REVIEW

RNAs as extracellular signaling molecules

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Abstract

RNA is emerging as a major component of the regulatory circuitry that underpins the development and physiology of complex organisms. Here we review recent evidence that suggests that RNA may supplement endocrine and paracrine signaling by small molecules and proteins, and act as an efficient and evolutionarily flexible source of sequence-specific information transfer between cells, both locally and systemically. As such, RNA signaling may play a central but previously hidden role in multicellular ontogeny, homeostasis, and transmitted epigenetic memory.

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Introduction

The emergence of non-protein-coding RNA (ncRNA) as an important regulatory molecule within the cell (Mattick & Makunin 2006, Prasanth & Spector 2007) forces a reconsideration of the role of RNA in a number of cellular processes previously considered to be the predominant or exclusive domain of proteins. This includes the potential of RNA to mediate cell-to-cell communication. The ability of RNA to compactly store and transmit information makes it an ideal medium to communicate between and co-ordinate the functions of cells in multicellular organisms. Indeed, the ability of RNA to propagate information between the cells and even between the organisms has been illustrated to devastating effect by the existence of retroviruses such as HIV.

Although only 1.2% of the mammalian genome encodes protein, a significant fraction is under evolutionary selection (Pheasant & Mattick 2007) and most is transcribed in complex interleaved patterns of protein-coding and non-coding RNAs (ncRNAs; Mattick & Makunin 2006, Birney *et al.* 2007, Kapranov *et al.* 2007a). The latter comprise a diverse range of classes (summarized in Table 1) that include small regulatory RNAs, such as microRNAs (miRNAs), small interfering RNAs (siRNAs), and small nucleolar RNAs, as well as long ncRNAs that can be sourced from genomic regions that are intergenic, intronic, anti-sense, or overlapping with protein-coding genes. In mammals, tens of thousands of long ncRNAs have been identified, many of which exhibit distinct expression

profiles in different cells and tissues (Ravasi *et al.* 2006, Mercer *et al.* 2008). There is also evidence for very large number of small RNAs (Berezikov *et al.* 2006, Mineno *et al.* 2006, Kapranov *et al.* 2007b). Although the role of a limited number of ncRNAs has been determined (Mattick & Makunin 2006, Prasanth & Spector 2007), the function of the vast majority is unknown.

The physicochemical properties of RNA enable it to fulfill a diverse range of structural and catalytic roles through various mechanisms (Scott 2007). These characteristics are fundamental to key cellular processes, such as translation and splicing, and are also central to the hypothesis that RNA formed the initial molecular basis for life on Earth (Joyce 2002, Orgel 2004). In addition to the ribosomal and spliceosomal RNAs, the ncRNAs have been shown to be involved in a diverse range of biological pathways and processes (Mattick & Makunin 2006), including cellular differentiation and development (Martinho *et al.* 2004, Wienholds *et al.* 2005, Zhao *et al.* 2005), chromatin modification (Bernstein & Allis 2005, Rinn *et al.* 2007), imprinting (Hatada *et al.* 2001, Sleutels *et al.* 2002, Okamoto *et al.* 2005), alternative splicing (Kishore & Stamm 2006), nuclear factor trafficking (Willingham *et al.* 2005), RNA modification (Kiss 2002), and mRNA translation and stability (Bartel 2004).

Recent years have seen that RNA move into mainstream thinking in biology as a key player in information transfer and regulatory control (Mattick 2007). This is beyond the established role of RNA as an intermediate between gene and protein or as an infrastructural

Table 1 Summary of major classes of non-coding RNAs with regulatory roles

	Description
ncRNA	
miRNA	MicroRNAs (miRNAs) are small (~21–23 nt) single-stranded RNAs that regulate gene expression by partial complementary base pairing to specific mRNAs. This annealing inhibits protein translation and in some cases facilitates degradation of the mRNA
siRNA	Small interfering or silencing RNAs (siRNAs) are small (~20–25 nt) double-stranded RNAs that act through the RNA interference (RNAi) pathway to interfere with the expression of a specific gene containing a complementary sequence. RNAi can down-regulate expression by inducing degradation of the targeted RNA, interfering with transcription or inducing epigenetic changes to the gene
snoRNA	Small nucleolar RNAs (snoRNAs) are small (~70–240 nt) RNAs that guide the methylation or pseudouridylation of rRNAs and other RNAs. SnoRNAs contain a 10–20 nt antisense sequence that is complementary to the sequence adjacent to the base targeted for modification
long ncRNAs	Long ncRNAs are generally defined as RNAs longer than 200 nt with little or no protein-coding capacity. Long ncRNAs can regulate gene expression through diverse mechanisms
piRNAs	Piwi-interacting RNA (piRNA) are small (27–30 nt) RNAs that are specifically expressed in the <i>Drosophila</i> and vertebrate germ line. piRNAs interact with Piwi proteins to direct chromatin modifications and transposon silencing
Riboswitches	A riboswitch is a part of an RNA that binds a small molecule ligand, such as an amino acid or co-factor, to cause a structural change that alters the function or expression of the RNA, such as modification of pre-mRNA splicing or mRNA translation. Most known riboswitches occur in bacteria, but they have also been discovered in eukaryotes

component in splicing and translation. Indeed, the widespread developmentally regulated expression of ncRNAs, both intronic and intergenic, has led to the proposal that these RNAs form a hidden layer of regulation that controls various cellular processes, in particular developmental trajectories (Mattick 1994, 2003, 2004, 2007) and brain function (Mehler & Mattick 2007, Mercer *et al.* 2008). Here we discuss the emerging evidence that one aspect of such a non-coding regulatory network may be its ability to mediate extra-cellular communication in multicellular organisms.

RNA as a signaling molecule

RNA has several advantages as a signaling molecule. First, it can encode sequence-specific interactions using base-pairing rules and specificities. Such sequence-specific interactions may be likened to digital signals and can occur over short stretches of nucleotides (e.g., ~22 nt miRNAs and siRNAs that regulate mRNA translation and turnover) in a more precise and efficient manner than can be achieved by proteins, which require complex tertiary structures to target-specific nucleotide sequences. Secondly, RNAs can act as molecular adaptors to connect incoming analog signals to sequence-specific outcomes. This is exemplified by ‘riboswitches’ which are mRNA elements that alter their structure in response to ligands, enabling them to sense and react to environmental parameters (Winkler & Breaker 2005, St Laurent & Wahlestedt 2007). RNAs can also function as adaptors to connect

the complexes formed by RNA:RNA and RNA:DNA interactions to a generic protein infrastructure that can act upon these signals in a way that is determined by the characteristics of the regulatory RNA or the resulting complex (Mattick 2007). This capacity enables the flexibility to create new members of various classes of RNA signaling molecules that vary in their target specificity, but utilize a common infrastructure and output pathways, thereby providing latitude to explore new connections in RNA-based regulatory networks (Mattick & Gagen 2001, Mattick 2007). Well-characterized examples include the recruitment of the RNA-induced silencing complex to mRNA:miRNA/siRNA complexes (Bartel 2004, Sontheimer 2005) and the evolution of lineage-specific miRNAs (Berezikov *et al.* 2006), as well as the snoRNA-mediated recruitment of generic enzyme complexes to impose site-specific base modifications on target RNAs (Bachellerie *et al.* 2002). Similarly, the promoter-directed RNAs that have been shown to regulate gene expression (Han *et al.* 2007, Martianov *et al.* 2007) presumably interact with relatively generic transcription factors and/or direct chromatin-modifying complexes to induce epigenetic changes at loci targeted via sequence-specific interactions (Mattick 2007), in one case involving RNA:DNA triplex formation (Martianov *et al.* 2007). Triplexes (Ohno *et al.* 2002) and promoter-associated RNAs (Kapranov *et al.* 2007b) occur genome wide and it seems plausible that many genes may be regulated by such signals and associated general mechanisms.

This intracellular signaling paradigm may be extended to include extracellular signaling whereby a

generic infrastructure is employed to transport and receive specific RNA signals in the extracellular milieu, independently of their information content, analogous to a postal system whereby an RNA 'cargo' is delivered by the protein 'courier'. Such systems would also involve generic infrastructural components such as cell surface receptors and transport vesicles (see below) that may transmit different RNA molecules regardless of their precise sequence. As such, RNA could transmit a range of signals to other cells without having to evolve novel signal-specific pathways, a further advantage of RNA in the context of lineage-specific developmental and physiological regulation.

RNA as an extracellular signal

Clear evidence that RNA can act as an extracellular signal is emerging. Studies in plants first showed that the phenomenon of co-suppression in response to transgene expression is mediated by RNA signaling (Napoli *et al.* 1990, van der Krol *et al.* 1990, Smith *et al.* 1994), and that this effect, which involves the RNA interference (RNAi) pathway, can be transmitted plant wide following grafting of an affected rootstock onto a scion (Palauqui *et al.* 1997, Voinnet & Baulcombe 1997, Garcia-Perez *et al.* 2004). The graft-transmissible signal, presumed to be RNA, is delivered into the nucleus where it causes transcriptional down-regulation of homologous chromatin, as well as degradation of homologous transcripts (Brosnan *et al.* 2007). Plants use the same or closely related pathways to help co-ordinate developmental processes (Vaucheret & Fagard 2001), and translocated RNA has been demonstrated to affect leaf development (Kim *et al.* 2001). The transport of these RNA signals occurs in local proximity via plasmodesmata or systemically via phloem (Himber *et al.* 2003).

The molecular nature of the graft-transmissible regulatory RNA signal in plants is yet to be determined but recent results indicate that it may not be simply miRNA or siRNA (Brosnan *et al.* 2007). In plants, there are four DICER-like (DCL) proteins that process double-stranded RNA (dsRNA) into miRNA and siRNA. DCL1 produces miRNAs, whereas siRNAs that are responsible for the silencing of viruses, transposons, and transgenes are produced by the combined action of DCL2, DCL3, and DCL4 (Bouche *et al.* 2006, Deleris *et al.* 2006, Fusaro *et al.* 2006). A *dcl1* mutant and a *dcl2-dcl3-dcl4* triple mutant that are compromised in miRNA and siRNA biogenesis, respectively, are not affected in transmission of the mobile silencing signal from the rootstock into the scion (Brosnan *et al.* 2007). These results indicate that the graft-transmissible regulatory RNA signals in plants may be another form of RNA (Brosnan *et al.* 2007).

There is also evidence for systemic RNA signaling in animals. The discovery of RNAi in the nematode *Caenorhabditis elegans* showed that RNA signals can be transmitted both from the environment (via ingestion) and systemically throughout the organism (Fire *et al.* 1998). Injection of exogenous dsRNA into a few cells was followed by whole organism knockdown of the homologous target mRNA sequences, an effect that can be inherited for several generations (Tavernarakis *et al.* 2000). This process involves a set of proteins, including cell surface receptors, at least some of which are conserved in most animals, including mammals (Jose & Hunter 2007; see below). Extracellular RNA is also present and circulates in the vascular system of mammals (Tsang & Lo 2007) and fetal mRNA has even been found within the blood of pregnant women (Lo 2005). Although the circumstantial evidence is strong, there is as yet no direct evidence that systemic RNA signaling occurs in mammals, nor that extracellular RNA signaling networks participate in normal physiological processes.

Transport of RNA across cell membranes

The phospholipid membrane of eukaryotic cells presents a strong barrier to the passive diffusion of nucleic acids. Consequently, it would be expected that cells would require pathways for the import of RNA from other cells and the extracellular milieu. Interestingly, while cells are relatively impermeable to exogenous mononucleotides, oligonucleotides can easily be taken up by cells, where they can modulate internal cellular processes such as splicing (Kole & Sazani 2001). Although mechanisms of RNA uptake by mammalian cells are not well understood, the RNAs are known to enter cultivated mammalian cells by natural processes, including direct cell-to-cell contact, membrane receptors, and channels (Feinberg & Hunter 2003, Valiunas *et al.* 2005).

In cases where cells are in close proximity they may connect physically to allow direct communication and RNA transfer. For example, mRNA required for *Drosophila* oocyte specification and growth is transcribed in neighboring nurse cells and trafficked through connecting ring canals to the oocyte in the egg chamber (Steinhauer & Kalderon 2006). Similarly, RNA within chromatid bodies is transferred between germ cells via cytoplasmic bridges during mouse spermatogenesis (Ventela *et al.* 2003), and there is evidence to suggest that axoplasmic and pre-synaptic RNAs in neurons derive from nearby glial cells (Eyman *et al.* 2007). Long membranous tubes, known as tunneling nanotubes, were recently discovered connecting a number of somatic cells (Rustom *et al.* 2004). While the physiology and extent to which these tubes

occur between cells is unknown, they have been shown to permit the exchange of cytoplasmic contents between the cells. Gap junctions, small channels that allow small molecules to be transferred directly between the cytoplasm of neighboring cells, were recently shown to permit movement of siRNAs between adjacent human embryonic stem (ES) cells with resultant effects on gene expression (Wolvetang *et al.* 2007).

Genetic screens have identified a number of genes required for the spread of systemic RNAi in *C. elegans*. The best characterized to date, *Sid-1*, encodes a transmembrane protein that allows the import of dsRNA into the cell (Winston *et al.* 2002, Feinberg & Hunter 2003). Another gene, *Sid-2*, encodes an intestinal luminal transmembrane protein required for environmental RNAi, but interestingly this protein is not present in all nematodes (Winston *et al.* 2007). Orthologs of *Sid-1* are present in most animals (Li *et al.* 2006). Mammals contain two paralogs of *Sid-1* (*Sidt1* and *Sidt2*), which suggests that mammals have evolved additional specialized RNA transport functions. The human *Sid-1* ortholog, *SIDT1*, has been confirmed to import dsRNA similarly across the cell membrane (Duxbury *et al.* 2005, Wolfrum *et al.* 2007), and microarray analyses indicate that both *Sid-1* paralogs are expressed in most tissues and cell types in humans and mice (Su *et al.* 2004). In mice, *Sidt1* and *Sidt2* exhibit specific expression patterns in the brain, suggesting that dsRNAs are transported intercellularly in the brain (Fig. 1) and that these two proteins fulfill distinct functions. Further, given the impermeability of the blood–brain barrier to even small RNAs, it may be that this barrier functions in part to segregate such an intercellular RNA signaling network from systemic circulation.

RNA signaling in the germ line

One exciting finding to emerge from genetic screens of *C. elegans* was the discovery of three genes, *rsd-2*, *rsd-3*, and *rsd-6*, required for the transfer of dsRNA into germ line cells (Tijsterman *et al.* 2004). Although little is known about the function of these genes, the protein encoded by *rsd-3*, which has both mouse and human orthologs, contains an ENTH (epsin NH2-terminal homology) domain that is commonly found in proteins involved in vesicle trafficking. However, *rsd* mutants do not exhibit generalized defects in endocytosis, suggesting the existence of a specific pathway for the import of silencing signals into the germ line (Tijsterman *et al.* 2004), and providing a means by which environmental factors may lead to heritable changes. Indeed, RNAi-mediated silencing can be inherited for several generations in *C. elegans* (Vastenhouw *et al.* 2006) and, since this process involves

a number of chromatin remodeling factors, it is likely that the intergenerational transmission of the signal requires epigenetic modifications.

There is abundant evidence that ncRNAs regulate chromatin architecture (Bernstein & Allis 2005, Mattick 2007, Rinn *et al.* 2007). The extracellular trafficking of such ncRNAs may impart heritable epigenetic modifications on the recipient cell, and these extracellular signals may themselves be potentially inherited through generations in both plants and animals. The existence of such pathways is supported by the recently discovered phenomenon of paramutation that involves the epigenetic transfer of information from one allele of a gene to another and is thought to be primarily mediated by *trans*-acting RNAs (Alleman *et al.* 2006, Rassoulzadegan *et al.* 2006, Chandler 2007). This process can be directed by miRNAs as well as other small RNAs and appears to require the action of the RNA methyltransferase Dnmt2 (Cuzin 2007). Similar to the blood–brain barrier, the blood–testis barrier (Russell 1977) may also have a role in privileging organ-specific RNA signaling or quarantining the male reproductive organ from non-specific systemic RNA signaling. It may also provide a checkpoint to regulate the transfer of specific RNA signals into the testes, which may be crucial if extracellular RNA signals are indeed able to induce heritable effects.

Vesicle-mediated RNA transport

RNA is labile in the mammalian circulatory system, as illustrated by the rapid degradation of exogenous RNA in plasma or blood (Tsui *et al.* 2002, 2006). However, some forms of circulating endogenous RNA seem to be stable and protected from degradation during systemic transport (Tsui *et al.* 2002). Endogenous RNA is unable to pass through size-exclusion filters permeable to free RNA or DNA, and the addition of detergents results in the immediate degradation of endogenous RNA (Ng *et al.* 2002, Tsui *et al.* 2002). This suggests that RNA is associated with, and protected by, lipid and/or protein in the systemic circulation.

Microvesicles are spherical fragments of plasma membrane that are emitted from a wide range of cells (Thery *et al.* 2002, Fevrier & Raposo 2004). They may originate directly from the cell surface and co-opt host membrane components and engulfed cytoplasmic contents during their formation, or they may also be secreted from the endosomal compartment. Microvesicles are known to play important roles in a number of biological processes, including oncogenesis (Ratajczak *et al.* 2006a), coagulation (Barry & Fitzgerald 1999), immune responses (Barry & Fitzgerald 1999), and modulation of susceptibility/infectability

Figure 1 *Sidt1* and *Sidt2* expression in the adult mouse brain. (a) No probe control of adult mouse brain in sagittal section (with detail of hippocampus). (b) *In situ* hybridization of *Sidt1* showing expression in the cerebral cortex, mitral layer of the olfactory bulb, and hippocampus. Close-up image (right) shows heterogeneous expression of *Sidt1* in the hippocampus. (c) *In situ* hybridization of *Sidt2* showing expression broadly throughout the brain. Close-up image (right) shows specific expression of *Sidt2* in the Purkinje cell layer of the cerebellum. Images courtesy of the Allen Brain Atlas (Lein *et al.* 2007).

of cells to retroviruses (Wiley & Gummuluru 2006) or prions (Fevrier *et al.* 2005).

Evidence is emerging that microvesicles may contain RNA and are involved in cell-to-cell communication (Baj-Krzyworzeka *et al.* 2006, Ratajczak *et al.* 2006a). A recent study investigating interactions between tumor-cell derived microvesicles and human monocytes found that mRNAs are contained within microvesicles of that then adhere to and are engulfed by monocytes (Baj-Krzyworzeka *et al.* 2007). Similarly, microvesicles obtained from mouse ES cells were shown to contribute to an up-regulation of pluripotency markers within recipient hematopoietic stem/progenitor cells and subsequently enhance their survival and expansion.

The ES cell-derived microvesicles were enriched for mRNAs encoding several pluripotent transcription factors, suggesting that RNA exchange occurs between stem cells, and presumably other cell types, during development (Ratajczak *et al.* 2006b).

A recent study examined the RNA content of 'exosomes' (small microvesicles that are processed and released from late endosomal compartments of many different cells) released by mast cells and identified functional mRNAs corresponding to ~1300 genes, many of which were not present within the donor cell (Valadi *et al.* 2007). It was also demonstrated that mouse exosomal mRNAs were translated in recipient human mast cells. Over 120 miRNAs were

also identified within the mast cell-derived exosomes (Valadi *et al.* 2007). Some of the RNAs were expressed at higher levels or uniquely within the exosomes relative to the donor cell. This suggests the existence of dedicated cellular control mechanisms to collect and package specific RNAs into the exosome. Furthermore, our own analysis of the microarray results from this study revealed that many longer ncRNAs were also present in exosomes, including a number of ncRNAs associated with important genes and several known ncRNAs, such as *Copg2as* and *Nespas* (Supplementary Table 1, which can be viewed online at <http://jme.endocrinology-journals.org/content/vol40/issue3/>).

The response of the immune system to RNA

In vertebrate cells, the introduction of RNA elicits an innate-immune response known as the interferon response, which leads to a prevention of mRNA translation and increased RNA degradation (Karpala *et al.* 2005). The potency of this interferon response is variable and seems to be cell-type specific (Reynolds *et al.* 2006). For example, neurons (Gan *et al.* 2002) and undifferentiated cells, such as oocytes (Stein *et al.* 2005)

and ES cells (Yang *et al.* 2001), do not mount an interferon response upon the introduction of long dsRNAs. Furthermore, the introduction of mammalian RNA into human dendritic cells does not activate the interferon response (Kariko *et al.* 2005), suggesting that the innate immune response is able to distinguish between endogenous host RNA and exogenous (e.g., viral) RNA and is therefore unlikely to prevent the import of systemic RNA signals. It is thought that post-transcriptional RNA modifications (such as editing, methylation, 5' capping, and polyadenylation) as well as compartmentalization of the innate immune response within the cell prevent this self-recognition (Ishii & Akira 2005, Sioud 2006). This would help explain why synthetic unmodified RNA is immuno-stimulatory, but natural host RNA is not.

The efficient transmission of retroviruses, such as HIV, between cells and organisms illustrates the efficacy of RNA to transfer information. Indeed, some retroviruses may exploit natural mechanisms of RNA extracellular signaling to spread throughout the host organism. This notion is supported by a recent study showing that HIV may hijack exosomes emitted from dendritic cells (Wiley & Gummuru 2006) and thereby potentially allow it to evade immune recognition.

Figure 2 Schematic showing known and putative extracellular signaling pathways mRNA (blue) and ncRNA (red) are (a) transcribed in the donor cell. These RNAs are then (b) trafficked and packaged into vesicles, which are emitted into (c) the extracellular environment. (d) The vesicles then dock and fuse with the target cell, releasing their RNA content. The mRNA may then be (e) translated in the donor cell and the ncRNA may impart regulatory functions, such as guiding the catalytic function of chromatin modifying proteins (ChM) to mediate (f) epigenetic modifications. In addition (g) extracellular RNA signals, such as siRNAs or miRNAs, may be transferred across the plasma membrane by specific receptors and channels, such as Sidt1. (h) These signals may fulfill regulatory functions in the cell such as translation inhibition or mRNA degradation.

Conclusion

While perhaps not surprising in retrospect, there is increasing experimental evidence to suggest that RNA has been widely adopted as a regulatory molecule not just within cells, but also systemically, as summarized in Fig. 2. The harnessing of the power of RNA to transmit information between the cells, and between the generations, adds an exciting and previously hidden dimension to the intercellular communication systems that operate in development and physiology, whose details are still hazy and whose enormous implications have yet to be explored.

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