Chapter 8
RARs and MicroRNAs

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Abstract MicroRNAs (miRNAs) are small noncoding RNAs acting as endogenous regulators of gene expression. Their discovery is one of the major recent breakthroughs in molecular biology. miRNAs establish a multiplicity of relationships with target mRNAs and exert pleiotropic biological effects in many cell physiological pathways during development and adult life. The dynamic nature of gene expression regulation by Retinoic Acid (RA) is consistent with an extensive functional interplay with miRNA activities. In fact, RA regulates the expression of many different miRNAs, thus suggesting a relevant function of miRNAs in RA-controlled gene expression programmes. miRNAs have been extensively studied as targets and mediators of the biological activity of RA during embryonic development as well as in normal and neoplastic cells. However, relatively few studies have experimentally explored the direct contribution of miRNA function to the RA signalling pathway. Here, we provide an overview of the mechanistic aspects that allow miRNA biogenesis, functional activation and regulation, focusing on recent evidence that highlights a functional interplay between miRNAs and RA-regulated molecular networks. We report examples of tissue-specific roles of miRNAs modulated by RA in stem cell pluripotency maintenance and regeneration, embryonic development, hematopoietic and neural differentiation, and other biological model systems, underlining their role in disease pathogenesis. We also address novel areas of research linking the RA signalling pathway to the nuclear activity of miRNAs.

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Abbreviations

Ago	Argonaute
ALDH1A2	Aldehyde dehydrogenase-1a2
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
C/EBPα	CCAAT/enhancer-binding protein-alpha
ceRNAs	Competing endogenous RNAs
DGCR8	DiGeorge syndrome critical region gene 8
ESC	Embryonic stem cells
HDAC	Histone deacetylase
Hox	Homeobox
HPCs	Hematopoietic progenitor cells
HSC	Hematopoietic stem cells
lncRNA	Long non-coding RNA
miRNAs	MicroRNAs
NFI-A	Nuclear factor I-A
PACT	Protein kinase R (PKR) activator
P-bodies	Processing bodies
PcG	Polycomb group proteins
Pitx3	Paired-like homeodomain transcription factor 3
pre-miRNA	MicroRNA precursor
pri-miRNA	Primary microRNA
PTB	Polypyrimidine tract binding protein
RA	Retinoic Acid
RALDH2	Retinaldehyde dehydrogenase 2
RAR	Retinoic acid receptor
RISC	RNA-induced silencing complex
RNA polymerase II	RNA polII
RXR	Retinoid X receptor
TF	Transcription factor
TRBP	Trans-activation response (TAR) RNA-binding protein
UTR	Untranslated region

It is now clear an extensive miRNA world was flying almost unseen by our genetic radar. As much as geneticists like to think that nothing can escape genetic analysis, the miRNA genes are so small that they almost escaped our notice [124].

History

The hypothesis of genomically-encoded regulators of protein expression acting in the cytoplasm as intermediate molecules between mRNA and proteins was formulated more than 50 years ago by Jacob and Monod. In a seminal paper they affirm that:

> …the most firmly grounded of these conclusions is the existence of **regulator** genes, which control the rate of information-transfer from **structural** genes to proteins…the regulator gene acts via a specific cytoplasmic substance whose effect is to **inhibit** the expression of the structural genes …the chemical identification of the **repressor as an RNA fraction** is a logical assumption. [62]

Thus, Jacob and Monod had already envisaged the existence of non-coding regulatory RNAs. More than 30 years later, in 1993, Victor Ambros' laboratory cloned the first microRNA (miRNA), *lin-4,* in *Caenorhabditis elegans*, and identified *Lin14* as one of its target mRNAs [81]. Seven years later, Gary Ruvkun's group cloned the second miRNA, *let-7*, and reported the conservation of miRNAs across species [117, 122].

The canonical miRNA genes, *lin-4* and *let-7*, were identified by genetic screening of mutant phenotypes that caused developmental timing defects in stage specific lineages of worm larvae [81, 122]. Since then, genetic cloning, bioinformatics, gene expression analysis and computational algorithms defining phylogenetic conservation and structural characteristics of miRNA precursors have allowed large-scale identification of miRNAs from various life forms, such as plants, *Drosophila melanogaster,* and vertebrates, including mammals. Animal viruses also use miRNA [2, 9, 77].

Global gene expression analyses have shown that miRNAs and their mRNA targets often have mutually exclusive expression in contiguous developmental stages or across tissues [38, 134, 137]. miRNAs participate in transcriptional programs that control development, cellular pluripotency, and differentiation. They affect expression levels of lineage-specific TFs in integrated transcriptional regulatory circuits, and, in turn, are regulated by the activities of these factors [39, 114, 151].

miRNAs are well-preserved in blood plasma or serum, urine, and formalin-fixed tissue blocks, and can be measured with much greater sensitivity than proteins [79, 103]. miRNA expression profiling is now considered to be a relevant approach in a broad range of biological and medical studies and may have many molecular diagnostic and therapeutic applications.

Development of the field

At present, the miRBase release 20 (June 2013) (http://www.mirbase.org/) reports 24,521 entries of hairpin precursor miRNAs which give rise to 30,424 mature miRNA products. MiRBase estimates 1872 precursors and 2578 mature miRNAs in humans. Well over half of the human transcriptome is computationally predicted to be subjected to miRNA regulation [43]. Expression analysis of miRNAs by commercially available oligonucleotide microarrays or quantitative polymerase chain reaction (qPCR) arrays are now powerful approaches for monitoring tissue specific, developmental, physiological and disease state miRNA expression across the whole genome.

The mechanisms related to miRNA biogenesis have been revealed in detail. Genes encoding miRNAs are an integral component of a cell's genomic program, and are generally conserved through evolution [1, 38]. They can be encoded in intergenic transcription units, in polycistronic clusters, within intronic sequences of protein-coding genes, or within the introns and exons of non-coding RNAs [1, 72, 85, 123]. For genes sharing the same promoters, the "host" transcript and miRNAs usually have similar expression profiles [5, 123]. Moreover, sequence variations in miRNA genes potentially influence the processing and/or target recognition of miRNAs [101]. miRNA gene mutation or mis-expression have been observed in various human cancers and indicate that miRNAs can function as tumor suppressors and oncogenes [35].

Most miRNA genes are initially transcribed by RNA polymerase II (Pol II) as long primary transcripts (pri-miRNA) that contain one or more hairpin-shaped structures (Fig. 8.1). The primary transcripts contain a 5′ cap and a poly(A) tail similar to that of mRNAs [15, 86]. RNA polymerase III can drive miRNA transcription from dense human clusters interspersed among repetitive Alu elements [13]. In the nucleus, the pri-miRNA is cleaved by a microprocessor complex consisting of the RNase III enzyme, Drosha, and the double-stranded RNA binding protein "DiGeorge syndrome critical region gene 8″ (DGCR8, also known as Pasha). Drosha and DGCR8 release an imperfect hairpin-structured precursor miRNA (pre-miRNA) of about 70 nucleotides [28, 55, 83, 85].

The Drosha complex includes several auxiliary proteins, such as RNA binding protein fused in sarcoma (FUS), Ewing sarcoma breakpoint region 1 (EWSR1), heterogeneous nuclear ribonucleoproteins (hnRNP), and DEAD (Asp-Glu-Ala-Asp) box helicases p68 (DDX5) and p72 (DDX17) [49]. Whereas some hnRNPs and p68/72 promote the fidelity and activity of Drosha processing, the exact role of these proteins in fine regulation of miRNAs expression is still largely unknown [50].

The Drosha-processed pre-miRNA is exported from the nucleus to the cytoplasm by the Exportin-5 [12, 93, 166]. The Exportin-5 recognizes the 2-nucleotide 3′ overhang structure and the double-stranded stem of the pre-miRNA [109]. In the cytoplasm, the pre-miRNA is captured by a second RNase III enzyme, Dicer. Dicer and its related double-stranded RNA binding partners, the transactivating response RNA-binding protein and protein kinase R activator, cleave the pre-miRNA at the stem-loop junction [20, 52, 61, 84, 95, 125]. This generates a miRNA:miRNA duplex of approximately 22 nucleotides with overhangs of 2-nucleotides at their 3′ ends. Generally, the strand whose 5′ end has a less tight base pairing (also known as the "*guide*" strand) is favored for incorporation into a functional protein complex called "*RNA-induced silencing complex*" (RISC), which is active in the repression of mRNA function. Within this riboprotein complex, miRNAs are driven to the target mRNA [129, 133]. The other miRNA strand [also known as "*passenger*" strand or star miRNA (miRNA*)] is usually degraded [100], although in some physiological conditions both strands of the miRNA duplex can be detectable in the RISC [111].

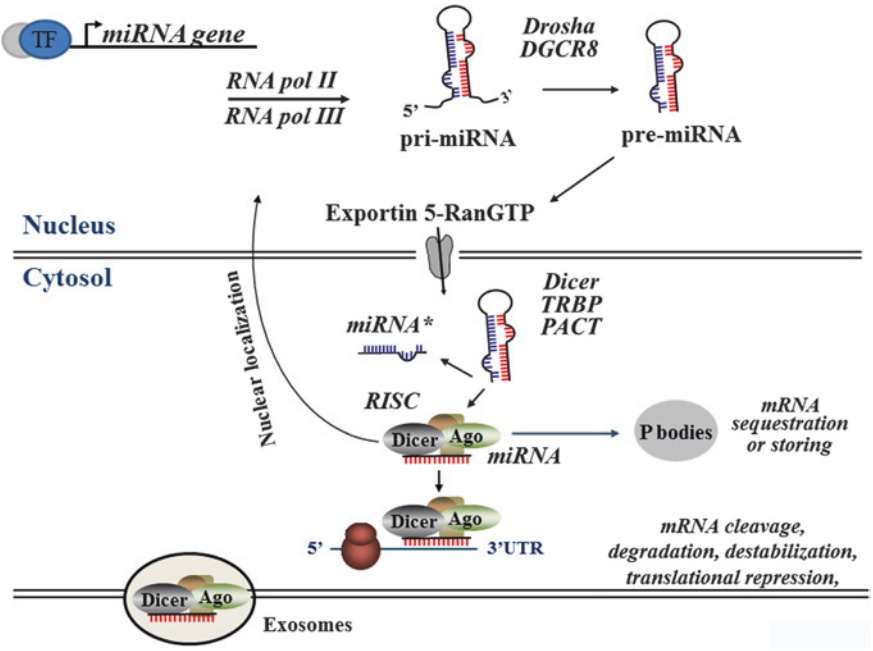

Fig. 8.1 miRNA biogenesis and function. miRNA genes are regulated by transcription factors (*TF*) and transcribed by RNA pol II and III into primary miRNA transcripts (*pri-miRNAs*). The pri-miRNA is processed into a precursor miRNA (*pre-miRNA*) stem-loop of ~70 nucleotides in length by the nuclear RNase III enzyme Drosha and its partner DiGeorge syndrome critical region gene 8 (*DGCR8*). Exportin-5 actively transports the pre-miRNA into the cytosol, where it is processed by the Dicer RNaseIII enzyme and its partner TRBP, into a double strand miRNA. The miRNA strand (in *red*) is recruited as a single-stranded molecule into the RNA-induced silencing (*RISC*) effector complex and assembled through processes that are dependent on Dicer and other double strand RNA binding domain proteins (*dsRBD*), as well as on members of the Argonaute (*AGO*) family. The other strand (miRNA*) is usually degraded. Mature miRNA guides the RISC complex to the 3′UTR of the complementary mRNA targets and repress their expression by several mechanisms: repression of mRNA translation, destabilization of mRNA transcripts through cleavage, de-adenylation, and localization in the processing body (*P-body*), where the miRNA-targeted mRNA can be sequestered from the translational machinery and degraded or stored for subsequent use. Nuclear localization of mature miRNAs has been described as a novel mechanism of action for miRNAs

The RISC contains a number of different proteins, including Argonaute (Ago) and Dicer proteins (Fig. 8.1). In humans, there is only one Dicer and four Ago proteins (Ago 1, 2, 3, and 4) [102]. Human Ago2 is the only Ago protein embodying the endonucleolytic "*slicer*" activity of RISC which catalyzes the cleavage of target mRNA [132]. The structure of human Ago2 in complex with miRNA-20A was recently determined. The RNA confers remarkable stability to the enzyme, most likely as a consequence of multiple interactions that spread along the entire protein [31]. All four Ago proteins and other proteins, such as a trinucleotide repeat

containing 6 A, B and C subunits (TNRC6, also known as GW182) are essential in the silencing process facilitated by miRNAs in the cytoplasm [20, 54, 102, 110]. Some evidence also suggests that TNRC6A may direct Ago proteins into the nucleus via a nuclear localization signaling activity [107]. Indeed, miRNAs and Ago proteins have been found in the nucleus of human cells (see below) [8, 168, 169].

Mature miRNAs serve as guides, directing RISC to mRNAs containing their complementary sequences (Fig. 8.1). Ago proteins serve as platforms for interacting proteins making base-specific contacts with the first nucleotides of the miRNA guide strand and, consequently, contributing to target recognition [110, 102]. This recognition between miRNA and mRNA targets mainly involves a limited base-pairing between the 5′-end '*seed*' region (2–8 nucleotides from the 5′-end) of the miRNA and the complementary sequences present in the 3′ untranslated region (UTR) of their mRNA targets. In some cases, miRNAs can efficiently bind to the 5′ UTR of the target mRNA, or even coding sequences [4, 73, 94, 80]; however, these non-canonical miRNA binding sites are less frequently used and less effective in suppressing mRNA function, possibly because the silencing complexes can be displaced by the translation machinery [4].

Reduced protein expression is brought about by mRNA translational repression, mRNA destabilization, or a combination of the two [37, 81, 90]. Complete complementarity between a miRNA and a mRNA target site is rare in animals, and cleavage of a target mRNA can only occur if a catalytically active Ago is bound [37]. When targeted for silencing by miRNAs, mRNA can be sequestered from the translational machinery, degraded or stored in large cytoplasmic foci, named processing bodies (P-bodies). The P-bodies contain a wide range of enzymes involved in RNA turnover [36]. Recent evidence indicates mRNA degradation is the major determinant of miRNA activity, being responsible for >84 % of the effects on protein expression [51]. However, in some model systems a block of translation initiation may precede mRNA decay and mediate the miRNA repressor activity [7]. Evidence also indicates that miRNAs induce translational up-regulation of target mRNAs [37, 152].

The regulatory potential of each miRNA is rendered even more complex as indicated by recent findings showing that miRNAs can also display a decoy activity that interferes with the function of regulatory proteins [30]. Moreover, endogenous RNA, and long non-coding RNA (lncRNA) complementary to miRNAs, can act as competing endogenous RNAs (ceRNAs) in both normal and pathological conditions. By binding a common pool of miRNAs, ceRNAs prevent them from binding their mRNA targets, thus blocking their activities [18, 68, 140, 146]. Overall, these studies reveal a new level of regulation of gene expression, controlled by an extensive interacting network of both coding RNAs, lncRNAs and miRNAs, which can be predicted on the basis of the overlap of miRNA-binding sites [67].

Recently it has been shown that miRNAs are also secreted from cells through the exosomal pathway, suggesting a new potential action of miRNA in cell-to-cell communication during the complex events that regulate development and differentiation [108].

Current State of the Field

MicroRNA and Retinoic Acid Regulatory Networks in Embryo Development

Since their discovery in *Caenorhabditis elegans* mutants, miRNA activities have consistently turned up as players in cell fate determination in other animals, including mammals [81, 122]. Interestingly, RA-regulated miRNAs are now known to influence mammalian development and embryonic stem cell (ESC) self-renewal and differentiation.

RA and miRNAs may act through common transcriptional pathways to regulate the balance between ESC self-renewal or differentiation capacity. miR-145 expression can be induced by RA treatment of human ESC [63], leading to suppression of mRNAs encoding the transcription factors, octamer-binding transcription factor 4 (Oct4), Sox2, and Kruppel-like factor 4 (Klf4), which are associated with the preservation of pluripotency in stem cells. Re-expression of these factors in human somatic cells reprograms them to a pluripotent stem cell state [143, 164]. The molecular mechanism for terminating the pluripotent state of ESC is an example of the complex interactions between RA, miRNAs and TFs. In response to RA, CBP/p300 acetylates p53 at lysine 373, which leads to its dissociation from E3-ubiquitin ligases HDM2 and TRIM24 and causes p53 protein stabilization. This key step activates the production of miR-145 and miR-34a, which, leads to repression of Oct4, Klf4, Lin28a, and Sox2, which, in turn, prevents the hESC from backsliding to pluripotency [63]. Interestingly, Oct4 transcriptionally represses miR-145 [164], an indication that this circuit is under stringent reciprocal control.

However, Oct4 gene transcription is also downregulated by complex epigenetic mechanisms induced by RA as shown by studies where proliferating stem cells were exposed to RA [22, 128].

RA treatment also induces the expression of miR-134, which enhances mouse ESC differentiation along ectodermal lineages [147]. This is due, in part, to miR-134 direct translational attenuation of genes, including Nanog and LRH1, both of which act as positive regulators of Oct4/POU5F1 and ESC growth [147]. Overall, these data establish that both the RA and miRNA signalling pathways affect ESC differentiation through their potential to regulate, or target, multiple genes that play a central role in ESC maintenance and differentiation.

Homeobox Genes

Homeobox (Hox) genes are classic targets of RA signaling [74, 75, 118]. Precise temporal and spatial activation of their transcription is required for proper specification of regional identities along the body's main axes. The miR-10 and miR-196 families of miRNA genes are embedded within the vertebrate Hox clusters

and their expression patterns are markedly similar to those of the Hox genes [76, 89, 97]. For example, the miR-196 gene is expressed in spatially non-overlapping domains with its conserved targets, Hoxa7, Hoxb8, Hoxc8 and Hoxd8 [97, 165]. Tabin's group made a conditional knockout of Dicer (a key enzyme required for producing functional mature miRNAs) that specifically removed Dicer from mouse limb buds. Using this approach, they found that miR-196 acts upstream of Hoxb8 and Sonic Hedgehog in mouse and chicken limb development [57]. They observed that expression of miR-196 is lower in the forelimb than in the hindlimb, where the miRNA acts as an inhibitor of Hoxb8, preventing it from being induced by RA [57]. Thus, in normal limb development, miR-196 appears to pre-empt inappropriate Hoxb8 induction by RA.

Over-expression of miR-196 alters Hox genes expression patterns that are required for the proper development of pectoral fin buds in zebrafish embryos [56]. Interestingly, this effect is a consequence of the direct activity of miR-196 on the RA signalling pathway. miR-196 targets the 3′UTR of the retinoic acid receptor ab (rarab). In fact, knocking down rarab mimicked the pectoral fin phenotype induced by miR-196 over-expression [56]. This is one of the few known examples of direct targeting of RAR transcripts by miRNAs.

miR-10 represses Hoxb1a and Hoxb3a within the spinal cord, and this repression works cooperatively with Hoxb4 [161]. Transcription of miR-10 is activated by RA. Overexpression of miR-10 induces phenotypes similar to those caused by the loss of Hoxb1a and Hoxb3a [161]. Interestingly, cluster genes of miR-10 Hox targets are located in close proximity to this miRNA gene, suggesting a coordinate wave of transcriptional and post-transcriptional regulation of Hox genes expression.

Heart and Muscle Development

The circulatory system is the first functional unit in the developing embryo, and the heart is the first functional organ. The essentiality of RA in cardiogenesis has been established by experiments demonstrating that heart-looping, development of posterior chambers, and differentiation of ventricular cardiomyocytes are all severely impaired in mice lacking RA [106] as well as by experiments showing that RA limits the cardiac progenitor pool [69].

RA and miRNAs display multiple interactions during cardiogenesis [162].

For example, disruption of miR-138 function leads to expansion of gene expression in the ventricular region that is normally restricted to the atrio-ventricular valve region. This aberrant expression of genes ultimately disrupts ventricular cardiomyocyte morphology and cardiac function [105]. In this study, miR-138 was found in specific domains of the zebrafish heart, where it was observed to repress the expression of the RA synthesis enzyme RALDH2 in the ventricle. This activity was complemented by miR-138-mediated ventricular repression of the gene encoding versican (cspg2), the core protein of the chondroitin sulfate

proteoglycan, which is positively regulated by RA overall suggesting an antagonism between miR-138 and RA signalling pathway [105].

During the development of zebrafish ventricles, miR-143 expression is dependent on heartbeat. Like miR-138, miR-143 negatively controls mRNA expression of RALDH2 and retinoid x receptor alpha b (rxrab), which affect the correct development of the heart tubes. Hence, miR-143 and RA signalling pathways are targets of heartbeat-dependent physical control, highlighting heartbeat as an essential epigenetic factor during cardiogenesis [104]. Interestingly, transcriptional networks that establish heart chamber-specific gene expression are highly conserved across species, from zebrafish to humans, implying that this mechanism may be active in mammals [135].

Analysis of miRNA profiling showed that miR-10a and miR-1 expression is gradually increased during in vitro RA-induced differentiation of ESC into smooth muscle cells. Functional studies showed that, subsequently, miR-10a and miR-1 repress histone deacetylase 4 (HDAC4) and Klf4, respectively, and play critical roles in the determination of smooth muscle cell fate [59, 163].

Polycomb Group Proteins

miR-214 expression impacts transcription controlled by polycomb group proteins (PcGs) [66]. PcGs are a group of proteins that contribute to cell commitment and differentiation by repressing the transcription of genes that regulate development [82]. In undifferentiated skeletal muscle cells, PcG proteins such as Suz12-polycomb repressive complex 2 subunit, embryonic ectoderm development (Eed), and Bmi1-polycomb ring finger oncogene, occupy and repress transcription of miR-214 which is a direct target of MyoD and myogenin. PcGs are released in differentiating myoblasts and during RA-induced ESC differentiation leading to the recruitment of MyoD and myogenin to the miR-214 genomic region and subsequent transcription of miR-214. Transcribed miR-214, in turn, negatively feeds back on the enhancer of zeste homolog 2 (Ezh2) by directly inhibiting translation of its mRNA. Ezh2 is the catalytic subunit of the PcG complex that mediates histone 3 lysine 27 trimethylation (H3K27me3). Reduced levels of Ezh2 cause a derepression of developmental regulators that are PcG targets, which then leads to an accelerated differentiation of skeletal muscle cells [66]. This network may exist to increase the effectiveness of the system to rapidly reduce Ezh2 availability at critical stages, such as those regulating muscle cell differentiation.

Nervous System Development

The RA signaling pathway and miRNAs are implicated in many aspects of central nervous system development and function. Numerous miRNAs were revealed in a comprehensive analysis of RA-induced differentiation of human NT2 cells, an in

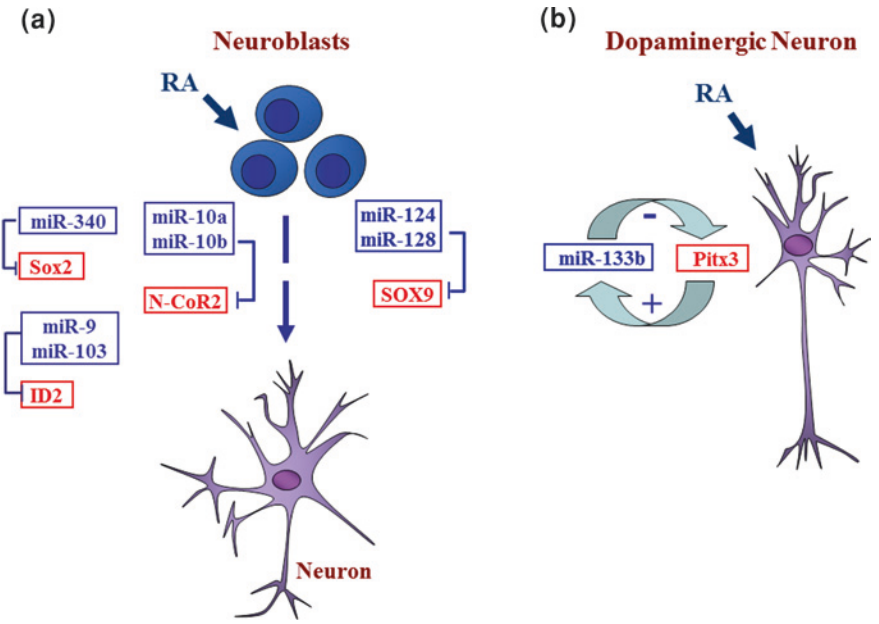

Fig. 8.2 miRNAs and neurogenesis. **a** Examples of RA-regulated miRNAs during neural differentiation (*blue*) and their targets (*red*). On the *right* miR-124 and miR-128, preferentially expressed in neurons, have been indicated as regulators of neural development and neuronal cell specification. miR-124 represses the expression of the transcription factor SOX9 that is linked to the stem cell phenotype. On the *left* miR-340, 10a, 10b, 9 and 103 act on genes involved in the maintenance of the undifferentiated state, thus favouring neural differentiation. **b** A miRNA based feed-back circuit regulates dopaminergic neurogenesis. miR-133b inhibits the expression of the transcription factor Pitx3, which, in turn, is a transcriptional inducer of miR-133b expression

vitro model of neurogenesis [131]. For example, miR-124 regulates adult mouse neurogenesis via suppression of Sox9 in the subventricular zone stem cell niche [21], miR-430 is associated with brain morphogenesis in zebrafish [47], and miR-124 and miR-128 play roles in neuronal cell specification [24, 130] (Fig. 8.2a).

miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing [96]. *Splicing regulator polypyrimidine tract binding Protein 1* (PTB1) blocks the expression of *neural-specific splicing regulator* (PTB2) by introducing an alternative exon carrying a stop codon into the PTB2 transcript. miR-124 suppresses PTB1 which indirectly activates PTB2. In the presence of RA, miR-124 levels are increased, resulting in widespread splicing regulation of essential neural mRNAs that trigger neural differentiation (Fig. 8.3).

miR-133b is expressed in adult mammalian midbrain dopaminergic neurons where it regulates their maturation and function [71]. miR-133b takes part in a negative feedback circuit that includes the paired-like homeodomain transcription factor 3 (Pitx3), a known regulator of neuronal gene expression [71]. Moreover,

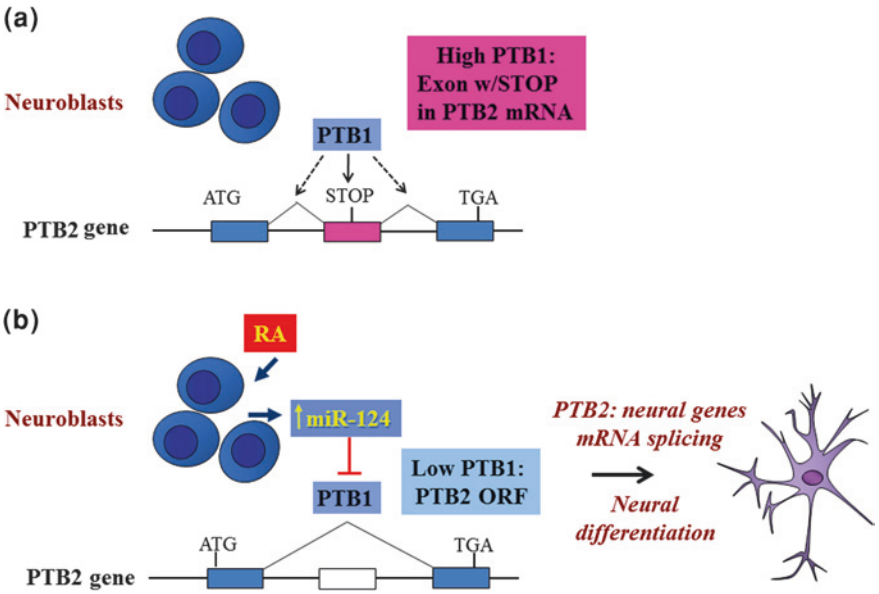

Fig. 8.3 miRNAs and neuroblasts. **a** In neuroblasts, the splicing regulator PTB1 introduces an alternative exon carrying a stop codon in the mRNA of the neural-specific splicing regulator PTB2, resulting in a block of PTB2 protein expression. **b** RA increases miR-124 levels in neuroblasts. This miRNA represses PTB1 protein expression, resulting in a change in mRNA splicing of the PTB2 gene, generating an open reading frame (*ORF*). PTB2, induces neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing

miR-133b is a direct transcriptional target of Pitx3, and its induction post-transcriptionally suppresses Pitx3, resulting in a functional fine-tuning circuit for dopaminergic behaviours such as locomotion (Fig. 8.2b).

Relevance

MicroRNA and Retinoic Acid Regulatory Networks in Adult Life and Disease

Many of the differentiation promoting and growth suppressing activities of RA appear to utilize miRNAs as fundamental components of their molecular mechanisms. Most of the data on RA-regulated miRNAs during adulthood derive from two biological processes regulated by RA: hematopoiesis and neuronal differentiation. Research in these two fields is relevant to two neoplastic diseases, acute promyelocytic leukemia (APL) and neuroblastoma, where patients

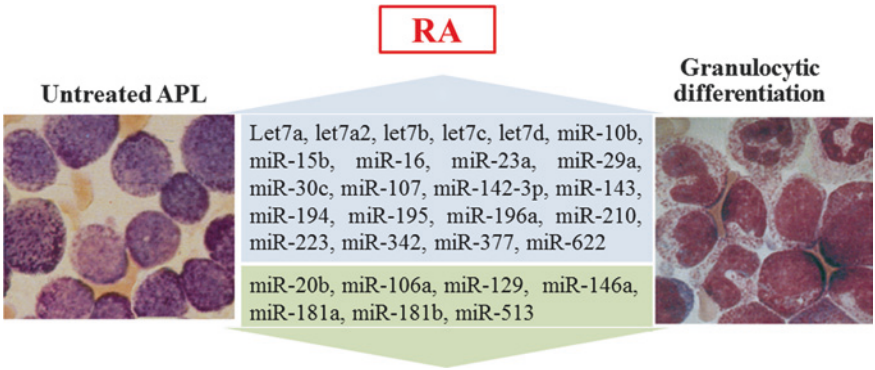

Fig. 8.4 miRNAs that are either up-regulated (*azure box*) or down-regulated (*green box*) in APL blasts undergoing granulocytic differentiation by RA treatment

being treated with RA have shown a notable clinical response. The introduction of all-*trans*-RA treatment has radically improved the prognosis of APL, whereas *13-cis*-RA significantly improves survival of neuroblastoma patients [98, 126]. The existence of two relevant disease model systems has encouraged research on effects of RA on target genes, including miRNA genes. The mechanism of miRNA regulation, be it direct or indirect, could have great functional relevance to the pathogenesis and, possibly, cure, of APL and other neoplastic diseases at the level of transcriptional repression of miRNA expression by PML/RARα or transcriptional activation by RA (Fig. 8.4). Moreover, the therapeutic effects of RA on neuroblastoma could be mediated in part by miRNA regulation.

Direct or Indirect Mechanisms: Insights from the RA-Responsive Leukemia Models

Many studies of RA signaling pathways regulated by miRNAs were performed using NB4 cells, an APL-derived cell line [78]. The results obtained in this cell line have been replicated in APL clinical samples and appear to be highly reliable [6, 16, 41, 45, 127]. For example, the repression by PML/RARα and up-regulation by RA of let-7c, miR-23a, miR-107, miR-210 and miR-342 have been confirmed in fresh APL blasts in two separate studies [16, 127], further suggesting that these miRNA activities are relevant in the biology of APL.

A number of miRNAs change their expression levels when hematopoietic cells are exposed to RA. The mechanism underlying the regulation of these miRNAs can be both direct and indirect, since RA-induced differentiation causes dramatic phenotypic changes in the cells.

Relatively few miRNAs have been shown to be directly regulated by RA through the activity of RARs or the APL-associated PML/RARα fusion oncoprotein on their target gene promoters [16, 127]. The let-7 family of miRNAs, commonly reported as RA-induced, does appear to be directly regulated by PML/RARα in APL cells [16]. Similarly, binding of the fusion protein, PML/RARα, to the miR-342 promoter in APL cells is induced by RA [16]. Thus, these miRNAs are examples of direct transcriptional regulation by RARs.

The regulation of miRNAs is also dependent upon indirect mechanisms, which are nonetheless affected by RA. The activity of the transcription factor *nuclear factor kappa B* (NFkB) is increased by RA treatment of APL cells [99]. One NFkB proximal binding site appears essential for the transactivation of the let-7a-3/let-7b cluster gene [45]. miR-342 is also under the control of the transcription factor PU.1, which is induced by RA treatment and binds to its responsive element on miR-345 gene promoter [27]. Indirect mechanisms are also involved in miRNA repression. For example, miR-145 is repressed during RA induced granulocytic differentiation. This miRNA gene is a transcriptional target of p73, which in turn is activated by RA treatment [6].

Other PML/RARα-regulated miRNAs were discovered through an *in-silico* search for potential PML/RARα binding sites in their gene promoters [127]. Sixty five intergenic miRNA genes were found to carry a predicted PML/RARα binding site in their promoter. However, experimental proof of regulation was obtained for relatively few of these miRNAs (miR-10b, miR-23a, miR-194, miR-195, miR-196a, miR-210, miR-377, and miR-622). The presence of PML/RARα on their promoter, indicating a direct regulation by the oncoprotein, was only shown for miR-23a and miR-210 [127].

The myeloblastic leukemia cell line HL60 is a classic model for RA-induced granulocytic differentiation [14]. miRNAs regulated by RA treatment in HL60 cells are largely the same as those regulated in APL blasts, although some miRNAs that are up-regulated (miR-22, miR-29a, miR-142-3p, miR-363, miR-494, miR-663) or down-regulated (miR-10a, miR-125b, miR-612) by RA in HL60 cells are not reported as modified by RA in the APL system. This possibly indicates the specificity of PML/RARα-mediated regulation in the APL context [91, 64].

Normal Hematopoiesis

Leukemia models of RA-controlled miRNAs have provided insight into their relevance in normal hematopoiesis. Several miRNAs that are induced by RA in leukemia cell lines increase their expression in differentiating normal $CD34^+$ hematopoietic progenitor cells (HPCs). Up-regulation of miR-29a and miR-142-3p was reported as a consequence of RA-induced differentiation of the myeloid cell lines, HL60, THP1 and NB4. The same miRNAs are also up-regulated during growth factor-induced myeloid differentiation of $CD34^+$ HPCs, suggesting that their regulation is differentiation-dependent rather than RA-specific [156]. Along the same lines, miR-15/16, which are up-regulated by RA in leukemia cell

lines, display a higher expression level in mature peripheral blood leukocytes isolated from APL and Acute Myeloid Leukemia (AML) patients who are in clinical disease remission [44].

One significant example of an RA-induced miRNA is miR-223. This miRNA has been widely studied in normal hematopoiesis and leukemia models. miR-223 was originally identified as up-regulated in mouse mature bone marrow hematopoietic cells [19]. It is also induced by RA during granulocytic differentiation of human APL cells [41]. Moreover, miR-223 null mice develop a myeloproliferative hematopoietic disorder [65].

miR-223 up-regulation in myeloid precursors is indirectly caused by the RA-induced activation of RARs. In fact, RA treatment triggers a regulatory circuit involving miR-223 and two transcription factors, the CCAAT/enhancer-binding protein-alpha (C/EBPα) and the Nuclear Factor I-A (NFI-A). These transcription factors compete for binding to the miR-223 promoter. NFI-A maintains miR-223 at low levels, whereas its replacement by RA-induced C/EBPα up-regulates miR-223 expression [41]. NFI-A is also a post-transcriptional miR-223 target. Its RA-induced down-regulation is required to fulfill miR-223 key role in granulopoieisis [40, 41]. In fact, miR-223 is sufficient to reprogram granulocytic differentiation in distinct myeloid leukemia subtypes, independently from the presence of a specific genetic lesion [40, 41], whereas NFI-A acts as a novel developmental gene directing HSC/HPC lineage choice. NFI-A, per se, is indeed able to induce an erythroid transcriptional program in both primary HSC/HPCs and human myeloid cell lines where it acts directly at the proximal promoter regions of fundamental myeloid genes [138, 139]. NFI-A is also a predicted target of miR-107 whose expression is induced by RA in APL cells, perhaps contributing to transcription factor down-regulation during myeloid differentiation [45].

The fine tuning of miR-223 expression levels can dictate lineage fate decision and differentiation/maturation of CD34 + HPCs into erythroid, granulocytic and monocytic/macrophagic lineages. A high expression of miR-223 increases granulopoiesis and impairs erythroid—and monocytic/macrophagic—differentiation. Monocytic/macrophagic differentiation occurs when miR-223 levels are moderately increased, whereas erythroid commitment and differentiation require stably low levels of miR-223 [155].

miRNA-Mediated Tumor Suppressive Activities of RA in the Hematopoietic System

RA may act through miRNAs to control the proliferation and self-renewal of hematopoietic cells. The failure of this regulation may lead to leukemogenesis or an increase in leukemia malignant behaviour. The expression level of the urokinase-type plasminogen activator receptor (uPAR) protein, which is involved in the regulation of chemotaxis, adhesion and proteolysis, correlates with a significant lower remission rate after chemotherapy and a higher risk for relapse in

AML patients [48]. uPAR was experimentally shown to be a target of miR-195, miR-377 and miR-622, which are among the miRNAs up-regulated by RA in APL [127] (Fig. 8.4). Thus, by regulating these miRNAs, RA activity could contribute to the blockage of factors that are implicated in crucial pathways linked to leukemogenesis.

RA-induced miRNAs also suppress other pathways involved in neoplastic transformation or progression. The let-7c miRNA targets the pre-B-cell leukemia homeobox 2 (PBX2) mRNA, which encodes a homeodomain transcription factor, forming complexes with Hox and Meis. This complex contributes to leukemia cell proliferation and stemness [119], but is suppressed by the RA-induced let-7c. Moreover, the let-7 family members target Ras and Bcl-2 mRNAs, thus potentially decreasing proliferation and increasing apoptosis of hematopoietic cells [45]. Several other RA-regulated miRNAs (miR-27a, miR-196a, miR-377, miR-520d, miR-524) are predicted to target Hoxb8, impairing the stem properties of hematopoietic cells [127]. Conversely, RA down-regulates miR-146a, which targets Smad4 [171]. Thus RA could increase Smad4 expression, a target gene of miR-146a, increasing the growth suppressive effect of TGFβ signaling and APL cells proliferation [171].

Normal and Neoplastic Neural Differentiation

Similar to the hematopoietic system, RA activity in neural tissue has been studied in depth by taking advantage of neoplastic model systems. RA is able to induce in vitro differentiation of neuroblastoma cells. This finding has a clinical counterpart in the response of patients with neuroblastoma to *13-cis*-RA. Retinoid treatment produces a significant improvement in patient survival and is now a clinical standard. Thus, it is not surprising that an abundant body of literature has developed on the effects of retinoids on miRNAs in neural tissue differentiation. As recently summarized in an excellent review [136], these studies employed a fairly heterogeneous array of methodologies that yielded different results. Despite the discrepancies, the overall data show that a large amount of miRNA is regulated during RA-induced differentiation of neuroblastoma cells. Interestingly, many RA-regulated miRNAs are the same as those modified in the hematopoietic system.

Several RA-regulated miRNAs in the neural differentiation system deserve further discussion since their targets are well defined and relevant (Fig. 8.2a). miR-10a and miR-10b are powerfully induced by RA, although a specific RARE in their promoters has not been identified. miR-10a targets include the nuclear receptor co-repressor 2 (NCoR2), one of the co-repressors bound by RARα on DNA [42, 142]. In the absence of RA, this co-repressor drives HDACs on the promoter of RARα target genes, maintaining an inactive chromatin structure. In addition, in smooth muscle cells [59], it has been shown that miR-10a targets HDAC4, and in T-cells, the repressor Bcl-6 [142]. Thus, the induction of miR10a and miR10b

by RA may trigger an activation loop by suppressing a co-repressor (NCoR2) and an effector (HDAC), favouring the recruitment and activity of co-activator complexes upon RA binding to RARα. In pancreatic cancer cells, miR-10a also targets Hoxb1 and Hoxb3 [160], possibly contributing to blockade of their anti-differentiation activity.

During neuroblastoma cell differentiation induced by RA, miR-340 is upregulated by demethylation of an upstream genomic region and directly represses the SOX2, a transcription factor linked to the stem cell phenotype [25]. The induction by RA of miR-9 and miR-103, both targeting the mRNA encoding the Helix-Loop-Helix transcription factor ID2, a differentiation inhibitor, contributes to the maintenance of the stem cell pool in the nervous system [3].

The miR 17-5p-92 cluster is suppressed by RA in Glioma and Neuroblastoma cell differentiation [10, 34]. This miRNA cluster is well known for its proliferative, anti-apoptotic and differentiation suppressive activity, an effect obtained by the coordinated suppression of a complex target network [112]. This underlines the potential role of miR 17-5p-92 in tumour development and maintenance and the effect of RA treatment in the regulation of this circuitry.

An interesting network elicited by RA in neuroblastoma cell differentiation involves the induction of miR-152. Overexpression of this miRNA decreases neuroblastoma cell invasiveness and growth. Interestingly, miR-152 targets DNA methyltransferase 1 (DNMT1). This may help to explain the changes in DNA methylation that occur during RA treatment of neuroblastoma cells [26]. Overall the activity of RA-induced miRNAs in neural cells seems to play a role in the suppression of the stem state and proliferation, while supporting differentiation. These biological functions are remarkably similar to those exerted by RA-regulated miRNAs in hematopoiesis.

Other Biological Model Systems

RA affects miRNA expression levels and functions in many other biological model systems. Retinoids have been used for their differentiation and anti-proliferative activities in several tumours, although their clinical activity is relatively limited outside APL and neuroblastoma. The activity of retinoids on miRNAs in other model systems is complex and sometimes difficult to correlate to molecular and biological networks. Sometimes the results appear contradictory.

In estrogen receptor positive breast cancer cells, RA induces the expression of miR-21, which is believed to increase cell proliferation [148]. However, the cells display a reduced motility, and proliferation possibly due to the suppression of various miR-21 targets, including tissue plasminogen activator, (t-PA), interleukin 1β (IL1B), intracellular adhesion molecule 1 (ICAM1) and maspin. On the other hand, the expression levels of Maspin, a "*non-inhibitory*" member of serin protease inhibitors (SERPIN), may serve as a prognostic marker for clinical outcomes in some tumours such as breast cancer [11].

In pancreatic cancer cells, RA increases the expression of miR-10a, which targets Hoxb1 and Hoxb3. The expression levels of these two genes is increased in cells derived from pancreatic cancer metastasis. However, RA-antagonists also suppress their expression levels, implying that inhibition of RA activity could be desirable in pancreatic cancer [160].

Recently, the activity of RA in inducing miR-10a has been studied in T(reg) lymphocytic cells. The repression of the miR-10a targets, Bcl-6 and NCoR2, decreased conversion of inducible T(reg) cells into the follicular T(H)17 subset of helper T cells [142]. This effect limits the plasticity of helper T-cells, indicating that RA acts through miRNAs in the determination of cell fate in the immune system.

miRNAs could play an important role in the differentiation activity of RA on spermatogonial stem cells. The induction of let-7 miRNAs by RA [149] may decrease the expression of the proliferative miRNA downstream targets Mycn, Ccnd1, and Col1α2, while the repression by RA of the miRNA clusters 17-5p-92 and 106b-25 may contribute to increasing their protein expression levels [150]. The sum of these effects is possibly, the induction of spermatogonial differentiation.

Finally, modulation of miRNAs by RA has unexpected effects on viral infection. RA-induced miR-23b targets the very low density lipoprotein (VLDL) receptor, which is a receptor for a minor group of rhinoviruses, resulting in decreased sensitivity to the infection by these viruses [113].

Retinoic Acid-Induced Nuclear Roles of miRNAs

Hundreds of miRNAs are distributed both in the nucleus and in the cytoplasm of normal and cancer cells [88, 116, 121]. Some miRNAs can even display a preferential nuclear localization [88]. In fact, the miRNA-associated RISC proteins Ago and Dicer have been found in the nucleus [17, 92, 153, 157], and contribute to control of transcription through the epigenetic regulation of chromatin structure [8, 168, 169]. Interestingly, importin 8, a specificity factor in the miRNA pathway, required for binding of Ago proteins to a variety of mRNA targets, also affects the nuclear localization of Ago proteins [159].

The nuclear activity of miRNAs can result in both gene transcriptional activation or silencing [60, 70, 114, 120, 144, 167, 168, 169, 170]. A series of interesting observations indicates that the transcriptional functions of miRNA depend on miRNA-complementary sequences present in the DNA of target gene promoters [8, 114, 168, 169]. It remains to be determined whether miRNAs directly interact with DNA or, alternatively, with nascent RNA sequences originating from the promoter of transcribed genes [141].

RA can be the trigger for this novel mechanism of transcriptional regulation by miRNAs. We have shown [168, 169] that during RA-induced granulocytic differentiation of immature myeloid precursor cells, miR-223 translocates to the nucleus,

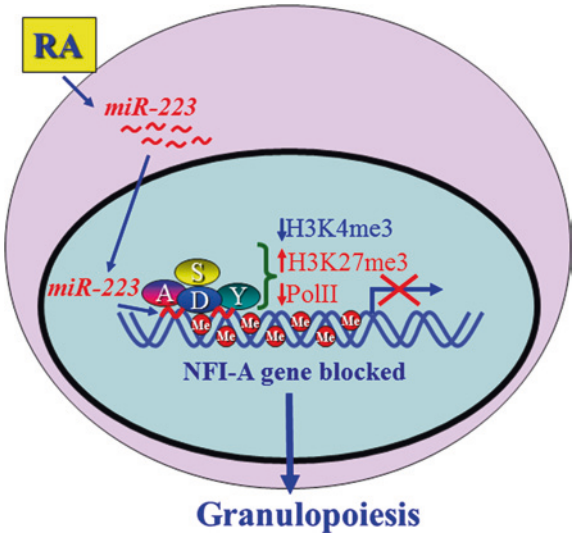

Fig. 8.5 Transcriptional activity of miR-223. Upon RA treatment of myeloid precursors, the expression level of miR-223 increases. A fraction of this miRNA translocates into the nucleus where it binds, either directly or indirectly, to the promoter region of the NFI-A gene. miR-223 aggregates with a transcriptional complex including Ago1 (*A*), Dicer1 (*D*) and the Polycomb Group proteins, Suz12 (*S*) and YY1 (*Y*). This increases H3K27 methylation, decreases H3K4 methylation, increases DNA methylation (*Me*) and blocks NFI-A PolII-dependent transcription, allowing granulocytic maturation of the cells

where it binds chromosomal chromatin. We have studied the transcriptional effect of miR-223 on the gene encoding NFI-A, a post-transcriptional target of its activity [168, 169]. miR-223 causes transcriptional repression of this gene. This repressor activity occurs through miR-223 binding to DNA sequences on the NFI-A promoter that are complementary to those of the miR-223 "*seed*" region. At these sites, miR-223 recruits a protein complex involving Ago1, Dicer1 and the PcG proteins YY1 and Suz12. The final effect is DNA hypermethylation and a change in chromatin epigenetic status, causing an increase in lysine 27 of histone 3 (H3K27) methylation and a decrease in H3K4 methylation (Fig. 8.5). "*Bivalent*" chromatin marks, depicted by the simultaneous presence of activator H3K4 trimethylation (H3K4me3) and repressive H3K27me3 histone modifications characterize developmentally regulated gene promoters and are also present in the NFI-A promoter. In the early phases of myeloid differentiation, they are repressive due to the RA-induced activity of miR-223, and the NFI-A gene transcription is inhibited. The biological consequence of these molecular events is progression towards myeloid differentiation and blockade of entry into the erythroid lineage [168, 169].

Among the other RA-regulated miRNAs, the let-7 family has shown the ability to mediate transcriptional gene silencing. In senescent human fibroblasts, endogenous let-7f contributes to transcriptional gene silencing of E2F target genes acting on those promoters where let-7f complementary sequences were found. This

miRNA contributes to aggregate a repressor complex including the RISC protein Ago2 and pRb1 [8]. This complex increases H3K27me3 and DNA methylation, resulting in transcriptional repression of target genes. Notably, diverse let-7 family members are induced by RA in myeloid hematopoietic cells [16, 45, 119] (Fig. 8.4) and some of them are targets of the APL-associated PML/RARα fusion protein [16]. In principle, therefore, a transcriptional effect of the let-7 may mediate part of the physiological and pathologic biological effects produced respectively by RARα or its leukemic mutant.

Future Directions

Retinoids and miRNAs are morphogens and essential regulators of embryogenesis, normal cell differentiation, and proliferation. During adult life, both are required for proper organ functioning. Considering the importance of miRNA—and RA-induced regulatory networks, the deregulation of any factor or pathway is highly likely to contribute to disease development. Currently, many aspects of their interactions remain unexplored and a number of questions are still unanswered. For instance, the complex sequence of miRNA biogenesis can be affected by retinoids at multiple steps, and any component of the retinoid pathway could be a target of the activity of miRNAs, as suggested by the initial findings reported in this chapter and further addressed, below.

MicroRNA Targeting of Retinoid Receptors

miRNA targeting and regulation of RARs and RXRs is almost completely unexplored. This is therefore a major area of future research. Various transcription factors, including nuclear receptors, are involved in complex feedback circuits with miRNAs, and it is likely that retinoid receptors are no exception [23, 29, 115]. miRNAs regulating the expression of RARs and/or RXRs may affect any biological area of retinoid activities. Thus, this research theme deserves further attention and will probably provide new, interesting information that will help to complete the picture of retinoid functions.

Most certainly, miRNAs make fundamental contributions to the fine tuning of developmental and differentiation pathways that are regulated by retinoids. However, very few miRNAs have been shown to act directly on the major RARs and proteins that contribute to RA signaling. Table 8.1 reports the results of a bioinformatic survey showing miRNA targeting of the major RARs and RXRs that is limited to conserved sites in miRNA families of mammals and vertebrates. Table 8.1 is based on two algorithms (PicTar and TargetScan Human 6.2). Shown in bold are the few experimentally verified miRNAs with activity on RARs and RXRs as indicated by the Tarbase 6.0 database [154]. Accepted verification

Table 8.1 Predicted and validated miRNAs targeting major RA receptor mRNAs

Gene name	Refseq ID	3′UTR length (nt)	miRNA families broadly conserved among vertebrates (conserved sites)	miRNA families conserved among mammals (conserved sites)
RARA	NM_000964	1,417	miR-27abc/27a-3p, miR-128/128ab, miR-135ab/135a-5p, miR-138/138ab, miR-194, miR-205/205ab, **miR-218/218-5p** [53], *miR-337, miR-199a, mir-199b, miR-220*	
RARB	NM_000965	1,315	miR-1ab/206/613, miR-15abc, miR-16-2-3p [58], miR-16abc/195/322/424/497/1907, miR-29abcd, miR-30abcdef/30abe-5p/384-5p, miR-34ac/34bc-5p/449abc/449c-5p, miR-133abc, miR-141/200a, miR-216a, miR-135ab/135a-5p, miR-144, miR-17/17-5p/20ab/20b-5p/93/106ab/427/518a-3p/519d, *miR-16, miR-101, miR-103, miR-107, miR-133b, miR-136, miR-146, miR-193, miR-195, miR-199a, miR-200a, miR-368*	miR-376c/741-5p
RARG	NM_000966	1,107	miR-24/24ab/24-3p, miR-30abcdef/30abe-5p/384-5p, miR-96/507/1271, **miR-182** [87], miR-142-3p, **miR-124/124ab/506** [90], miR-143/1721/4770, **miR-335-5p** [145], *miR-22, miR-34abc,* miR320, miR-331, miR-9*	miR-285p/708/1407/1653/3139, miR-326/330/330-5p
RXRA	NM_002957	3,971	miR-9/9ab, miR-27abc/27a-3p miR-128/128ab, miR-124/124ab/506, miR-216b/216b-5p, miR-219-5p/508/508-3p/4782-3p	miR-320abcd/4429, miR-197, miR-876-5p/3167
RXRB	NM_021976	1,094	miR-101/101ab, miR-144, miR-490-3p, *miR-17-3p, miR-199a**, **miR-26b-5p** [46], **miR-197-3p** [158], **miR-346** [158]	miR-488*
RXRG	NM_006917	341	miR-23abc/23b-3p	

The indicated genes (Refseq ID sequences) were analysed with TargetScan human 6.2 (http://www.targetscan.org/) and PicTar (http://pictar.mdc-berlin.de/). miRNAs identified by both algorithms are underlined. miRNAs identified only by PicTar are in italics. miRNAs, derived from the Tarbase 6.0 database [154] are in boldface; studies reporting some of these miRNAs are indicated in parenthesis

methods include microarray screenings of mRNA expression upon overexpression of miRNA or knockdown of their function [46, 56, 87, 90, 145, 158] and high throughput screenings of mRNA co-immunoprecipitated with miRNAs by several RNA-binding proteins (RBPs) including Ago protein [53]. Thus, a direct confirmation of miRNA interactions with the 3′UTR of these receptor mRNAs is not required by this database. As previously described, miR-196 does indeed target the 3′UTR of rarab in zebrafish [56].

In summary, the evidence supporting a functional interplay between miRNAs and RA signaling appears to be robust. However, our understanding of the molecular basis for and physiological significance of such interactions is far from complete. Numerous conceptual and experimental questions remain. Future research should focus on understanding whether and how retinoid and miRNA actions can trigger a cascade of dynamic events that result in fine-tuned, specific, control of gene expression. We will also be on the watch for important new developments that involve the recently discovered RA-induced nuclear functions of miRNAs.

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