Life Sciences 91 (2012) 475–489

Contents lists available at SciVerse ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Review

MicroRNAs: Small but amazing, and their association with endothelin

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open access

ARTICLE INFO

Article history:
Received 19 December 2011
Accepted 22 June 2012

Keywords:
Endothelin-1
miRNA
miRNA regulation
Biogenesis
Human disease

ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNA molecules involved in the expressional regulation of genes by inhibiting gene translation. MicroRNAs are recruited and incorporated into the miRISC, ribonucleoprotein complex, targeting specific mRNAs through mechanisms specific for a miRNA sequence. Here we review the biogenesis, regulation, and monitoring of miRNAs, as well as the current evidence for potential roles of miRNAs in human diseases associated with activation of the endothelin system. These diseases include cancer, kidney disease, cardiovascular diseases, inflammatory diseases, infectious diseases, and blood diseases, that may all be aggravated by aberrant miRNA expression. In this review we will also discuss regulatory mechanisms determining production of miRNA as well as measuring or targeting miRNAs as potential novel approaches for diagnosis and treatment. Targeting miRNAs possibly will allow one to detect diseases or to interfere with the progression of diseases associated with activation of the endothelin system.

Contents

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0024-3205
doi:10.1016/j.lfs.2012.06.025

Introduction

The field of molecular biology has undergone dramatic changes over the last decade, due to the advent of new technologies (e.g., different PCR techniques, microarrays, among others) and due to the accomplishments of the *human* genome project. These technologies have enabled us to identify new genes, analyze their functional importance, and link them to human diseases. These successes have raised our expectations to the point where we also aspire to finally unravel the complexity of disease at the level of molecular interactions in and between cells. An important discovery was the analysis of microRNAs (miRNAs). Since their first discovery in 1993 (Lee et al., 1993), they have come to be considered, by some, as the long awaited regulators of gene function and expression. Not surprisingly, their detection and analysis, among them is endothelin-1 (ET-1), have been adopted by nearly every research area. In its first section, this review summarizes important aspects of biogenesis/regulation of miRNAs and the current ways of monitoring these molecular tools. In the second part, the association between ET-1 and miRNAs is highlighted, with particular emphasis on the importance of different miRNAs for the ET-1 signaling axis. Promises as well as challenges for the future are discussed.

Biogenesis and regulation of miRNAs

Discovery of miRNAs

An excellent system to study developmental and biological processes is the nematode *Caenorhabditis elegans* (*C. elegans*), which was first used in 1974 by Sydney Brenner. The first important steps regarding the discovery of miRNAs were performed in *C. elegans* (Chalfie et al., 1981; Horvitz and Sulston, 1980). These works reported a mutation of the so-called lin-4 gene (e912), which results in lin-4's loss of function. This in turn leads to a developmental retardation of the *C. elegans* with typical appearances as thin, long adult nematodes with larval skin (Chalfie et al., 1981). This "worm" presents a phenotype in which early developmental features happen at later time points (Chalfie et al., 1981). The Horvitz group also found that null mutations, and therefore the loss of function of the lin-14 gene, results in defects in developmental timing, such as some cells expressing fates earlier in the development than normal (Ambros and Horvitz, 1984). Ruvkun worked on cloning lin-14 in the Horvitz lab (Ruvkun et al., 1989). They found that loss/ null mutations of the lin-14 gene results in exactly the opposite phenotype than loss of the lin-4 gene (Ambros and Horvitz, 1984). Ultimately, the group detected that a correlation exists between lin-14 and lin-4. The group of Ambros (Lee et al., 1993) found in their later experiments the first currently called "pri-miRNA (primary miRNA)", a 700 bp fragment that contains the lin-4 sequence and either a start or a stop codon. They concluded that lin-4 did not encode a protein (Lee et al., 1993). Furthermore, Han et al. (2004) identified the first currently called "pre-miRNA (precursor miRNA)," which is a 61 nucleotide long transcript. They also identified the first "miRNA," which has a size of 22 nucleotides. In parallel, Ruvkun et al. (1989) found that lin-14 was down-regulated at the transcriptional level. They also detected that the 3′-UTR of the lin-14 gene was necessary for regulation and that the 3′-UTR sequence has some conserved regions complementary to lin-4 (Ruvkun et al., 1989; Ambros et al., 2003a). Due to their complementary data, Ruvkun and Ambros (Ruvkun et al., 1989) shared their results and the two groups independently came to the same conclusion, that the small lin-4 gene regulates the lin-14 expression by binding through the conserved regions at the 3′-UTR (Wightman et al., 1993). This was the first time a small RNA (currently called "miRNA") coming from the intron region of gene transcripts and its biological function was described in the literature (Lee et al., 1993; Ambros et al., 2003a; Agami, 2010; Zamore et al., 2000). Since that time, there has been a dramatic increase in the number of newly identified miRNAs (currently numbering 1500) and respective targets (Agami, 2010).

miRNA regulation

In 2001 an important factor in the regulation of miRNAs, Dicer, was analyzed by two independent groups (Zamore et al., 2000; Grishok et al., 2001). In the same year, Lau et al. (2001) described another necessary factor in the regulation of miRNAs, called Drosha. Dicer and Drosha belong to a class of RNA III endonucleases and process pri- and pre-miRNA to mature miRNA (Kuehbacher et al., 2007). As already mentioned, there are three different types of miRNA: the pri-miRNA, pre-miRNA and miRNA. miRNAs can be transcribed from a single polycistronic transcription unit, so that only one polycistronic sequence produces more than just one functional miRNA and functions as a "miRNA family", with the same seed sequence but with different flankings (Lau et al., 2001; Carthew and Sontheimer, 2009; Kim et al., 2009; Lagos-Quintana et al., 2001; Nelson et al., 2004). In contrast, a miRNA cluster defined a region in which more than just one miRNA can bind, all miRNAs which bind in this specific region were called clustered miRNAs (Yu et al., 2006).

Canonical miRNA biogenesis

The transcription of the miRNA is mediated by RNA polymerase II (Lee et al., 2004) as well as RNA polymerase III (Borchert et al., 2006). pri-miRNAs contain a structure that is typical for RNA polymerase II transcripts. They are 5′-capped, spliced and poly-adenylated at the 3-end (Han et al., 2004). Since 2006 it is known that also RNA polymerase III promoters can transcribe miRNAs via the interaction with Alu sequences, mammalian wide interspersed repeat (MWIR) or near upstream tRNA sequences (Borchert et al., 2006). Two classical steps are of great importance in order to produce the mature miRNA; one taking place in the nucleus and the other one in the cytoplasm. The primary miRNA (pri-miRNA) is present in a stem-loop hairpin structure in the nucleus and is several base pairs long (Fig. 1; Han et al., 2004). Due to the binding of a micro-processing complex consisting of a nuclear RNase III type protein, Drosha, and a further protein, Pasha (DGCR8), the double stranded pri-miRNA is cleaved at the stem of the hairpin structure into the precursor miRNA (pre-miRNA) (Han et al., 2004). The DGCR8 was initially described in the literature in regard to a disease called "DiGeorge syndrome" in which children have several developmental deformities (Dodson et al., 1969; Kirkpatrick and DiGeorge, 1968).

The aforementioned microprocessor complex has a size of ~650 kDa in humans. The pre-mRNA binding protein DGCR8 is important for the orientation and cleavage of the pri-miRNA, due to the position of Drosha (Han et al., 2004). The cleavage occurs approximately 11 nucleotides from the basal segment (Denli et al., 2004). Drosha is important for the 2 nucleotide overhang on the 3′ end and for adding phosphates on the 5′ end (Han et al., 2004). The newly formed, double stranded

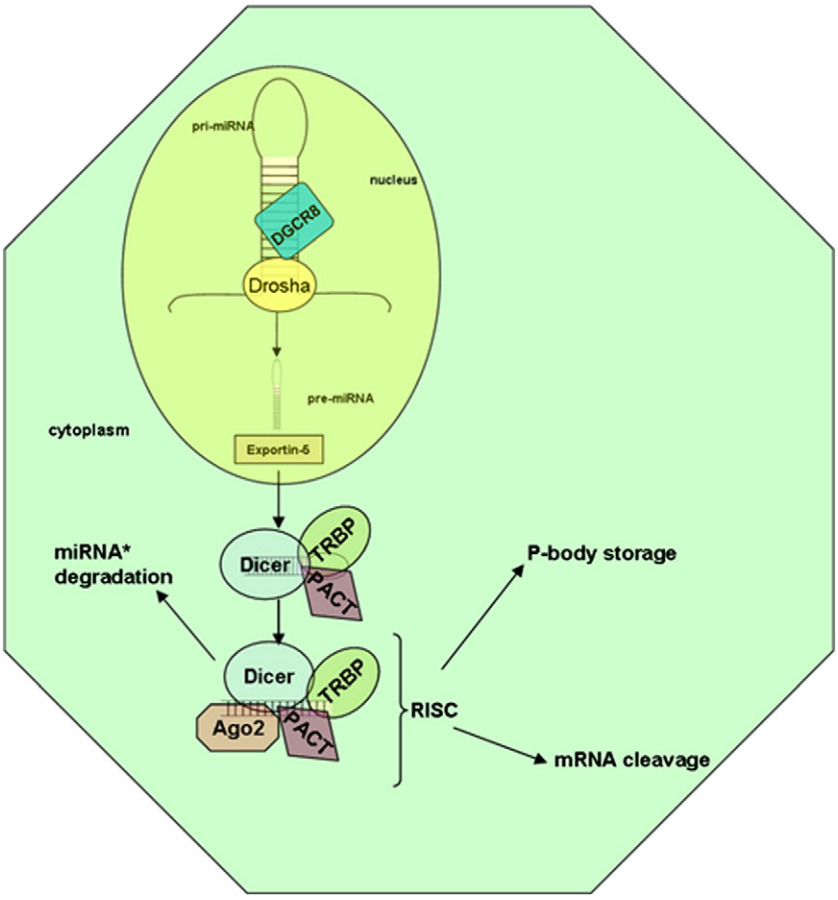

Fig. 1. General miRNA regulation loop. After the formation of the primary-miRNA (pri-miRNA), two proteins, called DGCR8 and Drosha, are involved in the processing of precursor miRNA (pre-miRNA). The pre-miRNA is transported from the nucleus to the cytoplasm by the binding of Exportin-5. In the cytoplasm, several different factors (Dicer, TRBP, Ago2 and PACT) form a RISC complex (RNA-induced silencing complex) where the fate of the miRNA is determined by miRNA degradation, storage, or cleavage of the mRNA.

structure is approximately 70 bp in length and is called pre-miRNA (Fig. 2a; Lee et al., 2002; Ambros et al., 2003b).

Further primary miRNA regulation mechanisms

To date, several different pri-miRNA processing steps have been discovered. The first processing step, which is also considered the classical or canonical step, is the cleavage by the microprocessor complex already described above.

A further pri-miRNA processing step is DEAD-box RNA helicase dependent (Han et al., 2004). However, except for the pri-miRNA processing step, Drosha and DGCR8 are involved. DEAD proteins are proteins with a specific amino acid codon corresponding to the letters (Asp-Glu-Ala-Asp) (Linder et al., 1989). DEAD family members present a specific binding site that can be identified in over 500 proteins and is important for several different RNA processing steps (Denli et al., 2004; Banerjee and Slack, 2002). The RNA helicase subunits, p68 and p72, were identified as DEAD box family members in 1988 and 1996 (Ford et al., 1988; Lamm et al., 1996). Lamm et al. (1996) showed that both proteins localize at the same region in the nucleus of HeLa cells, and therefore concluded that these proteins are involved in the regulation of nuclear processes. In 2007, Fukuda et al. published that p68 and p72 are important for the recognition of primary miRNAs. They designed mice with knockouts of either p68 or p72, resulting in an early developmental dysregulation of these mice (Fukuda et al., 2007). They concluded that some specific miRNAs need the formation of a complex consisting of Drosha/DGCR8 and the RNA helicase subunits p68 and p72 (Fig. 2b; Fukuda et al., 2007).

The third important pri-miRNA regulation step is the complex formation between Drosha/DGCR8-p68/p72 and p53 (Fig. 2c; Fuller-Pace and Ali, 2008; Silverman et al., 2003; Suzuki et al., 2009). p53 is a tumor suppressor gene and is frequently found dysregulated in tumors (Levine et al., 2004). The tumor suppressor p53 and the DEAD box protein p68 and p72 build a complex; however, the p53/p72 complex formation is weaker (Fuller-Pace and Ali, 2008). Due to the binding of p53 to DEAD box proteins, different pri-miRNAs processing steps are regulated, especially after DNA damage — where p53 is often found upregulated (Oba et al., 2010).

The fourth pri-miRNA processing step described in the literature also includes the Drosha/DGCR8 complex. In this processing step the complex is combined with ligand-specific signal transducers (SMAD). SMAD are downstream subunits in the activation cascade of transforming growth factor, TGF-β and BMPs, and are involved in the pri-miRNA processing by interacting with a complex consisting of Drosha/DGCR8 and p68 (Davis and Hata, 2011). Davis et al. published in 2008 that the R-SMAD alone can regulate the pri-miRNA processing and that several different miRNA were produced in this SMAD dependent manner (Fig. 2d; Lamm et al., 1996; Davis et al., 2008; Davis and Hata, 2011). They claim that a specific sequence, called the RNA-SMAD binding element (R-SBE), which is similar to the promoter region of TGF-β target genes, is important for the correct positioning of the microprocessor complex (Drosha/DGCR8/p68) (Davis and Hata, 2011).

The fifth pri-miRNA processing pathway depends on mRNA splicing (Fig. 2e; Berezikov et al., 2007). This mechanism was first described in 2007 by Ruby et al. in *Drosophila melanogaster* and *C. elegans* (Ruby et al., 2007). This group observed that some introns mimic the structural

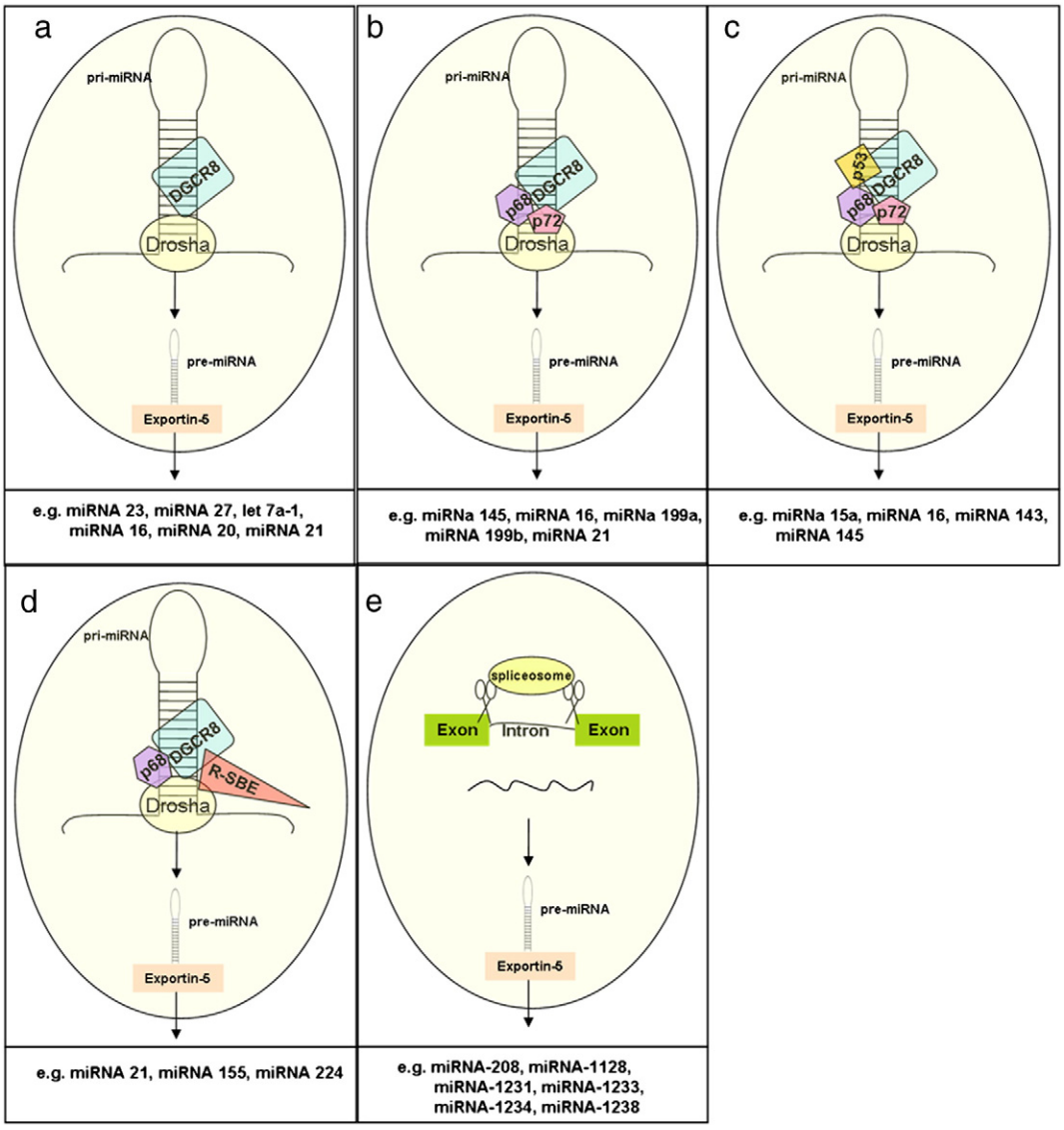

Fig. 2. Schematic illustration of positive pri-miRNA regulation. Beyond the classical miRNA biogenesis pathway (a), several different factors (p68, p72, p53 and R-SBE) have been discovered joining the omnipresent Drosha/DGCR8 proteins in the generation of the precursor miRNA (pre-miRNA). However, an alternative pre-miRNA producing mechanism has been demonstrated where introns are directly processed into pre-miRNA. These introns are called "mitrons" (e).

features of pre-miRNAs and that this processing step is Drosha independent. They called them "mitrons" and identified 18 mitrons in the above mentioned organism (Ruby et al., 2007). After splicing, these mitrons behave like pre-mRNAs where the Exportin-5 binds through and mediates the transport from the nucleus to the cytoplasm where the pre-miRNA matures to miRNA (Gwizdek et al., 2003; Kim, 2004; Okamura et al., 2007; Yi et al., 2003; Zeng and Cullen, 2004). Berezikov et al. published in 2007 that the same mechanism exists in mammals. The group also identified some miRNAs resulting from splicing, i.e., miR-1129, 1230, and 1231 (Berezikov et al., 2007). Besides the 5 positive pri-miRNA/miRNA processing ways described above, four pri-miRNA/miRNA negative regulation pathways have been analyzed to date (Davis et al., 2008; Sakamoto et al., 2009; Van Wynsberghe et al., 2011; von Brandenstein et al., 2011; Yamagata et al., 2009).

The first pri-miRNA processing step involves estradiol (E2) stimulation and is estrogen receptor α (ERα) dependent (Suzuki et al., 2009). In the case of ERα activation via E2 binding, the complex binds to the Drosha protein and therefore inhibits the binding of Drosha to the pri-miRNAs. Thus, resulting in the reduction of a subset of miRNAs (Fig. 3a; Davis-Dusenbery and Hata, 2010; Yamagata et al., 2009; Castellano et al., 2009).

The second negative regulation loop (Fig. 3b; Sakamoto et al., 2009; Suzuki and Miyazono, 2010) was analyzed in 2009 by Sakamoto et al. (2009). The nuclear factors (NF90 and NF45) form a heterodimer in the nucleus and bind to Drosha and the primary miRNA structure. This interaction interrupts the processing step from pri-miRNA to pre-miRNA. The authors claimed that the complex formation increases the pri-miRNA levels; however, the pre-miRNAs levels decrease (Sakamoto et al., 2009; Van Wynsberghe et al., 2011). This negative regulation step is important for let-7a family members (Davis-Dusenbery and Hata, 2010; Davis and Hata, 2009).

The third pri-miRNA negative processing step described in the literature involves the Lin-28 family. It is often up-regulated in primary human tumors, while let-7 miRNA is down-regulated (Fig. 3c; Iliopoulos et al., 2009; Roush and Slack, 2008). A correlation between Lin-28 and let-7 was first described in 2000 in *C. elegans* (Reinhart et al., 2000). Van Wynsberghe et al. published in 2011 that Lin-28 can interact with the primary let-7 product and therefore inhibits the maturation to let-7 (Van Wynsberghe et al., 2011; Newman et al., 2008).

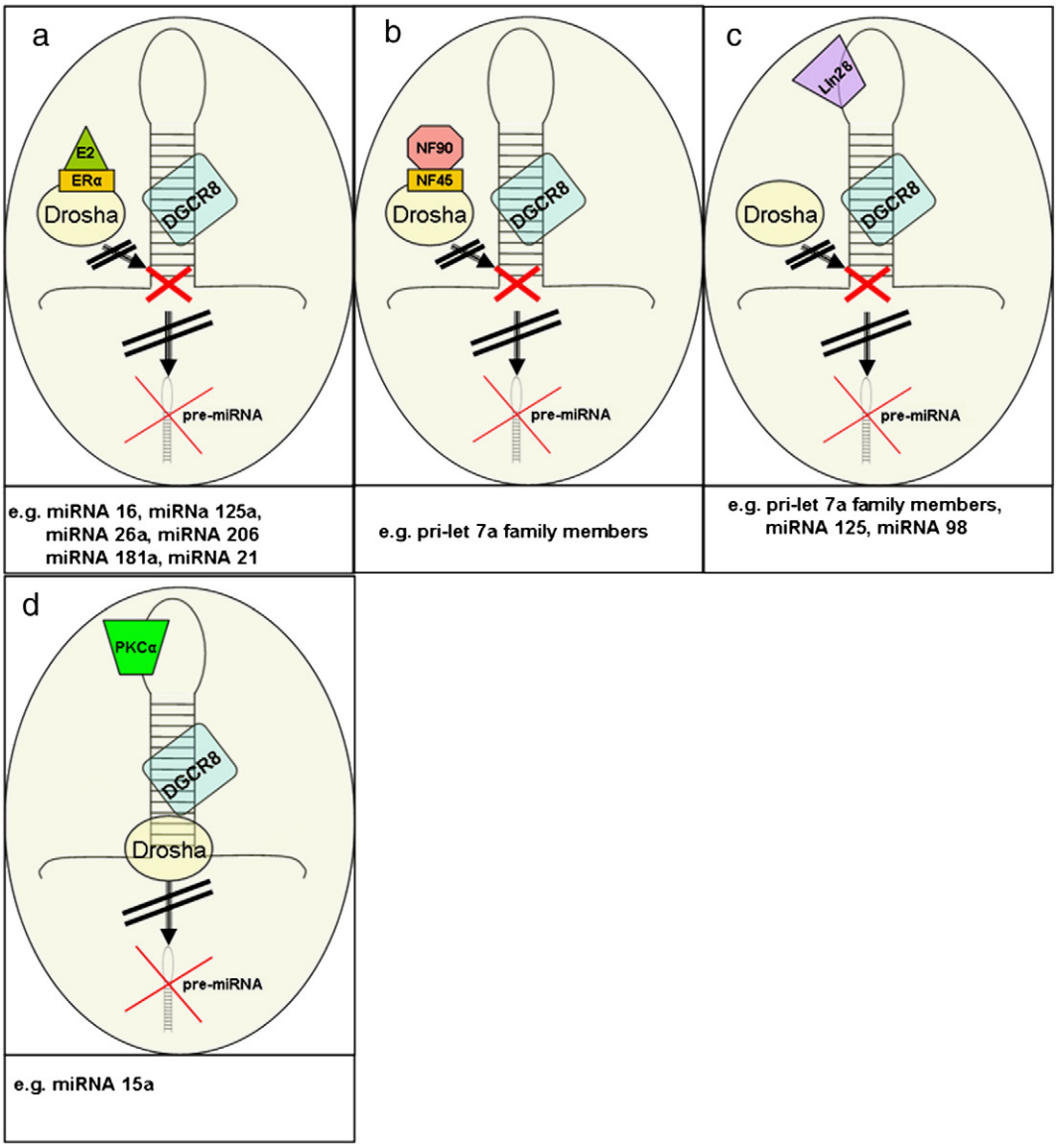

Fig. 3. Schematic illustration of negative pri-miRNA regulation. Different proteins are known [ERα and E2 (a); NF90 and NF45 (b); Lin28 (d); PKC α (c)] to inhibit the formation of pre-miRNA and are therefore responsible for decreasing miRNA levels.

Our group recently identified the third negative pri-miRNA processing step involving protein kinase C alpha (PKC α) (Fig. 3d; von Brandenstein et al., 2011). PKC α builds a complex with NF-κB and MAPK p38 in the cytoplasm and then they migrate together into the nucleus. In the nucleus PKC α binds to the primary miRNA and inhibits the maturation to miRNA. Our group also showed that after the treatment with endothelin-1, the nuclear PKC α levels decrease, and therefore the miRNA levels increase (von Brandenstein et al., 2011). Nevertheless, it is uncertain whether Drosha and DGCR8 were somehow involved in the PKC α regulation. Our hypothesis is that the binding of PKC α to the loop of the pri-miRNA 15a has a similar effect as the binding of Lin28 to pri-let7a-1, namely the inhibition of Drosha binding.

After the cleavage of pri-miRNA to pre-miRNA, with one of the previously mentioned mechanisms, the pre-miRNA leaves the nucleus.

The nuclear-cytoplasmic shuttling

The ~70 nucleotides long pre-miRNA is produced in the nucleus and binds to a transport protein, called Exportin-5. This pre-miRNA then migrates from the nucleus to the cytoplasm (Zeng and Cullen, 2004; Lund et al., 2004). Exportin-5 builds a GTP-Ran complex and is also important for the transport of small RNAs from the nucleus to the cytoplasm (Lee et al., 2003). The previously Drosha-produced 3′ and 5′ overhangs are important for the recognition and binding of Exportin-5 (Gwizdek et al., 2003; Zeng and Cullen, 2004; Lee et al., 2003). The nuclear/cytoplasmic transport via Exportin-5 is cell type dependent, however, the mechanism behind this is still unknown (Davis-Dusenbery and Hata, 2010).

Canoncial pre-miRNA to miRNA maturation process

Once in the cytoplasm, the pre-miRNA matures to miRNA. The so-called RNase III Dicer protein is the most important factor during this maturation process. The so-called RNase III Dicer protein is the most important factor during this maturation process. (Davis et al., 2010; Zhang et al., 2002). Dicer is a highly conserved protein and is present in every mammalian cell (Zhang et al., 2002). This protein is important for the proliferation of stem cells, at least in mice, and the differentiation of neurons and other tissues (Bernstein et al., 2003; Yang et al., 2005). The Dicer protein cleaves the pre-miRNA near the base of the hairpin formation, producing the approximately 20 to 22 bp long, double-stranded miRNA (Fig. 1; Zhang et al., 2002;

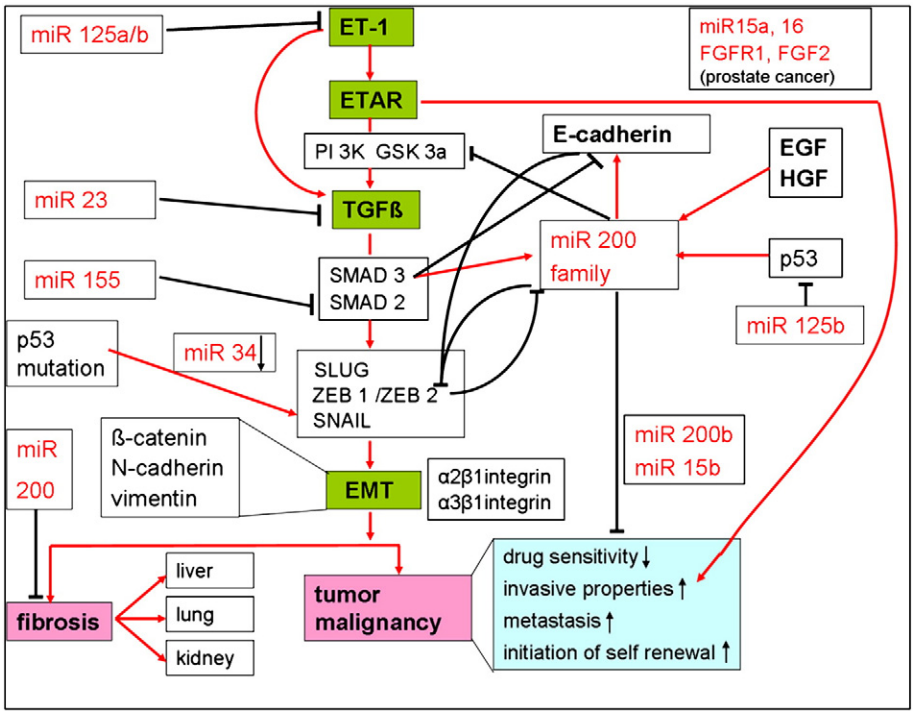

Fig. 4. Endothelin signaling axis and miRNAs. The ET axis can be influenced on different levels by known miRNAs, such as: miR125a/b (inhibiting ET-1), miRNA 23 (inhibiting TGF-β), and miRNA 155 (inhibiting SMAD3). On the other hand, the miRNA 200 family negatively influences the signal progression of TGF-β via the ZEB transcription factors and is able to influence tumor properties in concert with miRNA 15b. EMT, via the endothelin axis, plays an important role in the organ fibrosis of different organ systems such as the liver, the lung and the kidney, as well as for tumor malignancy. It is identified by the upregulation of β-catenin, N-cadherin and vimentin. For invasive behavior, integrins and the integrin-linked kinase activated by ET-1 play an additional modifying role. In prostate cancer, ET-1 via ETAR and the miRNA 15a/16 cluster influences invasive properties of tumor cells by modifying the behavior of tumor-surrounding stromal cells. Red arrows = induction of pathway; black bars = blockade of pathway.

Koscianska et al., 2011), which it also unwinds. The double-stranded miRNA has a characteristic cleavage site at one end (Gregory et al., 2005; Zeng and Cullen, 2005). This cleavage site is necessary for the further processing of pre-miRNA into miRNA. Sometimes, it is possible that both arms are cleaved to mature miRNAs. Depending on the arm (either 3′ or 5′) the miRNA is derived from, the miRNAs are called either miRNA-3p or miRNA-5p.

However, in general only one strand matures to miRNA, whereas the other miRNA strand (without characteristic cleavage site), indicated as miRNA*, is degraded by a RNA induced silencing complex, i.e., "RISC". The RISC complex is responsible for the cleavage of the target mRNA or translation silencing (P-body storage) (Liu et al., 2005). The newly formed miRNA can bind to the 3′UTR and triggers the mRNA cleavage. Alternatively, the target gene is stored in the so-called P-bodies (processing-bodies), which are involved in the repression of translation (Brennecke et al., 2005). P-bodies are special cytoplasmic structures which contain untranslated mRNAs and serve as translational inhibition sites (Liu et al., 2005; Teixeira et al., 2005).

Dicer formation in detail

The newly formed miRNA has a terminal structure typical for RNase III enzymes cleavage. The PAZ domain of Dicer recognizes this site and binds through it (MacRae et al., 2007; Saito et al., 2005). The PAZ (Piwi/Argonaute/Zwille) domain is a 110 amino acid long, evolutionarily conserved region (Yan et al., 2003). In 2005 Haase et al. published that Dicer builds a complex with TRBP, a human immunodeficiency virus (HIV)-1 transactivating response (TAR) RNA-binding protein. Truncation of TRBP, and therefore binding behavior change, is found in tumors frequently correlated with decreasing miRNA levels (Melo et al., 2009). Another Dicer binding partner that can also form a complex with TRBP, is a further double-stranded RNA binding protein called PACT (protein kinase R-activating protein) (Rossi, 2005). The binding of these two complex partners is necessary for Dicer stability. TRBP and PACT are closely related (Melo et al., 2009) and share the same binding partners, namely PKR and Dicer. The PACT and TRBP proteins are possible homologues to Loquacious (Loqs) (Kok et al., 2007). This protein was first identified in *Drosophila* by (Saito et al., 2005) and is conserved in all mammals. In *Drosophila*, two Dicer proteins exist and Loqs is important for the stabilization of the Dicer complex. Nevertheless, only the Dicer1 protein is necessary for the production of miRNAs in *Drosophila*. The Dicer2 protein, in contrast, is important for the siRNA production (Saito et al., 2005; Kim et al., 2006). In humans, only one Dicer protein (Dicer1) exists and is necessary for the production of all small RNAs (Haase et al., 2005). A further protein, called Argonaute (Ago) can bind to the complex mentioned above. After the addition of this protein, the whole complex is called RISC (RNA-induced silencing complex) (Gregory et al., 2005; MacRae et al., 2007; Faehnle and Joshua-Tor, 2007). So far, only one miRNA (miRNA 451) has been described as being processed independently from Dicer (Cheloufi et al., 2010). However, there are several different regulation mechanisms in the cytoplasm that can also disturb the maturation process.

Regulation of Dicer

Dicer itself can be regulated by several different miRNAs. Forman et al. (2008) showed that the miRNA let-7 binds to the 3′UTR of Dicer and is therefore responsible for the Dicer dysregulation. In 2010, Martello et al. showed that in the miRNA-family, miRNA 103/107 is in the position to regulate Dicer activity. Furthermore, miRNA expression in human breast cancer is due to binding through the 3′ UTR (3′ untranslated region). This repression of Dicer is associated with poorer prognosis for patients with this type of cancer (Martello et al., 2010). An estrogen dependent regulation mechanism was described by Cochrane et al. in 2011. The group showed that ERα negative breast cancer cells express high levels of miRNAs (miRNA

221, miRNA 222 and miRNA 29a), which negatively regulates Dicer, due to a direct interaction with the 3′UTR (Cochrane et al., 2011). Recently, Tao et al. (2011) identified a further miRNA binding site at the 3′UTR of Dicer protein. This group showed that the miRNA 18a binds to the 3′ UTR in urinary bladder cancer cells. They reported that this association has a tumor suppressive effect (Tao et al., 2011). A positive regulation of Dicer was found in correlation with miRNA 141 in patients with liver metastasis (Stratmann et al., 2011). Nevertheless, Cochrane et al. (2011) showed that the miRNA 200c also positively regulates Dicer. This group also claimed that this positive regulation can be the reason for the appearance of the well differentiated epithelial phenotype of breast cancer.

The RISC complex and target genes

The RISC complex, as mentioned above, consists of Dicer, the double-stranded RNA binding proteins TRBP and PACT, and Argonaute 2 (see Fig. 1; Rossi, 2005; Faehnle and Joshua-Tor, 2007). The Argo family consists of 8 different genes. However, Argonaute 2 is the only protein which is known to cleave the mRNA targets and therefore leads to mRNA degradation (Faehnle and Joshua-Tor, 2007). An important role of this complex formation is, as previously mentioned, the unwinding of the miRNA: miRNA* double strands, and the resulting degradation of the miRNA* strand by the RISC complex (Faehnle and Joshua-Tor, 2007; Miyoshi et al., 2009). During processing, the miRNA strand with less thermodynamic stability guides the RISC complex to the 3′UTR of target mRNAs (Krol et al., 2004). The recognition of the target gene depends on a so-called seed sequence of the miRNA (Brennecke et al., 2005). The seed sequence of each miRNA has a size of 6 to 8 nucleotides and usually starts from the first or the second nucleotide (Brennecke et al., 2005; Doench and Sharp, 2004; Lewis et al., 2003). The seed sequence is the docking site of miRNA on the 3′ UTR of the target mRNA. Several different databases exist which take into account the miRNA-mRNA homology and identify possible binding sites (Lewis et al., 2003; Griffiths-Jones, 2006; Maragkakis et al., 2009a; Kong et al., 2008).

Monitoring miRNAs

Computational approaches to miRNA target identification

There are several, freely available programs, which assist in the prediction of a potential binding site for a specific miRNA. The ones listed below are primarily for human and mammalian sequences. Since the programs incorporated in their algorithms differently defined criteria, a search may retrieve different results than the obtained gene target list. Thus, it is recommended to combine the results from one's search in different programs to look for a potentially beneficial overlap (Bernardo et al., 2011).

DIANA-microT is a software program for detection of miRNA binding sites, coordinated by Artemis Hatzigeorgiou: (agh@pcbi.upenn.edu) and Benoit Corda: (cordabe@pcbi.upenn.edu). It is based on several parameters calculated individually for each microRNA and combines conserved and non-conserved microRNA recognition elements into a final prediction score, which correlates with protein production fold change. Specifically, for each predicted interaction the program reports a signal to noise ratio and a precision score which can be used as an indication of the false positive rate of the prediction (Maragkakis et al., 2009b). In addition, the DIANA-microT web server is available as the user interface to the DIANA-microT 3.0 miRNA target prediction algorithm, which offers links to nomenclature, sequence and protein databases. Thus, users are facilitated by being able to search for targeted genes using different nomenclatures or functional features, such as the genes' possible involvement in biological pathways (Maragkakis et al., 2009a).

MicroInspector is a software for detection of miRNA binding sites. It is run by the Tabler lab at the Institute of Molecular Biology and Biotechnology (IMBB), Heraklion, Greece and University of Plovdiv, Bulgaria, Dept. of Plant Physiology and Molecular Biology (www.plantgene.eu). The description of the program is published in the 2005 web issue of Nucleic Acid Research (http://bioinfo.uni-plovdiv.bg/microinspector). As described by Rusinov et al., the program will analyze a user-defined RNA sequence, which is typically an mRNA or a part of an mRNA, for the occurrence of binding sites for known and registered miRNAs (Rusinov et al., 2005). It allows variation of temperature, the setting of energy values as well as the selection of different miRNA databases to identify miRNA-binding sites of different strength. MicroInspector could spot the correct sites for miRNA-interaction in known target mRNAs. Using other mRNAs, for which such an interaction has not yet been described, potential miRNA binding sites of similar quality are reported to be detected frequently, which can subsequently be analyzed experimentally.

miRanda is an algorithm for finding genomic targets for miRNAs. This algorithm has been written in C and is available as an open-source method under the GPL. miRanda was developed at the Computational Biology Center of Memorial Sloan-Kettering Cancer Center. This software will be further developed under the open source model, coordinated by Anton Enright and Chris Sander (miranda@cbio.mskcc.org).

miRNA.org (http://www.miRNA.org) is a comprehensive resource of miRNA target predictions and expression profiles (Betel et al., 2008). Target predictions are based on a development of the miRanda algorithm, which incorporates current biological knowledge of target rules and the use of an up-to-date compendium of mammalian miRNAs. miRanda reads RNA sequences (such as microRNAs) from file1 and genomic DNA/RNA sequences from file2. One or more miRNA sequences from file1 are scanned against all sequences in file2 and potential target sites are reported. Potential target sites are identified using a two-step strategy. First a dynamic programming local alignment is carried out between the query miRNA sequence and the reference sequence. This alignment procedure scores based on sequence complementarity and not on sequence identity. The second phase of the algorithm takes high-scoring alignments (Those above a score threshold, defined by -sc) detected from phase 1 and estimates the thermodynamic stability of RNA duplexes based on these alignments.

PicTar is an algorithm for the identification of miRNA targets. PicTar is a project of the Rajewsky lab at NYU's Center for Comparative Functional Genomics and the MDC, Berlin.

1. miRNA target predictions in vertebrates (Krek et al., 2005)
2. miRNA target predictions in seven *Drosophila* species (Grun et al., 2005)
3. miRNA targets in three nematode species (Lall et al., 2006)
4. Human miRNA targets that are not conserved, but co-expressed (i.e., the miRNA and mRNA are expressed in the same tissue) (Chen and Rajewsky, 2006)

RNAhybrid is a tool for finding the minimum free energy hybridization of a long and a short RNA. The hybridization is performed a domain-like fashion i.e., the short sequence is hybridized to the best fitting part of the long one. The tool is primarily meant as a mean for miRNA target prediction.

TargetScan, from the Whitehead Institute for Biomedical Research (wibr-bioinformatics@wi.mit.edu), predicts biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA (Lewis et al., 2005). As an option, non-conserved sites are also predicted. Also identified are sites with mismatches in the seed region that are compensated by conserved 3′ pairing (Friedman et al., 2009).

In addition there are programs specifically tailored to miRNA: 3′ UTR intermolecular interactions such as “Sylamer” (Alexiou et al., 2009; Selbach et al., 2008).

miRNA detection

As already mentioned, several different databases exist which can be used to detect possible miRNA–mRNA interaction sites. However, it is necessary to show this interaction via appropriate experiments. It is not enough to show a correlation between the increases or decreases of target gene levels and miRNA levels in culture or tissues, because this correlation can be "true or true and unrelated". The databases are usable for the identification of possible binding sites, but a regulation of a possible target sequence should be supported with experiments, e.g., use of mimics, si-RNAs and reporter assays.

Isolation and monitoring of miRNA

miRNA can be isolated from tissue (either fresh or paraffinized), cell culture, and body liquids. Until now it was possible to isolate miRNAs from: serum, plasma, cerebrospinal fluid, sputum, ejaculate, vaginal secret, menstruation blood, breast milk, sweat, and urine (Etheridge et al., 2011; Gilad et al., 2008; Hanson et al., 2009, Kosaka et al., 2010a, 2010b; Mitchell et al., 2008). In general, monitoring the miRNA gene expression profiles was used for the identification of miRNAs. Different methods can be used for analysis, however, the most common ones in monitoring miRNA expression profiles are, e.g., Northern blot analysis, quantitative Real-time PCR (qRT-PCR), microarray analysis, and *in situ* hybridization (de Planell-Saguer and Rodicio, 2011).

Detection of miRNAs for diagnostic purposes

To date, several methods have been in use to detect miRNAs. These include: Northern blot analysis with radiolabeled or biotinylated probes, microarray based technology, single-molecule detection in liquid phase, *in situ* hybridization, and high-throughput sequencing. Since all techniques have their specific limitations, the one "ideal" for the planned experiment depends upon the specific experimental setting.

Microarray and next generation/deep sequencing

With microarray technology, the relative change of expression (rather than the absolute abundance) is determined mostly within the context of healthy versus diseased tissue or untreated versus treated disease. Its setback is the requirement of sequence information for probe design. Here, next generation sequencing, also called "deep sequencing", being able to sequence shorter fragments of DNA/RNA, is able to overcome the sequence limitation of microarrays. This technology provides information on all RNA species and their absolute abundance, even allowing detection of previously unknown small RNAs/miRNAs. However, since terabytes of data are generated, extensive computational data storage and analysis capacities are required to analyze the result. Furthermore, functional experiments have to be performed to substantiate the data.

Quantitative real-time PCR (qRT-PCR) is probably currently the most frequently and widely used technique. It can be applied with 2 different technologies: i) stem-loop specific primers binding to the 3′ end of the miRNA followed by individual reverse transcription and a TaqMan miRNA assay, or ii) adding a poly(A) tail to the 3′ end of all miRNAs by using a universal primer. The first approach, being more expensive, is able to differentiate between mature and pri/pre-miRNAs. The second approach, being cheaper, is useful if different miRNAs have to be analyzed in a small starting material, but does not differentiate between precursor miRNAs.

Northern blotting, being time consuming and requiring a relative large amount of RNA (at least 10 μg), is not only a robust and well established method, but it also remains the only one to differentiate mature from pre-miRNA. With the invention of the so-called locked nucleic acid (LNA) probes, the sensitivity over common radiolabeled DNA probes for the detection of RNA molecules of small size and low abundance is greatly improved (for further details see Varallyay et al. (2008)). Finally, *in situ* hybridization is advantageous, if the discovery of spatiotemporal expression of miRNAs is requested, in helping to unravel the biologic role. The method works well for highly abundant miRNAs, but is inconsistent for miRNAs in low abundance (for further details see Jorgensen et al., 2010; Obernosterer et al., 2007; Pena et al., 2009; Silahtaroglu et al., 2007; Song et al., 2010). Less common methods include electrochemical detection, such as nanotechnology, bioluminescence miRNA detection as competitive solid-phase hybridization based method, surface-enhanced Raman spectroscopy method, or surface plamon resonance imaging (SPRI)-based methods (for further details see de Planell-Saguer and Rodicio, 2011).

Regulation and functional analysis

Further experimental analyses are imperative in order to validate a result as the direct effect of one specific miRNA. There are several reasons for this demand: i) the same miRNA is potentially able to regulate different genes at the same time, as well as one gene being potentially regulated by different miRNAs; ii) sequence homology between the gene region and the so-called seed-sequence (+/− its flanking sequence) does not necessarily mean that an interaction will actually take place; iii) there may be several miRNAs with cross-matching sequence homology, of which only one may actually bind. The latter two phenomena are as yet unexplained. They can be observed when comparing miRNA binding between species: the putative binding site for miRNA 17-3p, being well conserved in different animal species and in humans, only allows interaction with the expected miRNA in animals, but not in humans (unpublished data). miRNAs having the same seed sequence may still not bind to their respective binding site at a given gene.

To study functional effects, the primary choice will be the *in vitro* cell culture to study the efficacy of miRNAs in question. For further analysis of more complex pathophysiologic interactions, *in vivo* animal models may have to be employed.

Antisense oligonucleotides (ASO) and *anti-mRNA oligonucleotides* (AMO) have been used to modulate endogenous miRNA levels. The work principle is identical: the single-stranded oligonucleotide pairs with its complementary part of the miRNA and prevents mRNA target interaction. Different modifications have been used to prevent nuclease degradation and endonucleolytic damage by the RISC complex (Lennox and Behlke, 2011). The study by Esau et al. (2004) which analyzed miRNA patterns in adipocyte differentiation is a recent example of experimental work using ASOs targeting. The potential for using AMOs in a variety of human diseases, such as: neurodegenerative illnesses, cancer, viral infections, and metabolic disturbances, has been outlined by Weiler et al. (2006).

For in vitro experiments, different experimental aspects have to be considered: i) the specific characteristics of each cell line used, ii) optimizing inhibitor concentration depending on potency and efficiency of delivery, iii) the assay time itself, iv) cell type variations in loss or gain of function when comparing results, and v) the need for stressing cells to visualize phenotypic changes induced by miRNA inhibition.

For *in vivo* inhibition in animal models, anti-miRNAs have been proposed, which are partially or fully complementary to the mature miRNA target. Their effect should be a successful suppression of the miRNA in question and leading to increased target mRNA and protein levels. These reagents have to fulfill several requirements: i) high cell-permeability, ii) slow excretion, iii) stability *in vivo*, iv) high binding specificity and an affinity to the target miRNA, and v) low toxicity and side effects in other organs or off-targets. Particularly the last requirement has to be carefully considered because of the systemic delivery of these reagents. Furthermore, the desired knockdown effect has to be confirmed in the target tissue by qRT-PCR or Northern blotting, the protein upregulation monitored by Western blot, and

potential side effects, such as inflammatory reaction, have to be excluded by a histopathologic analysis particularly of the liver and kidneys.

Two principle antimiRNA modifications are currently in use:

1) 2′-0-Methyl group-modified oligonucleotides, also called *antagomiRs*

2) Locked-nucleic-acid (LNA)-modified oligonucleotides

The antagomiRs have been observed after tail vein injection in mice as being able to induce as well as suppress genes *in vivo* (Krutzfeldt et al., 2005) in a variety of organs, except the brain, while an immune response was not reported. However, relatively high doses (~80 mg/kg) and occasionally up to three injections are required (Bernardo et al., 2011).

LNA-oligonucleotides, have an improved resistance to degradation, and at the same time stabilize the miRNA-target duplex structure that is crucial for silencing activity. They have been preferably used in *in situ* hybridization (Doench et al., 2003). For *in vivo* experiments in non-human primates (Elmen et al., 2008) or by injection into the hearts of mice (Patrick et al., 2010; Porrello et al., 2011) usability has been reported. The use of lower doses due to their high affinities (Petersen et al., 2000) and a long lasting effect (60 days) was described.

Viral vectors expressing miRNAs

Viral vectors have been used for gene therapy and pharmaceutical target validation. While retroviruses need genomic integration and dividing cells, lentiviral vectors integrate into nondividing cells. In contrast, adenoviral or adenoassociated viral (AAV) vectors remain cytoplasmatic. These methods, however, while potentially able to produce the desired miRNA for a short or a long term, still pose several problems for their use. The most important problem is targeting the delivery into the desired cells/tissue by injection. At the same time, side effects, due to expression in other organ systems, pose a potential threat. Integration will provide prolonged, but not controllable production in either rapid proliferation cells (tumor), suitable for retrovirus delivery, while lentiviruses are more suited for organs with low cell division (heart, brain). Adenoviral or AAV vectors will only allow short term gene production, due to their non-integration. Secondly, limiting the expression to a certain cell type would greatly improve the specificity of the miRNA effect. Here, expression constructs with target cell specific or inducible (Epanchintsev et al., 2006) promoters could prove effective and prevent side effects.

Diagnostic relevance

Over the last years, since it became known that miRNAs: can regulate a variety of developmental processes, are involved in cell proliferation, in tumor development and in other diseases, this research area has exploded (Alvarez-Garcia and Miska, 2005). Due to the fact that miRNAs can release the cells either via vesicles, or as recently shown, in complex with Ago2, miRNA is accessible for diagnostic purposes (Arroyo et al., 2011; Hunter et al., 2008). In the recent past, studies monitoring the miRNA levels have been done in several different diseases and tumors. In 2010, Brase et al. (2010) published that two miRNAs (miRNA 375 and miRNA 141) are detectable in patient blood samples and that miRNA detection can be used as a diagnostic marker. Wulfken et al. (2011) published that the free circulating miRNA 1233 is a potential biomarker for the detection of clear cell renal carcinoma (RCC). Furthermore, Roth et al. published in 2010 that the analysis of two miRNAs (miRNA 10b and miRNA 34) in patients with breast cancer can possibly be used as a diagnostic marker.

Our group recently showed the regulation mechanism of miRNA 15a, which is protein kinase C alpha (PKC α) dependent (von Brandenstein et al., 2011). Furthermore, we also showed that the detection of miRNA 15a in urine samples from patients with RCCs, the malignant variant of kidney tumors, compared to oncocytoma, the benign variant, can possibly be used as a diagnostic marker (von Brandenstein et al., 2012).

Endothelin signaling and associated miRNAs

While in 2011, hundreds of articles were published reporting the new roles of miRNAs in many pathophysiologically different human disease entities, only a few articles can currently be found linking endothelin with miRNAs. In the following review section, these articles will be briefly discussed. They will be used as a starting point in analyzing other disease entities in which the respective miRNA may play a role although a possible relationship with endothelin itself has not been investigated in these articles. In many of these disease entities endothelin is in fact involved in the underlying pathophysiologic process, and may therefore indicate the need for new avenues of research.

miRNA 15

This miRNA has been implicated in different pathophysiologic events, being either up- or downregulated. Recently, von Brandenstein et al. (2011) have shown that ET-1 is able to induce a transcription complex consisting of NF-κB p65, MAPK p38 alpha, and PKC alpha in different tumor cell lines from renal cell carcinoma, cervical cancer, and melanoma. This complex migrates into the nucleus, where PKC alpha is able to prevent pri-miRNA 15a from transmigrating into the cytoplasm by binding to the pri-miRNA stem loop. Subsequently, cytoplasmic levels of miRNA 15a drop, since miRNA maturation is prevented. If miRNA 15a is stimulated in abundance (and PKC alpha levels low) as in clear cell renal cell carcinoma, it can be released in the urine. Here, the miRNA can be detected by qRT-PCR. This provides a diagnostic tool for renal cancer derived from the proximal tubule (von Brandenstein et al., 2012), because tumor removal by nephrectomy drops miRNA levels to background values. In contrast, in the benign renal tumor, the so-called oncocytoma, the situation is reversed: PKC alpha levels are high and miRNA levels are low.

miRNAs encoded by the miRNA 15/16 cluster are known as tumor suppressors. They inhibit cell proliferation, promote apoptosis of cancer cells, and suppress tumorigenicity both in vitro and in vivo (Aqeilan et al., 2009). Multiple oncogenes have been described as targets, including: BCL2, MCL1, CCND1, and WNT3A (Bonci et al., 2008). These miRNAs are downregulated in chronic lymphocytic lymphoma (Calin et al., 2008), pituitary adenoma, multiple myeloma (Roccaro et al., 2009) and prostate carcinoma, and in a subset of non-small cell lung cancer (Bandi et al., 2009).

In the prostate, both endothelin receptors are present on stromal and epithelial cells (Kobayashi et al., 1994). The ETB receptor is repressed by methylation, while activation of the ETA receptor provides an advantage for survival. Here, ET-1 treatment in prostate cancer inhibits apoptosis of tumor cells, in part through the Bcl-2 family. Family members Bad, Bax, and Bak are decreased; however, Bcl-2 itself remains unchanged (Nelson et al., 2005). In the tumor-supportive stromal cells, miRNAs 15a and 16 are downregulated in cancer-associated fibroblasts. Such downregulation promotes tumor growth and progression through the reduced post-transcriptional regression of Fgf-2 and its receptor FgfR1, which both act on stromal and tumor cells alike to enhance cancer cell survival, proliferation, and migration (Musumeci et al., 2011).

In autosomal-dominant polycystic kidney disease, Hocher et al. (1998) have demonstrated that the endothelin system plays a role in a paracrine manner. It participates in the regulation of mean arterial blood pressure, glomerular filtration rate, and renal blood flow. Furthermore, Ong et al. (2003) have found that ET-1 is expressed in cyst epithelia, mesangial cells, and vascular smooth muscle cells. ETA receptor was also expressed in glomeruli, cysts, and medium-sized renal arteries by microautoradiography. Recently, Lee et al. (2008) have associated

cystogenesis in polycystic liver and kidney diseases with miRNA 15a. They found decreased amounts of miRNA 15a in livers of patients with autosomal recessive and autosomal dominant kidney disease, as well as in congenital hepatic fibrosis. This resulted in the increased expression of the cell-cycle regulator Cdc25A. Cdc25 is a direct target of miRNA 15a, and increased cellular proliferation and cystogenesis in vivo.

miRNA 23

In the literature, endothelin-1 (besides phenylephrine and pressure overload) has been described as upregulating the endogenous miRNA 23a in cardiac muscle cells. When overexpressed in transgenic mice, cardiac hypertrophy ensues by direct inhibition of the translational activity of Foxo3a 3′UTR by miRNA 23a (Wang et al., 2010). In addition, miRNA 23a is able to alter expression levels of Manganese Superoxide Dismutase and the consequent reactive oxygen radicals. This observation was reported by Lin et al. (2011), who likewise discovered oxygen radical protection by miRNA 23a in the context of human retinal pigment epithelial cells. This is mediated by interaction of this miRNA with a binding site at the 3′UTR of the FAS gene. In endothelial cells, miRNA 23a, together with other members of its cluster (miRNA 27, miRNA 24), may also play an important role in the regulation of murine angiogenesis and neovascularization; through targeting SPRY2 and Sema6A proteins (Zhou et al., 2011). In addition, miRNA 23a has a role in restricting cardiac valve formation, since it is both necessary and sufficient for restricting the number of endocardial cells that differentiate into the endocardial cushion (Lagendijk et al., 2011). Thus, it can be speculated that a miRNA 23a dysregulation may be involved in the development of the so-called endocardial cushion defects as described by Rastelli et al. (1966). In addition, Lagendijk et al. (2011) have described that miRNA 23a is able to inhibit a transforming growth factor-β induced endothelial-to-mesenchymal transition in mouse endothelial cells, thus preventing a further contribution to potential fibrosis (see Fig. 4). However, miRNA 23a is also known to participate in the progress of cellular senescence: in (human cord-blood derived) multi-potent stem cells it participates in the down-regulation of the high mobility group A2, while helping to upregulate $p16^{(INK4A)}$, $p21^{(CIP1/WAF1)}$ and $p27^{(KIP)}$ (Lee et al., 2006). In this context, one finds miRNA temporally and spatially regulated in (neuroepithelial) stem cells (Farrell et al., 2010). Thus, in the brain miRNA 23 participates in the myelination process through regulation of laminin B1 and appears crucial for oligodendrocyte development (Aqeilan et al., 2009).

miRNA 125

In cardiovascular disease, different mediators are able to stimulate endothelial cells causing dysregulation, among them are oxidized low-density lipoproteins. One particular reaction is the upregulation of endothelin-1. Le et al. (2009) have shown that miRNA 125a-5p and 125b-5p prevent this upregulation by directly targeting the 3′UTR of pre-pro-endothelin mRNA (Aqeilan et al., 2009). Thus, this miRNA may be important because it is able to prevent endothelin production. This will prohibit the signaling cascade via ETA receptor leading to epithelial–mesenchymal transformation which is the basis for fibrosis. Since this miRNA is also reported as a regulator of p53, by binding to its 3′UTR in humans and zebrafish (Aqeilan et al., 2009), it should play a role in preventing the epithelial–mesenchymal transformation (EMT) (see Fig. 4). p53 upregulates members of the miRNA 200 family, which in turn decreases the TGF-β-mediated-transcription factor ZEB1, leading to reduced EMT formation. Another role of miRNA 125 is its participation in the downregulation of p53 in oral lichen planus (Aqeilan et al., 2009), a condition in which endothelin is upregulated (Chang et al., 2011).

miRNA 155

This miRNA is necessary to upregulate endothelin-1 in liver sinusoidal endothelial cells in humans but not in the rat (Yeligar et al., 2009), after ethanol-treatment, potentially modifying the influence of ET-1 in this micro-environment. At the same time, ethanol damage resulting in progressive liver fibrosis is potentially attenuated by the negative modulating effect of this miRNA on SMAD2 expression (see Fig. 4), since Bala et al. (2010) have found two predicted target sites in SMAD2 3′UTR. In non-alcoholic steatohepatitis (NASH) patients, where fat accumulation leads to inflammation and subsequent fibrosis, Degertekin et al. (2007) observed that the serum ET-1 level showed a statistically significant increase as fibrosis severity increased. This ET-1 increase could well be mediated by the difference in expression of hepatic miRNAs such as miRNA 155, as was observed in a mouse model of dietary, non-alcoholic steatohepatitis (Pogribny et al., 2010). Since inflammation is a part of the development of NASH, it is not surprising that this miRNA is involved in the different aspects of the inflammatory signaling pathways in which ET-1 participates. In the pathogenesis of alcoholic activation of von Kupffer cells, miRNA 155 as well as NF-κB being upregulated will contribute to an increased tumor necrosis factor alpha production. Furthermore, Thompson et al. (2010) showed that miRNA 155 was an actual target for the NF-κB pathway, and leads to the reduced expression of different transcription factors in several B-lymphoma cell lines. Since ET-1 induces a transcription complex containing NF-κB in different normal and tumor human cell lines (von Brandenstein et al., 2008), this miRNA should be considered as playing an important, yet unidentified, role in these cells. Not surprisingly, miRNA 155 has been shown as a pro-inflammatory regulator in clinical and experimental arthritis, a disease entity in which ET-1 is regarded as playing a major role and NF-κB is activated [rheumatoid arthritis, (Kurowska-Stolarska et al., 2011)]; [osteoarthritis, (Roy-Beaudry et al., 2003)]. Furthermore, this miRNA has been shown to participate in autoimmune diseases, where it can promote autoimmune inflammation by enhancing inflammatory T-cell development (O'Connell et al., 2010). In systemic lupus nephritis (Wang et al., 2010) miRNA 155 is elevated in the urine, just as ET-1 levels are (Dhaun et al., 2009). Finally, immortalization of B-cells by Epstein–Barr virus (EBV) is dependent upon virally induced miRNA 155 (Linnstaedt et al., 2010), while as a negative regulator of EBV disease, miRNA 155 can prevent bone morphogenic protein (BMPs: 2, 6, and 7)-mediated EBV reactivation (Yin et al., 2010). Regarding bone morphogenic proteins, miRNA 155 could also play an important part in early autoimmune diabetes, where it may support the role of the ETA receptor in negatively regulating activated BMPs 2 and 7 (Nett et al., 2006).

miRNA 195

Sickle cell disease is a model for a chronic inflammatory state with increased circulatory levels of pro-inflammatory cytokines. Here, placental growth factor induces endothelin-1 in the pulmonary microvasculature. ET-1 was able to significantly downregulate miRNA 195. This miRNA has a complimentary binding site in the 3′UTR of the chemokine CCL4. Signaling occurs via the activation of: PIK3A, p38 MAPK, NF-κB, and hypoxia-inducible factor-1 alpha (Gonsalves and Kalra, 2010). miRNA 195 is also known for its involvement in cell cycle regulation. Although not yet proven, ET-1 and miRNA 195 may work together in achieving apoptosis. Recently, von Brandenstein et al. (2012) described the upregulation the tumor suppressor gene $p16^{INK4a}$ by the activation of the ETS-binding site of the $p16^{INK4a}$ promoter through an ET-1 inducible, ETS/ERK2/Mxi-2 transcription complex. It is also known that $p16^{INK4a}$ shows aging dependent changes in its phosphorylation profile and increasingly associates itself with cdk4- and cdk6. This leads to a loss of cyclin D1 binding, preventing cell cycle progression (Sandhu et al., 2000). Since miRNA 195 is able to downregulate several G(1)/S transition-related molecules (cyclinD1, CDK6, and E2F3) as its direct

targets (Sandhu et al., 2000), at least a concerted action of ET-1 and miRNA 195 has to be assumed.

miRNA 199 and the miRNA 200 family

Different research groups have recognized the importance of the endothelin signaling axis in the progression of different diseases leading to fibrosis. This particular process is based on the epithelial–mesenchymal transition, so-called EMT (see Fig. 4). It could be identified in the liver (Yeligar et al., 2009; Degertekin et al., 2007), lung (Crestani, 2007; Jain et al., 2007) and kidney (Oba et al., 2010) as well as in tumors, such as ovarian cancer (Bagnato and Rosano, 2007; Rosano et al., 2011; Vergara et al., 2009). Epithelial–mesenchymal transition (EMT) is a prime example of a reversible differentiation process that occurs during normal development, such as neural tube and mesoderm formation during embryogenesis [Type I EMT, (Carew et al., 2011)]. Two other types of EMT have been recently described: type II in organ fibrosis, wound healing, and regeneration; and type III in cancer metastasis (Carew et al., 2011). In type III, EMT has been highlighted as a key process in tumor invasion, metastasis and tumorgenicity (Kong et al., 2008; Wellner et al., 2009), enhancing the invasive properties of epithelial tumor cells and promoting tumor metastasis (Berx et al., 2007; Thiery, 2002). Mani et al. (2008) have shown that this process is also associated with the acquisition of tumor initiating and self-renewal properties. The potential for a forward-and-back conversion between the epithelial and the mesenchymal state has also been recognized as influencing tumor malignancy (Gupta et al., 2009; Polyak and Weinberg, 2009).

EMT is promoted by transforming growth factors (TGFs), such as TGFB1 (TGFβ1) and TGFB2 [TGFβ2; (Thiery and Sleeman, 2006)]. TGF-β has been found to play an important role in particular stages of development and in disease processes, such as fibrosis and cancer metastasis (Derynck and Akhurst, 2007; Pardali and Moustakas, 2007; Zeisberg and Kalluri, 2004). Several EMT-activating transcription factors: ZEB1 (also called dEF1, TCF8, and AREB6), ZEB2 (also called SIP1), SNAI1 (also called Snail), SNAI2 (also called Slug), and TWIST involved in this process have been previously identified (Kong et al., 2008). They are TGF-β induced, and mediate its effects at least in part through repression of E-cadherin and initiation of EMT (Peinado et al., 2007).

It has been recently reported that miRNAs may play a crucial role in EMT. The miR-200 family was found to target ZEB1 and ZEB2, while being themselves significantly down-regulated in TGFβ-induced mesenchymal cells and cancer cells with mesenchymal characteristics (Mitchell et al., 2008; Bracken et al., 2008). Several investigations have shown this family of miRNAs involvement in progressive fibrosis and epithelial-to-mesenchymal-transformation in a variety of organs.

In the liver, sinusoidal endothelial cells have been demonstrated to be the source of increased mRNA expression of endothlien-1, hypoxia-inducible-factor-1 alpha, and inflammatory cytokines in ethanol-fed rats. However, the regulatory mechanism to control transcription was unknown. In a rat model of liver damage by ethanol-feeding, Yeligar et al. (2009) demonstrated that miRNA 199 plays a major role as a negative regulator to control transcription and homeostatic levels of ET-1, leading to a controlled microcirculatory tone. In non-alcoholic liver steatohepatitis (NASH), ET-1 levels have been found to be upregulated in the serum of NASH patients (Degertekin et al., 2007). In a respective murine model, upregulation of miRNA 200b has been observed, among others, indicating that the severity and susceptibility of NASH may be determined by variations in miRNA expression response. Furthermore, in a CCL(4)-induced mouse model, miRNA 199a and 200a and b were found positively and significantly correlated to the progressed liver fibrosis (Murakami et al., 2011). In addition, these miRNAs could be detected in liver biopsy specimens from 105 chronic hepatitis type C patients without a history of anti-viral therapy (Murakami et al., 2011).

It is known that ET-1 is an initiator of EMT (Fukuda et al., 2007) in the lung, where it helps to transform alveolar type II cells (Crestani, 2007). This process is mediated through the ETA-receptor, leading to the upregulation of TGF-β (Jain et al., 2007). The participation of miRNAs was not investigated in these experimental settings, since their potential role was still unknown at the time. However, given the almost identical signal-setting, members of the miRNA 200 family and other miRNAs can be expected to participate in this process.

In the kidney, the severity of tubulointerstitial fibrosis correlates not only with the risk of progressive renal dysfunction, but is also the most important factor for long term prognosis, particularly in diabetic nephropathy (Gilbert and Cooper, 1999). In proteinuric diseases, the activation of proximal tubules (Laghmani et al., 2002) by protein and other mediators leaking through the damaged glomerular capillary wall have been shown to induce ET-1 with tubulointerstitial fibrosis and EMT (Hocher et al., 1997). Thus, it is not surprising that in a model of unilateral ureteral obstruction mimicking EMT formation, the use of a miRNA 200b precursor led to an ameliorating effect on tubulointerstitial fibrosis with prevention of increased collagen types I and III as well as fibronectin deposition. This miRNA could be shown to act via the suppression of TGF-β induced ZEB 1 and 2 transcription factors.

Currently, the only tumor studies investigating the effects of the endothelin axis on EMT have been performed in ovarian cancer (Bagnato and Rosano, 2007). The expression of an EMT phenotype as well as the acquisition of chemoresistance could be linked to the activation of the endothelin A receptor pathway (Rosano et al., 2011). Activation of the ETAR by ET-1 enhanced the expression of α2 β1 and α3 β1 integrins. This is associated with an increase in the activity of the integrin-linked kinase once ovarian cancer cells adhered to type 1 collagen (Rosano et al., 2006). Specific blockage of the ETAR was able to downregulate SNAIL activity and to restore sensitivity to cytotoxic-induced apoptosis as well as inhibiting the invasiveness of resistant tumor cells.

MicroRNAs: promises, challenges, and future directions

The discovery of miRNAs holds promises and challenges for the future of clinical practice. First impacts have affected the fields of therapeutic targeting and disease detection. miRNAs may be able to be therapeutically controlled without the adverse side effects of current molecular approaches (Fasanaro et al., 2009; Montgomery and van Rooij, 2010; Seto, 2010). Toward that end, the report of a successful Phase I trial using LNA anti-miRs for the treatment of hepatitis C (see Bernardo et al., 2011) is encouraging, particularly since its quality was so convincing that a Phase II clinical trial was added (Santaris Pharma A/S, clinical trial identifier NCT01200420) (see Bernardo et al., 2011). In the field of cancer, one could envision analyzing the miRNA pattern of a patient's tumor sample by microarray as the first step of a multistep process in individualizing cancer therapy. Combined with additional molecular studies in the patient and even in relevant family members, this analysis could greatly help in tailoring the patient's future therapy. Furthermore, miRNA analysis may aid in cancer detection. Recently, Barker et al. (2009) has reported that miRNA pattern recognition could be able to identify the histogenetic origin of a CUP syndrome (cancer of unknown primary).

In the field of disease detection, miRNAs may play a role as biomarkers. Again, limitation of the current biomarkers may be avoided, such as reduced sensitivity, specificity, detectability only in already advanced lesions, and costly methods employed. miRNAs appear to be ideal biomarkers compared to currently available ones because of their: presence and stability in serum/blood (Gilad et al., 2008), and in urine (von Brandenstein et al., 2012), relatively easy detectability (by qRT-PCR), stability even after freeze-thaw cycles, and potential specificity to tissue or disease states. Several studies have been published which use the detection of miRNA in serum/blood as potential diagnostic or even prognostic indicators, such as in diabetes, cardiovascular disease or cancer (references — see Bernardo et al. (2011)). We have recently proposed that miRNA 15a could serve as biomarker for differentiation

of malignant versus benign renal cell carcinoma using patients' urine samples (von Brandenstein et al., 2012).

In spite of these encouraging developments, challenges on at least 3 different levels remain before a widespread use of miRNAs can be employed:

i) *Understanding of the processing of miRNA itself*

The mechanisms of modification, stabilization, degradation, and turnover are largely unknown. Additional regulatory pathways may yet to be discovered [such as PKC α regulating miRNA 15a (von Brandenstein et al., 2011)]. New discoveries of unconventional pathways even in the regulation of miRNA biogenesis, such as the one without Drosha and DGCR8 (Fig. 3e) may change our thinking of miRNA processing in the future.

ii) *The promiscuous effect of miRNAs and their interactive generation cascades*.

A single miRNA may be able to regulate different genes as well as several miRNAs that are involved in the regulation of the same gene (Suzuki and Miyazono, 2010). Here the specificity of gene–miRNA interaction has to be defined, and the potential functional effects of different miRNA interactions for gene expression characterized to prevent undesired or damaging side effects by losing tissue specificity.

iii) *Functional miRNA redundancy and interaction*

Quite similar to transcription factors, miRNAs are often clustered in families of highly related members, carrying out potentially distinct biological functions. Binding characteristics and the miRNA–miRNA interactions have to be defined, as a fundamental necessity, prior to any efforts of therapeutic targeting.

The challenges in unraveling the mysteries of the miRNA micro-cosmos will be numerous, but the potential rewards will be equally unimaginable. Surely, the roads of exploration will provide us with a new understanding of biology and the intricacies of nature.

Abbreviations

AAV adeno-associated virus
Ago Argonaute
AMO anti-microRNA oligonucleotides
AREB6 Atplal regulatory element, zinc finger protein
ASO antisense oligonucleotide
Bad Bcl-2-associated death promoter
Bak Bcl-2 antagonist/killer-1
Bax Bcl-2-associated X protein
BCL2 B-cell lymphoma 2
BMPs bone morphogenetic protein
bp base pairs
CCL4 Chemokine (C–C motif) ligand 4
CCND1 cyclin D1
Cdc25a Cell division cycle 25 homolog A
CDK Cyclin Dependent Kinase
C. elegans *Caenorhabditis elegans*
DEAD amino acid sequence D-E-A-D (Asp-Glu-Ala-Asp)
dEF1 Differentiation-enhancing factor 1
DGCR8 DiGeorge syndrome critical region gene 8
D. melanogaster *Drosophila melanogaster*
DNA deoxyribonucleic acid
E2 estradiol
E2F3 E2 promoter binding factor 3
EBV Epstein Barr virus
EMT epithelial–mesenchymal transition
ERα estrogen receptor alpha
ERK extracellular signal-regulated kinase
ET-1 endothelin-1
ETS E-twenty six family
ETAR endothelin A receptor
ETBR endothelin B receptor
FAS TNF receptor superfamily member 6
FgfR1 Fibroblast Growth Factor Receptor 1
Foxo3a forkhead box O protein 3a
HIF1α hypoxia-inducible transcription factor 1α
let letal
Lin lineage defective
LNA locked nucleic acids
Loqs Loquacious
MAPK Mitogen-Activated Protein Kinase
MCL1 Myeloid Cell Leukemia 1
miRNA microRNA
mRNA messenger RNA
Mxi2 Max interacting protein 2
NASH non-alcoholic steatohepatitis
NF45 nuclear factor 45
NF90 nuclear factor 90
NFkB nuclear factor beta
$p16^{(INK4a)}$ Cyclin-dependent kinase inhibitor 2A
$p21^{(CIP/WAF)}$ cyclin-dependent kinase inhibitor 1
$p27^{(KIP)}$ Cyclin-dependent kinase inhibitor 1B
p53 tumor suppressor
p68 interferon-induced, double-stranded RNA-activated protein kinase
p72 DEAD box family member of putative RNA-dependent ATPases and ATP-dependent RNA helicases, homologue to p68
PACT protein activator of the interferon induced protein kinase
P-bodies processing bodies
PIK3A Phosphatidylinositol 3-kinase A
PKC α protein kinase C alpha
PKR RNA-dependent protein kinase
Pol III polymerase III
pre-miRNA precursor microRNA
pri-miRNA primary microRNA
3p 3′ arm of the pre-miRNA
5p opposite arm of the 3p mature miRNA
qRT-PCR quantitative real time PCR
RISC RNA-induced gene silencing complex
RNA ribonucleic acid
R-SMAD receptor regulated small mothers against decapentaplegic
Sema6A Semaphorin-6A
SIP Smad interacting protein 1
SMAD small mothers against decapentaplegic
SNAIL zinc-finger transcription factor
SPRY2 Sprouty homolog 2
TCF8 transcription factor 8
TGF-β transforming growth factor beta
TGF-2 transforming growth factor-2
TRBP TAR (trans-activation responsive element) RNA binding protein
TWIST class A basic helix-loop-helix protein 38
UTR untranslated region
WNT3A wing signaling pathway 3A
ZEB1 zinc-finger E-box binding homeobox 1

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This review article is part of the "*ET-12 Best Presentation Awards*" for best oral and best poster presentation, both received by Dr. Melanie von Brandenstein at the *Twelfth International Conference on Endothelin*, Cambridge, UK, 2011, and is co-sponsored by Elsevier Publishers, Amsterdam. The support from Anne M. Schofield-Fries is gratefully acknowledged. The work was supported by the Koeln Fortune Program/Faculty of Medicine, University of Cologne to Melanie von Brandenstein and from the Walter and Marga Boll Foundation to Jochen W.U. Fries

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