Inhibition of rat corneal angiogenesis by a nuclease-resistant RNA aptamer specific for angiopoietin-2

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Angiopoietin-2 (Ang2) appears to be a naturally occurring antagonist of the endothelial receptor tyrosine kinase Tie2, an important regulator of vascular stability. Destabilization of the endothelium by Ang2 is believed to potentiate the actions of proangiogenic growth factors. To investigate the specific role of Ang2 in the adult vasculature, we generated a nuclease-resistant RNA aptamer that binds and inhibits Ang2 but not the related Tie2 agonist, angiopoietin-1. Local delivery of this aptamer but not a partially scrambled mutant aptamer inhibited basic fibroblast growth factor-mediated neovascularization in the rat corneal micropocket angiogenesis assay. These *in vivo* data directly demonstrate that a specific inhibitor of Ang2 can act as an antiangiogenic agent.

Angiogenesis, the growth of new blood vessels from existing ones, is regulated at multiple steps by interactions between pro- and antiangiogenic factors. During early angiogenesis, endothelial cells proliferate, migrate, and form tubular structures. In subsequent stages of angiogenesis, neovessels undergo vascular maturation, a process that appears to be regulated by the endothelial transmembrane receptor tyrosine kinase, Tie2 (1, 2). Tie2 is unique among receptor tyrosine kinases in that its extracellular ligands, the angiopoietins, seem to have opposing actions on endothelial cells despite sharing ≈60% amino acid sequence identity (3). Angiopoietin-1 (Ang1) is an activating ligand that induces phosphorylation of Tie2 (4) and promotes endothelial cell survival and vascular impermeability (5–9). Tie2 is activated in the quiescent adult endothelium, an effect that is likely mediated by Ang1 (10, 11). In contrast, angiopoietin-2 (Ang2) appears to be a naturally occurring Tie2 antagonist that is expressed only at sites of active angiogenesis, such as the ovary (3) and vascularized tumors (12). Destabilization of the endothelium by Ang2 is believed to potentiate the actions of proangiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). In support of this model, exogenous Ang2 has been demonstrated to increase pupillary membrane angiogenesis in the presence of VEGF and to induce regression if VEGF is inhibited in neonatal rats (13). In addition, exogenous Ang2 enhanced VEGF-mediated corneal neovascularization (14), and ectopic overexpression of Ang2 in cancer cell lines produced larger, more vascular tumors in mice (15, 16).

To investigate the specific role of Ang2 in the adult vasculature, we used an iterative *in vitro* selection strategy (SELEX, systematic evolution of ligands by exponential enrichment) to generate nuclease-resistant RNA ligands (aptamers) that bind Ang2 with high affinity. To enhance the specificity of the Ang2 selection, RNAs that could bind to recombinant human Ang1 were discarded (negative selection) before rounds of positive selection for RNAs that could bind to recombinant human Ang2 (Fig. 1). Using this approach, we identified aptamers that selectively bind and inhibit Ang2 activity *in vitro*. Moreover, we have demonstrated that such aptamers can inhibit angiogenesis *in vivo*, by using a well characterized, quantitative model of angiogenesis, the rat corneal micropocket angiogenesis assay (17).

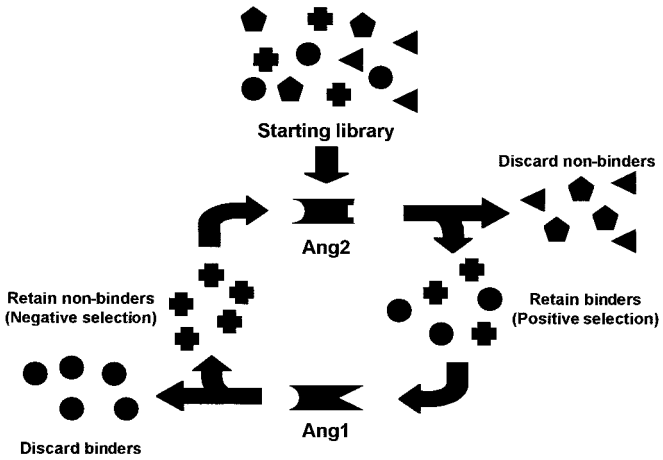

Fig. 1. ''Positive/negative'' systematic evolution of ligands by exponential enrichment (SELEX). Aptamers that selectively bind Ang2 over Ang1 were generated by performing ''negative'' selection against Ang1* before rounds of ''positive'' selection for Ang2.

Methods

Systematic Evolution of Ligands by Exponential Enrichment (SELEX). A random pool of RNA oligonucleotides of sequence 5′-GGGAGGACGATGCGG-N_{40}-CAGACGACTCGCTGAG-GATCCGAGA-3′ (where N_{40} represents 40 random nucleotides with equimolar 2′-hydroxy-ATP, 2′-hydroxy-GTP, 2′-fluoro-CTP, and 2′-fluoro-UTP content) was generated as described (18). For ''positive'' selection, RNA was incubated with recombinant human Ang2 (R&D Systems) for 15 min at 37°C in a selection buffer with ionic composition similar to extracellular vascular fluid (20 mM Hepes, pH 7.4/150 mM NaCl/2 mM $CaCl_2$/0.01% BSA). RNAs that bound Ang2 were isolated by vacuum filtration through a 0.45-μm nitrocellulose membrane (Schleicher and Shuell). Bound RNAs were recovered and amplified, and the process was repeated as described (18) with modifications. For ''negative'' selection, we incubated RNA with recombinant human Ang1* (provided by G. Yancopoulos, Regeneron Pharmaceuticals, Tarrytown, NY). RNAs that bound Ang1* were removed by filtration through a 0.45-μm nitrocellulose centrifugal filter (Schleicher and Shuell) followed by phenol-chloroform extraction of the filtrate to remove residual Ang1*. Successive rounds of selection were performed with decreasing concentrations of Ang2 and increasing concentrations of Ang1*. After round 11, selected RNAs were reverse-transcribed, amplified, cloned, and sequenced as described (18).

Abbreviations: Ang1, angiopoietin 1; Ang2, angiopoietin 2; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.

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Table 1. Round 11 RNA random region sequences

Clone	5′-GGGAGGACGATGCGG-N_{40}-CAGACGACTCGCTGAGGATCCGAGA-3′	Frequency
Family A		
11-1	ACUAGCCU*CAUCAGCUCAUG*UGCCCCUCCGCCUGGAUCAC	11/32
11-3	UUAAC*CAUCAGCUCAUG*GCCCCUGCCCUCUCAAGGACCAC	1/32
11-4	CACCAGACCGA*CAUCAGCUUAUG*GCCCCUCACCCACACCG	1/32
11-15	CCACCGAUCG*CAUCAGCUCAUG*GCCCCUCCCGACCCGCCA	1/32
11-19	CCAGACGUUCUCGCCCCGCCGAU*CAUCAGC*GCUGGCCCUAU	1/32
11-26	CACUACCACGCCAU*AUCAGCU*AAUGGCCCCUCCCUACGCA	1/32
11-32	ACUCACCAGUCAC*CAUCAGCUCAU*GCGCCCCUCCCCCGAC	1/32
Family B		
11-2	UGACCAAGCCUCACGUUGAACCUGCCAGUAGACCCCGCCCA	1/32
11-6	GGAGCGCAAUUCGCCUCGCAAGUUGAACUCCGCUGGCGG	1/32
11-9	UAAGCUCUUUGGCUUAGCCCGACACGUUGAACUCCAGAGU	1/32
11-10	CACGGUACCACCAAGUCACACGUUGAACUCCAUGCAGCUG	1/32
11-21	CAUGUCUACAACAAUCUCGCCCGUUGAGUCUCGUCGAAUU	2/32
11-30	CACUCAGCGCCCUGCGAAACGUUGCCGCCUCCCAACGUCU	1/32
11-34	CUCUUUUUGUCCCCGCACGUUGAACUCCUGUCCCUCUACU	1/32

Binding Affinity Assays. RNA–protein equilibrium dissociation constants (K_d) were determined by the double-filter nitrocellulose-filter binding method as described (19) with modifications (18, 20). Direct binding assays were performed by incubating ^{32}P-labeled RNA (<0.1 nM) with a range of protein concentrations in selection buffer at 37°C. Competition binding assays were performed by incubating ^{32}P-labeled 11-1 RNA (<0.1 nM), 10 nM Ang2 protein, and the indicated concentrations of unlabeled competitor RNA. The fraction of bound RNA was quantified and corrected for nonspecific background binding to the nitrocellulose filter as described (19). The K_d of the competitor RNA was estimated to be equivalent to its 50% inhibitory concentration (IC_{50}) as described (20). K_d were reported as the mean and 95% confidence interval (CI) of three experiments.

Oligonucleotide Synthesis. Small-scale synthesis of full-length RNAs was performed by *in vitro* transcription of synthetic DNA templates and purification by denaturing gel electrophoresis as described (18). Truncated RNAs were synthesized with an inverted 3′ deoxythymidine cap for exonuclease protection, gel-purified, and deprotected (Dharmacon Research, Lafayette, CO).

Immunoprecipitation and Western Blotting. The 293 cells stably expressing full-length recombinant human Tie2 (293-hTie2 cells) were provided by K. Peters, Procter & Gamble Health Care Research Center, Mason, OH. Recombinant human Ang2 (30 nM) or Ang1 (15 nM; R&D Systems) was preincubated with 150 nM RNA for 5 min at 37°C in selection buffer without BSA. Confluent 293-hTie2 cells in 60-mm dishes were treated with the angiopoietin-RNA mixtures for 8 min at 37°C. The cells were lysed in Triton lysis buffer, and tyrosine-phosphorylated proteins were immunoprecipitated with mouse monoclonal antiphosphotyrosine (PY99, 1:100) and immobilized on Protein A agarose beads (Santa Cruz Biotechnology). Protein complexes were separated by SDS/PAGE, transferred to nitrocellulose, and analyzed by Western blotting with mouse monoclonal anti-Tie2 (Ab33, 1:5,000) (21).

Rat Corneal Pocket Assay. Animal experiments were performed according to protocols approved by the Duke University Institutional Animal Care and Use Committee. The rat corneal micropocket assay was performed as described (22) with modifications. Briefly, sustained-release pellets were prepared the day before surgery by combining a solution of recombinant human bFGF (R&D Systems), sucrose octasulfate aluminum complex (sucralfate, Sigma), and RNA in selection buffer without BSA with an equal volume of 12% (wt/vol) Hydron polymer (poly2-hydroxylethylmethacrylate, Sigma), such that each dried pellet contained 50 ng bFGF, 15 μg sucralfate, and 120 pmol RNA.

Female Fischer 344 rats (8–10 weeks of age; Charles River Breeding Laboratories) were anesthetized, and superficial corneal micropockets were surgically created in both eyes. Hydron pellets containing 11-1.41 RNA were inserted into left-sided pockets, and pellets containing Scramble RNA were inserted into right-sided pockets. In total, 31 pellets were successfully implanted into one or both corneas of 19 rats. On the sixth postoperative day, animals were anesthetized and administered 300 units of heparin via tail vein injection. The thoracic aorta was cannulated with PE 100 tubing, and 10 cc Higgins waterproof India ink was injected (Sanford, Bellwood, IL). After enucleation, the eyes were fixed in 10% neutralized buffered formaldehyde for 24 h, then the corneas were dissected and mounted onto slides.

Corneal images were captured and analyzed by using Scion IMAGE software (Scion, Frederick, MD) by an investigator who was blinded to the treatment assignment. Four corneas with evidence of nonincisional corneal trauma or nonhealing of the surgical incision were excluded, leaving 27 corneas (16 control and 11 treatment) for analysis. Length of the longest neovascular sprout, the average length of six vessels evenly distributed across the neovascular arc, and total neovascular area were measured. Statistical significance was determined by the Student's t test.

Results

Isolation of an RNA Aptamer That Binds Ang2 with High Affinity and Specificity. We used an iterative *in vitro* selection strategy to select from a large ($>10^{14}$ molecules) combinatorial library, nuclease-resistant RNAs that bind Ang2 but not Ang1*, a genetically modified version of Ang1 that retains its biological activity (3). Over successive rounds of positive selection for Ang2 and negative selection against Ang1*, the affinity of the RNA pool for Ang2 increased while the affinity for Ang1* remained low (data not shown). We cloned and sequenced amplification products from round 11 of selection (Table 1). The majority of RNA sequences from these rounds could be grouped into families that shared one of two conserved motifs: 5′-CAUCAGCUCAUG-3′ (family A) or 5′-CACGUUGAAC-3′ (family B). All RNAs assayed from these two families bound Ang2 with similarly high affinity in direct binding assays (K_d = 3–10 nM). Furthermore, RNAs from family A and family B cross-competed for binding to Ang2, suggesting that these

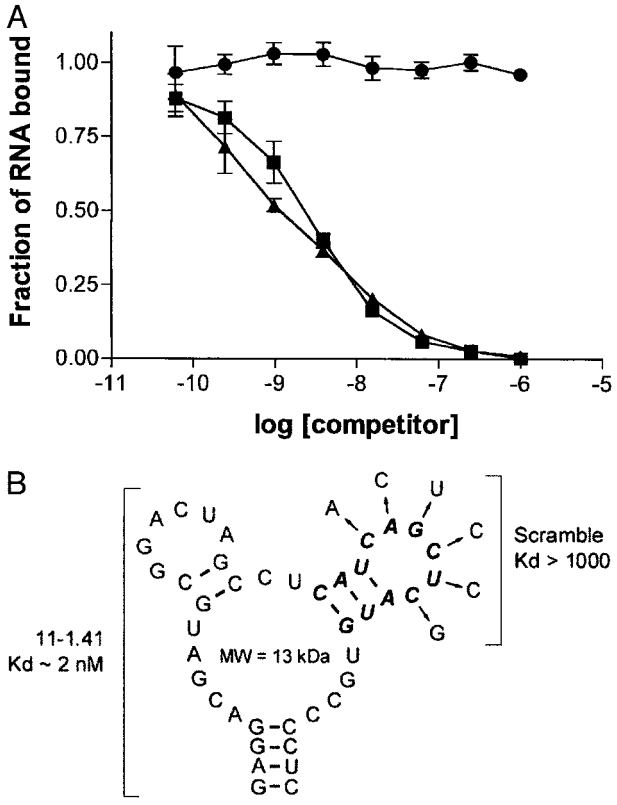

Fig. 2. Analysis of RNA sequence, structure, and binding. (*A* and *B*) A stem-loop secondary structure was predicted for clone 11-1, based on sequence information from family A. A 41-nt truncate of clone 11-1 (11-1.41; ▲) competed for binding to Ang2 with affinity similar to the full-length aptamer (■). Scrambling six of the nucleotides within the conserved sequence of 11-1.41 completely disrupted the ability of this RNA (Scramble; ●) to compete for binding to Ang2.

families bind to the same or overlapping regions of Ang2 (data not shown). The dominant sequence in the final round of selection, aptamer 11-1, bound Ang2 with a K_d of 3.1 nM (95% CI 2.2–4.3 nM) and Ang1* with a $K_d > 1$ μM. Thus, this aptamer possesses >200-fold specificity for Ang2 versus Ang1*. Therefore, we focused subsequent studies on aptamer 11-1.

A 41-nt Truncate of Aptamer 11-1 Retains High-Affinity Binding to Ang2. To allow for efficient chemical synthesis, full-length aptamers (>80 nt) must be shortened ideally to fewer than ≈50 nt. Application of an RNA-folding algorithm (23) to aptamer 11-1 suggested a common stem-loop structure that incorporated the conserved sequence motif, when the folding was constrained based on covariation analysis of the RNA sequences in family A (24). A 41-nt truncate of aptamer 11-1 (11-1.41), corresponding to the possible secondary structure (Fig. 2*A*), bound to Ang2 with a K_d of 2.2 nM (95% CI 1.3–3.8 nM), similar to the full-length aptamer (Fig. 2*B*). Scrambling the six nucleotides that comprise the terminal loop of 11-1.41 completely disrupted its ability to bind Ang2, and this RNA (Scramble) served as a negative control for 11-1.41 in cell-based and *in vivo* assays.

11-1.41 Inhibits Binding of Ang2 Activity in Cell Culture. Ang2 does not induce tyrosine phosphorylation of Tie2 in endothelial cells. However, it is capable of inducing Tie2 phosphorylation when Tie2 is ectopically expressed in nonendothelial cell lines (3). The exact mechanism of this seemingly paradoxical activity of Ang2 has not been elucidated, but it provides an assay with which to test effects of the aptamer on Ang2 function. We treated

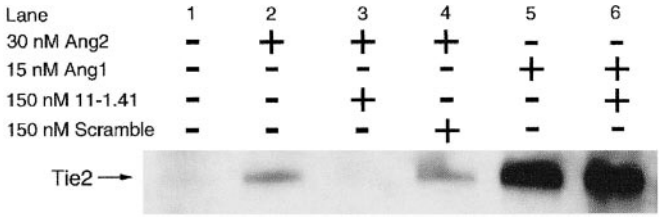

Fig. 3. Inhibition of Ang2 function by 11-1.41. Treatment of 293 cells ectopically expressing Tie2 with either Ang2 (lane 2) or Ang1 (lane 5) results in the activation of Tie2, as demonstrated by immunoprecipitation of tyrosine-phosphorylated proteins followed by Western blotting for Tie2. A molar excess of 11-1.41 RNA completely inhibited the activation of Tie2 by Ang2 (lane 3) but not by Ang1 (lane 6). Scramble RNA had no effect on the activation of Tie2 by Ang2 (lane 4).

293-hTie2 cells with Ang2 or Ang1 in the presence or absence of a molar excess of RNA (Fig. 3). A 5-fold excess of 11-1.41 RNA completely inhibited the activation of Tie2 by Ang2, whereas Scramble RNA had no effect. A 10-fold excess of 11-1.41 RNA did not inhibit the activation of Tie2 by Ang1, demonstrating the functional specificity of this aptamer for Ang2. Although the site of aptamer binding to Ang2 is unknown, the ability of this aptamer to inhibit the activation of Tie2 by Ang2 in cell culture suggests that its binding interferes with ligand oligomerization and/or receptor binding. Negative selection against Ang1*, which contains the first 73 aa of Ang2, may have provided the additional benefit of driving the selection away from the functionally less important signal peptide region of Ang2 and toward the more important coiled-coil and fibrinogen-like domains, which mediate ligand oligomerization and receptor binding, respectively (25).

11-1.41 Inhibits bFGF-Induced Corneal Angiogenesis in Rats. We used the rat corneal micropocket assay to examine the effect of inhibiting endogenous Ang2 in the presence of other growth factors, as occurs in most cases of physiological and pathological angiogenesis. Although the angiopoietins alone do not promote angiogenesis in the mouse corneal micropocket model, Ang2 enhances the angiogenic effects of VEGF (14). We used bFGF rather than VEGF, as bFGF is a more potent stimulator of angiogenesis than is VEGF in the rat cornea, through a process that likely involves VEGF (26). Moreover, the 11-1.41 aptamer and the control Scramble RNA bound equally poorly to bFGF ($K_d \approx 100$ nM). We inserted slow-release pellets containing bFGF and either 11-1.41 or Scramble RNA into bilateral corneal micropockets. Pellet sizes and pellet–limbus distances were similar between treatments (data not shown). Of the 10 animals in which bilateral pellets were implanted, average neovascular length was decreased by >30% in six of the corneas treated with the 11-1.41 aptamer and in only one of the corneas treated with the Scramble control aptamer (Fig. 4*A*). In addition, we compared neovascularization between treatment groups in the 27 unpaired corneas. Despite variability between animals, neovascularization was reduced by up to 40% in the 11-1.41 aptamer treated group. Longest vessel length (Fig. 4*B*; $P = 0.02$) and average vessel length (Fig. 4*C*; $P = 0.03$) were significantly shorter in corneas treated with 11-1.41, and a similar trend was observed for vascular area (Fig. 4*D*; $P = 0.07$), indicating that the Ang2 aptamer can impede angiogenesis *in vivo*.

Discussion

Multiple steps are required for the formation of a functional blood vessel, and numerous factors have been identified that regulate this complex process. The endothelial transmembrane receptor tyrosine kinase Tie2 is essential for normal embryonic vascular development (1, 2). Tie2's extracellular ligands, the

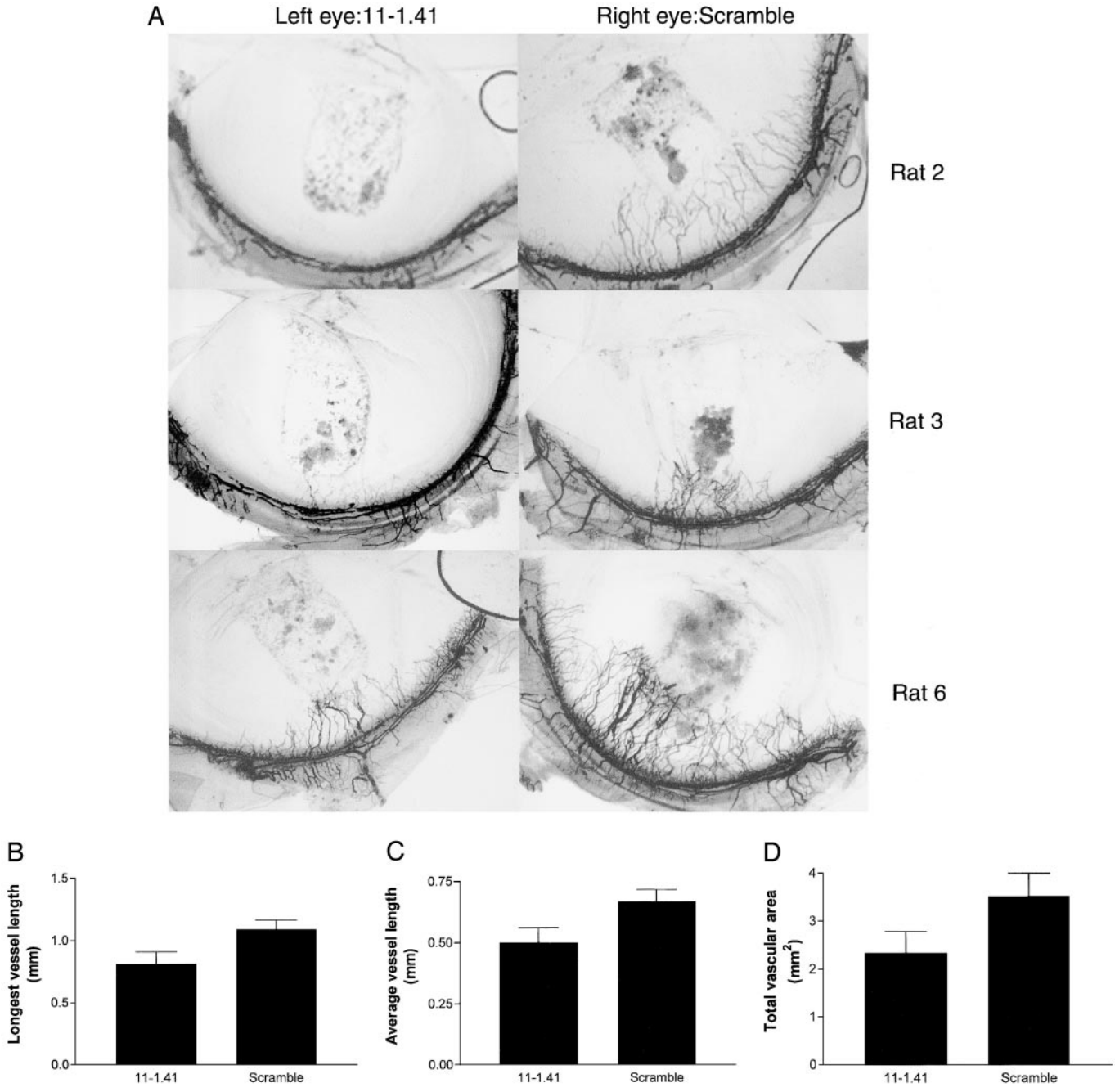

Fig. 4. Rat corneal pocket angiogenesis assay. Slow-release pellets containing bFGF and either 11-1.41 or Scramble RNA were inserted into bilateral corneal micropockets. (*A*) Images from three individual animals demonstrated dramatic differences in neovascularization between corneas treated with 11-1.41 and corneas treated with the Scramble control aptamer. Longest vessel length (*B*), average vessel length (*C*), and total vascular area (*D*) were measured in 11 corneas treated with 11-1.41 and in 16 corneas treated with Scramble RNA, and data are presented as mean values with error bars representing standard errors.

angiopoietins, play opposite roles in vascular development (3, 5), but their roles in the adult vasculature remain unclear. Studies demonstrating expression of Ang1 in the quiescent vasculature and Ang2 at sites of active tumor angiogenesis suggest that Ang2 may promote angiogenesis by destabilizing the mature endothelium (15, 27–30). This hypothesis is supported further by overexpression studies in which Ang2 enhanced tumor angiogenesis and/or growth while Ang1 inhibited it (15, 16, 31). Inhibition of Tie2 with soluble receptor has been shown to inhibit tumor angiogenesis, growth, and metastasis (32–34). However, soluble receptor studies do not distinguish between the actions of Ang1 and Ang2, as both ligands are inhibited by this strategy, and evidence exists that Ang2 does not act simply as a dominant negative inhibitor of Ang1 (3, 35, 36). Studies in which administration of exogenous Ang1 has enhanced neovascularization (14, 37) indicate that Ang1 can also be proangiogenic in the adult vasculature. These seemingly contradictory results suggest that Ang1 and Ang2 are context-dependent modulators of angiogenesis and highlight the importance of targeting these factors in an appropriate spatial and temporal context.

With the explosion of genomics and proteomics, families of proteins such as the angiopoietins will likely become even more

extensive, and specific tools with which to dissect their precise roles *in vivo* will become even more important. Systematic evolution of ligands by exponential enrichment (SELEX) and other iterative *in vitro* selection strategies using combinatorial libraries provide powerful techniques through which reagents with desired properties can be "made-to-order." To clarify the role of Ang2 *in vivo*, we generated an aptamer (11-1.41) that binds with high affinity to Ang2 and with >200-fold specificity for Ang2 over Ang1*. Local delivery of a single dose of 11.1–41 RNA significantly inhibited bFGF-mediated corneal neovascularization. This effect should only be enhanced by the optimization of this aptamer for *in vivo* applications. For instance, modification of 11-1.41 with 2′-fluoro-pyrimidines and addition of the 3′ inverted nucleotide cap improve resistance to degradation by plasma nucleases (38, 39), but the truncated aptamer's small size (≈13 kDa) allows rapid tissue diffusion and renal clearance (40, 41). Therefore, modifications such as attachment of polyethylene glycol or liposomes, which enhance aptamer bioavailability, will likely enhance the aptamer's efficacy for both local and systemic applications in relevant disease models. Further, the highly specific nature of aptamers renders them quite capable of discriminating between protein homologs from different species (20). Therefore, the affinity of the human Ang2 aptamer for nonhuman Ang2 may be less than its affinity for human Ang2, and its efficacy in humans may be underestimated by its efficacy in rodent models.

These data join growing evidence that Ang2 is proangiogenic but are the first to directly demonstrate that specific inhibition of Ang2 is antiangiogenic. This aptamer not only validates Ang2 as a target for other inhibition strategies but may also be adapted itself as a therapeutic agent. Like monoclonal antibodies, many of which have successfully entered the clinical realm, aptamers bind their targets with high affinity and specificity, and the attachment of fluorescent, radioactive, or other functional groups may expand the repertoire of potential applications for these versatile molecules (42). However, unlike monoclonal antibodies, oligonucleotides are intrinsically poor antigens, and they are amenable to large-scale chemical synthesis. A small but growing number of aptamers have now progressed to animal and/or human studies. A DNA aptamer against thrombin has been successfully substituted for heparin in a canine cardiopulmonary bypass model (43). A DNA aptamer against platelet-derived growth factor has been used to inhibit intimal hyperplasia in a rat carotid injury model (44) and matrix protein accumulation in a rat model of proliferative glomerulonephritis (45). Both DNA and nuclease-resistant RNA aptamers against human L-selectin have been shown to inhibit human lymphocyte trafficking in severe combined immunodeficient (SCID) mice (46, 47). The most progress has been made with a polyethylene glycol-conjugated version of a nuclease-resistant RNA aptamer against VEGF, which has now shown promising results in both preclinical and clinical studies for the exudative form of age-related macular degeneration (41, 48, 49).

Clinical trials of antiangiogenesis therapy for cancer have revealed that tumor regression is uncommon when antiangiogenic agents are used as monotherapy (50). Tumors express a wide array of proangiogenic molecules that may compensate for each other if only one is blocked (51). Furthermore, there is considerable angiogenic heterogeneity among individuals, between tumors, and even within single tumors (52–55). Therefore, the Ang2 aptamer described here also represents a promising lead compound for use in an aptamer "mixture" that block multiple proangiogenic targets, which may be a more effective approach for the treatment of the many malignant and benign disorders associated with pathological angiogenesis.

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1. Sato, T. N., Tozawa, U., Deutsch, U. & Wolburg-Bucholz, K. (1995) *Nature* **376,** 70–74.
2. Dumont, D. J., Gradwohl, G., Fong, G. H., Puri, M. C., Gertsenstein, M., Auerbach, A. & Breitman, M. L. (1994) *Genes Dev.* **8,** 1897–1909.
3. Maisonpierre, P. C., Suri, C., Jones, P. F., Bartunkova, S., Wiegand, S. J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T. H., Papadopoulos, N., *et al.* (1997) *Science* **277,** 55–60.
4. Davis, S., Aldrich, T. H., Jones, P. F., Acheson, A., Compton, D. L., Jain, V., Ryan, T. E., Bruno, J., Radziejewski, C., Maisonpierre, P. C. & Yancopoulos, G. D. (1996) *Cell* **87,** 1161–1169.
5. Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N. & Yancopoulos, G. D. (1996) *Cell* **87,** 1171–1180.
6. Papapetropoulos, A., Garcia-Cardena, G., Dengler, T. J., Maisonpierre, P. C., Yancopoulos, G. D. & Sessa, W. C. (1999) *Lab. Invest.* **79,** 213–223.
7. Thurston, G., Suri, C., Smith, K., McClain, J., Sato, T. N., Yancopoulos, G. D. & McDonald, D. M. (1999) *Science* **286,** 2511–2514.
8. Suri, C., McClain, J., Thurston, G., McDonald, D. M., Zhou, H., Oldmixon, E. H., Sato, T. N. & Yancopoulos, G. D. (1998) *Science* **282,** 468–471.
9. Thurston, G., Rudge, J. S., Ioffe, E., Zhou, H., Ross, L., Croll, S. D., Glazer, N., Holash, J., McDonald, D. M. & Yancopoulos, G. D. (2000) *Nat. Med.* **6,** 460–463.
10. Wong, A. L., Haroon, Z. A., Werner, S., Dewhirst, M. W., Greenberg, C. S. & Peters, K. G. (1997) *Circ. Res.* **81,** 567–574.
11. Hanahan, D. (1997) *Science* **277,** 48–50.
12. Holash, J., Maisonpierre, P. C., Compton, D., Boland, P., Alexander, C. R., Zagzag, D., Yancopoulos, G. D. & Wiegand, S. J. (1999) *Science* **284,** 1994–1998.
13. Lobov, I. B., Brooks, P. C. & Lang, R. A. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 11205–11210.
14. Asahara, T., Chen, D., Takahashi, T., Fujikawa, K., Kearney, M., Magner, M., Yancopoulos, G. D. & Isner, J. M. (1998) *Circ. Res.* **83,** 233–240.
15. Tanaka, S., Mori, M., Sakamoto, Y., Makuuchi, M., Sugimachi, K. & Wands, J. R. (1999) *J. Clin. Invest.* **103,** 341–345.
16. Ahmad, S. A., Liu, W., Jung, Y. D., Fan, F., Wilson, M., Reinmuth, N., Shaheen, R. M., Bucana, C. D. & Ellis, L. M. (2001) *Cancer Res.* **61,** 1255–1259.
17. Polverini, P. J., Bouck, N. P. & Rastinejad, F. (1991) *Methods Enzymol.* **198,** 440–450.
18. Fitzwater, T. & Polisky, B. (1996) *Methods Enzymol.* **267,** 275–301.
19. Wong, I. & Lohman, T. M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5428–5432.
20. White, R., Rusconi, C., Scardino, E., Wolberg, A., Lawson, J., Hoffman, M. & Sullenger, B. (2001) *Mol. Ther.* **4,** 567–574.
21. Peters, K. G., Coogan, A., Berry, D., Marks, J., Iglehart, J. D., Kontos, C. D., Rao, P., Sankar, S. & Trogan, E. (1998) *Br. J. Cancer* **77,** 51–56.
22. Shan, S., Lockhart, A. C., Saito, W. Y., Knapp, A. M., Laderoute, K. R. & Dewhirst, M. W. (2001) *Clin. Cancer Res.* **7,** 2590–2596.
23. Matthews, D. H., Sabina, J., Zuker, M. & Turner, D. H. (1999) *J. Mol. Biol.* **288,** 911–940.
24. Davis, J. P., Janjic, N., Javornik, B. E. & Zichi, D. A. (1996) *Methods Enzymol.* **267,** 302–314.
25. Procopio, W. N., Pelavin, P. I., Lee, W. M. & Yeilding, N. M. (1999) *J. Biol. Chem.* **274,** 30196–30201.
26. Seghezzi, G., Patel, S., Ren, C. J., Gualandris, A., Pintucci, G., Robbins, E. S., Shapiro, R. L., Galloway, A. C., Rifkin, D. B. & Mignatti, P. (1998) *J. Cell Biol.* **141,** 1659–1673.
27. Stratmann, A., Risau, W. & Plate, K. H. (1998) *Am. J. Pathol.* **153,** 1459–1466.
28. Zagzag, D., Hooper, A., Friedlander, D. R., Chan, W., Holash, J., Wiegand, S. J., Yancopoulos, G. D. & Grumet, M. (1999) *Exp. Neurol.* **159,** 391–400.
29. Ahmad, S. A., Liu, W., Jung, Y. D., Fan, F., Reinmuth, N., Bucana, C. D. & Ellis, L. M. (2001) *Cancer* **92,** 1138–1143.
30. Etoh, T., Inoue, H., Tanaka, S., Barnard, G. F., Kitano, S. & Mori, M. (2001) *Cancer Res.* **61,** 2145–2153.
31. Hayes, A. J., Huang, W. Q., Yu, J., Maisonpierre, P. C., Liu, A., Kern, F. G., Lippman, M. E., McLeskey, S. W. & Li, L. Y. (2000) *Br. J. Cancer* **83,** 1154–1160.
32. Lin, P., Polverini, P., Dewhirst, M., Shan, S., Rao, P. S. & Peters, K. (1997) *J. Clin. Invest.* **100,** 2072–2078.
33. Lin, P., Buxton, J. A., Acheson, A., Radziejewski, C., Maisonpierre, P. C., Yancopoulos, G. D., Channon, K. M., Hale, L. P., Dewhirst, M. W., George, S. E. & Peters, K.G. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 8829–8834.

34. Siemeister, G., Schirner, M., Weindel, K., Reusch, P., Menrad, A., Marme, D. & Martiny-Baron, G. (1999) *Cancer Res.* **59,** 3185–3191.
35. Teichert-Kuliszewska, K., Maisonpierre, P. C., Jones, N., Campbell, A. I., Master, Z., Bendeck, M. P., Alitalo, K., Dumont, D. J., Yancopoulos, G. D. & Stewart, D. J. (2001) *Cardiovasc. Res.* **49,** 659–670.
36. Kim, I., Kim, J. H., Moon, S. O., Kwak, H. J., Kim, N. G. & Koh, G. Y. (2000) *Oncogene* **19,** 4549–4552.
37. Chae, J. K., Kim, I., Lim, S. T., Chung, M. J., Kim, W. H., Kim, H. G., Ko, J. K. & Koh, G. Y. (2000) *Arterioscler. Thromb. Vasc. Biol.* **20,** 2573–2578.
38. Pieken, W. A., Olsen, D. B., Benseler, F., Aurup, H. & Eckstein, F. (1991) *Science* **253,** 314–317.
39. Beigelman, L., McSwiggen, J. A., Draper, K. G., Gonzalez, C., Jensen, K., Karpeisky, A. M., Modak, A. S., Matulic-Adamic, J., DiRenzo, A. B., Haeberli, P., *et al.* (1995) *J. Biol. Chem.* **270,** 25702–25708.
40. Willis, M. C., Collins, B. D., Zhang, T., Green, L. S., Sebesta, D. P., Bell, C., Kellogg, E., Gill, S. C., Magallanez, A., Knauer, S., *et al.* (1998) *Bioconjug. Chem.* **9,** 573–582.
41. Tucker, C. E., Chen, L. S., Judkins, M. B., Farmer, J. A., Gill, S. C. & Drolet, D. W. (1999) *J. Chromatogr. B Biomed. Sci. App.* **732,** 203–212.
42. Hicke, B. J. & Stephens, A. W. (2000) *J. Clin. Invest.* **106,** 923–928.
43. DeAnda, A., Jr., Coutre, S. E., Moon, M. R., Vial, C. M., Griffin, L. C., Law, V. S. & Komeda. (1994) *Ann. Thorac. Surg.* **58,** 344–350.
44. Leppanen, O., Janjic, N., Carlsson, M. A., Pietras, K., Levin, M., Vargeese, C., Green, L. S., Bergqvist, D., Ostman, A. & Heldin, C. H. (2000) *Arterioscler. Thromb. Vasc. Biol.* **20,** E89–E95.
45. Ostendorf, T., Kunter, U., van Roeyen, C., Dooley, S., Janjic, N., Ruckman, J., Eitner, F. & Floege, J. (2002) *J. Am. Soc. Nephrol.* **13,** 658–667.
46. Hicke, B. J., Watson, S. R., Koenig, A., Lynott, C. K., Bargatze, R. F., Chang, Y. F., Ringquist, S., Moon-McDermott, L., Jennings, S., Fitzwater, T., *et al.* (1996) *J. Clin. Invest.* **98,** 2688–2692.
47. Watson, S. R., Change, Y. F., O'Connell, D., Weigand, L., Ringquist, S. & Parma, D. H. (2000) *Antisense Nucleic Acid Drug Dev.* **10,** 63–75.
48. Ruckman, J., Green, L. S., Beeson, J., Waugh, S., Gillette, W. L., Henninger, D. D., Claesson-Welsh, L. & Janjic, N. (1998) *J. Biol. Chem.* **273,** 20556–20567.
49. The Eyetech Study Group (2002) *Retina* **22,** 143–152.
50. Kerbel, R. S. (2001) *J. Clin. Oncol.* **19,** 45S–51S.
51. Carmeliet, P. & Jain, R. K. (2000) *Nature* **407,** 249–257.
52. Rohan, R. M., Fernandez, A., Udagawa, T., Yuan, J. & D'Amato, R. J. (2000) *FASEB J.* **14,** 871–876.
53. Kumar, R., Kuniyasu, H., Bucana, C. D., Wilson, M. R. & Fidler, I. J. (1998) *Oncol. Res.* **10,** 301–311.
54. Bergers, G., Javaherian, K., Lo, K. M., Folkman, J. & Hanahan, D. (1999) *Science* **284,** 808–812.
55. Yu, J. L., Rak, J. W., Carmeliet, P., Nagy, A., Kerbel, R. S. & Coomber, B. L. (2001) *Am. J. Pathol.* **158,** 1325–1334.