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Genome Analysis

MicroRNA regulation of messenger-like noncoding RNAs: a network of mutual microRNA control

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Metazoan microRNAs (miRNAs) are commonly encoded by primary mRNA-like characteristics (mlRNAs). To investigate whether mlRNAs are subject to miRNA control, we compared the expression of mlRNAs to that of tissue-specific miRNAs. We show that, like mRNAs, the expression levels of predicted mlRNA targets are significantly reduced in tissues where a targeting miRNA is expressed. On the basis of these results, we describe a potential network for posttranscriptional miRNA-miRNA control.

Messenger-like noncoding RNAs show microRNA-related reductions in expression

Extensive analyses over the recent years strongly suggest that most transcribed sequences in eukaryotes are noncoding RNAs (ncRNAs) [1]. One particularly interesting group is mammalian messenger-like ncRNAs (mlRNAs) that have been extensively mapped in the genomes of both rodents and humans over the past few years (Box 1). However, few such transcripts have been functionally characterized. Although mlRNAs can be targeted by small interfering RNA (siRNAs) [2], it is not yet clear if they are regulated by microRNAs (miRNAs). In animals, miRNAs can either repress or activate translation, depending on the physiological state of a cell [3], or they can degrade and sequester their mRNA targets (for a review, see Ref. [4]). The reduction in mRNA levels seems to be independent of translational repression because mRNAs with a disrupted translational potential are still subject to miRNA-induced degradation [5]. An example of miRNA control of noncoding transcripts has yet to be reported in animals, but five *trans*-acting siRNA-generating transcripts were identified in *Arabidopsis thaliana*. These transcripts were targeted by miR-173 or miR-390, leading to the generation of specific sets of phased siRNAs [6]. Recently, plant ncRNA IPS1 was shown to interfere with the activity of miR-399 through target mimicry [7], which is a new twist on the miRNA–ncRNA phenomenon.

Several mammalian miRNAs have strong tissue-specific expression. Analyses have shown that predicted mRNA targets of human tissue-specific miRNAs have significantly lower expression levels in the tissues where the miRNAs are expressed [8,9]. There are 10 236 mouse mlRNAs with known expressional profiles from a range of different tissues. To test whether miRNA expression influences mlRNA expression levels, we applied the methodology of Sood *et al.* [9] to the predicted mlRNA targets of eight tissue-specific miRNAs [10] (Table S1 in Supplementary Material).

The functional domains of mlRNAs are generally not known. We therefore predicted miRNA-binding sites along the entire sequence of the 10 326 mlRNAs (see Supplementary Material for details), selecting the top 100 miRNA targets for analysis of tissue-related mlRNA expression. Following the method used by Sood *et al.* [9], we used Wilcoxon's rank sum test to estimate whether the expres-

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Box 1. Messenger-like noncoding RNAs[0]

Messenger-like noncoding RNAs (mlRNAs) are RNAs that resemble mRNAs in length and in biogenetic characteristics but lack extended open reading frames (ORFs). They are transcribed by RNA polymerase II, capped, polyadenylated and often spliced, and are sufficiently stable to be cloned as full-length cDNAs [22]. Messenger-like RNAs are generally poorly conserved among mammals but nevertheless display characteristics of purifying selection [23]. Only a few transcripts have been functionally characterized. These include noncoding RNAs (ncRNAs) that have been implicated in transcriptional regulation [24] and linked to ultraconserved elements [25] or to subcellular transcriptional factor localization [2]. Several longer ncRNAs have also been implicated in prostate function and cancer, psoriasis, retinal development and taurine regulation (reviewed in Ref. [12]). In addition, an increasing number of mlRNAs seem to encode functional microRNAs [14].

Table 1. Analysis of the relative expression levels of predicted targets of eight tissue specific miRNAs[a]

miRNA	Tissue	mlRNA targets		mRNA targets	
		Rank[b]	P[c]	Rank[b]	P[c]
miR-133a	Heart	1*	0.023	10	0.542
miR-133a	Muscle	2*	0.050	1*	0.005
miR-153	Brain	5	0.254	18	0.944
miR-206	Heart	2*	0.007	3	0.116
miR-206	Muscle	3	0.071	2	0.056
miR-375	Pancreas	2	0.168	12	0.648
miR-376a	Pancreas	1*	0.0004	18	0.808
miR-122a	Liver	16	0.714	2	0.0828
miR-124a	Brain	12	0.458	1*	0.0007
miR-208	Heart	5	0.249	2	0.178

[a]miRNA, microRNA; mlRNA, nessenger-like noncoding RNA.
[b]The value in the 'Rank' column denotes the reduction of mRNA/mlRNA target expression in the tissue of miRNA expression relative to 19 other tissues.
[c]An asterisk (*) denotes that target expression levels are significantly ($P < 0.05$) reduced in the tissue of miRNA expression compared with other tissues.

sion level of the target genes was significantly reduced (Table 1). This resulted in three miRNAs whose predicted mlRNA targets had significantly reduced expression (Figure 1): miR-133a, which is expressed in heart and muscle; miR-206, which is also expressed in heart and muscle; and miR-376a, which is expressed in the pancreas.

The main difference between the results obtained for the mouse mlRNA targets here and previous results obtained with human mRNA targets (Sood *et al.* [9]), is that the significance levels are lower (i.e. the *P* values are higher) for the mlRNA targets. The main reason for these differences is the software available for prediction of miRNA target sites in mlRNAs. To evaluate the effect of this, we repeated the analysis on mouse mRNAs and found that, when subjected to the same analytical procedure, the results obtained for mRNA targets were similar to those obtained for mlRNA targets (Table 1; see also Supplementary Material for details). It is therefore reasonable to assume that the negative expressional correlation between tissue-specific miRNAs and their mlRNA targets is no weaker than the corresponding correlation for mRNA targets.

It can further be argued that tissue-specific downregulation of target RNAs might be caused by reciprocal transcriptional of a (pri-)miRNA and its targets and not to a direct effect of the miRNA on the stability of its RNA targets [11]. This model is plausible for protein-coding genes, for which the miRNA-induced downregulation can also occur at the translational level. However, there is no *a priori* reason why untranslated mlRNAs should be preferentially downregulated in the tissue where a targeting miRNA is expressed, unless there is a direct effect of the targeting miRNA on the mlRNA target stability. Thus, the most reasonable explanation for the observed negative correlation is that the mlRNA targets are subject to miRNA-induced degradation in the tissues where the targeting miRNA is expressed.

miRNAs and primary transcripts form a regulatory network

Predicting targets for all the 461 known mouse miRNAs (miRBase 10.0) in the 10 326 mlRNAs resulted in a total of >158 000 potential miRNA–mlRNA interactions. The average mlRNA was targeted by 15 (0–79) different miRNAs, and the average miRNA targeted 343 mlRNAs. On a sequence-length basis, this is comparable to similar data for mRNAs (see Supplementary Material for further details). Of the few mlRNAs that have been studied in detail (reviewed in Ref. [12]), ncRNA NRON (RIKEN cDNA Clone ID: A630081L07), which affects nuclear import of a transcription factor (NFAT) involved in T-cell activation, is particularly interesting [2]. NRON is predicted to be targeted by 28 miRNAs, and of these, 4 (miR-135a and b, miR-431 and miR-125a) are specifically expressed in the central nervous system. NRON is preferentially expressed in lymphoid tissue, and although it has not been demonstrated to be absent from the brain [2], it would not be surprising if NRON were under negative regulation in this immunologically silent organ.

Some mlRNAs have been shown to serve as pri-miRNAs (e.g. *H19* [13], RIKEN cDNA Clone ID: I0C0030C13), and a substantial proportion of miRNA loci is found within exons of uncharacterized noncoding transcripts [14]. The possibility that miRNAs could influence pri-miRNA expression or activity implies the existence of subnetworks in which miRNAs exert posttranscriptional control of (pri-)miRNA expression. There is also accumulating evidence that posttranscriptional miRNA processing is subject to regulatory activity. In *Caenorhabditis elegans*, the let-7 pri-miRNA stably accumulates as a *trans*-spliced and polyadenylated transcript. Also, during mouse embryo development, variation in pri-miRNA and mature miRNA let-7 is not coordinated, suggesting posttranscriptional regulation during neural cell specification [15]. Additional analysis of mouse miRNAs also demonstrated that many primary miRNA transcripts are present at high levels without being processed by Drosha [16]. Similarly, the precursor of the mammalian miR-138 (pre-miR-138-2) is ubiquitously expressed in all analyzed tissues, whereas the mature miR-138 is only expressed in distinct cell types [17].

To characterize the predicted miRNA–miRNA posttranscriptional regulatory system, we constructed a network from the 36 miRNAs that are encoded by defined 33 mlRNA precursors (Figure 2a; see Supplementary Materials for details). When topological characteristics were analyzed, the network displayed meaningful subgraphs, such as the feed-forward loops, feedback loops and multiple input motifs, which are generally associated

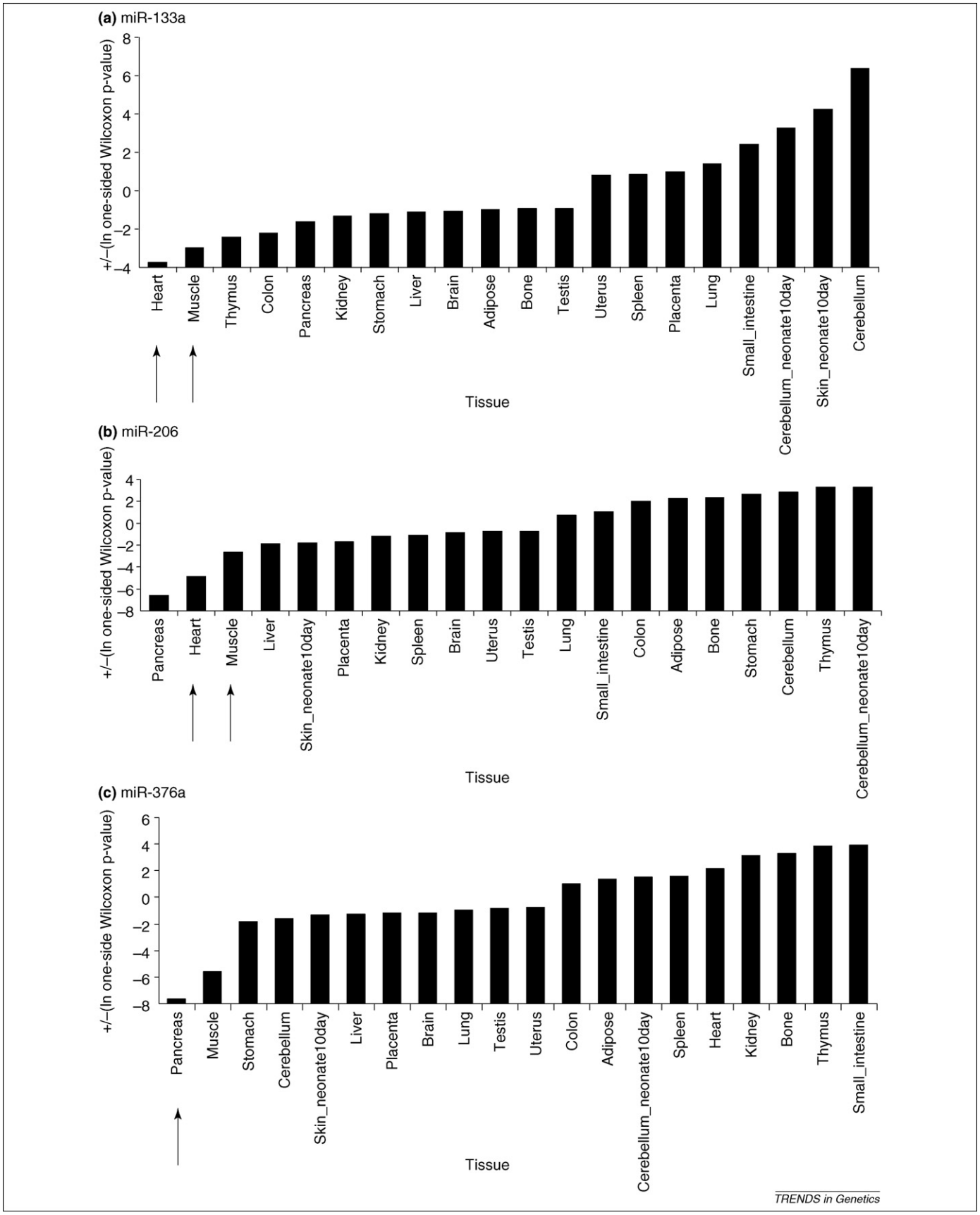

Figure 1. Tissue-specific effects of microRNAs (miRNAs) on messenger-like noncoding RNA (mlRNA) target expression. Arrows indicate the tissues in which the targeting miRNAs are expressed. The figure shows an analysis of the mouse mlRNAs expression of predicted targets of three tissue-specific miRNAs across 20 tissues. The ordinate indicates the probability (i.e. statistical significance value) as calculated by Wilcoxon rank sum test of the predicted mlRNA targets being expressed at lower levels in a tissue compared with a background set of mlRNAs (see Supplementary Material for details). The tissues are sorted by their probability values. **(a)** miR-133a targets. **(b)** miR-206 targets. **(c)** miR-376a targets.

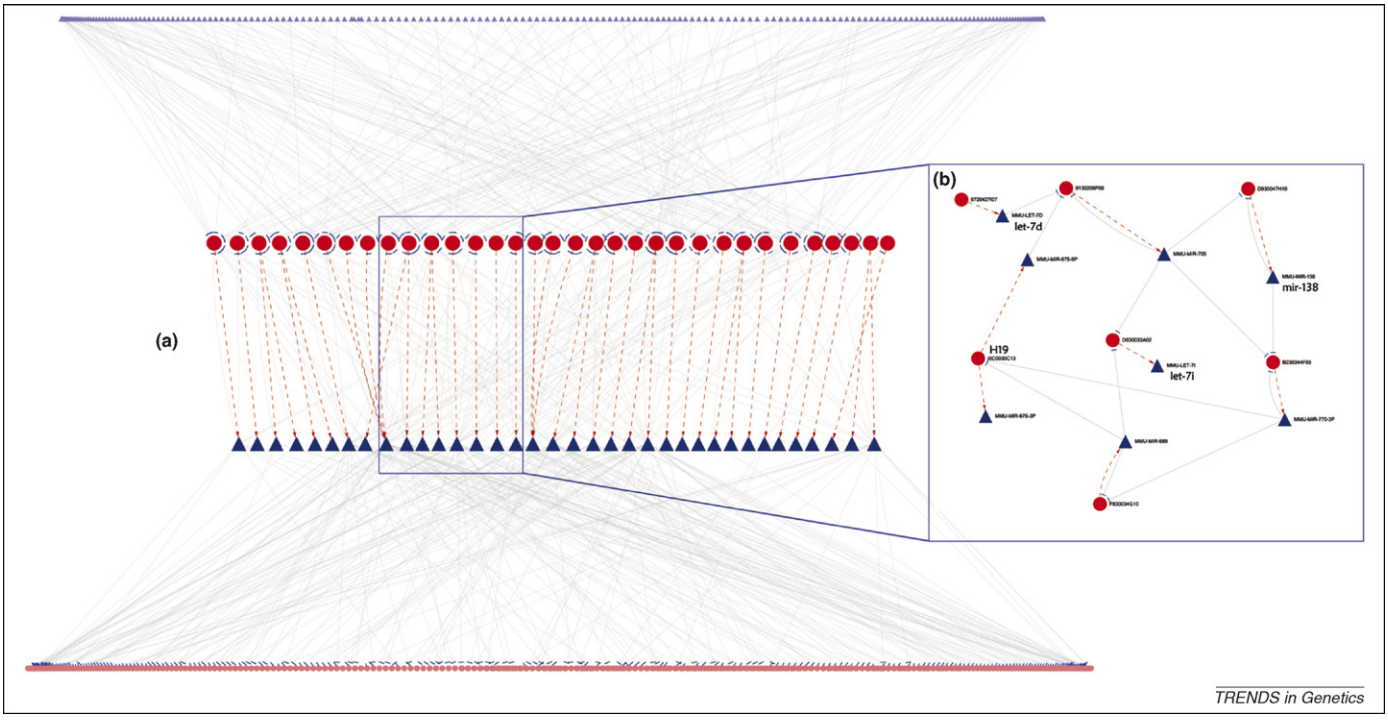

Figure 2. A network of mutual microRNA (miRNA) posttranscriptional control. **(a)** A network of 33 pri-miRNAs (indicated by the red circles) and their derived miRNA (indicated by the dark blue triangles) are shown. Other miRNAs [i.e. miRNAs not known to be encoded by primary transcripts with mRNA-like characteristics (mlRNAs)] are depicted by light blue triangles and other (non-miRNA encoding) ml-RNAs are depicted by pink circles. 'Off-spring' miRNAs are located below their encoding pri-mRNAs, their relationship indicated by a red, dashed arrow. A miRNA having a predicted target site in another pri-miRNA sequence is indicated by a grey edge. **(b)** A subnetwork of seven pri-miRNAs and their miRNA 'offspring' forming a putative regulatory loop. Analysis of the network revealed several forms of biologically informative subgraphs in which different miRNAs can cooperate to mutually enhance or inhibit the activity of each separate miRNA. Among these subgraphs is also a network of interlinked miRNAs and pri-miRNAs that forms a continuous feed-back loop. The loop consists of several miRNAs that are linked to cancer and whose processing have been shown to be under posttranscriptional control.

with molecular regulatory networks (Figure S3 in Supplementary Materials), demonstrating that refined control is a possible function of the network. We identified a small group of seven mlRNAs in the network that, together with their derived miRNAs, form a putative loop of mutual posttranscriptional control (Figure 2b). Intriguingly, the loop includes the imprinted H19 (primary transcript of miR-675), which is known to have distinct developmental and tissue-specific regulation [18], as well as the putative tumor-suppressive let-7 and the brain-specific miR-138; the second two were recently shown to be under posttranscriptional control [17]. According to the network, the average miRNA-encoding mlRNA is potentially under control of ~20 miRNAs, and the encoded miRNAs can in turn control ~300 other mlRNAs.

The suggestion that miRNAs regulate the posttranscriptional levels of other noncoding RNAs, including their own primary transcripts, might also explain the complex mode of biogenesis of many miRNAs. Although a primary transcript of ~100 nucleotides seems to be sufficiently long to specify correct processing of the miRNA, most independently transcribed pri-miRNAs are much longer than this [14]. It is also common that pri-miRNAs resemble mRNAs in that they carry a 7-methyl-guanosine cap, are polyadenylated and are often also spliced. The accepted version of miRNA biogenesis starts with processing of the pri-miRNA transcript by Drosha in the nucleus, followed by export of pre-miRNA hairpin to the mature miRNA in the cytosol. The H19 ncRNA (primary transcript of miR-675) and pri-miR-138 are both reported to accumulate in cytoplasm [17,19], and a recent microarray survey [20] indicated that the cellular localization of a substantial proportion of human pri-miRNAs might be less distinctly nuclear than previous studies suggested. Therefore, although pri-miRNA processing by Drosha might be confined to the nucleus, its capping, polyadenylation and possible export to the cytoplasm might secure the primary transcripts for storage, posttranscriptional regulation of miRNA-processing or even other functions not directly linked to the encoded miRNA.

Concluding remarks

Here we described a potential network for posttranscriptional microRNA (miRNA)–miRNA control. There are several caveats to our analysis. The most important applies to the reliability of the miRNA target site prediction, because target prediction was not strongly supported by a conservation filter. Second, the specificity of the miRanda algorithm seems to be lower than for comparable target prediction software, and it is reasonable to assume that a proportion of the predicted target sites might not be biologically active (see Supplementary Material for details). Nonetheless, estimates suggest that a random miRNA target site has a 50% chance of being effective [8], and degradation of mRNA targets seems to be independent of translational activation [5,21], which suggest that noncoding transcripts are amenable to miRNA control. Furthermore, messenger-like noncoding RNAs (mlRNAs) targeted by tissue-specific miRNAs show expressional signatures reminiscent of those for mRNAs [8,9].

The network constructed from target predictions in miRNA-encoding mlRNAs resembles a molecular regulatory network, supporting the proposition that miRNA regulatory influence is not limited to protein-coding transcripts.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tig.2008.04.004.

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Erratum

Corrigendum: Adapting to environmental changes using specialized paralogs

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