

Small RNA and its application in andrology and urology

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Abstract: Small non-coding RNAs such as small interfering RNA (siRNA), microRNA (miRNA) and piwi-interacting RNA (piRNA) exist in almost all kingdoms of organisms and have recently emerged as master regulators of gene expression to affect a diverse range of important biological processes. They exert their functions largely through two related but opposing mechanisms: RNA interference (RNAi) mediated by siRNA, miRNA and piRNA, and RNA activation (RNAa) mediated by small activating RNA (saRNA) and miRNA, leading to silencing and overexpression of target genes respectively. Dysregulation of these mechanisms have been implicated in a variety of human diseases including urological and andrological diseases. Importantly, both mechanisms can be readily harnessed for therapeutic purposes for a variety of diseases by using small RNA molecules as the "ribodrug". In this review, we highlight recent advances in the applications of small RNA as therapeutics for urological cancer, male infertile and erectile dysfunction.

Keywords: Small RNA; microRNA; RNAi; RNAa; urology; andrology; prostate cancer; bladder cancer; erectile dysfunction

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Introduction

RNA molecules can be divided into two major categories: protein-coding RNA and non-coding RNA (ncRNA). In human, around 98% of all transcriptional output is ncRNA (1). ncRNA can be further divided into two main subgroups based on their function: housekeeping ncRNA such as transfer RNA (tRNA), ribosomal RNA (rRNA), and regulatory ncRNA which includes both short and long ncRNA. It has become increasingly clear in recent years that regulatory ncRNA has profound biological function by regulating protein coding genes through a diverse array of molecular mechanisms. The best studied regulatory ncRNA is a class of tiny RNA molecules which have a size ranging from 20 to 30 nucleotides (nts) and highly depend on a family of conserved proteins known as Argonaute (Ago) for function. They include microRNA (miRNA), piwi-interacting RNA (piRNA) and small interfering RNA (siRNA). These small ncRNAs are mainly involved in an evolutionarily conserved gene silencing mechanism known as RNA interference (RNAi). Apart from their main

function as silencers, we and other groups have recently reported that small RNA can also activate gene expression by targeting gene promoter sequences in a process termed RNA activation (RNAa) (2-4). These observations thus revealed a new class of small RNA molecules and a new layer of complexity of small RNA-mediated gene regulation (Table 1). It has become increasingly evident that various small RNA-guided gene regulations play important roles in normal physiological processes and in disease. It is also clear that these magnificent cellular mechanisms which were not known to us until recent years can be harnessed as powerful therapeutics for disease treatment. In this article, we review recent advances in the research of small RNA in urological and andrological diseases and focus on their potential application in the treatments of these diseases.

miRNA and its applications

miRNA and its biological functions

MicroRNA (miRNA) is a class of endogenous small RNA

Table 1 Classes of small RNA

Properties	microRNA (miRNA)	Piwi-interacting RNA (piRNA)	Small interfering RNA (siRNA)	Small activating RNA (saRNA)
Size (nt)	20-24	24-30	21-23	21
Source	Endogenous(ubiquitous)	Endogenous(restricted largely to spermatocytes and spermatids)	Exogenous or endogenous	Exogenous or endogenous
Conservation	Eukaryotes	Vertebrates and invertebrates	Eukaryotes	Mammals
Strandedness	Single-stranded	Single-stranded	Double-stranded	Double-stranded
Biogenesis	Dicer dependent	Dicer independent	Dicer dependent	Dicer dependent
Ago dependence	Ago subfamily	Piwi subfamily	Ago subfamily	Ago subfamily
Target	3'-UTR, 5'-UTR, gene promoter, coding regions, pseudogenes and competing endogenous RNA	Transposon	mRNA or gene promoter	Gene promoter
Functions	mRNA degradation,transcriptional silencing, posttranscriptional silencing, translation inhibition	Transposon silencing, transcriptional regulation, post-transcriptional regulation	mRNA degradation, transcriptional silencing, posttranscriptional silencing	Gene activation

(20-24 nt), which is transcribed from the genome and processed into its mature form by RNase III enzymes Drosha and Dicer via a multi-step process. Upon loaded by Ago proteins, miRNA binds to homologous sequences on the 3'untranslated regions (UTRs) of target messenger RNA (mRNA), resulting in translational repression and/or mRNA degradation (5). miRNA has also been shown to regulate gene expression by targeting gene promoters (6), 5'-UTR (7), coding regions (8), pseudogenes (9) and competing endogenous RNA (ceRNA) (10). A recent report showed that miRNA could regulate gene translation in a "seed" sequence-independent manner (11). Various models of action for miRNA revealed so far imply that the miRNA has diverse functions in biology and disease.

The application of miRNA in urological cancers

Urological malignancies including cancer of the prostate, bladder and kidney are the leading causes of urological patient death. The molecular mechanisms underlying their development and progression remain poorly understood. Human miRNA genes are frequently located at fragile sites and genomic regions implicated in cancers (12), suggesting the involvement of miRNAs in this complicated biological processes (Table 2). With the improvement of RNA-sequencing technology, hundreds of miRNAs have been identified and each miRNA are predicted to have hundreds of target genes.

According to their roles in cancer, miRNAs can be classified into oncogenic miRNAs (oncomiRs) and tumor suppressor miRNAs (tsmiRs). OncomiRs are generally

upregulated in most tumor types and are able to promote malignant transformation and cancer progression. The well-known oncomiR is the miR-17-92 cluster consisting of several members including miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1. The miR-17-92 cluster is located at the 13q31 locus and amplified in many types of cancer (40). PTEN (phosphatase and tensin homologue) is one of the most frequently dysregulated tumor suppressors for several human urological cancers (41). Members of miR-17-92 cluster have been found to be able to inhibit PTEN expression via binding to its 3'UTR (13). mir-17-92 can also target and suppress the expression of BCL2L1(BIM) (13), TSP1 and CTGF genes (14). The miR-106b-25 cluster (miR-106b, miR-93 and miR-25), which shares high degree of homology with the miR-17-92 cluster, can promote cell proliferation and resistance to apoptosis by inhibiting BIM, p21 (15), PTEN (16) and p53 (17). The miR-221/222 locus, located on chromosome X, is another widely recognized oncomiR cluster. The two miRNAs in this cluster are transcribed from the same promoter and share identical seed sequences. Functional studies showed these oncomiRs negatively module many tumor suppressor genes, including p27, p57 (18), DDIT4 (19), PTEN and TIMP3 (20). Although different types of human cancer may have different miRNA expression signatures, some oncomiRs are consistently associated with a high risk for malignancies, such as miR-21 (40). Overexpression of miR-21 could suppress apoptosis, induce cell proliferation and survival, and facilitate cell migration and invasion by suppressing such target genes as PDCD4 (21), PTEN (22), RHOB (23), RECK and TIMP3 (24).

Table 2 Involvement of miRNA in urological cancers

Role	microRNA	Validated targets	References
OncomiRs	miR-17-92	PTEN, BIM, TSP1 and CTGF	(13,14)
	miR-106b-25	BIM, p21, PTEN and p53	(15-17)
	miR-221/222	p27, p57, DDIT4, PTEN and TIMP3	(18-20)
	miR-21	PDCD4, PTEN, RHOB, RECK and TIMP3	(21-24)
	miR-15a/miR-16-1	BCL2, CCND1, CCND3, CCNE1, CDK6, VEGF, FGF2 and FGFR1	(25-30)
	miR-143/145	RAS, c-Myc, BNIP3, FSCN1, OCT4, SOX2 and KLF4	(31-36)
TsmiRs	miR-200 family and miR-205	ZEB1 and ZEB2	(37)
	miR-101	EZH2	(38)
	miR-449a	HDAC-1	(39)

TsmiRs may inhibit cancers by suppressing oncogenes. Similar to protein-coding tumor suppressor genes, they are frequently deleted, mutated, or methylated in many human tumors (12,42). miR-15a and miR-16-1, located at human chromosome 13q14 and transcribed as a cluster, are the first identified tumor-suppressor miRNAs. Deletion or mutation of 13q14 locus was observed in many solid tumors and chronic lymphocytic leukemia (CLL) (12). Initially, miR-15a and miR-16-1 were found to be able to induce cell apoptosis by suppressing anti-apoptotic factor BCL2 in chronic lymphocytic leukemia (CLL) cells (25). Later studies showed that this cluster of miRNAs can also inhibit cell proliferation, induce apoptosis and suppress tumorigenicity by targeting multiple additional oncogenes in several signaling pathways, such as cell-cycle regulators (CCND1, CCND3, CCNE1 and CDK6) (26,27) and angiogenic factors and their receptors (VEGF, FGF2 and FGFR1) (28-30).

miR-143/145 is another important tsmiR cluster and is involved in suppressing the RAS and c-Myc signaling pathways (31,32). Systematic analysis of miRNA expression profile revealed that downregulation of miR-143 and miR-145 was associated with aggressive phenotype (43) in prostate cancer patients. BCL2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) is widely upregulated in human tumors and miR-145 negatively regulates BNIP3 by targeting its 3'-UTR. Artificial restoration of miR-145 reduced cell growth in prostate cancer cells (33). In bladder tumor tissues, miR-145 is one of the most underexpressed miRNAs (44) and is capable of inhibiting tumor cell growth and invasion by targeting FSCN1 *in vitro* (34). Interestingly, three key pluripotency genes (OCT4, SOX2 and KLF4) essential for stem cell fate decision were demonstrated to be direct targets of miR-145 in both human embryonic stem cells (35) and cancer cells (36).

Beyond their role in cell growth control, miRNAs can also affect tumorigenesis and cancer progression via additional signaling pathways. For instance, miR-200 family and miR-205 which are frequently downregulated in invasive bladder and prostate cancer act as Epithelial-mesenchymal transition (EMT) repressors by targeting ZEB1 and ZEB2 (37). Moreover, miRNAs have been implicated in the regulation of epigenetics. Loss of miR-101 and miR-449a in prostate cancer cells led to overexpression of histone methyltransferase EZH2 (38) and histone deacetylase HDAC-1 (39), respectively.

Several studies have revealed that tumor suppressor factors (i.e. tumor suppressor genes, tsmiRs) and oncogenic factors (i.e. oncogenes, oncomiRs) are exquisitely regulated in organisms. On one hand, miRNAs are critical downstream effectors of classic oncogene/tumor suppressor networks. For examples, the transcription of miR-17-92 cluster and miR-34 family is directly activated by c-Myc and p53, respectively (45,46). And p53 can also module the biogenesis of miR-16-1, miR-143 and miR-145 (32,47). On the other hand, miR-145 suppresses c-Myc via targeting its 3'UTR (32), while miR-25 (a homologous miRNA of the miR-17-92 cluster) inhibits p53 by its 3'UTR (17).

Supplementation of tsmiRs or inhibition of oncomiRs by antagomirs could be a new class of targeted molecular therapy. For instance, miR-15a and miR-16 are significantly decreased in cancer cells of advanced prostate tumors. Delivery of antagomirs specific for miR-15a and miR-16 to normal mouse prostate caused hyperplasia, but reconstitution of miR-15a and miR-16-1 expression led to growth arrest, apoptosis and regression of prostate tumor xenografts (27). Another independent study also reported that systemic delivery of synthetic miR-16 via tail vein injection significantly inhibited the growth of metastatic prostate tumors in a prostate cancer xenograft model in nude mice (48).

The application of miRNA in andrology

Although miRNAs have been intensively studied in cancer, their physiological roles in andrology are much less clear. Some recent works suggest that miRNAs may have regulatory role in testicular and epididymal development and spermatogenesis. A systematic cDNA array and miRNA array analysis showed that neonatal epididymis expressed more miRNAs than that of young adults and aged men. Some of the miRNAs expressing high in young adults include miR-143, miR-7a, miR-21, miR-23a, miR-24, miR-27a/b and miR-29a (49). In young adults, male infertility may be associated with dysexpression of certain miRNAs. Compared with fertile males, miR-34c-5p, miR-122, miR-146b-5p, miR-181a, miR-374b, miR-509-5p and miR-513a-5p are dramatically decreased in azoospermia patients, but increased in asthenozoospermia (50). Another work performed in non-obstructive azoospermia patients identified 19 differentially up-regulated and 154 downregulated miRNAs. Among the downregulated ones are some testicular miRNAs (miR-181a, miR-29c, and miR-34b*) and several members of the miR-17-92 cluster (51). It is interesting that miR-17-92 cluster is highly expressed in primordial germ cells and spermatogonia during development of mouse germ cells (52). It is known that single-nucleotide polymorphisms (SNPs) inside miRNA target sites (miR-TS-SNPs) may influence miRNA biogenesis and susceptibility to tumorigenesis (53). Recently, these SNPs were found to be implicated in male infertility. Some fertility related genes contain miR-TS-SNPs, and the most polymorphic were identified in the 3'UTR of KITL, ACTB, ACE, CAMK4, ESR1 and MTHFR (54). Ogorevc *et al.* also analyzed the relationships between selected infertile candidate genes and dysexpressed miRNAs in non-obstructive azoospermic patients and found 3 upregulated and 10 downregulated miRNA contained miR-TS-SNPs (54). These works suggested that miRNA expression profiles may serve as noninvasive molecular markers for male infertility diagnosis.

piRNA and its applications

piRNA and its biological functions

Piwi-interacting RNA (piRNA) is a class of endogenous single-stranded small RNAs (24-30 nt) found in both vertebrates and invertebrates. Distinct from miRNAs, piRNAs are transcribed from specific genomic regions containing repetitive elements, such as transposable

elements, and processed into its mature form by a Dicer-independent pathway. At the assistance of Piwi and Aub proteins, piRNA is implicated in transposon silencing at both the transcriptional and post-transcriptional levels (55). Opposite to the well known piRNA-mediated silencing, piRNA is also able to function as an epigenetic activator. 3R-TAS1 piRNA, a piRNA transcribed from a region of the telomere-associated sequence (TAS) on the right arm of chromosome 3 (3R-TAS) in *Drosophila*, promotes the euchromatic character of 3R-TAS heterochromatin and its transcriptional activity (56).

The application of piRNA in andrology and urology

Different from miRNA's ubiquitous expression in different tissues and organs, piRNA is specifically present in spermatocytes and spermatids during spermatogenesis (57). Thus, it is believed that piRNA plays an essential role in germline development. In mice, PIWI-like proteins subfamily consists of MIWI, MIWI2, and MILI, and deficiencies of these members cause aberrant male germ cell development. MIWI-deficient mice display spermatogenic arrest at the beginning of the round spermatid stage (58). Spermatogenesis in the MILI-null mice is blocked at the early prophase of the first meiosis (59). MIWI2-deficient mice display a defect in early prophase of meiosis I and a marked and progressive loss of germ cells with age (60). Despite considerable knowledge has been gained about the function of piRNA in *Drosophila* and mice, their function in human is obscure. Recently, nine SNPs of four human Piwi genes (PIWIL1/HIWI, PIWIL2/HILI, PIWIL3/HIWI3 and PIWIL4/HIWI2) were identified in patients with idiopathic azoospermia or oligozoospermia. Among them, an SNP in the 3'UTR region of HIWI2 and a non-synonymous SNP in HIWI3 were significantly associated with an altered risk of oligozoospermia (61). Furthermore, Hiwi (human Piwi ortholog) has been found to be aberrantly expressed in a variety of human cancers (62) and its overexpression correlates with poor clinical prognosis of soft-tissue sarcoma patients (63), while there has been no report of its association with urological cancers.

siRNA and its applications

siRNA and its biological functions

Small interfering RNA (siRNA) is a class of 21- to 23-nt double-stranded RNAs (dsRNAs) endogenously generated

Table 3 The application of siRNA in urology and andrology

Disease	Target gene	Trigger RNA	Applications	References
Prostate cancer	Survivin	shRNA	<i>In vitro, in vivo</i> (xenograft)	(67)
	Stat3	shRNA	<i>In vitro, in vivo</i> (xenograft)	(68)
	FOXO3/ <i>Foxo3</i>	siRNA	<i>In vitro</i>	(69)
	Integrin αv	siRNA	<i>In vitro, in vivo</i> (xenograft)	(70)
	PLK1 and BCL2	aptamer-siRNA chimeras	<i>In vitro, in vivo</i> (xenograft)	(71)
Bladder cancer	Survivin	siRNA	<i>In vitro, in vivo</i> (xenograft)	(72)
	BCL2	siRNA	<i>In vitro</i>	(73)
	EphB4	siRNA	<i>In vitro, in vivo</i> (xenograft)	(74)
	PLK1	siRNA	<i>In vitro, in vivo</i>	(75)
Renal cancer	SOCS3	siRNA	<i>In vitro, in vivo</i> (xenograft)	(76)
	PLK1	siRNA	<i>In vitro</i>	(77)
	Survivin	siRNA	<i>In vitro</i>	(78)
Erectile dysfunction	PDE5/ PDE5	shRNA/siRNA	<i>In vitro, in vivo</i>	(79-81)
	<i>PIN</i>	shRNA/siRNA	<i>In vitro, in vivo</i>	(82)
	CX43	shRNA	<i>In vitro</i>	(83)

or artificially designed and is the trigger of the well-known RNA interference (RNAi) mechanism. RNAi can be induced by exogenously introduced dsRNAs (64), or endogenous dsRNAs (65). In addition, plasmid-expressed short hairpin RNA (shRNA) can also be used to silence gene expression via RNAi pathway (66). It is well known that many human diseases are caused by abnormal overproduction of specific gene products, such as oncogenes. Therefore, siRNA or shRNA targeting disease-causing gene is promising therapeutic for many diseases (Table 3).

The application of siRNA in urological cancers

Apoptosis (programmed cell death) is a crucial biological process in cancer initiation and progression. Cancer cells overexpress many anti-apoptotic genes (i.e. survivin, BCL2, etc) or underexpress pro-apoptotic genes (i.e. caspase family) to maintain aggressive cell proliferation. Resisting cell death caused by dysregulation of apoptosis related genes is one of the fundamental hallmarks of cancer and is a major target for cancer therapy (84). Abnormal upregulation of survivin may inhibit caspase activity and enable cancer cells to escape programmed cell death and promote resistance to radiation and chemotherapy. Vector-based siRNA expression effectively suppressed survivin expression and led to decreased tumor formation in nude mice bearing prostate cancer xenografts and enhanced chemosensitivity (67).

Knockdown of survivin Null by siRNA in bladder cancer cells also suppressed their growth *in vitro* and *in vivo* (72). Similarly, BCL2 downregulation by siRNA enhanced mitomycin C induced apoptotic cell death in bladder cancer cell lines (73). Stat3 belongs to the STAT transcription factor family and functions as an oncogenic transcription factor by promoting proliferation, anti-apoptosis and cell cycle progression. RNAi of Stat3 led to downregulation of its downstream genes including BCL2, cyclin D1 and c-Myc in prostate cancer cell lines and in tumors implanted in nude mice (68). EphB4 is a receptor protein tyrosine kinase aberrantly expressed in bladder cancer cell lines and tumor specimens. EphB4 knockdown *in vitro* using siRNA suppressed cell viability by inducing apoptosis via activation of caspase-8 pathway and by inhibiting anti-apoptotic factor, Bcl-xl. EphB4 siRNA delivered into tumors was able to alleviate xenograft tumor burden *in vivo* (74).

Overactivated mitosis is another essential cause for the aberrant proliferation signaling in cancer cells. Polo-like kinase-1 (PLK1) is a critical regulator of mitotic progression. Elevated PLK1 expression in patients with bladder cancer and renal cell carcinoma is frequently associated with poor prognosis. siRNA targeting PLK1 could disrupt cell mitosis and cell cycle progression and thus reduced cell proliferation in bladder cancer and renal cancer cells (75,77). In an orthotopic bladder cancer mouse model, PLK1 siRNA delivery successfully prevented bladder cancer

cell growth (77).

Evading immune destruction is also one of the hallmarks of cancer development and progression (84). Interferons are essential parts of the primary immune system by effectively triggering protective response, such as activating natural killer cells and macrophages, and facilitating antigen presentation to T lymphocytes. SOCS3 have been identified as an inhibitor of the IFN-mediated JAK/STAT signaling pathways. Suppression of SOCS3 by siRNA promoted IFN- α -induced cell death in renal cell carcinoma xenografts in nude mice (76). Dendritic cells (DCs) are the most potent antigen-presenting cells, while DCs can also induce tolerance, rather than immune activation. In tumors from human prostate cancer patients and transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, tumor-associated dendritic cells (TADCs) possess increased FOXO3/*Foxo3* expression resulting in suppressed T cell function. Silencing FOXO3/*Foxo3* with siRNAs could block its inhibitory effect on T cells, promote expression of costimulatory molecules and proinflammatory cytokines, and diminish expression of tolerogenic factors (69).

High pathological grades of malignancy is usually reflected in local invasion and distant metastasis. Integrins are one of major cell adhesion components, which can determine cells fate by controlling cell replication, migration and differentiation, especially in cancer metastasis (41). siRNA targeting Integrin α_v , an ECM receptor gene, inhibited growth of human prostate cancer in a bone xenograft imaging model (70).

Clinical application of siRNA for cancer therapy highly depends on the efficiency and cell-type specificity of siRNA delivery. Sato *et al.* reported that Topotecan, a topoisomerase I inhibitor able to enhance survivin siRNA, caused renal cancer cell growth suppression by increasing cellular uptake of the siRNA (78). Furthermore, siRNAs targeting PLK1 and BCL2 were linked to an aptamer which specifically recognized prostate specific membrane antigen (PSMA) could inhibit tumor growth and mediate tumor regression in a xenograft prostate cancer model, by selectively binding to prostate cancer cells and tumor vascular endothelium (71).

The application of siRNA in andrology

Erectile dysfunction (ED) is a common andrological disorder, especially in aged men or men with diabetes. During physiological process of erection, nitric oxide (NO) release triggers the accumulation of cyclic guanosine

monophosphate (cGMP) in cavernous smooth muscle cells (CSMCs), which subsequently induces increased blood flows into the corpus cavernosum and finally stimulates an erection by loosing smooth muscle cells. Dysregulation of NO synthesis and cGMP accumulation may result in erectile dysfunction (ED). Phosphodiesterase type 5 (PDE5) can catalyze the degradation of cGMP and suppress erectile function. Lin *et al.* reported that a lentiviral vector-based siRNA expression caused the knockdown of *PDE5* and prolonged accumulation of cGMP in cultured rat CSMCs. Injection of the lentiviral vector into rat penis significantly enhanced erectile function (79). Synthetic siRNA or shRNA vector-mediated PDE5 silencing also led to enhanced cGMP in cultured human CSMCs (80,81). siRNA or shRNA-mediated knockdown of *PIN* (protein inhibitor of NOS), a key repressor of nNOS (neuronal nitric oxide synthase), elevated cGMP concentration *in vitro* and improved erectile dysfunction in aged rats (82). Additionally, aberrant upregulation of CX43 (connexin43) is usually associated with ED. CX43 siRNA could decrease GJIC (gap junction intercellular communication) in cultured human CSMCs (83). Taken together, siRNA may have a therapeutic application in ED treatment; however, delivery of siRNA into corpus cavernosum tissue may be challenging and awaits further development.

saRNA and its applications

SaRNA and its biological functions

RNA activation (RNAa) is a newly discovered mechanism of gene regulation triggered also by small dsRNA that targets gene promoter regions instead of coding sequences. These promoter-targeted dsRNAs that can activate gene expression are referred to as small activating RNA (saRNA) (2,4). saRNA has been shown to activate the expression of endogenous genes (2-4), presenting a novel and natural tool of overexpressing functionally important genes for disease treatment. Vector-based systems offer robust gene overexpression and are being tested as a promising gene therapy strategy for the treatment of a variety of diseases. However, serious concerns of insertional mutagenesis in clinical settings are associated with the use of viral vectors. As an alternative, RNAa may offer a safer approach for gene overexpression and disease treatment. Several recent studies have shown that saRNA-mediated gene activation can suppress cancer cell growth by upregulating tumor suppressor genes and improve smooth muscle function by

Table 4 The application of saRNA in urology and andrology

Disease	Species	Gene	Trigger RNA	Applications	References
Prostate cancer	Human	p21	saRNA	<i>In vitro, in vivo</i> (xenograft)	(2)
	Human	E-cadherin	saRNA and miRNA	<i>In vitro</i>	(2,6,85)
	Human	KLF4	saRNA	<i>In vitro</i>	(86)
	Human	NKX3.1	saRNA	<i>In vitro, in vivo</i> (xenograft)	(87)
	Mouse	<i>Ccnb1</i>	saRNA and miRNA	<i>In vitro, in vivo</i> (xenograft)	(87,88)
Bladder cancer	Human	p21	saRNA	<i>In vitro, in vivo</i> (xenograft)	(89,90)
	Human	E-cadherin	saRNA	<i>In vitro</i>	(91)
Renal cancer	Human	p21	saRNA	<i>In vitro</i>	(92)
ED	Human	VEGF	saRNA	<i>In vitro</i>	(93)

activating VEGF gene (Table 4).

The application of saRNA in urological cancers

It is well known that tumorigenesis and cancer progression correlates with inactivation of tumor suppressor genes. p21 is a cyclin-dependent kinase inhibitor that functions as a key mediator of cell-cycle arrest and down-regulation of p21 is frequently observed in different types of cancers. Several studies showed that restoration of p21 expression by saRNA can inhibit cell proliferation, cell cycle progression, and induce apoptosis in prostate, bladder and renal cancer cells (2,89,90,92). It is believed that EMT plays an important role in the initiation and progression of cancer. E-cadherin, a key mediator in EMT signaling, can serve as a potent inhibitor of cancer cell growth and invasion (84). Overexpression of E-cadherin by saRNAs led to inhibited cell proliferation and suppression of migration and invasion of prostate and bladder cancer cells (2,85,91). NKX3-1 is a prostate specific tumor suppressor gene and could be activated by its promoter-targeted saRNA (87).

Recently, our group has utilized RNAi as a laboratory tool to investigate the function of KLF4, a member of the Krüppel-like family of transcription factors, in prostate cancer cells in which KLF4 expression is significantly downregulated (86). *In vitro* studies indicated that saRNA-mediated overexpression of KLF4 inhibited prostate cancer cell proliferation and survival, and altered the expression of its downstream cell-cycle-related genes including p21, p27, p57 and CCNB1. Reactivation of KLF4 by saRNA also suppressed migration and invasion of prostate cancer cells (86).

It is largely recognized that miRNA functions as a silencer of gene expression. Our recent work indicates that miRNAs may also positively-regulate gene expression

by targeting promoter sequences and thus act as saRNA. In human prostate cancer PC-3 cells, introduction of pre-miR-373/miR-373 could activate E-cadherin and CSDC2 (cold-shock domain-containing protein C2) (6). Cyclin B1 is an essential protein that drives cell cycle entry into mitosis and is a cancer antigen highly expressed in a majority of human cancer (88). In mouse prostate cells (i.e. TRAMP C1), we recently identified three miRNAs (miR-744, miR-1186 and miR-466d-3p) that target the cyclin B1 promoter to induce *Ccnb1* expression in a physiological context. Short-term overexpression of miR-744 and miR-1186 resulted in enhanced cell proliferation, while long-term expression caused chromosomal instability and tumor suppression *in vivo* (88). This study thus provided the first example that RNAi mechanism functions as an endogenous cellular process and is exploited by cancer cells to gain a growth advantage.

The application of saRNA in andrology

Vascular endothelial growth factor (VEGF) is a well-known cytokine with strong angiogenic properties and can also stimulate cell proliferation, delay senescence, suppress apoptosis and promote nerve regeneration. Vector-based overexpression of VEGF has been shown to improve erectile function (94). Our group identified a saRNA with the capacity to activate VEGF in human (2) and nonhuman primates cells (87). Very recently, this saRNA was demonstrated to induce endogenous VEGF expression in primary human corpus cavernosum smooth muscle cells (CCSMCs) *in vitro* (93). Delivering an saRNA targeting mouse VEGF by lentivirus into mice resulted in improved vascularity and blood flow in an ischemic mouse hindlimb model (95). These studies imply that saRNA-mediated

VEGF upregulation may present a new avenue for treating ED and other vascular disorders.

Prospectives

Despite tons of efforts have been made to develop small RNA-based therapeutics, the scientific and pharmaceutical communities are still facing significant hurdles in bringing small RNA drugs (ribodrugs) to the clinic. Among them, efficient delivery to target tissue and cells is the greatest challenge (96). Recently, promising results have been reported from the first-ever phase I clinical trial of siRNA delivered by nanoparticles for treating melanoma (97). Many other clinical trials of siRNA-based drugs for a large array of diseases are ongoing with some having advanced into phase II. With the rapid progression in the development of small RNA delivery technology, we believe that in the future not far away from now ribodrugs will be an indispensable weapon in the arsenal of urologists and andrologists for fighting against a variety of diseases.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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