

Tumor angiogenesis and anti-angiogenic gene therapy for cancer (Review)

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Received January 11, 2016; Accepted July 11, 2017

DOI: 10.3892/ol.2018.8733

Abstract. When Folkman first suggested a theory about the association between angiogenesis and tumor growth in 1971, the hypothesis of targeting angiogenesis to treat cancer was formed. Since then, various studies conducted across the world have additionally confirmed the theory of Folkman, and numerous efforts have been made to explore the possibilities of curing cancer by targeting angiogenesis. Among them, anti-angiogenic gene therapy has received attention due to its apparent advantages. Although specific problems remain prior to cancer being fully curable using anti-angiogenic gene therapy, several methods have been explored, and progress has been made in pre-clinical and clinical settings over previous decades. The present review aimed to provide up-to-date information concerning tumor angiogenesis and gene delivery systems in anti-angiogenic gene therapy, with a focus on recent developments in the study and application of the most commonly studied and newly identified anti-angiogenic candidates for anti-angiogenesis gene therapy, including interleukin-12, angiostatin, endostatin, tumstatin, anti-angiogenic metargidin peptide and endoglin silencing.

1. Introduction

Angiogenesis is a complex multi-step process. Angiogenesis is a biological process in which novel capillary blood vessels grow from pre-existing vasculature (1), providing tissues with oxygen and nutrients. As it is correlated with numerous complicated interactions between various biological components, such as several cell types, soluble angiogenic factors and extracellular matrix components, the process of angiogenesis is complex, and primarily consists of four distinct sequential steps: i) Degradation of basement membrane glycoproteins and other components of the extracellular matrix surrounding the blood vessels by proteolytic enzymes; ii) endothelial cell activation and migration; iii) endothelial cell proliferation; and iv) endothelial cells transforming into tube-like structures and forming capillary tubes, and developing into novel basement membranes (2). In normal conditions, angiogenesis only occurs during embryonic development, the female reproductive cycle and wound repair (3). However, aberrant angiogenesis is a key mediator and a major process in cancer development.

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Key words: tumor angiogenesis, gene therapy, angiogenesis inhibitor, interleukin-12, angiostatin, endostatin, endoglin silencing

Tumor angiogenesis. In 1971, Folkman (4) suggested the hypotheses that angiogenesis is required for the development and growth of solid tumors beyond the size of 1-2 mm³. Subsequently, they showed specific fragmentary evidence to indicate that solid tumors were dependent upon neovascularization for sustained growth (5). Following this, an anti-angiogenic strategy, which may develop into a novel therapeutic approach for the treatment of solid tumors, has become a focus of study groups. Over the previous 40 years, a vast volume of data has accumulated, supporting Folkman's hypothesis (6). Concurrently, the intricate mechanism of tumor angiogenesis has been gradually exposed as efforts have been put into this field of study. The normal process of angiogenesis is under a relatively dynamic homeostasis, tightly controlled by pro-angiogenic and anti-angiogenic regulators. Once this homeostasis is disrupted, the 'angiogenic switch', which refers to the phenotype, will become active and initiate angiogenesis (7).

Through numerous studies investigating tumor angiogenesis, different types of regulators have been defined (8-13). These regulators are separately released from endothelial cells, tumor cells, stromal cells, blood and the extracellular

matrix (14-17). These modes of tumor angiogenesis may coexist or shift from one to another during tumor growth and proliferation (18-20). Certain well-known pro-angiogenic regulators include vascular endothelial growth factor (VEGF), basic fibroblast growth factor, transforming growth factor- α and - β (TGF- α and - β), epidermal growth factor, platelet-derived growth factor, placental-derived growth factor and angiopoietin 1 and 2. Specific, commonly studied anti-angiogenic regulators include angiostatin, endostatin, tumstatin, platelet factor-4, interleukin (IL)-12, thrombospondin-1 (TSP-1), tissue inhibitors of metalloproteinases (TIMPs) and interferon- α , - β and - γ . Various biological activities trigger this angiogenic switch. Genetic mutations (activation of oncogenes or loss of tumor-suppressor genes that control production of angiogenesis regulators), metabolic stress (hypoxia, low pH or hypoglycemia), mechanical stress (pressure generated by proliferating cells) and the immune/inflammatory response (immune/inflammatory cells that have infiltrated the tissue) are important stimuli of angiogenic signaling and tend to cause tumor formation (21,22). Among them, hypoxia is one of the primary factors that drive tumor angiogenesis, causing increased expression of VEGF and other angiogenesis stimulators from hypoxic cells (23). Concurrently, matrix-remodeling enzymes, particularly matrix metalloproteinases, mediate a number of the changes in the microenvironment of the tumor tissue by degrading the extracellular matrix (24). Once hypoxia induces the upregulation of VEGF, angiogenesis is initiated with additional activation of hypoxia-inducible factor (HIF) signaling, to provide oxygen supply (25), which stimulates the endothelial cells (ECs) of the preexisting vasculature to sprout and migrate into the hypoxic tissue, led by a gradient of VEGF (26). Subsequently, the endothelial cells differentiate into several cell types, consisting of the tip, stalk and tube cells (27). The tip cells, which express delta-like 4 (DLL4), are non-proliferative cells located at the top of the novel vessels and guide the direction of the novel vessel in response to VEGF signals (28,29). The stalk cells, which express Notch-1, are highly proliferative, with the ability to elongate the sprouting vessel through proliferation when they receive DLL4/Notch signaling (30). The tube cells are non-proliferating, which shape the final appearance of the vessels (6). During additional vascular formation, endothelial progenitor cells (EPCs) are involved in the construction of the inner layer of the novel blood vessels, with pericytes such as specialized muscle cells stabilizing the vessel tubes by providing structural support and forming an outer layer around the ECs (31,32). Subsequently, the ECs connect with each other to form a continuous endothelium, which is characterized by complex, tight junctions (32) and create loops that allow the blood to circulate through adhesion molecules, followed by the construction of the basement membrane. Finally, the vessel is mature and capable of transporting oxygen and nutrition to meet the requirements of the hypoxic tumor tissues (33).

2. Gene therapy

Gene therapy is a therapeutic technique used to correct or alleviate the symptoms of disease by transferring the exogenous genes into the cells of an individual, which may supplement or alter a defective gene, or induce cell death. In total, there

are 4 major strategies exploited in gene therapy, consisting of: Gene replacement; gene modification; gene augmentation; and gene blockage (34). By July 2015, >2,200 clinical trials on gene therapy had been conducted or approved worldwide (35-37). Among these trials, >60% are associated with cancer gene therapy, indicating that gene therapy is not limited to hereditary diseases, but may be used for acquired diseases such as cancer, and it has already become a promising approach in cancer therapy.

In previous years, various gene therapy strategies for cancer have been developed, such as anti-angiogenic gene therapy, suicide gene therapy, immunomodulatory gene therapy, siRNA therapy, pro-apoptotic gene therapy and oncolytic gene therapy (38,39). However, as tumorigenesis is an intricate process that involves various signaling pathways and different mechanisms, and often a single gene may evoke several biological processes and activate diverse signaling pathways, occasionally there is no explicit boundary between these aforementioned gene therapies. For example, gene tumor protein p53 may not only elicit apoptotic activities in tumor cells (40-42), but also has demonstrated anti-angiogenic efficacy in a number of studies (43,44). Therefore, gene therapy exploiting the p53 gene may be characterized as an anti-angiogenic and pro-apoptotic therapy.

Generally, whether gene therapy may be implemented successfully or not will depend on two conditions: i) A suitable gene must be identified to relieve the disease symptoms; and ii) this gene must be delivered to the right location for the gene expression product to treat the disease without causing side effects. As gene therapy is such a precise and delicate therapeutic intervention at the molecular level, there remain a number of technical difficulties to overcome, one being the ability to develop a suitable delivery system for the gene therapy.

3. Delivery systems for gene therapy

Constructing an efficient, safe and specific delivery system is the fundamental basis for gene therapy. Ideal gene delivery systems should possess several attributes: i) A relatively broad range of insertion capacity, with high transfection rates and a non-invasive administration method; ii) it allows for sustained gene expression; iii) a good target-specific selectivity for the tumor type; iv) safety-associated features, including biocompatibility, stability and non-immunogenicity; v) easy availability. At present, numerous different vectors have been constructed and applied in clinical trials: Table I has listed the top 10 most used vectors in clinical studies (35-37). Generally, the delivery systems in gene therapy may be categorized into two groups, namely viral and non-viral vectors systems (45).

Viral vectors. Viral vectors were the first studied and are the most commonly applied gene delivery systems, as they are derived from viruses with a natural ability to transfection (46). In order to make viral vectors more suitable for delivering heterologous genes into targeted cells, they are often genetically optimized for improved efficiency, increased safety and enhanced uptake (46,47). During previous decades, the understanding of viral vectors has increased, concomitant with improvement in their design and production. Based on

Table I. Top 10 most used vectors in gene therapy clinical trials.

Vectors	Advantages	Disadvantages	Number of clinical trials	Proportion in the total clinical trials %
Adenovirus	High efficiency in transfection and transgene expression; ability to transfect a broad spectrum of cell types; independent of active cell division; high titers; does not integrate into host DNA	Expression is transient and the vector itself will elicit inflammatory and immune response	503	22.2
Retrovirus	Transduces a broad range of cells; allows a sustainable and stable expression of genes up to 12 kb; high titers	Requires cell division for successful transduction; no targeting ability and specificity	417	18.9
Plasmid DNA	Low production cost; easy to manufacture; higher transfection rate mediated by electroporation or ultrasound	Naked plasmid only transfects muscle cells; susceptible to enzymatic degradation	397	17.9
Adeno-associated virus	High efficiency in transfection and transgene expression; replication defective; easily transduced	Integrates into the host-cell genome; only allows a gene insert up to ~5 kb	137	6.2
Vaccinia virus	Self-replicating; tumor-selective; effective in apoptosis-defective cells	High immunogenicity	121	5.5
Lipofection	Selective targeting ability toward angiogenic endothelial cells; easier to manufacture, purify, chemically modify and scale-up than viral vectors and bacterial vectors; increased gene transfection compared with naked plasmid DNA	Toxicity; non-uniformity in targeting tumor vasculature	115	5.2
Lentivirus	Transduces both proliferating and non-proliferating cells; prolonged transgene expression with a maximum 8 kb gene insertion	Risk of viral infection and insertional mutagenesis	114	5.2
Poxvirus	Broad spectrum host range of infection; no integration into host genome; high efficiency of gene transfection and expression	High Immunogenicity	101	4.6
Herpes simplex virus	Highly efficient in transduction and gene expression; allows gene inserts ≥ 50 kb	No cell targeting specificity; transient transgene expression; cytotoxic to host cells	73	3.3
RNA transfer	Various ways to transfer, including using cationic polymers, cationic lipids, carbon nanotubes and cell penetrating peptides	Hard to condense; low transfection efficiency	39	1.8

Source: (16-18).

this progress, a number of viral vectors have been identified and explored for gene delivery, including commonly used viral vectors, such as adenovirus, adeno-associated virus (AAV), retrovirus, herpes simplex virus (HSV), lentivirus, and

poxvirus (45), and certain novel developed viral vectors, such as alphavirus vectors (48). Among these, lentiviral vectors and AAV vectors have been the subject of focus in previous years (20), and a recent patent has provided novel methods to

shield the lentiviral vectors with a thin polymer shell, conferring the shielded virus novel binding ability with additional characteristics, including higher thermal stability, resistance to serum inactivation and the ability to infect cells with high efficiency (49). Generally, compared with traditional transfection methods, viral vectors confer a higher transduction efficiency with long-term gene expression. However, certain weaknesses exist in terms of the immunogenicity, mutagenicity, toxicity and high cost of these vectors and the limited size of the transfected gene (50). Therefore, additional studies are required for optimal use of viral vectors in gene therapy.

Non-viral vectors. In order to circumvent the limitations of viral vectors, there has been a focus on developing non-virus-mediated gene delivery modalities, including physical mediated methods, and chemical and biological vectors. Physical methods primarily consist of microinjection, microparticle bombardment, ultrasound mediated microbubble and electroporation. Compared with viral vectors, ultrasound-targeted microbubbles (51,52) and gene electrotransfer plasmids (53) have received the majority of the attention in previous years as they are more safe and efficient in terms of gene delivery. Commonly used chemical vectors may be classified into 2 major types based on the nature of the synthetic material, namely cationic polymers and cationic liposomes (45). Despite the promising prospect that cationic liposomes presented with several studies in clinical trials, the low transfection efficiency and side effects, including toxicity, are the primary obstacles preventing its widespread use (54-57). Therefore, the newly-described cationic core, the shell nanoparticles, appears to be an alternative to liposomes, as it offers a greater number of advantages, including high gene transfection efficiency and the ability of the concurrent delivery of drugs and genes to the same cells (58). Biological vectors generally refer to bacteria and specific mammalian cells. The types of bacteria used as vectors include attenuated strains of *Bifidobacteria*, *Clostridia*, *Listeria*, *Salmonella*, *Shigella*, *Yersinia* and non-pathogenic *Escherichia coli* (34). As for mammalian cells, hematological cells and mesenchymal stem cells (MSCs) are usually used as carriers of gene therapy vectors (59). Additionally, gene-transfected EPCs may be useful as a tumor-specific drug delivery system (60). Compared with viral vectors, non-viral vectors provide advantages, including relative safety, ability to transfer large size genes and less toxicity. They may also be constructed and modified by simple methods for tissue- or cell-specific targeting (54). However, non-viral vectors exhibit limitations of a low transfection efficiency and poor transgene expression (61). In conclusion, all of these methods have been investigated and each of them presents distinct advantages and disadvantages.

4. Anti-angiogenesis gene therapy and angiogenesis inhibitors

Anti-angiogenic gene therapy targeting endothelial cells (ECs). For the majority of cancer therapy strategies, the tumor vasculature has provided issues for drug delivery, as it is a barrier that prevents drugs from reaching tumor cells. However, tumor angiogenesis is an easily accessible target for anti-angiogenic cancer therapy, particularly when the

anti-angiogenic drugs are administered by delivery systems with specificity for tumor endothelial cells. Notably, compared with the anti-angiogenic therapies directly targeting tumor cells, targeting ECs may be more practical when compared with tumor cells, as endothelial cells have been identified to be genetically more stable (62). The inhibition of EC proliferation, migration and EC apoptosis by anti-angiogenic agents may damage the viability of numerous tumor cells, due to destruction of ECs not only limiting the supply of oxygen, nutrients and growth factors produced by ECs to the surrounding tumor cells, but also leading to the lack of structural support for tumor cells, eventually resulting in the disassembly of tumor tissues (Fig. 1). In addition, the same anti-angiogenic molecule may be efficient in various types of cancer (63). Based on those therapeutic advantages, efforts have been made to explore tumor treatments that target angiogenesis. Furthermore, the comprehensive study of various angiogenesis growth factors and inhibitors with demonstrated therapeutic effects as administered anti-angiogenic drugs have provided evidence for anti-angiogenesis therapy. Table II summarizes the anti-angiogenic drugs approved for clinical use. However, during the long-term process of cancer treatment, the efficacy of pharmaceutical proteins is limited due to their short half-life, high cost and vulnerability to interference by endogenous substances (64). Compared with monoclonal antibodies and engineered antibodies, gene therapy has the advantages of sustained and localized expression of the therapeutic gene product, lower cost and fewer side effects (65,66). Therefore, anti-angiogenesis cancer gene therapy and combination of gene and anti-angiogenesis therapy have become required.

Principles of anti-angiogenic gene therapy. At present, anti-angiogenic cancer gene therapies primarily adopt the following two principles: Gene augmentation; and gene blockade. The former involves introducing exogenous anti-angiogenic genes into targeted cells so that through their expression tumor angiogenesis is halted, while the latter results in the inhibition of the excessive expression of pro-angiogenic genes in endothelial cells, and other tissue cells, of the tumor. Therefore, the genes of interest may be divided into equivalent categories: Anti-angiogenic genes utilized for gene augmentation; and pro-angiogenic genes for gene blockade (Fig. 2).

Angiogenesis inhibitors. With the development of biotechnology and an improved understanding of angiogenesis mechanisms, numerous pro- and anti-angiogenesis genes have been identified and utilized in studies investigating cancer gene therapy (63,67). In total, >300 angiogenesis inhibitors have been identified at present (68); among them, >30 agents have been extensively studied in gene therapy (Table III). As various papers have already reviewed a number of these anti-angiogenic molecules (63,64,68,69), only the most commonly discussed inhibitors will be examined in this paper, to avoid repetition.

IL-12. IL-12, first recognized as a pro-inflammatory cytokine with immunoregulatory functions (70,71), has been suggested to exert an anti-angiogenesis effect in several experiments (72-74). Due to its ability to stimulate immunity and inhibit tumor angiogenesis, IL-12 has been identified as one

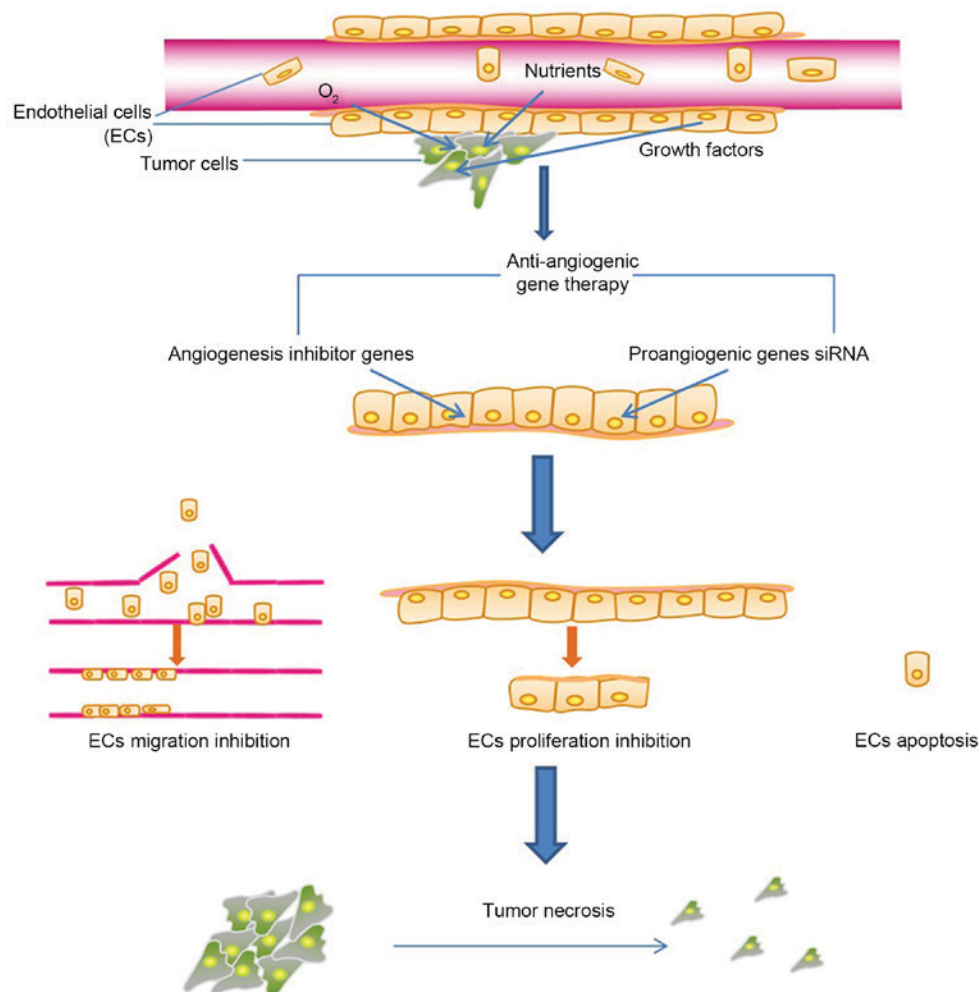


Figure 1. Gene therapy targeting angiogenesis in ECs. The figure depicts the advantages of gene therapy targeting angiogenesis in ECs, as a single vessel may support the growth of numerous tumor cells by providing them with oxygen, nutrients and growth factors produced by surrounding ECs. The inhibition of EC proliferation, migration, and an increase in EC apoptosis by anti-angiogenic agents may lead to the destruction of blood vessels, which may additionally initiate tumor necrosis. ECs, endothelial cells.

of the most potent antitumor candidates not only for cancer immunotherapy (75), but also for anti-angiogenic therapy (76). Although previous evidence has indicated its anti-tumor activities in *in vitro* and *in vivo* experiments (77), the anti-tumor effect of IL-12 evidently varies between mouse strains (78), and the mechanism that leads to the various responses remains unclear. However, a previous study demonstrated that the higher expression of IL-12 receptor (IL-12RB1) by C3H/HeJ mouse splenocytes resulted in a significantly stronger response to IL-12 compared with other mouse strains, providing a potential explanation for the variation of IL-12 anti-tumor efficacy between different individuals (78). Although unsatisfactory side effects, including toxicity, have been identified in several early clinical trials using systemically delivered recombinant human IL-12 (rhIL-12) (79-81), interests in gene therapy approaches have increased due to its potential in achieving high drug concentrations in the local tumor environment, with low systemic levels. Apart from several early clinical trials of gene therapy using IL-12 in previous decades, a more recent study provided long-term overall survival results from a phase I study of intratumoral electroporation (EP) of plasmid (p)IL-12, which was completed in 24 patients with malignant melanoma. This study suggested that improved survival is

correlated with systemic disease stabilization with pIL-12 EP (82). An additional biomarker analysis study investigating the efficacy of intratumoral electroporation of pIL-12 from a phase 2 study in melanoma also demonstrated that pIL-12 EP monotherapy induces tumor responses in 31% of patients, and no severe local or systemic toxicity was observed in the treatment (83). Concurrently, certain gene therapies involving IL-12 use different delivery systems to explore therapeutic methods with low systematic toxicities, high tumorous specificities and sustained local expression of IL-12, such as plasmid (84,85), HSV-1 (86), Semliki forest virus vector (87), T-cells (88), a novel helper-dependent adenoviral vector (89) and *Lactococcus lactis* (90). Other strategies in previous studies have focused on combining IL-12 with other anti-tumor genes, including suicide genes (91), or other therapies, such as chemotherapy (92), to explore its preclinical efficacy and safety prior entry of these methods into clinical trials.

Melanoma differentiation-associated gene-7 (MDA-7). MDA-7, also termed IL-24, was identified through subtraction hybridization from a human melanoma cell line (93), and has demonstrated efficacy as a potent tumor suppressor gene in initial studies in the 1990s (93-95). As an anti-cancer

Table II. List of antiangiogenic drugs approved for clinical use.

Drug	Target/mechanism	Type of cancer	(Refs.)
Avastin (Bevacizumab)	Monoclonal antibody targeting VEGF	Ovarian, colorectal, renal, breast and prostate cancers, NSCLC and glioblastoma	(172-175)
EYLEA (Aflibercept)	Fused protein consists of VEGFR1 and VEGFR2	Colorectal cancer, prostate cancer, NSCLC and SCLC	(176-179)
Erbix (Cetuximab)	EGFR monoclonal antibody	Colorectal cancer, gastric cancer and NHSCC	(180-183)
Endostar (endostatin)	Recombinant protein of the angiogenesis inhibitor endostatin	NSCLC, melanoma, nasopharyngeal carcinoma and colorectal cancer	(184-187)
Nexavar (Sorafenib)	Molecular inhibitor of VEGFR, PDGFR and Raf kinases	Hepatocellular carcinoma, thyroid cancer, ovarian cancer and renal cancer	(188-191)
Sunitinib (Sutent/SUNITINIB MALATE)	Molecular receptor tyrosine kinase inhibitors	Renal cancer, NSCLC, hepatocellular carcinoma and prostate cancer	(192-195)
Sprycel (Dasatinib)	Multi-BCR/ABL and Src family tyrosine kinase inhibitor	Chronic myeloid leukemia, melanoma and adenoid cystic carcinoma	(196-198)
Iressa (gefitinib)	EGFR tyrosine kinase inhibitor	NSCLC, squamous cell carcinoma of the head and neck, and esophageal cancer	(199-201)
Tarceva (Erlotinib)	EGFR tyrosine kinase inhibitor	Hepatocellular carcinoma, pancreatic cancer and NSCLC	(188,202-203)
Votrient (pazopanib)	RTK inhibitor targeting VEGFR, PDGFR and c-Kit	Renal cancer, soft-tissue sarcoma, ovarian cancer and thyroid carcinoma	(204-206)

VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; RTK, receptor tyrosine kinase; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.

agent, MDA-7 functions through diverse modalities, including anti-angiogenesis (96), tumor-specific apoptosis (97) and immunotherapeutic activity (98). Previously, a study examining the effect of MDA-7 on Her2/Neu-induced mammary tumors concluded that MDA-7 inhibited tumor growth of HER2+ breast cancer cells partially through p53 apoptosis effector related to PMP-22, which is a member of the PMP-22 family, with growth arrest and apoptosis-inducing capacities (99). In an additional study, the human MDA-7 gene was transfected into the human laryngeal cancer Hep-2 cell line and human umbilical vein endothelial cells with adenovirus vector (100), and the results demonstrated that MDA-7 exerted anti-tumor functions in the laryngeal carcinoma cell lines, whereas no harmful effect was observed in the healthy cells. As for gene delivery, a study has introduced a method for increasing the expression level of MDA-7 in osteosarcoma (OS) using a novel oncolytic adenovirus, where an increased sensitivity of OS to doxorubicin induced by MDA-7 was also observed (101). Finally, 3 vectors expressing MDA-7 in fusion with the arginine-glycine-aspartic acid (RGD) peptide, which is considered to exhibit the most significant effect on the binding specificity of integrin receptors, were constructed. With a stronger expression potency observed and integrity

validated, MDA-7 with RGD peptide appears to be a more appealing therapeutic option, when compared with the administration of MDA-7 alone (102), indicating a future direction for cancer gene therapy.

Angiostatin. Angiostatin is the first of four Kringle domains of a 38-kDa internal proteolytic fragment of plasminogen, which has been recognized as a potent endogenous angiogenesis inhibitor, and its anti-tumor effect has also been widely demonstrated (103). However, the primary obstacle preventing its future application in clinical trials is that it exhibits a limited therapeutic efficacy with a short half-life (104). To resolve this problem, studies have focused on elucidating efficient delivery systems, and experiments investigating various non-viral and viral methods delivering angiostatin gene have been conducted. At present, angiostatin has been expressed in HSV (105,106), vaccinia virus (107), oncolytic measles virus (108), adenovirus (109), adeno-associated viral vectors (110,111) and lentivirus (112), or mediated by plasmids (113) and cationic liposomes (114). Concurrently, angiostatin is often co-transfected with other genes for an enhanced anti-tumor efficacy, like antisense HIF-1 α (115), p53 (116), IL-12 (117), Fas gene (113), soluble form of vascular

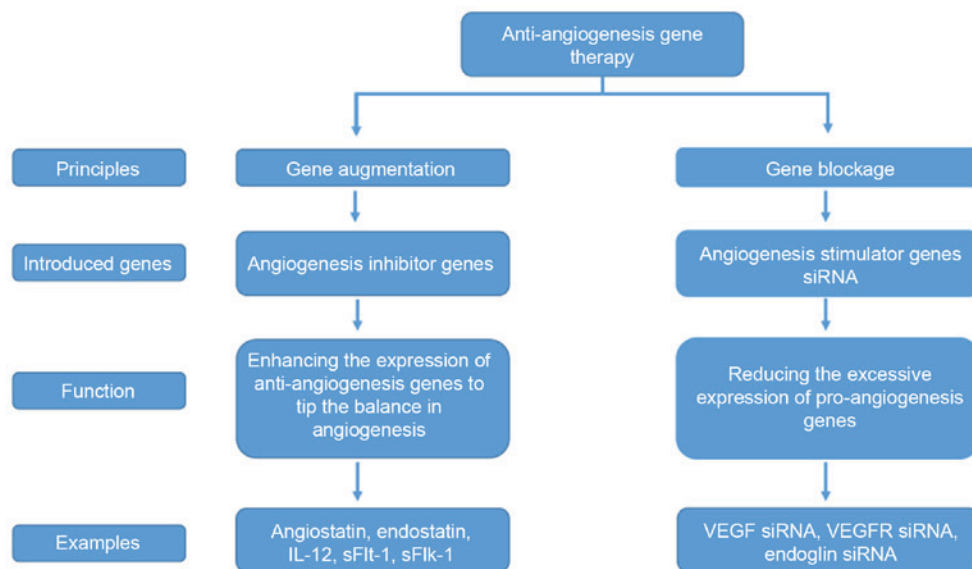


Figure 2. Principles of anti-angiogenesis gene therapy. The flowchart depicts the two major principles of anti-angiogenic gene therapy. It highlights the major differences between the principles, and indicates representative examples in each category. IL-12, interleukin 12; sFlt-1, soluble fms-like tyrosine kinase-1; sFlk-1, soluble form of vascular endothelial growth factor receptor 2; VEGF, vascular endothelial growth factor, siRNA, small interfering RNA; VEGFR, VEGF receptor.

endothelial growth factor receptor 2 sFlk1 (112) and most commonly used endostatin-angiostatin fusion gene due to the fact that they were identified to act synergistically when used in combination (106-108). Previous studies (118-120) suggested that angiostatin mimic (kringle1-5) appears more attractive compared with angiostatin in terms of tumor suppression and metastasis inhibition, potentially due to the synergistic effect of the Kringle 5 domain of plasminogen.

Endostatin. Endostatin, a 20 kDa C-terminal cleavage fragment from the $\alpha 1$ chain of type XVIII collagen, is one of the most extensively studied endogenous angiogenesis inhibitor that was originally identified by O'Reilly (121,122). Endostar (YH-16), a protein drug of recombinant human endostatin, was approved by China's State Food and Drug Administration for the treatment of non-small cell lung cancer in 2005 (123), indicating the potential of endostatin in cancer treatment. A gene-based endostatin approach has also received attention, and has made its progression within a pre-clinical context over previous decades, along with breakthroughs in clinical trials. Previous studies primarily focused on two categories: The joint method, combining endostatin with other genes or with other cancer therapeutics; and the exploration of a more suitable delivery system for endostatin to be expressed in a more efficacious, tumor-targeted way. For example, Huiqi *et al* (124) examined the therapeutic effect of combining endostatin gene therapy with ^{32}P colloid radiotherapy on hepatocellular carcinoma (HCC) cells, and concluded that the combination of these two treatments demonstrated an improved therapeutic effect on HCC compared with either treatment alone. Kubo *et al* (112) also investigated a combinatorial anti-angiogenic gene therapy with endostatin, angiostatin and sFlk1, and an improved therapeutic efficacy was demonstrated compared with that of a single-agent regimen, due to the ability of three genes targeting different pathways of endothelial growth factor signaling. An additional study identified that the combination of human

endostatin and soluble tumor necrosis factor (TNF)-related apoptosis-inducing ligand gene transfer indicated an enhanced tumor suppressing effect through anti-angiogenic and pro-apoptotic mechanisms (125). As for delivery systems, a previous study has demonstrated that ultrasound-targeted microbubble destruction (UTMD)-mediated gene therapy may enhance the transfection efficiency of endostatin, indicating that the UTMD-mediated delivery system exhibits potential as a gene therapy targeting retinal neovascularization (126). Additionally, certain other previous experiments concerning the efficacy of gene delivery or the efficiency of combinatorial therapy utilizing endostatin have all demonstrated progress in cancer treatment to a certain level (108,127-130).

Tumstatin. As an alternative appealing endogenous angiogenesis inhibitor, tumstatin, a cleavage fragment of the $\alpha 3$ chain of type IV collagen (131), is an exciting candidate for cancer gene therapy, due to the fact that its anti-angiogenic ability is 10-fold higher compared with that of endostatin (132). By binding to $\alpha_v\beta_3$ and $\alpha_3\beta_1$ integrins (133), tumstatin exerts its anti-angiogenic effects through diverse modalities, including the induction of endothelial cells apoptosis, inhibition of cell proliferation and tube formation in endothelial cells, and a previous study has identified that tumstatin stimulates endothelial cell apoptosis through the Fas signaling pathway (134). The anti-angiogenic and anti-tumorigenic effects of tumstatin have been widely demonstrated by gene transfer experiments conducted in various xenograft models, such as hepatocellular carcinoma (135), S180 tumor (136), lung carcinoma (137) and renal carcinoma cell (138). In previous years, efforts have been made to develop and test diverse delivery systems in tumstatin gene therapy. For example, lentivirus-mediated signal peptide TNF- α -Tumstatin (45-132)-expressing mesenchymal stem cells (SPTT-MSCs) have been used as a novel delivery approach in human prostate cancer cells *in vitro* and *in vivo*, and

Table III. Angiogenesis inhibitors for gene therapy.

Angiogenesis inhibitor	Mode of action
16-kDa a prolactin fragment	Inhibits EC proliferation, induces apoptosis
2-methoxyestradiol	Inhibits angiogenesis by inhibiting hypoxic inducible factor-1 α
Angiostatin	Inhibits EC proliferation and migration
Antiangiogenic metargidin peptide	Inhibits angiogenesis by binding to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins
Arresten	Inhibits angiogenesis by binding to $\alpha_1\beta_1$ integrin
Canstatin	Induces proapoptotic activities in EC
Cleaved antithrombin III	Potently inhibits angiogenesis and tumor growth
Endostatin	Inhibits angiogenesis by binding $\alpha_5\beta_1$ integrin
Endothelial-monocyte activating polypeptide II	Inhibits angiogenesis through upregulating TNF receptor-1, induces EC apoptosis
HGFK1	Kringle 1 domain of human hepatocyte growth factor, a more effective anti-angiogenesis molecule than angiostatin
Human ribonuclease inhibitor	Inhibits the activity of pancreatic RNase
IL-12	Potent cytokines in stimulating antitumor immunity, which also showed significant inhibitory activity on angiogenesis
IL-18	Cytokine with antiangiogenic activity via induction of IFN- γ
IL-24	Cytokine with antitumor ability including tumor specific apoptosis, anti-angiogenesis, and immunotherapeutic activity
Interferon-inducible protein-10	Member of CXC chemokine family, potent immunomodulatory and antiangiogenic activity
Interferons	Multifunctional cytokines that regulate antiviral, antitumor, and cellular immune responses, potent antiangiogenic properties via inhibition of bFGF
Kallistatin	Inhibits proliferation, migration, and adhesion of ECs
NK4	Inhibits angiogenesis by inhibiting HGF signaling
p53	Inhibits angiogenesis by increasing thrombospondin-1 expression and decreasing VEGF expression
Pigment epithelium-derived factor	Inhibits angiogenesis through interfering with VEGF signaling
Platelet factor-4	Inhibits ECs proliferation and migration
Restin	Inhibits ECs migration, induces apoptosis
sFlk-1	Soluble VEGFR-2, inhibiting VEGF signaling passage
sFLT-1	Soluble VEGFR-1, inhibiting VEGF signaling passage
Tetrahydrocortisol	Most potent naturally occurring angiostatic steroid
Thrombospondin-1	Inhibits ECs proliferation and migration by interactions with CD36
Tissue inhibitors of metalloproteinases	Block the activity of MMPs, inhibits tumor angiogenesis and tumor growth
TNF- α	Potent vessel virulent effects on tumors, inhibits angiogenesis through activity mediated by TNF receptor
Tumstatin	Inhibits angiogenesis by binding $\alpha_v\beta_3$ integrin
Vascular endothelial growth inhibitor	Induces EC cell cycle arrest and apoptosis
Vastatin	Induces cell cycle arrest and apoptosis of ECs
Vasostatin	Inhibits ECs proliferation, induces tumor cell apoptosis
Endoglin siRNA	Suppresses multiple angiogenic signaling pathways by inhibiting endoglin expression
VEGF siRNA	Inhibits VEGF expression
VEGFR-2 siRNA	Inhibits VEGFR-2 expression
HGF siRNA	Suppresses the HGF-induced angiogenesis by inhibiting HGF expression
Survivin siRNA	Induces apoptosis within the vascular wall by inhibiting survivin expression

EC, endothelial cell; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; TNF, tumor necrosis factor; IL, interleukin; si, small interfering; CD36, cluster of differentiation 36; HGF, hepatocyte growth factor; HGFK1, hepatocyte growth factor kringle domain 1; bFGF, basic fibroblast growth factor; sFlk1, soluble form of vascular endothelial growth factor receptor 2; sFLT, soluble fms-like tyrosine kinase-1; IFN- γ , interferon γ ; p53, tumor protein 53.

results have demonstrated significant anti-tumorigenic effects on prostate cancer cells, indicating that SPTT-MSCs may represent a promising solution for prostate cancer (139). In an additional experiment, gene electrotransfer of naked plasmid DNA containing the tumstatin cDNA has been adopted to investigate the anti-tumor effect of tumstatin in B16F1 melanoma-bearing mice: A marked decrease in tumor growth and an increase in mouse survival was observed, indicating that this strategy appears appealing in terms of gene delivery and tumor suppression (140). In addition, the pET-15b vector generated to express a synthetic fusion protein, VTF, which is composed of vasostatin and tumstatin with a (Gly-Ser-Gly)₂ bridge, demonstrated the suppression of B16 melanoma growth and the potent inhibition of tumor blood vessels formation *in vivo*, when compared with a single inhibitor, the fusion proteins of different angiogenesis inhibitors targeting different pathways exhibited improved therapeutic effects (141). Additionally, as T42, which was derived from two active domains of tumstatin, has demonstrated anti-tumor efficacy, a previous study constructed two adenoviral vectors with T42 and 4xT42 peptide genes to evaluate their anti-cancer effects on breast cancer *in vitro* and *in vivo*; the results suggested evidence that this modality may be a potential alternative for the treatment of breast cancer (142).

Anti-angiogenic metargidin peptide (AMEP). AMEP, the disintegrin domain of human metargidin, is a novel anti-cancer agent that exerts its effect by binding to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins via its Arg-Gly-Asp (RGD) integrin binding sequence (143-145). The antitumor and anti-angiogenic effects of AMEP were first suggested *in vitro* using a recombinant protein (143). Subsequently, it was also demonstrated *in vivo* using an AMEP-coding plasmid (146), and a higher anti-tumor efficiency of AMEP compared with TSP-1 and soluble fms-like tyrosine kinase-1 was observed, with a significant decrease in tumor metastasis, suggesting that AMEP may not only inhibit the proliferation of tumor cells but may also suppress tumor metastasis. Following this, a phase I clinical trial study was conducted to investigate the safety and tolerability of the AMEP plasmid mediated by intratumoral electrotransfer into cutaneous metastatic melanoma. Results indicated a good safety profile and also, to a certain extent, the efficacy of AMEP plasmid gene electrotransfer in metastatic melanoma (147). Additionally, a previous study indicated that the anti-tumor activities of gene electrotransfer of the AMEP plasmid in murine melanoma cells were correlated with the integrin quantity within the melanoma cells, rather than the expression level of AMEP; however, the anti-angiogenic effect was only partly associated with the quantity of integrins, and appeared to be dependent on the dose of the AMEP plasmid (148). In addition, a previous study confirmed that the integrin quantity within melanoma cells may serve as a biomarker for the antitumor efficacy of therapies targeting integrins, whereas the anti-angiogenic effectiveness of the AMEP plasmid may be predicted by the expression levels of AMEP in the treatment of melanoma (149). It also suggested that intratumoral delivery of the AMEP plasmid was more effective compared with an intramuscular method. Based

on these aforementioned studies, it may be predicted that future studies investigating the electrotransfer of the AMEP plasmid will be more focused on particular types of cancer, in which the overexpression of integrin is observed.

NK4. First isolated as a proteolytic digestion product of hepatocyte growth factor (HGF) (150), NK4 is a novel anti-tumor agent through its bifunctional activities of HGF antagonism and anti-angiogenesis. Studies have also demonstrated that NK4 exerts potent anti-angiogenic action via indirectly inhibiting VEGF expression of tumor cells concomitant with direct effects on endothelial cells (151). Although the marked anti-angiogenic effect and anti-tumor ability of NK4 has been confirmed in a diverse number of cancer models, such as malignant pleural mesothelioma, melanoma, lung and pancreatic carcinomas, and colon, biliary gastric and gall bladder cancers (152-156), this individual anti-angiogenic agent alone is not therapeutically sufficient, due to the fact that human cancers are more intricate, and require treatment with multiple targets. Therefore, subsequent studies have explored the potential of NK4 in combination with conventional chemotherapeutic agents or with other inhibitors targeting different signaling pathways. Matsumoto *et al* (157) identified that the anti-tumor efficacy of combining the NK4 plasmid with cisplatin to treat squamous cell carcinomas was increased compared with NK4 gene therapy alone. An additional study demonstrated that 5-fluorouracil enhanced the NK4-induced apoptosis of colon cancer cells by down-regulating the intracellular signaling of the HGF/c-Met pathway (158). Previously, studies have explored a more efficient and suitable way to deliver NK4. Zhu *et al* (159) demonstrated that MSC-based NK4 gene therapy may markedly inhibit the growth of gastric cancer xenografts, and MSCs are a better vehicle for NK4 gene therapy compared with lentiviral vectors. Additionally, a preliminary clinical trial in humans has been designed to examine the safety and possible clinical benefits of adenoviruses expressing NK4 (160).

Endoglin. Endoglin, a TGF- β co-receptor, is involved in the activation of a complex signaling pathway regarding the proliferation, migration and adhesion of endothelial cells (161,162), particularly in tumor vasculature, due to the fact that the expression of endoglin is markedly increased in the endothelial cells of tumor vessels, making it a potential predictive factor for tumor prognosis (163). As such, endoglin has been hypothesized to serve as a promising target for cancer therapy, and several studies using different anti-endoglin antibodies, including monoclonal antibodies (164,165), immunotoxin-conjugated antibodies (166) or radiolabeled antibodies (167) have all demonstrated good anti-angiogenic and antitumor responses. In the case of gene therapy, silencing endoglin by RNA interference is considered to be an alternative potential approach for endoglin targeting, and one study group have conducted a series of experiments to explore the potential of this approach. Dolinsek *et al* (168) first investigated the therapeutic effectiveness of small interfering RNA (siRNA) molecules against endoglin *in vitro* and *in vivo*, and the results indicated that siRNA molecules targeting endoglin exhibited good anti-angiogenic and antitumor efficacy

on endothelial cells *in vitro*, and on tumors *in vivo*. However, as the effect of siRNA against endoglin exhibited a short half-life a plasmid DNA encoding shRNA against endoglin was constructed and delivered into murine endothelial cells *in vitro* and tumors *in vivo* using gene electrotransfer to determine its antitumor and vascular-targeted effects (169). Furthermore, in order to specifically silence endoglin within the tumor vasculature, the same study group also prepared a plasmid that silenced endoglin with a tissue-specific promoter (hTERT) (170), which was endothelin-1-dependent and was involved in migration of endothelial cells (171). The results of the study indicated that this plasmid may achieve higher levels of specificity and safety with the same efficacy as a plasmid with a constitutive promoter. An additional previous study demonstrated that endothelial and melanoma cells expressed high levels of endoglin, and that subsequent to endoglin silencing with gene electrotransfer, cell viability was specifically decreased; whereas in tumor cells with low expression of endoglin, only a non-specific decrease in cell viability was observed following electrotransfer (172), providing novel possibilities for melanoma treatment with targeted gene therapy approach.

5. Conclusion and future direction

The previous 4 decades have witnessed the feasibility of Folkman's theory in cancer treatment. Anti-angiogenesis therapy, which used to be described as a novel and potential method waiting to be verified of its efficacy in treatment of various diseases, particularly cancer, now represents one of the most significant and promising treatment modalities in clinical oncology. With numerous efforts exploring the various possibilities in utilizing anti-angiogenesis therapy, gene therapy has become an attractive alternative to conventional protein drugs due to its ability to achieve prolonged and localized gene expression, without the issues of high cost and complex processes of production associated with protein drugs.

With increased interest in angiogenesis during the previous two decades, studies examining gene-based anti-angiogenic approaches have made progress in the following three aspects. Firstly, there has been continuous identification of targets for anti-angiogenic gene therapy due to the additional understanding of tumor angiogenesis. Secondly, further improving the efficacy of existing gene delivery systems, with detailed optimization, including the use of tissue-specific promoters or peptides specifically targeted to tumor ECs, and exploring novel methods to better facilitate gene transfer, particularly in the field of non-viral delivery method, such as ultrasound and gene electrotransfer. Thirdly, constantly improving anti-tumor efficacy by combining anti-angiogenic genes with other genes, including different angiogenesis or suicide genes, or genes that neutralize anti-angiogenic resistance such as antisense HIF-1 α . As an increasing number of studies have identified that using the anti-angiogenic gene approach as a monotherapy is not sufficient for tumor eradication, and that certain angiogenesis inhibitors may make tumor cells more susceptible towards chemotherapy and radiotherapy, subsequent studies have investigated anti-angiogenic gene therapy in combination with chemotherapy or radiotherapy.

Although a number of individual angiogenesis inhibitors have demonstrated the ability to suppress tumor progression and metastasis in a variety of cancer models, the efficacy of tumor regression varies between different types of cancer when using the same angiogenesis inhibitor, indicating that a future direction for anti-angiogenic gene therapy is to identify prognostic biomarkers to assist in determining the most efficient angiogenesis inhibitor gene for each type of cancer, which will largely rely on an improved understanding of the biological mechanisms of tumor angiogenesis. In addition, as anti-angiogenic gene therapy has demonstrated more potent effectiveness in small tumors compared with large ones, future application of anti-angiogenic gene therapy may be more involved in preventing and treating early-stage cancers. Notably, the methods of gene delivery and the limited therapeutic effect of monotherapy are major obstacles to anti-angiogenic gene therapy; therefore, efforts to develop more efficient gene delivery methods, and to explore additional possibilities in combination therapy, are required. Furthermore, a better understanding of the mechanisms of action and a better selection of the clinical trial patient population should also be performed by future studies.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Nature Science Foundation of China (grant no. 31470967).

Availability of data and materials

Not applicable.

Authors' contributions

HH gave guidance on the conception and design of the article. TL and GK contributed their ideas on this topic and were involved in planning the structure of this review. GK and TW participated in the collection and organization. TL was a major contributor in writing the review. HH made critical modifications to important knowledge content within the manuscript. GK was a major contributor in revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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