBS (control). Ten-week-old mice were anesthetized with Avertin (0.015–0.017 ml/g body weight), and under an operating microscope two pellets were surgically implanted

into the corneal stroma of one eye at a distance of 0.7 mm from the corneo-scleral limbus. Ten NG2 wild-type mice received pairs of pellets containing bFGF and NG2 antibody. Another 10 NG2 wild type mice received pairs of pellets containing bFGF and PBS. Thirteen NG2 knockout mice received pairs of pellets containing bFGF and PBS. Over an 8 day-period after recovery from surgery, the mice were examined under a Leica GZ6 stereomicroscope (Leica, Allendale, New Jersey) to evaluate the progress of corneal angiogenesis in the operated eyes. On day 8, angiogenesis was quantified by determining the area of vascularization, as described previously [33, 34].

Tissue processing, immunohistochemistry, and imaging

Tissues were fixed in 4% paraformaldehyde for 6 h, cryoprotected in 20% sucrose overnight, and frozen in OCT embedding compound (Miles, Inc., Elkhardt, Indiana). Cryostat sections (40 μm) were air-dried onto Superfrost slides (Fisher Scientific, Pittsburgh, Pennsylvania). Immunohistochemical labeling was carried out as previously described [17–19]. Pericytes were identified by labeling with affinity-purified rabbit polyclonal antibodies against the NG2 proteoglycan or the PDGF β-receptor [13, 17–19, 35]. Both NG2 and PDGF β-receptor are regarded as specific markers for pericytes [36, 37]. An affinity-purified rabbit antibody against the alpha subunit of hypoxia-inducible factor-1 (HIF1α) was a generous gift from Dr. Robert Abraham (The Burnham Institute, La Jolla, California).

Since endothelial cells express different cell surface markers as a function of developmental age [38], we identified them using a cocktail of antibodies against endoglin (CD105), PECAM-1 (CD31), and VEGF receptor-2 (flk-1) (Pharmingen, San Diego, California). This strategy has been previously utilized to maximize labeling of all vascular endothelial cells, both immature and mature [17, 39].

Vascular cells in S (synthesis) phase of the cell cycle were identified by means of BrdU (Sigma, St. Louis, Missouri) incorporation and subsequent labeling with sheep anti-BrdU antibody (Fitzgerald Industries, Concord, Massachusetts) [40–42]. Briefly, frozen sections were digested with 0.005% pepsin (Sigma, St. Louis, Missouri) in 0.01 HCl for 30 min at 37 °C followed by treatment with 4 N HCl for 30 min at room temperature. Sections were then blocked by incubation in 5% goat serum in PBS for 30 min [43] prior to incubation with antibody. Fluorescence microscopic imaging of endothelial (CD31 + CD105 + flk1) [38, 39], pericyte (NG2 or PDGF β-receptor) [13, 18, 19], and nuclear (BrdU) markers [43] was performed according to the published methods.

Statistical analysis

Prism 4.0 software (GraphPad, San Diego, California) was used for statistical analyses. Systematic random sampling of serial histological sections was carried out according to previously described methods [32].

Results

Ischemic angiogenesis is diminished in the NG2 null retina (intrinsic targeting)

The stereotyped laminar architecture of the retina makes it an ideal tissue for quantification of ischemic neovascularization[4, 31]. In the hyperoxia model, the return from exposure to 75% oxygen to a normal atmosphere represents relative hypoxia, resulting in the sprouting of new blood vessels from the primary vascular plexus at the inner face of the retina. Many of these new vessels protrude into the vitreous, where they are easily recognized as pathological angiogenic tufts composed of endothelial cells positive for endoglin (CD105), PECAM-1 (CD31), and VEGF receptor-2 (flk-1) and pericytes positive for PDGF β-receptor. In the wild type mouse retina, the profusion of abnormal vascular protrusions beyond the inner limiting retinal membrane is readily apparent (outlined area in Figure 1b). By contrast, relatively few ectopic vessels are present in the NG2 null retina after parallel hypoxic induction (Figure 1a). The enlargement and morphological distortion of the hypoxic wild type retina are reproducible phenomena caused by edema due to leakage of fluid from the extensive pathological neovasculature. Edema is not apparent in the hypoxic NG2 null retina, presumably due to the relative scarcity of pathological vessels.

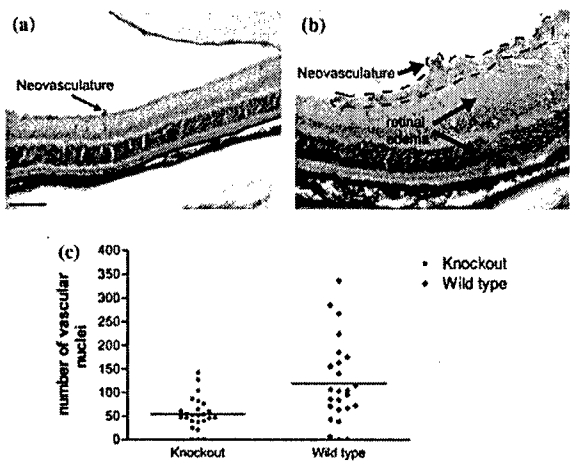

Figure 1. Ischemic retinal neovascularization. Sections of NG2 null (a) and wild type (b) retinas from the ischemia protocol were examined at P17 after PAS/hematoxylin staining. In the wild type retina there is a profusion of vascular tufts protruding past the internal limiting membrane into the vitreous (area enclosed by dashed line). These ectopic vessels are much less common in the NG2 null retina (arrow indicating neovasculature). The wild type retina is also characterized by swelling and distortion from edema caused by leakage from the extensive pathological vasculature. Bars in A and B = 50 μm. (c) Retinal sections from wild type and NG2 knockout mice subjected to the ischemia protocol were analyzed for vascular cell nuclei associated with angiogenic tufts protruding into the vitreous. The number of ectopic vascular nuclei per section (5 mice × 5 sections per mouse = 25 sections) is shown for both genotypes to illustrate the distribution of the data. Wild type mice show a clear trend toward increased ischemic retinal angiogenesis. The average numbers of nuclei per section are shown by the solid bars through each data set (119.8 for wild types vs 54.9 for knockouts. $P = 0.0019$).

Figure 1c presents a quantitative comparison of ischemic neovascularization in the wild type and NG2 null retinas. Each of the data points represents the number of ectopic vascular nuclei (endothelial cells plus pericytes) counted in one of the 25 wild type and 25 knockout slides selected by systematic random sampling from the two sets of serial retinal sections. It is immediately apparent that wild type retinas have an increased tendency towards larger numbers of ectopic vascular nuclei. The averages for the entire data set are 119.8 nuclei per wild type section vs. 54.9 nuclei per NG2 null section (statistically significant by the Mann–Whitney test, $P = 0.0019$). Genetic ablation (intrinsic targeting) of NG2 therefore diminishes the angiogenic response of retinal vasculature to a hypoxic stimulus.

The results of a separate experiment designed to evaluate BrdU labeling suggest that cell proliferation offers at least a partial explanation for the observed difference between the responses of the wild type and NG2 null retinas to hypoxia. In this trial, pathological vascular tufts were again more numerous in wild type than in knockout retinas, confirming the results shown in Figure 1. In addition, BrdU-labeled nuclei were seen more frequently in wild type angiogenic sprouts than in NG2 null counterparts. Double-labeling of hematoxylin-stained sections for BrdU and the pericyte marker PDGF β-receptor allowed us to determine mitotic indices for pericytes in wild type and NG2 null angiogenic tufts. We have previously demonstrated the specific expression of PDGF β-receptor by pericytes in hypoxic retinal tufts [18]. This analysis revealed a large decrease in pericyte proliferation in the ischemic knockout retina. Figure 2a shows that 45.2% of pericytes in angiogenic tufts are labeled with BrdU in the ischemic wild type retina vs. 18.7 % of pericytes in the knockout retina (statistically significant, $P = 0.0068$ Mann–Whitney test). Counting BrdU-positive nuclei in PDGF β-receptor-negative cells also allowed us to compare mitotic indices for endothelial cells. Interestingly, 38.3% of endothelial cells are BrdU-positive in the wild type retina, vs. 22.8% in the NG2 null retina ($P = 0.0147$ Mann–Whitney test).

The validity of these results was confirmed in a separate set of experiments in which BrdU-positive nuclei were counted in conjunction with staining with the endothelial antibody cocktail to identify endothelial cells. The mitotic indices for both endothelial cells and pericytes were reduced in NG2 null retinas. Both types of labeling paradigms (endothelial cocktail and PDGF β-receptor) therefore demonstrate a reduction in proliferation of pericytes and endothelial cells in the NG2 null retina. Reduced proliferation of these cell populations is likely to be an important factor in the sub-normal angiogenic response of the NG2 null retina to hypoxia.

We have previously demonstrated in wild type mice that the extensive investment of ischemic retinal vessels by NG2-positive, PDGF β-receptor-positive pericytes is comparable to the high pericyte:endothelial cell (P/E) ratio normally seen in the central nervous system [18, 19]. An additional important distinction between pathological vessels in the wild type and NG2 null retinas is the relative scarcity of pericytes relative to endothelial cells in the knockout neovasculature. Determination of the respective numbers of pericyte and endothelial cell nuclei associated with the angiogenic tufts allows us to determine the pericyte to endothelial cell investment ratio in these clusters of vessels (Figure 2b). In wild type neovasculature the P/E investment ratio is 0.86 (i.e. close to one pericyte per endothelial cell), while in the knockout retina the P/E value falls to 0.24 (only one pericyte for every four endothelial cells) ($P = 0.0011$ Mann–Whitney test). The observation that pericyte proliferation is more adversely affected than endothelial cell proliferation in knockout retinas may partially account for this difference in P/E investment ratios.

It has been shown that an early step in the angiogenic response of the retinal vasculature to withdrawal from hyperoxia is up-regulation of the HIF-1 transcription factor. HIF-1 plays a critical role in the induction of VEGF expression and subsequent steps in the angiogenic process [44]. Immunostaining for the HIF-1α subunit was used to

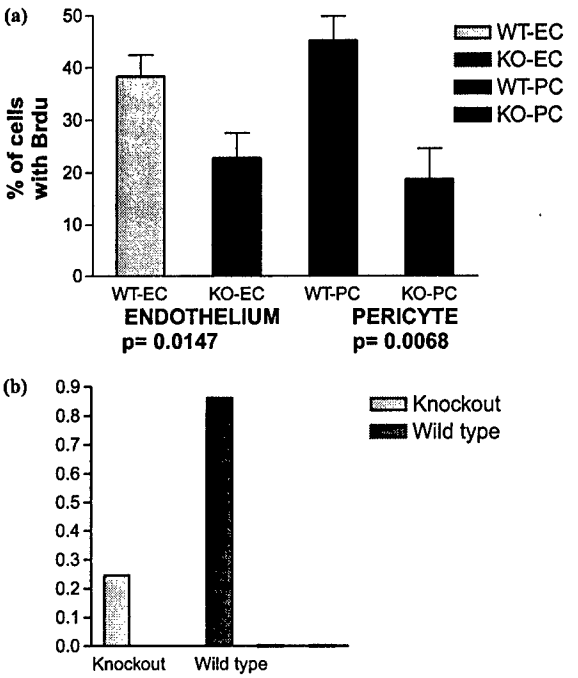

Figure 2. Pericyte/endothelial cell mitotic indices and investment ratios. (a) By combining hematoxylin counter-staining with double staining for PDGF β-receptor and BrdU, we were able to calculate the mitotic index for pericytes and endothelial cells associated with vascular tufts. The number of BrdU-positive pericyte nuclei divided by the total number of hematoxylin-stained pericyte nuclei $\times$ 100 = the percentage of mitotic pericytes. Thus, 45.2% of pericytes are BrdU-positive in the wild type retina (WT-PC) vs. only 18.7% in the knockout retina (KO-PC) ($P = 0.0068$). Total and BrdU-positive nuclei were also counted in PDGF β-receptor negative cells to provide endothelial cell data. In the wild type retina 38.3% of endothelial cells are BrdU-positive (WT-EC), compared to 22.8% in the NG2 null retina (KO-EC) ($P = 0.0147$). (b) The data used to construct (a) were also used to determine the pericyte/endothelial cell investment ratio in ischemic vascular tufts (total number of pericyte nuclei/total number of endothelial cell nuclei). For wild type retinas this ratio was almost one pericyte per endothelial cell (0.86), while in NG2 null retinas the ratio fell to 0.24 ($P = 0.0011$). The high ratio of pericytes to endothelial cells in the wild type retina is characteristic of microvasculature in the central nervous system.

evaluate this initial response of wild type and NG2 null retinas to hypoxia. Very low HIF-1α levels were observed in control retinas from P13 wild type and knockout mice. In contrast, 16 h after removal of experimental P13 pups from 75% oxygen, HIF-1α was up-regulated in similar fashion in the inner layers of both the wild type and NG2 null retinas (data not shown). Differences in pathological retinal neovascularization between the two genotypes therefore are not due to the initial response of retinal cells to hypoxia, but to subsequent neovascularization events downstream of HIF-1α expression.

Corneal angiogenesis is reduced by both intrinsic and extrinsic targeting of NG2

Implantation of a bFGF-containing pellet along with a control PBS-containing pellet induces a robust angiogenic response in the wild type mouse cornea (Figure 3a). We have shown that these corneal microvessels are richly invested by NG2-positive, PDGF β-receptor-positive pericytes [18]. If the second pellet contains anti-NG2 antibody instead of PBS, the angiogenic response to bFGF is substantially reduced (Figure 3b). A diminished response to bFGF is also observed in the NG2 null cornea (Figure 3c). These qualitative observations were quantified by measuring the extent of the vascularized areas in each of the three experimental situations (Figure 3d). The mean area of corneal neovascularization was 0.3863 mm^2 in the control group of wild type mice ($n = 10$), compared with 0.087 mm^2 in the NG2 knockout animals ($n = 13$). In the presence of an NG2 antibody-containing pellet, corneal vascularization in wild type mice was reduced to 0.1445 mm^2 ($n = 10$). Both of these differences were statistically significant ($P = 0.0006$ for wild type mice vs. knockout mice, and 0.0039 for control wild type mice vs. antibody treated wild type mice). Thus both intrinsic (genetic ablation) and extrinsic (antibody blocking) targeting of NG2 result in diminished corneal angiogenesis.

Discussion

Since the cellular processes underlying neovascular sprout formation remain incompletely understood [45, 46], increased attention to pericytes and their interaction with endothelial cells will be required not only to attain a better understanding of neovascularization in general, but also to realize the full potential of anti-angiogenic therapy. The critical contribution of pericytes during angiogenesis has been well established by observation of the pathological phenotypes of mice in which pericyte development is blocked [11, 13, 47]. The functional importance of pericytes has been attributed largely to their ability to stabilize and provide structural support to pre-existing endothelial tubes. They are thought to accomplish this by controlling endothelial cell proliferation and motility, and by contributing to the establishment of a permeability barrier and the regulation of blood flow [36, 48–53]. However, it is now becoming clear that pericytes can play a much earlier role in microvascular development than previously realized. The use of NG2 and other markers for nascent pericytes has revealed the participation of these cells in the earliest stages of angiogenesis [6, 36, 16–20, 53–56]. Pericytes may even be important for the stimulation and guidance of nascent vascular tubes. Strategies for targeting pericytes may therefore be able to affect not only existing vessels, but also the formation of new vessels.

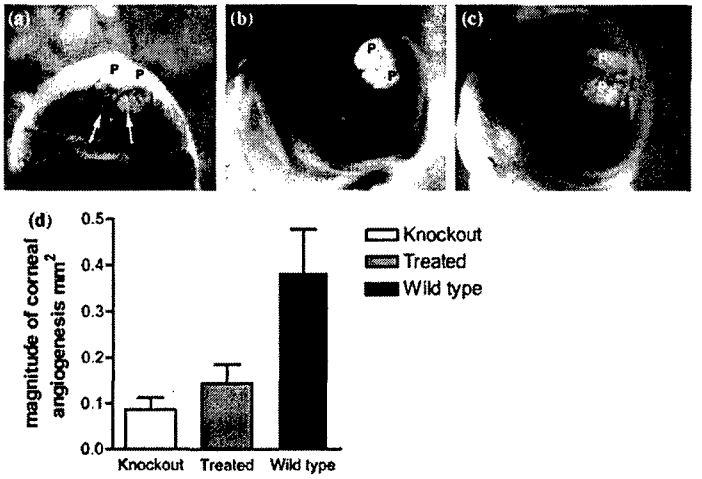

Figure 3. Corneal angiogenesis. Corneal angiogenesis was compared in wild type corneas implanted with a bFGF-containing pellet and a PBS-containing pellet (a), wild type corneas implanted with a bFGF-containing pellet and an NG2 antibody-containing pellet (b), and NG2 null corneas implanted with a bFGF-containing pellet and a PBS-containing pellet (c). Pellets are labeled with the letter P. Invasion of the cornea by new blood vessels in response to bFGF (arrows in a) is greatly reduced in the knockout mouse and by the presence of antibody against NG2 in the wild type mouse. (d) The angiogenic responses shown in (a–c) were quantified by measurement of the area (in mm^2) occupied by new vasculature. Compared to bFGF-induced vascularization of the wild type mouse cornea, vascularization of the NG2 null cornea is reduced by a factor of 4.4 ($P = 0.0006$). Vascularization of the wild type cornea is reduced by a factor of 2.7 by inclusion of anti-NG2 antibody to inhibit invading pericytes ($P = 0.0039$).

Our current studies show that intrinsic targeting of NG2 (by genetic ablation) leads to decreased ischemic angiogenesis in the mouse retina in response to hypoxia. The wild type mouse retina contains more than twice as many pathological vascular tufts as the retina of the NG2 null mouse. Since HIF-1α induction is similar in wild type and knockout retinas, we know that the defect in the null mouse lies not in the initial response of retinal cells to hypoxia, but probably in later stages of vascular cell responsiveness to HIF-1-induced factors such as VEGF. This seems reasonable in light of the fact that NG2 is not expressed by cells of the retina per se, but instead by pericytes in the microvasculature [17–19].

A major factor in the sub-normal angiogenic response of the NG2 null retina appears to be reduced vascular cell proliferation. Only 41% as many mitotic pericytes are present in the ischemic vasculature of the NG2 null retina as in the wild type retina. These data represent the first direct *in vivo* evidence in support of a role for NG2 in cell proliferation. The ability of NG2 to sequester growth factors such as bFGF and PDGF-AA and possibly assist in presentation of these factors to their respective signaling receptors could represent one mechanism by which the proteoglycan promotes cell proliferation [21, 28].

Interestingly, the absence of NG2 and the decreased number of mitotic pericytes is accompanied by a 1.7-fold decrease in the number of mitotic endothelial cells, suggestive of a stimulatory effect of pericytes on endothelial cell proliferation. This idea is somewhat at odds with previous reports that pericytes can inhibit endothelial cell proliferation in cell culture models [48] and that the absence of pericytes is accompanied by endothelial cell hyperplasia *in vivo* [11]. However, the pericyte/endothelial cell relationship is a complex, dynamic one that is likely to vary depending on the specific model under investigation. An excellent example of this is provided by a recent study of the proliferative retinopathy that results from endothelial cell-specific ablation of PDGF-B [47]. The general conclusion from this work was that reduction of pericyte density below 50% of normal, invariably led to the development of proliferative retinopathy. Nevertheless, localized instances were also encountered in the same investigation [47] in which increased pericyte density promoted the formation of chaotic, endothelial cell-rich vasculature, demonstrating that under certain conditions pericytes can have pro-angiogenic properties. The ability to use pericytes as effective anti-angiogenic targets also is suggestive of the pro-angiogenic nature of these cells [9, 10].

Our current data support a pro-angiogenic role for pericytes in the formation of ischemic retinal microvessels. Our results with wild type mice show that endothelial cells are richly invested by NG2-positive, PDGF β-receptor-positive pericytes in this pathological vasculature (see also [18]). Coupled with our documentation of the early participation of NG2-positive pericytes during neovascularization [17], these observations suggest the possibility that pericyte-derived factors or NG2-dependent sequestration of growth factors might act to promote the proliferation of endothelial cells. Alternatively, the ability of NG2 to neutralize the growth-inhibitory effects of angiostatin [22, 57] may promote endothelial cell proliferation in the wild type mouse, an effect that would be absent in the NG2 null mouse.

In addition to the quantitative reduction of ischemic angiogenesis in the NG2 knockout mouse, capillaries in the null mouse also have an altered cellular composition. The pericyte:endothelial cell investment ratio in ischemic vessels of the wild type retina is 0.86, or almost one pericyte per endothelial cell. This high investment ratio is characteristic of capillaries in the central nervous system in general, and the retina in particular, possibly contributing to the integrity of the blood–brain barrier and the high metabolic needs of neural tissues [5, 36]. This investment ratio falls to 0.24 in the ischemic neovasculature of the NG2 null retina. The relative changes in pericyte and endothelial cell proliferation in the NG2 knockout mouse would not appear to account for the magnitude of this decrease. Thus other factors that we have not yet investigated, such as decreased cell motility or increased apoptosis, may contribute to the large decrease in pericyte number relative to that of endothelial cells. While a specific role for NG2 in apoptosis has not been explored, there are numerous indications of NG2 involvement in cytoskeletal reorganization and cell motility [23–28]. In future work it will therefore be important for us to investigate the impact of NG2 on these processes in the context of neovascularization.

Intrinsic targeting of NG2 by genetic ablation leads to an even more pronounced decrease in bFGF-induced corneal angiogenesis. Neovasculature covers a 4.4-fold greater surface area in the wild type cornea than in the NG2 null cornea, once again supporting the idea that NG2 plays a role in pericyte development and/or function, and in the development of new vasculature. Interestingly, extrinsic targeting of NG2 through the use of a neutralizing antibody also produces a significant decrease in corneal angiogenesis (2.7-fold). While our data do not allow us to determine which aspects of pericyte function are blocked by the antibody, previous studies have shown that anti-NG2 antibodies are capable of blocking growth factor-induced cell proliferation [58] and both growth factor-induced and extracellular matrix-induced cell motility [27, 59] in cell culture models.

Our demonstration of the functional importance of NG2 during pathological ocular angiogenesis logically raises the question of the proteoglycan's function during normal developmental neovascularization. How can we rationalize the observation that the NG2 knockout mouse possesses functional vasculature? More than one answer is possible. First, pathological angiogenesis may differ in some respects from normal angiogenesis. During pathological angiogenesis, the vasculature may be responding to combinations of signals that are not normally experienced during development or else are occurring out of their normal sequence (multiple factors released by tumor cells would be a good example). The role of NG2 may be magnified under these abnormal circumstances. Additional experiments with wild type and NG2 knockout mice are planned in order to examine NG2-dependent aspects of angiogenesis in other types of pathological models such as tumor progression and

wound healing. Second, both of our pathological angiogenesis models have utilized postnatal animals, whereas the bulk of developmental neovascularization takes place during embryogenesis. It seems possible that embryonic development involves a higher degree of plasticity than events that occur postnatally. In other words, the ability to compensate for the loss of NG2 may be greater during embryogenesis. In response to postnatal challenges, such compensatory mechanisms may not be available, thus facilitating our ability to detect the contribution of NG2. Examination of normal angiogenic events that occur postnatally (for example in normal retinal development) may therefore reveal the effects of NG2 ablation. It has required detailed and careful experimentation to detect changes in pathological retinal and corneal angiogenesis in the NG2 null mouse. The same type of painstaking analysis may be required to detect subtle deficiencies in developmental neovascularization in the knockout mouse. Such studies remain to be undertaken, but in light of our current results would appear to offer great promise.

Acknowledgements

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