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The Varied Roles of Nuclear Argonaute-Small RNA Complexes and Avenues for Therapy

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Argonautes are highly conserved proteins found in almost all eukaryotes and some bacteria and archaea. In humans, there are eight argonaute proteins evenly distributed across two clades, the Ago clade (*AGO1-4*) and the Piwi clade (*PIWIL1-4*). The function of Ago proteins is best characterized by their role in RNA interference (RNAi) and cytoplasmic post-transcriptional gene silencing (PTGS) – which involves the loading of siRNA or miRNA into argonaute to direct silencing of genes at the posttranscriptional or translational level. However, nuclear-localized, as opposed to cytoplasmic, argonaute-small RNA complexes may also orchestrate the mechanistically very different process of transcriptional gene silencing, which results in prevention of transcription from a gene locus by the formation of silent chromatin domains. More recently, the role of argonaute in other aspects of epigenetic regulation of chromatin, alternative splicing and DNA repair is emerging. This review focuses on the activity of nuclear-localized short RNA-argonaute complexes in a mammalian setting and discusses recent *in vivo* studies employing nuclear-directed sRNA for therapeutic interventions. These studies heed the potential development of RNA-based drugs which induce epigenetic changes in the cell.

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Introduction

The argonautes are a family of highly evolutionarily conserved proteins that bind small-RNAs (sRNA) and have essential roles in the RNA interference and microRNA pathways (reviewed in ref. 1). Argonaute proteins are broadly classified into two main evolutionary clades; the Ago clade binds small ~20–30 nt non-coding RNAs which include microRNAs (miRNAs) and small interfering RNAs (siRNAs), while the Piwi clade Ago binds P-element induced wimpy testis (PIWI)-interacting RNAs (piRNAs). Wago, a third clade, is particular to nematode worms.[2] Argonautes are characterized by a Piwi-Argonaute-Zwille (PAZ) domain and a PIWI domain. This PAZ domain functions by binding specifically to the 3′ end of single-stranded RNA and is also found in the Dicer enzyme.[3] The Piwi pathway is thought to be confined to regulating RNA expression in germ cells (reviewed in ref. 4). The Ago pathway is ubiquitously expressed and in mammals is composed of four enzymes, Ago1, Ago2, Ago3, and Ago4 which are all capable of loading small RNA. This review will focus on the Ago pathway.

Ago2 is well studied as it is the "catalytic engine" that drives mRNA cleavage at miRNA target sites[5,6] and is required for embryonic development.[5,7] Ago2 is best characterized in its role in post-transcriptional gene silencing (PTGS). PTGS is a cytoplasmic process which involves the loading of the guide strand of the ~20–24 nt sRNA species, siRNA or miRNA, into the Ago2-containing RNA induced silencing complex (RISC), which can then recognize a complementary mRNA target and bind to inhibit translation or destabilize through the Ago2 endonuclease activity (reviewed in ref. 8). While PTGS was first characterized as a genome/viral defence, there are many small endogenous RNAs, in particular, miRNAs and endo-siRNA in mammalian embryonic stem cells.[9] siRNA are generated by Dicer RNase cutting of long dsRNA, such as that produced in virus replication, while miRNA are processed from nuclear hairpin dsRNA into short hairpin pre-miRNA by DGCR8 and Drosha, an RNAse III–type endonuclease, with subsequent transport to the cytoplasm and further cleavage by Dicer (reviewed in ref. 10) (**Figure 1a**). Except, in the case of miR-451, as its pre-miRNA is processed directly by Ago2 instead of Dicer.[11,12] The loading of the sRNA guide strand into Ago requires the Dicer and TRBP containing RISC, with subsequent activation requiring the C3PO complex for efficient passenger strand removal.[13,14] In humans, it is thought C3PO may be the sole loader and activator of Ago2.[15]

While Ago2 is well studied, much less is known about the function of the other argonautes. Ago1 is 80% identical to Ago2 but lacks a key catalytic residue and cannot cleave RNA efficiently. It is associated with the loading of specific sRNAs derived from the Epstein–Barr virus[16] and Ago1 and/or Ago3 is required for optimal resistance to influenza-A in mice.[17] Little is known about the function of Ago1 except that its overexpression slows neuroblastoma growth.[18] Ago3, like Ago2, contains the catalytic residues essential for cleavage and is required to induce human embryonic stem cell proliferation arrest through binding of sRNA generated from transcribed *Alu* repeats and subsequent PTGS of critical stem cell mRNAs.[19] Ago4 has been reported to localise to mouse spermatocyte nuclei during meiotic prophase and regulate meiotic entry.[20]

Sequencing or microarray analysis of endogenous sRNA bound to human Ago1, Ago2 and Ago3 shows that the great majority is miRNA, which has been loaded with no particular strand bias towards the pre-miRNA 3p (antisense) or 5p (sense) strand.[21–23] However, those miRNA preferentially

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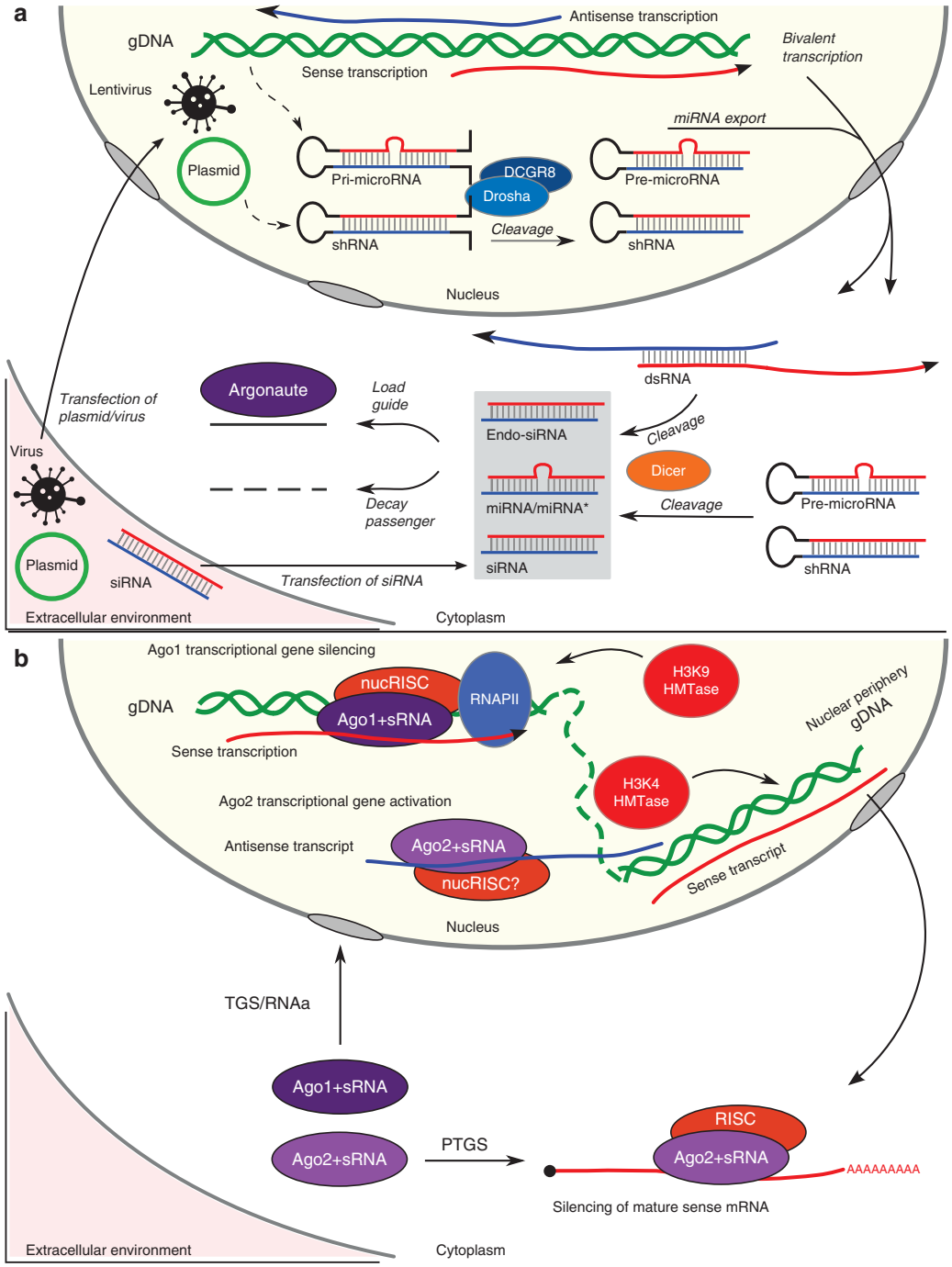


Figure 1 The role of argonaute in transcriptional gene silencing (TGS) and activation (RNAa). (**a**) The dicer-generated small RNA (sRNA) species which are loaded into argonaute (gray box) can be derived from shRNA expressed from plasmids or viral vectors transfected into the cell, or from endogenous processes, such as miRNA biogenesis or dsRNA resulting from bivalent transcription. Alternatively, siRNA directly transfected into the cell may be loaded. The sRNA species have a single "guide" strand loaded into argonaute while the other "passenger" strand is degraded. (**b**) Loaded Ago1 (Ago1+sRNA) may enter the nucleus and together with a nuclear RISC (nucRISC) and in association with active RNAPII and sense transcripts, initiate chromatin remodelling and transcriptional gene silencing (TGS) by such enzymes as histone 3 lysine 9 methyltransferases (H3K9 HMTase). The loaded Ago2 (Ago2+sRNA) is catalytically active and may direct the silencing of mature mRNA in the cytoplasm via the post-transcriptional gene silencing (PTGS) pathway, or it may be migrate to the periphery of the nucleus and (perhaps in association with a nucRISC), degrade primarily antisense transcripts. This process is associated with remodeling to active chromatin by histone 3 lysine 4 histone methyltransferases (H3K4 HMTase) and subsequent transcriptional gene activation (RNAa). There is also evidence nuclear Ago2 is associated with TGS.

loaded into Ago1 or Ago2 had distinct antisense and sense bias, respectively.[23] Other endogenous sRNA are also bound to Ago, as well as sRNA from intronic and exonic coding gene regions and promoter regions of coding genes.[24] Many of these sRNA species were longer than the 21 nt canonical length, in particular promoter-derived RNAs were mostly of 21–24 nucleotides in length and mostly associated with Ago1 and Ago3. The differential loading between Ago1 and Ago2 might be due, in part, to editing of the 3′-end, with miRNA terminating in 3′ adenine and uracil preferentially loaded into Ago1 and Ago2, respectively.[22] This differs from piRNA loaded into PIWI-clade Ago, as these are 2′-O-methylated at the 3′ termini and often include uracil at the 5′ end.[25–27] Recent findings show that in Ago1-4 knockout mouse embryonic stem cells, expression of inducible Ago2 confers miRNA stability.[28] Interestingly, this same work unveiled a new class of Ago/Dicer-dependent miRNAs which arise from the transcription start site (TSS-miRNA) of RNAPII protein-coding gene promoters.

Function in the Nucleus

Over the last decade or so, Ago has been recognized as having a role in the nucleus. RNA-induced silencing complexes (RISCs) containing Ago1 and Ago2 are present in both the cytosolic and nuclear fractions of human cells[29–32] and Ago1 and Ago2 are known to associate with promoter DNA.[33] Argonaute–RNA complexes can regulate nuclear events such as transcriptional silencing and activation as well as alternative splicing and DNA repair.[34–36] Nuclear Ago1 directly interacts with RNA polymerase II (RNAPII)[30,37,38] and binds to the promoters of actively transcribed genes.[30,38] There are two contrary reports that describe human nuclear Ago2 as part of a multiprotein complex together with Dicer, TRBP and TRNC6A/GW182,[39] or conversely in solitary form.[40] However, both of these studies agree that Ago2 is loaded in the cytoplasm and imported into the nucleus (**Figure 1b**).

Using immunofluorescence microscopy, two groups have shown that tagged Ago1 and Ago2 have different nuclear distributions. Ago1 is scattered throughout the nuclear interior, whereas Ago2 co-localizes with siRNA primarily in the inner nuclear envelope.[30,41] ChIP-seq data shows that Ago1 is associated with thousands of chromosomal loci throughout the genome, but in particular with the promoters of actively transcribed genes and distributed in highly punctate peaks mostly overlapping with histone 3 lysine 4 tri-methylation (H3K4me3).[30] In the same study, there was no evidence to suggest Ago2 interacts with chromatin. Sequencing of sRNA bound to argonaute in whole-cell lysate from mouse T cells, shows that ~0.02% of reads map to promoter regions, with twofold more bound to Ago1 and Ago3, than to Ago2. Around 0.2% of Ago1 tags mapped to coding regions, which was six- and twofold more than Ago2 and Ago3, respectively. Over 80% of tags mapping in the antisense direction were bound to Ago1, while for sense tags, the split was closer across Ago proteins.[22]

Dicer also has a nuclear localization signal, in the dsRNA binding domain[42] and there is clear evidence it is nuclear-imported. Nuclear Dicer has been variously reported as located in the nuclear periphery in association with NUP153, a component of the nuclear pore complex,[43] or spread throughout the nucleoplasm with a punctate distribution.[44] Nuclear Dicer is associated with RNAPII at actively transcribed genes and is required for Ago1-mediated TGS.[44] Furthermore, nuclear Dicer is catalytically active and will cleave target RNA,[29,39,44,45] with knockdown resulting in accumulation of endogenous double-stranded RNA (dsRNA), induction of the interferon-response pathway and cell death.[44] Dicer is known to be important for the epigenetic regulation of the cell as its activity is required for the correct formation of heterochromatin structure in mammalian cells.[46]

Collectively, evidence suggests nuclear Ago1 binds mostly antisense sRNA and is distributed throughout the nucleus at promoters of active genes in association with RNAPII and Dicer, while nuclear Ago2 complexes are at the nuclear periphery and contain both sense and antisense sRNA.

Exogenous Small RNA Transcriptional Silencing and Activation

RNA-directed transcriptional gene silencing (TGS) is an important means of regulation, evident by the conservation of mechanism from yeast to plants to higher mammals (reviewed in ref. 47). The phenomenon of mammalian TGS was first reported by Morris *et al*. in 2004.[48] They described transcriptional silencing of both endogenous and integrated proviral elongation factor 1-alpha 1 (*EEF1A1*) by delivery of a siRNA targeting the promoter region. This study was closely followed by those from several other groups also reporting TGS driven by promoter-directed siRNA targeting the HIV-1 viral LTR,[49] *CDH1*,[50] *RASSF1*,[51] *PGR*, *MVP*, both *AR* and *PTGS2* (ref. 52) and *NOS3*.[53] The phenomenon has continued to be observed and clarified (**Table 1**).[29,33,37,54–72]

The counter-phenomenon of mammalian sRNA-induced transcriptional gene activation (RNAa) was reported in 2006 by Li *et al*.[73] who observed that transfecting siRNA complementary to the promoter regions of *CDKN1A*, *CDH1*, and *VEGFA* resulted in increased transcription of the respective gene. Unlike the early TGS studies, their promoter-directed dsRNA designs avoided promoter CpG-rich regions. These observations were soon supported by work from two other groups that reported RNAa of the HIV-1 viral LTR[74] and *PGR*,[75] respectively. RNAa resulting from a single transfection could be observed for between 9 and 13 days.[73,75] Paradoxically, Janowski *et al*. showed that 21-mer ds siRNA designed against the progesterone receptor (*PGR*) as little as a few nucleotides apart could profoundly affect the activation potential and in some cases, cause TGS instead of RNAa.[75] Subsequently, there are many more reports of small-RNA directed RNAa, including by groups also demonstrating TGS (**Table 2**).[29,51,54,64,65,72,76–89]

Results showing suppression or activation of transcription by promoter-directed siRNA need to be interpreted with some caution. It is possible that alteration of observed transcriptional levels attributed to RNAa or TGS may in fact be the result of sequence-specific off-target effects. These off-target effects have been reported for siRNA designed against the *VEGFA* promoter[90] and the HIV-1 LTR promoter.[74]

Table 1 List of transcriptional gene silencing (TGS) studies

Study	Gene symbol	Cell line	Effector name	Effector type	Transfection	Location (wrt TSS)[a]	Year	Citations[b]	Reference
1	CCR5/RASSF1	HEK293T	R61	ds siRNA	MPG	NS	2006	247	37
2	CDH1	HCT-116/MCF-7	dsCDH1-1, 2	ds siRNA	Lipid	NS	2005	193	50
3	CDKN1A	MCF-7	Bx332409	ds siRNA	Lipid	NS	2008	133	54
4	CDKN2A	HEK293T	p16siRNA	U6shRNA-cassette	Calcium phosphate	NS	2007	16	55
5	EEF1A1	293FT	EF52	ds siRNA	MPG	–106	2004	570	48
6	EEF1A1	Many	EF52	ds siRNA	Lipid	–106	2007	160	56
7	EEF1A1/HIV-1 (Viral LTR)	HEK293T	EF52/siRNA-247	ds or ss siRNA	MPG	–125/viral 247	2006	151	57
8	HIV-1 (Viral LTR)	TZM-bl	247as, 362as	antisense-RNA	Lipid	NS	2009	27	58
9	HIV-1 (Viral LTR)	MAGIC-5	HIV-prom-A	siRNA	Lipid	–100	2005	74	49
10	HIV-1 (Viral LTR)	MAGIC-5	HIV-prom-A	ds siRNA	Electroporation	–100	2008	46	59
11	HIV-1 (Viral LTR)	Molt-4	shKB	U6shRNA	Retrovirus	NS	2009	25	60
12	HPA	PC3/EJ/SGC-7901	siH3	siRNA, shRNA	Lipid	–9	2012	15	61
13	MSTN	Mouse C2C12	siMstn-P2	siRNA	Lipid	–153	2012	3	62
14	MYC	PC3, DU145, LNCaP	myc13	ds siRNA	Lipid	–13	2009	58	63
15	NOS3	HAEC	"microRNA"	27mer-siRNA	Lipid	Intron-4	2005	74	53
16	PGR	T47D	PR9	ds siRNA	Lipid	–9	2008	121	64
17	PGR	T47D	PR9	ds siRNA	Lipid	–9	2010	41	29
18	PGR	T47D	PR13580	ds siRNA	Lipid	+13580	2010	34	65
19	PGR, AR, HTT	T47D/MCF-7	Many	ds siRNA	Lipid	Many	2006	176	33
20	PGR, IGSF1	T47D/MCF-7	miR-423-5p	ds miRNA	Lipid	–59	2011	41	66
21	PGR, MVP, AR, PTGS2	T47D	Many	ds siRNA	Lipid	Many	2005	98	52
22	PLAU	PC3/DU145	siuPA pool of 4	ds siRNA	Lipid	–213, –131, –111, –50	2007	50	67
23	POLR3D	HEK293	miR-320	ds miRNA	Lipid	–132	2008	254	68
24	RASSF1	HeLa	ShPr21	U6shRNA	Vector	–28	2005	94	51
25	SIV (Viral LTR)	MAGIC-5, CEMx174	Many	ds siRNA	Lipid	NS	2008	20	69
26	TGFbRII	Rat SBC10	siRNA-p-412	U6shRNA	Vector	–412	2007	23	70
27	UBC	HEK293T	UbC167	ds siRNA	Lipid	–167	2009	86	71
28	VEGFA	C166	LV-856	U6shRNA	Retrovirus	NS	2009	43	72

[a]5′-most location with respect to the transcriptional start site of the target. A negative number implies an upstream location and "NS" denotes the location was not stated. [b]Citations according to Web of Science (July 2014).

Requirement for Argonaute in Nuclear Processes

Knockdowns of argonaute protein have shown it is required for small-RNA induced TGS or RNAa (**Tables 1** and **2**). Some controversy exists about whether Ago1 or Ago2 is the main argonaute recruited to enact TGS. The first studies reported Ago1 as the key argonaute driving TGS, while some latter studies show evidence that only knockdown of Ago2 resulted in abrogation of TGS, with knockdown of Ago1, Ago3, or Ago4 not overly reducing the effect. In an early study, the Corey group reported that both Ago1 and Ago2 were required for TGS,[33] however in later work they rationalized that off-target knockdowns may explain their earlier results.[29] In the case of RNAa, fewer studies have examined the requirement for argonaute; however, there is no disagreement; all suggest that only Ago2 is required (**Figure 2**).

Association with Transcription, Epigenetic Regulation, and RISC Subunits

Early models speculated that siRNA might directly base-pair with DNA and recruit DNA methyltransferase (DNMT), but work by ourselves and others has shown that DNMTs cannot methylate DNA:RNA hybrid structures[91] and that instead, Ago-loaded nuclear siRNA binds to nascently transcribed RNA.[56,64] There are a number of reports that TGS requires active transcription by RNAPII and expression of sense-strand mRNA through the gene promoter.[56,57,63,71] However, other data shows that Ago1 and Ago2 bind to antisense transcripts during TGS.[29] Interestingly, transfection of only the siRNA anti-sense strand is sufficient to silence the *EEF1A1* gene.[56,57] Matsui *et al.*[89] report Ago2-dependent transcriptional gene activation of *PTGS2* by the endogenous miRNA, miR-589. As only one strand of miR-589 is complementary

Table 2 List of transcriptional gene activation (RNAa) studies

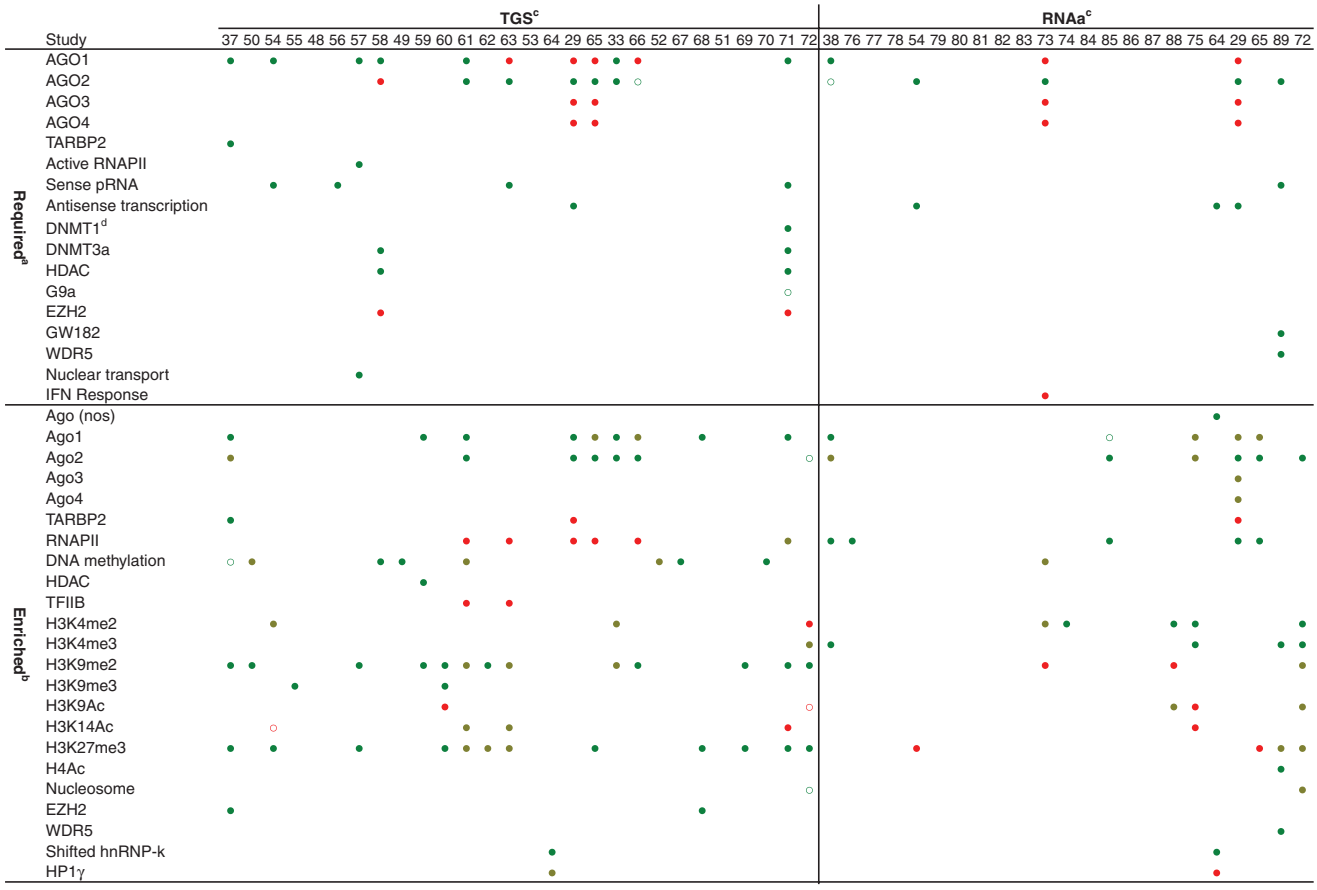
Study	Gene symbol	Cell line	Effector name	Effector type	Transfection	Location (wrt TSS)[a]	Year	Citations[b]	Reference
1	CCNB1	TRAMP C1	miR-744, miR-1186	ds miRNA	Lipid, Retro-virus	–192/–699	2012	34	38
2	CDH1	PC3	miR-373	ds miRNA	Lipid	–645	2008	358	76
3	CDH1	MB-453/MCF-7, *in vivo*	dsEcad-215	ds siRNA	Lipid	–215	2010	12	77
4	CDKN1A	A549 and *in vivo*	dsP21-322	ds siRNA	Lipid	–322	2010	18	78
5	CDKN1A	MCF-7	p21-322	ds siRNA	Lipid	NS	2008	133	54
6	CDKN1A	T24/J82	dsP21	ds siRNA	Lipid	–322	2008	48	79
7	CDKN1A	A498	dsP21-322	ds siRNA	Lipid	–322	2009	18	80
8	CDKN1A	T24	dsP21-322	ds siRNA	Lipid	–322	2008	24	81
9	CDKN1A	5637	dsEcad-215	ds siRNA	Lipid	–215	2008	20	82
10	CDKN1A	PC3	dsEcad-215	ds siRNA	Lipid	–215	2010	6	83
11	CDKN1A, CDH1, VEGFA	Many	Many	ds siRNA	Lipid	Many	2006	297	73
12	HIV-1 (Viral LTR)	1G5	LTR-247as+7	siRNA-cassette	Vector	Viral 254	2007	21	74
13	KLF4	Many	dsKLF4-496	ds siRNA	Lipid	–496	2010	40	84
14	LDLR	HepG2	LDLR-24, LDLR-28	ds siRNA	Lipid	–24/–28	2010	24	85
15	Many	Mammalian cell lines	Many	ds siRNA	Lipid	NS	2010	49	86
16	NANOG	NCCIT	dsNanog-752	ds siRNA	Lipid	–752	2012	9	87
17	PAWR	Many	dsPAWR-435	ds siRNA	Lipid	–435	2013	2	88
18	PGR	T47D/MCF-7	PR11	ds siRNA	Lipid	–11	2007	214	75
19	PGR	MCF-7	PR11	ds siRNA	Lipid	–11	2008	121	64
20	PGR	MCF-7	PR11	ds siRNA	Lipid	–11	2010	41	29
21	PGR	MCF-7	PR13515	ds siRNA	Lipid	+13515	2010	34	65
22	PTGS2	A549	miR-549/RNA12	ds miRNA/ siRNA	Lipid	–57/–34	2013	5	89
23	VEGFA	C166	LV-451	U6shRNA	Retrovirus	NS	2009	43	72

[a]5'-most location with respect to the transcriptional start site of the target. A negative number implies an upstream location and "NS" denotes the location was not stated. [b]Citations according to Web of Science (July 2014).

to the promoter RNA, they posit that the sense strand is the only potential partner for gene activation. In agreement, microarray data shows that in MCF7 cells, miRNA with biased Ago2 association are predominantly derived from sense strands of the corresponding pre-miRNA, while the majority of Ago1-associated miRNAs originate from the anti-sense strand.[23]

In accord with expectation, there is clear evidence that, after TGS, less RNAPII is found at promoters,[29,33,53,61–66] while after RNAa, there is enrichment for RNAPII.[29,38,65,76,85] Most studies that have examined chromatin regulation found promoters after TGS to be enriched for histones with silencing marks and those after RNAa with active marks (**Figure 2**). In particular, TGS is associated with enrichment for the repressive histone 3, lysine 9 di- and tri-methylation (H3K9me2, H3K9me3) and lysine 27 tri-methylation (H3K27me3) marks, with loss of the active histone 3 lysine 9 acetylation(H3K9Ac), lysine 14 acetylation (H3K14Ac) marks and in some cases loss of the active H3K4me3 mark. For RNAa, histone regulation is reversed, with loss of repressive H3K9me2 and H3K27me3 marks and gain of the activating H3K4me2 and H3K4me3 marks. Intriguingly, like TGS, RNAa is associated with the loss of H3K9ac and H3K14ac marks. These marks are associated with active and bivalent promoters in mouse cells.[92] Some TGS studies report an increase in DNA methylation at the targeted promoter (**Figure 2**). The *de novo* methyltransferase Dnmt3a is required to establish DNA methylation at the targeted region of the promoter and Dnmt1 is required for maintenance of that methylation.[58,71]

Knockdown experiments suggest a TGS requirement for the chromatin condensation-associated histone deacetylase HDAC, perhaps the H3K9 methyltransferase G9a, but not the polycomb-group H3K27 methyltransferase, EZH2.[58,71] However, in ChIP experiments from two TGS studies, EZH2 is enriched at target promoters.[37,68] There is also a requirement in TGS for the RISC-loading complex subunit, TARBP2.[37] For RNAa, it has been reported that activation of the *PTGS2* locus required the WD repeat-containing protein 5 (WDR5) and the argonaute-interacting, GW182.[89] Interestingly, the long intergenic ncRNA (lncRNA), HOTTIP, transcribed from the 5′-end of the *HOXA* locus, binds to WDR5 and in turn, mixed-lineage leukaemia (MLL) histone methyltransferase, which drives gene activation and H3K4 trimethylation (H3K4me3). This same histone mark is known to be enriched in RNAa (**Table 2**).[72,75,89] This suggests transcriptional gene activation by short RNA and lncRNA may share a number of features in common.



[a] 'Required' defined as having knockdown evidence for their involvement in TGS or RNAa

[b] 'Enriched' defined as having immunoprecipitation evidence for involvement

[c] Filled green circles correspond to a positive association and red circles the converse. Grey circles indicate a factor was tested but no association was found. Unfilled circles denote a lesser degree of evidence.

[d] Dnmt1 was found to be required for maintenance, but not establishment of TGS.

Figure 2 Required and enriched factors for transcriptional gene silencing and activation.

Endogenous Small RNA Transcriptional Silencing and Activation

miRNA

The initial observation of exogenous small-RNAs directing TGS suggested endogenous sRNAs such as miRNA or endo-siRNA may elicit the same phenomenon. The first examples of endogenous TGS and RNAa in mammals were of genes with predicted miRNA binding sites in their promoters; in particular the silencing of *POLR3D* by miR-320 (ref. 68) and *PGR* and *IGSF1* by miR-423-5p[66] and the activation of *CDKN1* transcription by miR-373, (ref. 76) *PTGS2* transcription by miR-549 (ref. 89) and *CCNB1* by miR-744 and miR-1186 (ref. 38) (**Tables 1** and **2**). Huang *et al*. found Ago1 was enriched at the *CCNB1* promoter, just proximal to the predicted miR-744-binding site, but did not find any enrichment for Ago2.[38] Particular miRNA 5′ sequence elements may direct import of the mature miRNA back into the nucleus[93] with this re-importation dependent on the association of Importin8 with Ago2.[31,94] There is evidence that Ago2 is involved in control of chromatin structure at a genomic miRNA site,[95,96] with Ago2 knockdown correlated with upregulation of expression of the miRNA-155 host gene primary transcript (miR-155HG), the overlapping antisense long noncoding RNA transcript and an increase in acetylation of histone 4 in the promoter region.[95]

Benhamed *et al*. describe senescence-associated transcriptional gene silencing (SA-TGS); during cellular senescence miRNA such as let-7 direct Ago2 to promoters of the tumor suppressor gene/transcription factors repressor complex (RB1/E2F) target genes (such as *CDC2* and *CDCA8*). These miRNA/Ago2 complexes block RNAPII engagement at target promoters and cooperate with E2F/RB1 complex to repress E2F-target promoters, resulting in increased H3K9me2 and H3K27me3 heterochromatin associated marks and a decrease in the active mark, H3K4me3.[96] Ago2 accumulates in the nucleus of senescent cells, with Ago2 knockdown resulting in delayed senescence. This transcriptional repression of proliferation-promoting genes by SA-TGS may contribute to tumor suppression.

lncRNA

miRNA have an epigenetic TGS role either in *cis* as antisense targeting its own genomic location to silence adjacent gene transcription, or by associating with a methyltransferase such as EZH2, they may also function more broadly in TGS to target gene promoters in *trans*.[68] lncRNA can also have both a *cis* and *trans* epigenetic role and can function in *cis* as antisense at the transcriptional level, regulating protein coding gene expression or in *trans* acting as scaffolds to mediate interactions that guide enzyme complexes to specific RNA or DNA target sites in order to exert their effect.

Despite lncRNA frequently being nuclear-localized, many have been found to interact with miRNA,[97,98] suggestive of a role for nuclear Ago-miRNA complexes; however, direct evidence is lacking. Functionally, these miRNA-binding lncRNA, known as competing endogenous RNA (ceRNA), act like a "microRNA sponge" effectively reducing available miRNA.[99] By antagonizing miRNAs, lncRNA are known to regulate several developmental processes.[100–102] lncRNA interacting with miRNA can also result in histone modification and subsequent gene repression.[103,104] For example, the lncRNA-miRNA complex of HULC and miR-372 recruits the histone modifying enzyme P300, a histone acetyltransferase and subsequently causes heterochromatin formation resulting in TGS.[105]

Sense-antisense transcription frequently results in formation of dsRNA.[106] It has long been known in *Schizosaccharomyces pombe* that dsRNAs target complementary mRNAs for degradation via the Ago1 RNAi pathway (reviewed in ref. 107) and these interactions result in heterochromatin formation at specific DNA loci. Recently, antisense ncRNAs have been implicated in the silencing of tumor suppressor genes through epigenetic remodeling events in humans.[54,57] miRNAs can recruit Ago2 to antisense lncRNA transcripts that overlap their target gene promoter.[66,73,108] Younger *et al*. found miR-423-5p binds to RNA within the target progesterone receptor (PR) gene promoter, as well as ncRNA transcribed from the *PR* promoter, with TGS associated with recruitment of Ago2 to this ncRNA with subsequent decrease in promoter RNAPII occupancy and increase in the H3K9me2 mark.[66] Other data shows nuclear Ago2-mediated regulation of the lncRNAs MALAT1 and the star-strand HOTAIR* by miR-9 and miR-141, respectively.[99,109] It appears that both MALAT1 and HOTAIR and most likely other lncRNAs may be functioning as a ceRNA[110] and competing for miRNA target sites, mediated by Ago2. Evidence for this appears in recent global analyses of Ago-bound transcripts.[111–113]

In the human genome, antisense transcription is widespread,[106] which suggests a large potential for convergent transcription of overlapping transcripts to form endogenous dsRNA and to regulate the transcriptome. In *S. pombe* and mammals, convergent transcription induces TGS in *trans*.[45] Antisense ncRNAs have been implicated in the silencing of tumor suppressor genes through epigenetic remodeling events in humans.[54,57]

Argonaute Association with Other Endogenous Processes

Alternative splicing

The introduction of exogenous duplex RNAs into the nucleus has been shown to redirect exon splicing of aberrant splice sites of the disease-associated *SMN2* and dystrophin genes.[35] The duplex RNAs recruited Ago2 to pre-mRNA transcripts and altered splicing without nuclear cleavage of the pre-mRNA. The first reported involvement of endogenous sRNA pathways in alternative-splicing was of the regulation of pre-mRNAs by MALAT.[114] Subsequently, Ago1 and Ago2 have been identified in physical association with MALAT, chromatin modifiers and splicing factors.[36] Using the *CD44* gene as a model, the authors show that Dicer-dependent recruitment of Ago1 and Ago2 facilitated spliceosome recruitment and modulated RNAPII elongation rate thereby affecting alternative splicing. The recruitment of Ago1 and Ago2 to *CD44* transcribed regions required the Dicer and histone modifying enzymes which resulted in increased H3K9 methylation on variant exons associated with heterochromatin and TGS.[36]

Double strand break repair

The double strand break (DSB) repair role of Ago in plants is well known, however, this function has been recently found conserved in humans, with a reduction of DSB repair ability observed after knockdown of Dicer or Ago2.[115] These DSB-induced small RNAs (diRNA) are produced from sense and antisense strands of DSB proximal sequence. In mammals, like in plants and *Drosophila*, diRNAs may function as guide molecules directing chromatin modifications which cause heterochromatin formation or the recruitment of protein complexes to DSB sites to facilitate repair. diRNA binding and catalytic activity of Ago2 are required for recruitment of the repair protein Rad51 to DSBs.[34]

Model of Action

In the literature, there is conflicting information about the involvement of Ago1 and Ago2 in enacting transcriptional gene silencing and activation (see previous sections). More recent evidence suggests reconciliation into one central model is unnecessary. Instead, there is evidence for two discrete but overlapping pathways involved in TGS and RNAa (**Figure 1b**).

Generally, exogenous promoter-directed sRNA data shows that Ago1 is associated with TGS and transcription of sense promoter-associated RNA, while Ago2 is associated with both TGS and RNAa and antisense transcription (**Figure 2**). With regard to endogenous nuclear miRNA bound to Ago, the evidence suggests overlapping functionality of Ago1 and Ago2, but with some clear exceptions. Some miRNA species exhibit pre-miRNA antisense strand loading bias into Ago1 and the sense strand into Ago2.[23] The interactions and location of argonautes within the nucleus reinforce the idea of discrete pathways of action. Ago1, but not Ago2, interacts directly with RNAPII and binds to the promoters of actively transcribed genes[30,37] and only Ago1 has readily observable interactions with chromatin.[30] Furthermore, Ago1 is dispersed throughout the nucleus, whereas Ago2 is primarily localized to the inner nuclear envelope.[30,41] At the nuclear periphery are lamina associated domains (LADs), DNA which is known to be mostly maintained in a silent state with activation upon cellular differentiation (reviewed in ref. 116). The distribution of Ago2 in the vicinity of LADs is consistent with its involvement primarily in RNAa.

Ago2-induced RNAa has some seemingly paradoxical observations, in particular, the observation that the *PGR* promoter-directed siRNAs PR9 and PR11 (**Tables 1** and **2**), designed only two nucleotides apart, may induce TGS in high PGR-expressing T47D cells and RNAa in low PGR-expressing MCF-7 cells, respectively.[29,33,64] RNAa at the *PGR* locus is known to depend upon antisense transcription and involves the PR11-loaded Ago2.[29,64]

Perhaps these results can be explained by consideration of the findings by Morris *et al*.[54] In their 2008 study, they provide evidence that sense transcription from the p21 (*CDKN1A*) locus is held in balance by transcription from overlapping antisense transcripts; *i.e.*, Ago2-mediated PTGS of antisense transcription results in activation of p21 sense transcription, while—conversely—PTGS of p21 sense mRNA results in activation of p21 antisense transcription and the recruitment of Ago1 at the p21 promoter. They also show the presence of p21 antisense transcript and Ago1 are required for regulation of promoter-associated sense RNA and suppression of the p21 promoter. Their findings imply that apparent RNAa is, in fact, the result of post-transcriptional repression of antisense RNA at bidirectionally transcribed loci. If instead PTGS is directed towards the sense RNA, this may result in increased promoter RNA transcription and reinforcement of silencing through recruitment of Ago1. This suggests that at bidirectional promoters, Ago1 and Ago2 may work in concert in the nucleus to reinforce cytoplasmic PTGS of gene loci. Indeed, genome-wide data shows that candidate antisense promoter-associated ncRNAs (pancRNAs) are associated with active chromatin marks, with the forced expression or knockdown of these pancRNAs causing DNA demethylation and methylation at the gene promoter, respectively.[117,118]

The association of Ago1, active RNAPII, Dicer and promoter sense transcription with TGS suggests a rapid monitoring system for aberrant transcription such as might be expected by transcription from transposons, repeated sequences and proviruses, or from chromosome abnormalities.[29,48,117–119]

Presently, it is unknown why there is a distinct strand loading bias between Ago1 and Ago2. These argonautes are structurally very similar and it remains to be seen whether the loading bias is an intrinsic property of argonaute or of cofactors in a loading complex.

Disease Therapy

The demonstrated specificity and potentially long-term efficacy of exogenous sRNA *in vitro*, raises the potential for a new class of RNA-based drugs. It may be possible to develop cancer therapies or to stably suppress HIV-1 replication in the CD-4$^+$ T cells of HIV patients. Indeed, many TGS and RNAa studies target genes associated with cancer and the HIV-1 virus in cultured cells (**Tables 1** and **2**). To this end, the field has now matured to the point where *in vivo* experiments are being undertaken. The efficacy *in vivo* of sRNA-directed transcriptional silencing or activation to control tumor growth has been demonstrated using mouse xenograft models. *In vivo*, stable RNAa activation of *CCNB1* by miRNA constructs resulted in tumors with reduced size compared to controls.[38] Similarly, treating established tumors with lipid transfections of siRNA targeting *CDKN1A*[78] or *CDH1* (ref. 77) every three days showed reduced tumor growth, relative to controls. In addition, dsRNA chemically modified for lipidoid-encapsulated nanoparticle delivery is efficacious in promoting RNAa of *CDKN1A* in mouse xenografts.[120,121]

Effective lentiviral delivery of shRNA has also been demonstrated *in vivo*. The lentiviral transfer of *VEGF* promoter targeted shRNAs has been observed to increase blood flow in the hindlimbs of ischemic mice[72] and recently, in this journal, Suzuki *et al*. showed that shRNA targeting the HIV-1 LTR was able to inhibit HIV-1 replication in lentiviral transduced human peripheral blood mononuclear cells circulating in humanized mice.[122] These results show promise in the translation of exogenous sRNA therapy to the clinic. It is foreseeable that RNA-based drugs may eventually be used to reprogram epigenetic state at a targeted locus.

Also, as we better understand endogenous nuclear sRNA processes, the mechanisms behind some diseases may be uncovered. In a now classic case, Tufarelli *et al*. documented a rare α-thalassaemia resulting from a deletion truncating *LUC7L* and bringing it, juxtaposed, within proximity of the *HBA2* hemoglobingene. Expression of the *LUC7L* antisense transcript resulted in epigenetic silencing of *HBA2*.[123]

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