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Modulation of angiogenesis with siRNA inhibitors for novel therapeutics

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Cancer and many other serious diseases are characterized by the uncontrolled growth of new blood vessels. Recently, RNA interference (RNAi) has reinvigorated the therapeutic prospects for inhibiting gene expression and promises many advantages over binding inhibitors, including high specificity, which is essential for targeted therapeutics. This article describes the latest developments using small-interfering RNA (siRNA) inhibitors to downregulate various angiogenic and tumor-associated factors, both in cell-culture assays and in animal disease models. The majority of research efforts are currently focused on understanding gene function, as well as proof-of-concept for siRNA-mediated anti-angiogenesis. The prospects for siRNA therapeutics, both advantages and looming hurdles, are evaluated.

Introduction

Angiogenesis is the process of generating new capillary blood vessels from pre-existing blood vessels which involves multiple gene products expressed by various cell types and an integrated sequence of events. This uncontrolled process of new blood vessel growth from the preexisting circulation network is an important pathogenic cause of tumor growth, many blinding ocular conditions and inflammatory diseases [1]. Angiogenesis can be characterized distinctly as hemangiogenesis (HA; blood neovascularization) and lymphangiogenesis (LA; lymphatic neovascularization), the latter being an important initial step in tumor metastasis and transplant sensitization [2]. During recent years, much has been learned about the stimulators and inhibitors of HA and LA, and members of the vascular endothelial growth factor (VEGF) family have emerged as prime mediators of both processes [3]. Therefore, identifying and evaluating the specific inhibitors of pro-angiogenesis factors has been the focus of anti-angiogenesis research with a goal for therapeutic development. The emergence of RNA interference (RNAi; Box 1), a natural mechanism for post-transcriptional gene silencing (PTGS) [4], offers a promising approach to develop a powerful class of inhibitors applicable to angiogenesis, with either a chemically synthesized small-interfering RNA (siRNA) oligonucleotide or a gene expression vector producing short-hairpin RNA (shRNA) as the therapeutic agent (Figure 1) [5]. Here, the latest developments for using RNAi agents to regulate angiogenesis are reviewed, including studies to identify the genes involved in controlling the angiogenesis process and efforts to develop novel anti-angiogenic therapeutics for the treatment of cancer, ocular neovascularization and rheumatoid arthritis.

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RNAi-mediated functional analysis of angiogenesis factors

Hypoxia (inadequate oxygen), which is one of the key early initiators of angiogenesis, is followed by the production of nitric-oxide synthetases that are responsible for governing vascular tone and regulating growth factors, such as VEGF, angiopoietins, fibroblast growth factors (FGFs) and their receptors. Genes involved in matrix metabolism, including matrix metalloproteinases (MMPs), plasminogen-activator receptors and inhibitors and collagen prolyl hydroxylase, have also been reported as crucial in angiogenesis. The functional validation of angiogenic factors for their specific role has been greatly facilitated by the use of RNAi inhibitors, revealing a network involving the early activation of the VEGF pathway and interactions among MMPs and adhesion molecules, leading to the regulation of signal transduction pathways.

The VEGF pathway

Several pathologies are associated with the upregulation of the VEGF pathway. The VEGF family consists of five growth factors that bind to and activate three distinct receptors. VEGF-A binds to VEGFR1 and VEGFR2, whereas placental growth factor (PIGF) and VEGF-B bind only to VEGFR1. VEGF-C and VEGF-D bind to VEGFR2 and VEGFR3.

Box 1. RNA interference

Active intermediates of the endogenous RNA-interference process, small-interfering RNA oligos, or siRNAs, have enabled an easy-to-make and easy-to-use gene inhibitor that can be used intracellularly by an RNA-induced silencing complex (RISC) to degrade homologous mRNA with high specificity and potency (Figure 1) [4]. Using siRNA to inhibit genes *in vitro* and *in vivo* has improved studies on the mechanism of action for many disease genes, including those involved in the angiogenesis process [5]. The capability of using siRNA *in vivo* to validate angiogenesis factors as drug targets is uniquely important, because its pathological impact can only be characterized accurately in animal disease models. With the emergence of clinically viable delivery vehicles, anti-angiogenesis RNAi agents appear to have a promising and unprecedented role for the treatment of many serious human diseases that result from excessive angiogenesis.

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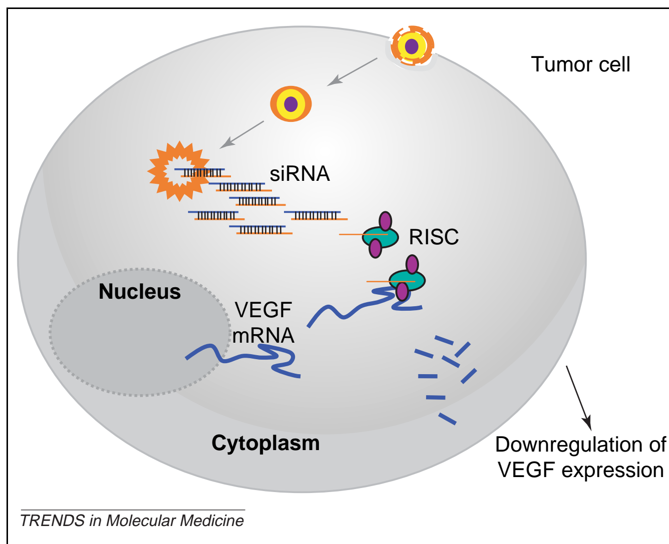

Figure 1. Delivering VEGF-specific siRNA into tumor cells resulted in the downregulation of VEGF gene expression. In the cytoplasm of the transfected tumor cell, the VEGF-specific siRNAs released from the delivery carrier are incorporated into a multi-protein RNA-inducing silencing complex (RISC). The siRNA duplex is unwound within the RISC in a process that requires ATP. Once unwound, the single-stranded antisense strand guides RISC to its homologous target: VEGF mRNA that has a complementary sequence. This results in the endonucleolytic cleavage of the target VEGF mRNA and a consequent knockdown of VEGF protein levels in the transfected tumor cells.

VEGF has received considerable attention. The transcription factor hypoxia inducible factor (HIF)-1 is a key determinant of hypoxia-regulated gene expression, including VEGF. The inhibition of HIF-1α by siRNA markedly attenuated the induction of VEGF and several other key genes, including heme oxygenase I (HO-1) and phosphoglycerate kinase (PGK) [6], indicating a role for VEGF in oxygen-dependent cell-cycle regulation. Progesterone receptor (PR) B also preferentially regulates VEGF expression in breast cancer cells, identified using siRNA [7]. In cell-culture-based assays, the expression of $VEGF_{165}$ was specifically inhibited using siRNA in HeLa cells, ovarian carcinoma cells and melanoma cells [8]. $VEGF_{165}$ is the predominant protein among the major splice variants of VEGF-A, which include: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ amino acids, each one comprising a specific exon addition. In a different study, siRNAs selectively blocked a splice variant of VEGF ($VEGF_{165b}$) showing its inhibitory function; antibodies against VEGF cannot distinguish this variant from other isoforms [9], giving an example of siRNA selectivity. The existence of this splicing to switch VEGF into an inhibitor potentially represents an interesting point for therapeutic intervention; that is, using $VEGF_{165b}$-specific siRNA to reduce the levels of VEGF protein. The inhibition of breast tumor growth by the intratumoral delivery of siRNA targeting VEGF has been demonstrated in an MDA-435 xenograft model [10]. In parallel studies of VEGF-siRNA intratumoral administration, the suppression of tumor angiogenesis and growth was also observed in a PtdCho-3 xenograft model [11]. In eye disease, VEGF-siRNA was used as an inhibitor of choroidal neovascularization (CNV) induced by laser photocoagulation in a murine retina model [12]. Using a ligand-directed nanoparticle that was amenable to systemic administration, VEGF-siRNA mediated anti-angiogenesis activity and suppressed ocular neovascularization induced by herpes simplex virus (HSV) infection in mice [13].

Other studies have used siRNA to inhibit factors upstream of VEGF. When Src homology 2 domain adaptor protein (Shb) was inhibited with siRNA, VEGF-dependent cellular migration was reduced, resulting from a loss of stimulated phosphatidylinositol 3-kinase, the phosphorylation of focal adhesion kinase, the generation of focal adhesions and stress fiber formation [14]. Another protein, IQGAP1 (IQ-motif-containing GTPase-activating protein 1), which is expressed in endothelial cells (ECs), was found, through siRNA-mediated IQGAP1 knockdown, to be involved in VEGF-stimulated reactive oxygen species production, Akt phosphorylation, endothelial migration and proliferation [15].

Studies of VEGF receptors have also benefited from siRNA technology. Using a neuroblastoma syngenic tumor model [16] and HSV-induced ocular neovascularization [13], siRNAs specific to murine VEGFR2 inhibited angiogenesis in both tissues, reducing tumor growth and ocular neovasculature, respectively. Similarly, siRNAs specific to murine VEGFR1 also demonstrated anti-angiogenic effects in the mouse ocular model [13].

Matrix metalloproteases and adhesion molecules

Although most vascular basement membrane (BM) components sustain the growth and survival of the vascular endothelium, the MMPs and vascular integrins have emerged as key regulators of angiogenesis. MMP9 and MMP2 are important for the mobilization of sequestered VEGF and initiation of tumor angiogenesis [17], whereas specific integrins mediate interactions between endothelial cells and the BM by activating the integrin receptor signaling that controls many key functions, such as proliferation [18].

The knockdown of MMP-9 with siRNA [19] concurrently resulted in increased levels of surface E-cadherin and a redistribution of β-catenin at the plasma membrane, in addition to its physical association with E-cadherin. In a cell-culture assay, the reduction of bovine aortic smooth muscle cell (BASMC) migration caused by the incubation with conditioned media can be completely reversed by siRNA knockdown of MMP-2, but not MMP-9, expression [20]. To indirectly block the induction of MMP activity, a novel MMP regulator, RECK (reversion-inducing cysteine-rich protein, with Kazal motifs), was downregulated using siRNA [21], causing diminished activation of MMP-2. In a separate study, siRNA knockdown of a ubiquitous Mint isoform protein, Mint-3, inhibited membrane type 5 (MT5)-MMP activity [22], indicating that Mint proteins might be the adaptor proteins that regulate the trafficking of MT-MMPs.

The cleavage of CD44, which is a cell-surface glycoprotein that is involved in cell adhesion and migration and contributes to the migration and invasion of tumor cells, is regulated by MMPs. It can be suppressed by the metalloproteinase inhibitor KB-R7785 and tissue inhibitor of metalloproteinases-1 (TIMP-1) or enhanced by the metalloproteinase disintegrin ADAM10, as demonstrated by siRNA-mediated knockdown [23]. A different cell-surface

molecule, CD13/aminopeptide N (CD13/APN), has been identified as a potent regulator of angiogenesis and its transcription in endothelial cells is induced by VEGF through the RAS–MAPK (mitogen-activated protein kinase) pathway. The role of Ets-2 in the induction of CD13/APN was revealed when Ets-2 mRNA and protein were downregulated using Ets-2-specific siRNA [24]. Another metalloprotease disintegrin, ADAM12, was characterized by siRNA knockdown in C2C12 myoblast cells, suggesting that ADAM12-mediated adhesion and/or signaling might have a role in the determination of the pool of reserve cells during myoblast differentiation [25]. This protein has also been recognized as a key enzyme implicated in ectodomain shedding of membrane-anchored heparin-binding proHB-EGF-dependent epidermal growth factor receptor (EGFR) transactivation [26]. When an siRNA for Pacsin 3, which is a protein bound to ADAM12, was used in HT1080 cells, the inhibition of this gene attenuated the shedding of proHB-EGF induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) and angiotensin II. FAK has a crucial role in the adhesion to collagens. Using FAK siRNAs to inhibit its expression resulted in a strong inhibition of adhesion not only to collagen I but also to collagen IV and fibronectin [27].

Integrins initiate signal transduction through intracellular molecules and collaborate with other membrane receptor-mediated signal pathways, including the transforming growth factor (TGF)β1 pathway. When the effects of cell adhesion status on the TGFβ1-mediated Erk1/2 regulation was evaluated in a gastric carcinoma cell variant [28], the role of Smad protein in enhancing TGFβ1-mediated Erk1/2 activation was revealed with a Smad2 siRNA. Smad2 protein is part of a signal transduction pathway leading to the transcriptional regulation of multiple cellular processes, such as cell proliferation, apoptosis and differentiation. The effects of carcinoembryonic antigen-related cell-adhesion molecule 6 (CEACAM6) crosslinking on pancreatic adenocarcinoma cellular interaction with extracellular matrix (ECM) components, vitronectin (αvβ3 integrin) and fibronectin (α5β1 integrin) were characterized using siRNA knockdown of CEACAM6, leading to increased ECM component adhesion [29]. Integrin-linked kinase (ILK) stimulated the expression of VEGF through the stimulation of HIF1α expression in a PKB–Akt- and mTOR–FRAP-dependent manner. With siRNA inhibition of ILK, many significant effects were observed, including the inhibition of HIF-1α and VEGF expression, in addition to reduced VEGF-mediated endothelial cell migration and capillary formation *in vitro*, culminating in reduced angiogenesis *in vivo* [30]. These data demonstrated an essential role for ILK in two key aspects of tumor angiogenesis: VEGF expression by tumor cells and VEGF-stimulated blood vessel formation. Interestingly, a widely expressed focal adhesion protein, PINCH-1, was found to form a ternary complex with ILK and α-parvin [31]. Using siRNAs, PINCH-1 and ILK, but not α-parvin, were found to be crucial for promoting cell spreading and motility.

The cooperative roles of platelet-derived growth factor receptor (PDGFr) and αvβ3 integrin in the glioblastoma cell migration have been shown by PDGF stimulation of vitronectin-adherent cells, promoting the recruitment of αvβ3 integrin [32]. The Lck/yes-related novel protein (Lyn) was preferentially associated with αvβ3 integrin in the victronectin-adherent cells, both in the presence and absence of PDGF stimulation. The downregulation of Lyn expression using siRNA inhibits the cell migration that is mediated by αvβ3 integrin in PDGF-stimulated cells. In fibrotic liver, the fate of stellate cells was influenced by the ECM through an intermediary of αvβ3 integrin, shown using siRNA to silence the α(v) subunit [33]. In a different study, microfilaments were found to associate with αvβ3-integrin-positive focal contacts in endothelial cells. When vimentin expression was inhibited by siRNA, the cells assembled focal contacts smaller than normal and showed decreased adhesion [34]. A laminin adhesion receptor, α6β4 integrin, was demonstrated, using an siRNA targeting either subunit of α6β4, to have a key role in the invasive phenotype of MDA-MB-231 breast carcinoma cells when surface expression was inhibited [35].

Receptors involved in angiogenesis

In addition to receptors for the VEGF family, which includes PDGF, the inhibition of other receptors, such as EGFR (erbB1), is a promising anti-angiogenesis approach. When erbB1 expression was blocked (90% inhibition) in A431 human epidermal carcinoma cells using siRNA, EGF induction of tyrosine phosphorylation was lost along with cell proliferation, and apoptosis was induced [36]. Similarly, using retrovirus-mediated transfer of Her-2/neu siRNA, breast and ovarian tumor cells exhibited slower proliferation, increased apoptosis, increased G_0–G_1 arrest and decreased tumor growth [37]. Her-2/neu siRNA also increased the expression of the antiangiogenic factor thrombospondin-1 and decreased the expression of VEGF, suggesting that Her-2/neu stimulates tumor growth in part by regulating angiogenesis. When a pSUPER plasmid expressing shRNA for S100A10, a key plasminogen receptor on the extracellular cell surface, was transfected into colorectal (CCL-222) cancer cells, the RNAi-mediated downregulation of S100A10 resulted in a 45% loss of plasminogen binding and a complete loss in plasminogen-dependent cellular invasiveness [38]. An inducible RNAi mediated the inhibition of endogenous CXCR4, a receptor with pleiotropic roles in angiogenesis, host immune response, homing and tumor metastasis, significantly inhibited a breast cancer cell migration *in vitro* [39]. Furthermore, a pro-apoptotic protein, Bim, was identified, using siRNAs, as a crucial mediator of anoikis in epithelial cells [40]. The communication between G-protein-coupled receptor (GPCR) and EGFR signaling systems that are involved in the cell-surface proteolysis of EGF-like precursors was revealed using siRNA, which demonstrated the role of amphiregulin (AR) and prevented GPCR-induced EGFR tyrosine phosphorylation, downstream mitogenic signaling events, cell proliferation, migration and activation of the survival mediator Akt/PKB [41]. In the same study, siRNA for the metalloprotease-disintegrin TNFα-converting enzyme (TACE) suppressed GPCR-stimulated AR release, EGFR activation and downstream events.

Box 2. siRNA, antisense, ribozyme and DNAzyme

RNAi is a newly discovered cellular pathway for the silencing of sequence-specific genes at the mRNA level by the introduction of cognate double-stranded RNA. Similarly, gene-silencing nucleic acids, such as ribozymes, DNA enzymes (DNAzymes), antisense oligonucleotides (ODNs) and siRNAs, depend on hybridization to accessible sites within target mRNA for activity. However, the accurate prediction of accessible sites as a means to facilitate reagent design is problematic. In a study attempting to evaluate the use of scanning arrays for the effective design of ribozyme, DNAzyme and siRNA sequences targeting the EGFR mRNA [67], all three types complementary to the same site were effective in a dose-dependent manner, using Lipofectin-mediated transfection. In fact, the effects achieved correlated in all cases with concomitant dose-dependent reduction in EGFR protein expression. However, the ribozyme and DNAzyme exhibited similar but low potencies, with IC_{50} values of ~750 nM, whereas siRNA was significantly more potent, with an IC_{50} of ~100 nM. In a separate comparison, the combination of plasmid-expressed antisense (AS)- and sense (S)-RNA sequences was more effective than expressed AS-RNA alone for target gene knockdown. Expressed S-RNA alone had no effect [68]. Furthermore, the potency of 22-nucleotide siRNAs targeting the reporter gene luciferase was one order of magnitude stronger than that of a phosphorothioate AS-DNA. Overall, many more studies comparing the efficiencies of gene silencing by siRNA with other nucleic acid inhibitors find, in most cases, better siRNA potency [5].

Factors in signal transduction pathways

VEGF promotes angiogenesis through the activation of signaling pathways that include the activation of a series of relay proteins by the binding of VEGF to its appropriate receptor, which transmits a signal to the nucleus of the endothelial cells; the nuclear signal ultimately prompts a group of genes to make products needed for new endothelial cell growth. VEGF stimulation of sphingosine kinase (SPK) affects not only endothelial cell signaling but also tumor cells expressing VEGF receptors [42]. In T24 bladder tumor cells, VEGF inhibition reduced cellular sphingosine levels and raised those of sphingosine-1-phosphate. siRNA targeting SPK1, but not SPK2, blocked VEGF-induced accumulation of Ras-GTP and phospho-ERK, but not EGF-induced accumulation of phospho-ERK1/2. The involvement, revealed by siRNA, of diacylglycerol kinase α (Dgkα) in hepatocyte growth factor (HGF)-stimulated cell migration, which impaired angiogenesis *in vitro*, indicated its essential role in both proliferative and migratory response to VEGF, and suggested that it is a novel therapeutic target for controlling angiogenesis [43]. In a different study, the inhibition of Mcl-1 by siRNA decreased proliferation and induced apoptosis, supporting the notion that VEGF-induced multiple myeloma cell proliferation and survival is mediated by Mcl-1, and provided the preclinical framework for novel therapeutics targeting both Mcl-1 and/or VEGF to improve patient outcome in multiple myeloma [44]. Growth-arrest-specific protein 1 (Gas1), which is upregulated by the junction membrane protein vascular endothelial cadherin (VEC) in 293 cells, was inhibited by siRNA in cell and allantois organ cultures, which resulted in endothelial cells that could not be protected from apoptosis by VEGF, once again providing evidence for another potential drug target [45].

One of the key factors in the downstream signal transduction pathway of VEGF receptors is B-Raf kinase, which constitutively activates the MEK–ERK pathway. Cell proliferation of melanoma cells with increased B-Raf levels was strongly inhibited by siRNA-mediated depletion of the mutant B-Raf protein [46]. When FAK was silenced using siRNA in primary human colon cancer cells or SW620 colonocytes, pressure-stimulated adhesion was prevented and pressure-activated FAK397, Src and FAK576 phosphorylation were also ablated [47]. Integrin-linked kinase-associated serine–threonine phosphatase 2C (ILKAP) selectively associates with ILK to modulate cell adhesion and growth factor signaling. When ILKAP is suppressed with siRNA, cell entry into S phase increased, consistent with its antagonism of ILK [48]. The role of diphosphoinositol polyphosphates (DIP) was also characterized by siRNA-mediated downregulation, indicating that DIP inactivates Rho and activates Rac following EGF stimulation [49]. Using siRNA to inhibit Disabled-2 (DAB2) protein expression in K562 cells modulated both cell–cell adhesion and the phosphorylation of MAPK [50]. Knocking down 3-phosphoinositide-dependent protein kinase-1 (PDK1) with siRNA demonstrated that PDK1 maintains steady-state phosphorylated MEK levels and cell growth [51]. Interestingly, the downregulation of PDK1 reduced MEK and MAPK activities but could not prolong MAPK signaling. The interaction of (thyroid receptor interacting protein 6) TRIP6/ZRP-1 (zyxin-related protein 1) with lysophosphatidic acid (LPA) receptor was induced by LPA and associated with the activation of actin rearrangement, focal adhesion assembly and cell migration [52], as revealed by TRIP6 siRNA in SKOV3 ovarian cancer cells.

RNAi-based anti-angiogenesis therapeutics

RNAi is an endogenous mechanism for the potent and specific inhibition of gene expression, which can be diverted to act on cellular genes by introducing siRNA agents. Their success as a powerful research tool, illustrated by the many advances described above, is fueling enthusiasm for siRNA as a novel modality for anti-angiogenesis therapeutics, a large unmet clinical need (Box 2).

Anti-VEGF siRNA to treat cancer

There are two distinct approaches to achieve anti-angiogenesis activity for cancer treatments: (i) activating endogenous or exogenously delivering anti-angiogenesis factors; or (ii) delivering inhibitors to reduce the activities of endogenous pro-angiogenesis factors. siRNA is a particularly useful inhibitor with both potency and sequence-specific selectivity that has been demonstrated to induce a phenotypic response in cell-culture studies of anti-angiogenesis (Tables 1–4). However, their therapeutic potential will only be realized when the *in vivo* anti-angiogenesis efficacy of the siRNA agents can be achieved with clinically feasible delivery systems [53].

Using intratumoral delivery of VEGF-targeted siRNA, the inhibition of tumor growth was observed in MDA MB-435 and MCF-7 human xenograft breast cancer models [10]. In tumors expressing both thrombospondin-1 (TSP1) and VEGF, the effect of TSP1 to reduce vascularization and tumor growth were restored using the

Table 1. Anti-angiogenesis RNAi for revealing functions of cytokines and receptors

Cytokines and receptors	Cells	RNAi agent	RNAi phenotype
VEGF	HeLa, MM66, ovarian carcinoma primary and immortalized human podocytes	SiRNA and vector-based siRNA	Knockdown of VEGF did not affect tumor cell proliferation [8]. Reduction of VEGF by siRNA against $VEGF_{165b}$ [9]
EGFR (erbB1)	A431	SiRNA	Inhibited EGF-induced tyrosine phosphorylation and induced cell apoptosis [38]
Her-2/neu	SK-OV-3, BT-474, MDA-MB-453	Retroviral vector based	Slower proliferation, increased apoptosis, increased G_0–G_1 arrest [39]
VEGF R1/R2	SVR (CRL-2280), 293	SiRNA	Downregulation of endogenous mVEGFR1 in SVR cells and co-transfected mVEGFR2 in 293 cells [14]
Amphiregulin (AR)	SCC-9	SiRNA	Inhibited cell proliferation, migration and activation of survival mediator Akt/PKB [43]
Mcl-1	MEFs	SiRNA	Reduced FBS-, VEGF- and IL-6-induced proliferation, induced apoptosis [46]
SPK-1/SPK2	T24	SiRNA	SPK-1 siRNA, but not SPK-2 siRNA, blocks VEGF-induced accumulation of Ras-GTP and phospho-ERK [44]
PRB (progesterone receptor B)	T47-Dco	SiRNA	Abrogated estradiol induced VEGF expression [7]

systemic administration of aqueous VEGF siRNA but failed to show signs of target inhibition [54]. In the same report, local delivery of the siRNA into the tumors had no effect on VEGF expression. In a different study, intratumoral delivery of VEGF siRNA with a cationic carrier resulted in dramatically suppressed angiogenesis and growth [11]. These preclinical studies illustrate the growing interest in siRNA-based anti-angiogenesis therapeutics for the treatment of cancers.

It has been recognized that the local delivery of anti-tumor agents is limited to only a few tumor types with clinical relevance. Therefore, systemic administration of siRNA will provide the greatest clinical benefit as a treatment, especially for disseminated metastatic cancer. To that end, VEGF-pathway siRNA agents with *in vivo* validated activity were further evaluated by systemic administration to mice bearing neuroblastoma tumors using a ligand-directed nanoparticle carrier, and exhibited the inhibition of target expression, angiogenesis and tumor growth after repeated dosing [16]. The observed efficacy was achieved with a potency amenable to clinical application, further strengthening the promise of siRNA as a therapeutic modality.

Anti-VEGF siRNA to treat ocular neovascularization diseases

New and uncontrolled blood vessel development in eyes is a pivotal process in the pathogenesis of several ocular neovascularization (NV) diseases, including age-related macular degeneration (AMD), diabetic retinopathy (RA)

Table 2. Anti-angiogenesis RNAi for revealing functions of matrix proteins and adhesion molecules

Matrix proteins and adhesion molecules	Cells	RNAi agent	RNAi phenotype
Integrin α-6-β4	MDA-MB-231	SiRNA	Decreased invasion of tumor cells and promoted decreased migration on non-laminin substrata [37]
Integrin α-v-β3	Primary rat or fist passage human HSCs	SiRNA	Inhibited proliferation and increased apoptosis of cultured stellate cells [35]
MMP2	BAECs	SiRNA	Reversed the inhibitory effect of BAECs' conditioned media on BASMC cell migration [22]
MMP9	COH	SiRNA	Increased levels of surface E-cadherin, redistribution of β-catenin [21]
CXCR4	MDA-MB-231	Inducible-shRNA vector	Decreased cell invasion *in vitro* [41]
TACE	SCC-9	SiRNA	Suppressed GPCR-stimulated AR release, prevented chemotactic migration response to LPA [43]
Mint3	HEK293	Vector based	Decreased MT5-MMP activity [24]
ADAM10	U251MG	SiRNA	Suppressed CD44 cleavage [25]
ADAM12	C2C12	SiRNA	Lower expression levels of p130, p27, myogenin, and integrin α 7A isoform [27]
Smad2	Gastric carcinoma	SiRNA	Abolished the enhanced activation of Erk1/2 by TGF-β1 [30]
DAB2 (Disabled-2)	K562	Vector based	Modulation of cell–cell adhesion and MAPK phosphorylation [52]
Vimentin	TrHBMEC	SiRNA	Assembled smaller than normal focal contacts and showed decreased adhesion to the substratum [36]
S100A10	CCL-222	Vector based	45% loss of plasminogen binding, 65% loss in cellular plasmin generation and complete loss of plasminogen-dependent cellular invasiveness [40]
RECK	CL-1	SiRNA	Abolished the inhibitory effect of TSA on MMP2 activation [23]
PINCH-1	HeLa	SiRNA	PINCH-1 is essential for prompt cell spreading and motility, and is crucial for cell survival [33]
CEACAM6	BxPC3	SiRNA	Abolished the increase in ECM-component adhesion induced by antibody-mediated crosslinking [31]

Table 3. Anti-angiogenesis RNAi for revealing functions of cellular signaling factors

Cellular signaling factors	Cells	RNAi agent	RNAi phenotype
Ets-2	KS1767	SiRNA	Inhibited CD13/APN transcription [24]
PDK-1	293T, A549	SiRNA	Lower levels of the steady-state phosphorylated MEK and phosphorylated MAPK, inhibited cell growth [53]
Akt1/2	293T, HT1080	SiRNA	No effect on MEK–MAPK pathway [53]
Gas1 (growth arrest specific 1)	HUVEC	SiRNA	Reduced the anti-apoptotic protective effect of VEGF [47]
cRaf-1	293T, HT1080	SiRNA	Lower levels of the steady-state phosphorylated MEK and phosphorylated MAPK, inhibited cell growth [53]
Lyn (member of Src family)	U-87MG	SiRNA	Inhibited the cell migration mediated by $\alpha v\beta 3$ integrin in PDGF-stimulated cells [35]
PACSIN3	HC1080	SiRNA	Attenuated the shedding of proHB-EGF induced by TPA and angiotensin II [28]
ILKAP (integrin-linked kinase-associated phosphatase 2C)	LNCaP	SiRNA	Increased entry of cells into the S phase [50]
DIP	HeLa	SiRNA	Diminished phosphorylation of p190RhoGAP and Vav2 upon EGF stimulation, affected cell movement [51]
ILK (integrin-linked kinase)	PtdCho-3, DU145	SiRNA	Inhibition of HIF-1 α and VEGF expression [32]
	HUVEC	SiRNA	Inhibition of VEGF-mediated cell migration and capillary formation *in vitro* [32]
	HeLa	SiRNA	ILK is essential for promoting cell spreading and motility, and is crucial for cell survival [33]
c-Src	BxPC3	SiRNA	Abolished the increase in adhesion induced by CEA-CAM6 antibody mediated crosslinking [31]
Bim	MCF-10A	SiRNA	Inhibition of anoikis [42]
FAK	Primary colon-cancer cells	SiRNA	Prevented pressure-stimulated adhesion [49]
	SW620	SiRNA	Inhibition of adhesion to collagen I, collagen IV and fibronectin [29]
TRIP6	SKOV3	Vector based	Reduced the LPA induced cell migration [54]
MAPKKK (B-Raf)	OCM-1	SiRNA	Diminished the cell proliferation induced by B-Raf [48]
IQGAP1	Cultured endothelial cells	SiRNA	Reduction of VEGF-stimulated ROS production, Akt phosphorylation, endothelial migration, and proliferation [16].
DgK-α	PAE-KDR		Impaired *in vitro* angiogenesis on matrigel [45]
HIF-1 α	Primary vascular smooth muscle cell	SiRNA	AsIII-induced VEGF was not inhibited [6]
	A549	SiRNA	Suppressed the S–G_1-phase shift in response to hypoxia
Shb	Porcine aortic endothelial cells	SiRNA	Inhibition of VEGF-induced cell migration [15]

and herpes simplex virus (HSV)-induced stromal keratitis (SK). These ocular diseases often lead to blindness; unfortunately, managing them therapeutically is challenging. The systemic administration of ligand-directed nanoparticles carrying anti-VEGF siRNA in a mouse SK model was applied to measure its anti-angiogenesis therapeutic potential [13]. In the study, potent siRNAs targeting VEGFA, VEGFR1 and VEGFR2 were validated first *in vitro*, and mixed with a polymer conjugate to form nanoparticles 100–120 nm in diameter (Box 3). The

Table 4. Anti-angiogenesis RNAi for developing novel therapeutics

Therapeutic applications	Target gene	Model	RNAi phenotype
Tumor angiogenesis	VEGF siRNA and VEGF R2 siRNA	MCF-7/nude and MDA-MB-435/nude	Reduction of VEGF and inhibition of tumor growth [5,10,16]
	VEGF siRNA	PtdCho-3/nude	Suppressed tumor angiogenesis and tumor growth [11]
	VEGF siRNA	JT8/nude	Inhibition of tumor growth [54]
	Her-2/neu-retroviral-vector-based siRNA	SK-OV-3/nude and BT-474/nude	Inhibition of tumor growth [39]
Ocular neovascularization	VEGF siRNA	Mice/laser photocoagulation	Inhibited choroidal neovascularization [12]
	VEGF siRNA	Monkey/laser photocoagulation	Inhibited the growth and vascular permeability of laser-induced choroidal neovascularization [13]
	VEGF R1 siRNA and VEGF R2 siRNA	Mice/HSV induction	Anti-angiogenesis effect demonstrated by reduction of the neovasculature areas [14]
Rheumatoid arthritis	GG2–1 siRNA	Rheumatoid arthritis synovial fibroblasts (RASFs)	Enhanced apoptosis, decreased proliferation, and decreased production of MMP-1 in TNF-α-stimulated RASFs [61]
	Akt siRNA	Rheumatoid synovial cells	Increased TRAIL-mediated apoptosis [62]
	ASC siRNA	THP-1 cells	Abolished the cryopyrin-mediated secretion of IL-1β [63]

Box 3. A cocktail of siRNAs targeting multiple genes

Many human diseases result from the overexpression of multiple endogenous and exogenous pathogenic genes. Angiogenesis-related diseases represent this typical characteristic caused by the abnormal overexpression of multiple genes. Moreover, disease progression and the development of drug resistance can also circumvent the effect of single-drug treatments. One strategy to overcome those hurdles is using a combination of multiple drugs. siRNAs should provide a unique advantage for combination therapy by combining multiple siRNA oligos targeting multiple disease-causing genes without dramatic differences in the drug chemical properties. The combination of siRNAs targeting VEGFA, VEGFR1 and VEGFR2 has demonstrated an improved anti-angiogenesis potency compared with siRNAs targeting only one factor [13]. The combination appears to have achieved a synergistic effect by the knockdown of all three targets simultaneously, resulting in a potent anti-angiogenesis efficacy. Anti-angiogenic molecules can fall into five main categories according to their mode of action: (i) inhibitors of pro-angiogenic growth factors and their corresponding receptors, such as VEGF, VEGFR1 and VEGFR2, bFGF and FGF receptors and PDGF; (ii) protease inhibitors that prevent the breakdown of the surrounding matrix, which is needed for blood-vessel growth; (iii) endogenous inhibitors of angiogenesis, such as endostatin; (iv) inhibitors of cellular adhesion molecules; and (v) molecules with undefined mechanisms. Owing to the complications of angiogenesis pathways, combining multiple siRNAs targeting angiogenic factors in each category might enable the identification of potent anti-angiogenic agents for potential therapeutic applications. The same strategy was applied during an attempt to achieve stronger anti-SARS coronavirus activity with remarkable success [69].

tri-functional polymer was composed of branched polyethylenimine at one end, polyethylene glycol (PEG) in the middle and an RGD peptide motif at other end. This polymer was found to self assemble with negatively charged siRNA into a particle with the RGD peptide exposed on its surface [16]. The RGD peptide is a specific ligand for the $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins of activated endothelial cells of the neovasculature in the diseased tissue, and PEG helps to prevent nonspecific binding to other tissues, such that the VEGF siRNA can be delivered in a ligand-directed endocytosis. By comparison, subconjuctival administration of siRNA resulted in weaker and shorter anti-angiogenesis efficacy. In addition, the combination of siRNAs targeting three pro-angiogenesis genes, *VEGF*, *VEGFR1* and *VEGFR2*, achieved stronger efficacy than that of siRNA only targeting one of the three pro-angiogenesis genes.

Using a different model, the feasibility of siRNA-mediated anti-angiogenesis in retinal cells *in vitro* and in the murine retina *in vivo* was evaluated [12]. siRNAs specific to murine and human VEGF were tested *in vitro* using chemically induced hypoxia to modulate endogenous VEGF expression. *In vivo* studies evaluating the effects of naked siRNA on levels of VEGF were performed first by co-injecting recombinant adenoviruses carrying enhanced green-fluorescent protein (eGFP) or hVEGF cDNAs, then by using laser-induced choroidal neovascularization (CNV) along with subretinal delivery of the appropriate siRNAs in mice. The siRNAs effectively and specifically inhibited hypoxia-induced hVEGF levels in human cell lines or with adenoviral-expressed hVEGF *in vivo*. Subretinal delivery of mVEGF-siRNA significantly inhibited CNV after laser photocoagulation in mice.

Anti-angiogenesis siRNA to treat rheumatoid arthritis

Rheumatoid arthritis (RA) is one of the most severe articular diseases, characterized by synovial hyperplasia followed by impaired joint function with a severe impact on quality of life. Although the mechanisms that regulate synovial cell outgrowth are not fully understood, there is evidence that VEGF has an important role in the development of RA [55–58]. In patients with RA, both serum basic fibroblast growth factor (bFGF) and VEGF concentrations were increased, and when those patients were treated with steroids, their synovial fluid levels of bFGF and VEGF were reduced [56]. The investigation of polymorphisms of the *VEGF* gene in patients with RA and their relationship with clinical features and the radiographic progression of joint disease indicated that the frequencies of two haplotypes (CGCT and AAGT) were significantly increased in patients with RA compared with controls [55]. The VEGF receptor inhibitor PTK787/ZK222584 caused a dose-dependent reduction in the vascularity of a mouse RA model and it inhibited knee swelling by 40% in antigen-induced arthritis and inhibited both severity scores (by 51%) and global histological scores in the mouse model [57]. Furthermore, treatment using infliximab, an anti- TNFα antibody resulted in the reduction of soluble adhesion molecules and VEGF and achieved a rapid suppression of disease activity [58]. This indicates that the pathology of RA is closely related to the overexpression of VEGF. One signature in RA joints is the overexpression of TNF which, with other cytokines, induced angiogenesis in inflammation tissue. Therefore, using siRNA inhibitors to modulate the relevant pathways might provide therapeutic potentials for the treatment of RA.

After primary RA synovial fibroblasts (RASFs) were treated by TNFα, several genes were selected, using microarray analysis, based upon their differential expression status [59]. Using siRNA to downregulate one of these genes, *GG2-1*, which is a TNFα-inducible FLICE-like inhibitory protein (FLIP)-like gene, significantly enhanced apoptosis, decreased proliferation and decreased the production of MMP-1 in TNFα-stimulated RASFs. Akt protein kinase B had an important role in TNFα-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in rheumatoid synovial cells. Using siRNA to inactivate Akt significantly increased TRAIL-mediated apoptosis in synovial cells [60]. Mutations in the human *CIAS1* gene, which codes for Cryopyrin, have been identified in a continuum of inflammatory disorders. Cryopyrin mutants induce potent NF-κB activity that is dependent on the expression of ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), an adaptor protein previously suggested to mediate Cryopyrin signaling. The induction of Cryopyrin activity resulted in ASC binding and the secretion of IL-1β, an effect that was abolished by the inhibition of ASC expression with siRNA [61]. Even though no efficacy data have been reported using siRNA to reverse RA pathology in animal disease models, the potential application is very promising; the downregulation of RA-causing cytokines and their receptors and of VEGF and its signaling factors,

Box 4. Dual-targeted anti-angiogenesis therapeutics

Inhibiting the expression of drug-target genes with siRNAs is quite different from the inhibitory actions of conventional drugs such as monoclonal antibodies and small-molecule inhibitors, which bind to and inhibit existing proteins. This represents a novel and distinct class of therapeutic inhibitors that will probably have a different range of applicable drug targets, albeit with substantial overlap. Most importantly, the selectivity conveyed by the gene sequence, which could potentially be as stringent as a single nucleotide difference between two genes, promises to aid in therapeutic approaches by providing much better target selectivity. In addition, using a tissue-targeting nanoparticle delivery with ligand-directed tissue selectivity enables the potential for a dual-targeted therapeutic approach, and one that is uniquely useful for enhancing the extracellular stability of the siRNA agent, avoiding effects on the targeted gene in normal tissues and increasing the activity of siRNA in disease tissue (Figure I). Using this systemic delivery of siRNAs targeting VEGF pathway factors at sites of neovascularization, anti-angiogenesis efficacies were achieved in a neuroblastoma tumor model [16] and in an HSV-induced mouse ocular SK model [13]. Identifying ligands binding to adhesion molecules and integrin receptors for tissue-targeted delivery of siRNA agents will provide synergistic effects to further improve anti-angiogenesis efficacy.

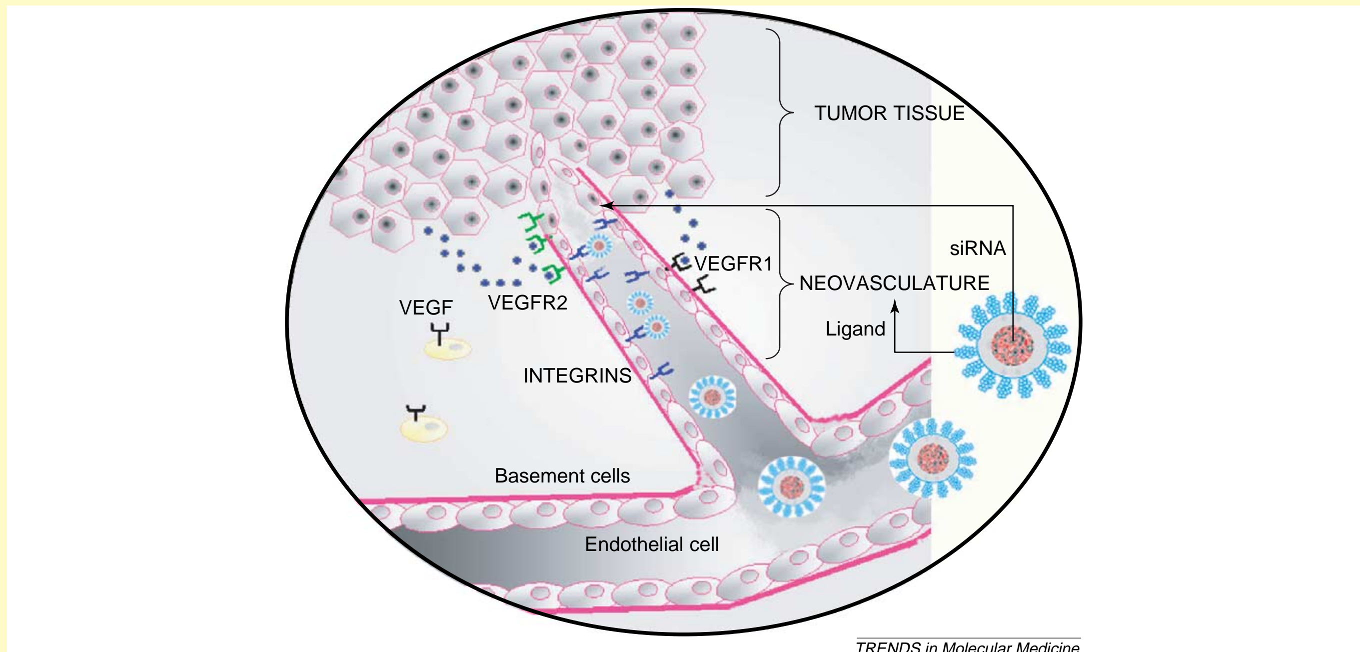

Figure I. Dual-targeted anti-angiogenesis siRNA systemic delivery using ligand-directed nanoparticles. A layered, self-assembled siRNA nanoparticle system [16] is used to deliver siRNAs specific to genes involved in the VEGF pathway to achieve a dual-targeted antiangiogenesis therapeutic effect. The first targeted effect is achieved by using the RGD peptide as the surface ligand of the nanoparticle to target integrin expression on tumor neovasculature and thus avoid effects on the targeted gene in normal tissues. The second target effect is achieved by using siRNA specifically against genes involved in VEGF pathway, such as VEGF, VEGFR1, and VEGFR2, to inhibit the angiogenesis activity in the targeted tumor tissue and thus generate anti-tumor efficacy.

either individually or with siRNAs in combination, represents a novel approach for the treatment of RA.

Concluding remarks: siRNA, a powerful anti-angiogenesis agent

The modulation of angiogenesis pathways using siRNA inhibitors of gene expression has proven to be a powerful approach for validating gene functions of the relevant factors *in vitro* and *in vivo*. The rapid development of experimental evidence improving our understanding of angiogenesis and approaches for intervention has clearly demonstrated the power of RNAi, which has unique advantages over previous methods for gene-function studies [62], including nucleic acid inhibitors (antisense, ribozyme and DNAzyme). The information obtained for each individual factor not only contributes to the global understanding of angiogenesis but also provides strong support for efforts to develop novel therapeutics for the treatment of various angiogenesis diseases, including cancer, ocular neovascularization diseases and rheumatoid arthritis. Nonetheless, although the *in vivo* studies described in this article provide the groundwork for potential therapeutic applications of RNAi technology, thorough preclinical studies, such as pharmacology and toxicology, of specific siRNA therapeutic candidates remain. Some reports have revealed the occurrence of incomplete selectivity of siRNA oligos, including 'off-target effects' [63] and the activation of interferons [64,65], which raises concerns for safety and unwanted side effects. It is fair to say that all those studies were based upon cell-culture experiments without support from the *in vivo* study. A recent study using systemic delivery (intraperitoneal and intravenous) of naked siRNA duplexes into mice indicated a lack of interferon response compared with that of polyinosinic acid: polycytidylic acid [poly(I:C)], an analog of the long-dsRNA ligand for Toll-like receptor 3 [66]. With clinically viable delivery emerging, siRNA inhibitors have achieved therapeutic efficacy in several animal disease models of angiogenesis. The dual-targeted therapeutics (Box 4) and the combination of siRNAs targeting multiple pro-angiogenesis factors have further demonstrated the advantages of this

novel therapeutic modality. As with any emerging technologies, many issues related to siRNA therapeutics must be addressed diligently before the success of this novel approach can be achieved in clinics.

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