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MicroRNAs in embryonic stem cells and early embryonic development

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Abstract

MicroRNAs (miRNAs) are involved in the regulation of a broad range of biological processes. There is an extensive literature on the roles of miRNAs in metazoan development, from early embryogenesis to lineage commitment, and formation and maturation of cellular subtypes and complex tissues. This review will present the evidence that miRNAs are essential for the earliest stages of metazoan development, including establishment and maintenance of the pluripotent embryonic stem cells (ESCs) from which all foetal tissues arise. We will also discuss specific miRNAs that have been associated with early embryogenesis and pluripotency. Finally, we will point out evidence showing that there is strong and highly redundant miRNA regulation of a common set of target genes in the early stages of embryonic development and in ESCs.

Keywords: embryonic stem cells • microRNAs • gene expression • epigenetic • embryonic development

Introduction

MicroRNAs (miRNAs) are small noncoding RNAs implicated in regulation of a wide array of biological processes in plants and metazoans, including metabolic pathways, development and many disease processes including cancers. There is an extensive literature on the roles of miRNAs in organismal development, from early embryogenesis to lineage commitment, and formation and maturation of cellular subtypes and complex tissues. In this review, we will focus on the roles of miRNAs in the earliest stages of metazoan development, and in the establishment and maintenance of pluripotent progenitor cells from which all foetal tissues arise.

miRNAs in early embryogenesis

It is likely that miRNAs are essential at the earliest stages of development, although there appears to be somewhat conflicting evidence on this point. It may be that there are some species-specific differences in the roles of miRNAs in development. However, many of the discrepancies can be attributed to differences in experimental design, the contribution of maternal proteins and maternal miRNAs, and differences in the life cycles of specific organisms.

Many experiments have been performed by knocking out dicer and dgcr8, two enzymes involved in miRNA biogenesis, in various organisms. In the nematode C. elegans, dcr-1 −/− worms are viable, but infertile, and have some partially penetrant developmental abnormalities, such as absent alae and a burst vulva phenotype [1, 2]. dicer1 −/− zebrafish hatch and appear normal for the first week after fertilization, then start having decreased activity and growth arrest, and eventually die between 14 and 21 days after fertilization [3]. Mouse embryos lacking dicer1 or dgcr8 expression displayed more severe defects than their nematode and zebrafish counterparts. By embryonic day E7.5, there were 50% fewer than expected dicer1 −/− embryos, and the dicer1 −/−

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embryos that were observed were stunted and morphologically abnormal, with conspicuous absence of expression of both Oct4 and brachyury, which suggests depletion of the pool of pluripotent stem cells, as well as arrest of development prior to gastrulation [4]. Mouse embryos deficient in zygotic dgcr8 were early embryonic lethal and showed gross morphological defects by E6.5 [5].

Although there is developmental arrest at quite different stages in these three organisms (the adult stage in *C. elegans*, the larval stage in the zebrafish and prior to gastrulation in the mouse), impairments in development appear at similar times after fertilization, suggesting that the arrest may not be stage-specific, but rather occurs when falling levels of maternally contributed dicer or dgcr8 reach critical thresholds. Therefore, the organism with the shortest life cycle, *C. elegans*, which develops from egg to adult in 2.5 days can progress to adulthood without production of new dicer mRNA or protein. In contrast, the zebrafish has an embryonic period of 2 days and a larval stage lasting 27 days, and arrests 5 days into the larval stage, whereas the mouse arrests at day 6.5–7.5 out of its 21-day gestational period.

Evidence that maternally contributed dicer1 mRNA is functional for the first week of development, and that maternally contributed dicer1 protein and/or miRNAs are present for ~3 days was provided by a study using microinjection of morpholinos into one-cell stage zebrafish zygotes. Morpholino knockdown of dicer1 mRNA resulted in arrest at approximately 3 days after fertilization and death at 7 days after fertilization [3]. Further evidence was provided using a germline replacement method in zebrafish to eliminate both maternal and zygotic dicer1. Germ cells from dicer1 −/− embryos were transplanted into germ-cell-depleted wild-type embryos. Crossing these fish containing dicer1 −/− germ cells resulted in embryos that lack both maternal and zygotic dicer1, termed MZdicer embryos. These embryos had normal axis formation and regionalization, but displayed growth and developmental defects starting from the first day, including defects in gastrulation, somitogenesis, and brain and heart development [6]. Interestingly, microinjection of members of the mir-430 family of miRNAs, which are highly expressed in the wild-type early embryo [7], into MZdicer embryos at the one-cell stage rescued brain morphogenesis, but not heart development [6].

The critical role of miRNAs starting from the very first cell division in the mouse was demonstrated by crossing mice with a conditionally floxed dicer1 allele with mice that carried the Cre recombinase under the control of the oocyte-specific Zp3 promoter. In this way, animals that lacked dicer1 only in their oocytes were generated. Fertilizing these dicer1-depleted oocytes with sperm from wild-type males resulted in zygotes that were unable to undergo their first cell division, demonstrating that maternal dicer1 was necessary at the first cleavage stage [8].

miRNAs in embryonic stem cells

Embryonic stem cells (ESCs) are derived from the inner cell mass of blastocyst-stage embryos. The cardinal features of ESCs are the capacity for indefinite self-renewal, pluripotence and a normal karyotype. In the mouse, the definitive proof of pluripotence is germline transmission, whereby progeny whose genetic material is entirely of ESC origin are produced. Common assays used to demonstrate pluripotence in hESCs include the formation of embryoid bodies (EBs) *in vitro* and transplantation into immunocompromised mice to generate mature teratomas.

As on the organismal level, the functions of miRNAs in ESCs have been probed using gene knockout strategies. Dicer1-null mESCs continued to display typical mouse ESC morphology and express the pluripotence marker Oct-4/Pou5f1, but were deficient in centromeric silencing. Moreover, they had a slowed proliferation rate, and were unable to differentiate, shown by the failure to contribute to the tissues of chimeric mice, form teratomas in immunodeficient mice, or generate EBs in suspension culture [9, 10].

Dgcr8 null mouse ESCs also showed preserved ESC morphology and expression of pluripotence markers, as well as a longer doubling time and defects in EB and teratoma formation [5]. However, the defect in differentiation appeared to be more severe in the dicer1 null cells than the dgcr8 null cells, because the dgcr8 null EBs arrested later in the differentiation process (16 days, rather than 8), and did express some markers of differentiation, albeit at low levels. This might have been due to the fact that dicer1 is necessary for processing of both miRNAs and siRNAs, whereas dgcr8 is specific to the miRNA biogenesis pathway.

These data suggest that while miRNAs promote (but are not required for) the self-renewal of ESCs, they are essential for pluripotence. Further evidence supporting this conclusion was shown by reintroducing the dicer1 gene into dicer1-null mESCs, which had previously been shown to be unable to differentiate, and rescuing the ability to differentiate [9].

Roles of specific miRNAs in early embryogenesis

Evidence that a small number of specific miRNAs are critical for early embryonic development was provided by a series of experiments from the Plasterk laboratory. miRNA expression profiling of zebrafish embryos at different stages of development [7] revealed that the mir-430 family of miRNAs were highly expressed between 5 and 48 hrs after fertilization. Several members of the mir-430 family were able to rescue the brain morphogenesis defect when injected at the one-cell stage into embryos deficient in maternal and zygotic dicer [6]. It was also proposed that the mir-430 family contributes to clearance of maternal mRNAs, an important step in the maternal-to-zygotic transition [6]. The mRNA expression profiles of dicer-deficient embryos and dicer-deficient embryos complemented with injected mir-430 were compared to identify putative mir-430 target mRNAs. The set of identified mRNAs was enriched both for mRNAs with mir-430 target sequences in their 3′UTRs and for maternally expressed mRNAs. There was also evidence that the targeted mRNAs were destabilized by deadenylation.

Table 1 Summary of large-scale miRNA sequencing papers including mESCs and hESCs. The number of samples analysed, the species and embryonic stem cell lines represented, the number of known miRNAs observed and the number of novel miRNAs found are shown. J1aza indicates J1 mESCs treated with the DNA methyltransferase inhibitor 5'-aza-deoxycytidine

	Landgraf *et al.* 2007 [18]	Calabrese *et al.* 2007 [19]	Morin *et al.* 2008 [20]	Bar *et al.* 2008 [21]
#Samples	256	4	2	2
Species	Human, mouse, rat	Mouse	Human	Human
ESC samples (# reads/sample)	Male mESC (1254)	Dicer +/+ (57,834)	H9 hESC (6,147,718)	H1-Undiff (280,185)
	Female mESC (335)	Dicer −/− (54,339)	H9 EB (6,014,187)	H1-Diff (143,124)
		J1 (149,986)		
		J1aza (155,934)		
Known miRNAs	340 human/303 mouse/205 rat	360	334	191
Novel miRNAs	12 human/23 mouse/14 rat	46	83	13

In *Drosophila*, temporal reciprocity of expression of the mir-309 family and their predicted target mRNAs across the maternal-to-zygotic transition has been observed [11].

The zebrafish model has also been used to elucidate functional targets of the mir-430 family. Nodal is a TGFb family member that activates a signalling pathway involved in left-right axis determination and mesoderm induction (reviewed in [12]). The mir-430 family has been implicated in the tuning of Nodal activity by down-regulating both the Nodal agonist squint and the Nodal antagonist lefty [13].

Roles of specific miRNAs in embryonic stem cells

miRNA profiling of mouse and human embryonic stem cells (mESCs and hESCs) has been performed using a variety of methods, and has been used to identify candidate miRNAs that may be important in establishing or maintenance of the pluripotent phenotype. Cloning and sequencing of novel miRNAs using standard methods has been used to identify miRNA expressed in mESCs [14] and hESCs [15]. These authors used qualitative Northern blot to demonstrate differential expression of a subset of the cloned miRNAs in ESCs. Two studies measured miRNA expression in murine ESCs by qRT-PCR [16, 17]. In all four studies, two clusters of miRNAs were found to be strongly up-regulated in ESCs (mir-302, mmu-mir-290/hsa-mir-371/372/373).

There have been four papers describing large-scale small-RNA sequencing studies including ESC samples (Table 1). Landgraf *et al.* sequenced ~330,000 clones from 256 small RNA libraries from a variety of mouse, rat and human cells and tissues, including two mESC lines [18]. The results of this study included the discovery of a small number of novel candidate miRNAs, as well as evidence that each cell type expressed only a limited repertoire of approximately 70 miRNAs. However, these findings might have stemmed from the shallowness of the sequencing performed (only ~1000 clones per library were sequenced). The low clone counts also precluded the collection of statistically significant expression information from this dataset, but the results were largely consistent with the previous four studies described above.

Calabrese *et al.* sequenced four mESC samples, two from a floxed Dicer mESC cell line before (Dicer +/+) and after (Dicer −/−) deletion of one of the Dicer catalytic domains, and two from the J1 mESC line before (J1) and after (J1aza) treatment with the DNA methyltransferase inhibitor 5-aza-deoxycytidine [19]. This group found that approximately 70% of the small RNAs found in the Dicer +/+ and J1 samples were encoded in 6 genomic clusters, including the mir-290, mir-17, mir-467 clusters, an imprinted cluster on chromosome 12, the mir-21 cluster and the mir-15b/16 cluster. Noting that the mir-17, mir-15b/16 and mir -290 clusters had been associated with the cell cycle or oncogenesis, the authors suggested that a major role for miRNAs in ES cells was in cell cycle regulation. One drawback of this study was the absence of non-ESCs for comparison.

Morin *et al.* performed high-throughput sequencing on a sample of undifferentiated hESCs and a sample of EBs differentiated from hESCs [20]. Of the four large-scale sequencing studies, this paper reports the greatest depth of sequencing. However, this study shows the greatest discrepancy from all the other reports. Many of the miRNAs found to be overexpressed in ESCs compared to differentiated cells in other studies were found to be expressed more highly in the EBs than the undifferentiated hESCs in this study. In addition, there were examples of miRNAs that might be expected to be coordinately expressed (*e.g.* those located in the same genomic cluster, or those that have similar seed sequences) displaying opposing expression patterns. A possible explanation for these findings lies in the choice of EBs as the comparison sample. The generation of EBs from ESCs is an

unpredictable process, which results in a mixed population of variable undifferentiated, differentiating and differentiated cells in varying ratios. The EBs analysed in this study were collected at 15 days, at which time there can still be many undifferentiated or partially differentiated cells present. It is possible that these authors have found an interesting pattern of miRNA expression that is present in cells undergoing early differentiation, but further studies that carefully dissect out the timecourse of miRNA expression in a homogeneous population of differentiating cells will need to be done to discern whether or not this is the case.

The most recent sequencing paper, by Bar *et al.*, performed high-throughput sequencing on undifferentiated and spontaneously differentiated hESCs. Results of this study were consistent with most of the other expression studies, with members of the mir-302, mir-17 and mir-519 clusters showing higher expression in the undifferentiated sample than the differentiated sample [21].

Two common limitations among these four sequencing studies were insufficient depth of sequencing and lack of replication, with a single library constructed and sequenced for each sample. Given that the error rates seen in high-throughput sequencing sets can be quite substantial, and that different miRNAs can display vastly different copy numbers, ranging from one to tens of thousands of copies per cell, the expression levels of relatively rare miRNA species are not well measured until the number of reads is in the millions, which was only achieved in the Morin *et al.* study. The actual variance in the sequencing data cannot be estimated without adequate technical and biological replication, which was not achieved in any of the sequencing studies. Each group used a different method for assessing whether candidate mature miRNA sequences represented 'real' miRNAs. These factors may explain why the overlap between lists of novel miRNAs reported in these four sequencing studies was so poor, with only a handful of sequences in common.

A comprehensive microarray profiling analysis of miRNAs in hESCs in the context of a variety of multipotent and differentiated cell lines from our group [22] confirmed many of the results of previous studies, as well as introducing a number of new findings. It was observed that the group of miRNAs up-regulated in hESCs was enriched for oncogenic miRNAs, whereas the group of miRNAs down-regulated in hESCs was enriched for tumour suppressor miRNAs. A large cluster of miRNAs on chromosome 19 adjacent to the mir-371/372/373 cluster containing 54 miRNAs across 96 kb, as well as the small mir-302, mir-371/372/373, mir-17, mir-106a and mir-106b clusters were significantly up-regulated in hESCs. The chromosome 19 cluster had initially been described as a placenta-specific cluster [23]. This study also found that a large imprinted cluster on chromosome 14 containing 57 miRNAs was significantly down-regulated in hESCs. This cluster of miRNAs is coordinately regulated with the upstream noncoding RNA GTL2/MEG3 [24]. Down-regulation of this cluster of miRNA has been associated with high tumour cell proliferation and poor survival in ovarian cancer patients, leading to the hypothesis that members of this cluster are tumour suppressor genes [25]. The homologous mouse chromosome 12 cluster was found to be highly expressed in the paper by Calabrese *et al.* discussed above, but because there were no non-ESC comparison cells in that study, it was not observed in that report that the expression of this cluster is actually lower in ESCs than in differentiated cells.

A recent report was published using ChIP-Seq to study chromatin occupancy of miRNA genes in mESCs [26]. Mapping of H3K4me3, which binds preferentially to transcription initiation sites, H3K36me3, which is a chromatin mark associated with transcriptional elongation was used to define the promoter regions and infer the transcript lengths of the miRNA genes. Mapping of the polycomb group protein Suz12, and the pluripotence-associated transcription factors Oct4/Pou5f1, Sox2, Nanog and Tcf3 was also performed. Interestingly, the promoter regions of both the pluripotence-associated miRNA genes (mir-290 cluster, mir-302 cluster, mir-92 cluster and let-7g) and the lineage-specific miRNA genes (mir-9, mir-124a, mir-155 and mir-708) were co-occupied by the pluripotence-associated transcription factors. However, the lineage-specific miRNA gene promoters were also occupied by Suz12. These findings are consistent with results from the same group suggesting that the pluripotence-associated transcription factors bind to promoters of genes that are highly differentially expressed between pluripotent and differentiated cells, and that binding of polycomb group proteins distinguishes the genes that are repressed in pluripotent cells from those that are active in pluripotent cells.

miRNAs with common seed sequences

Target specificity of miRNAs for cognate mRNAs is strongly influenced by Watson-Crick base-pairing between a 'seed' sequence located at positions 2-8 in the mature miRNA sequence and target sequences in the mRNA 3′UTR [27–29]. Eighteen out of 76 miRNAs significantly up-regulated in hESCs share a dominant seed sequence. These miRNAs share at least 6 out of 7 nucleotides in the seed sequence. In humans, the miRNAs containing this dominant seed sequence are located in the mir-302 cluster, the mir-371/372/373 cluster, the chromosome 19 cluster, and three homologous clusters on chromosome 13 (the tumour-associated mir-17 cluster), chromosome 7 (the mir-106b cluster) and the X chromosome (the mir-106a cluster) (Table 2).

The large miRNA cluster on chromosome 19 is primate-specific, and is interspersed with primate-specific Alu repeat elements. It appears that other organisms have independently experienced massive expansions of miRNAs containing the same dominant seed sequence. Examples include the *Drosophila* mir-430 family, which has been shown to be important in early embryogenesis, as discussed above, as well as a large cluster found on mouse chromosome 2, containing multiple members of the mir-467 family, which was first described in the report by Landgraf *et al.* [18], and the mir-427 family in Xenopus tropicalis, of which there are ~80 copies clustered in the genome [30]. As noted previously, the lack of depth of sequencing in the Landgraf *et al.* study

Table 2 Table of human, mouse, zebrafish and frog microRNAs with seed sequences sharing at least 6 out of 7 nucleotides. The miRNAs significantly up-regulated in hESCs are highlighted in blue. Mature miRNA sequences are shown, with positions 1, 2-8, 9 and 10-end denoted. The chromosomal locations of the homologous human miRNA cluster are indicated at the left, and the arm of the precursor hairpin encoding the mature miRNA sequence is listed to the right of each sequence.

Species: human		**Mature miRNA nucleotide number**				
	miRNA name	1	2-8	9	10-end	arm
chr 4	hsa-miR-302a	T	AAGTGCT	T	CCATGTTTTGGTGA	3′
	hsa-miR-302b	T	AAGTGCT	T	CCATGTTTTAGTAG	3′
	hsa-miR-302c	T	AAGTGCT	T	CCATGTTTCAGTGG	3′
	hsa-miR-302d	T	AAGTGCT	T	CCATGTTTGAGTGT	3′
chr 7	hsa-miR-106b	T	AAAGTGC	T	GACAGTGCAGAT	5′
	hsa-miR-93	A	AAGTGCT	G	TTCGTGCAGGTAG	5′
chr 13	hsa-miR-17-5p	C	AAAGTGC	T	TACAGTGCAGGTAGT	5′
	hsa-miR-18a	T	AAGGTGC	A	TCTAGTGCAGATA	5′
	hsa-miR-20a	T	AAAGTGC	T	TATAGTGCAGGTAG	5′
chr 19	hsa-miR-372	A	AAGTGCT	G	CGACATTTGAGCGT	3′
	hsa-miR-373	G	AAGTGCT	T	CGATTTTGGGGTGT	3′
	hsa-miR-519a	A	AAGTGCA	T	CCTTTTAGAGTGTTAC	3′
	hsa-miR-519b	A	AAGTGCA	T	CCTTTTAGAGGTTT	3′
	hsa-miR-519c	A	AAGTGCA	T	CTTTTTAGAGGAT	3′
	hsa-miR-519d	C	AAAGTGC	C	TCCCTTTAGAGTGT	3′
	hsa-miR-519e	A	AAGTGCC	T	CCTTTTAGAGTGT	3′
	hsa-miR-520a	A	AAGTGCT	T	CCCTTTGGACTGT	3′
	hsa-miR-520b	A	AAGTGCT	T	CCTTTTAGAGGG	3′
	hsa-miR-520c	A	AAGTGCT	T	CCTTTTAGAGGGTT	3′
	hsa-miR-520d	A	AAGTGCT	T	CTCTTTGGTGGGTT	3′
	hsa-miR-520e	A	AAGTGCT	T	CCTTTTTGAGGG	3′
	hsa-miR-526b*	A	AAGTGCT	T	CCTTTTAGAGGC	3′
X chr	hsa-miR-106a	A	AAAGTGC	T	TACAGTGCAGGTAGC	5′
	hsa-miR-18b	T	AAAGTGC	A	TCTAGTGCAGTTA	5′
	hsa-miR-20b	C	AAAGTGC	T	CATAGTGCAGGTAG	5′
Species: mouse		**Mature miRNA nucleotide number**				
	miRNA name	1	2-8	9	10-end	arm
chr 4	mmu-miR-302a	T	AAGTGCT	T	CCATGTTTTGGTGA	3′
	mmu-miR-302b	T	AAGTGCT	T	CCATGTTTTAGTAG	3′
	mmu-miR-302c	A	AGTGCTT	C	CATGTTTCAGTGG	3′
	mmu-miR-302d	T	AAGTGCT	T	CCATGTTTGAGTGT	3′

Continued

Table 2 Continued.

chr 7	mmu-miR-106b	T	AAAGTGC	T	GACAGTGCAGAT	5′
	mmu-miR-93	C	AAAGTGC	T	GTTCGTGCAGGTAG	5′
chr 13	mmu-miR-17	C	AAAGTGC	T	TACAGTGCAGGTAG	5′
	mmu-miR-18a	T	AAGGTGC	A	TCTAGTGCAGATAG	5′
	mmu-miR-20a	T	AAAGTGC	T	TATAGTGCAGGTAG	5′
chr 19	mmu-miR-290-3p	A	AAGTGCC	G	CCTAGTTTTAAGCCC	3′
	mmu-miR-291a-3p	A	AAGTGCT	T	CCACTTTGTGTGC	3′
	mmu-miR-291b-3p	A	AAGTGCA	T	CCATTTTGTTTGT	3′
	mmu-miR-292-3p	A	AAGTGCC	G	CCAGGTTTTGAGTGT	3′
	mmu-miR-294	A	AAGTGCT	T	CCCTTTTGTGTGT	3′
	mmu-miR-295	A	AAGTGCT	A	CTACTTTTGAGTCT	3′
X chr	mmu-miR-106a	C	AAAGTGC	T	AACAGTGCAGGTAG	5′
	mmu-miR-18b	T	AAGGTGC	A	TCTAGTGCTGTTAG	5′
	mmu-miR-20b	C	AAAGTGC	T	CATAGTGCAGGTAG	5′
	mmu-miR-105	C	CAAGTGC	T	CAGATGCTTGTGGT	5′
Species-specific	mmu-miR-467a	T	AAGTGCC	T	GCATGTATATGCG	5′
	mmu-miR-467b	G	TAAGTGC	C	TGCATGTATATG	5′
	mmu-miR-467c	T	AAGTGCG	T	GCATGTATATGTG	5′
	mmu-miR-467d	G	AAGTGCG	C	GCATGTATATGCG	5′
Species: zebrafish		**Mature miRNA nucleotide number**				
	miRNA name	1	2-8	9	10-end	arm
chr 7	dre-miR-93	A	AAAGTGC	T	GTTTGTGCAGGTA	5′
chr 13	dre-miR-17a	C	AAAGTGC	T	TACAGTGCAGGTA	5′
	dre-miR-18a	T	AAGGTGC	A	TCTAGTGCAGATA	5′
	dre-miR-20a	T	AAAGTGC	T	TATAGTGCAGGTAG	5′
X chr	dre-miR-18b	T	AAGGTGC	A	TTTAGTGCAGATA	5′
	dre-miR-18c	T	AAGGTGC	A	TCTTGTGTAGTTA	5′
	dre-miR-20b	C	AAGGTGC	T	CACAGTGCAGGTAG	5′
Species-specific	dre-miR-430a	T	AAGTGCT	A	TTTGTTGGGGTAG	3′
	dre-miR-430ab	A	AAGTGCT	A	TCAAGTTGGGGTAG	3′
	dre-miR-430c	T	AAGTGCT	T	CTCTTTGGGGTAG	3′
	dre-miR-430i	T	AAGTGCT	A	TTTGTTGGCGTAG	3′
	dre-miR-430j	A	AAGTGCT	A	TCAAATTGGGGTA	3′
Species: frog			**Mature miRNA nucleotide number**			
	miRNA name	1	2-8	9	10-end	arm
chr 7	xtr-miR-93a	A	AAGTGCT	G	TTCGTGCAGGTAG	5′
	xtr-miR-93b	A	AGTGCTG	T	TCGTGCAGGTAG	5′

Continued

Table 2 Continued.

chr 13	xtr-miR-17-5p	C	AAAGTGC	T	TACAGTGCAGGTAGT	5′
	xtr-miR-18a	T	AAGGTGC	A	TCTAGTGCAGATAG	5′
	xtr-miR-20a	T	AAAGTGC	T	TATAGTGCAGGTAG	5′
X chr	xtr-miR-106	A	AAAGTGC	T	TATAGTGCAGGTAGA	5′
	xtr-miR-18b	T	AAGGTGC	A	TCTAGTGCAGTTAG	5′
	xtr-miR-20b	C	AAAGTGC	T	CATAGTGCAGGTAG	5′
Species-specific	xtr-miR-427	G	AAAGTGC	T	TTCTGTTTTGGGCG	3′

precluded rigorous expression analysis. However, there was a trend toward higher expression of the mir-467 cluster of miRNAs in mESCs and embryonic cells. It is clear that the numerous miRNAs that contain this ESC-dominant seed sequence are descended from at least two unrelated ancestral DNA sequences, because some of them are encoded on the 5′ arm of the precursor hairpin, whereas others are encoded on the 3′ arm (Table 2).

Overlapping functions of the mir-106b cluster and the mir-17 cluster were demonstrated in the mouse. Deletion of the mir-106b cluster did not result in any detectable phenotype, and deletion of the mir-17 cluster resulted in neonatal lethality, with lung hypoplasia, ventricular septal defect and defects in B cell development [31]. Double mutants lacking both the mir-106b cluster and the mir-17 cluster displayed a more severe phenotype, with embryonic lethality at midgestation. It would be interesting to discover whether additional deletions of the mir-106a cluster, the mir-290 cluster and the mir-467 cluster in mouse would lead to a yet more pronounced phenotype.

High expression of miRNAs containing this dominant seed sequence in several different types of highly proliferative cells, including hESCs, mESCs, early embryonic cells, placental cells and tumour cells in a variety of different organisms, suggests that down-regulation of a common set of target mRNAs may enhance the proliferative properties of many diverse cell types. However, the variation in sequences outside the seed among the specific miRNAs in this set (see Table 2) and in the relative amounts of the specific miRNAs among different cell types points to the likelihood that there will be quantitative and qualitative differences in the set of mRNAs targeted by these miRNAs in different cells types.

Conclusion

miRNAs play key roles at several stages in metazoan development. They are essential for early embryogenesis and cellular differentiation, and enhance proliferation and the maternal-to-zygotic transition, although they do not appear to be required for these latter functions. In short, they seem to be essential for differentiation, but not self-renewal. This is consistent with the observation that miRNAs appear to have arisen around the same time as multicellularity, when cellular differentiation became a more central concern of the organism. Only one unicellular organism, *Chlamydomonas reinhardtii*, has been shown to produce miRNAs [32], and these miRNAs appear to be quite different from those found in other organisms.

The possibility that one of the primary functions of miRNAs is to effect changes in cell state leads us to the notion that miRNAs might be used as tools to efficiently direct cellular differentiation. The ability to control the phenotypes of cells would be very useful in the development of desired cell types for clinical and preclinical applications, such as cell therapies or *in vitro* testing of pharmaceuticals. In addition, one could imagine the use of miRNAs directly as therapeutic agents, such as signals to direct cancer cells into a nonproliferative terminally differentiated state.

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