STARLING REVIEW

MicroRNAs and endocrine biology

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Abstract

microRNAs (miRNAs) are highly conserved, non-coding RNAs that powerfully regulate gene expression at the post-transcriptional level. These fascinating molecules play essential roles in many biological processes in mammals, including insulin secretion, B-cell development, and

adipocyte differentiation. This review provides a general background regarding current knowledge about miRNA biogenesis and the potential contributions of these RNAs to endocrine function.

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Introduction

A whole new class of molecules has been discovered that potently regulate gene expression. These molecules are non-protein-coding small RNAs aptly referred to as microRNAs (miRNAs), as they are approximately 21–23 nucleotides in length, and act by negatively regulating gene expression at the post-transcriptional level (Lagos-Quintana *et al.* 2001, Lau *et al.* 2001, Bartel 2004). Accumulating studies have confirmed that hundreds of these RNAs are encoded in animal genomes, and the most recent computational studies estimate that there may be as many as 1000 distinct miRNAs in humans, together potentially regulating a large portion of protein-encoding genes (and possibly non-protein-encoding genes as well) (Berezikov *et al.* 2005). More than a decade after the discovery of the first miRNA, lineage-deficient-4 (lin-4) (Lee *et al.* 1993), the important roles of these intriguing molecules in diverse biological processes are just beginning to surface. In mammals, they have been shown to regulate adipocyte differentiation, insulin secretion, B-cell development and neural stem cell fate, and they may be important for proper immune function (Esau *et al.* 2004, McManus 2004, Poy *et al.* 2004, Chen & Lodish 2005, Smirnova *et al.* 2005). Given their critical roles in these processes, it is conceivable that the dysregulation of these small RNAs may participate in the pathogenesis of prevalent human diseases, including cancer and diabetes. Indeed, in the past couple of years, there has been a flurry of papers suggesting that miRNAs participate in tumorigenesis (Calin *et al.* 2004, Metzler *et al.* 2004, Takamizawa *et al.* 2004, He *et al.* 2005, Karube *et al.* 2005,

Lu *et al.* 2005, O'Donnell *et al.* 2005) and recently, it has been proposed that the *lethal-7* (*let-7*) family of miRNAs may target the RAS oncogene (Johnson *et al.* 2005).

History

When the founding member of the miRNA family, lin-4, was discovered in *Caenorhabditis elegans*, most investigators viewed this RNA as an oddity in worm genetics (Lee *et al.* 1993). Seven years passed before the discovery of another *C. elegans* miRNA, let-7, which also was found to regulate developmental timing in these animals (Reinhart *et al.* 2000). This time, however, the discovery was particularly intriguing because let-7 is highly conserved among bilaterally symmetrical animals (Pasquinelli *et al.* 2000). This finding led to the discovery by several groups that hundreds more of these miRNAs exist in cells (Lagos-Quintana *et al.* 2001, Lau *et al.* 2001, Lee & Ambros 2001). The discovery of this new class of molecules has captivated the scientific community, because tantalizing evidence suggests that they have an essential role in many biological processes. Their recent rise in popularity might be partly attributed to the fact that they feed into the same pathway as short-interfering RNAs (siRNAs), double-stranded RNAs (dsRNAs) that mediate gene silencing by RNA interference (RNAi) (Hamilton & Baulcombe 1999). In fact, the development of techniques for utilizing RNAi as a laboratory tool (to perform reverse genetics studies) and as potential therapies has almost outpaced our understanding of the biology of this process.

Figure 1 MicroRNA biogenesis pathway. Two major processing events lead to the production of mature microRNAs (for simplicity, only catalytic components of the pathway are depicted in this diagram). They are first processed by the RNase III enzyme, Drosha, and are then exported out of the nucleus. Once in the cytoplasm, they are subjected to another processing step by another RNase III enzyme, Dicer. These mature microRNAs can then undergo unwinding and a single strand can enter the RISC complex, where they can act by repressing the translation of their mRNA targets or by inducing their degradation, mediating RNA interference.

Biogenesis of miRNAs

Mature miRNAs are derived from two major processing events, driven by sequential cleavages by the RNase-III enzymes Drosha and Dicer (Fig. 1) (for comprehensive reviews on this subject see He & Hannon 2004 and Kim 2005). Briefly, miRNAs are transcribed by RNA polymerase II, producing primary-miRNAs (pri-miRNAs) (Lee *et al.* 2002, 2004). Pri-miRNAs, often several kilobases long, are poly-adenylated and capped, similar to the production of mRNAs from protein encoding genes (Cai *et al.* 2004, Lee *et al.* 2004). These pri-miRNAs are then subjected to processing by the microprocessor complex, composed of Drosha and its associated binding partner, Pasha (also known as DGCR8), which results in the excision of a 65–75 nucleotide stem-loop precursor called a pre-miRNA (Lee *et al.* 2003, Landthaler *et al.* 2004). These pre-miRNAs are then recognized and transported from the nucleus to the cytoplasm via the Ran-GTP dependent nuclear transmembrane protein, Exportin5, where they are then subjected to a second cleavage step by Dicer (Yi *et al.* 2003, Lund *et al.* 2004). Processing by Dicer results in the production of a small double-stranded miRNA duplex containing 2-nucleotide-long 3' overhangs (Bernstein *et al.* 2001). These double-stranded products are thought to be quickly unwound by an as yet unidentified helicase, and a single mature strand can

be asymmetrically incorporated into the RNA-induced silencing complex (RISC) (Khvorova *et al.* 2003, Schwarz *et al.* 2003) where they can then act by translational repression (by a cleavage-incompetent RISC) or mRNA degradation (by a cleavage-competent, Slicer-containing RISC) (Khvorova *et al.* 2003, Schwarz *et al.* 2003, Cullen 2004, Tang 2005).

miRNAs: two mechanisms for post-transcriptional gene silencing

How do miRNAs negatively regulate gene expression? The current philosophy is that they act by mediating translational repression or degradation of the mRNA targets. In animals, most miRNAs are thought to function by translational repression, whereas in plants mRNA degradation is thought to be the primary mechanism by which they exhibit their silencing effects. Translational repression is thought to occur when the miRNA imperfectly pairs to the target mRNA, although the precise mechanism has yet to be elucidated (Fig. 1). The degree of repression is often correlated with the number of miRNA binding sites, most of which are thought to occur in the 3' untranslated regions (UTRs) of the targets, but which can potentially bind to complementary sequences in the open reading frame (ORF) or 5'UTR (Doench & Sharp 2004,

Figure 2 Roles of miR-375 and miR-143. (A) miR-375 has been shown to be expressed in pancreatic β -cells and is thought to play a role in insulin secretion. In addition, 67 other β -cell-specific miRNAs have been discovered, although their functions have yet to be elucidated. (B) miR-143 has been proposed to have a role in adipocyte differentiation, and inhibition of this miRNA leads to down-regulation of proteins that have a known role in this process (GLUT4, HSL, aP2, and PPAR- γ 2 (PPAR-g2)). It is unclear if there are additional miRNAs that are critical for adipocyte differentiation and maturation.

Zeng *et al.* 2002). In contrast, mRNA degradation is thought to occur by siRNA-acting miRNAs. siRNA-acting miRNAs form perfect/near perfect interactions with their target mRNAs, which results in the cleavage of the mRNA known as RNA interference (Fig. 1) (Rhoades *et al.* 2002). This perfect/near perfect association presumably triggers the action of Slicer, which in mammals is putatively thought to be the Argonaute2 (Ago2) endonuclease acting either alone or in addition to other Argonaute or as yet unknown proteins within the RISC complex (Liu *et al.* 2004b, Meister *et al.* 2004, Okamura *et al.* 2004). Slicer functions by cleaving the target mRNA between the 10th and the 11th nucleotide from the 5' end of the miRNA (Tuschl *et al.* 1999, Liu *et al.* 2004a). Thus far, only a handful of mammalian miRNAs have been shown to act in a siRNA-like manner. One example is miR-196, which directs the cleavage of the HOXB8 transcript (Mansfield *et al.* 2004, Yekta *et al.* 2004), and it will be interesting to determine if there are others that can act by this mechanism.

Intriguingly, knockout of *Ago2* in mice is lethal as it is required for development, suggesting that a cleavage-competent RISC is essential in mammals (Liu *et al.* 2004b). This finding, along with the evolutionary conservation of a cleavage-competent RISC suggests that there may be more siRNA-acting microRNAs.

miRNA function

What are all of these microRNAs doing in cells? Very few miRNAs have actually been characterized and most of their functions remain unknown. There has been a tremendous effort to identify miRNAs and their targets computationally using bioinformatics, but there is very little experimental data to validate these findings (for a recent review see Brown & Sanseau 2005). A major flaw of all these studies is that the biology of miRNAs is not well understood, and it is therefore difficult to determine what constitutes a valid mRNA target. This has led to

discrepancies in the literature, which only future experimental validation can resolve. Further complicating the accuracy of the predicted targets is that little is known about what regulates miRNA expression. It has been shown that some miRNAs display developmental and tissue specificity, demonstrating that their expression can be regulated both temporally and spatially. Thus, caution must be taken when predicting targets, as a putative target may contain binding sites for a particular RNA, but it may not be expressed in the same cells as the miRNA. However, it may be possible that miRNAs can be shuttled locally between adjacent cells as seen with injected dsRNAs in *C. elegans*, although this is currently speculative.

Recently, a group has found that pre-miRNAs can contain polymorphisms. In one instance a polymorphism in the mature miRNA sequence of miR-32c-2, which can potentially alter the biological function (Iwai & Naraba 2005). It will be important to determine whether this example constitutes a rare situation or if many miRNAs contain polymorphisms. If the latter is true, then the regulation of miRNAs may be as wildly complex as that seen in our protein encoding genes.

Despite all that is not known in vertebrates, much experimental data suggests the important roles miRNAs have, which is most prominently suggested by the lethality seen in *Dicer* knockout mice due to their inability to develop properly (Bernstein *et al.* 2003, Harfe *et al.* 2005, Yang *et al.* 2005). This data is augmented by the few mammalian miRNAs that have been characterized, the most recent being miR-122a, which is thought to play a role in the development of mouse testis (Yu *et al.* 2005), and others, such as miR-143, which has been proposed to regulate adipocyte differentiation (Esau *et al.* 2004, Yu *et al.* 2005).

miRNAs and endocrine biology

Might miRNAs be important for endocrine function and their mis-expression responsible for aberrations in hormone regulation? Last year, the discovery of a pancreatic islet-specific miRNA, miR-375, that inhibits insulin secretion in mouse pancreatic β -cells, uncovered a novel component of the insulin secretion machinery (Fig. 2A) (Poy *et al.* 2004). miR-375 is believed to act by inhibiting the expression of myotrophin (also known as V-1), a cytoplasmic protein that induces the exocytosis of insulin granules (Poy *et al.* 2004). In normal pancreatic β -cells, glucose rapidly enters the cell through facilitative glucose transporters, and is then phosphorylated by glucokinase, the rate-limiting step in β -cell glucose metabolism (reviewed by Stumvoll *et al.* 2005). The ATP produced from glucose oxidation in the β -cell causes depolarization of the cell by closing ATP-sensitive potassium channels on the cell surface (Bottcher *et al.* 2005). Depolarization

opens voltage-sensitive calcium channels on the cell surface, and the entry of calcium into the cell triggers insulin release (Stumvoll *et al.* 2005). As part of the calcium-regulated insulin secretory machinery, myotrophin plays a part in the final step of glucose-stimulated insulin secretion.

Type 2 diabetes results from combined defects in insulin secretion and insulin action. The disease has reached epidemic proportions, affecting over 153 million people worldwide and imposing a huge health care burden (Zimmet *et al.* 2001). Although the mechanisms are not completely understood, insulin secretory capacity declines over the course of the disease in patients with type 2 diabetes, possibly due to accumulated damage caused by hyperglycaemia, hyperlipidaemia, and oxidative stress (Wallace & Matthews 2002, Robertson *et al.* 2003). A better understanding at the molecular level of β -cell biology and the mechanisms of insulin secretion may provide insight into this disease and lead to the development of better treatments. We do not yet know if aberrations in the function of miR-375 occur in diabetic patients, but continued study of the role of this miRNA may reveal new targets for drug therapy.

In addition to miR-375, the authors found another 67 miRNAs expressed in β -cells, and future studies should determine if these other miRNAs are involved in pancreatic β -cell development or in the regulation of insulin production or secretion. Taken together, the study of these molecules in the pancreas may lead not only to enhanced understanding of diabetes pathophysiology and the development of treatments, but also to an increase in our understanding of pancreatic development and may thus aid in the development of better protocols to generate β -cells in culture for transplantation.

Another miRNA that may play a role in endocrine function is miR-143, which has been proposed to play a role in adipocyte differentiation (Fig. 2B) (Esau *et al.* 2004). Investigators found that reducing the level of this miRNA by transfecting 2'-O-methoxyethyl phosphorothioate-modified antisense RNA oligonucleotides into human pre-adipocytes *in vitro* inhibited their differentiation, as determined by the decreased expression of 4 adipocyte-specific genes (GLUT4, HSL, fatty acid-binding protein aP2 and PPAR- γ 2) and their inability to accumulate triglycerides. Computationally, miR-143 has been predicted to have several targets (Lewis *et al.* 2003), and the authors determined that knockdown of miR-143 led to upregulation of one of the predicted targets, ERK5/BMK1. ERK5 is thought to promote cell growth and proliferation, which is consistent with the phenotype observed (inhibition of differentiation), but it still remains to be determined whether other targets of miR-143 also contribute to the phenotype. In addition to miR-143 having a proposed role in human adipocyte differentiation, another miRNA, miR-14, was found to regulate adipocyte droplet size and triacylglycerol levels in the fruit

fly, *Drosophila* (Xu *et al.* 2003). Thus, miRNAs may be important regulators of fat metabolism in both flies and humans. Since excess adiposity is rampant in western countries, and contributes to several common diseases including type 2 diabetes, hypertension and coronary heart disease, new insights provided by the study of miRNAs in adipocyte biology and long-term energy storage could have a tremendous clinical impact.

While only a few miRNAs have been shown to play a role in endocrine biology, miRNAs have been predicted to target many genes important for proper endocrine function and metabolism. Many groups are working diligently to uncover the roles of these remarkable molecules. These studies will likely bring many more surprises, and encourage us to think about endocrine regulation in new ways.

Concluding remarks

miRNAs are a fascinating new class of molecule, which are powerful regulators of gene expression. While our knowledge of how these molecules function is growing daily, we are far from fully understanding their basic biology. To date, target predictions have been largely computational and again it is important to reiterate that they need to be validated experimentally. Future studies should focus on determining how their expression is regulated and elucidate the exact mechanisms by which they exert their gene silencing effects. Accumulating data demonstrates the vast roles miRNAs play and it can be expected that alterations in their expression will participate in the pathogenesis of human diseases.

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