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Mechanisms of control of microRNA biogenesis

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MicroRNAs (miRNAs) are a class of ~22 nt non-coding RNAs that control diverse biological functions in animals, plants and unicellular eukaryotes by promoting degradation or inhibition of translation of target mRNAs. miRNA expression is often tissue specific and developmentally regulated. Aberrant expression of miRNAs has been linked to developmental abnormalities and human diseases, including cancer and cardiovascular disorders. The recent identification of mechanisms of miRNA biogenesis regulation uncovers that various factors or growth factor signalling pathways control every step of the miRNA biogenesis pathway. Here, we review the mechanisms that control the regulation of miRNA biogenesis discovered in human cells. Further understanding of the mechanisms that control of miRNA biogenesis may allow the development of tools to modulate the expression of specific miRNAs, which is crucial for the development of novel therapies for human disorders derived from aberrant expression of miRNAs.

Keywords: biogenesis/Dicer/Drosha/processing/miRNA/microRNA.

Abbreviations: ds, double strand; miR, microRNA; nt, nucleotide; Pre-miRNA, precursor miRNA; pri-miRNA, primary microRNA; ss, single strand.

Introduction to the Basics of MicroRNA Biogenesis

The mechanism of microRNA (miRNA) biosynthesis is evolutionarily conserved and involves sequential endonucleolytic cleavages mediated by two RNase III enzymes, Drosha and Dicer (Fig. 1) (*1*). Following transcription by RNA polymerase II (RNA Pol II), Drosha processes the primary miRNA transcript (pri-miRNA) into a ~60–100 nt hairpin structure termed the precursor-miRNA (pre-miRNA) in the nucleus (Fig. 1) (*2–5*). Following cleavage by Drosha, the pre-miRNA is transported out of the nucleus through the interaction with Exportin-5 and Ran-GTP. The pre-miRNA then undergoes further processing catalysed by Dicer (Fig. 1) (*6*, *7*). This cleavage event gives rise to a ~22 nt double-stranded (ds) RNA product containing the mature miRNA guide strand and the passenger (miRNA*) strand (Fig. 1). The mature miRNA then promotes the association of a large protein complex, termed the RNA-induced silencing complex (RISC), with specific regions in the 3′-untranslated region (3′-UTR) of target genes (Fig. 1) (*8*).

Selection of miRNA targets is mediated by imperfect base pairing between the miRNA and miRNA-recognition site present in the 3′-UTR of the target mRNA. The imperfect nature of the miRNA:mRNA interaction means that a single miRNA can potentially target tens to hundreds of mRNAs (*9–11*). Association of the miRNA–RISC results in the repression of the target gene by promoting mRNA degradation and/or translational inhibition (*12–16*). Through the repression of targets, miRNAs elicit critical changes in gene expression programmes which have been reported to underlie diverse aspects of biology, including developmental timing, differentiation, proliferation, cell death, and metabolism (*17–19*).

Regulation of miRNA Genes

Transcriptional control of miRNA genes

Transcription is one of the major regulatory steps in the biosynthesis of miRNAs. The majority of miRNAs are transcribed by RNA Pol II and bear the 7-methylguanylate cap at the 5′-end and poly (A) tail at the 3′-end characteristic of mRNAs (*2*, *20*). Large-scale mapping of the promoters of 175 human miRNA genes through nucleosome positioning and chromatin immunoprecipitation-on-genomic DNA microarray chip (or ChIP-on-chip) analysis suggests that many characteristics of miRNA gene promoters, such as the relative frequencies of CpG islands, TATA box, TFIIB recognition, initiator elements and histone modifications, are similar to the promoters of protein coding genes (*21*, *22*). The DNA-binding factors that regulate miRNA transcription largely overlap with those that control protein-coding genes, including c-myc and p53, as well as cell-type specific transcription factors such as MEF2, PU.1 and REST (*23–26*). Furthermore, transcription of primary miRNA transcripts can be dynamically regulated in response to growth factor stimulation, including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and bone-derived neurotrophic factor (*26–29*).

The proto-oncogene *c-myc* encodes a transcription factor which regulates ~10–15% of human genes and plays a central role in control of cell growth and apoptosis (*30*). It is clear that c-Myc binds to E-boxes and activates transcription of the miR-17-92 cluster (*31*). Consistent with the prominence of *c-myc* activation

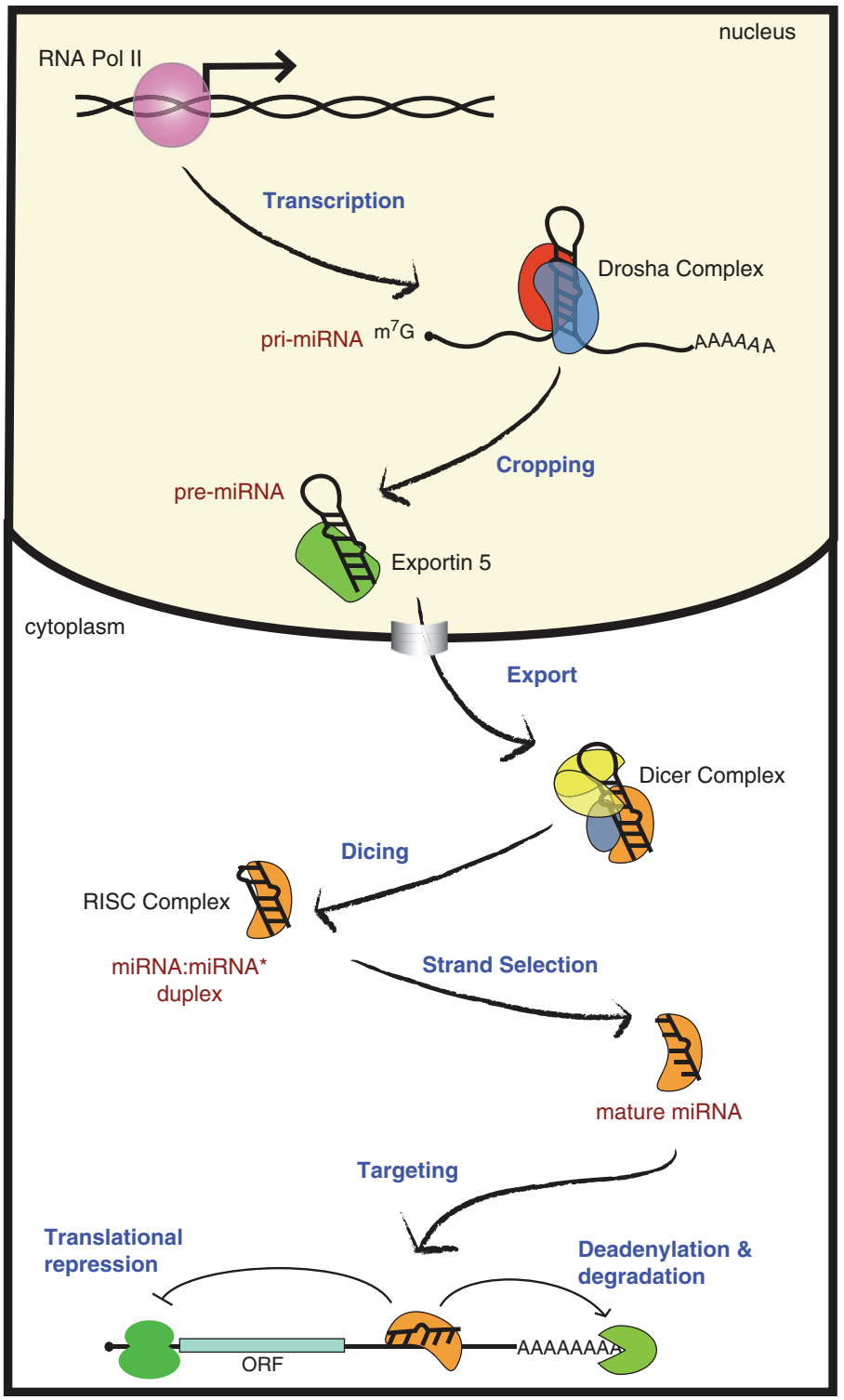

Fig. 1 miRNA biogenesis pathway. miRNAs are initially transcribed as a long, capped and polyadenylated pri-miRNA. The Drosha complex crops the pri-miRNA into a hairpin-shaped pre-miRNA. Next, Exportin-5 promotes the nuclear translocation of the pre-miRNA which is further processed by the Dicer complex. Following Dicing, the resulting miRNA : miRNA* is dissociated and the mature miRNA is incorporated into the RISC where it functions to mediate gene silencing either by translational inhibition or by promoting the degradation of target mRNAs.

in tumours, miR-17-92 is often highly expressed in tumours (*32*). Furthermore, amplification of the 13q31-q32 gene locus, which contains the miR-17-92 cluster, is commonly observed in *c-myc*-rearranged lymphomas, suggesting that *c-myc* and the miR-17 cluster may cooperate to promote transformation of cells (*33*). The miR-17-92 cluster contains six miRNAs, including miR-17-5p and miR-20, which are known to repress the cell cycle regulator E2F1 (*31*). Interestingly, c-Myc also promotes E2F1 transcription, suggesting that c-Myc is likely to fine tune cell cycle progression via the regulation of both

miRNA and mRNA expression (*34*). In addition, c-Myc also decreases the expression of several tumour suppressor miRNA genes, including the miR-15a, -29, -34 and let-7 families (*35*). Exogenous expression of c-Myc-repressed miRNAs in lymphoma cells reduced cell growth, indicating that down regulation of a subset of miRNAs is an important mechanism of c-Myc-mediated tumourigenesis (*35*). Finally, c-Myc is shown to increase transcription of Lin-28B which mediates the post-transcriptional repression of let-7 family members (*36*). In summary, c-Myc activity contributes to tumourigenesis by both positively and negatively regulating the expression of diverse miRNAs.

Epigenetic control of miRNA genes

Many of the mechanisms of epigenetic control known to regulate protein-coding genes, such as DNA methylation and modifications of histones, seem to be also applied to miRNA genes. For example, in bladder cancer, the expression of miR-127 is reduced due to promoter hypermethylation (*37*). Several additional miRNA loci, such as miR-9-1, -193a, -137, -342, -203 and -34b/c, are also found to be hypermethylated in multiple human cancers similar to known tumour suppressor gene loci (*38*, *39*). miRNA gene promoters are also regulated by histone modifications during development and pathogenesis. For example, low expression of miR-1 in lung cancer cells can be rescued by treating with histone deacetylase (HDAC) inhibitors, suggesting that the promoter of miR-1 may be aberrantly acetylated in tumours. Restored miR-1 expression reduced cell growth, mobility and tumour formation *in vivo*. Inhibitors of HDAC have the potential to promote downregulation of some miRNAs. For example, in the breast cancer cell line SKBr3, 32 miRNAs are significantly downregulated following treatment with HDAC inhibitors (*40*). Additionally, miR-27a was strongly repressed by HDAC inhibitors and was found to target genes previously shown to be upregulated by HDAC treatment (*40*).

Regulation of the First Processing Step from Pri- to Pre-miRNA

miRNA processing by the Drosha complex

Long-primary transcripts of miRNA genes (pri-miRNAs), which are generally several thousand nucleotides, undergo two sequential cleavages to become the mature ~22 nt miRNAs (Fig. 1). The first step of miRNA processing is catalysed in the nucleus by the RNase III enzyme, Drosha (*3*, *5*, *41*). Pri-miRNAs contain a distinctive stem-loop structure. Drosha cleaves at the base of the stem to generate a ~60–100 nt hairpin pre-miRNA with a characteristic 2 nt overhang at the 3′-end (Fig. 1) (*3*). Although Drosha contains a highly conserved RNase III domain, purified recombinant Drosha fails to efficiently generate pre-miRNAs *in vitro* (*42*). This observation suggests that other cofactors might be required to enhance the catalytic activity of Drosha. Mass-spectrometric analysis indicates that Drosha associates with at least 20 distinct polypeptides (*4*) and forms a large complex known as the 'Drosha microprocessor complex'. The Drosha microprocessor complex minimally includes Drosha and a required cofactor, DGCR8, which is able to promote the efficient cleavage of pri-miRNA *in vitro* (*4*, *42*). DGCR8 is thought to recognize the region between the single-stranded RNA (ssRNA) and the stem in order to direct Drosha cleavage one helical (~11 bp) turn away from the ssRNA–dsRNA junction (*42*). Although the cropping of many miRNAs can be mediated by a complex of purified DGCR8 and Drosha *in vitro*, the pri-miRNA to pre-miRNA processing of some miRNA is relatively inefficient (*43*). Therefore, the efficient processing of some miRNAs by the Drosha/DGCR8 complex may require the involvement of accessory factors.

Control of Drosha/DGCR8 expression

The total levels of Drosha and DGCR8 in the cell are tightly controlled and may play a role in the regulation of pri-miRNA processing. Increased Drosha expression is observed in late-stage cervical cancer samples and is associated with poor prognosis in oesophageal cancer patients (*44*, *45*). Interestingly, despite a 2- to 7-fold increase in Drosha expression in cervical cancer samples, only miR-31 is increased, while the other differentially expressed miRNAs were decreased (*45*). DGCR8 was originally identified as a gene located within a common chromosomal deletion at ch22q11, which gives rise to a syndrome characterized by learning disabilities and heart defects termed DiGeorge syndrome (*46*). Mouse models of a homologous chromosomal deletion exhibit a moderate decrease of only 59 miRNAs (*47*). Given the critical role of Drosha and DGCR8 in mediating pri-miRNA processing, it is surprising that relatively minor changes are found in miRNA levels upon dramatic alteration of DGCR8/Drosha levels (*47*). These findings may be explained, at least in part, by the observation that the total levels of Drosha and DGCR8 are coupled in an intricate feed-back circuit. In addition to cleaving pre-miRNA hairpins, the Drosha microprocessor complex can also promote cleavage of hairpin structures within annotated protein coding genes which are not further processed by Dicer (*48*, *49*). This type of cleavage allows Drosha to modulate protein-coding gene expression independent of miRNA production. Indeed, tiling microarray analysis in *Drosophila* cells following depletion of Drosha indicates that many annotated protein-coding genes, including *DGCR8*, are targeted for degradation through this mechanism (*48*). Therefore, Drosha maintains a highly regulated level of DGCR8 through the Drosha microprocessor-mediated cleavage of *DGCR8* mRNA. Furthermore, DGCR8 stabilizes Drosha protein levels and ensures the tight coupling of the core microprocessor proteins. This regulatory loop seems to be highly effective *in vivo* as in the DGCR8 heterozygous knockout mouse, DGCR8 protein level is only modestly reduced despite a 50% decrease in genomically encoded DGCR8 (*49*). Thus the relative levels of DGCR8 and Drosha may need to be closely coupled for accurate pri-miRNA processing. Interestingly, *in vitro* pri-miRNA

processing experiments using recombinant proteins indicate that as little as a 3-fold excess of DGCR8 over Drosha dramatically inhibits processing activity of Drosha (*4*).

In addition to alteration of protein levels, the overall activity of the Drosha/DGCR8 complex may be altered under some circumstances. Cells grown to high confluency exhibit increased pre- and mature-miRNA expression without alteration of Drosha or DGCR8 protein level (*50*). Furthermore, extracts from high-confluency cells promoted the more efficient conversion of pri- to pre-miRNA in *in vitro* processing assays (*50*). Although the mechanism of this increased processing is unknown, it is possible that post-transcriptional modifications or association with accessory factors could alter the activity of Drosha or DGCR8. For example, it is interesting to note that DGCR8 is a heme-binding protein and binding of heme promotes the dimerization of DGCR8 and facilitates pri-miRNA-processing activity (*51*).

Control of Drosha processing by the DEAD-box RNA helicase-interacting proteins

The DEAD-box RNA helicases p68 (DDX5) and p72 (DDX17) were initially identified as components of the Drosha microprocessor complex by immunoprecipitation-mass spectrometry analysis and subsequently shown to also associate with DGCR8 (*4*, *52*). Knockout of either p68 or p72 in mouse results in embryonic lethality (E11.5 or P2, respectively) and animals with double knockout of p68 and p72 show earlier lethality without obvious specific degeneration of organogenesis (*53*). Microarray analysis indicates that the steady state levels of ~35% (94 out of 266 surveyed) of mature miRNAs in p72 null mouse embryonic fibroblasts (MEFs) are reduced verses control, wild-type MEFs. Most of the miRNAs which are reduced in p72 knockout mice are similarly reduced in p68 knockout mice, while combined knockout of p68 and p72 does not further reduce mature miRNA levels. This observation suggests that the two proteins are largely functionally redundant or act as a heterodimer in the Drosha complex. Interestingly, while the level of pre-miRNA was also reduced in p72 or p68 knockout MEFs, the level of pri-miRNA was unchanged. Furthermore, *in vitro* pri-miRNA-processing assays indicate that the Drosha processing activity of extracts depleted of p68 and p72 is attenuated compared to control extracts. Additionally, *in vivo* RNA-immunoprecipitation studies show reduced Drosha association with pri-miR-199a in MEFs derived from p68 and p72 knockout animals. Together, these results strongly support a role of p68 and p72 in promoting the Drosha processing of a sub-set of miRNAs (*53*). The precise mechanism of p68/p72-mediated processing is unclear, but it may involve the rearrangement of the pri-miRNA hairpin structure which leads to enhanced Drosha recruitment or association with the pri-miRNA. Alternatively, as p68 andp72 are known to interact with a variety of proteins, including RNA processing enzymes and transcription factors, they may serve as a scaffold for the recruitment of other protein factors in the Drosha microprocessor complex (*54*, *55*).

A role for p68/p72 in miRNA processing is further supported by the regulation of pri-miRNA processing mediated by multiple p68-interacting proteins, including the Smads, p53 and estrogen receptor α (ER-α) (Fig. 2). The Smads are the signal transducers of the TGF-β family of signalling pathways. In the canonical pathway, ligand binding to the types I and II TGF-β receptors promotes the nuclear accumulation of receptor-specific Smads (R-Smads) in complex with the common-Smad (co-Smad), Smad4. The complex of R-Smad and co-Smad bind to specific DNA sequences within the promoter of target genes and regulates gene transcription either positively or negatively. TGF-β and its family member, bone morphogenetic protein 4 (BMP4) are particularly important for the differentiation of vascular smooth muscle cells (VSMCs). Treatment with either BMP4 or TGF-β increases expression of VSMC-specific genes; this process is due, at least in part, to the miR-21-mediated repression of programmed cell death protein-4 (PDCD4). miR-21 is rapidly induced by BMP4 or TGF-β in VSMC which results in a subsequent decrease in PDCD4 and increased VSMC gene expression (*56*). Interestingly, although knockdown of the R-Smads prevents upregulation of mature and pre-miR-21 in response to BMP4, no alteration in pri-miR-21 transcription is detected, suggesting that R-Smads post-transcriptionally affect the level of miR-21. Furthermore, BMP4 could increase the expression of pre- and mature miR-21 derived from an expression construct driven by a cytomegarovirus promoter, suggesting that miR-21 is post-transcriptionally regulated at the Drosha processing step. The identification of R-Smads as binding partners of p68 by yeast-two-hybrid system suggests that R-Smads could associate with the Drosha complex (*57*). Consistently, co-immunoprecipitation and RNA-ChIP studies indicate that R-Smad is present in a complex with Drosha and p68 on the pri-miR-21 hairpin following BMP4 or TGF-β stimulation (*56*). An increase in Drosha binding to pri-miR-21 is also observed after growth factor treatment, suggesting that R-Smads may promote the association of Drosha with miRNA hairpins (*56*). These results indicate that in addition to the transcriptional regulation mediated by the R-Smads, TGF-β can also regulate gene expression through miRNA processing. In addition to miR-21, several other miRNAs are post-transcriptionally induced by BMP and TGF-β, suggesting that rapid modulation of miRNA levels plays an important role in cellular response to cytokine signalling (Fig. 2) (*56*).

The tumour suppressor protein p53 was recently identified to modulate miRNA processing through association with p68 and Drosha, similarly to the R-Smads (*58*). Under conditions of DNA damage, several miRNAs, such as miR-143 and miR-16, are post-transcriptionally induced (Fig. 2) (*58*). p53 is essential for this process as p53-null HCT116 cells do not induce miRNAs in response

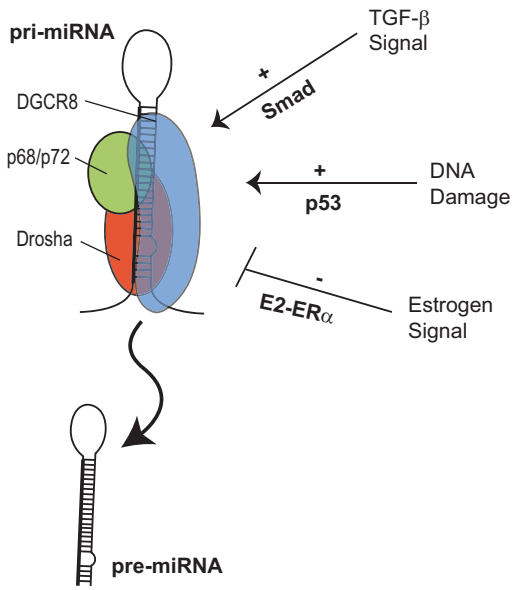

Fig. 2 DEAD box RNA helicase-dependent miRNA processing pathways. The RNA helicases p68 and p72 play a critical role in the post-transcriptional regulation of numerous miRNAs in response to cellular signals, including TGF-β stimulation, DNA damage and estrogen stimulation. The downstream mediators of TGF-β stimulation and DNA damage, the Smads and p53, act to promote miRNA processing. Conversely, when bound to E2, ER-α reduces the processing of a subset of miRNAs. For clarity, some potential members of the Drosha processing complex are not shown.

to DNA damage (*58*). Co-immunoprecipitation studies indicate that p53 is present in a complex with both Drosha and p68, and addition of p53 to *in vitro* pri-miRNA processing assays could enhance the processing reaction by Drosha (Fig. 2) (*58*). Interestingly, several mutants of p53 which are linked to oncogenesis show reduced post-transcriptional miRNA expression (*58*).

More recently, it was reported that estrogen receptor-α (ER-α) also associates with p72 and Drosha upon estradiol (E2) stimulation (*59*). Unlike the Smads or p53, however, association of E2-bound ER-α with the Drosha complex inhibits the association of Drosha with a subset of pri-miRNAs (Fig. 2) (*59*). In summary, these results indicate that the association of p68/Drosha with transcription factors, such as p53, R-Smads and ER-α, is important for the rapid regulation of miRNA expression in response to extracellular stimuli. It is intriguing to speculate that other known p68-interacting transcription factors, such as MyoD (*60*), and Runx2 (*61*), androgen receptor (*62*) and β-catenin (*63*), might also play a role in the Drosha microprocessor complex. Furthermore, although each of the described mechanisms requires the RNA helicases p68 or p72, the miRNAs that are regulated are not completely overlapping. We recently showed that R-Smads recognize and directly associate with ~20 pri-miRNAs which contain a conserved sequence (5′-CAGAC-3′) within the stem region (*64*). We found that direct association of R-Smads with pri-miRNA facilitates the recruitment of Drosha and DGCR8 and leads to efficient cropping of the pri-miRNA and elevated expression of mature miRNA (*64*). It is intriguing to speculate that p53 and/or ER α similarly select pri-miRNAs based on a specific sequence or structure of pri-miRNAs.

Control of Drosha processing by other auxiliary proteins

In addition to the mechanisms described above, several processing mechanisms independent of the DEAD-box RNA helicases have been studied. For example, hnRNP A1 is required for the processing of a member of the miR-17-92 cluster, miR-18a. Addition of hnRNP A1 to *in vitro* pri-miRNA-processing assays dramatically increases the conversion of pri-miR-18a to pre-miR-18a (Fig. 3A). Conversely, depletion of hnRNP A1 lowers pre-miR-18a levels, but does not alter the expression of the other five clustered miRNAs (*65*). Further studies indicate that direct binding of hnRNP A1 to the loop region of miRNA hairpins causes the structural rearrangement of the hairpin to generate a more favourable Drosha cleavage site (Fig. 3A) (*66*). The loop region of miR-18a is well conserved throughout evolution (*66*), emphasizing the importance of the hnRNP A1-loop interaction for the processing of pri-miRNA. Interestingly, ~14% of human miRNAs contain highly conserved loop regions, suggesting that processing regulation by hnRNPs may extend well beyond miR-18a. Consistently, the processing of two other pri-miRNAs bearing conserved loops, miR-101 and let-7a-1 is also prevented by complementary looptomiRNAs, suggesting that the loop represents a critical regulatory region for the generation of these miRNAs (*66*).

The importance of the loop region in the regulation of pri-miRNA processing is further supported by recent studies which indicate KH-type splicing regulatory protein (KSRP) directly interacts with G-rich regions present within the loop of a sub-set of pri-miRNAs to promote both Drosha and Dicer mediated miRNA processing (Fig. 3B) (*67*). Transient knockdown of KSRP in HeLa cells strongly reduced the expression of a group of mature miRNAs, including let-7a and miR-206. Furthermore, knockdown of KSRP altered cellular responses mediated by these miRNAs, including proliferation and skeletal muscle differentiation. KSRP was found to be present in both the Drosha and Dicer complexes, and knockdown of KSRP reduced the cropping activity of Drosha and Dicer (*67*). The interaction of KSRP with sequential pri-miRNA and pre-miRNA cleavage steps may serve to promote the synergistic activation of processing for a subset of miRNAs (Fig. 3B). Alternatively, KSRP association with either the Drosha or Dicer complexes may be sufficient to alter the miRNA processing in response to cellular stimuli. For example, lipopolysaccharide (LPS) treatment of macrophages dramatically increases mature miR-155 without significantly altering the expression of the pri-miR-155 (*68*). Mobility shift assays indicate KSRP interacts with pri-miR-155 and knockdown of KSRP prevents LPS-mediated elevation of miR-155 (*68*). Altogether these results suggest a role of KSRP in promoting the post-transcriptional regulation of miR-155 in

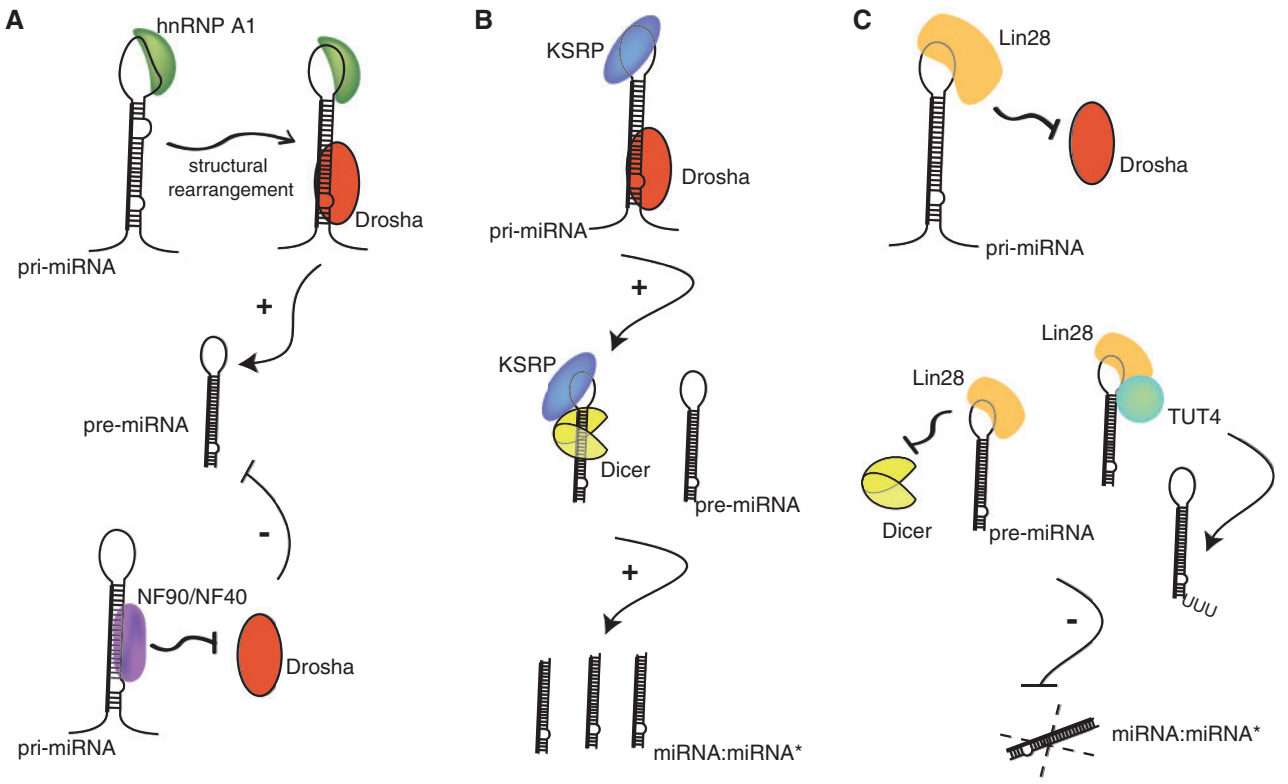

Fig. 3 RNA helicase-independent mechanisms of miRNA processing regulation. (A) hnRNP A1 recognizes the terminal loop of miRNAs and promotes the structural remodelling of the stem region. Structural rearrangement generates a favourable Drosha-binding site and enhances pri- to pre-miRNA processing. NF90 and NF40 are strongly associated with the stem region of pri-miRNAs, which precludes association with the Drosha processing complex and thus inhibits the processing of miRNAs. (B) Similarly to hnRNP A1, KSRP binds to the loop region of miRNAs and promotes both the Drosha and Dicer processing. (C) Binding of Lin28 to the terminal loop prevents the association of both Drosha and Dicer with the pri- and pre-miRNAs, respectively. Additionally, Lin28 acts as a scaffold to promote the association of TUT4 with the pre-miRNA. TUT4 promotes the 3′-uridinylation of pre-miRNA which is then rapidly degraded.

response to LPS. Interestingly, miR-155 is derived from the non-coding BIC RNA which has long been associated with induction of lymphomas (*69*). BIC (pri-miR-155) is strongly induced by B-cell receptor signalling in a variety of cell types. Interestingly, in the Burkitt-lymphoma-derived Ramos cell line, induction of BIC does not give rise to an increase in mature miR-155 (*70*). This effect seems to be unique to the Ramos cell line as expression of pri-miR-155 in HEK293 or Hodgkin's lymphoma cell lines gives rise to very high levels of miR-155 (*70*). The mechanism of this cell type-specific effect is unclear, however, in light of the results above, it may be interesting to examine if KSRP levels or activity is altered in some lymphomas.

In addition to the positive regulation of miRNA processing mediated by hnRNPs and KSRP discussed above, negative regulation of the Drosha processing of some pri-miRNAs has also been reported. The let-7 family of miRNAs is encoded as multiple copies in the genome; for example, the human genome contains nine mature let-7 sequences derived from 12 precursors (*71*). While most let-7 primary transcripts are highly expressed throughout development, mature let-7 is detectable only in highly differentiated cells, suggesting a mechanism of post-transcriptional regulation during differentiation (*71*). Analysis of the pri-miRNA sequences of the human let-7 family, as well as let-7 from different species indicates the presence of conserved nucleotide sequences in the hairpin loop. This observation suggests that this region might be critical for the post-transcriptional regulation of let-7 family of miRNAs (*72*, *73*). While pri-let-7 is processed efficiently *in vitro* using cell extracts derived from differentiated cells, processing is reduced in extracts derived from undifferentiated cells. Affinity purification followed by mass spectrometry shows that Lin-28 interacts with let-7 and inhibits processing (Fig. 3C) (*72*, *74*). The physiological relevance of let-7 regulation by Lin-28 is supported by the reciprocal expression of Lin-28 and mature let-7 during development.

The precise mechanism of Lin-28 inhibition of Drosha processing is still unclear. As Lin-28 has a strong affinity for let-7, it is plausible that Lin-28 prevents interaction with Drosha/DGCR8 by mutual exclusion (Fig. 3C). Alternatively, similarly to hnRNP A1 discussed above, the interaction of Lin-28 with the loop region could rearrange the secondary structure of the hairpin and inhibit Drosha processing. Furthermore, as discussed further below (see: Control of Dicer Activity), recent reports suggest that Lin-28 inhibits the biogenesis of let-7 through multiple mechanisms (Fig. 3C) (*75*, *76*). It is interesting to note that all three proteins (Lin-28, KSRP and hnRNPs) interact with conserved nucleotides present within the loop of pri-miRNAs (Fig. 3). The balanced

interaction of proteins with the pri-miRNA loop may allow for the fine tuning of miRNA levels during development or in response to extra-cellular cues, such as growth factors. For example, although KSRP interacts strongly with pri-let-7g in differentiated cells, high levels of Lin28 block KSRP association in embryonic carcinoma p19 cells (*67*).

Repression of Drosha processing of let-7 family members is also mediated by the nuclear factor 45 (NF45) and nuclear factor 90 (NF90) proteins (*77*). *In vitro* and *in vivo* pri-miRNA processing assays indicate that the NF90/NF45 complex reduces pre-miRNA while increasing pri-miRNA levels. The NF90/NF45 family of proteins shows strong association with small ds RNAs, including the adenovirus VA RNA as well as some pri-miRNAs (*78*). Electrophoretic mobility shift assay analysis suggests that the high-affinity association of NF90/NF45 complex with pri-miRNA precludes interaction with the Drosha Complex, thus reducing production of pre- and mature miRNA (Fig. 3A) (*77*). Unlike Lin-28, which specifically regulates let-7, the NF90/NF45 complex seems to be more promiscuous as the processing of three additional miRNAs is also modulated by the NF90/45 complex (*78*).

Potential Control of Nuclear Export of pre-miRNA

The export of pre-miRNA from the nucleus to the cytoplasm may be differentially regulated under certain physiological conditions. For example, the hairpins of pre-miR-105, -128 and -31 are detected in many cells at relatively high abundance. However, under some conditions the mature miRNA is undetectable (*79*). For example, pre-miR-31 is expressed at comparable levels in the pancreatic cancer cell line HS766T and the MCF7 breast cancer cell line. However, while HS766T cells show high levels of mature miR-31, no expression is detected in the MCF7 cells. *In situ* hybridization of pre-miR-31 shows the expected cytoplasmic localization in HS766T cells, while in MCF7 cells pre-miR-31 accumulated in the nucleolus (*79*). Together, these results indicate that the nuclear-cytoplasmic shuttling of pre-miR-31 is regulated in a cell-type dependent manner; however, the mechanism of altered pre-miRNA export is unknown.

Regulation of the Second Processing Step from Pre- to Mature miRNA

Control of Dicer expression

Following translocation into the cytoplasm, the pre-miRNA is cleaved near the terminal loop by the RNase type III protein Dicer to generate a ~22-nt ds mature miRNA (Fig. 1) (*80*, *81*). Dicer is highly conserved throughout evolution and present in nearly all eukaryotic organisms. Similarly to Drosha, several Dicer-associated proteins have been identified, including TAR RNA-binding protein (TRBP) and protein kinase R-activating protein (PACT) (*82*). Association of TRBP and PACT with Dicer enhances Dicer stability and processing activity (*82*). The total levels of Dicer may serve as an important control point in miRNA biogenesis. A careful analysis of the Dicer 5′-UTR identified multiple alternatively spliced leader exons that are expressed in a tissue specific manner (*83*). Although differential splicing alters the translation efficiency of Dicer *in vitro*, the specific roles of the various Dicer isoforms are unclear. Altered expression of Dicer is observed in several types of human cancer, for example, Dicer is increased in prostate tumours, as well as in a Burkitt's lymphoma derived cell line (*84–86*). Conversely, Dicer expression is decreased in non-small cell–lung carcinoma, and reduced Dicer levels correlate with poor prognosis (*87*). These conflicting trends may be indicative of cell type differences or tumour stage. Analysis of precursor lesions of lung adenocarcinoma showed increased Dicer expression, while advanced invasive adenocarcinoma showed decreased Dicer levels (*88*).

Depletion of the Dicer cofactors, TRBP or PACT, decreases the steady state levels of Dicer protein (*89*, *90*). Truncation mutations of TRBP are found in carcinoma and are associated with decreased miRNA levels and dramatic destabilization of Dicer (*91*). Dicer protein levels can be rescued by full-length TRBP, suggesting that the interaction between Dicer and TRBP is a critical determinant of Dicer stability. Furthermore, the expression or activity of Dicer can also be modulated by cellular-signalling pathways. For example, MAPK/ERK signalling was found to promote the phosphorylation of TRBP (*92*). Phosphorylated TRBP enhances miRNA production by increasing the stability of Dicer. Interestingly, increased abundance of Dicer is correlated with the increase in growth-promoting miRNAs and decrease of let-7 which has a tumour suppressor activity (*92*).

Control of Dicer activity

Pre-miRNAs are often expressed at a low level relative to pri-miRNA or mature miRNA, suggesting that Dicer is generally very efficient at processing pre-miRNA to mature miRNA (*79*, *93*). Unlike Drosha which requires many accessory factors to promote processing, regulation of miRNAs at the Dicer step often involves inhibition of Dicer activity. Early evidence for post-transcriptional control of miRNA at the level of Dicer processing comes from studies of miRNA expression in colorectal neoplasia. Mature miR-143 and miR-145 are expressed at much lower levels in tumour samples than normal tissue. However, the expression of pre-miR-143 or pre-miR-145 is not significantly different in the tumour samples, suggesting that the Dicer processing step is inhibited in the colorectal tumour samples (*94*). miRNA processing by Dicer is also differentially regulated during normal development and tissue specification. For example, while pre-miR-138 is ubiquitously expressed in all tissues and HeLa cells, the mature miR-138 is only detected in adult mouse brain and foetal liver (*95*). Potential alteration in nuclear export is ruled out as the pre-miR-138 is effectively exported into the cytoplasm, therefore it is suggested that the regulation of Dicer processing of pre-miR-138 might be inhibited in many tissues. Furthermore, while

pre-miR-138 is processed efficiently by Dicer *in vitro*, addition of HeLa cell extracts inhibits the processing of pre-miR-138 (*95*). HeLa extracts do not alter the processing of other pre-miRNAs which are normally expressed in HeLa cells (*95*). These results suggest that HeLa cells, and other most tissues, express a yet to be identified factor which inhibits Dicer activity specifically on miR-138.

In addition to inhibition of the Drosha processing step discussed above, Lin-28 also inhibits the Dicer processing of let-7 family members (Fig. 3C) (*74*, *96*). Lin-28 shuttles between the nucleus and cytoplasm, but is commonly enriched within the cytoplasm, suggesting that the cytoplasm may be the primary location of the Lin-28-let-7 interaction. Addition of purified Lin-28 decreases the association of let-7 with Dicer, and results in reduced processing by Dicer, suggesting that Lin-28 can compete with Dicer for access to let-7 (Fig. 3C) (*74*). Furthermore, Lin-28 also promotes the 3′-polyuridylation of pre-let-7 by acting as a scaffold between let-7 and the terminal uridine transferase TUT4 (Fig. 3C) (*75*, *76*). Polyuridylation of pre-let-7 inhibits Dicer processing and promotes the degradation of pre-let-7 (*76*). Analysis of the Lin-28-binding site within the loop of let-7 identified a tetra-nucleotide sequence required for Lin-28/TUT4-mediated uridylation. Interestingly, this motif is located within the terminal loop of 15 additional pre-miRNAs, some of which have also been shown to undergo uridylation (*76*).

Control of the Argonaute

Dicer is associated with several other proteins in a complex termed the RISC-loading complex (RLC) which allows the tight coupling of Dicer cleavage to the incorporation of miRNA into the RISC (*90*). Cleavage by Dicer results in an unstable dsRNA composed of the active guide strand (miRNA) and the passenger (miRNA*) strand (Fig. 1). Selection of the miRNA strand which is incorporated into the RISC can be regulated in a cell type-specific manner. The Argonaute (Ago) proteins are the primary component of the RISC complex, and the effectors of miRNA-mediated repression of target mRNAs (*97*). The human genome contains eight Ago-family proteins; Ago1-4 and Piwi1-4 (*97*). While all of the Ago proteins have the ability to interact with miRNAs and siRNAs, Ago2 is the only one with RNA cleavage activity and is thought to play a critical role in miRNA-mediated mRNA silencing (*97*). Total levels of the Ago proteins within the cell also contribute to global miRNA regulation and biogenesis. Ectopic expression of Ago proteins results in a dramatic increase in mature miRNAs (*98*). In contrast, MEFs and hematopoietic cells deleted in *Ago2* gene exhibit reduced levels of all mature miRNAs examined (*98*, *99*). The dramatic increase in mature miRNA mediated by increased Ago expression could indicate that Ago proteins are limiting in the cell and serve to stabilize miRNA. If this is the case, one might expect that Ago level may be linked to the level of miRNAs or siRNAs present within the cell. Indeed, a retrospective study of micro-array data from siRNA treated cells indicates an increase in Ago2 mRNA independent of the siRNA sequence (*100*). Ago2 mRNA and protein expression is also found to be increased in a subset of breast cancer cells. Epidermal growth factor treatment enhances Ago2 stability, suggesting that Ago2 levels can be regulated at both the transcriptional and post-transcriptional level (*101*). Furthermore, increased Ago2 expression was associated with a transformed phenotype in breast cancer cells (*101*). Interestingly, Ago2 is found to be targeted for ubiquitination and degradation by Lin-41 (*102*). Lin-41 is expressed primarily in stem cells and undifferentiated cell types. Co-immunoprecipitation studies indicate that Lin-41 directly interacts with Ago2 and overexpression of Lin-41-reduced miRNA-mediated mRNA silencing (*102*). Reduction of Ago2 by Lin-41 may serve to prevent expression of miRNAs and inhibit cellular differentiation, thus maintaining a stem-cell-like phenotype (*102*). Interestingly, Lin-41 is a target of let-7 which is highly regulated and important for cellular differentiation (*74*). Similarly to lin-28, the levels of Lin-41 are inversely correlated with the level of let-7 (*102*). It is interesting to speculate that in conditions which are characterized by low levels of let-7, such as lung cancer, Lin-41 may be increased which could in turn result in reduced expression of Ago2. Indeed, immunohistochemical analysis of 68 gastric and colorectal tumours indicates a reduction in Ago2 protein in 40 and 35% of gastric and colorectal cancers, respectively (*103*). It is interesting to speculate that alterations in Ago2 might be a universal characteristic of poorly differentiated tumours.

Perspectives

miRNA biogenesis can be regulated at multiple steps by different regulatory mechanisms. Further investigation is expected to uncover many more mechanisms and upstream signalling pathways that are critical for the maintenance or induction of miRNA levels. In the future it will be important to investigate how multiple mechanisms of miRNA biogenesis control cooperate to give rise to spatially and temporally controlled miRNA expression. Furthermore, an understanding of how miRNAs are regulated under physiologic conditions will allow better understanding of the pathogenesis of various human diseases and facilitate the development of a novel therapeutic approaches to modify specific miRNA expression *in vivo*.

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Conflict of interest
None declared.

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