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Mammalian MicroRNAs: A Small World for Fine-Tuning Gene Expression

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Abstract

The basis of eukaryotic complexity is an intricate genetic architecture where parallel systems are involved in tuning gene expression, via RNA-DNA, RNA-RNA and RNA-protein and DNA-protein interactions. In higher organisms, about 97% of the transcriptional output is represented by non-coding RNA (ncRNA) encompassing not only rRNA, tRNA, introns, 5' and 3'-untranslated regions, transposable elements and intergenic regions, but also a large rapidly emerging family, named microRNAs. MicroRNAs are short 20-22 nucleotide RNA molecules that have been shown to regulate the expression of other genes in a variety of eukaryotic systems. MicroRNAs are formed from larger transcripts that fold to produce hairpin structures and serve as substrates for the cytoplasmic Dicer, a member of the RNase III enzyme family. A recent analysis of the genomic location of human microRNA genes suggested that 50% of microRNA genes are located in cancer-associated genomic regions or in fragile sites. This review focuses on the possible implications of microRNAs in post-transcriptional gene regulation in mammalian diseases, with particular focus on cancer. We argue that developing mouse models for deleted and/or overexpressed microRNAs will be of invaluable interest to decipher the regulatory networks where microRNAs are involved.

What are microRNAs?

MicroRNAs are a group of small non-coding RNA (ncRNA) molecules, distinct from but related to small interfering RNAs (siRNAs), that have been identified in a variety of organisms (for reviews see He and Hannon 2004; Bartel 2004). These small 20-22 nucleotides (nt) RNAs are transcribed as parts of longer molecules of several kilobases (kb) in length that are processed in the nucleus into hairpin RNAs of 70-100 nt by the double-stranded RNA-specific ribonuclease Drosha (Cullen 2004). The hairpin RNAs are transported to the cytoplasm, via an exportin-5 dependent mechanism, where they are digested by a second, double-stranded specific ribonuclease called Dicer. These mechanisms are described in more detail in the next section. In animals, single-stranded microRNA binds specific messenger RNA (mRNA) through sequences that are significantly, though not completely, complementary to the target mRNA, mainly to the 3' untranslated region (3' UTR). By a mechanism that is not fully characterized, the bound mRNA remains untranslated, resulting in reduced levels of the corresponding protein; alternatively, the bound mRNA can be degraded, resulting in reduced levels of the corresponding transcript (Fig. 1).

The central dogma of classical biology is that genetic information flows from DNA to RNA to proteins. Therefore "genes" are synonymous with proteins and a gene is defined as a protein-coding region with associated regulatory signals (Mattick 2003). MicroRNAs represent new stars in the gene regulation galaxy, and there is a strong interest among researchers in different fields to understand their mechanism of action and identify their targets. The definition of a gene in the genomics era should be expanded as a "transcription unit" or "a complete chromosomal segment responsible for making a functional product" (Okazaki et al. 2002). Due to the complex genetic architecture of eukaryotic cells, this definition is still not complete, and additional issues such as overlap, alternative splicing, or transcribed pseudogenes have to be considered (Snyder and Gerstein 2003). Thus, the term "gene" could be expanded to include microRNAs (and other functional ncRNAs).

The number of microRNAs is growing rapidly, especially after using "in silico" cloning (Table 1). One such bioinformatics tool is MiRscan: it ranks predicted hairpins that are conserved in the genome of two related animals based on several criteria (Lim et al. 2003). Initially, it was estimated that there could be from 200 to 1,000 microRNA genes in the mammalian genome (~1-3% of known genes are represented by microRNAs). Today the number of microRNAs, including those electronically cloned, is over one thousand and still growing (Berezikov et al. 2005; Xie et al. 2005). Mammalian microRNA genes have been distinguished by using the prefix *mir* followed by a number; the prefixes *lin* and *let*refer to microRNAs originally identified in *C. elegans*. (see below). The MicroRNA Registry website contains a comprehensive list of microRNAs from all species (Table 1).

How are microRNAs produced?

More than a decade ago, Victor Ambros and colleagues, discovered that *lin-4*, a gene known to control the timing of *C. elegans* larval development, does not code for any protein, but instead codes for a pair of small RNAs (Lee et al. 1993). One RNA is longer (~70 nt) and can fold in a stem-loop structure, as the precursor of the shorter RNA (~22 nt). The Ambros and Ruvkun laboratories later discovered that the small *lin-4* RNAs had an antisense complementarity to multiple sites in the 3' UTR of the *lin-14* protein-coding gene (Lee et al. 1993; Wightman et al. 1993). The sequence complementarity between the non-coding *lin-4* RNA and the 3' UTR of the protein-coding *lin-14* gene suggested that *lin-4* regulated translation of *lin-14* by binding to the 3' UTR. It was later shown that *lin-4* regulated the *lin-14* gene during early larval development as well as the *lin-28* gene during late larval development by a mechanism involving RNA-RNA binding that led to suppression of translation (Moss et al. 1997). The shorter *lin-4* RNA is now known as the founding member of the class of microRNA genes (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001).

The majority of microRNAs (70%) are located in introns and/or exons, and ~30% are located in intergenic regions (Rodriguez et al. 2004) (Fig. 1). The first group of microRNAs from introns and/or exons are oriented in sense with the exon-coding "host" gene, and therefore, may be transcribed as part of annotated genes. The second group of microRNAs

are transcribed from intergenic regions (gene deserts) (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001), indicating that they form independent transcription units (Lee et al. 2002). The third group of microRNAs are derived from introns and/or exons of annotated genes, but are transcribed in the antisense orientation, suggesting that they too form their own transcription units. Tightly linked microRNAs may be transcribed as polycistronic messengers; however, microRNAs separated by more than 50 kb tend to represent independent transciption units (Baskerville and Bartel 2005). RNA polymerase II and III are candidates for pri-microRNAs (primary) transcription (Fig.1).

In animals (Fig.1), the first step in microRNA maturation is the nuclear cleavage of the several kb long pri-microRNA, which releases a ~70 nt cropped hairpin-shaped intermediate, known as pre-microRNA (precursor). The Drosha RNase III endonuclease is responsible for this nuclear processing (Bartel 2004; Lee et al. 2003; Zeng et al. 2005). Drosha cannot cleave without a partner, forming a complex with DGCR8 (a product of the DiGeorge syndrome critical region gene 8), which contains two double-stranded RNA-binding domains (Tomari and Zamore 2005; Han et al. 2004). The hairpin precursor is actively transported from the nucleus to the cytoplasm by Exportin-5 (Yi et al. 2003; Lund et al. 2004). A second enzyme, an RNase III endonuclease called Dicer, is responsible for generating a ~21 nt short single-stranded RNA that is the mature microRNA (Lee et al. 2003). Dicer was first recognized for its role in generating siRNAs that mediate RNA interference (RNAi) (Bernstein et al. 2001). After cleavage in the cytoplasm, the microRNA pathways of plants and animals share some other steps with RNA silencing, and the cleavage products become incorporated as single-stranded RNAs into the ribonucleoprotein RNA-induced silencing complex, RISC (Hammond et al. 2000). The RISC has been purified from fruit fly and human cells and in both cases contains a member of the Argonaute protein family (essential for gene silencing in *Caenorhabditis elegans, Neurospora*, and *Arabidopsis*), which is thought to be a core component of the complex (Hammond et al. 2001; Mourelatos et al. 2002). Once incorporated in the cytoplasmic RISC complex, the microRNA will specify cleavage if the mRNA has sufficient complementarity to the microRNA, or it will block translation if the microRNA does not have sufficient complementarity, resulting in reduced expression of the corresponding protein. MicroRNA

regulation of the microRNA:mRNA duplex is mainly mediated through multiple complementary sites in the 3' UTRs, but there are many exceptions. MicroRNAs may also bind the 5' UTR and/or the coding region of mRNAs, resulting in a similar outcome (Fig. 1).

How are microRNAs working?

The functions of microRNA are various, such as the control of leaf and flower development in plants (Aukerman and Sakai 2003) or the modulation of hematopoietic lineage differentation in mammals (Chen et al. 2004). Several groups have uncovered roles for microRNAs in the coordination of cell proliferation and cell death during development, and in stress resistance and fat metabolism (Ambros 2003). For example, the *Drosophila* microRNA gene (*mir-14*) suppresses cell death and is required for normal fat metabolism (Xu et al. 2003), while the *bantam* locus encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene *hid* in *Drosophila* (Brennecke et al. 2003). It was recently shown that, in addition to their regulatory functions, cellular microRNAs mediate antiviral defenses in human cells (Lecellier et al. 2005).

However, the function of most microRNAs is not known. The antisense single-stranded microRNAs can bind specific mRNA transcripts through sequences that are significantly, though not completely, complementary to the target mRNA. This process is also known as post-transcriptional gene regulation (PTGS). Some microRNAs can downregulate large numbers of target mRNAs (Lim et al. 2005) and it has been speculated that microRNAs could regulate ~30% of the human genome (Bartel 2004). MicroRNAs seem to be responsible for fine regulation of gene expression, "tuning" the cellular phenotype during delicate processes like development and differentiation in all organisms, from plants to mammals. Many microRNAs are conserved in sequence between distantly related organisms (see The MicroRNA Registry in Table 1), suggesting that these molecules participate in essential processes (Lagos-Quintana et al. 2003; Pasquinelli et al. 2000). One example is the cluster of *mir-16-1* and *mir-15a*, which is highly conserved in 9 out of 10 primate species (Berezikov et al. 2005).

Target identification has been hampered by the fact that in animals, in contrast to plants, microRNAs do not bind perfectly to their targets. A few nucleotides typically remain unbound, yielding complex secondary structures. Mammalian genes can have more than one microRNA target site in their 3' UTRs and one microRNA can target more than one mRNA. Bioinformatics approaches have been developed to search for the most thermodynamically favorable microRNA:mRNA duplex interactions (Table 1). Several computational procedures are available to predict microRNA targets such as DianaMicroT, TargetScan, and miRanda (Lewis et al. 2003; Kiriakidou et al. 2004; Enright et al. 2003). Another set of programs developed for target identification is RNAhybrid, RNAcalibrate, and RNAeffective (Rehmsmeier et al. 2004), which work by searching for the most energetically favorable hybridization sites for the smaller microRNA within the larger mRNA (Table 1). Only a few target mRNAs have been experimentally proven and studied *in vitro*, but the numbers of such confirmed interactions is expected to sharply increase, as prediction tools become more sophisticated. Generally, the proven mRNAs targets are produced from different chromosomes as the corresponding microRNAs (such as *mir-19a* on chromosome 13q31.3 having as a target the mRNA of the PTEN tumor suppressor gene located on chromosome 10q23.3), but examples of microRNAs targeting "host" antisense exon-coding genes are also known. An important example is that of *mir-196*, which leads to the cleavage and degradation of mRNA from the homeobox gene *HOXB8* (Yekta et al. 2004).

MicroRNAs are not the only ncRNA sequences that have been shown to play a role in the regulation of gene expression. A large set of ncRNAs are known as "gene regulators" and the list in mammals includes *Air, H19*, *Ipw, NTT, Tsix* and *XIST* (Table 2). These ncRNAs have a variety of functions, from potential involvement in the imprinting process to X-chromosome inactivation in mammals. It is likely that other ncRNAs will be found, albeit with additional effects on critical biological processes.

MicroRNAs and Human Cancer

Several papers have now shown the possibility that small ncRNAs play an important role in cancer (Table 3). We show in Figure 2 diagrams of several proposed mechanisms for

microRNAs as cancer players (Calin et al. 2004a, McManus 2003). Inherited or somatic mutations, amplification, deletion, or epigenetic silencing of microRNA genes may not only cause certain cancers, but may contribute to an individual's risk of developing cancer (Fig. 2).

The first report linking microRNAs and cancer (Calin et al. 2002) involves chronic lymphocytic leukemia (CLL), the most common form of adult leukemia in the Western world. Hemizygous and/or homozygous loss at chromosome 13q14 occurs in more than half of CLL cases. Loss of chromosome 13q14 is also found in more than 50% of mantle cell lymphomas (Stilgenbauer et al. 1998), ~30% of multiple myeloma (Elnenaei et al. 2003), and ~69% of prostate cancers (Dong et al. 2001), suggesting that one or more tumor suppressor genes located at chromosome 13q14 are involved in the pathogenesis of human tumors (Calin et al. 2002). However, detailed genetic analysis, including extensive loss of heterozygosity (LOH), mutation, and expression studies failed to demonstrate the consistent involvement of any of the 12 protein-coding genes located in or close to the deleted region. A cluster of two microRNAs, *mir-15a* and *mir-16-1,* was found within the minimal region of deletion (~30 kb) at 13q14, and to be deleted or downregulated in ~70% of CLL samples. Similarly, a t(12;13) chromosome translocation in a patient with CLL was found to decrease the *mir-15a* and *mir-16-1* precursors (Calin et al. 2002). A similar cluster of microRNAs, named *mir-15b* and *mir-16-2*, is located on human chromosome 3, but is expressed at very low levels in lymphoid cells.

The first genome-wide systematic search for correlations between the genomic positions of microRNAs and cancer-associated genomic regions (CAGRs) provided a catalog of microRNAs possibly involved in cancer (Calin et al. 2004a). 186 microRNAs were mapped to the human genome and compared to the location of previously reported nonrandom genetic alterations identified in human tumors, as well as with cloned fragile sites (FRAs). Minimal regions of LOH are suggestive of the presence of tumor suppressor genes, minimal regions of amplification for the presence of oncogenes, while common breakpoint regions are found close to both types of cancer genes. Some common fragile sites predispose to DNA instability in cancer cells, indeed they are preferential sites of sister chromatid exchange, translocation, deletion, amplification, or integration of plasmid DNA and tumor-

associated viruses. This study concluded that 19% (35 of 186) of microRNAs are located inside or near FRAs and the relative incidence of microRNAs inside FRAs occurred at a rate 9-times higher than in non-FRAs. A significant proportion, 52.5% (98 of 186) of microRNAs are in CAGRs described in a variety of tumors such as lung, breast, ovarian, colon, gastric, and hepatocellular carcinoma, as well as leukemias and lymphomas.

Little was known about the expression levels of microRNA genes in normal and neoplastic cells until 2002. To assess cancer-specific expression levels for hundreds of microRNAs is time-consuming, requires a large amount of total RNA, and the use of radioactive isotopes. cDNA microarrays are useful tools to identify different patterns of expression in large number of samples. The first developed oligonucleotide microRNA microarray chips (Liu et al. 2004), containing hundreds of human precursor and mature microRNA probes identified distinct patterns of microRNA expression in human and mouse tissues (tissue-specific microRNA expression signatures). Another method to determine microRNA expression levels involves the use of a bead-based flow cytometric technique (Lu et al. 2005a). Since these two methods of assessing global microRNA expression, several commercially available platforms have been developed for microRNA gene expression profiling.

Specific differences in microRNA expression between CLL samples and normal CD5+ B-cells, the latter representing normal cells corresponding to CLL malignant cells, were found. Two clusters of CLL samples were identified that were associated with the presence or absence of ZAP-70 expression, a predictor of early disease progression. Low levels of ZAP-70 (identified by both Western blots and flow cytometry) are associated with good prognosis. Two microRNA signatures were associated with the presence or absence of mutations in the expressed immunoglobulin variable-region genes or with deletions at chromosome 13q14, respectively. Different microRNA expression profiles suggested that specific microRNAs can have prognostic significance in CLL, thus expanding the spectrum of prognostic markers (Calin et al. 2004b). A unique microRNA expression signature composed of 13 genes can differentiate cases with low or high ZAP-70 expression and cases with unmutated or mutated IgVH. Furthermore, both *mir-15a* and *mir-16-1* are members of

this signature, suggesting an important functional role in the pathogenesis of human CLL (Calin et al. 2005).

The list of microRNAs reported to be involved in cancer is growing continuously (Table 3). One report showed that *mir-143* and *mir-145* exhibited reduced levels of the mature microRNA in colon cancer patients, colon cancer cell lines, and precancerous adenomatous polyps when compared to normal tissues by Northern Blot (Michael et al. 2003). On the contrary, levels of unprocessed hairpin precursors were not altered. It is possible that the reduced accumulation of the mature microRNA reflects early changes in neoplastic cells or that this depletion is related to their activity in binding and inactivating target mRNAs in the cytoplasm. Putative targets (human mRNAs that display imperfect complementarity of 70-100%) for *mir-143* and *mir-145* include several genes that encode components of signal transduction pathways (such as RAF, RHO, and G-proteins) and chromatin-mediated control of gene expression were found by computer search (Table 3).

In an independent study, it was shown that *mir-143* regulates adipocyte differentiation (Esau et al. 2004). When antisense oligonucleotides (ASOs) targeting human microRNAs were transfected into cultured human primary subcutaneous pre-adipocytes, *mir-143* was up-regulated as adipocytes differentiated. Bioinformatics approaches predicted several putative microRNA targets. ERK5/BMK1 (which promotes cell growth and proliferation in response to tyrosine kinase signaling) was predicted to be a *mir-143* target and in fact, ERK5 protein levels were up-regulated in *mir-143* ASO-treated adipocytes. But because each microRNA can regulate multiple target genes, *mir-143* could directly inhibit *ERK5* by binding the 3' UTR of *ERK5*, or indirectly, by binding to another target gene (Esau et al. 2004).

Another study reported reduced expression of the *let7* microRNA in human lung cancer and the correlation with clinicopathological features (Takamizawa et al. 2004). Reduction in the expression (>80%) levels of *let-7* was observed in 60% (12 of 20) of cancer cell lines and 44% (7 of 16) of lung tumors when compared with normal lung tissues by Northern blots. The reduced expression was significantly associated with shortened survival of patients after potential curative resection. 143 cases had been followed for more than 5

years after surgery and were used to study the prognostic significance of *let-7*. 143 non-small cell lung carcinoma (NSCLC) cases were classified into clusters 1 and 2 (cluster 1 with low *let7* expression and higher disease stages; cluster 2 with high *let-7* expression and lower disease stages). The difference in postoperative survival between clusters 1 and 2 was highly significant. In addition, overexpression of *let7* in the A549 lung adenocarcinoma cell line inhibited lung cancer cell growth *in vitro* (Takamizawa et al. 2004). Several observations led to the conclusion that *let-7* acts as a tumor suppressor gene in lung tissue. Several *let7* family members, including *let 7a-2*, *let 7c* and *let 7g*, have been mapped to minimally deleted regions in lung cancers (Calin et al. 2004a). *Let-7* expression is lower in lung tumors than in normal lung tissue, while RAS protein is significantly higher in lung tumors (Johnson et al. 2005). Finally, expression of the RAS oncogene is regulated by *let7*, and overexpression of *let7* has been shown to inhibit lung tumor cell line growth (Johnson et al. 2005).

High expression of human *BIC* RNA has been found in Hodgkin lymphoma (van den Berg 2003). In contrast, an analysis of non-Hodgkin lymphoma (NHL) did not reveal any remarkable up-regulation of *BIC* expression. The *BIC* locus was originally identified as a common retroviral integration site in avian-leukosis virus-induced B-cell lymphomas (Tam et al. 1997). The human *BIC* locus also encodes a microRNA, *mir-155* (Tam 2001; Lagos-Quintana et al. 2002). The *conditio sine qua non* for the development of Burkitt's lymphoma is activation of the MYC oncogene, mostly by chromosomal translocations in which MYC is juxtaposed next to an immunoglobulin enhancer. But activation of MYC alone is not sufficient for full malignancy; in fact, *MYC* cooperates with other oncogenes. In childhood Burkitt's lymphoma, there has been reported a 100-fold up-regulation of the hairpin precursor *mir-155* (Metzler et al. 2004). It could be speculated that *mir-155* directly down-regulates one of the MYC antagonists, such as MAD1, MXI1 or ROX/MNT. Accumulation of *mir-155* and BIC RNA was also reported in other B-cell lymphomas (Eis et al. 2005).

It was recently reported that *mir-21* is upregulated in both human gliobastomas as well as cultured glioblastoma multiforma cells and cell lines compared to primary astrocyte cultures derived from normal tissue; inhibition of *mir-21* expression led to the activation of

caspases and cell death *in vitro* (Chan et al. 2005). These data suggest a role for *mir-21* in malignant glioblastoma, most likely by acting to influence expression of genes involved in apoptosis (Chan et al. 2005).

Overall, the studies to date clearly implicate a causative role for microRNAs in human cancer. The intricate network of signals regulating gene expression requires a delicate balance between protein-coding genes and microRNA genes. Disruption of this delicate relationship by mutation can tip the balance in favor of the development of cancer.

MicroRNAs and other Human Diseases

Several papers have been published showing a probable link between microRNAs and other human diseases, but the precise mechanisms are still not known (Table 4). Fragile X syndrome is the most common inherited mental retardation disease. In the U.S., conservative estimates report that Fragile X syndrome affects approximately 1 in 4000 males and 1 in 8000 females; the rate of the female carrier state has been estimated to be as high as 1 in 250 and the male carrier state has been estimated to be 1 in 1000. The Fragile X mental retardation protein (*FMRP*) is an RNA-binding protein that can function as a translational suppressor. *FMRP* can form a messenger ribonucleoprotein (mRNP) complex, interacting with specific RNA transcripts and proteins. The complex is transported out of the nucleus and into the cytoplasm, where it can either associate with the ribosome or interact with RISC; in both cases, it will lead to protein synthesis regulation. FMRP can interact with microRNAs and other components of the microRNA pathways, including Dicer and the mammalian ortholog of Argonaute 1 (*AGO1*), a component of RISC (Jin et al. 2004; Peng et al. 2004).

Spinal muscular atrophy (SMA) is an inherited neurodegenerative disease with neuromuscular symptoms. Survival of Motor Neurons protein (*SMN*) is a component of the SMN complex that has critical functions in the assembly and restructuring of diverse ribonucleoprotein (RNP) complexes. Components of the SMN complex (*GEMIN3* and *GEMIN4*) are also in a separate complex that contains eIF2C2, a member of the Argonaute protein family. This novel complex contains numerous microRNAs that form novel RNPs named miRNPs (Mourelatos et al. 2002). This link between microRNAs and a devastating

neurodegenerative disease is intriguing and will be of great interest to determine what effect these microRNAs have on miRNPs in SMA patients.

DiGeorge Syndrome is characterized by few specific cardiac malformations, facial deformity and certain endocrine and immune anomalies. The DGCR8 (DiGeorge syndrome critical region gene 8) protein and Drosha interact in human cells. Knockdown of the *Drosophila* dmDGCR8 resulted in a 5- to 23-fold accumulation of some pri-microRNAs, confirming a role in mediating the genesis of miRNAs from the pri-microRNA transcript (Landthaler et al. 2004; Gregory et al. 2004).

MicroRNAs have also been shown to regulate insulin secretion (Poy et al. 2004). Overexpression of *mir-375* (a pancreatic islet-specific microRNA) suppresses glucose-induced insulin secretion, and conversely, inhibition of endogenous *mir-375* by ASOs enhances insulin secretion in murine pancreatic α- and β-cell lines. Bioinformatics approaches predicted several putative targets with a potential role in insulin secretion, such as *MAPK4*, *MKI1*, *VTI1A*, and *MTPN*; the last two proteins are known to be involved in vesicle transport of neurons and in neurotransmitter release.

These studies represent the beginning links between microRNAs and human disease. Further investigations are likely to reveal the involvement of additional microRNAs and their targets in simple as well as complex genetic diseases.

Mouse models for microRNA studies

Almost all mammalian microRNAs are highly conserved and more than 220 mouse microRNAs were cloned by conventional methods (Lagos-Quintana et al. 2002; Seitz et al. 2004) (see The MicroRNA Registry in Table 1). It has been shown that a tissue-specific microRNA dominates the population of expressed microRNAs, suggesting important roles in tissue differentiation. For example, in mouse liver, *mir-122a* and *mir-122b* represent 72% of all cloned microRNAs; these microRNAs were not detected in the other tissues analyzed. In spleen, *mir-143* was the most abundant microRNA. In colon, *mir-142* was cloned several

times; however, due to the strong RNase activity in small intestine and pancreas, too few microRNA sequences were obtained from these tissues to reach statistical significance.

Using a microRNA microarray approach, microRNA expression was analyzed in 17 mouse organs and tissues (Babak et al. 2004). More than half of the 78 microRNAs detected were expressed in specific adult tissues. The results were confirmed by Northern blotting and were consistent with microRNA expression data reported in the literature. Furthermore, this study is consistent with microRNA expression profiling in human tissues (Liu et al. 2004). For example, seven of eight microRNAs were brain-specific in both mice and humans.

Another report gave new light on the world of microRNA regulation in humans and mice (Seitz et al. 2004). By bioinformatics approaches, 46 potential microRNAs located in the human imprinted 14q32 domain have been identified. 40 of these microRNAs are clustered and most of them are arranged in tandem arrays. The majority of these genes map within a ~40 kb region, making it the largest microRNA cluster that has been described. In mouse, this microRNA cluster is conserved at the homologous region on chromosome 12. The detected microRNAs are expressed in embryos and placenta, whereas in the adult their expression pattern is limited to the brain. Expression of these microRNAs comes solely from the maternally-inherited chromosome which is imprinted. Regulation of their expression is dictated by a region located ~200 kb upstream from the microRNA cluster (Seitz et al. 2004).

Research has shown how microRNAs have a role in fine-tuning specific *Hox* mRNA expression patterns during mouse development (Mansfield et al. 2004). A novel approach was used to monitor microRNA tissue-specific expression patterns during embryogenesis. Until now, microRNA expression studies were done by Northern blots, tissue-specific RNA cloning and microarrays, but all of those methods are not useful to display spatio-temporal expression patterns. *In situ* hybridization methods are still difficult to adapt in vertebrate embryos because of the small size of microRNAs. An alternative approach, previously used in *Drosophila melanogaster*, was used where several reporter transgenes ("sensors") were made to detect microRNAs in the embryo. The sensor was a constitutively expressed reporter

gene (lacZ) containing microRNA complementary sequences in the 3' UTR. In cells lacking the corresponding microRNAs, the reporter transgene is stable and will express b-galactosidase. In contrast, cells expressing the corresponding microRNA degrade the lacZ message by an RNA interfering pathway (RNAi), thus resulting in absence of b-galactosidase activity.

To prove the biological significance of microRNAs in human biology and cancer in particular, the development of mouse models for deleted and/or overexpressed microRNAs will be of invaluable interest to decipher the regulatory networks where microRNAs are involved. Several groups have started this task by disrupting Dicer 1 in mice. Loss of Dicer1 by replacement of exon 21 with a neomycin-resistance cassette leads to lethality early in embryogenesis at day 7.5 of development; Dicer1-null embryos were found to be depleted of pluripotent stem cells (Bernstein et al. 2003). Another group generated Dicerex1/2 mice that have a deletion of the amino acid sequences from the first and second exons of the dicer gene (Yang et al. 2005). Dicerex1/2 homozygous embryos died between days 12.5 and 14.5 of gestation, again demonstrating that Dicer is necessary for normal mouse development. In addition, blood vessel formation and maintenance in Dicerex1/2 embryos and yolk sacs were severely compromised, with altered expression of *Vegf*, *Flt1*, *Kdr*, and *Tie1* being detected in the mutant embryos. This study suggests that Dicer has a role in embryonic angiogenesis, probably through processing of microRNAs that regulate expression levels of key angiogenic regulators (Yang et al. 2005).

Although Dicer-null ES cells are viable, they are defective in RNA interference (RNAi) and the generation of microRNAs (Kanellopoulou et al. 2005). These mutant ES cells displayed severe defects in differentiation both *in vitro* and *in vivo*. An increase in centromeric repeat transcripts was detected, whereas the expression of homologous small double-stranded RNAs was markedly reduced in Dicer-deficient ES cells (Kanellopoulou et al. 2005; Murchison et al. 2005). Re-expression of Dicer in the knockout cells rescued these phenotypes. These Dicer-deficient cells were able to undergo gene silencing when provided with synthetic siRNAs (Kanellopoulou et al. 2005; Murchison et al. 2005), indicating that Dicer function is required for processing of microRNAs, but not required for the action of

microRNAs once they are produced. However, the status of cytosine methylation of centromeric satellite sequences remains in question. Taken together, these studies suggest that the essential role of Dicer in the production of mature microRNAs is central for biological processes in mammalian organisms, ranging from stem cell differentiation to the integrity and function of centromeric heterochromatin (Kanellopoulou et al. 2005).

Critical insights into the roles of individual proteins of the microRNA processing machinery have been provided by *in situ* hybridization studies of Argonaute (*Ago1 - Ago4*) family members in mouse embryos (Liu et al. 2004; Lu et al. 2005b). Studies in embryos at days 9.5 to 14.5 of development have shown that Argonaute family members are restricted in both their temporal and spatial patterns of expression. However, Ago2 shows the broadest expression in mouse embryos and is consistent with the fact that Ago2-deficient mice die early in development (Liu et al. 2004). Thus, the differential expression of *Ago* family members suggests specific roles for these proteins during organogenesis, most likely involving the production of microRNAs that are controlled at different times and in specific tissues by the presence of Ago proteins.

One example of the use of mouse models to study the roles of specific microRNAs in cancer was provided by He et al. (2005). Their initial observation was that the *human mir 17-92* cluster was overexpressed by more than five-fold in a majority of human B-cell lymphomas. To determine whether overexpression of this cluster could drive B-cell lymphomagenesis in mouse, the authors derived hematopoeitic stem cells from a susceptible *Eμ-myc* transgenic mouse and transduced them with a retrovirus carrying the *mir 17-92* cluster. Injection of transduced cells into normal mice resulted in acceleration of the development of B-cell lymphomas; in contrast, other subsets of microRNAs expressed in these cells did not have similar effects (He et al. 2005).

The ability to manipulate the genome of mice coupled with the multitude of inbred strain backgrounds provides invaluable tools to genetically dissect the biological functions of individual microRNAs. Further studies of mouse models will provide significant insights into the roles of different microRNAs in development and disease processes.

Conclusions

A link between microRNAs and human diseases, in particular with cancer as focused on in this review, has been recently shown. To better understand the biological significance of microRNAs and of alterations found in human diseases (such as the effect of microRNA mutations or variations in expression) the development of mouse models is mandatory. Regulation of gene expression in the mammalian genome, during development, differentiation, and disease is a complex and multitasked system. To make transcripts, instead of proteins, is energetically “less expensive” for cells and could be the reason why regulation at the RNA level is “cheaper” and more efficient than at the protein level. Penetrance in gene expression requires a fine regulation and microRNAs could have a role in different phenotypic expressions of the same gene. The ability to simultaneously regulate large sets of genes by a single microRNA appears to be at the heart of control of multiple pathways that include morphogenesis and cell fate decisions, response to infectious organisms, and centromeric heterochromatin structure. MicroRNAs appear to be at the center of the balance between apoptosis and proliferation in cancer cells and thus could provide novel approaches for therapeutics. MicroRNAs are young stars in the genome galaxy with many surprises still in store.

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FIGURE LEGENDS

Fig. 1. Biogenesis of microRNAs and hypothetical mechanisms in regulation of gene expression. RNA polymerases II and III are believed responsible for microRNA transcription, although a recent publication indicates that polymerase II is the main RNA polymerase for microRNA transcription (Lee et al. 2004). **(A)** Exonic microRNAs in sense orientation as a part of annotated host genes are transcribed as parts of longer molecules that are processed in the nucleus into hairpin RNAs of 70-100 nt by the dsRNA-specific ribonuclease Drosha. The hairpin RNAs are transported to the cytoplasm, where they are digested by a second, double-strand specific ribonuclease called Dicer. In animals, single-stranded miRNA binds specific mRNA through sequences that in most of cases are significantly, though not completely, complementary to the target mRNA. **(B)** The excision of intronic microRNAs out of the precursors is completed through the process of RNA splincing, followed by Dicer digestion. MicroRNAs are finally incorporated in to an RNA-induced silencing complex (RISC) to induce translation suppression or degradation depending of the degree of complementary with the target mRNA. **(C)** and **(D)** Exonic and Intronic microRNAs in antisense orientation as a part of annotated host genes can be trancribed as independent transcription units. The mature microRNA sequence, in our hypothetical mechanisms, can lead to translation or transcription suppression of the host gene of other target mRNAs. MicroRNA genes located in intergenic regions (gene desert) are transcribed as independent transcription units and their biogenesis can be described as in Fig.1a.

Fig. 2. **MicroRNAs as cancer players.** MicroRNAs may act as tumor suppressors or oncogenes in cancer. Orange triangles represent *mir* promoters and blue ovals represent their corresponding *mir* genes. Orange rectangles represent promoters of protein-coding genes and blue rectangles represent the actual coding sequences of their corresponding genes. One mechanism for the downregulation of "suppressor-microRNAs" that has been identified is **(A)** Homozygous deletion of microRNA coding regions, as exemplified by deletion of the *mir-15a/mir-16a* cluster in B-CLL (Calin et al. 2002). Hypothetical mechanisms for downregulation of "suppressor-microRNAs" in cancer include **(B)** the combination of

Deletion plus Promoter Hypermethylation, and **(C)** Deletion plus Mutation. Mechanisms for the upregulation of "onco-microRNAs" that have been identified are: **(D)** Amplification and overexpression of pre-microRNAs, as exemplified by *mir-155/BIC* in pediatric Burkitt's Lymphoma (Metzler et al 2004), and **(E)**translocations of either protoconcogenes near the promoter of microRNAs or translocation of microRNAs near the promoters of oncogenes (modified after Calin et al PNAS, copyright 2004, National Academy of Sciences, USA).