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Animal models to study microRNA function

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Abstract

The discovery of the microRNAs, *lin-4* and *let-7* as critical mediators of normal development in *Caenorhabditis elegans* and their conservation throughout evolution has spearheaded research towards identifying novel roles of microRNAs in other cellular processes. To accurately elucidate these fundamental functions, especially in the context of an intact organism various microRNA transgenic models have been generated and evaluated. Transgenic *C. elegans* (worms), *Drosophila melanogaster* (flies), *Danio rerio* (zebrafish), and *Mus musculus* (mouse) have contributed immensely towards uncovering the roles of multiple microRNAs in cellular processes such as proliferation, differentiation, and apoptosis, pathways that are severely altered in human diseases such as cancer. The simple model organisms, *C. elegans*, *D. melanogaster* and *D. rerio* do not develop cancers, but have proved to be convenient systesm in microRNA research, especially in characterizing the microRNA biogenesis machinery which is often dysregulated during human tumorigenesis. The microRNA-dependent events delineated via these simple *in vivo* systems have been further verified *in vitro*, and in more complex models of cancers, such as *M. musculus*. The focus of this review is to provide an overview of the important contributions made in the microRNA field using model organisms. The simple model systems provided the basis for the importance of microRNAs in normal cellular physiology, while the more complex animal systems provided evidence for the role of microRNAs dysregulation in cancers. Highlights include an overview of the various strategies used to generate transgenic organisms and a review of the use of transgenic mice for evaluating pre-clinical efficacy of microRNA-based cancer therapeutics.

Keywords

microRNA; function; mouse; model systems; zebrafish; fruit fly; *in vivo*; *Caenorhabditis elegans*; *Drosophila melanogaster*; *Danio rerio*; *Mus musculus*; GEMMs; transgenic; cancer

1. Introduction

The central dogma of molecular biology, laid down by Francis Crick in 1958, stated that the fundamental role of RNA molecules is to transmit the genetic code into proteins[1,2]. However, with the characterization of the first transfer RNA (tRNA) in 1965[3], additional

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RNAs emerged that violated the central dogma. For example, ribosomal RNAs (rRNAs)[4], small nuclear RNAs (snRNAs)[5] and small nucleolar RNAs (snoRNAs)[6] are not translated into protein products like a messenger RNAs (mRNAs), but indirectly influence the process of protein synthesis. Such RNA molecules were collectively termed “non-coding RNAs” (Review[7]). While the novel roles performed by some non-coding RNAs were being assimilated as imperative molecular mechanisms, a study conducted to identify genes in heterochronic signaling incidentally led to the discovery of an additional non-coding RNA, a 22 nucleotide RNA molecule, *lin-4*[8]. The discovery of *lin-4* further defied the central dogma of molecular biology via an unprecedented mechanism and led to the establishment of a new class of small non-coding RNAs called “microRNAs”[9–11].

Lin-4 was the first microRNA (miRNA) identified, which was determined to be indispensable for the normal development of *Caenorhabditis elegans*[8]. Functionally, *lin-4* interacts with the 3′-untranslated region (3′-UTR) of the mRNA transcript *lin-14*, resulting in a marked repression of the lin-14 protein[12]. Unfortunately, due to limited knowledge in RNA biology at the time, *lin-4* and its peculiar role were overlooked to be a worm-specific phenomenon. Seven years later, a second *C. elegans* miRNA, *let-7* was discovered which encouraged further miRNA investigations[13]. It became apparent that *let-7* was not only critical for the development of *C. elegans*, but is also evolutionarily conserved in other organisms, including humans[14]. Currently ~2,500 human encoded miRNAs have been identified, which are listed in a miRNA database, miRBase (http://www.mirbase.org/, Release 21)[15]. In addition to their identification, biochemical and molecular studies have determined that the canonical function of miRNAs is to post-transcriptionally regulate a repertoire of protein-coding mRNA transcripts, whereas a few miRNAs perform unanticipated or “non-canonical” functions (Review[16,17]).

Following the identification of these first two miRNAs, *lin-4* and *let-7*, our understanding of miRNAs in normal physiology and diseased states, such as cancer (Review[18]) has advanced remarkably. Advancements in the field have been possible due to state-of-the-art technologies such as high throughput screening and deep sequencing, but majorly due to the development of appropriate *in vivo* model systems (Review[19]). Therefore, the focus of this review will be on the various model systems that have been instrumental in elucidating the roles of miRNAs in cancers and the technologies that have been extensively applied to generate these animal model systems. Briefly, the utility of *in vivo* models in evaluating the potential of miRNAs as therapeutic agents or targets for treatment of various cancers will also be touched upon.

1.1. MicroRNA biogenesis, mechanism of action and function

1.1.1. Biogenesis

1.1.1.1 Expression of miRNA genes: The transcription of miRNA genes is regulated by multiple mechanisms eventually dictating the level of expression of a particular miRNA in normal or diseased states (Reviews[20–22]).

(a) Regulation mediated by availability of transcription factors: A transcription factor can enhance or repress the expression of a miRNA gene depending on the availability of the

particular factor (Reviews[23,24]). The prominent tumor-suppressor p53 which functions as a transcription factor for several genes, also enhances the transcription of miRNA genes. Examples of miRNAs that are directly induced by p53 include *mir-34a* and *b/c*[25–28] and two miR-200 subfamilies, *mir-200c/141* and *mir-200a/200b/429*[29,30]. In the case of *mir-15a~16-1* and mir-107 a p53-indirect effect leads to upregulation of the miRNAs through activation of the host genes *DLEU2*[28] and *PANK1*[31], respectively. Conversely, MYC, a well-studied oncogenic transcription factor, negatively regulates the tumor suppressive miRNA, *let-7a-1*[32,33].

(b) Regulation mediated by genomic location of miRNA genes

(i) Location in the epigenome: Transcriptional activation or inactivation of specific miRNA genes is largely influenced by epigenetics. Such epigenetic regulation includes the proximity of the miRNA gene promoter to a CpG island, various histone modifications to the chromatin, and availability of factors that maintain and regulate expression from the epigenome. *The expression of mir-127*, a miRNA located near a CpG island is dependent on the methylation status of the promoter, implying epigenetic control on the expression of miRNAs[34,35]. MiRNAs also undergo massive upregulation when the DNA methylatransferases 1 and 3b (DNMT1, DNMT3) are downregulated[34], lending further support to the role of DNA methylation in regulating miRNA expression (Reviews[23,24]).

(ii) Location relative to host genes: The origin of a miRNA gene from a specific chromosomal location impacts the extent of expression of the miRNA. In the context of other genes, miRNAs genes are either intragenic where they are embedded within a host gene, or intergenic if they are located between two genes on a chromosome. Expression of an intragenic miRNA is dependent on the expression of the host gene (Reviews[23,24]). MiR-126 is one such miRNA whose expression is concomitantly controlled by epigenetic regulation of its host gene *EGFL7*[36]. Intragenic miRNAs are also regulated by canonical mechanisms that influence host gene expression such as transcription factor occupancy at the promoter of the host gene[28,31] (Review[37]). MiRNAs that are not directly regulated by a host gene are still subject to nearby epigenetic influence. For example, *let-7a-3* and miR-129-1 expression are dependent on a nearby region of the genome that is prone to altered methylation states during the onset of cancer. The proximity to this differentially methylated region severely impacts their expression[24,38,39].

(iii) Regulation by miRNA copy number: A single mature miRNA can be expressed and processed from multiple loci in the genome. For example, three individual genes encoding human *mir-7* produce an identical mature miRNA product[40]. Conversely, miR-21 is generated from a single genomic locus[41,42]. The advantages of miRNAs originating from various loci relative to one originating from a single locus is discussed in a later section.

(iv) Cancer-Associated Genomic Regions (CAGRs): Specific regions in the human genome that are prone to amplification or loss upon the onset of cancers are referred to as Cancer-Associated Genomic Regions (CAGRs). CAGRs contain amplified or deleted miRNA and/or protein-coding genes. Many of these genetic aberrations are required for tumorigenesis. MiRNAs that are lost are frequently located in either fragile sites of the genome or regions susceptible to loss of heterozygosity (LOH). For example, the *mir-15a~16-1* cluster located

in a fragile region of the genome at 13q14.3 is frequently deleted in Chronic Lymphocytic Leukemia (CLL) patients[28,43,44]. Whereas other miRNAs are commonly amplified in multiple cancers due to their location in fragile regions. For example, the 17q23-25 chromosomal region containing *mir-21* gene, a commonly overexpressed miRNA in multiple cancers[41] is an amplified CAGR (Reviews[23,24]).

1.1.1.2 Process of Biogenesis: The primary miRNA (pri-miRNA) transcript produced as a result of RNA Polymerase II/III dependent transcription containing a single miRNA or as a cluster of miRNAs, produces a monocistronic or polycistronic pri-miRNA transcript, respectively[45]. Pri-miRNA transcripts form stem-loop structures flanked by single-stranded (ss) RNA ends. For RNA Polymearse II transcripts, the ends contain a canonical 5′ 7-methylguanosine cap and a polyadenylation signaling at the 3′-end. The size of a typical pri-miRNA can range from a hundred to a few kilobases in length and can originate from either intragenic or intergenic miRNA genes[45,46] (Figure 1).

Processing of most pri-miRNAs begins with the association of the RNaseIII enzyme DROSHA and its cofactor Di George Syndrome Critical Region 8 (DGCR8) forming the microprocessor complex[47,48]. The microprocessor complex recognizes the ssRNA regions of the pri-miRNA sequence flanking the stem-loop and cleaves the ends. The resultant ~60–80 nucleotide long hairpin structure is referred to as a precursor miRNA (pre-miRNA)[47,48]. The pre-miRNA is translocated into the cytoplasm via Exportin-5 where another RNase III enzyme, DICER1 performs additional processing[49,50]. DICER1 cleaves the pre-miRNA to generate a ~22 nucleotide duplex molecule containing the guide and the passenger miRNA strands. Following cleavage, the DICER1-miRNA duplex associates with Transactivation-Responsive RNA-binding protein (TRBP) that mediates a stable transfer of the miRNA duplex into an Argonaute protein (AGO) [51,52]. Selective incorporation of the miRNA duplex into either AGO1, AGO2, AGO3 or AGO4 is dictated by the presence of bulges or mismatches in nucleotides 9–12 of the duplex[53,54]. Incorporation of a miRNA duplex in AGO2, an AGO protein with endonuclease activity, results in selective cleavage of the passenger strand. The ssRNA guide strand is retained, and with AGO forms the mature miRNA-induced silencing complex (miRISC)[55,56]. On the other hand, the endonuclease activity deficient AGOs, AGO1, AGO3 and AGO4, generate a functional miRISC by binding to the guide strand and separating the passenger strand based on thermodynamic instability. The released passenger strand is shunted for further degradation[57] (Biogenesis reviews[20,22,46], Argonautes reviews[54,58]).

1.1.2. Mechanisms of action and functions of miRNAs

(a) Incorporation into miRISC and targeting: The well-established role of functionally active miRISC is to negatively regulate transcription of the target protein-coding transcripts. The canonical mechanism by which miRISC performs its function depends on the extent of complementary binding between the 5′-end “seed region” of the miRNA, the 3′-UTR of the target mRNA transcript, and the enzymatic activity of the AGO protein[17,54,59,60]. Perfect complementarity between the seed sequence, nucleotides 2–7 of the guide miRNA strand, and the target results in either degradation or translational repression of the target. The fate of the target transcript is dictated by whether the incorporated AGO displays catalytic

activity or not, and whether additional complementarity occurs between the target and the miRNA. If a catalytically active AGO is incorporated into miRISC and the binding between the miRNA and the target are complementary between nucleotides 9–12 of the miRNA, then target cleavage will occur[56,61]. Translational inhibition in the absence of target degradation occurs when the miRNA binds to its target via partial complementarity (Review[17,54,60]) or if an endonuclease deficient AGO is included in mRISC. The partial complementarity between the miRNA and its target is highly conserved across species, providing the basis for a combinatorial interactome. A combinatorial interactome is the mechanism by which a single miRNA regulates multiple targets, thus simultaneously exerting its regulatory effects on various signaling pathways. For example, the very well-studied miRNA miR-21 simultaneously targets transcripts of proteins that regulate cell division and apoptosis, such as phosphatase and tensin homolog (PTEN)[62], and programmed cell death 4 (PDCD4)[63] to drive the process of tumorigenesis (Review[42]). Partial complementarity between the miRNA and the target also facilitates targeting of a single transcript mRNA by multiple miRNAs resulting in enhanced repression of the target. This resulting moderate-to-severe downregulation of target transcripts via a miRISC is the canonical mode of action of miRNAs (Reviews[46,60,64]).

(b) The role of family members in expression and targeting: The mechanism of action of miRNAs originating from a single locus, or a single mature miRNA originating from multiple loci remain largely unchanged. In these instances the same cohort of target mRNA transcripts is repressed[42,62,65,66]. However, miRNAs originating from several loci that contain subtle variations in their mature sequences [46,67] can exert their repressive functions on a larger repertoire of target transcripts. These miRNA families have acquired an evolutionary advantage relative to miRNAs with a single mature miRNA sequence. In addition to an increased pool of potential targets, the presence of multiple miRNA family members across the genome may allow at least one of the family members to evade transcriptional or epigenomic regulation. Therefore, the presence of multiple genetically distinct miRNA family members may prevent the depletion of an entire pool of a specific mature miRNA during the onset of a diseased state. For example, transcription of the twelve *let-7* miRNA genes produces nine unique mature miRNA sequences that differ by at most three nucleotides (Reviews[67,68]). These minor nucleic acid changes can potentially alter the targeting affinity of the various family members (Figure 1 depicts miRNA family members). It has also become apparent that the promoter of *let-7a3* resides in a heavily methylated region of the genome in normal cells resulting in low levels of *let-7a3* in a normal cell. This is however not the case for the other *let-7* family members which are highly expressed under normal conditions leading to a stably differentiated state of the cell. Nonetheless, upon the onset of tumorigenesis, the methylation state of cells become severely disrupted, and except for *let-7a3* all the other *let-7* isoforms become repressed[24,38,67]. Thus, the presence of multiple genetic loci encoding miRNA family members and slight variations in sequence between members adds an additional layer of complexity in the regulation of miRNAs in adverse cellular conditions.

Similar to the *let-7* family, another well-studied family of miRNAs is the miR-34 family. The three canonical miR-34 family members include miR-34a that arises from a

monocistronic locus, and miR-34b/c, which are expressed from a polycistronic transcript[25,27,34] (Figure 1 depicts mono- and polycistronic miRNA genes). The function of miR-34 in normal physiology is well established as an inducer of cellular senescence and cell cycle arrest[69] (Review[27]). Nevertheless, the advantage of multi-loci encoding miR-34 family members is that miR-34a and miR-34 b/c can be differentially regulated in tissue specific context[70–72].Recent reports suggest that the tissue-specific expression of the miR-34 paralogues miR-449a/b/c add an additional level of complexity to the control of cancer cell proliferation, invasion, and migration[73–75]. Indeed, it was not until the paralogue *mir-449a/b/c* cluster was deleted in mouse models that the *mir-34a*, *mir-34b/c* double mutant displayed a phenotype[76–78].

(c) The role of miRNA clusters and paralogous in targeting: Analogous to the overlapping role that miRNA family members have on gene expression, some paralogous clusters can also have overlapping roles while others have gained novel functions. A paralogous miRNA cluster is generated when a cluster undergoes duplication and translocates to another area of the genome (Figure 1 depicts miRNA paralogues). The resultant paralogue may express miRNAs similar to the parent cluster, located in relatively analogous positions[79–81]. One such miRNA cluster, miR-17~92 has been extensively studied due to its implication in the human developmental syndrome, Feingold disease. Loss of *mir-17~92* results in severe skeletal abnormalities, and learning and developmental disabilities associated with Feingold disease[82]. However, similar developmental defects were not observed following the knockout of two *mir-17~92* paralogous clusters– *mir-106b~25* and *mir-106a~363*[83]. Additionally, the presence of a single wild-type *mir-17~92* allele was capable of mitigating the deleterious effects of the loss of *mir-17~92*, despite the absence of its paralogues[84]. Collectively the three paralogous clusters encode a total of fifteen miRNAs that can be sub-classified into four miRNA families that are presumed to target analogous target transcripts. However in this case it can be inferred that alterations in certain nucleotides of the paralogues may have ceased their ability to compensate for *mir-17~92* deletion[80,83,84] (Figure 1 depicts miRNA paralogues). Therefore, in order to dissect the function of each miRNA in a family of miRNAs or within paralogues demands the generation of appropriate model systems to advance the field forward.

(d) Use of bioinformatics to elucidate miRNA function: Recently there has been a surge in the development of bioinformatic tools to precisely predict targets of a miRNA, or predict miRNAs that target a particular transcript[19]. Several computationally predicated miRNA-target pairs based on complementarity between the miRNA seed sequence and the 3′-UTR of transcripts have been experimentally validated[8,46,60,67,85–87]. Thus, implying that the developed algorithms are powerful in predicting relevant targets of novel miRNAs with unknown functions based on the canonical mode of action of miRNAs. However, there is increasing evidence that demonstrates some non-canonical mechanisms of actions of miRNAs[17,64,88]. For example, a passenger strand (also known as miRNA*) may not always be released for degradation once the miRNA: miRNA* duplex is incorporated into AGO. The miRNA* strand and may have an equal or a higher potential of becoming incorporated in an active miRISC[17,89]. Moreover, certain miRNAs modulate the expression of targets either positively or negatively by physically interacting with the 5′-UTR of transcripts[90,91],

the coding sequences (CDS) of transcripts[91,92], or with gene promoters[93]. Mature miRNAs have also been experimentally validated to interact with other non-coding RNAs such as other miRNAs, long non-coding RNAs (lncRNAs)[91,94], or circular RNAs (circRNAs)[91,95]. The canonical "seed-sequence" binding hypothesis has also been challenged as miRNAs can bind some targets independently of the seed sequence[96,97]. Data from these studies, along with the identification that certain miRNAs are contained in extracellular vesicles such as exosomes, has added an additional level of complexity in the mechanism by which miRNAs function, including non-autonomous mechanisms[98].

1.2. MiRNA function and relevance in cancer

MiRNAs are important players in the normal developmental processes of animal species. As such, disruption in the normal physiological levels of certain miRNAs can lead to the development of multiple diseases, including cancers.

Detailed characterization of various miRNAs have revealed many important properties of these powerful post-transcriptional modulators in both normal and diseased states. In the context of cancers, certain miRNAs have been identified as functional "drivers of cancer", whereas others are regarded as mere "passengers" in the tumorigenic process. A few known miRNA drivers of cancer become upregulated while others are severely downregulated or lost. The miRNAs that promote hallmarks of cancer are referred to as oncogenic miRNAs (oncomiRs). Those that prevent or reduce tumorigenesis are collectively called tumor suppressive miRNAs (Figure 1).

1.2.1. OncomiRs—OncomiR coding genes are frequently located in regions of the genome that are aberrantly amplified, or are subject to increased expression[99]. Increased expression of an oncomiR can be attributed to enhanced transcription of the oncomiR gene due to (i) availability of transcription factors, (ii) hypomethylation of its promoter, or (iii) its location in an intra- or intergenic region that is subject to increased expression in cancer via other mechanisms. OncomiRs can also be upregulated due to defects in biogenesis and/or stability of the mature miRNA[22,23,46,64,84]. The way by which an oncomiR typically functions is through targeting tumor suppressive protein-coding transcripts via canonical mechanisms, or through other less understood non-canonical mechanisms.

The first oncomiR to be validated was the miR-17~92 cluster (oncomiR-1). Overexpression of the cluster led to the development of lymphoproliferative and auto-immune diseases in mice via targeting of BIM, a pro-apoptotic protein[100]. Other targets of miR-17~92 that support the oncogenic role for this cluster include *PTEN*, *E2Fs*, and *MYC*. More detailed analysis of this cluster confirmed that the cell-type and context specific processing of individual miRNAs from the cluster adds an additional level of complexity to the function of the oncomiR[80,101]. Co-operatively the individual miRNAs processed from miR-17~92 functions as an oncomiR. However, miR-92 alone can antagonize an additional cluster member, miR-19 and also negatively regulates the oncogenic effects of c-Myc[84,101,102]. Because miR-19 alone can recapitulate the oncogenic role of the intact mir-17~92 cluster[102,103], negative regulation by miR-92 suggest that miR-92 may be functioning as a tumor suppressor. The function of the miR-17~92 miRNA cluster is extremely intriguing

and is currently under active investigation. Specifically, molecular roles and tissue specific effects of individual miRNAs of the miR-17~92 cluster are being determined in appropriate model systems[83,84,102–105]. These positive findings highlight the importance of carefully dissecting individual miRNAs from clusters so as to accurately identify the functions of each of the miRNAs contained within them.

Other miRNAs that have been well established as oncomiRs due to their implication in multiple solid tumors and hematological malignancies are miR-21 and miR-155[106–108]. Independent studies determined that overexpression of individual miRNAs such as miR-21 and miR-155 are sufficient to cause lymphoproliferative diseases. The mechanism by which miR-155 initiates cancer is not well understood, however, in leukemic mouse models it was determined that miR-155 promotes cancer progression, perhaps through gradual downregulation of its targets, SHIP and C/EBP[109]. In miR-21-dependent mouse models of lung cancer or pre-B-lymphoma, downregulation of the miR-21 targets PTEN and PDCD4 (negative regulators of cell death and cell-cycle, respectively) contributed to enhanced proliferation and growth[42,62,63].

1.2.2. Tumor Suppressive miRNAs—About 50% of the miRNAs involved in repressing oncogenic protein-coding genes are located in or are close to fragile regions of the genome that are frequently deleted in cancer. Additional mechanisms elicited by cancer cells to repress tumor suppressive miRNAs include LOH, hypermethylation of the promoter, or the activation of transcriptional repressors that specifically downregulate the expression of the miRNA gene[23,25,43,69]. In the case of most tumor suppressive miRNA genes, identification of their role in development preceded their characterization as tumor suppressors. The most striking example of such a tumor suppressive miRNA is *let-7. Let-7* was identified as a crucial differentiation factor in *C. elegans* prior to its identified role in tumorigenesis. Indeed, the development of cancer requires a reversal of a well-differentiated state to an undifferentiated state, thus, it is perhaps not surprising that downregulation or loss of *let-7* family members is common in tumorigenesis[67,68].

High levels of *let-7* expressed from multiple genomic loci are expected in normal fully differentiated cells[67,68]. This results in repression of *let-7* targets which are important oncogenes, such as *KRAS*, *NRAS*[85,110], *HMGA2*[110,111], *LIN28*[112–115], and *MYC*[116]. A candid tumor-suppressive miRNA, such as *let-7* has multiple loci of origin in order to maintain an appropriate level of the tumor suppressive miRNA as a defense mechanism against developing cancers[67,117]. However, since most *let-7* isoforms are located in regions of the genome frequently deleted in cancer, *let-7* is severely downregulated in multiple cancers[43]. One anomaly to this rule is the expression of *let-7a-3* gene. In lung adenocarcinoma, epigenetic regulation of the gene encoding *let-7-a3* results in hypomethylation of the promoter enhancing the accumulation of the *pre-let-7a-3* transcript in lung cancer cells, and subsequently its potential oncogenic effects[24,38].

Additional miRNAs that have been well established as tumor suppressors include miR-15a and miR-16-1, which were among the first miRNAs that demonstrated a negative correlation with the development of cancers[43,118]. *Mir-15a~16-1* is located in 13q14.3, a region that is homozygously or hemizygously lost in over 50% of CLL cases[118]. Early reports correlated

loss of *mir-15a~16-1* with an increase in expression of *BCL2*, a pro-survival factor that normally prevents cell death[119–122]. In addition to targeting *BCL-2*, targets of miR-15a~16-1 include several cell-regulatory proteins, such as MCL1[122,123], another BCL2-family member, CCND1[121], a cell cycle regulator, and WNT3A[121], a protein that induces several tumorigenic features including survival, proliferation, and invasion. Hence it is speculated that the simultaneous overexpression of these pro-survival onco-proteins, as a result of the loss of *mir-15a~16-1* cluster may synergistically contribute to the development of cancers[118,124]. To further evaluate the tumor-suppressive potential of each miRNA in the *miR-15a~16-1* cluster, modelling CLL in more sophisticated model systems is required.

1.3. Tools that advanced miRNA research

Research in the miRNA field exponentially increased following the discovery of the second miRNA in *C. elegans*, *let-7. Let*-7 was experimentally found to be homologous in a few species such as *Drosophila melanogaster* (fruit fly) and *Danio rerio* (zebrafish)[14,67]. However, additional computational evidence showed that *let-7* was conserved further throughout evolution in all metazoans evaluated, and therefore, the discovery of *let-7* was regarded as one of the most significant breakthroughs in the history of miRNA research[18,67]. Moreover, bioinformatic analysis corroborated with molecular studies also established that the *let-7* target, *lin-41* is conserved across species[10,12,14,125]. These studies were fundamental in establishing the proposed global mechanism of action of miRNAs, that miRNAs negatively regulate protein coding gene expression through miRNA:target interactions. The more recent utility of computational target prediction algorithms in miRNA research has further advanced the field in two ways: (i) a miRNA and its targets can be predicted *in silico* such that a biologically relevant target is putatively identified prior to in depth biochemical and molecular analysis, and (ii) computational methods spread awareness about similarities between various species. This knowledge can be used to identify appropriate, and perhaps simpler organisms that can be used as an adequate model system in miRNA research. *In silico* analysis combined with biochemical and molecular studies conducted in *in vivo* models, and *in vitro* from cells isolated from the *in vivo* models, or from human samples, have remarkably advanced our understanding of miRNAs in development and disease.

While the functional significance of miRNAs in various developmental stages of *C. elegans* was emerging, and the correlation between miRNA levels and disease such as cancer were being reported, a spontaneously occurring mouse model of CLL, the New Zealand Black (NZB) mouse was discovered. This discovery incidentally highlighted the importance of mouse models in delineating the role of miRNAs in cancers. It was determined that the endogenous loss of the *mir-15a~16-1* cluster in this mouse led to the spontaneous development of CLL as the mouse aged[126,127]. This discovery followed by bioinformatic developments that described ~60% of miRNA loci remain conserved from mouse to humans spearheaded the use of murine models in miRNA research[125,128]. Indeed, from worms to mice many miRNAs and the components of the miRNA biogenesis machinery are conserved. The benefits of using simple model organisms such as worms, zebrafish and drosophila in miRNA-based biochemical studies have indirectly paved the path towards characterization of miRNAs that have relevance in cancer (Figures 4,5). Validation and

clinically relevant studies become possible due to the increased use of very robust and durable mouse model systems (Figures 4,5). Therefore, in this review, a short background on the historical perspective of using various model systems will be followed by an detailed explanation on the current technology used to generate appropriate model systems. Some of the most successful models that have faithfully aided in uncovering the roles of various tumor suppressive or oncogenic miRNAs involved in cancer will be discussed. In closing, the pros and cons of the most widely used model systems in miRNA functional studies in cancer will be elaborated upon.

2. Generation of model organisms and their use in miRNA functional studies

2.1. Caenorhabditis elegans

Although *C. elegans* do not develop cancer, they have been extensively used as model organisms to identify the functions of molecules and delineate pathways involved in normal cellular processes that are severely impaired in cancer, such as cell proliferation, differentiation, metabolism and death[129–131]. The completely sequenced *C. elegans* genome revealed that ~60% of its miRNAs have a human orthologue[132]. However, since *C. elegans* have a reduced number of miRNA family members for miRNAs that are conserved, studying miRNA function in *C. elegans* excludes redundancy as a hurdle to overcome. Moreover, *C. elegans* are self-fertilizing hermaphrodites that can produce a large number of genetically identical offspring. Additionally, their visually-traceable, well-organized transparent body make *C. elegans* an excellent model system[129–131]. Phenotypic and genetic screens, application of molecular techniques, and development of transgenic *C. elegans* have identified a few critical miRNAs, including their mechanism of action. Studies in *C. elegans* have also been an instrumental in understanding the molecular basis of miRNA biogenesis[133–135]. For example, the *C. elegans* ortholog of DICER, *dcr-1*, involved in RNA-mediated silencing, was identified as a critical component necessary for the processing of mature *let-7* from its precursor molecule[133].

2.1.1. Generation of transgenic *C. elegans* for use in miRNA functional studies

—The most common mechanism used to generate transgenic *C. elegans* is transformation via either (i) microinjection or (ii) DNA bombardment. Transformation is widely used to ectopically introduce a transgene or fragment of DNA of interest into the animals to rescue a mutant phenotype or to over-express or silence a gene. The DNA is typically co-delivered with a scorable marker to determine successful transformation. For example, a scorable marker such as a promoter driven gfp::transgene allows for the selection of GFP positive worms when the promoter is positively regulated[136].

Microinjection: Microinjection is a precise mechanism of introducing DNAs into the distal end of the worm gonad, which is composed of a syncytium of cells sharing cytoplasmic material. The transgene is usually a plasmid, cosmid, phage, Yeast artificial chromosome (YAC), or PCR product co-injected into the gonad with a scorable marker. Injected DNAs undergo efficient homologous recombination with each other to generate large extrachromosomal arrays. The extrachromosomal arrays contain multiple copies of the

transgenic DNA that do not usually integrate into the genome, but can become inheritable by a fraction of the F1 generation. However, when integration is essential, random incorporation of the extrachromosomal DNA can be induced using radiation (gamma or UV) or through the use of a single DNA oligonucleotide that facilitates random integration and suppresses array formation [136,137].

DNA bombardment: DNA bombardment is a specialized technique where the transgene and co-injected transformation plasmid DNA mix is coated onto a gold microparticle and is bombarded into the worm using a gene-gun. The advantage of this technique is that it produces a considerable number of non-homologous integrants post-transformation[136,138,139].

Apart from the above-mentioned techniques, additional newer strategies are now being applied towards generating transgenic *C. elegans*, such as the CRISPR-Cas9 system, discussed in a later section.

2.1.2. *C. elegans* as a model system for studying the function of miRNAs in cancer—The first discovered miRNA, *lin-4* was identified in *C. elegans* via a conventional method of forward genetic mutagenesis screen, with the intent to mutate heterochronic genes resulting in phenotypic developmental defects. *Lin-4 lof* worms reiterate early phases of developmental fates (L1) at later stages of development, resulting in the absence of well differentiated adult phenotypes such as the adult cuticle, and a developed vulva. Development into adulthood in *lin-4* null mutants was rescued by microinjection of *lin-4* PCR products confirming that lin-4 *lof* was responsible for the mutant heterochronic phenotype. The phenotype of *lin-4 lof* mutants is completely opposite to that of *lin-14 lof* mutants attributed to the fact that the *lin-4* miRNA negatively regulates the *lin-14* mRNA transcript by binding to several complementary sequences in the *lin-14* 3′-UTR. When the *lin-4* complementary sequences were mutated abnormally high *lin-14* protein levels led to worms with retarded developmental phenotypes at late developmental stages[8]. In addition to suppressing *lin-14*, *lin-4* also post-transcriptionally regulates the heterochronic genes, *lin28* and *hbl-1*. Thus, *lin-4* is regarded as a critical switch in nematodes for the development of well-differentiated adult structures[140,141]. Nevertheless, its homologs remained unidentified in higher organisms for many years, and *lin-4* was presumed to have been lost during the course of evolution. But recent advances in bioinformatics have provided researchers with the tools needed to identify the human homolog for *lin-4,* miR-125. Analogous to the role of *lin-4* in worm development, miR-125 in human cells targets LIN28 resulting in the acquisition of a differentiated state in normal cells[142,143]. In multiple human cancers, the two miR-125 family members, miR-125a/b are severely under-expressed, specifically in leukemia[144] and melanoma[145], and ovarian[146], breast[147], oral[148] and thyroid[149] carcinomas. In concert, ectopic miR-125 prevents cellular proliferation and migration in bladder cancer[150], inhibits epithelial–mesenchymal transition (EMT) of triple-negative breast cancer cells[151], and induces radiosensitivity and chemosensitivity in breast cancer and osteosarcoma, respectively[152,153].

Seven years after the identification of *lin-4* the second miRNA, *let*-7 was subsequently discovered, again through genetic analysis of the heterochronic pathway in *C. elegans*[8,13].

Mutants with severely retarded developmental phenotypes were identified and used to map the sequence of *let-7. let-7* is expressed in the later stages of worm development, and regulates the transition of L4 larval stage worms to adulthood. A striking phenotype of *let-7 lof* worms is lethality as they fail to transition from larval to adults, at non-permissive temperatures. Most worms die due to bursting of the vulva. However, microinjecting the worms with PCR fragments containing the *let-7* sequence rescued the progenies. Further analysis via northern blotting verified that *let-7* did not encode a protein but instead encoded a 22-nucleotide RNA molecule in the rescued progenies. The offspring were selectively scored via co-injecting a GFP reporter plasmid, goa-1::GFP. The microinjected worms developed normally, supporting a role for the *let-7* RNA product in inhibiting the bursting vulva phenotype. Due to the *in silico* finding that *let-7* is complementary to the 3′-UTR of lin-41, target validations that *let-7* regulates lin-41 were performed. A lacZ reporter gene was fused to the 3′-UTR of lin-41 and was co-injected with goa-1::GFP in a *let-7* wild-type organism. Reduced luciferase levels and subsequent validation studies confirmed that *let-7* exerts negative post-transcriptional regulation of *lin-41*. Moreover, overexpression of *let-7* was shown to be implicated in premature development of *C. elegans,* therefore, acting as a critical developmental switch in worms[13].

After the discovery of the first two miRNAs in *C. elegans*, it was speculated that nematodes had invented a novel mechanism to sequentially control their developmental course. This notion was nonetheless challenged by simple bioinformatic analysis conducted to investigate if *let-7* was conserved in other organisms. Sequence analysis in *Drosophila melanogaster* (fruit fly), *Danio rerio* (zebrafish), *Mus musculus* (mouse), *Gallus gallus* (chicken), and *Homo sapiens* (Humans) confirmed that *let-7* had been preserved throughout evolution[14,125]. Similarly, the *let-7* target, *lin-41* was also conserved[14]. Moreover, other detectable *let-7* family members, miR-48, miR-84, and miR-241, were also identified as heterochronic miRNAs crucial for the temporal patterning of development in *C. elegans* [154]. Both bioinformatic and biochemical analysis of *let-7* family members revealed a considerable redundancy in target specificity between miRNA family members, suggesting a complex mechanism by which family members function. Although miRs-48, -84 and -241 lack human homologs, several *let-7* loci are present in humans generating nine mature *let-7* family members, therefore, there is a need to dissect the individual functions or functional redundancy between human encoded *let-7* family members (Reviews[67,68]).

Despite the widespread acceptance of the role of *let-7* in development, not much is known about *let-7* in human embryonic development due to ethical constraints. However, shortly after the finding that miR-15a/16-1 is a *bona fide* tumor suppressive miRNA cluster, investigators evaluated the potential of *let-7* as a tumor suppressive miRNA. *Lof* of *let-7* in seam cells of *C. elegans*, leading to the inability of the seam cells to exit the cell cycle and become terminally differentiated, indicated that *let-7* may play a role in maintaining the balance between cell differentiation and proliferation[13,67]. In human cancers *let-7* expression is often reduced[155], confirmed by the finding that multiple *let-7* family members are located in fragile regions of the genome that are often lost in various malignancies[43], signifying that *let-7* is a tumor suppressive miRNA.

To delineate the molecular mechanism of *let-7* in cancers, additional *let-7* targets were computationally predicted in worms which identified *let-60* as a putative target. Reporter plasmids containing the 3′-UTR of *let-60* with or without the putative *let-7* binding sites verified that *let-60* was indeed a *let-7* target. Moreover, *let-7* mutant worms that usually die at non-permissive temperatures, when fed with silencing RNAs (RNAi) directed to *let-60* survived. This was the first experimental evidence suggesting a novel role for *let-7* in negatively regulating *let-60* via a post-transcriptional mechanism. This breakthrough suggested that additional miRNAs may be dysregulated in cancer, other than the initially discovered mir-15~16 cluster lost in CLL. Indeed, *let-60* is the human homolog of the RAS proto-oncogenes, proteins that are amplified and constitutively activated in multiple human cancers[85]. Multiple studies have since validated the negative regulation of RAS exerted by *let-7* in various model systems and in cells derived from human patients, which has led to the advancement of *let-7* family members as potential cancer therapeutics to target such oncogenes[110,156–159].

With the emergence of the fact that miRNAs exist not merely as key developmental switches in nematodes, but are also crucial for normal cellular behavior in multiple species, including humans, severely dysregulated miRNAs are in the limelight for their roles in cancer initiation, development and progression. One such miRNA, miR-34, frequently lost in various cancers, has been associated with stress-response in normal cells, and with radio- and chemotherapeutic response in human cancer cells[27,69,160,161]. *In vitro* studies in wild-type and p53-mutated mouse and human cells determined that miR-34 was capable of suppressing the cell-cycle via a p53-mediated pathway[25]. However, the effects of miR-34 modulation in response to radio- and chemotherapies *in vivo* were unknown.

To determine the biochemical changes imposed by miR-34 and to record miR-34 dependent molecular observations *in vivo*, a mir-34-promoter::gfp transgene was constructed, and microinjected in *C. elegans*[162]. GFP signals from miR-34 transgenic animals were invariably identified in somatic tissues, including the vulval cells. Although, miR-34 expression was detected in the vulva in late larval stages and in the adult worm, miR-34 *lof* did not affect the development of vulva indicating that miR-34 was not a heterochronic miRNA[162]. Moreover, although miR-34 was upregulated in wildtype transgenic worms following exposure to radiation, miR-34 was not transcriptionally activated by the p53 worm homologue *cep-1*[162]. This observation in worms was contradictory to that predicted from human cell lines, and may be attributed to evolution of higher organisms[161,162]. Nevertheless, since miR-34 was upregulated in worms following exposure to radiation, the next big question was, how does miR-34 affect apoptotic and non-apoptotic pathways post-radiation exposure? *C. elegans* have been characterized as an excellent model to study both apoptosis in germline cells, and necrosis (non-apoptotic pathway) in vulva cells [163,164]. Upon exposure of miR-34 mutant worms to radiation it was observed that the germline cells of the worms were unaffected, yet the vulva cells were radio-sensitized. Thus, miR-34 is an essential miRNA for induction of apoptosis, but is a suppressor of necrosis in nematodes. The data also provided evidence that the single miRNA, miR-34 can independently function as an apoptotic inducer, irrespective of p53 status[162]. Additionally, *C. elegans* express only one isoform of miR-34 while miR-34 in humans is encoded by four family members miR-34a/b/c and mir-449a[73–75] indicating a

possible evolutionary advantage towards enhancing miR-34 mediated tumor suppressive function. This notion requires further evaluation in an appropriate model system.

Conventionally, transformation has been utilized to generate transgenic worms, however, with new sophisticated techniques, such as transgenesis via the CRISPR-Cas9 system, targeting an endogenous locus has now become the preferred approach. The use of CRISPR-Cas9 system to knock out *let-7* in *C. elegans* validated the role of *let-7* in maintaining vulval integrity via the regulation of lin-41. This study exemplified the importance of ablating a miRNA to holistically understand its effects in normal physiology via reverse genetics[165].

C. elegans have provided a platform for investigators to biochemically delineate the functions of some important miRNAs that are also crucial players in human cancers. However, during the time that experiments were being conducted in *C. elegans,* the use of computational knowledge to elucidate *let-7* homology between species opened up the possibility of using additional simple organisms to identify the intricate functions of miRNAs. Conceptually, gathering functional knowledge for a single conserved miRNA in various species will aid in appropriately documenting the function of the miRNA, ultimately enhancing our understanding of the molecular biology of human diseases, such as cancer.

2.2. Drosophila melanogaster (fruit flies)

Fruit flies are a simple yet useful model system in cancer research, since cellular alterations in flies leads to the development of a few of the hallmarks of cancers, unlike in *C. elegans*. Although *D. melanogaster* lacks an intact angiogenic system, they have been very useful models to study cell survival and proliferation, cell death and apoptosis, and invasion and metastasis in the presence of normal cells. Additional advantages of using fruit flies as a model organism include i) the large brood size, one fly can lay ~100 eggs per day for about 20 days, ii) the series of conspicuous developmental stages that flies go through from embryogenesis through adulthood over the course of 10 days, and iii) the fact that genotypic changes are easily phenotypically tractable[166].

2.2.1. Generation of transgenic *D. melanogaster* to model cancer phenotypes

—Transgenesis procedures in flies rely on the flies' endogenous transposition mechanism, P-element mediated transposition. The two-vector transposition system includes a helper vector referred to as the P-transposase vector, and the P-element transposon backbone containing the transgene and a scorable marker or a reporter vector (Figure 2). The transgene can be constitutive or inducible, or tissue-specific depending on the promoter (Figure 3). Simultaneously, the scorable marker that is expressed may be a gene that is easily detectable as a physical change – for example white eye color, small wings, GFP-wings, or lethality. The two vectors are co-injected into a generation zero (G0) embryo after which the transgene and the P-transposase are randomly incorporated into the genome. Using P-elements flies have been generated to express Gal4, a yeast derived transcription factor gene, in a tissue-specific manner. Gal4 associates with the UAS response elements to drive transcription of genes containing them. As such, UAS driven-transgenic lines have also be generated. Crossing UAS-transgene expressing lines with Gal4 expressing flies yield flies

that express the transgene in the pattern of the Gal4 activator, i.e. expressed conditionally and in a tissue-specific manner (Reviews [166,167] (Figure 3).

Although the conventional method for generating transgenic flies via transposition has not become obsolete, newer strategies for more efficient transgenesis are gaining attention, such as the Flp-FRT, Cre-loxP[166] and CRISPR-Cas9 systems[168] (Figure 2). Such innovative and novel technologies have significantly advanced the development of transgenic animal models in miRNA functional studies, especially in the context of elucidating the function of miRNAs in various cancer-related events.

2.2.2. *D. melanogaster* as a model for studying the functions of miRNAs in cancer—After the discovery of *let-7* in *C. elegans*, *let-7* research was extended to *D. melanogaster* since flies only express a single *let-7*. In flies, *let-7* is produced as a polycistronic pri-miRNA encoding miR-100, *let-7,* and miR-125, also conserved in humans[148,169–171]. To evaluate if the progression of juvenile flies to adulthood is spatiotemporally controlled via the expression of *let-7*, Gal4 was cloned into the *let-7* locus removing *let-7* and putting Gal4 was under the regulation of the *let-7* promoter. Mutant pupae underwent normal morphogenesis into adult flies. However, the mutant adults displayed severe flight, motility and fertility defects[170]. This study demonstrated that although loss of *let-7* in juvenile flies did not phenocopy the dramatic larval lethality observed in *C. elegans*, *let-7 lof* resulted in severe detrimental effects in the developing flies.

An important contribution to the miRNA field made through the use of *D. melanogaster* was achieved using the yeast Flp-FRT system to delineate the consequence of knocking-out a miRNA via homologous end recombination[170,171]. Using this system, the individual miRNAs from the *let*-7/mir100/mir-125 cluster were knocked out, resulting in data that supported that *let-7* was sufficient for the normal development of *D. melanogaster*, whereas miR-100 and miR-125 were dispensable[170]. In humans the cluster exists as three paralogs, miR-100/*let-7a-2*/miR-125b-1, miR-99a/*let-7c*/miR-125b-2, and miR-99b/*let-7e*/miR-125a. These paralogues exist on chromosomal regions that are frequently lost in multiple cancers[43,67]. In a few recent studies, one or more of the individual miRNAs in each cluster has been confirmed to be negatively associated with cancers due to their potent tumor-suppressive functions[67,172–174].

Until recently, transgenic *D. melanogaster* generation was accomplished using technologies such as the Flp-FRT or the GAL4-UAS systems that mediated successful knock-in or knock-out of miRNA genes in a spatio-temporal pattern. The transgenic *D. melanogaster* models so generated have contributed immensely towards identifying the roles of novel miRNAs in cellular functions of flies, and miRNAs that have a potential role in human cancers. However, the state-of-the-art technology for modeling human cancers *in vivo* is the CRISPR-Cas9 system, which in a recent study was successfully used to knock-out miR-219 and miR-315 in *D. melanogaster*[168]. Although the roles of miR-219 and miR-315 were not evaluated biochemically in the mutant flies, other investigations have reported that miR-219 is an essential neurodifferentiation factor[175–177], and is suppressed in several human cancers, of which the reduced expression ultimately drives the acquisition of tumorigenic properties via diverse mechanisms[178–181]. Regardless, this pioneering study was the first to highlight

the power of the CRISPR-Cas9 technology in developing transgenic *D. melanogaster* models to study miRNA *lof*, which will likely lead to new and innovative miRNA functional studies.

D. melanogaster have proven to be an instrumental models in understanding the fundamentals of miRNA biogenesis including studies identifying the molecular mechanisms of components such as *dicer*[182] and *locquacious* (TRBP homologue)[183]. Indeed, studies in *D. melanogaster* validated that certain miRNAs remained conserved across evolutionary history, such as *let-7*[184]. However, due to the failure to find orthologous miRNAs for *bantam*[185] and miR-14[186], and the lack of common targets or overlapping pathways regulated by miRNAs such as miR-7, current miRNA studies are mostly being conducted in other model systems discussed in this review. This difference in miRNA conservation among flies and humans may be as a result of evolutionary canalization. Evolutionary canalization suggests that *D. melanogaster* encoded miRNAs are constantly evolving, acquiring distinct properties, and gaining robustness in their conspicuous functionalities[40,65,66,187,188] (Review[189]).

2.3. *Danio rerio* (Zebrafish)

A model system that has been, and continues to be a major contributor in elucidating the functional role of miRNAs in both normal and cancerous cells, is zebrafish. Ever since *in silico* predictions identified *let-7* as a conserved miRNA across species, additional miRNAs initially identified in zebrafish have displayed a striking homology in composition and function to miRNAs encoded by humans and other vertebrates[14,125]. *D. rerio* has been a useful model to conduct miRNA-based studies because of the homology with human-encoded miRNAs and additional features of zebrafish such as i) the small size, an adult zebrafish is about 2–3 cm in length, ii) the large brood size, a female lays about 100 eggs every 2–3 days, iii) the ability to easily visualize the forming embryos since fertilization is external, and iv) the short 3 month generation time of the progeny. Additionally, due to an intact angiogenic and immune system, and a well-developed organ system, successful engraftment of human cancer cells into zebrafish embryos has enabled tumor growth in the host microenvironment. Thus, zebrafish have also demonstrated to be successful model systems to assess tumoral response to anti-cancer treatments *in vivo*[190,191]. Taken together, although zebrafish is a simplistic model system, the compelling features of *D. rerio* have contributed towards the use of this organism to better understand the effects of aberrantly altering the endogenous levels of certain miRNAs[190,191]. Without question zebrafish has proven to be a successful model system that has led to the identification of multiple miRNAs via basic biochemical and molecular studies and holds immense potential to be utilized as a model to identify clinically relevant miRNAs (Figure 5).

2.3.1. Generation of transgenic *D. rerio* for use in miRNA functional research

—There are multiple ways to generate transgenic zebrafish via microinjection at the one or two cell stage. In addition to its use in flies, the previously described Gal4-UAS system via P-element mediated transposition has been used to conditionally express transgenes in zebrafish[166,167,191,192] (Figure 2 and 3). With regard to miRNA overexpressing lines, injection of a plasmid or a linearized transgene inclusive of the miRNA driven by a

constitutive or conditional promoter[192], or injection of miRNA mimics[193] are common strategies used to overexpress a miRNA.

However, most of the zebrafish lines generated for studying the function of a miRNA have been created using a reverse genetics approach via knock-out or knock-down strategies. The tools used to knock-out miRNAs include i) Transcription Activator-Like Effector Nuclease (TALENs)[194] or ii) the CRISPR-Cas9 system[195]. Whereas, most reported knock-down studies make use of i) synthetic anti-sense RNA-analogues called morpholinos[196], or ii) heavily modified anti-sense RNA oligonucleotides, Locked-Nucleic Acids (LNA)[197] (Review[191]). Additional approaches include combinations of the above-mentioned strategies incorporating inducible vectors, or the use of Cre-LoxP and Flp-FRT systems (Figure 2 and 3).

2.3.2. *D. rerio* as a model for studying the function of miRNA in cancer—

Zebrafish serve as a model that is simple for understanding the basic mechanisms of miRNAs in human diseases such as cancer[14,67], similar to *C. elegans* and *D. melanogaster.* However, *D. rerio* is evolutionarily closer to humans[14,67]. One of the founding miRNAs, *let-7*, is absent in zebrafish embryos during the first ~48 hours post fertilization. Overexpressing *let-7* during this critical time in zebrafish embryos causes severe developmental defects. However, upon countering the overexpressed *let-7* with morpholinos, the defects are reversed. The absence of *let-7* during the first ~48 hours of development, but continuous expression until adulthood describes the heterochronic nature of *let-7*[198]. This observation made in invertebrate models is also typical in the case of vertebrates[197,198]. However, due to ethical concerns, similar experiments cannot be conducted in human embryos, therefore, the temporal nature of *let-7* in humans has yet to be validated. Importantly, *let-7* family members are highly conserved between zebrafish and mammals. There are eleven mature *let-7* miRNAs expressed in zebrafish while in humans there are nine. Hence zebrafish is an excellent model to begin to dissect the individual contributions of miRNA family members. This observed conservation also suggests that *let-7* family members may display redundancy in activities during vertebral development[14,67]. Although *let-7* family members are implicated as tumor-suppressors, functional redundancy due to the presence and expression of other family members presents a challenge towards precisely discerning the function of each miRNA in the *let-7* family. Currently, researchers using the CRISPR-Cas9 system have successfully generated knockouts of each of the *let-7* family members in zebrafish. Since the knockouts are viable, these zebrafish transgenics may be useful to uncover the functions of individual members of the *let-7* family. However, detailed biochemical characterization of the individual knockouts has yet to be performed *in vivo*[199].

The finding that *let-7* is conserved in zebrafish, but that *let-7* does not have a role in early zebrafish development has prompted researchers to identify other miRNAs that contribute to zebrafish developmental. One groundbreaking contribution to the miRNA field using zebrafish was the elucidation that miRNAs are dispensable for cell-fate determination, despite their indispensable roles in highly related cell-fate specification, tissue, and organ-formation[197]. Using microarray analysis conducted on whole organisms at different stages of development, it was determined that most miRNAs are not expressed during the first 12 hours post fertilization; however, heightened expression is observed post organogenesis at 96

hours. More detailed *in situ* analysis showed that during development many miRNAs are expressed in a tissue-specific manner[197]. For example, in *Dicer* mutant fish with global miNRA downregulation, the importance of miR-430 in brain morphogenesis was demonstrated. Mimics of the miR-430 family were injected into one-cell stage *Dicer* mutant embryos, which successfully rescued the defective brain morphology of *Dicer* mutant fish[193]. This investigation also highlighted that mature miRNA mimics can form functional miRISC complexes in the absence of active Dicer, shedding light on an unknown mechanism of miRNAs biogenesis with immense applicability in therapeutics. Another significant conclusion drawn from this study was that miRNA expression can be discriminated based on specialized cell-types within a specific organ. For example, miR-217 and miR-7 are highly expressed in exocrine and endocrine cells of the pancreas, respectively[197,200,201]. This observation solidified the notion that miRNAs are not always required for cell-fate determination, but may also be crucial for cellular differentiation, tissue formation, and maintenance of tissue-identity in a whole organism.

With the findings from zebrafish research that miRNAs are involved in differentiation, tissue formation, and maintenance of tissue identity, zebrafish have indirectly contributed to the characterization of tumor-suppressive miRNAs that are frequently lost in poorly differentiated human cancer cells originating from a specific organ. For example, developmental studies performed in zebrafish demonstrated that miR-122 is a liver-specific miRNA that is only expressed during organogenesis[197]. Analysis of miR-122 in mouse models of liver cancer and in human hepatocellular carcinoma (HCC) patients verified that miR-122 is liver-specific and functions as a tumor suppressor; loss of miR-122 correlates with aggressive HCC and poor prognosis[202]. Similar, although slightly more controversial results were found for miR-126. MiR-126 was shown to be specifically expressed in differentiating endothelial cells during zebrafish organogenesis[197]. To dissect the function of miR-126 in endothelial cell biology, morpholinos knockdown of miR-126 confirmed that miR-126 is essential for several aspects of endothelial cell biology including cell survival, migration, tissue organization, and vascular integrity and stability[203]. This study suggests that enhancing the expression of miR-126 in endothelial cells may inhibit migration and invasion of tumor cells through a well-integrated endothelium by enhancing endothelial cell-differentiation. However, additional contradictory reports in multiple human cancers also suggest that overexpression of miR-126 in cancer cells may induce proliferation of tumor cells via increased vascularization of tumors[204]. Therefore, more comprehensive studies are required to further delineate the role of miR-126 in human cancers.

An additional miRNA identified in zebrafish that showed tissue specificity was miR-200[197]. MiR-200 was determined to be involved in the development of sensory organs of epithelial origin in both zebrafish and mouse[205]. Functionally, loss of miR-200 in zebrafish during organogenesis results in the generation of embryos with underdeveloped olfactory neurons, due to terminal differentiation of olfactory progenitor cells. This finding identified that expression of miR-200 is critical for the development of sensory epithelial in zebrafish through preventing differentiation[205]. Consequently, several studies confirmed that loss of expression of miR-200 family members is responsible for epithelial-to-mesenchymal transition (EMT) of multiple human cancer cells[206].

In a more clinical context, a recent study used zebrafish to assess the emerging role of exosomes as vehicles for drugs delivery. Exosomes are an integral mode of cellular communication, and a mechanism that is often hijacked by cancer cells. Resent literature suggests that one of the major macromolecules contained within exosomes that mediate the cancerous phenotypes that exosomes promote are miRNAs[207,208]. Therefore, current preclinical research is being directed towards exploring the potential use of exosomes as *in vivo* drug delivery vehicles for conventional therapeutics and for delivery of therapeutic miRNAs. In this pioneering study, DiD labelled human brain cancer cells were xenotransplanted in the zebrafish brain ventricle to generate a model for primary glioblastoma-astrocytoma. Following which, exosomes derived from mouse brain endothelial cells were loaded with a fluorescently labelled drug –doxorubicin, and the loaded exosomes were injected into the cardinal vein of zebrafish embryos. *In vivo* fluorescent imaging confirmed penetration of the blood-brain barrier (BBB). Moreover, the therapeutic effects of doxorubicin were confirmed by a reduction in vascular endothelial growth factor (*VEGF*) mRNA levels, and a dramatic reduction in size of the xenotransplanted brain cancer cells[209]. These studies provide evidence that exosomes can be used as efficient drug delivery systems, at least in a simple model system and that the exogenously added non-self-exosomes do not produce an inflammatory response . This research also opens avenues to assess exosome-mediated delivery of miRNAs that have therapeutic potential, specifically as anti-cancer drugs using zebrafish as a model system.

Zebrafish is by far the simplest model system that contains a well-developed blood circulatory system that can help to recapitulate the presence of the tumor microenvironment. Therefore, *D. rerio* has an immense potential to contribute towards a better understanding of the role of miRNAs and exosomes in influencing the tumor microenvironment and their potential altered behavior in a model with an intact immune system. Thus, based on the contributions that zebrafish research had in unfolding the functions of a few crucial miRNAs in cancer, it can be accepted that zebrafish is a powerful model organism and further studies using zebrafish will likely result in more breakthroughs in the field.

2.4. *Mus musculus* (mouse)

Considerable progress in miRNA research can be attributed to the use of the previously described *in vivo* models. However, the ability to closely recapitulate human cancers in mouse models, and the conservation between humans and mice suggest that studies in mice provide the most meaningful insights on the role of miRNAs in the molecular pathogenesis of human cancers. Evolutionarily, ~60% of mouse miRNA loci are conserved between mouse and humans[125,128]. Additionally, mice are widely used because i) they are smaller in size than other mammals that are closer in evolutionary history to humans, ii) are relatively inexpensive and easy to maintain, and iii) they produce a fairly large number of offspring in a reasonable amount of time. The use of transgenic mouse models to evaluate the contribution of miRNAs in cancers has proved to be a robust and experimentally tractable system. Recent developments in the field have resulted in the generation of new mouse models that better recapitulate the clinical outcomes of patients treated with various therapeutics than previously used conventional mouse models (Reviews [210,211]), and

therefore current efforts are also being directed towards the development of useful mouse models to evaluate miRNAs therapeutics pre-clinically.

2.4.1. Generation of transgenic *M. musculus* for use in miRNA functional research

(i) Genetically Engineered Mouse Models (GEMMs) generated via transgenesis: The most simple and straightforward method to generate a genetically engineered mouse model (GEMM) entails microinjecting a transgene into the male pronucleus of a fertilized egg, followed by transplanting the fertilized egg into a pseudopregnant female to generate offspring expressing the randomly incorporated transgene, at variable copy numbers. Littermates that have successfully incorporated the transgene into the germline are screened and crossed to generate homozygous mice. Generation of transgenic mice using this method represents the first generation of GEMMs expressing a transgene that is expressed from an exogenous promoter or an enhancer element, resulting in constitutive or tissue-specific overexpression of the transgene[212]. Knockout models using this method are accomplished using DNA cassettes, or knockdown of an endogenous protein-coding or miRNA gene via transgenesis of shRNAs or miRNA sponges, respectively (Reviews[210,213,214]) (Figure 2).

(ii) GEMMs generated via homologous recombination: To exchange an endogenous gene via site-specific homologous recombination gene-targeting vectors containing the gene of interest and a selectable marker, flanked by homologous DNA sequences of insertion, are transfected *in vitro* into embryonic stem (ES) cells isolated from a blastocyst. ES cells are utilized to generate GEMMs because they are pluripotent and thus retain the capacity to generate into any cell type, including cells of the germline. Additionally, ES cells maintain a normal karyotype in culture post *in vitro* gene manipulation, and exhibit a higher rate of homologous recombination. The genetically engineered ES cells are then re-implanted into the blastocyst of a surrogate female to generate chimeric mice. Chimeric appearance of the animals' coat is indicative of successful incorporation of the gene-targeting vector. Chimeras are bred to generate germline transmitted GEMMs. GEMMs with knock-in, knock-out, or conditionally overexpressed genes are successfully generated with this method (Reviews[210,213,215])

2.4.2. Strategies utilized to generate gene-targeting vectors—

A variety of strategies have been employed to fine-tune the design of gene-targeting vectors that are used to recapitulate human cancers in multiple model systems. The application of gene-targeting vectors used to uncover the *in vivo* functions of miRNAs involved in the development of multiple human cancers have undeniably advanced this field of research. The most common and current technologies used to design gene-targeting vectors for generating GEMMs via homologous-recombination of these vectors into ES cells, are described in this section.

i) Cre-LoxP system: The Cre-LoxP system is derived from the bacteriophage, Coliphase P1. Cre, cyclization recombinase is a 38-kDa site-specific DNA recombinase that specifically recognizes the 34-bp sites of LoxP, locus of X-over of P1, enabling site-specific recombination. The interaction of Cre with paired LoxP sites results in excision or inversion of a DNA fragment, depending on the same or opposite orientations of the LoxP sites,

respectively. LoxP sites can be located in *cis* surrounding a specific gene, or can be located in *trans*, where the two LoxP sites are located in separate areas of the genome. One of the advantages of using the Cre-LoxP system is the ability to manipulate the transgene *in vivo*. The Cre-LoxP system can be utilized to knock-out or knock-in a transgene constitutively, or conditionally with both spatial and temporal control. Incorporation or excision of a reporter gene cassette, such as LacZ or GFP can be included to indicate successful loss or gain of function of the gene. To generate a tissue-specific conditionally expressing transgenic model either a tissue-specific Cre expressing line is crossed with a constitutively floxed line, i.e. LoxP-transgene-LoxP line, or Cre is expressed in the specific tissue of the floxed line via administration of Cre expressing lentivirus or adenovirus (Reviews[192,210,213,215,216]) (Figure 3).

ii) Flp-FRT system: The Flp-FRT system uses the yeast Saccharomyces cerevisiae derived flippase (Flp) recombinase that allows site-specific recombination with a pair of 34-bp Flp recombinase target (FRT) sites that flank the transgene or a reporter gene cassette. This system is analogous to the Cre-LoxP system, and has made marked contributions towards generation of GEMMs (Reviews[214,215,217])(Figure 3).

iii) Inducible systems: An inducible system is a tremendously powerful technology that enables a researcher to precisely control the expression of a transgene that can allow for a more accurate recapitulation of human cancer development and progression. Moreover, knock-out GEMMs generated using inducible systems as opposed to the conventional knock-out of a developmentally essential gene, bypass the potential lethal consequences that may be observed at early developmental stages. Inducible systems also allow for evaluating oncogene addiction in tumor maintenance, and effects of oncogene ablation on tumor progression. An inducible transgene can be generated by multiple mechanisms, however, in this review, a few of the widely used inducible systems to generate GEMMs will be focused on.

a) Tet ON/OFF system: The Tetracycline (Tet)-inducible system is typically used to turn a transgene on or off. The Tet technology is a binary system that includes tetracycline controlled transcription factors (Tet transactivator (tTA) or reverse tTA (rtTA)) and an operator sequences of the bacterial Tet Operon (TetO). TetO is fused upstream of the transgene, and when crossed with a tTA expressing line, a Tet-OFF circuit is generated such that the tTA is unable to bind TetO in the presence of tetracycline (Tet), or the less toxic derivative doxycycline (Dox). On the contrary, the Tet-ON system is generated when a TetO-regulated transgene expressing line is crossed with a rtTA line. In this case the transgene function only occurs in the presence of Tet or Dox. Dox bound rTtA binds to the TetO inducing transgene expression. When Tet or Dox is withdrawn from the diet rtTA ceases to bind to TetO, terminating the downstream transgene expression (a similar but converse mechanism occurs with the Tet-OFF system). Therefore, the reversible nature of the Tet-ON/OFF systems under the control of a tissue-specific promoter, and/or in conjugation with Cre-LoxP or Flp-FRT has been especially instrumental in modeling spatially and temporally controlled gene expression in various model organisms (Review[213,215]) (Figure 3).

b) Cre-ER(T) system: Another powerful tool that has wide applications in reversibly controlling transgene expression is the Cre-ER(T) system. The Cre-ER(T) system uses a mutated Estrogen receptor (ER)-ligand binding domain fused to Cre recombinase. The Cre-ER(T) fusion protein is expressed constitutively, but remains sequestered in the cytoplasm unless hydroxytamoxifen (OHT) is added. OTH allows Cre-ER(T) to translocate into the nucleus where it acts on the transgenes. Thus, the Cre-ER(T) system can allow for gene expression in either a tissue-specific manner or constitutively via local or systemic administration of OTH, respectively. When a Cre-ER(T) line is bred with a line containing a LoxP flanked gene of interest, the Cre-ER(T) fusion protein can be temporally induced via OHT resulting in translocation of Cre-ER(T) into the nucleus to exert homologous recombination at that specific site (Review[213,215]) (Figure 3).

The above mentioned Cre-LoxP and Flp-FRT systems have been created in complex combinations with the inducible systems to generate gene-editing vectors that are not only spatially and temporally controlled, but also enable reversible expression of the gene of interest (Figure 3). A few examples of mouse models that have been used to study the function of miRNAs in cancers have successfully been generated using these technologies. Specific use of these systems are highlighted in the following section.

2.4.3. Using *M. musculus* for miRNA functional studies in cancer—The first evidence of a mouse that spontaneously developed a cancer homologous to humans was the New Zealand Black (NZB) mouse strain that developed CLL. Similar to the molecular alterations occurring in human CLL patients, these NZB mice had lost the *mir-15~16 cluster*[43,124,126,218]. This discovery was the founding premise of miRNA involvement in cancer and demonstrated the power in using murine models to gain a better understanding of the contribution of miRNAs in carcinogenesis. Moreover, using this naturally occurring model of CLL, it was determined that exogenous delivery of miR-15~16 to NZB derived malignant CLL cell lines could reverse CLL phenotypes through the induction of apoptosis[43,124,126,127,218]. This investigation shed light on the importance of modeling cancers in appropriate organisms and on the use of mouse models to evaluate miRNAs with potential therapeutic application.

Discovering that loss of the miR-15~16 miRNA cluster in both mice and human patients was driving CLL, resulted in an exponential increase in the identification of miRNAs that are misregulated in cancer. This fueled *in vivo* studies to determine if these misregulated miRNAs had a significant role in promoting or maintaining disease. Initially focusing on the miR-15~16 cluster, both constitutive and conditional knock-out mouse models for *mir-15a~16-1* were generated. A GEMM containing a floxed *mir-15a~16-1* locus was crossed to mice constitutively expressing Cre to generate *mir-15a~16-1*$^{+/-}$chimeric mice. To conditionally knock-out *mir-15a~16-1* exclusively from B-cells, *mir-15a~16-1*$^{flox/+}$ mice were crossed with CD19-Cre transgenic mice. Intercrossing the F1 chimeras generated constitutively null mice, or mice with *mir-15a~16-1* deleted only in B-cells[218]. Both models demonstrated manifestations of CLL phenotypes, and cellular and molecular alterations in the models displayed a striking resemblance to human CLL, depicting the accuracy of transgenic mouse models to study the development and stage of miRNA-mediated cancer progression.

Following the generation of robust models used to evaluate the loss of expression of tumor suppressive miRNAs, the oncogenic potential of the first speculated oncomiR, *oncomiR-1* was explored. *OncomiR-1* is a polycistronic gene that gives rise to a single transcript containing seven miRNA precursors, commonly known as the miR-17~92 cluster[101]. In patients suffering from various hematologic cancers, the genomic locus of *mir-17~92* was observed to be amplified, and has since been extensively investigated via bioinformatic and biochemical approaches to confirm its oncogenic property[84,100,219–221]. Although initially identified as a oncomiR in hematopoietic malignancies, miR-17~92 is also involved in the development of solid cancers such medulloblastoma[222], and hepatocellular[223] and malignancies of the lung[224] and breast[225]. Due to its pleotropic role in various malignancies, modeling the cluster in mouse models was a high priority. Since the knock-out model of *mir-17~92* produced via crossing floxed *mir-17~92* with Actin-Cre resulted in mice that suffered post-natal lethality due to severe birth defects[83], multiple conditional knock-outs and overexpression GEMMs were generated to investigate the oncogenic role of miR-17~92[84,100,104]. Targeted overexpression of the entire miR-17~92 cluster, specifically in B-cells using B-cell specific Ig-heavy chain promoter Eμ-enhancer, resulted in severe B-cell lymphomas and leukemias[104]. To elucidate roles for the individual miRNAs in the cluster, transgenic mice were generated that overexpressed each individual miRNA in the miR-17~92 polycistron. The results revealed that miR-19 is sufficient to exert the oncogenic potential of *oncomiR-1*[84]. MiR-92 overexpression, on the other hand, counters the effects of miR-19 in oncogenesis via a feedback mechanism[102,103]. Through the extensive use of mouse models, it is now evident that there exists a complex but delicate balance between miRNAs that are simultaneously expressed, but that may function individually as either potent oncomiRs or tumor-suppressive miRNAs. The balance in expression of such antagonistically functioning miRNAs likely plays a very critical role in maintenance of normal cellular physiology.

Very few phenotypes have been observed in mouse models following altered expression of a single miRNA. Indeed, overexpression of the miR-17~92 cluster or loss of the miR-15~16 cluster can both promote tumorigenesis, but in both cases, multiple miRNAs were altered. The miRNA field was further revolutionized following evidence that overexpression of either miR-21 or miR-155 is sufficient to induce tumorigenesis without the contribution of other oncogenic alterations[106,107]. For the first time, it was proven that hematologic cancer maintenance is dependent on a single oncomiRs, such that reducing expression of the miRNA results in cancer regression. In these studies, a transgenic vector expressing the pre-miRNA was placed downstream of a floxed STOP cassette under the control of a Tetracycline promoter, i.e. *miR-21*^{LSLrTA}. In the absence of doxycycline, the miRNA is not expressed. To conditionally express the transgene in the lymphoid tissues the miRNA lines were crossed to Nestin-Cre mice resulting in STOP cassette excision, overexpression of the miRNAs, and development of pre-B cell lymphomas. However, upon impregnating the mouse chow with doxycycline, there was a rapid regression of pre-B cell lymphomas and an increase in survival. While the miR-21 study was the first to show that tumors can be addicted to changes in miRNA expression, the miR-155 report reconfirmed the role of miR-155 in lymphoma as initially reported by the Croce group[106–108].

While these aforementioned studies focused on overexpressing or knocking out miRNAs independently of other genetic alterations, additional *in vivo* evidence has determined that miRNAs cooperate with both protein-coding genes and with each other and thus, suggests that miRNA-based therapeutics might have a place in the clinic. For example, miR-21 was found to enhance lung cancer in the inducible autochthonous model driven by the proto-oncogene, Kras, $Kras^{Lox\text{-}Stop\text{-}Lox(LSL)\text{-}G12D/+}$. In the absence of $Kras^{G12D}$ expression, miR-21 was insufficient to induce oncogenesis in the mice. However, following $Kras^{G12D}$ transgene expression, significantly more tumors were observed, compared to the $Kras^{G12D/+}$ control mice[226]. Similarly, in the pancreatic autochthonous $KRAS^{G12D/+}$ model it was suggested that miR-21 may be involved in pancreatic cancer development via a multi-step process[227]. Confirmation that miR-21 was essential for pancreatic cancer maintenance was verified in animals that were orthotopically transplanted with pancreatic ductal adenocarcinoma cells (PDACs). A single dose of intratumoral administration of lentiviral vectors expressing antisense-miR-21 severely impaired tumor cell growth via onset of necrosis due to miR-21 depletion[228].

The use of protein-coding transgenic models have also contributed to the miRNA field, specifically to evaluate miRNA-based therapeutics. For example, the $KRAS^{G12D/+}$ autochthonous lung cancer model was used to evaluate the tumor-suppressive roles of miR-34a and *let-7b,* and to assess their therapeutic efficacies. To this end, $Kras^{G12D/+}$ mice were tail-vein injected with synthetic formulations of each of the miRNAs (miR-34a or let-7b), or orthotopically administered adenoviral-encoded *let-7a*. The resulting regressed tumors demonstrated that each of the individual tumor-suppressive miRNAs could act as potential therapeutic agents[157,159,229]. Following this, additional autochthonous NSCLC mouse models have confirmed that combinatorial RNA therapeutics produce even greater effects than treatment with an individual miRNA, some of these studies include the use of miR-34 combined with *let-7b*[156], or miR-34 and an siRNA targeting *Kras*[230].

The above studies proved the tumor suppressive role of *let-7* through the use of exogenous *let-7*, however, the endogenous tumor suppressive activity of the *let-7* family in a transgenic model was only recently demonstrated. To delineate if *let-7* suppresses the Myc-driven tumorigenesis process, *let-7g* and Myc were simultaneously overexpressed using a triple transgenic, liver-specific, tet-off system. *Let-7g* was successfully overexpressed despite the high levels of Lin28 by innovatively cloning the mature *let-7g* sequence into a miR-21 stem-loop, retaining the miR-21 loop, which prevents Lin28B-mediated processing inhibition of *let-7* family members. Myc overexpression led to tumorigenesis in the absence of *let-7g* expression; however, proliferation and growth were markedly reduced when *let-7g* was overexpressed. This study also showed that *let-7* is transiently repressed in tissues undergoing repair and regeneration as the cells require enhanced proliferation. Further, through evaluating a conditional liver-specific knock-out of *let-7b* and *let-7c2,* investigators proved that reduced *let-7* levels resulted in higher liver mass relative to control, due to increased mTOR signaling activity. This study demonstrates that *let-7* is expressed in liver tissue, likely to suppress the development of liver cancer, but that controlled balance of *let-7* levels is the key to maintaining the regenerative capacity of liver[231].

Multiple studies have identified *let-7* as a potent tumor suppressor, and the consequences of *lof* of *let-7* results in loss of cellular differentiation, increased proliferation, and tumorigenesis. However, with the growing evidence that *let-7* is an important tumor suppressor, the mechanisms involved in *lof* of *let-7* has become a subject of interest in the field. Early studies evaluating *let-7* led to the intriguing finding that loss of mature *let-7* is not always associated with changes in transcription of the *let-7* gene, which encouraged characterizing *let-7* at the post-transcriptional level. With the ground-breaking finding that RNA-binding proteins such as LIN28A and B selectively inhibit *let-7* miRNA biogenesis[112–115], *in vivo* studies were conducted to delineate the role of LIN28B in *let-7* mediated tumorigenesis. Colon cancer cells constitutively expressing LIN28B were implanted into immunocompromised mice. Biochemical evaluation of the tumors indicated increased levels of endogenous Lin28B, which strikingly negatively correlated with *let-7* levels in tumor cells relative to surrounding normal cells[232]. This *in vivo* correlative study, along with additional cell-based and molecular assays confirmed the negative feedback loop that exists between *let-7* family members and Lin28A/B[112,113,232–234]. In transgenic mouse models expressing Lin28B under the *Vil1* promoter, which drives expression specifically in the intestines, tumorigenesis was directly dependent on the loss of mature *let-7*[233]. High Lin28B negatively correlated with the levels of *let-7*, whereas rescuing mature *let-7* levels led to reversion of tumorigenic phenotypes[233]. This study, followed by others suggests that miRNAs are downregulated by various mechanisms in the process of tumorigenesis[25–27,33,233]. For example, Myc, an important oncoprotein that upregulates the *oncomiR-1* cluster (*mir-17~92*) is predominantly associated with widespread depression of miRNA expression in humans and in cells obtained from mouse models of lymphoma through direct interaction with miRNA promoters [33].

While both MYC and LIN28 are involved in downregulation of a subset of miRNAs, global miRNA downregulation was identified as a common feature in human tumors, which could not simply be explained by MYC and LIN28. Thus, experimental designs turned to evaluating major components of the miRNA processing machinery. In mouse models where the *Dicer1* locus was floxed, Dicer1 was identified as a haploinsufficient tumor suppressor[235]. A single copy of *Dicer1* was necessary for tumorigenesis. Interestingly *DICER* is also lost in human cancers, and similar to the mouse model, only one allele is deleted[236]. Prior to this work multiple studies suggested that genetic mutations in components of the biogenesis machinery, such as DROSHA, DICER, and XPO5 may severely dysregulate miRNAs leading to cancer[237,238]. However, with the use of robust murine models, the anticipated pathophysiological consequences of a disrupted biogenesis pathway on global miRNA depression resulting in tumorigenesis is now regarded as a hallmark of cancer. With the growing understanding that miRNAs are globally downregulated in cancers, innovative targeting vectors have been generated to directly ablate several evolutionarily conserved miRNAs in mouse models, and to clone in reporter constructs downstream of the endogenous promoter to identify both temporal and spatial expression patterns. The targeting vectors contained floxed pre-miRNA sequences that were placed downstream of a FRT flanked promoter-less LacZ reporter to generate LacZ-STOP(Neo)-floxed miRNA transgenic vectors. Embryos generated by crossing LacZ-Neo-flox mice with Actin-Cre animals were evaluated for LacZ expression patterns in various

tissues. Data suggest that approximately one third of miRNAs exhibit a global expression pattern, whereas about two thirds of miRNAs demonstrate developmental stage or tissue-specific expression patterns. For example, miR-210 and miR-146a were undetected in earlier stages, but were expressed in the adult mouse, sub-compartmentalized in immune cells. This investigation re-confirms the spatio-temporal nature of miRNAs, as suggested by the conventional model organisms, *C. elegans* and *D. melanogaster*. The observations made from these reporter studies can also be extrapolated to appropriately select a Cre-mouse for tissue-specific ablation/expression of specific miRNAs. This idea was verified using mice expressing the transgene and crossing them to animals that temporally or constitutively expressed Cre, and phenotypic and developmental alterations were observed for a subset of miRNAs[239,240]. Overall, this strategy holds promise for uncovering the roles of independent miRNA family members in various cancers, demanding further characterization.

The advantage of using a multi-purpose targeting vector comes from the power to generate a conditional knock-out model of the miRNA of interest (Figure 2). The expression of the upstream STOP cassette in the LacZ-STOP(Neo)-floxed miRNA transgenic mouse post-embryonic stages, via Neomycin selection allows bypassing of embryonic lethality in the absence of the miRNA, without physically excising the miRNA transgene. Nevertheless, crossing the parental-transgene expressing strain with a constitutively expressed Flp-strain leads to miRNA-rescue in the progeny by excision of the FRT-flanked LacZ-STOP regions upstream of the miRNA transgene. Although these murine models have been successfully generated, they remain to be characterized, which may shed additional light on the loss of miRNAs and their re-expression in various tissues, and may aid in the quest to better understand the role of various miRNAs in tumorigenesis[239,240].

To understand the role of miRNAs in promoting metastatic potential of cancer cells *in vivo,* in a model with an intact immune system, investigators have relied on the syngeneic mouse model. In a recent study, primary and metastatic tumors from the $Kras^{LA1/+};p53^{R172H\Delta G}$ autochthonous lung cancer model were harvested, cultured, and subcutaneously injected back into the same immunocompetent host. When xenografts of cells derived from metastasized tumors were implanted in the syngeneic mice the cells metastasized, whereas animals xenografted with cells from the primary tumor only produced localized subcutaneous tumors. Upon miRNA profiling, it was observed that the metastatic tumors expressed low levels of miR-200 family members (miR-200a, miR-200b, miR-200c, miR-141, and miR-429), relative to the localized tumors. However, when the miR-200b~200a~429 cluster was overexpressed in the metastatically derived cells, only primary tumors were capable of growing as compared to control cells that metastasized to the lung, heart, liver, and kidneys[241].

3. Conclusion

Since the discovery of *lin-14*, scientists have come a long way in delineating the roles of miRNAs in higher vertebrates, including humans. Although the biogenesis of miRNAs and their biological role in maintaining normal physiology of cells have been fairly well established, this is a rapidly growing yet constantly evolving field of research. Currently,

efforts are being made to identify miRNAs that drive aberrant cellular events responsible for abnormal behavior of a cell, leading to pathogenesis of diseases such as cancer.

Initial insights into the miRNA world were furnished through the use of a simple, yet elegant model system, *C. elegans*. The miRNAs *lin-4* and *let-7* were identified through forward-genetic screens, that determined these miRNAs are temporally expressed and required for normal development of *C. elegans*[8,12,13,67]. Nevertheless, with the advent of transgenesis and the use of more complex systems with closer homology to humans, such as *D. melanogaster*, *D. rerio* and *M. musculus*, a greater understanding of miRNA biogenesis, function, and misregulation in disease has become evident. Advancements in bioinformatic, biochemical and molecular approaches have revealed that miRNAs are aberrantly expressed in multiple cancers, and that in many instances they function to promote and/or maintain the tumorigenic phenotype. The first miRNAs discovered to be involved in human disease, miR-15a/16-1, were determined to be downregulated in human CLL patients. Consequently, the discovery of NZB mouse that naturally developed CLL, and displayed severe downregulation of miR-15/16 expression resulted in the recognition that mice have the potential to serve as a robust model systems for miRNA functional studies in cancer[118,126,127].

Soon after this novel discovery and the realization that the mouse genome is ~90% homologous to that of humans, potent methods to generate GEMM prospered. These pioneering studies in the miRNA field led to implementing intriguing strategies to generate efficient gene targeting vectors that can not only replace an endogenous gene of interest (in this case, a miRNA gene), but also control its expression spatially and temporally. Various GEMMs have since been generated that have made immense contributions in uncovering the functions of numerous miRNAs as global and specific tumor-associated miRNAs, and have also supported the transition of miRNAs into the clinic. Models with intact immune systems are also be extensively evaluated as they more faithfully recapitulate human tumorigenesis and thus, are more accurate for studying cancer and therapeutic response, especially for agents targeted to cells of the microenvironment (Reviews[247,249–251]).

D. rerio or zebrafish is a vertebrate that has benefitted the research community through experiments that have helped to define the role of miRNAs as developmental factors, supported by biochemical experiments conducted in these visually traceable optically transparent embryos. Moreover, embryonic lethality, due to altered expression of critical heterochronic miRNAs is easily observed as fertilization of the egg is external, whereas in mice, lethal embryos are rapidly reabsorbed, thereby obstructing evaluation of miRNAs involved in development (Review[252]). Although zebrafish have proven to be convenient model organisms for identifying miRNA involvement in development, they have also indirectly contributed to the understanding of various aspects of cancer, including the role of miRNAs in the development of cancers. While zebrafish do not develop cancer, many of the hallmarks of tumorigenesis such as proliferation, migration, differentiation and apoptosis are conserved. As such, miRNAs ascertained through zebrafish studies have been validated and characterized in human cancer cell lines and/or in mouse models that recapitulate human cancer more closely. Mouse models, like zebrafish have gained immense recognition in modeling various human cancers, since they belong to the class of vertebrates. However, the

contributions made by invertebrates such as *C. elegans* and *D. melanogaster* have undeniably influenced this revolutionary area of research. *C. elegans* and *D. melanogaster* have been useful in dissecting the molecular machinery of miRNA biogenesis, and contributed towards the identification of miRNA-mediated alterations of cellular pathways. Such developments achieved in simple model systems have since been extrapolated to more complex *in vitro* and *in vivo* systems to understand the effect of disturbed expression patterns of critical miRNAs, leading to cancer initiation, development and progression.

Generation of transgenic mice have come a long way since using the conventional method of random integration of an exogenous DNA into the mouse genome, via non-homologous recombination. One major drawback with this method is the lack of specificity at the site of transgene incorporation and high propensity of off-targeting effects. On the contrary, the primary advantage of generating GEMMs via the contemporary methods of gene targeting vectors is that the endogenous gene becomes replaced by the transgene (contained in the targeting vector) via site-specific homologous recombination (Figure 2). This ensures that the transgene is incorporated precisely at a specific genomic location. This same method also allows for evaluating tissue-specific effects of the miRNA through integration of tissue-specific promoters in the targeting vector.

Spatially and/or temporally controlling the expression of a transgenic-miRNA has led to the discovery of miRNAs with tissue-specific or stage-specific roles in the development of cancers. A powerful contribution of GEMMs is the generation of autochthonous mice that spontaneously develop cancers at the true anatomical location once the conditional-mutant allele of a known oncogene is activated. These models, as well as multiple other GEMM and xenograft models have been utilized to uncover the contribution that miRNAs make in the presence of other genetic lesions, for example miRNA involvement in potentiating the process of tumor generation or regressing tumors growth. Thus, these pre-clinical models have applications in segregating miRNAs that may have potential therapeutic applications.

The importance of the immune system on tumorigenesis can only be appreciated using GEMM or syngeneic models. Injecting tumor cells derived from autochthonous mice into another mouse of the same genetic background has established that the ability of a tumor cell to become invasive and migrate requires a complete and efficient microenvironment, including an intact and functional immune system. Therefore, the advantage of using immunocompetent mouse models such as the autochthonous and syngeneic GEMMs over the immune-deficient xenograft models is that the former possesses an intact immune system recapitulating the natural scenario of tumor initiation, development, and progression. Each of the stages of tumor development typically require cancer cells to cross-talk with surrounding stromal cells, normal cells, and the immune cells. This is the possible reason why cancer cells show metastatic potential in syngeneic models, but often not in sub-cutaneous, or PDX xenograft mouse models (Table 1).

The most common strategy in the field to predict the function of a miRNA in cancer development is to first utilize the straight-forward and basic sub-cutaneous (SC) xenograft mouse model. Advantages of modeling human cancer in SC xenograft models include the ability of cells to grow inadvertently, develop into primary tumors *in vivo,* and the ease of

tracking and measuring the tumors. Although the associated disadvantage is that the genetic, histological, and therapeutic responsiveness of the xenografted tumors is not comparable to the source human tumor. Moreover, the reduced propensity of SC xenografted cancer cell lines to metastasize *in vivo* owing to the lack of a conducive environment for the implanted cancer cells is perhaps another disadvantage that provides evidence that this model does not completely recapitulate an endogenous human cancer. Such models have immensely contributed towards a collection of preliminary evidence in understanding the effects of oncomiRs such as miR-21[106], or tumor suppressive miRNAs such as *let-7*[158] in tumorigenesis. Despite the fact that SC xenograft models demonstrate a response to therapeutic anti-sense oncomiRs or tumor-suppressive miRNA mimics, follow-up studies in more advanced models with intact immune systems are essential prior to clinical advancement [156,230,245].

On the contrary, an orthotopic model is generated when cancer cells derived from a specific anatomical location are implanted into the same location to develop primary tumors that are genetically and histologically more representative of human tumors. Orthotopic models also lack an intact immune system, similar to xenograft model systems; however, the advantage here is that the cancer cells are introduced *in vivo* in their original anatomical location where the tumor was harvested from. Thus, the interaction between cells of similar origin, in the correct anatomical location may provide a moderately conducive environment for the orthotopically developed cancer to metastasize, and respond to miRNA based therapeutics. Additionally, another xenograft mouse model system, the PDX model, when treated with certain drugs have shown to favor predictability of patient response to the same. PDX models stably maintain the heterogeneity of the engrafted human tumor in the mice for multiple passages, owing to the animals' deficient immune system. Therefore, the potential of PDX models in pre-clinical evaluation of therapeutics, personalized-treatment development and useful biomarker identification is being extensively explored. The use of PDX models has recently been extended to the miRNA field. Exosomal miRNAs such as miR-21 and miR-1246 identified in PDX models of breast cancer are predicted as *bona fide* circulating miRNA biomarkers indicative of breast cancer in patients[243]. Moreover, the therapeutic potential of replacement of miR-100 has recently been evaluated in PDX models via targeted therapy of the oncogene, fibroblast growth gFactor receptor 3 (FGFR3), in FGFR3 driven PDX tumors[242]. Therefore, although it is becoming accepted that PDX models may be applicable to developing personalized miRNA-based cancer therapies, this model is at its infancy and requires more explicit evidence to be recognized as a faithful pre-clinical model system. With the recently accepted notion that accurate tumor progression, and therapeutic response requires an intact immune system, an emerging concept of using model systems to perform pre-clinical evaluations of miRNA-based therapies or other drugs is on the cutting edge. To this end, another mouse model that deserves a mention here is the humanized mouse model. A nude mouse is humanized by engraftment of human-donor derived immune cells. This model system is not yet in practice in miRNA functional research in cancers per se, but it may be beneficial in pre-clinical evaluation of miRNA-based therapeutics, which may eventually positively impact the time-span required for translating miRNA-based cancer treatments into the clinic.

Although advancements are being made to models for use in evaluating miRNA-based cancer therapeutics at the pre-clinical stage, many contributions in the field related to uncovering miRNAs that maintain normal archetype of a cell or miRNAs that drive the process of tumorigenesis emerged from the extensive use of GEMMs. Additionally, GEMMs have also made unparalleled contributions towards the discovery of miRNAs that confer therapeutic potential as replacement therapies or as therapeutic targets. While the technologies used to generate transgenic mice has advanced remarkably innovative strategies to generate genetically engineered pre-clinical models for miRNA-based therapeutic research requires attention. It is anticipated that additional use of the CRISPR-Cas9 system[244] will support miRNA research by rapidly accelerating the process of generating GEMMs by robustly overexpressing or knocking-out a miRNA gene of interest. This will not only aid in the precise knock-out of a miRNA gene of interest to evaluate replacement therapies, but will also facilitate knock-in/out of discrete miRNA family members to finally dissect the functions of individual miRNAs that was not as easily achievable using conventional methods. The CRISPR-Cas9 system is superior in many ways to the now more traditional methods of generating GEMMs (Reviews[244,245,250,253–255]). Genetic manipulation of multiple genes can be simultaneously achieved in a single-embryonic cell, not requiring the laborious selection of embryonic stem cells post transgenic vector injection. The CRISPR-Cas9 system is capable of producing miRNA knock-in/out mouse models in four weeks, and therefore, has immense potential in miRNA functional delineation in multiple human cancers. Efforts towards generating model systems utilizing the CRISPR-Cas9 system targeting miRNA genes *in vivo* in the correct anatomical location of existing autochthonous or syngeneic models is required, which can further accelerate the fate of modeling human cancer efficiently and robustly in model organisms.

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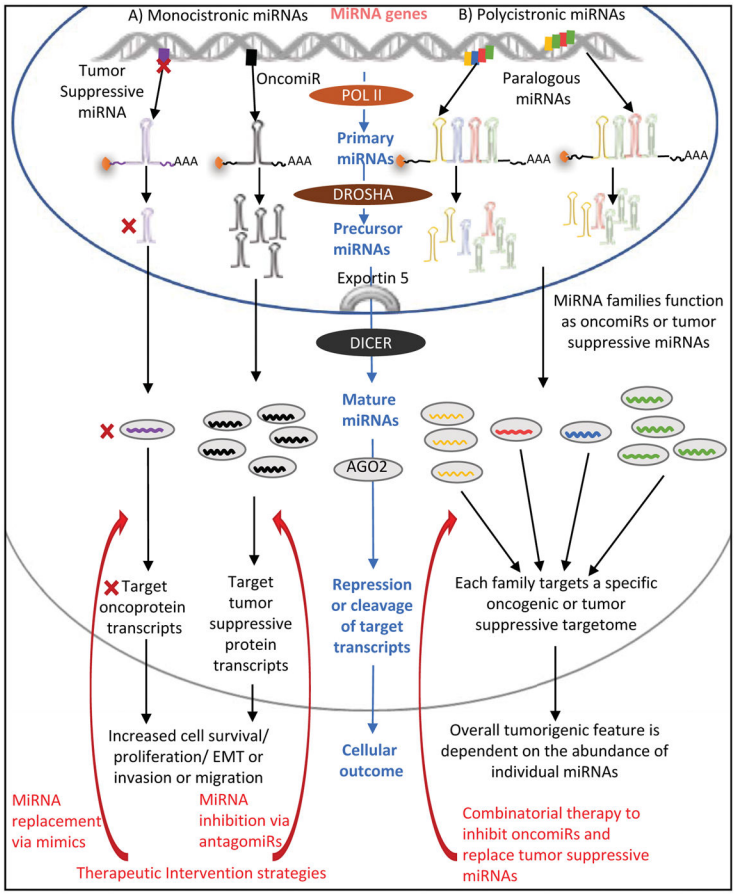

Figure 1. Overview of oncomiRs or tumor suppressive miRNAs encoded as monocistronic or polycistronic genes, their involvement in tumorigenesis, and potential use as miRNA-based cancer therapeutics

(A) A monocistronic miRNA gene encodes a transcript containing a single primary miRNA. In cancers, one mechanism to alter the abundance of a mature miRNAs is through changes in transcription of the primary miRNA, where the expression of a tumor suppressive miRNA is downregulated, while that of an oncomiR is enhanced. A tumor-suppressive miRNA typically targets transcripts encoding oncogenic proteins, therefore miRNA replacement therapies using tumor suppressive miRNA mimics are currently being tested. OncomiRs on the other hand target tumor suppressor protein transcripts, and hence their inhibition via antagomiRs is also a potential miRNA-based therapeutic strategy. **(B)** Transcription of a polycistronic miRNA gene or a miRNA cluster results in a primary miRNA transcript containing multiple miRNAs. The duplication of a cluster, and expression of a more or less intact cluster from multiple genomic loci generates paralogous miRNAs. The resultant miRNAs from paralogues can be predominantly tumor-suppressive or oncogenic; however, their function is often largely context dependent – i.e. tissue-specific, temporally regulated, etc. The potential therapeutic strategy targeting miRNAs expressed from clusters depends on the abundance of the individual tumor suppressive or oncogenic miRNAs. Combinatorial miRNA therapeutics is a potential strategy currently being evaluated to combat tumorigenesis where the altered ratio between oncogenic/tumor suppressive miRNAs drives cancer development.

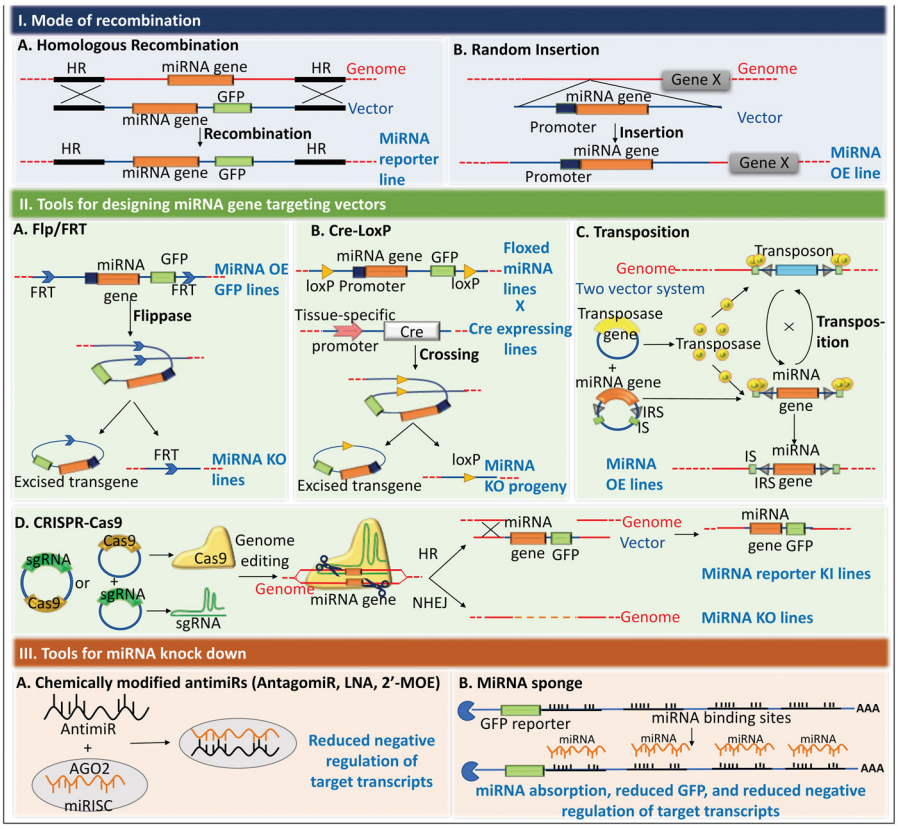

Figure 2. Strategies for the generation of targeting vector to knock-out miRNAs, and tools employed to knock-down miRNAs

(I) Modes of recombination that govern the genomic location of incorporation of the transgene. **(A)** Homologous recombination (HR) allows a site-specific insertion of the transgene via crossing-over of the specific HR sites between the genomic site and the vector, in the presence of the enzyme recombinase. **(B)** Random insertion results in incorporation of the transgene at a random site in the genome. **(II)** Various gene-editing used for the generation of gene-targeting vectors **(A)** Flp/FRT system: Exogenously added or endogenously expressed Flippase (Flp) recombinase allows site-specific recombination with Flp recombinase target (FRT) sites flanking the transgenic miRNA gene. This results in knocking-out the targeted miRNA. **(B)** Cre-LoxP system: This system is analogous to the Flp/FRT system. The Cre-recombinase catalyzes site-specific recombination between two-LoxP sites flanking the miRNA of interest, resulting in its excision and miRNA knock-out. **(C)** Transposition is the mode of transgenic vector incorporation widely used in *D. melanogaster*. This mode of insertion of transgene utilizes multiple mechanisms to insert the transgene at a specific transposon site on the genome. The mechanism here explains a two-vector system. Transposase, the enzyme that facilitates transposition is encoded by the transposase vector, and the miRNA gene to be transposed at the transposon site in the genome is encoded by the second vector, flanked by inverted repeat sequences (IRS) and insertional sites (IS), that mediate the transgene exchange with a random transposon. **(D)** CRISPR-Cas9 system: Cas9-vector and an sgRNA vector are expressed in cells. The Cas9 endonuclease associates with the expressed sgRNA, which guides Cas9 to a homologous

region in the genome to generate a double strand (ds) break. The ds break is repaired in an error-prone manner using Non-Homologous End Joining (NHEJ) or a targeting vector is inserted at the breakpoint via Homologous Recombination to generate either a miRNA knock-out or a knock-in of a miRNA and/or a reporter vector, respectively. **(III)** Strategies to exogenously or endogenously knock-down a mature miRNA (A–B). **(A)** Various chemical modifications on small miRNA-complementary oligonucleotides, double stranded or single stranded, have successfully been generated, to sequester functional mature miRNAs and inhibit their function. AntagomiRs are ssRNAs conjugated with cholesterol. Locked-Nucleotide Acid (LNA) are generated via the formation of a 2′, 4′methylene bridge in the ribose resulting in a stable bicyclic nucleotide. 2′-MOE are 2′-O-methoxyethyl phosphorothioate modified oligonucleotides. **(B)** A miRNA sponge depicted here contains multiple binding sites (6–8) for a specific miRNA in the 3′-UTR of a reporter vector. Sequestration of the miRNA results in negative regulation of the reporter and reduced regulation of the endogenous miRNA targets.

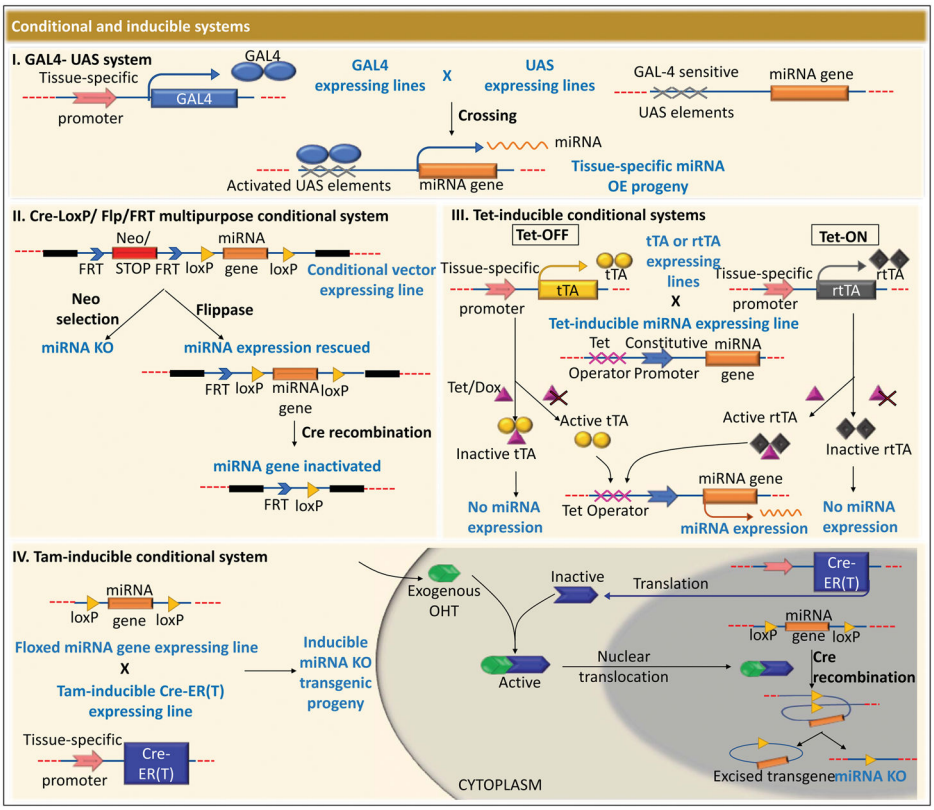

Figure 3. Conditional and inducible systems

(I) The GAL4/upstream activating sequence (UAS) system (GAL4-UAS) is an inducible system has been utilized in the generation of transgenic flies and zebrafish models. Tissue-specifically expressed GAL4 lines are crossed with a line constitutively expressing the transgene encoded downstream of a UAS element, allowing GAL4 mediated activation of UAS in a tissue-specific manner. Specific binding of GAL4 to UAS element allows the transcription of the miRNA gene, resulting in a tissue-specific overexpression (OE) of the miRNA in the offspring. **(II)** A combination of Cre-LoxP and Flp/FRT is a powerful tool to generate a multi-purpose conditional and inducible targeting vector. In this case, expression of the Neomycin (Neo)/STOP cassette generates a knock-out first vector, inhibiting the expression of the downstream miRNA gene. However, expression of Flp leads to excision of the STOP cassette through recombination of the two FRT sites, rescuing the miRNA gene expression. This system allows miRNA functional studies first in the absence of miRNA expression, following which the effects of rescuing the miRNA can be evaluated. Finally, the effects of the loss of miRNA can be confirmed upon complete inactivation of the miRNA gene, achieved via expression of Cre. **(III)** The Tetracycline-inducible systems (Tet-OFF and Tet-ON) have proved to be very versatile in the generation of transgenic model systems. Tet-OFF is mediated via the expression of the Tet transactivator (tTA), whereas the Tet-ON system is dependent on the expression of the reverse tTA (rtTA). Lines expressing tTa or rtTA in a tissue-specific manner are crossed with a transgenic strain expressing the miRNA gene under the control of a constitutive promoter incorporated downstream of a Tetracycline activated element, the Tet Operator (TetO). tTa binds to the TetO in the absence of

Tetracycline (Tet) or Doxycycline (Dox), leading to the constitutive expression of the transgenic miRNA gene, while the rtTA remains inactive and unable to bind to TetO in the absence of Tet/Dox inhibiting the expression of the miRNA gene. Upon the addition of Tet/Dox to the Tet-OFF system, tTa binds to Tet/Dox and the miRNA gene expression is turned off, whereas in the case of Tet-ON system, Tet/Dox binds to rtTa enabling it to induce the expression of the miRNA gene via direct interaction with TetO. **(IV)** Tamoxifen (Tam)-inducible conditional system is an extensively used inducible system in the generation of transgenic model organisms. A strain containing a floxed miRNA gene is crossed to a Tam-inducible Cre-ER(T) expressing line, generating an inducible miRNA knock-out strain. Cre-ER(T) is the Estrogen receptor (ER)-ligand binding domain fused to Cre recombinase, which remains inactive due to sequestration in the cytoplasm. However, upon exogenous addition of hydroxytamoxifen (OHT), the OHT-Cre-ER(T) complex translocates into the nucleus, and actively allows Cre-mediated recombination of the two LoxP sites to occur. The resulting Cre-LoxP recombination knocks-out the miRNA gene from the specific tissue expressing the Tam-inducible Cre-ER(T) vector.

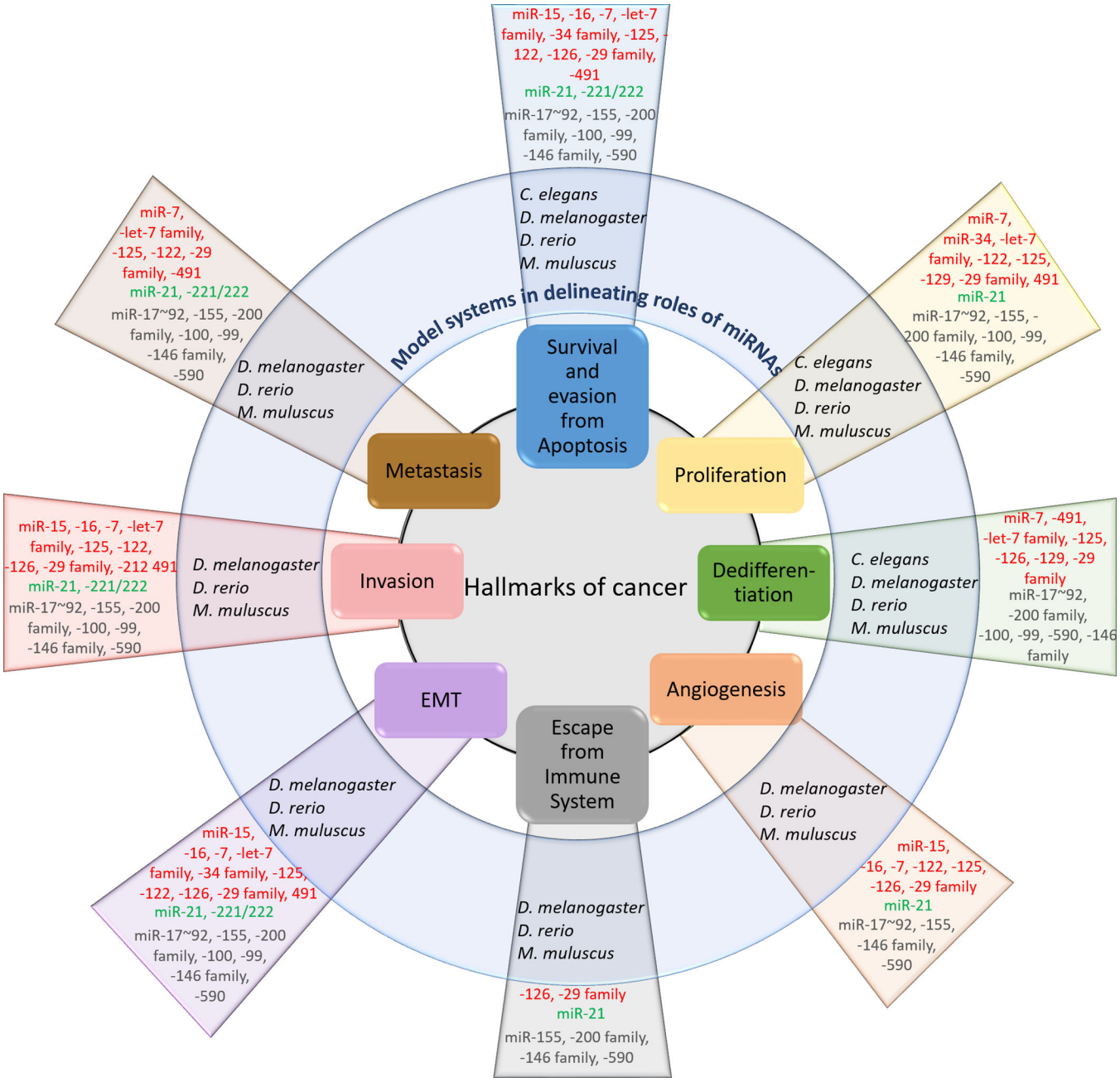

Figure 4. Functions of miRNAs in regulation of the hallmarks of cancer, identified via the use of *in vivo* model organisms

Hallmarks of cancer are the cellular processes that become severely dysregulated upon the onset of a cancer. The various model organisms, owing to their endogenous properties have been utilized to delineate the functions of the enlisted miRNAs that mediate the specific cancerous feature. MiRNAs represented in red are *bona fide* tumor suppressive miRNAs, in green are oncomiRs, while in grey represent miRNAs that, depending on their context, can function as either a tumor suppressive or oncogenic miRNA.

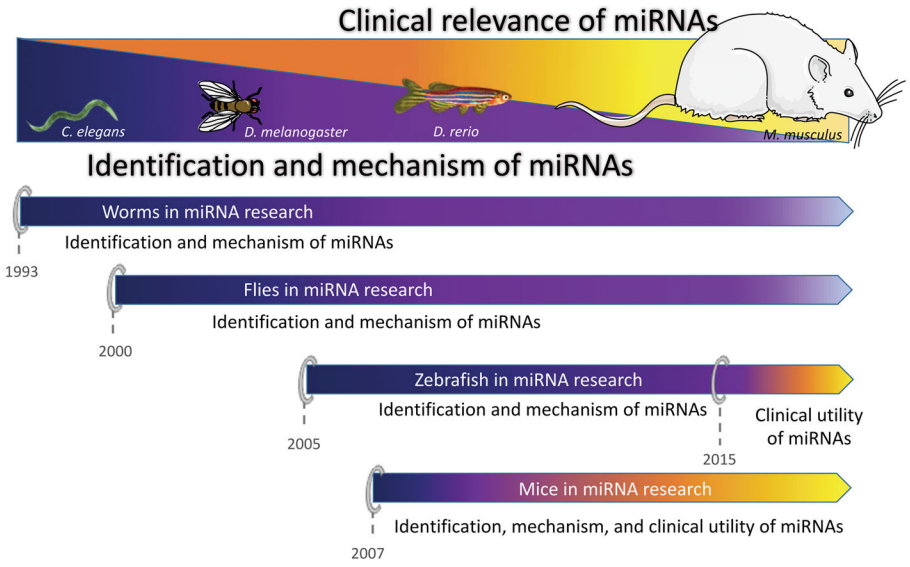

Figure 5. Utility of model systems in various aspects of miRNA research

The model systems highlighted in this review include *C. elegans* (worms), *D. melanogaster* (flies), *D. rerio* (fish) and *M. musculus* (mice). All the enlisted model systems have contributed immensely towards the identification of evolutionarily conserved miRNAs, and delineating their mechanism of action in normal cells. The simple organisms, worms and flies do not develop cancers hence they have mainly been utilized to delineate miRNA biogenesis and function. However, the more complex systems, fish and mice, with an intact immune and angiogenic system have shown immense robustness in identifying the normal functions of miRNAs, and their roles in driving tumorigenesis. Since the discovery of the first miRNA, *lin-4*, in 1993, these *in vivo* systems have come a long way, and have demonstrated their applicability as pre-clinical model organisms to predict therapeutic relevance of certain miRNAs. Illustration created using graphics from Library of Science & Medical Illustrations (http://www.somersault1824.com/science-illustrations/) and Servier Medical Art (http://www.servier.fr/servier-medical-art).

Table 1

The various mouse models that have been used to identify miRNAs that contribute to tumorigenesis.

Mouse Model	Immune status of mouse background	Generation Method	Applications
Spontaneous Models	Immune competent	Spontaneously occurring tumors[127].	To identify miRNA genes that are lost or amplified in nature, resulting in the development of cancers.
Xenograft Models	Immune deficient mice – SCID/NOD-SCID, NOG/NSG, RAG	Sub-cutaneous injection of human tumor-cell lines[106].	To evaluate *in vivo* oncogenic or tumor-suppressive potential of miRNAs. To determine delivery and efficacy of miRNAs that have therapeutic potential.
		Orthotopic implantation of human tumor-derived cells or cell lines[228].	The above applications. And, orthotopic xenograft mice also serve as a model for metastasis of tumor cells, therefore, they can be utilized to delineate miRNA functions in invasion and metastasis.
Patient-Derived Xenograft (PDX) Models	Immune deficient NOD-SCID mice	Primary tumor engraftment[242,243]	To maintain tumor heterogeneity, and allow personalization of treatment. Additionally, evaluate exosomal miRNAs released from tumors, and evaluate miRNAs that may have therapeutic potential.
Genetically Engineered Mouse Models (GEMMs)	Immune competent	Autochthonous mouse[156,230] – conditionally activated mutations in transgenic oncogenes that result in endogenous tumors evolving spontaneously from normal cells in the correct anatomical location	Evaluation of spontaneous tumors to identify miRNAs that drive tumorigenesis in normal cells. Evaluation of miRNAs with therapeutic potential in tumors arising in endogenous microenvironments.
		Transgenic mouse – Random incorporation of transgene, to overexpress or knock-down/out a miRNA, in the genome.	To analyze the *in vivo* roles of oncogenic or tumor-suppressive miRNAs.
		Conditional or inducible expression of gene targeting vectors, via homologous recombination[106,240].	To analyze the tissue-specific or cancer-stage specific roles of oncogenic or tumor-suppressive miRNAs.
		Site-specific recombination[157,240] Can also be virus mediated – retrovirus, lentivirus, adenovirus	The above, and to determine delivery and efficacy of miRNAs that have therapeutic potential.
		CRISPR-Cas9 mediated knock-in or knock out[244,245]	Has the potential to analyze the tissue-specific or cancer-stage specific roles of oncogenic or tumor-suppressive miRNAs, with greater efficacy relative to conventional site-specific recombination.
Syngeneic mouse models	Immune competent	Implantation of tumors cells derived from mice from the same strain of origin[241]	Intact immune system, and microenvironment support the development of a tumor in a bona fide cancerous environment. This model aids in the identification of miRNAs that potentiate metastasis of tumors.
Humanized Mouse Models	Immuno deficient mice – NSG or NOG	Engraftment of human immune cells (peripheral blood mononuclear cell (PBMCs) or CD34+ cells)[246,247]	Possible immune response in patients against a treatment can be evaluated in mice. Not utilized in pre-clinical studies of miRNA-based cancer therapies yet.
		GEMMs expressing a human gene in mouse genome[248]	Compensates for a gene that is missing in the mouse genome, to further study its role in oncology. MiRNAs that are not conserved can be evaluated. Has potential in human-specific miRNA functional studies.