Fazlul H. Sarkar *Editor*

MicroRNA Targeted Cancer Therapy

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Preface

The Nobel Prize in Physiology or Medicine could have been awarded to Victor Ambros, David Baulcombe and Gary Ruvkun in 2013; however, it did not come to fruition but it is my belief that these investigators may receive such honor in future years to come. Since the discovery of microRNA (miRNA) some 20 years ago, these three scientists worked to uncover the mystery of miRNA, the small segments of nucleotides that silence genes. While studying the development of the nematode worm, Ambros and Ruvkun discovered miRNA in animals, while Baulcombe discovered it in plants. Since their discovery, it took more than two decade to fully appreciate the value of miRNA in human health and diseases. The literature search conducted recently showed about 11,000 articles on "miRNA and Cancer" while "cancer specific miRNA" search yield only about 2,500 articles; however, "miRNA targeted therapy" showed about 500 articles although not all appears to be directly relevant to miRNA targeted therapy. The rapid growth seen especially in the last decade in the field of miRNA research clearly suggests that this is a very attractive field in biomedical research although we need to harness the fruit of this cutting-edge research area for cancer targeted therapy, which became the subject of the new book as illustrated below.

Activation of oncogenes and/or the inactivation of tumor suppressor genes are known to contribute to the development of tumors as well as progression of disease to a metastatic state. The regulation of genes is by far controlled by many transcription factors which are often deregulated during the development and progression of cancer. In addition, emerging evidence clearly suggests that the deregulation of microRNAs (miRNAs) or small non-coding RNAs could also regulate the expression of genes. Likewise, miRNA genes are also regulated by transcription factors. It is well known that miRNAs are a major class of small, noncoding RNA molecules that regulate gene expression by targeting mRNAs to trigger either translational repression or mRNA degradation. The most attractive feature of miRNA is that one miRNA can regulate many target mRNAs, and thus miRNA targeted therapy is highly promising because multiple genes could be regulated by targeting a single miRNA, which becomes very important for the killing of highly heterogeneous populations of cancer cells within a tumor mass. Moreover, miRNAs

have recently been more widely investigated due to their potential role as targets for cancer therapy especially for targeted elimination of cancer stem cells (CSCs) because the CSCs are the cells that are believed to be highly resistant to conventional therapeutics, and thus responsible for tumor recurrence and metastasis. Therefore targeted elimination of CSCs through targeting miRNAs appears to be highly promising to eradicate human malignancies. The chapters presented in this book are focused on discussing the role of miRNAs in the regulation of cancer cell function during tumor development and progression, which will arm us with knowledge that will allow us to design miRNA targeted cancer therapy in the near future, especially for overcoming therapeutic resistance which will drastically improve the treatment outcome of patients diagnosed with cancer.

In this book, we are providing an overview and an update of our current understanding of the mode of action of several of these well characterized miRNAs in human cancers and document known strategies for the development of miRNA targeted therapeutics. It is anticipated that this special book would stimulate further research in the field and educate young scientists to generate stimulating ideas for novel discovery towards de-programming or re-programming of genes through targeting miRNAs that are either silenced or activated in cancer, and such mechanistic insight would serve as a novel approach for the prevention and/or treatment of most human malignancies.

The first and the second chapter rightly focused on glioma and glioma stem cells as described by the laboratory of Drs. Mittal and Brodie, respectively. The next three chapters are focused on lung cancer and dedicated to tumor recurrence, drug resistance, and lung cancer stem cells by Drs. Gong, Ochiya and Ahmad, respectively. Next, Dr. Abdelmohsen's group has summarized the knowledge of miRNA in ovarian cancer diagnosis and therapy followed by a presentation by Dr. Banno and colleague who described the application of miRNA in the treatment and diagnosis of cervical cancer. The next chapter is focused on colorectal cancer and drug resistance as well as the biology of tumor recurrence as discussed in the chapter presented by Dr. Majumdar and his colleague. This is followed by chapters on liver cancer, renal cancer and pancreas cancer presented by Drs. Galle, Majid, and Cordelier, respectively. The next chapter is from the laboratory of Dr. Sarkar focusing on the role of miRNA in drug resistance, EMT, and cancer stem cells, both in prostate and pancreas cancer. The next two chapters are focused on thyroid cancer and pediatric solid tumors as presented by Drs. Kimura and Segura, respectively. The next three chapters are not focused on any particular tumor system, rather these are focused on general topic covering the aspects of immunology, DNA repair system, and the molecular signaling associated with VEGF and its receptor in the context of miRNA as presented by Drs. Hong and Farooqi, respectively. The last chapter, contributed by Dr. Azmi and his colleagues, is the latest in the field of system biology and network modeling and how these tools could be useful in understanding the complexities of miRNA networks. As such, this field would become the future of miRNA research toward the development of targeted therapy. Although not explicitly stated, the field of cell-cell communication, exosome and miRNA is an active area of research and some of chapters, especially Chapter 2

invoked their roles in the biology of cancer development and progression; however, focused chapters on this topic would be an important future project. A variety of cells release membrane vesicles, such as exosomes that are thought to play key roles in cell−cell communication by transportation of miRNAs. There have been some efforts to use exosomes as a carrier of miRNAs toward miRNA targeted therapy because exosomes provide for stability of miRNA in the body fluid and carry the functional miRNAs in remote cells. Furthermore, recent studies have demonstrated that the modification of ligands on the exosomal membrane permits the accumulation of the exosomes to target tissues such as cancer and the delivery of the therapeutic miRNAs into the target cells. This concept would provide, in the future, an overview of the potential roles of exosomes with respect to carrier of therapeutic miRNAs for miRNA targeted therapy, which awaits further exploration.

Finally, I would like to thank the Springer publishing group for their trust in me for organizing this special topic on the emerging role of miRNA in cancer therapy in the book entitled *MicroRNA Targeted Cancer Therapy*. This book illustrates the complexities of the regulation and deregulation of genes mediated through miRNAs and how miRNAs could be targeted for cancer therapy as documented by a series of chapters complied in this book as stated above. It is hoped that targeting miRNAs will not only target cancer cells and CSCs but it will also target the tumor microenvironment (more like the entire tumor environment such as the entire host; especially the exosome mediated re-distribution of miRNAs occurs in human) for enjoying the benefit of better treatment outcomes for patients diagnosed with cancer toward achieving the objectives of complete eradication of cancer. This book provides the tip of the iceberg of the collection of chapters on the state of our knowledge on miRNA in cancer and targeted therapy. This knowledge would likely be useful for bringing newer generations of scientists with broader perspectives in launching cutting-edge innovative molecular research and drug development that will certainly help in designing targeted clinical trials in order to realize the dream of targeted therapy for eradicating human malignancies.

In closing, I would like to thank all the authors for their cooperation, hard work, and talented contributions to bring this book to the readers in a timely fashion. I sincerely hope that the content of this book will be useful in educating younger scientists in the field of "miRNA and cancer" research so that they can carry the torch in innovative research to rip the benefit of miRNA targeted cancer therapy for better treatment of human malignancies. Finally, I would like to dedicate this book to my lovely wife, Arfatun H. Sarkar and my three wonderful children, Sarah, Sanila and Shaan for their understanding, unconditional love, support, and sacrifice to enhance my scientific career.

Fazlul H. Sarkar

Contents

Chapter 1
The Therapeutic Role of MicroRNAs in Human Gliomas

Sanila H. Sarkar, Aamir Ahmad, and Sandeep Mittal

1 Introduction

Central nervous system (CNS) tumors are classified based on their cell of origin and are graded based on standard histopathological features. The World Health Organization (WHO) classification, the most widely employed grading system for brain tumors, divides CNS tumors by predominant cell type and grade. The histopathological grading of gliomas accounts for presence of nuclear atypia, cellularity, mitotic activity, endothelial proliferation, necrosis, and proliferative index [1]. Tumors of the CNS can be broadly divided into primary brain tumors and brain metastases. With improved treatment and survival of cancer patients, there has been an increasing incidence of metastatic disease in the brain over the last decade. Primary brain tumors can arise from all cell types within the nervous system. Gliomas comprise a heterogeneous group of neuroectodermal tumors with unique clinical, histological, and molecular characteristics. Gliomas are the most common type of primary brain tumors and are broadly categorized into low-grade (WHO grade I and II) and high-grade (WHO grade III and IV) gliomas. Other common primary brain tumors include meningiomas, ependymomas, choroid plexus papillomas, medulloblastomas, pituitary adenomas and vestibular schwannomas.

Glioblastoma (GBM) is the most aggressive type of glioma and the most common malignant brain tumor. It is characterized by increased proliferation,

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robust angiogenesis and extensive invasion into surrounding brain tissue, with partial or complete disruption of the blood–brain barrier (BBB) [2, 3]. Although there is no specific set of symptoms that are diagnostic of brain tumors, one of the principal indicators that a brain tumor is presence of a leaky BBB, which can be detected by contrast-enhanced MRI or CT scan. The Cancer Genome Atlas Research Network has identified four subtypes of GBM: mesenchymal, classical, proneural and neural subtypes [4]. Proneural GBMs make up a large fraction of brain tumors.

Adding to the complexity of brain tumors is the highly infiltrating nature of malignant gliomas; these tumor invariably recur locally despite aggressive multimodality treatment with surgery followed by adjuvant chemoradiation therapy. As such, the prognosis of malignant gliomas remains extremely poor [2]. The median survival time after initial diagnosis of GBM is 14 months [5], and the 5-year survival rate is less than 10 % [6]. In combination with radiation therapy, the oral alkylating agent temozolomide (TMZ) is often given and has shown promise in improving the prognosis of GBM patients [7, 8]. TMZ crosses the BBB, and can help inhibit proliferation and induce apoptosis in glioma cells. In general, malignant gliomas are very infiltrative effectively negating the possibility of complete surgical resection. This highly infiltrative nature also explains the high rate of local tumor recurrence despite maximal multimodal treatment. Recurrent glioma cells, believed to arise from the infiltrating glioma stem cells (GSC), are highly resistant to both ionizing radiation and alkylating chemotherapeutic agents [2]. Tumor infiltration and presence of resistant GSCs are the major reasons for tumor recurrence. As such, considerable effort has been made to identify more effective ways to counter the invasiveness of gliomas. Recent studies have alluded to the hypothesis that GBM are maintained by a small population of GBM stem cells, which retain stem cell properties, are highly tumorigenic, and display increased resistance to radiation and chemotherapy [5]. Further complicating the problem, GSCs share a core developmental program with normal stem cells making them difficult to target [9]. Future studies should focus on finding molecular targets that regulate GBM stem cells while sparing normal stem cells, as well as identifying different biomarkers for early detection of tumor progression.

2 MicroRNAs in Gliomas

While the underlying causes of brain tumors remain largely unknown, some progress has been made in our understanding of gliomagenesis. Environmental factors that may induce tumorigenesis include exposure to vinyl chloride or ionizing radiation [10]. Genetic causes include overexpression of tumor oncogenes and mutations or deletions of tumor suppressor genes [11]. Moreover, there is mounting evidence that well-conserved, small non-coding segments of RNA called microRNAs (miRNAs) are involved in the transcriptional and post-transcriptional genetic regulation of these genes. These miRNAs are approximately 20–22

nucleotides in length and bind to the 3′ untranslated region (UTR) of multiple target messenger RNAs (mRNAs), resulting in genetic silencing of genes via translational repression or target degradation [12]. They are also known to play an important role in epithelial to mesenchymal transitions (EMT) required for embryonic development and cancer metastasis [13]. Thus, the impact of miRNAs in tumorigenesis has garnered considerable interest in recent years [14]. Since their discovery in 1993, research has exponentially increased on the impact of miRNAs in a variety of disease processes. Currently there are thousands of known miRNAs and many more continue to be discovered.

Deregulation of miRNA expression has been associated with many pathological states, including various cancers. Specific miRNAs have been reported to play vital roles in tumor initiation, proliferation, migration and invasion. Since malignant neoplasms can develop from either a reduction or deletion of a tumor suppressor miRNA or from amplification or overexpression of an oncogenic miRNA, we can divide miRNAs as either oncogenic or tumor suppressors. Overexpression of tumor suppressor miRNAs in GBM stem cells inhibits cell proliferation and induces neural differentiation [5]. Conversely, inhibition of tumor oncogenic miRNAs may result in decreased glioma growth and cell proliferation, and increased apoptosis [15–17]. Each miRNA can have hundreds of targets and therefore regulate a large number of diverse cellular functions [2]. Since every miRNA has many different targets, it is possible to regulate multiple gene networks by targeting a single miRNA. This demonstrates the vast potential of miRNAs in cancer therapy, and establishes a strong reason to investigate the use of miRNAs in glioma therapy [18, 19]. Tables 1.1 and 1.2 outline the oncogenic and tumor suppressor miRNAs involved in gliomagenesis, respectively. In the next sections, we will review in detail the role of some of the main miRNAs involved in gliomas.

3 Oncogenic miRNAs

3.1 miR-21

miR-21 is one of the most extensively studied miRNAs in cancer biology. It is highly expressed in embryonic and newborn central nervous system [28], and plays an important role during cerebral development. Its levels are often elevated in a variety of malignancies, including breast, colon, liver and pancreatic cancer [15–17]. miR-21 targets multiple components and plays an anti-apoptotic function in gliomas. Uncontrolled expression of miR-21 contributes to malignant transformation of glial cells, increases drug resistance, and is a major cause of tumor recurrence in high-grade gliomas [17]. Previous studies have confirmed that presence of miR-21 in cerebrospinal fluid (CSF) can be used to detect malignant gliomas [3]. However, CSF can only be collected by invasive methods and as such is not an ideal source for evaluation of miRNAs. Thus, the development of accurate blood

Table 1.1 Oncogenic miRNAs in glioma and their reported effects

miRNA	Target	Effects	Reference
miR-10b		In orthotopic human glioma mouse model, inhibition of miR-10b diminishes invasiveness, angiogenicity and growth of mesenchymal-like glioma cells and prolongs survival of glioma-bearing mice. Suppresses TP53, FOXO3, CYLD, PAX6, PTCH1, HOXD10 and NOTCH1	[4]
miR-10b	CSMD1	Upregulated in glioblastoma stem cells, compared to normal neural stem cells	[5]
miR-10b		Significantly elevated in glioblastoma	[20]
miR-17	PTEN, MDM2	Increases survival under nutrition-deproved conditions. Promotes cell motility and invasion	[21]
miR-19a/19b	PTEN	Overexpressed in glioma tumors and cell lines, and correlate positively with tumor grade	[22]
miR-20a	TGFβR2	Regulates TGF-β signaling and plays role in p53-Quaking pathway	[23]
miR-20a		Upregulated in pediatric brainstem glioma, compared to adult subtype	[24]
miR-21		Plasma levels significantly altered in glioblastoma patients compared to normal controls. Also, plasma levels in patients treated by operation and chemo-radiation almost revived to normal levels	[3]
miR-21	STAT3	Reduction of miR-21 decreases expression of hTERT and represses STAT3 expression and STAT3 phosphorylation; knockdown of miR-21 inhibits growth and diminishes expression of STAT3 in xenograft model	[25]
miR-21		Overexpression of PDGF-B in U87 glioblastoma and F98 rat glioma cells resulted in decreased miR-21 expression and overall increased cell proliferation	[15]
miR-21		Four-fold increase observed in the plasma of glioblastoma patient	[26]
miR-21		Inhibition of miR-21 sensitizes human glioblastoma U251 stem cells to chemotherapeutic drug temozolomide, enhancing apoptosis	[27]
miR-21		Circulating miR-21 in glioblastoma significantly higher than controls	[16]
miR-21		Chronic temozolomide exposure results in acquired temozolomide-resistance and elevated miR-21 expression. Concomitant treatment with miR-21 inhibitor and temozolomide resulted in a significantly higher apoptotic rate than temozolomide treatment alone	
miR-21		Significantly elevated in glioblastoma	[20]
miR-21		miR-21 and SOX2 upregulated in RCAS/tv-a generated mouse brain tumor specimens. Upon irreversible depletion of miR-21, expression of SOX2 strongly	[28]

(continued)

Table 1.1 (continued)

miRNA	Target	Effects	Reference
		diminished in both mouse primary glioma cultures and human glioma cell lines	
miR-21	PDCD4	Downregulation of miR-21/overexpression of PDCD4 inhibits metastasis via silencing of hnRNPC, resulting in suppressed Akt activation	[17]
miR-21	Tap63	High expression levels needed to maintain TRAIL-resistant phenotype. Impairs TRAIL-dependent apoptosis by inhibiting the expression of key functional proteins	[29]
miR-21		Expression found only in tumor cells and tumor-associated blood vessels, whereas no expression observed in adjacent normal brain parenchyma. miR-21 levels correlated significantly with histologic grade	[30]
miR-23a	PTEN	Oncogenic CREB (cAMP response element-binding protein) induces miR-23a which, in turn, suppresses PTEN	[31]
miR-23a	APAF1	High expression in tumor tissues. Inhibition results in suppression of proliferation and invasion	[32]
miR-23b	VHL	Downregulation of miR-23b triggers growth inhibition, induces apoptosis, and suppresses invasion of glioma *in vitro*. miR-23b deletion decreases HIF-1α/VEGF expression and suppresses β-catenin/Tcf-4 transcription activity by targeting VHL	[33]
miR-23b	Pyk2	Reduced expression of miR-23b enhances glioma cell migration *in vitro* and invasion ex vivo via modulation of Pyk2; increased expression of miR-23b results in decreased Pyk2 expression	[34]
miR-24-3p miR-27a-3p	MXl1	Overexpression of miR-24-3p and miR-27a-3p promotes cell proliferation, miR-23a~27a~24-2 and miR23b~27b~24-2 work synergistically to regulate MXl1	[35]
miR-27a		Stable expression reduces proliferation and increases the accumulation of glioma cells in sub-G1 arrest	[36]
miR-27a		Upregulated in glioma tissues and cell lines, potentially through adherens junction, focal adhesion, the neurotrophin pathway, MAPK signaling pathway, TGF-β pathway, cytokine-cytokine receptor pathway, or the p53 pathway	[37]
miR-30b/c	caspase-3	High expression levels needed to maintain TRAIL-resistant phenotype. Impairs TRAIL-dependent apoptosis by inhibiting the expression of key functional proteins	[29]
miR-30a-5p	SEPT7	Knockdown results in inhibition of cell growth and invasion in glioblastoma cells and induction of SEPT7 with downregulation of PCNA, cyclin D1, Bcl2, MMP2 and MMP9	[38]
miR-92b	NLK	Induces wnt/β-catenin signaling resulting in increased proliferation and invasion	[39]

(continued)

Table 1.1 (continued)

miRNA	Target	Effects	Reference
miR-106b		Upregulated in pediatric brainstem glioma compared to adult subtype	[24]
miR-106b	RBL2	Overexpressed in gliomas; antisense suppresses proliferation of glioma cells and xenograft tumors	[6]
miR-106b-5p	RBL1, RBL2, CASP8	Significantly high in glioma tumors and correlates with tumor grading	[40]
miR-125b	MMP9	Levels significantly higher in highly invasive glioma stem cell and progenitor cell lines	[41]
miR-125b	MAZ	Downregulated in glioblastoma associated endothelial cells, resulting in increased expression of MAZ, a transcriptional factor that regulates VEGF	[42]
miR-128		Overexpression of PDGF-B in U87 glioblastoma and F98 rat glioma cells results in decreased miR-21 expression and overall increased cell proliferation	[15]
miR-128		Plasma levels significantly altered in glioblastoma patients compared to normal controls. Also, plasma levels in patients treated by operation and chemo-radiation almost revived to normal levels. Positively correlated with histopathologic grades of glioma	[3]
miR143/145		Overexpressed in invasive subpopulation	[43]
miR-155	FOXO3a	Regulates Akt signaling and induces proliferation and invasiveness	[44]
miR-182	CYLD	Overexpressed in a set of gliomas. TGF-β induces miR-182 expression, leading to prolonged NF-κB activation both *in vitro* and *in vivo*.	[45]
miR-182		3.1 times high in glioma patients, compared to healthy persons	[46]
miR-183	IDH2	Upregulated in the majority of high-grade gliomas and glioma cell lines compared with peripheral, non-tumorous brain tissue. Downregulates IDH2 levels and upregulates HIF-1α levels	[47]
miR-183/96/182 cluster		Inhibition of cluster induced ROS-mediated AKT/survival, induced p53/apoptosis signaling independent of target genes FGF9, CPEB1 and FOXO1. Knockdown also enhanced the anticancer effect of temozolomide on glioma cells	[48]
miR-196b		Overexpressed; confers a poor prognosis via promoting cellular proliferation in glioblastoma	[49]
miR-221	TIMP3	Significantly increased in high-grade gliomas compared with low-grade gliomas. Overexpression increases cell invasion. Increased expression levels in high-grade gliomas confer poorer overall survival.	[50]
miR-222	TIMP3	Significantly increased in high-grade gliomas compared with low-grade gliomas. Overexpression increases cell invasion and confers poorer overall survival.	[50]

(continued)

Table 1.1 (continued)

miRNA	Target	Effects	Reference
miR-222	DKK2	Activates Wnt/β-catenin signaling and promotes tumorigenesis	[51]
miR-342-3p		Plasma levels significantly altered in glioblastoma patients. Plasma levels in patients treated by operation and chemo-radiation almost revived to normal levels. Positively correlated with histopathologic grades of glioma	[3]
miR-372		Upregulated in glioma tissues. Cumulative overall survival of glioma patients with advanced histologic grades significantly worse for high miR-372 expression group than for low miR-372 expression group	[52]
miR-376a*	RAP2A/ AMFR	Clinically, a significant correlation between accumulation of unedited miR-376a* and the extent of invasive tumor spread	[53]
miR-650		Possible prognostic marker with high expression in high grade gliomas	[54]

For miRNAs with multiple reports, major findings of reports are listed individually. The 'target' column is left blank in case no target was reported/validated in the study

miRNAs biomarkers will likely provide a less invasive and more practical way to diagnose gliomas, monitor therapeutic response, and detect tumor recurrence. A recent study showed that miR-21 may be used as a biomarker to detect GBM [3, 16]. Compared to control subjects, plasma levels of miR-21 were significantly higher in patients with malignant gliomas and correlated to histologic grade of glioma [16]. In addition, it was observed that the levels of miR-21 decreased after chemoradiation.

miR-21 works by targeting multiple genes. One study found that miR-21 inhibited cell growth in U87 and LN229 human GBM cell lines, accompanying a decrease in human telomerase reverse transcriptase (hTERT) mediated by signal transducer and activator of transcription 3 (STAT3) transcription [25]. This study showed that knockdown of miR-21 resulted in a significant increase in apoptosis and an induction of cells in cell cycle arrest. In addition, it showed that hTERT is necessary for cell survival, as it works to prevent the shortening of telomeres thereby delaying cell senescence. Furthermore, the study confirmed that STAT3 is critical for hTERT regulation of miR-21. These findings were also validated using a LN229 malignant glioma xenograft model [25].

Another study found that miR-21 works by regulating the Bax/Bcl-2 and caspase-3 activity [27]. Pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins are known to regulate apoptosis in glioma cells. Bcl-2 poses one of the biggest obstacles to radiation and chemotherapy. It has been observed that GBM patients have a low Bax/Bcl-2 ratio [27]. Treatment of U251MG cells with a miR-21 inhibitor resulted in an increase of Bax and a decrease of Bcl-2 expression, dramatically improving the Bax/Bcl-2 ratio. Caspase proteins, especially caspase-3, are known to work downstream of Bax/Bcl-2, and play a vital role in GBM apoptosis. The authors noted that

Table 1.2 Tumor suppressor miRNAs in glioma and their reported effects

miRNA	Target	Effects	References
miR-7	EGFR	Enhanced levels induce apoptosis, inhibit proliferation migration and invasion, and antagonize ERK, Akt and Stat3. Plasmid-mediated gene therapy with miR-7 resulted in glioma xenografts growth arrest	[12]
Let-7a	K-Ras	Inhibits cell growth *in vitro* as well as *in vivo*.	[55]
miR-15b	NRP-2	Decreases cell invasiveness and *in vitro* tube formation	[56]
miR-16-1	Zyxin	Lower in glioma cells than normal brain tissues. Inhibits migration and invasion	[57]
miR-23b		miR-23b is epigenetically downregulated (through increased methylation), and restoration of miR-23b can effectively suppress cell growth in GSCs, induce cell cycle arrest and inhibit proliferation	[58]
miR-23b	TFAM	Inhibits PI3K/Akt signaling leading to reduced cell proliferation and migration	[59]
miR-24	ST7L	Inhibits proliferation and invasion, and induces apoptosis. Deletion of miR-24 suppresses β-catenin/Tcf-4 transcription activity	[60]
miR-25	MDM2 and TSC1	Overexpression results in p53 accumulation by directly targeting MDM2 and TSC1, leading to inhibited cell proliferation through cell cycle arrest *in vivo*	[61]
miR-32	MDM2 and TSC1	Overexpression results in p53 accumulation by directly targeting MDM2 and TSC1, leading to inhibited cell proliferation through cell cycle arrest *in vivo*	[61]
miR-34a		Tumor suppressor in U87 GSCs. Downregulated in CD133-positive cells. Suppresses cell proliferation and induces apoptosis in GSCs	[62]
miR-34a	PDGF-R	Downregulated by PDGF signaling pathway activation	[63]
miR-34c		Downregulated in glioma patients and cell lines. Overexpression reduces Notch, leading to cell cycle arrest and induction of apoptosis	[64]
miR-106a	SLC2A3	Low levels correlate with poor prognosis. Inhibits glucose uptake and cell proliferation	[65]
miR-107	Notch2	Downregulated in glioma tissues and cell lines, overexpression leads to inhibition of migratory and invasive ability of glioma cells via direct targeting of Notch2	[66]
miR-107	CDK6 and Notch 2	Inhibits proliferation and cell cycle, specifically p53 mutated U251 and A172	[67]
miR-107		Inhibits growth and invasion of glioma cells by Targeting Notch2 and stem cell markers	[68]
miR-124	NRAS, PIM3	Downregulated in glioblastoma stem cells, compared to normal neural stem cells	[5]

(continued)

Table 1.2 (continued)

miRNA	Target	Effects	References
miR-124	Slug, Twist, Vimentin	Cell differentiation agent-2 (CDA-2) inhibits cell growth and induces differentiation of glioma cells through upregulation of miR-124, accompanied with decreased expression of SLUG, Twist and Vimentin	[69]
miR-124	SOS1	Regulates Ras/Raf/Erk pathway and inhibits proliferation	[70]
miR-124		Inhibits STAT3 signaling	[71]
miR-124	CLOCK	Regulates proliferation and migration through targeting of NF-κB	[72]
miR-125b	E2F2	Overexpression of miR-125b inhibits the proliferation of CD133 positive glioma stem cells and reduces the expression of stemness marker	[73]
miR-128	P70S6K1	Levels decreased in gliomas; overexpression suppresses p70S6K1 and its downstream targets, HIF-1 and VEGF	[74]
miR-128	EphB2	Promotes cell-cell adhesion and inhibits cell migration	[75]
miR-128a		Negative regulator of mesenchymal signaling -CD44, vimentin, YKL-40	[76]
miR-134	Nanog	Reduces proliferation and invasion, induces apoptosis	[77]
miR-136	AEG-1, Bcl-2	Downregulated in human glioma, and promotes apoptosis of glioma cells induced by chemotherapy. Restoration of AEG-1 and Bcl-2 suppresses miR-136 enhanced apoptosis	[78]
miR-137	COX2	Levels downregulated in gliomas; ectopic expression inhibited cell proliferation and invasion	[79]
miR-137	RTVP-1	Promotes neural differentiation and suppresses stem cell markers	[80]
miR-138	EZH2, CDK6	High levels correlate with longer progression free survival. Induces cell cycle arrest	[81]
miR143/145	CTGF	Low in astrocytic tumors compared to normal brain specimens, low expression results in poor prognosis	[2]
miR-145	ADAM17	Significantly downregulated in glioma cell lines compared to normal brain tissue and negatively regulates tumorigenesis. Restoration inhibits proliferation, migration and invasion via silencing of ADAM17	[82]
miR-145	Sox9, Adducin3	Negatively correlates with tumor grade	[83]
miR-146b	MMP16	Inhibits migration and invasion of glioma cells	[84]
miR-146b		Exosomes expressing miR-146b inhibit glioma xenograft growth	[85]
miR-149	Akt1, PCNA, cyclinD1, MMP-2	Reduces proliferation and invasion, and induces cycle arrest	[86]

(continued)

Table 1.2 (continued)

miRNA	Target	Effects	References
miR-152	MMP-3	Decreases cell invasiveness	[56]
miR-153		Downregulated in glioma tissues	[87]
miR-155	GABRA1	Decrease in miR-155 expression restores expression of GABRA1, making glioglastoma cells sensitive to signals and inhibit cell proliferation mediated by GABRA1	[88]
miR-181b	IGF1R	Modulates PI3K/Akt and MAPK/ERK1/2 pathways leading to suppression of proliferation, invasion and tumorigenesis	[89]
miR-181b	MEK1	Modulates sensitivity to temozolomide	[90]
miR-181d	MGMT	A predictive marker of temozolomide response	[91]
miR-193a	Mcl-1	Induces apoptosis	[92]
miR-195	Cyclin D1, Cyclin E1	Inhibits proliferation and anchorage-independent growth, downregulates pRB and PCNA	[93]
miR-196b		Low expression associated with occurrence of preoperative seizures in low-grade gliomas, and may predict seizure prognosis in patients without preoperative seizures	[94]
miR-200b	CREB1	Suppresses proliferation and colony formation	[95]
miR-203		Reduced in high grade gliomas, low levels associated with poor prognosis	[96]
miR-204	SOX4, EphB2	Downregulated in glioma and neural stem cells. Suppresses self-renewal, stem cell-associated phenotype and migration of glioma cells. Restoring via promoter hypermethylation suppresses tumorigenesis	[97]
miR-206	Otx2	Ectopic expression inhibits cell proliferation and promotes apoptosis; miR-206 inhibitor upregulates expression of Otx2	[98]
miR-211	MMP-9	Inhibits glioma cell invasion and migration via epigenetic silencing and suppression of MMP-9. Activates intrinsic mitochondrial/Caspase-9/3-mediated apoptotic pathway in both glioma cells and CSCs. Increases drug retention capacity	[99]
miR-218	ECOP	Overexpression induces glioma cell apoptosis and inhibits viability, proliferation and tumorigenicity. miR-218 sensitizes glioma cells to apoptosis by regulating ECOP-mediated suppression of NF-κB activity	[100]
miR-218	LEF1, MMP-9	Expression low in glioma tissues, especially in glioblastoma. Inverse correlation in 60 GBM tissues between the levels of miR-218 and MMP mRNAs (MMP-2, -7 and -9)	[101]
miR-219	EGFR	Downregulated in gliomas. Inhibits anchorage independent growth, proliferation and migration	[102]

(continued)

Table 1.2 (continued)

miRNA	Target	Effects	References
miR-223	NF1A	Negatively regulate tumorigenesis via regulation of p21	[103]
miR-329	E2F1	Interferes with cell cycle progression and inhibits cell proliferation	[104]
miR-375		Expression significantly decreased in glioma tissues with ascending histopathologic grade. Loss of miR-375 expression effectively predicted the decreased overall survival	[105]
miR-383	IGF1R	Downregulated in gliomas and inversely correlates with glioma grade. Regulates Akt signaling	[106]
miR-410	MET	Inhibits proliferation and invasion of glioma cells	[107]
miR-483-5p	ERK1	Significantly downregulated in gliomas; overexpression suppresses cell proliferation and induces cell cycle arrest	[108]
miR-504		Negative regulator of mesenchymal signaling	[76]
miR-524-5p	Jagged-1, Hes-1	miR-524-5p behaves as a tumor suppressor by negatively targeting Jagged-1 and Hes-1	[109]
miR-708		Induces apoptosis by affecting multiple pathways	[110]
miR-1275	Claudin-11	Consistently downregulated during GSC differentiation, along with the upregulation of its target, CLDN11.	[111]
miR-6165	Pkd1, DAGLA	Induces apoptosis	[112]

For miRNAs with multiple reports, major findings of reports are listed individually. The 'target' column is left blank in case no target was reported/validated in the study

miR-21 inhibitor in combination with TMZ resulted in an increase of caspase-3 activity, thereby improving the effectiveness of apoptosis after chemotherapy and decreasing the likelihood of glioma recurrence [27].

In addition to Bax/Bcl-2 and hTERT, miR-21 targets programmed cell death 4 (PDCD4) and phosphatase and tensin homolog (PTEN), which are frequently downregulated in GBM with a marked increase in miR-21 expression [17]. PDCD4 is a protein that is upregulated during apoptosis and suppresses tumorigenesis. One way to regulate PDCD4 is phosphorylation by Akt, leading to ubiquitination and degradation of PDCD4. PTEN is a tumor suppressor that negatively regulates the PI3K/Akt signaling pathway. This study also found that in T98G GBM cell lines, downregulation of miR-21 or overexpression of PDCD4 or PTEN can inhibit metastasis via silencing of heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNPC), resulting in suppressed Akt activation [17]. Suppressed Akt results in inhibited migratory and invasive activities, while silenced hnRNPC results in reduced proliferation and enhanced apoptosis. hnRNPC is involved in mRNA metabolism, including pre-mRNA processing, mRNA transport, mRNA stabilization, and can also enhance translation of proteins [17]. Further studies need to investigate the mechanism by which hnRNPC regulates miR-21 biogenesis.

Another interesting aspect of miR-21 is its connection to tumor necrosis factor (TNF) and tumor necrosis factor-related apoptosis-induced ligand (TRAIL). Novel research on TRAIL shows promise because it only induces apoptosis in cancer cells while sparing normal, healthy cells [113]. Many human cancers, including some gliomas, are TRAIL-resistant and do not respond to normal signals for programmed cell death [29]. One study found that miR-21 is markedly upregulated in TRAIL-resistant glioma cells (TB10 and LN229) and is downregulated in TRAIL-sensitive glioma cells (T98G and LN18) mainly by targeting the 3′ UTR region of Tap63, a member of the p53 family [29]. Sensitization of cancer cells to apoptosis is a valuable strategy to design novel treatment options. Thus, the relationship between miR-21 and TRAIL needs to be further elucidated as it may provide a mechanism for overcoming resistance to apoptosis.

Platelet derived growth factors (PGDF) are a vast family of pro-oncogenic growth factors. Alterations in the PGDF family, including overexpression of PGDF-A and B ligands on their receptors, are commonly observed in high-grade gliomas [15]. However, the connection between PGDF signaling and miRNAs remains to be elucidated. Interestingly, one study found that the expression of oncogenic miR-21 can be downregulated by activating PDGF-B, inducing GBM tumorigenesis and enhancing tumor proliferation [25]. In human U87 and rat F98 GBM cell lines, prolonged exposure of PDGF-B promoted downregulation of miR-21 expression [25]. Furthermore, small interfering RNA (siRNA)-mediated PGDF-B silencing increases the levels of miR-21 in U87 cells, confirming the relationship between PGDF-B signaling and miR-21 [25]. These findings conflict with the majority of studies on miR-21 and further demonstrate the complex balance of miRNAs in gliomas and the need for additional work to help clarify these intricate relationships.

Researchers are now investigating the effectiveness of miR-21 in synergy with other miRNAs and other drugs. One study found the combination of a miR-21 inhibitor and a miR-10b inhibitor could be an effective therapeutic strategy for controlling GBM growth [20]. Another study focused on the use of TMZ and a miR-21 inhibitor, finding that only the combination of both agents is effective in promoting GSC apoptosis thereby limiting the potential of tumor recurrence after chemotherapy [27]. This study observed that U251MG cells are normally resistant to TMZ alone, and the use of a miR-21 inhibitor or the use of TMZ alone had no effect on the stem cell population. The synergistic effects of miR-21 in combination with drugs and other miRNAs show great promise for glioma therapy and needs further investigation.

3.2 miR-182

A key regulator of cell fate is nuclear factor-κB (NF-κB), which mediates the inflammation pathway. The role of inflammation in promoting cancer is widely known and well documented [114–118]. Inflammation starts with the recruitment of

leukocytes by endothelial cells and their migration from plasma into tissue, caused by pro-inflammatory cytokines, protein kinase C activation, viruses or oxidants [117]. Any of these events can activate tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β), which in turn activate NF-κB and cyclooxygenase-2 (COX-2). NF-κB functions as a transcription factor for COX-2 and also activates many genes that cause inflammation in a feed-forward loop. NF-κB is a heterotrimer with three subunits (p50, p65 and IκBα). Upon activation of the complex, IκBα undergoes phosphorylation, ubiquitination, and eventually degradation, thus releasing the p50 and p65 heterodimer for translocation into the nucleus as the active NF-κB [115].

This pathway is also controlled by feedback mechanisms regulated by the anti-inflammatory cytokines IL-4, IL-10, transforming growth factor β (TGF-β), peroxisome proliferator activated receptor γ (PPAR-γ), manganese superoxide dismutase, glutathione, and catalase among others [117]. In response to pro-inflammatory cytokines such as TNF-α and IL-1, NF-κB activates the transcription of inhibitory Smad7, which in turn suppresses the TGF-β pathway [118]. Uncontrolled inflammatory responses via increased levels of NF-κB are associated with a number of neoplasms, including breast, prostate, ovarian, lung, colon and pancreatic cancer, head and neck cancer, melanoma and lymphoma [45, 118]. Investigating the multiple levels of NF-κB regulatory processes, as well as the crosstalk between NF-κB and TGF-β, may provide ways to prevent or treat cancers, including gliomas.

miR-182 is another oncogenic miRNA that is overexpressed in malignant gliomas [45, 46], with one study reporting 3-fold higher levels in glioma patients when compared to healthy control subjects [46]. Another study noted that miR-182 directly targets and suppresses cylindomatosis (CYLD) [45]. CYLD de-ubiquinates NF-κB, and acts as a negative regulator of the NF-κB pathway resulting in increased apoptosis. The authors noted a significant inverse correlation between CYLD levels and glioma tumor grade, which was also associated with shorter overall survival in GBM patients [45]. Restoration of CYLD resulted in inhibited glioma tumorigenesis, and inhibited glioma growth and angiogenesis *in vivo*. Suppressed CYLD resulted in ubiquitin conjugation of NF-κB and sustained NF-κB activity, which caused glioma cells to become more aggressive both *in vitro* and *in vivo*. In addition, suppression of miR-182 resulted in inhibited NF-κB activity. Furthermore, the authors found that TGF-β induced miR-182 expression and led to prolonged NF-κB activation, alluding to a possible regulatory mechanism by which NF-κB and TGF-β crosstalk. This study is critical for the development of more effective glioma therapies, as it discovered a possible mechanism for sustained NF-κB activation in malignant gliomas. Finding a way to regulate the NF-κB pathway will undoubtedly prove to be a powerful instrument in designing novel therapies for glioma patients.

3.3 *miR-10b*

miR-10b belongs to the tumor-oncogene family, and was one of the earliest discovered miRNAs. It is known to be upregulated in GSCs compared to normal neural stem cells [5, 20], as well as in GBM tissues with one study finding an average increase of 142-fold [5]. In U87-2M1 cells, an invasive type of the U87 subline that resembles the mesenchymal GBM cells, it was demonstrated that inhibition of miR-10b resulted in a distinct increase in apoptosis, with suppression of both glioma cell invasion and angiogenesis *in vitro* and *in vivo* [4]. After silencing miR-10b, invasive proteins such as MMP-13, MMP-2, CTNNB1 and HGF were downregulated. This finding was due to the suppression of multiple tumor suppressor genes, including TP53, FOXO3, CYLD, PAX6, PTCH1, HOXD10 and NOTCH1. Specifically, miR-10b targets genes HOXD10, NOTCH1, TP53 and PAX6, which may all regulate invasiveness of GBM via suppression of the proteins MMP-2 and CTNNB1 [4]. Additional studies should focus on the role of miR-10b in the invasion and angiogenesis of the other subtypes of GBM, and future mesenchymal glioma therapies should focus on silencing miR-10b.

In addition, miR-10b also targets tumor suppressor gene CSMD1 [5]. CSMD1 maps to chromosome 8p23, a region that is deleted in many tumor types. miR-10b binds to the predicted 3′ UTR region of CSMD1, resulting in a repression of the tumor suppressor gene. CSMD1 works in a complex regulatory framework centered on miR-10b in GBM stem cells and tissues. miR-10b is also known to be upregulated in breast cancer, leukemia, and pancreatic cancer, and promotes tumor invasion and metastasis in breast cancer. Combined with the information gathered on miR-10b in GBM, we can speculate that miR-10b functions as a global oncogene to stimulate tumorigenesis in multiple tumor tissue types [5].

3.4 *miR-106b*

miR-106b is a tumor oncogenic miRNA. Its levels are found to be overexpressed in the majority of gliomas, and its expression is significantly correlated to tumor grade [6]. One study found a 1.74-fold and 2.2-fold increase in miR-106b expression for WHO grade III and IV gliomas, respectively, when compared to low-grade tumors [6]. This was confirmed using three human malignant glioma cell lines: U251, LN229 and TJ905. When these cell lines were transfected with a miR-106b antisense oligonucleotide (ASON), cell proliferation was suppressed and cells were arrested in G0/G1. However, cell cycle arrest was only significant in the U251 and TH905 cell lines. Furthermore, tumor growth in a miR-106b ASON nude mouse xenograft model was significantly impaired; validating the claim that miR-106b is tumor oncogenic [6]. While previous studies have shown miR-106b to target the cyclin-dependent inhibitor p21/CDKN1A, this study proposed the cell

cycle regulator RBL2 as a direct target of miR-106b. It was found that cells are significantly shifted into S phase when RBL2 expression was suppressed. This study added to the growing body of evidence that miR-106b is tumor oncogenic, and proposes a pathway by which miR-106b affects cell cycle regulation.

In another study, the role of miR-106b in pediatric brainstem gliomas was investigated. It was found that the levels of miR-106b were significantly higher in pediatric brainstem gliomas and correlated with malignancy [24]. Brainstem gliomas are common in pediatric patients and the prognosis of these young children remains dismal. The importance of miR-106b in cell cycle regulation and its role in the development of malignancies cannot be overlooked as we continue to explore the potential role of miRNAs in glioma therapy.

3.5 miR-20a

Closely related to miR-106b, miR-20a is also tumor oncogenic and works in a complex pathway to affect tumorigenesis. Recently it was discovered that Quaking (QKI) is directly regulated by p53 and works to activate and stabilize miR-20a [23]. QKI is a tumor suppressor gene that is often deleted in GBM, resulting in an unstable miR-20a. miR-20a regulates TGF-β receptor 2 (TGF-βR2), the TGF-β signaling network and overall cell proliferation and differentiation. When miR-20a is unstable, it contributes to tumorigenesis and results in uncontrolled cell proliferation [23]. This p53-QKI-miR-20a-TGF-β pathway adds to the growing body of evidence that miRNAs can regulate tumorigenesis, and further shows the importance of proper regulation of miRNAs.

miR-20a also seems to play a causative role in malignant tumor progression of pediatric brainstem gliomas similar to miR-106b. Interestingly both these miRNAs are a part of the miR-17 family, which also is the precursor for miR-91 and miR-103. This group of miRNAs plays a crucial role in the development of breast cancer, further demonstrating the universal power of miRNAs in tumorigenesis and the global oncogenic effects of miR-20a and miR-106b [119]. Appropriate regulation of these miRNAs may prevent tumorigenesis, and may serve to be a powerful therapy for patients with gliomas.

3.6 miR-183

miR-183 is also upregulated in the majority of high-grade gliomas as well as U251, U87 and A172 malignant glioma cell lines [47]. This increase is associated with a decrease in isocitrate dehydrogenase (IDH) 2, which has complementary sequences to miR-183 in its 3′ UTR. Isocitrate dehydrogenases (IDHs) are a group of enzymes involved in the conversion of isocitrate to α-ketoglutarate during oxidative decarboxylation and IDH1 and IDH2 are known mutational targets in human cancers.

This is important because tumor cells obtain energy from aerobic glycolysis, with a defect in mitochondrial respiration. Recent studies have demonstrated that IDH1 and IDH2 mutations are frequently present in low-grade and anaplastic gliomas and represent a favorable prognostic biomarker [120]. Tanaka et al. noted that IDH2 is a direct target of miR-183, allowing the investigators to speculate that miR-183 might induce the mitochondrial dysfunction apparent in tumor cells [47]. Furthermore, the authors found that miR-183 upregulation resulted in an increased expression of HIF-1α and two downstream targets of HIF-1α, vascular epithelial growth factor (VEGF) and glucose transporter 1 (GLUT1). Both these targets were also upregulated as a result of miR-183. HIF-1α plays a role in angiogenesis, metabolism and survival in tumor cells, and the overexpression of its targets may affect tumorigenesis. This study identified a plausible mechanism by which miR-183 affects the way tumor cells get energy, and sheds light on another possible way to interfere with tumorigenesis of gliomas.

4 Tumor Suppressor miRNAs

4.1 miR-34a

miR-34a was originally identified as a likely tumor suppressor miRNA and a downstream transcriptional target of p53 [121, 122]. Prior reports have shown that miR-34a is downregulated in GBM compared to normal brain tissue, and that it inhibits cell proliferation, survival and invasion in adherent glioma cell lines [63, 123]. It works by targeting c-Met, Notch-1, Notch-2, and CDK6. The c-Met pathway is frequently expressed in gliomas and medulloblastomas, and overexpression of the c-Met pathway in tandem with the HGF pathway significantly correlates with poor prognosis. Notch signaling is a conserved pathway that controls differentiation, proliferation, EMT and migration, and consists of four members (Notch-1–4). The Notch pathway plays a critical role in glioma cell survival and cell proliferation [123]. CDK6 is a cell cycle regulator involved in cell proliferation, differentiation and transformation of many cancers including gliomas. It accelerates the transition of cells from the G0/G1 to S phase of the cell cycle. Its levels are often elevated when compared to normal brain tissue, and elevated levels again significantly correlate to poor prognosis. These downstream targets demonstrate the multiple pathways by which miR-34a can affect tumorigenesis, and the potential of miR-34a in glioma therapy.

A recent study found that the pathogenesis of proneural GBM is strongly linked to dysregulated PDGF signaling, another direct downstream target of miR-34a [63]. This study confirmed that miR-34a is downregulated by oncogenic PDGF signaling via PDGF receptors (PDGFRs), and that miR-34a inhibits growth in proneural GBM cells both *in vitro* and *in vivo*. Additionally, expression of miR-34a was negatively correlated with histologic grade [63]. While miR-34a

was originally discovered to be a downstream transcriptional target of p53, this study demonstrated that the regulation of miR-34a expression by PDGF signaling likely works independently of the p53 pathway. Again, this shows the multiple and complex pathways by which miR-34a functions as a tumor suppressor in glioma development. The advantage of a broad range of miRNA targets provides support for further research on miR-34a RNAs as therapy for high-grade gliomas.

4.2 miR-25 and miR-32

Overexpression of miR-25 and miR-32 result in p53 accumulation by directly targeting Mdm2 and TSC1, respectively, which are negative regulators of p53 and mammalian target of rapamycin (mTOR) [61]. This leads to inhibited cell proliferation through cell cycle arrest and inhibited growth of GBM in mouse brain *in vivo*. It was also found that miR-25 and miR-32 repress p53 through two feedback regulatory transcriptional factors E2F1 and MYC, respectively. In addition, it was found that p53 accumulates when Mdm2 is silenced resulting in GBM growth arrest. Mdm2 regulates p53 by negatively affecting ubiquitination degradation, and its levels are inversely correlated to GBM tissue in patients. It is well accepted that an active mTOR pathway can suppress PI3K-Akt signaling, which in turn affects the p53 activity via Akt-mediated phosphorylation of Mdm2 [124]. This crosstalk is important in growth and development, and consequently plays an integral role in tumorigenesis. miR-32 was also found to directly target TCS1, causing elevated TSC1 levels and p53 activation along with an increase in mTOR activity [61]. This was confirmed using MYC and E2F1 knockdown models, adding compelling evidence to the claim that miR-32 can stabilize p53 through activation of mTOR by targeting TSC1. Both miR-25 and miR-32 affect the very important p53 pathway whose deregulation plays one of biggest roles in tumorigenesis of all cancers, including gliomas.

4.3 miR-107

miR-107 is another tumor suppressor miRNA that has been shown to be downregulated in glioma tissues as well as U87, U251 and A172 glioma cell lines [66]. Conversely, overexpression of miR-107 lead to inhibited migration and invasive ability of glioma cells [66]. miR-107 works by directly targeting the 3′ UTR sequence of Notch-2, which is known to transactivate Tenascin-C, MMP-12 and COX-2. Tenascin-C, a large extracellular matrix of glycoprotein that acts as a tumor-specific antigen, is often upregulated in gliomas and Tenascin-C invasion is mediated by MMP-12. Knockdown of Notch-2 suppresses glioma cell invasion in U87 and A172 glioma cell lines, suggesting that Notch-2 is involved in glioma

invasion and that miR-107 exerts its anti-invasive tumor suppressive activity through Notch-2 signaling pathways.

Another study suggested that miR-107 targets CDK6 to induce cell cycle G1 arrest and inhibit invasion, in addition to targeting Notch-2 [67]. This study indicated that miR-107 is a transcriptional target of p53, and that miR-107 is downregulated particularly in p53-mutant U87 and A172 glioma cell lines. Moreover, the transfection of wild-type p53 into glioma cells stimulated miR-107 expression, and miR-107 expression inhibited cell proliferation and arrested cells in G0/G1 by targeting CDK6 and Notch-2. CDK6 is a cell cycle regulator involved in cell proliferation, differentiation and transformation of many cancers including gliomas, acting as an oncogene [125]. Proper regulation of CDK6 and Notch-2 is essential in controlling gliomas, showing the potential of miR-107 in glioma therapy.

4.4 miR-124

miR-124 is involved in the differentiation of brain tumor stem cells, making it an ideal target for therapy [5, 126]. The levels of miR-124 increase during differentiation of mouse embryonic stem cells. One study found that its levels are considerably decreased in glioma cell lines compared to normal neural stem cells, possibly by epigenetic modification such as promoter sequence hypermethylation [126]. miR-124 also induces differentiation in adult mouse neural stem cells, mouse oligodendroglioma-derived stem cells as well as in human GBM-derived U87 stem cells. Moreover, it can inhibit proliferation and induce G0/G1 cell cycle arrest in GBM-derived stem cells. This study also concluded that CDK6 is a downstream target of miR-124, and that its expression is inhibited by miR-124 in U251 cells [126]. This is another example of how miRNAs work in complex pathways, and how pathways can be targeted by different miRNAs. The ability to detect and regulate glioma stem cells will serve as early biomarkers for the disease providing better patient outcomes, and the role of miR-124 in early tumorigenesis cannot be overlooked.

Another study revealed two downstream targets of miR-124: NRAS and PIM3 [5]. NRAS is a small guanine-nucleotide binding protein and is one of the three RAS isoforms that play a crucial role in cell proliferation, differentiation and survival. The 3′ UTR region of NRAS is targeted by miR-124, and its levels are significantly increased in GBM stem cells. PIM3 is a proto-oncogene with serine/threonine kinase activity known to promote tumor cell growth through modulating cell cycle regulators. miR-124 represses PIM3 expression through directly targeting its 3′ UTR region. This shows the dual tumor suppressive activity of miR-124 and adds to the multiple pathways by which miR-124 works.

Adding to the body of evidence on miR-124, another study found for the first time that cell differentiation agent-2 (CDA-2) induces cell differentiation through suppressing Twist and SLUG in glioma cells [69]. miR-124 was found to be

upregulated by CDA-2. CDA-2, extracted from human urine, has shown promise in improving chemotherapy responses in many tumors including gliomas and has high anti-cancer properties. It was shown to suppress proliferation in U251 and SWO-38 glioma cells *in vitro*, and also promotes proper differentiation into mature astrocytes. Twist and SLUG are transcriptional repressors that are involved in both embryonic development and cancer metastasis. These repressors recruit histone deacetylases to condense chromatin and repress expression. It was found that inhibition of miR-124 upregulated levels of SLUG and Twist proteins in U251 glioma cells, and partially eliminated the function of CDA-2 on mesenchymal markers. This provides concrete evidence that miR-124 and CDA-2 regulation are correlated, which will prove to be valuable as we continue to discover better treatment options for glioma patients.

4.5 miR-218

miR-218 is also often downregulated in gliomas [100, 101]. One study found that overexpression of miR-218 induces glioma cell apoptosis and inhibits glioma cell viability, proliferation and tumorigenicity [100]. This study identified epidermal growth factor receptor-coamplified and overexpressed protein (ECOP) as a downstream target of miR-218, which can regulate the transcriptional activity of NF-κB and its associated apoptotic response. This study suggested that miR-218 sensitizes glioma cells to apoptosis by regulating ECOP suppression of NF-κB. Another study found lymphoid enhancer binding factor 1 (LEF1) and MMP-9 as downstream targets of miR-218 [101]. LEF1 is an oncogenic transcription factor involved in the Wnt signaling pathway, and affects cell proliferation and migration. This study showed that miR-218 directly targets LEF1, resulting in a reduced synthesis of MMP-9. Again, the multiple downstream targets of miR-218 demonstrate the high variability of miRNAs in glioma therapy, and further validate future research on multi-targeting miRNAs.

5 Conclusions

The role of miRNAs in gliomas is still vastly unclear, but research perseveres to discover the complex regulatory mechanisms by which miRNAs affect glioma tumorigenesis. The multiple pathways by which miRNAs work render them ideal therapeutic targets. Possible treatments for gliomas include overexpression of tumor suppressive miRNAs (e.g. miR-34a, -25, -32, -107, -124 and -218), as well as inhibition of tumor oncogenic miRNAs (e.g. miR-21, -182, -10b, -106b, -20a and -183). It is interesting to note that some miRNAs, such as miR-23b, miR-27a, miR-125b, miR-128, miR-143, miR-145 and miR-196b have reported oncogenic as well as tumor suppressor functions (Tables 1.1 and 1.2). This suggests a

context-dependent complex functionality of miRNAs which requires further elaborate studies. The importance of identifying all of the downstream targets to these miRNAs and further elucidating the complex mechanisms in these regulatory networks may be the key to developing novel drug therapies to be used in combination with radiation and chemotherapy. In addition to helping to regulate tumor initiation, invasion, growth, proliferation, metastasis and apoptosis, many miRNAs may function as early biomarkers for developing gliomas. The role of some miRNAs, such as miR-21, miR-124, miR-10b and miR-106b, in brain stem cell development make these miRNAs suitable targets for therapy. This will provide glioma patients with earlier diagnosis, in hopes of achieving improved prognosis and reduced incidence of tumor recurrence. Moreover, identifying global oncogenic miRNAs and global tumor suppressive miRNAs will offer targeted therapies for many different tumor types in addition to malignant gliomas. Future studies will further validate the profound effect that miRNAs have on glioma tumor development and, thus, prevention. Researchers continue to focus on miRNAs in gliomas, as well as in other cancers, since miRNAs play a multifaceted role in cancer stem cell development, early diagnosis, therapeutic treatment, and ultimately aiming to improve the prognosis of patients.

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Chapter 2
miRNA Expression and Functions in Glioma and Glioma Stem Cells

Chaya Brodie, Efrat Buchris, and Hae Kyung Lee

1 Introduction

Gliomas are the most common form of primary brain tumors, accounting for 80 % of malignant CNS tumors [1]. These tumors exhibit a marked malignant progression characterized by high level of infiltration throughout the brain, resistance to traditional chemotherapy and radiotherapy and high level of angiogenesis [1, 2]. Malignant gliomas are classified on the basis of histopathological features, clinical presentation and genetic alterations as astrocytomas, oligodendrogliomas, or tumors with morphological features of both astrocytes and oligodendrocytes, termed oligoastrocytomas [3]. Astrocytic tumors are graded based on the WHO consensus-derived scale of I to IV according to their degree of malignancy as judged by various histological features accompanied by genetic alterations [3, 4]. Grade I tumors are biologically benign and can be cured if they can be surgically resected; grade II tumors are low-grade malignancies that may follow long clinical courses, but their infiltration into the surrounding brain parenchyma renders them incurable by surgery; grade III tumors exhibit increased anaplasia and proliferation and grade IV tumors (glioblastoma, GBM), exhibit more advanced features of malignancy, including vascular proliferation, necrosis and resistance to radio and chemotherapy [5]. On the basis of clinical presentation, GBMs have been

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further subdivided into primary or secondary subtypes [6]. Primary GBMs account for the great majority of tumors in older patients, while secondary GBMs are less common and tend to occur in patients under the age of 45. Remarkably, primary and secondary GBMs are morphologically and clinically indistinguishable as reflected by a similar poor prognosis when adjusted for patient age. However, although these GBM subtypes share a common phenotypic endpoint, recent genomic profiles have revealed strikingly different transcriptional patterns and DNA copy number aberrations between primary and secondary GBMs [6]. Based on these studies, a recently expanded group of novel markers has been identified, which allow a better classification of gliomas and can serve as novel prognostic markers [7–9]. In addition, specific miRNAs have been also identified as diagnostic and prognostic markers in GBM and as novel therapeutic targets [10, 11].

1.1 The New Classification of GBM

A recent study by Phillips et al. [12] reported the identification of three high-grade astrocytoma subsets, identified by differential expression of markers associated with outcome, and named them proneural, proliferative and mesenchymal in recognition of the key features of the molecular signatures associated with each group. This study showed that the proneural subtype is distinguished by markedly better prognosis and expresses genes associated with normal brain and the process of neurogenesis. The two other subtypes, the proliferative and mesenchymal, are characterized by a resemblance to either highly proliferative cell lines or tissues of mesenchymal origin and show activation of gene expression programs indicative of cell proliferation or angiogenesis, respectively.

More recently, using an unsupervised approach to classify data from The Cancer Genome Atlas (TCGA) project, Verhaak [13] reported the existence of four GBM subtypes, termed proneural, neural, classical and mesenchymal. The four GBM subtypes have distinct molecular markers and three of the subtypes identified in this study were demonstrated to have a strong association with specific genomic alterations; the proneural subtype exhibiting IDH1 mutations and/or PDGFRA amplification, the classical subtype exhibiting amplification and/or mutation of EGFR, and the mesenchymal subtype showing loss and/or mutation of NF1.

While additional subtypes likely exist, only the proneural and the mesenchymal GBMs have been consistently identified in both supervised and unsupervised classification of GBM and have been reported to have both prognostic and predictive values [12, 13]. Recent studies also identified specific miRNAs that are characteristic of the mesenchymal GBM [14, 15].

1.2 The Mesenchymal Transformation of GBM

The epithelial to mesenchymal transformation (EMT) is a process that allows polarized epithelial cells to undergo multiple biochemical changes that enable them to acquire a mesenchymal cell phenotype which includes enhanced migratory capacity, invasiveness and increased resistance to apoptosis [16]. Activation of EMT is important for cancer cell dissemination and for the promotion of tumor metastasis in epithelial tumors [17]: however, the importance of this process in the neuro-epithelial context is much less characterized. The results from recent studies suggest that primary GBM, GSCs and glioma cells can undergo mesenchymal transformation [18–21]. During this process, which strongly resembles EMT of epithelial tumors, glioma cells acquire a mesenchymal stem cell-like properties which include increased ability to migrate, upregulation of mesenchymal markers and the ability to differentiate to the mesenchymal lineage.

The clinical relevance of the mesenchymal transformation of GBM is of utmost importance, as several studies showed that GBM patients whose tumors have a proneural phenotype have better survival compared with those that have mesenchymal phenotype [12, 13] and upon recurrence, tumors frequently transform toward the mesenchymal phenotype which is characterized as the most aggressive and therapy resistant subtype [12, 20, 22, 23].

1.3 The Factors Underlying the Mesenchymal Transformation of GBM

Using gene regulatory network analyses, the transcription factors signal transducer and activator of transcription 3 (STAT3) and CCAAT enhancer-binding protein β (C/EBPβ) have been recently identified as synergistic initiators and master regulators of the mesenchymal transformation in glioma [24]. Analysis of regulatory networks of available expression microarray data sets of GBM, the transcriptional co-activator TAZ has been identified as another major regulator of mesenchymal differentiation in malignant glioma [25]. In addition, a recent study reported that the TNF-1/NF-KB pathway activated by macrophages/microglia, also plays a role in the mesenchymal transformation of proneural glioma stem cells [23]. Similarly, specific miRNAs have been also identified as important regulators of the EMT process [26] and of the mesenchymal transformation of GBM [27, 28].

2 Cancer Stem Cells (CSCs)

2.1 General Aspects of Cancer Stem Cells

CSCs represent a subset of tumor cells that has the ability to self-renew, generate the diverse cells that comprise the tumor and to continually sustain tumorigenesis. CSCs share important characteristics with normal tissue stem cells, including self-renewal (by symmetric and asymmetric divisions) and differentiation capacity, albeit in an aberrant mode [29]. The first evidence for the existence of CSCs came from acute myeloid leukemia in which a rare subset comprising 0.01–1 % of the total population could induce leukemia when transplanted into immunodeficient mice [30]. CSCs are distinct from the cell of origin, which specifically refers to the cell type that receives the first oncogenic hit(s). Moreover, CSCs do not necessarily originate from the transformation of normal stem cells but may arise from restricted progenitors or more differentiated cells that have acquired self-renewing capacity [31]. One implication of this model is that there are mechanistic parallels between the self-renewal programs of normal stem cells and CSCs. It has been presumed in many cases that the cells in which cancer originate share committed cells that have undergone some degree of differentiation [32].

2.2 Glioma Stem Cells (GSCs)

Glioma stem cells (GSCs) were one of the first CSCs isolated from solid tumors. GBM contain a small subpopulation of self-renewing and tumorigenic CSCs which are implicated in tumor infiltration, resistance to conventional therapies and tumor recurrence [33–38]. Interestingly, GSCs isolated from human tumors and cultured in vitro showed remarkable similarities to normal neural stem cells (NSCs), expressing neural stem/progenitor markers such as Nestin, Sox2, and Olig2 and upon induction, could be differentiated to cells expressing neuronal or glial markers [36, 39]. Transplantation of GSCs into immunodeficient mice yielded tumors that shared similar histology and global gene expression patterns with their parental tumors [40]. Understanding the mechanisms associated with the stemness and oncogenic features of these cells is essential for the development of therapeutic approaches that can eradicate GSCs and may provide the basis for the development of novel therapeutic approaches for GBM patients [41–43].

2.3 miRNAs in Cancer

miRNAs are 19–25 nucleotide non-coding small RNAs that can play important regulatory roles in animals and plants by targeting mRNAs for cleavage or

translational repression [44–46]. miRNAs induce gene silencing by partial sequence homology and thus a single miRNA can have hundreds of targets and therefore regulate diverse cellular functions such as pathways involved in stem cell function, cell proliferation, migration and oncogenic transformation [47, 48]. Indeed, aberrantly expressed miRNAs have been described in various types of tumors including GBM [49–51]. The expression and function of specific miRNAs in astrocytic tumors have been studied with regards to mechanisms of gliomagenesis, patient prognosis and their use as novel therapeutic targets. [52, 53].

Mechanisms of miRNA deregulation have been described at various levels including genetic, epigenetic, transcriptional, and processing levels [54]. Amplification and deletion of many miRNAs are associated with their location at regions that are either amplified or deleted in human cancers [55]. Repression of some miRNAs is mediated by CpG hypermethylation in different tumors [56], whereas other miRNAs are regulated by transcription factors such as p53, E2F, STAT3 or via deregulation of Dicer or Drosha as has been observed in many cancers [57]. Various studies documented that miRNAs can act as tumor suppressor or oncogenes based on the function of their major target genes [58]. In addition, they have been shown to regulate a variety of cellular functions such as cell proliferation, cell death and apoptosis, cell migration and invasion, metastasis, angiogenesis, tumor microenvironment, tumor immunology and chemoresistance as well as many aspects of cancer stem cell biology [48–52, 59].

2.4 miRNAs as Biomarkers in GBM

Various studies have been performed to identify biological markers for the detection and risk stratification of gliomas. miRNA expression and signature have been also associated with the diagnosis and prognosis of patients with different types of tumors [60–62]. Genome-wide profiling studies indicated that the miRNA signature can contribute to the distinguishing of different types of cancers, the identification of tissue of origin of purely characterized tumors or of metastasis of undefined origin and the classification of tumor histological subtypes [63, 64]. In addition, specific miRNAs have been demonstrated to have great potential as early minimally invasive biomarkers in different tumors due to their stability, abundance and accessibility in various fixed and fresh tissues, different biologic fluids and circulating exosomes [65, 66].

Various gene profiling studies suggested the existence of multiple GBM classes but their characteristics are not fully defined. Recent studies employed also the use of miRNAs as biomarkers in GBM [66]. A study by Kim et al., analyzed 261 miRNA expression profiles from TCGA and identified five clinically and genetically distinct subclasses of glioblastoma based on their miRNA signature. These subgroups were related to different neural precursor cell types resembling those of radial glia, oligoneuronal precursors, neuronal precursors, neuroepithelial/neural crest precursors, or astrocyte precursors [11]. In addition, this study also

identified specific miRNAs as potent regulators of subclass-specific gene expression networks in GBM. One of the miRNAs, miR-9, was found to suppress the mesenchymal differentiation of glioblastoma by inhibiting JAK kinases and of STAT3 [11].

To identify significant miRNA-mRNA correlations in gliomas, a study by Ma et al. [67] analyzed the miRNA and mRNA signatures and the relationship between them in 82 glioma specimens. Statistical analysis showed that expression of miR-128a, -504, -124a, and -184 was negatively correlated with the expression of mesenchymal markers in GBM. Functional analysis of miR-128a and -504 demonstrated that mesenchymal signaling in GBM may be negatively regulated by miR-128a and -504 [67].

In another study, Li et al., described an integrated approach to identify miRNA functional targets during glioma malignant progression by combining the paired expression profiles of miRNAs and mRNAs across 160 glioma patients. They identified miR-524-5p and miR-628-5p as protective factors and their expression decreased during glioma progression [68]. miRNA profiling of glioma was also analyzed in cerebrospinal fluid (CSF) in a study by Baraniskin et al. [69] which identified miR-21 and miR-15b as potential markers in CSF for glioma that can distinguish these patients from normal individuals and from patients with brain lymphoma and metastatic brain tumors.

A recent review that summarized the expression of specific miRNAs in GBM based on the recent literature identified 253 upregulated and 95 downregulated miRNAs. Both of the oncogenic and tumor suppressor miRNAs were found to target genes involved in cell migration, invasion, angiogenesis and proliferation [28].

In addition to studies aiming at analyzing the miRNA signatures of GBM, there have been multiple studies reporting the expression of specific miRNAs in different tumor grades. miR-21 has been shown to exhibit both diagnostic and prognostic values in different tumors including gliomas [70]. The expression of miR-26a has been associated with poor prognosis [71]. The decreased expression of miR-328 is associated with unfavorable prognosis in glioma [72] and miR-181d expression acted as a predictive biomarker for temozolomide response [73]. We recently reported that both miR-145 [74] and miR-137 [75] were downregulated in glioblastoma as compared to normal brain specimens and that the decrease of miR-137 was attributed to hypermethylation of pre-miR-137 promoter [75].

2.5 miRNA Functions in GBM

In addition to their diagnostic application, specific miRNAs have been also implicated as potential therapeutic targets and tools [76, 77]. The most appealing advantage of using miRNAs for the treatment of cancer therapy is their ability to affect multiple target genes in the context of a network, making them suitable especially for the treatment of glioma that is a complex heterogeneous tumor. The

miRNAs that are overexpressed in a deregulated manner in tumors are considered as oncogenes and are called oncomiRs [71, 78]. They are associated with the inhibition of tumor suppressor genes or those which control cell proliferation, differentiation, migration and apoptosis.

Various studies have highlighted the importance of miRNA deregulation in several aspects of the pathogenesis of GBM including cell cycle control, invasion, migration, resistance to chemotherapy and radiotherapy and cell apoptosis [79–81]. Specific miRNAs control some of the core signaling pathways in GBM such as EGFR signaling and those related to p53, PTEN/PI3K/AKT and the Notch pathways. Moreover, several in vitro and pre-clinical studies demonstrated the therapeutic benefits of either expressing tumor suppressor miRNAs or inhibiting OncomiRs [82–85].

Various modification of anti-miRNA oligonucleotides have been reported to successfully decrease miRNA expression in pre-clinical models, including conjugation to peptides, addition of 2-O-methoxyethyl and locked nucleic acid (LNA)-oligonucleotides anti-miRNA oligonucleotides [86, 87]. In addition, various reports described the simultaneous silencing of multiple miRNAs that share the same seed by using seed-targeting tiny LNAs [88]. Similarly, miRNA sponges, which are transcripts with repeated miRNA antisense sequences that contain tandem siRNA binding sites can also sequester miRNA from their endogenous targets and can block an entire miRNA seed family [89].

The delivery of miRNA mimics can be used for increasing the expression of downregulated or tumor suppressor miRNAs [90]. Studies using adeno-associated virus, lipid-based nanoparticles, and the combination of miRNA expression vectors and lipid-based nanoparticles, demonstrated effective biological impact in various xenograft systems [91]. However, difficulties in the delivery of miRNA mimics may be due to difficulties in targeting a specific tissue and the fate and function of the passenger strand that sometimes acts as an anti-miRNA.

2.5.1 Oncogenic miRNAs in GBM

Different oncogenic miRNAs have been described in GBM. This paragraph highlights some of these miRNAs and their cellular functions.

The miR-21 – miR-21 is located on chromosome 17 within TMEM49, a transmembrane protein that is overexpressed in various tumors [70] and is also associated with the malignancy of glioma [92]. miR-21 is considered an oncogenic miRNA and it regulates cell migration by inhibiting RECK and TIMP3 [93] and cell apoptosis [94]. Silencing of miR-21 decreases glioma cell migration and growth, induces cell apoptosis and inhibits the growth of glioma xenografts [95].

miR-26a – miR-26a was reported to be amplified in glioma and to promote glioma cell growth and transformation. MAP3K2 and MEKK2 have been implicated as potential targets of miR-26a that eventually lead to cell

proliferation and inhibition of cell apoptosis via targeting of the RBI, Pi3K/AKT and the JNK pathways [95].
miR-221/222 – These two miRNAs are highly expressed in GBM and regulate glioma cell proliferation by targeting p27 and cell apoptosis by targeting the survivin 1 homologs BIRC1 and NIAP. These miRNAs also promote resistance of glioma cells to cytotoxic T cells by targeting ICAM-1 [96].

Some of the additional oncomiRs that were described in gliomas include miR-10b, miR-125b, miR-182, miR-296, miR-196a [52].

2.5.2 Tumor Suppressor miRNAs in GBM

Some miRNAs exhibit tumor suppressive activities by targeting oncogenes or signal pathways associated with proliferation, migration or resistance to cell apoptosis. Some of the known glioma tumor suppressor miRNA include the following miRNAs.

miR-181 – miR-181a, 181b and 181c were described as tumor suppressor miRNAs in glioma and overexpression of miR-181a sensitizes glioma cells to radiation by targeting Bcl_2 [97].
miR-124 – miR-124 inhibits glioma cell growth by downregulating SOS1. miR-124 also inhibits the STAT4 signaling pathway and enhances T cell mediated immune clearance of glioma [98].
miR-145 – This miRNA is downregulated in glioma specimens and have been shown to inhibit glioma cell migration by targeting CTGF [74] and NEDD9 [99]. Additional studies demonstrated that miR-145 also targeted Sox9 and adducin 3 in glioma cells [100].
miR-146a – miR-146a is another miRNA that inhibits the migration and invasion of glioma cells probably by targeting MMP16.

Additional miRNAs in this group include, miR-128, miR-137, miR-17 and miR-184, miR-218 and miR-219-5p [52].

2.6 miRNAs in Glioma Stem Cells

Despite the large number of publications related to the expression and function of specific miRNAs in glioma cells, much less is known about the role of miRNAs in GSCs. Three main approaches were undertaken to identify miRNAs important for the function of GSCs, comparative analysis of miRNA expression in GSCs vs. normal neural stem cells (NSCs), comparing subpopulations of GSCs such as CD133+ and CD133− and comparing undifferentiated (neurospheres) vs. differentiated GSCs.

2.6.1 GSCs Versus NSCs

As described in Sect. 2.2, a potential origin of GSCs is attributed to transformed NSCs [39]. Therefore, one of the approaches to identify miRNAs that play a role in the initiation, maintenance and function of GSCs is to study their expression and function in comparison with that of NSCs. The similarity in some of the characteristics of NSCs and GSCs raises the possibility that these two cell types share some common miRNA-based regulatory pathways but differ in others. Indeed, NSCs and GSCs share partially overlapping miRNA profiles in particular those related to self-renewal and proliferation, whereas miRNAs related to differentiation, migration and response to apoptotic stimuli are expected to be quite different. A study that compared the miRNA profiles of glial tumors, embryonic stem cells (ESCs), neural precursor cells (NPCs), and normal adult brain tissues of both human and mouse origin, reported that gliomas display a microRNA expression profile reminiscent of neural precursor cells [101]. Half of these miRNAs were clustered as miR-17-92, miR-106b-25, miR-106a-363, miR-183-96-182, miR-367-302, miR-371-373 and the large miRNA cluster in the Dlk1-Dio3 region [101]. Fifteen miRNAs exhibited disparate expression between stem cells and glioma specimens; ten were associated with stem cell specific clusters and five (miR135b, miR-141, miR205, miR-200c, miR-301c) were associated with malignancy [101].

In a recent study a genomic-wide miRNA expression profiling in GSCs and NSCs using combined miRNA microarray and deep sequencing analysis identified eight miRNAs (miR-10a, miR-10b, miR-140-5p, miR-204, miR-424, miR-34a, miR-193-aP and miR-455-5p) that were up-regulated and two miRNAs (miR-124 and miR-874) that were downregulated in GSCs relative to NSCs. These modified miRNAs inhibited the expression of respective target genes that were involved in either tumor suppression or progression [102].

2.6.2 miRNAs Associated with CD133+ and CD133− Cells

Another method for identifying miRNAs that play a role in the stemness of GSCs is to compare the expression of miRNA signature between CD133+ and CD133− cells. Using this approach a number of miRNAs were identified including miR-425, miR-451 and miR-486. miR-451 was reported to target the LKB1/AMPK pathway and to sensitize cells to glucose deprivation [52, 103].

2.6.3 miRNAs Associated with the Differentiation of GSCs

One of the approaches to target GSCs is by their differentiation which abolishes their stemness and tumorigenic potential. Therefore differentiation of GSCs in the presence of serum and absence of growth factors can reveal miRNAs that are associated with the stemness inhibition of these cells. Using this approach, Fareh

et al. [104] identified that the miR-302-367 cluster was induced during the differentiation of GSCs and that stable expression of this cluster inhibited self renewal and stemness of GSCs by targeting the CXCR4 pathway upstream of sonic hedgehog (SHH)-GLI-NANOG. Similarly, a recent study analyzing the miRNAs of differentiated GSCs identified miR-1275 as a major miRNA that was downregulated during the differentiation process along with up-regulation of its target gene claudin 11 [105]. miR-137 was also reported to be upregulated during NSC and GSC differentiation along with downregulation of its target gene RTVP-1/GLIPR1 [75].

Additional miRNAs have been identified as important in regulating various functions of GSCs.

miR-7 – miR-7 was reported to affect the proliferation and invasion of a GSC line and to inhibit the expression of EGFR and AKT [84].

miR-9/9* and miR-17 – These miRNAs, in addition to miR-106b, are highly abundant in CD133+ GSCs. Their inhibition decreases neurosphere formation and induces GSC differentiation. Both miR-9/9* and miR-106b target calmodulin-binding transcription activator 1 (CAMTA1) [106]. Interestingly, the inhibitor of differentiation 4 (ID4), induces dedifferentiation of human glioma cells to glioma stem-like cells and enhances SOX2 expression by suppressing miR-9* [107].

miR-124 – miR-124 is one of the major neuronal miRNA and has reported to promote neuronal differentiation by targeting the PTBP1, Sox9 and the REST pathways (27, 114). In addition, miR-124 decreases neurosphere formation, CD133+ population and stem cell marker expression in GSCs by targeting SNAI2 [108].

miR-137 – This miRNA has been also reported to promote the neuronal differentiation of NSCs and GSCs by targeting CDK6 [109]. In addition, we recently reported that miR-137 targets RTVP-1 that regulated the stemness of GSCs upstream of the CXCR4-Gli- pathway [75].

miR-34a – miR-34 which is downregulated in glioma, exerts tumor suppressive effects in GSCs by targeting c-Met, Notch-1 and Notch-2. The Notch pathways plays a major role in the stemness, proliferation and radioresistance of GSCs. miR-34 induced GSC differentiation, and inhibited GSC proliferation, migration and survival [110, 111].

miR-326 – miR-326 is another miRNA that targets the Notch pathway but very interestingly its expression is regulated by its target gene [112]. miR-326 exerted cytotoxic effects on GSCs in vitro and in vivo [112].

miR-17-92 cluster – This miRNA cluster has been implicated in the regulation of GSC apoptosis, proliferation and differentiation. Target genes of the miR-17-92 cluster including CDKN1A, E2F1, PTEN and CTGF have been identified [113].

miR-128 – miR128 has been identified as a tumor suppressor miRNA in glioma [27, 52] and was reported to inhibit the self-renewal and stemness of GSCs by targeting the polycomb transcriptional repressor Bmi [114].

miR-145 and miR-143 – miR145 expression is significantly decreased in GSCs compared to NSCs and it decreases the migration of these cells by targeting CTGF and its downstream signaling protein SPARC [74]. In addition, miR-145 has been reported to target the stemness-related protein SOX2 in GSCs [115]. Similarly, the expression of miR-143 was decreased in GBM and this miRNA inhibited glycolysis and the stemness of GSCs [116].

2.7 *miRNA Delivery into Brain Tumors*

The potential therapeutic use of miRNAs is hindered by the lack of effective delivery approaches into target tissues. The main challenges are associated with the ability of therapeutic miRNAs to enter the cell cytoplasm without encountering the endosomal vesicles, evading kidney filtration and excretion and removal from the bloodstream by phagocytic cells. This difficulty is further amplified in the case of brain tumors due to the presence of the blood brain barrier (BBB), which prevents the entry of RNA molecules and potential RNA-based therapy. Although the BBB is compromised in areas of tumors, it is still intact in areas of tumor infiltration. Various methods including the use of stereotactic or direct intra-tumoral injection, convection-enhanced delivery, intrathecal and intra-ventricular injection and intravascular infusion with or without modification of the blood-tumor-barrier were described for the delivery of drugs into the brain [117]. In addition, recent studies demonstrated intranasal delivery of stem cells, exosomes, nanoparticles and viruses to the brain in animal models of brain inflammation and glioma xenografts [117].

2.8 *Vectors for miRNA Delivery*

Vectors for gene therapy and the delivery of miRNAs can be divided into two categories: viral and non-viral vectors. Viral vectors include adenovirus, adeno-associated virus, lentivirus and retrovirus vectors. Indeed, various reports demonstrated the ability of viral vectors to successfully deliver miRNAs to tumor sites [118, 119]. However, despite multiple reports demonstrating that modified viral vectors are effective in gene delivery, the immune response to adenovirus vectors remains a problem and lentivirus vectors have the potential for insertional mutagenesis, which may be a concern when used for therapy [118, 119]. Therefore, non-viral vectors, which retain biocompatibility, targeting efficacy and enhanced transfection efficiency, are a more suitable alternative to viruses for achieving successful miRNA delivery without side effects [120, 121].

In addition, RNA nanoparticles, in which the scaffold, the ligand and the therapeutic tool are all composed of RNA, have been recently described to specifically target tumors with low toxicity and low immunogenicity [122].

2.9 Exosomes for miRNA Delivery

In addition to the biological and chemical vehicles described so far, a recent approach for RNA delivery has been recently explored using extracellular vesicles and in particular exosomes, due to their natural adaptation for the transport of various substances, including nucleic acids [123].

Exosomes are membrane vesicles of endocytic origin, 50–100 nm in diameter, and are secreted by most cells into the extracellular environment [124]. They can impact the function of neighboring cells through intercellular transfer of mRNAs, microRNAs, receptors and enzymes, and are involved in the communication of immune responses [125]. Exosomes have multiple advantages over existing microRNA delivery vehicles such as low immunogenicity since they can be derived from a patient's own cells [126]. More importantly, exosomes are natural carriers for miRNAs, which make them excellent delivery systems for these molecules [127]. We recently showed that synthetic miRNA mimics are delivered by mesenchymal stem cells (MSCs) to glioma cells via exosomes [115]. Similarly, exosomes isolated from MSCs have been reported to deliver miR-146 and miR-9 to glioma cells [128, 129].

These and other studies suggest that the future advances in the manipulation and targeting of miRNA delivery by exosomes may lead to the development of efficient cancer-specific therapy.

2.10 Stem Cells for the Delivery of miRNAs

An alternative approach to current therapies of GBM with a potential to target infiltrative tumor cells is the use of stem cells, which exhibit homing to tumor and injury sites in the brain. Indeed, numerous studies demonstrated tropism of NSCs to infiltrating glioma cells in the brain and their therapeutic benefits [130]. Another source of stem cells that exhibit tropism to tumor cells is adult human mesenchymal stromal stem cells (MSCs) that can be obtained from autologous bone marrow (BM) and adipose tissue or from cord or placenta. MSCs exhibit homing abilities, which enable them to migrate to sites of injury, inflammation and tumors [131, 132]. Specifically, MSCs have been shown to cross the blood brain barrier and migrate to sites of experimental GBM when administered intra-arterially and intravenously and can deliver cytotoxic compounds and exert anti-tumor effects [133, 134].

The ability of MSCs to cross the blood brain barrier, to home to tumor cells and deliver therapeutic molecules render these cells excellent delivery vehicles for the targeted therapy of brain tumors. We recently demonstrated the ability of MSCs to deliver miRNA mimic to glioma cells and GSCs in vitro and in vivo. The delivery of the miRNA mimic was mediated by exosomes and had an impact on the expression of target genes, cell migration and invasion [115]. In addition, recent

studies also demonstrated the use of MSC-derived exosomes to deliver miRNA mimics to glioma cells [128, 129].

2.11 Pre-clinical Studies in Glioma and GSC-Derived Xenografts

Different viral vectors have been utilized for the treatment of glioma in pre-clinical studies. A recent study combined adenoviral vectors expressing hTERT-targeting ribozyme controlled HSV-tk expression together with the overexpression of miR-145. Intratumoral injection of the adenovirus vector expressing the HSV-tk expression cassette plus miR-145, combined with intraperitoneal injection of ganciclovir increased animal survival [135]. In another study, Wang et al., employed adenovirus vector expressing siRNAs that silenced the expression of miR-221 and miR-222 in glioma cells and increased the expression of p27kip1 that led to cell cycle arrest in G1 and cell apoptosis [136]. Similarly, we recently demonstrated the effectiveness of lentivirus vectors expressing pre-miR-124, pre-miR-137 and pre-miR-145 in the transduction and function of glioma stem cells [74, 75, 115].

In addition to the use of viral vectors there have been studies describing the use of nanoparticles for the delivery of miRNA mimics or siRNAs. Stable nucleic acid lipid particles (SNALPs), which target GBM cells were generated by covalent coupling of the peptide chlorotoxin (CTX) to the liposomal surface [137]. These CTX-coupled SNALPs efficiently and specifically delivered encapsulated anti-miR-21 oligonucleotides to cultured U87 GBM cells and into established intracranial tumors.

In another study, Yang et al., employed cationic polyurethane (PU)-shortbranch PEI (PU-PEI) to deliver miR-145 into CD133+ GSCs which decreased their oncogenic potential and induced their differentiation into CD133-cells. Intravenous administration of the nanoparticle-formulated miR-145 increased the sensitivity of CD133+ GSC-derived xenografts to temozolomide and radiation and prolonged animal survival [138].

In another study, systemic administration of miR-7 encapsulated in cationic liposomes resulted in the decreased growth of glioma xenografts and metastatic nodules by targeting the EGFR [139].

3 Conclusions

miRNAs have been reported to play major roles in a variety of cellular processes and biological system and their deregulation has been implicated in the pathogenesis of many diseases including cancer. Multiple studies have demonstrated the use of miRNAs as potential diagnostic and prognostic markers in GBM and as important regulators of GSC functions. Moreover, preclinical studies demonstrated their impact on tumor growth and invasiveness and novel approaches have been

developed for the delivery of miRNA mimics or antagonists to tumor sites. However, despite these significant advances, there are still major issues that need to be addressed prior to the clinical application of miRNA-based therapy in GBM. These include specific target validations and prevention of undesired off-target effects, and the development of delivery approaches for the targeting infiltrating glioma cells and GSCs. Nonetheless, the increasing numbers of discoveries and reports contribute to our understanding of the mechanisms involved in the biology of GBM and GSCs and are likely to make a significant therapeutic impact in the near future.

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Chapter 3
The Role of MicroRNA in Lung Cancer Drug Resistance and Targeted Therapy

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1 Introduction

Based on the report in *Cancer Statistics 2013* in USA, lung and bronchus cancers in men and in women continue to be the leading common causes of cancer death, although cancer death rates have declined 20 % from their peak in 1991 (215.1 per 100,000 population) to 2009 (173.1 per 100,000 population) [1]. Chemotherapy and targeted therapy are also the mainstream in lung cancer treatment. However, the major problem in lung cancer therapy is the emergence of inherent and acquired drug resistance of the cancer cells [2]. MicroRNAs (miRNAs) are a new class of small, non-coding RNAs that range in size from 19 to 25 nucleotides (nt) and have important roles in a variety of biologic processes [3]. miRNAs are predicted to regulate the expression of up to one-third of human protein-coding genes, and they are involved pathogenesis, diagnosis, treatment and prognosis [4–7]. The accumulating evidences demonstrate that miRNAs regulate drug sensitivity and/or resistance to chemotherapeutic agents [8–11]. Therefore, to explore the role of miRNAs in drug resistance may accelerate novel therapeutic strategies for lung cancer treatment [12].

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2 Drug Resistance in Lung Cancer

Chemotherapy is a major treatment modality in both primary and palliative care for lung cancer patients. However, some patients do not respond to such therapy, or they respond well initially and then gradually relapse. This may lead to an increase in the drug dosage, which generally increases the adverse affects, yet fails to improve the clinical prognosis or outcome.

2.1 Current Opinion of MDR in Lung Cancer

Non-small cell lung cancer (NSCLC) cells are often intrinsically resistant to certain anticancer drugs, whereas small-cell lung cancer (SCLC) cells can acquire resistance with continued administration of the drug. Moreover, at the time of diagnosis, the majority of patients with lung cancer most often already have metastatic disease, making it difficult to use other therapeutic options, such as surgery or radiation [13]. Thus, a better understanding of the different mechanisms underlying multiple drug resistance (MDR) is of utmost importance if we are to develop strategies to overcome it. Although numerous mechanisms are associated with MDR in lung cancer, we are a long way from fully understanding how to overcome drug resistance.

2.2 Representative Features of MDR

There are three separate forms of MDR have been characterized in more detail [14]: (a) atypical MDR, (b) classical MDR and (c) non-Pgp MDR. Although all three MDR phenotypes have much in common with respect to cross-resistance patterns, the underlying mechanisms certainly differ. Atypical MDR is associated with quantitative and qualitative alterations in topoisomerase II alpha, a nuclear enzyme that actively participates in the lethal action of cytotoxic drugs [15]. Moreover, atypical MDR cells do not overexpress P-glycoprotein, and are unaltered in their ability to accumulate drugs. Given that classical and non-Pgp MDR is transcriptional activation of membrane-bound transport proteins, these transport proteins belong to the ATP-binding cassette (ABC) superfamily of transport systems. The classical MDR phenotype is characterized by a reduced ability to accumulate drugs, due to activity of an energy-dependent uni-directional, membrane-bound, drug-efflux pump with broad substrate specificity [16]. The classical MDR drug pump is composed of a transmembrane glycoprotein (P-glyco-protein, Pgp) with a molecular weight of 170 kDa, and is, in man, encoded by the so-called multidrug resistance (MDR1) gene. Typically, non-Pgp MDR has no P-glycoprotein expression, yet has about the same cross-resistance pattern as classical MDR. This

non-Pgp MDR phenotype is caused by overexpression of the multidrug resistance-associated protein (MRP) gene, which encodes a 190 kDa membrane-bound glycoprotein. MRP probably works by direct extrusion of cytotoxic drugs from the cell and/or by mediating sequestration of the drugs into intracellular compartments, both leading to a reduction in effective intracellular drug concentrations. Together, all these three types of MDR have much similar mechanism in drug resistance induction.

2.3 Molecular Mechanisms of MDR

Many molecular mechanisms have been identified, such as (a) drug transporters involved in the efflux of chemotherapeutic drugs, (b) drug inactivation by sulfur-containing macromolecules and role of antioxidants, (c) DNA repair pathways inducing resistance to chemotherapy, (d) modifications or alterations of drug target sites, (e) loss of intracellular death mechanisms, (f) resistance to small molecule inhibitors and (g) alteration of drug metabolism pathway (Fig. 3.1) [17–22]. Additional contributing factors include ineffective drug delivery to the tumor, increased metabolism and therefore a shortened half-life of the drug, lack of drug specificity to the tumor, and tumor vasculature [23, 24]. These factors make it even harder to pinpoint the exact mechanism underlying resistance to a particular drug. It is hoped that identification of new targets and understanding their contribution to lung cancer drug resistance will provide opportunities for innovative therapies in overcoming drug resistance.

2.4 miRNA Analysis – A Novel Screening Tool in Lung Cancer Chemotherapy

There is a need for the development of new tools to screen patients prior to beginning chemotherapy. More recently a tool known as lung metagene score was developed in an attempt to individualize treatment for lung cancer patients [25]. The lung metagene score (formerly known as the lung metagene predictor) is a screening tool developed to estimate the risk of recurrence in early stage NSCLC [26, 27]. By comparing microarray data of untreated and drug-treated NSCLC cells, Heller et al. identify 33 miRNAs whose expression is upregulated after drug treatment and which are associated with a CpG island [28]. Moreover, resveratrol, a plant phenolic phytoalexin that has been reported to have antitumor properties in several types of cancers, alters miRNA expression profiles in NSCLC cells [29]. Specifically, miR-21 acts a biomarker predictive for platinum-based adjuvant chemotherapy response in NSCLC patients [30]. Although mRNA analysis is a novel screening tool for lung cancer chemotherapy, this technique is still under

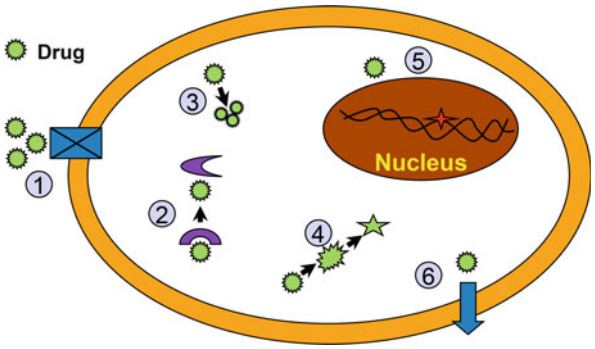

Fig. 3.1 The typical molecular cellular mechanisms for drug resistance. Drug resistance is often seen through: ① failure of the drug to enter the cell by loss of the cell surface receptors or transporters; ② alteration of drug target site; ③ change or inactivation of drug; ④ alteration of drug metabolism pathway; ⑤ increased repair of damaged DNA; ⑥ active transport out of the cell

development and needs extensive clinical trials before it can be used as standard tool in the management of patients with lung cancer. This will also allow initiation of early and aggressive chemotherapy in patients with a high risk of recurrence in early stage NSCLC. Moreover, such screening tools will also allow us to choose the most appropriate chemotherapy drug that will be most effective in the treatment of such patients, based on their miRNA profiling.

3 miRNAs in Lung Cancer: Tiny Molecules with Huge Impacts

miRNAs play important roles in the regulation of a wide class of cellular processes by sequestering target mRNAs and inhibiting translation of the proteins that they encode.

3.1 miRNAs Altered in Lung Carcinogenesis and Development

A large number of human miRNA genes are frequently located at fragile sites, as well as in minimal regions of loss of heterozygosity, minimal regions of amplification (minimal amplicons), or common breakpoint regions. Overall, more than one half of miR genes are in cancer-associated genomic regions or in fragile sites [31]. This phenomenon indicates miRNAs may act as oncogenes (oncomiRs) or tumor suppressors (tumor suppressor miRs) in lung carcinogenesis. For example, the mutations of tumor suppressor miRs result in carcinogenesis.

3.1.1 miRNAs as Oncogenes-OncomiRs

Aberrations within the cancer miRome were first described in B-cell chronic lymphocytic leukemia, where the locus for the tumor suppressor miR-15 is deleted in 68 % of patients [32]. miRNA-specific phenotypes have since been described for a number of other malignancies. Several 'oncomiRs'-miRNAs behaving as oncogenes have now been identified and validated to play different roles in lung cell transformation and carcinogenesis (Table 3.1). Specially, miR-21 is a prominent oncogenic driver in a variety of cancers [33] and a number of studies have now shown miR-21 to be up-regulated in NSCLC of various stages and histologies [34–38]. Three targets have been validated for this miRNA: phosphatase and tensin homolog (PTEN), human mutS homolog 2 (hMSH2) (in NSCLC) and programmed cell death 4 (PDCD4) (in breast and colon cancers). Among these, both PTEN and PDCD4 are tumor suppressors that control cell growth and proliferation [35, 39, 40]. PTEN is also shown to be a target of miR-221/222 [41]. Overexpression of these two miRNAs in NSCLC cell lines plays a role in resistance to tumor necrosis factor (TNF)-related apoptosis-inducing ligand [42] and enhances migration through activation of the serine/threonine-specific protein kinase-Akt signaling pathway [41]. Although these findings require *in vivo* validation, miR-221/222 are known to be frequently upregulated in several solid tumors, including lung cancer [43]. Overexpression of miR-155 in adenocarcinoma and squamous cell lung cancer has also been shown across multiple studies [36, 44]. Its prognostic value and to its potential targets are now being explored [45]. Oncogenic properties of the miR-17-92 cluster, which is housed in the intronic region of chromosome 13q31.3, enhance tumorigenesis by regulating the translational product of proto-oncogene c-Myc (c-Myc) [46, 47]. The miRNAs belonging to this cluster (miR-17, -18a, -19a, -20a, -19b-1 and -92-1) are frequently present in SCLC, and their potential therapeutic applications in SCLC are now undergoing study [48]. Inhibition of miR-17-5p and miR-20a with antisense oligonucleotides can induce apoptosis selectively in lung cancer cells overexpressing miR-17-92, suggesting the possibility of 'oncomiR addiction' to expression of these miRNAs in a subset of lung cancers [49]. Collectively, the present findings contribute towards better understanding of the oncomiRs, which might ultimately lead to the future translation into clinical applications by inhibition of oncomiRs.

3.1.2 miRNAs as Tumor Suppressors-Tumor Suppressor miRs

Downregulation of certain miRNAs in cancer is indicative of their tumor-suppressor characteristics. The miRNA let-7 is involved in cell cycle progression [50] and is a classic example of a miRNA behaving as a tumor suppressor in human cancers. Decreased expression of let-7 family members is often observed across many cancers, including lung cancer, and is often associated with poor prognosis [43, 51]. Putative mechanisms of let-7 down-regulation in cancer include genetic

Table 3.1 Deregulated miRNAs in lung cancer

miRNA	Expression change	Deregulated mechanism	Protein targets
let-7	Down	Proliferation/apoptosis	c-Myc, K-RAS/cyclins
miR-34	Down	DNA damage response	P53
miR-106	Down	Proliferation	SLC7A5
miR-200	Down	Cell adhesion	ZEB
miR-17-92	Up	Proliferation	E2F
miR-21	Up	Apoptosis	PTEN, PDCD4, hMSH2
miR-31	Up	DNA mismatch repair/ chemoresistance	hMLH1/ABCB9
miR-155	Up	Survival/chemoresistance	FOXO3a
miR-205	Up	Invasion	E2F1
miR-221/ 222	Up	Cell growth, proliferation and migration	PTEN, TIMP3

ABCB9, ATP-binding cassette, sub-family B (MDR/TAP), member 9; c-Myc, a translational product of proto-oncogene c-Myc; E2F, a group of genes that codifies a family of transcription factors (TF) in higher eukaryotes; FOXO3a, forkhead box O (FOXO) transcription factor 3a; hMLH1, human mutL homolog 1; hMSH2, human mutS homolog 2; K-RAS, a translational product of proto-oncogene K-RAS; PDCD4, programmed cell death 4; PTEN, phosphatase and tensin homolog; TIMP3, tissue inhibitor of metalloproteinase 3

alterations [31], regulation of K-RAS, c-Myc and HMGA2 oncogenes [52–54], direct targeting of *Dicer* mRNA [55] and cell proliferation control in a cyclin-dependent manner [56]. Increased levels of K-RAS and its hyperactivity can occasionally be attributed to a polymorphism in the K-RAS 3′ untranslated region (3′UTR) binding site for let-7 (let-7 complementarity site 6 or LCS6). The allelic frequency of LCS6 in a large population is reported to be 5.8 % (n = 2,433) and as much as 20 % in NSCLC [57]. Underexpression of another miRNA family, the miR-34 cluster, is correlated with poor survival in male smokers with squamous cell carcinoma (SCC) stages I–IIIa [58, 59]. Induction of miR-34 results in apoptosis of lung cancer cells [60]. Expression levels of miR-34a/b/c are shown to be directly correlated with expression of the p53 tumor-suppressor protein, whereas ectopic expression of these miRNAs induces cell-cycle arrest in a mouse fibroblast model [61]. Another study indicates miR-34 expression is inversely correlated with Axl, a receptor that induces proliferation, migration and invasion in cancer, in a panel of NSCLC cell lines and 44 NSCLC resection specimens [62]. Exogenous delivery (local and systemic) of lipid-formulated miR-34 is also shown to reduce tumor size in a mouse model of NSCLC. The expression of miR-29 family is inversely correlated to DNA methyltransferase (DNMT) 3A and DNMT3B in lung cancer tissues and that miR-29 directly targets both DNMT3A and -3B. The enforced expression of miR-29 in lung cancer cell lines restores normal patterns of DNA methylation, induces reexpression of methylation-silenced tumor suppressor genes, such as fragile histidine triad (FHIT) and WW domain-containing oxidoreductase (WWOX), and inhibits tumorigenicity *in vitro* and *in vivo*

[63]. Overexpression of miR-126 in a lung cancer cell line results in a decrease in Crk protein without any alteration in the associated mRNA. These lung cancer cells exhibit a decrease in adhesion, migration, and invasion [64]. Both miR-15a and miR-16 are frequently deleted or down-regulated in squamous cell carcinomas and adenocarcinomas of the lung. In these tumors, expression of miR-15a/miR-16 inversely correlates with the expression of cyclin D1. These two miRs are implicated in cell cycle regulation in an Rb-dependent manner [65]. Furthermore, miR-1 is downregulated in human primary lung cancer tissues and cell lines. Expression of miR-1 in nonexpressing A549 and H1299 cells reverses their tumorigenic properties, such as growth, replication potential, motility/migration, clonogenic survival, and tumor formation in nude mice. Exogenous miR-1 significantly reduces expression of oncogenic targets, such as MET, a receptor tyrosine kinase, and Pim-1, a Ser/Thr kinase, frequently upregulated in lung cancer [66]. Taken together, these findings suggest a possible therapeutic potential for artificially enhancing cellular levels of tumor suppressor miRs.

3.2 miRNAs as Biomarkers for Early Diagnosis in Lung Cancer

Recently the report from the National Lung Cancer Screening Trial demonstrates an improved survival attributable to early detection of lung cancer by screening [67]. This finding underscores the importance of lung cancer screening research. Current efforts at screening primarily involve clinical history such as smoking and asbestos exposure, imaging in the form of low-dose computed tomography (CT), and either white light or autofluorescence bronchoscopy. The presence of miRNAs in body fluids offers an avenue to improve on these methods by quantitation of miRNAs in these fluids to enhance these efforts.

The first significant effort in this direction comes from Chen and colleagues [68]. By deep sequencing of pooled sera from patients with and without lung cancer, 8 miRNAs are identified as differentially expressed in comparison populations. These miRNAs then are validated in a few clinical samples by RT-PCR. A convincing external validation of these results is still pending. Another exploratory study looks at the potential for microarray profiling of serum-derived miRNAs [69]. In this study, the authors demonstrate the technical feasibility of performing such profiling and demonstrate the high accuracy of predicting cancer presence versus absence by cross-validation analysis. In fact, screening for circulating miRNAs in the peripheral blood can be used as a potential diagnostic tool in lung cancer [70]. Although no independent validation of such a signature is performed and cancers of different histology are grouped into one class, this is an important proof-of-principle study that serum miRNA profiling can be used to classify patients with and without cancer. A different approach to this problem is to perform miRNA microarray profiling of exosomes isolated from serum. Such a study demonstrates a higher exosome content in patients with lung cancer compared with controls [71]. The miRNA expression content of exosomes parallels that

of the tumor using a panel of 12 miRNAs, supporting the hypothesis that these are tumor-derived exosomes. A third approach is to perform miRNA profiling of whole blood with the hypothesis that the body's response to the tumor may be used to detect the presence of the tumor. Such an approach in a small pilot study demonstrates promising results worthy of future investigation [72]. In this study, the investigators used Paxgenetubes (Becton, Dickinson and Company, NJ) to collect whole blood. These tubes have a lysis agent as well as an RNA stabilizer that limits the changes in RNA induced by specimen processing. The miRNA profile of whole blood of 17 patients with lung cancer is compared with those of 19 controls without lung cancer. A 24-miRNA signature using a support vector machine algorithm has a classification accuracy of 95.4 %. Problems common to all of these studies include the absence of suitable controls and the lack of strong external validation data. The first is particularly important as tobacco exposure leads to alteration of miRNAs [73], and if nonsmoking controls are chosen, then the differences noticed may be a result of smoking exposure and not necessarily the presence of lung cancer. The approach of using whole blood is limited by the fact the miRNAs are expressed robustly in nonnucleated cells such as red blood cells (RBCs) [74], and the effect of the presence of cancer on the miRNA expression of RBCs is largely unknown; therefore, the biologic basis of the signature is difficult to delineate. However, these are useful data worthy of future study. Extensive validation of such signatures is essential to be convincing.

As well as other body fluids, sputum is abundant in miRNAs. Endogenous miRNAs are present in sputum in a remarkably stable form and sensitively and specifically detected by real-time RT-PCR. Detection of mir-21 expression produces 69.66 % sensitivity and 100.00 % specificity in diagnosis of NSCLC, as compared with 47.82 % sensitivity and 100.00 % specificity by sputum cytology [75]. Another study evaluates the utility of miRNA biomarkers of squamous lung cancer in human sputa. A directed RT-PCR study using 6 miRNAs demonstrates reasonable sensitivity (76 %) and specificity (96 %) of a 3-miRNA signature (miR-205, miR-210, and miR-708) for the detection of squamous cell lung cancer [76]. A similar study by the same group indicates a 4-miRNA signature (miR-21, miR-486, miR-375, and miR-200b) has a sensitivity of 80.6 % and a specificity of 91.7 % [77]. The optimized five miRNAs panel (miR-21, miR-143, miR-155, miR-210 and miR-372) detects NSCLC with 83.3 % sensitivity and 100 % specificity in 30 prospectively accrued study patients [78]. Therefore, these findings suggest sputum miRNA profiling using cluster analysis is a promising approach for the early detection of lung cancer. For better diagnosis, further larger follow-up studies using this approach are warranted.

3.3 miRNAs in Lung Cancer Prognosis

Many studies have attempted to use miRNA expression patterns to predict the prognosis of lung cancer patients. For the most part, these experiments have been

performed with NSCLC. The clinical question is an important one as a significant proportion of patients with even early, adequately resected lung cancer recur. It would be useful to generate a prediction algorithm to identify those that will recur; if such a prediction can be made accurately, and then this subset of high-risk patients can be potentially treated by adjuvant chemotherapy to decrease their disease recurrence rate.

The miRNA profiling of lung cancers can predict outcome is first reported by Yanaihara et al. [36]. In this early study, the investigators compare the miRNA expression profiles of numerous lung cancers with their corresponding normal counterparts. As a secondary analysis, they demonstrate that miRNAs let-7 and miR-155 can predict disease outcome. The second major study to address this issue is published by Yu et al. [79]. In this well-designed study, RT-PCR-based miRNA profiling is performed on training and test sets to identify a 5-miRNA-based prognostic classifier for lung cancer. This finding also is validated in an independent validation set. After these early studies, two microarray-based studies [51, 80], also demonstrate the prognostic potential of miRNAs. The first study is in squamous cell lung cancer and finds miR-146b to be a significant prognostic miRNA. A similar study by Patnaik et al. also demonstrates miR-146b-3p to have prognostic value along with several other miRNAs previously not associated with lung cancer prognosis. The prognostic value of miR-21 and miR-205 overexpression is also validated in NSCLC [37]. Genetic variants of miRNA sequences are associated with NSCLC survival [81]. Additional two studies have validated this concept [58, 82]. Interestingly, one large study has looked at the ability of serum miRNAs to prognosticate lung cancer [83]. Although the ability to predict prognosis reaches the statistical significance, the prediction accuracy is modest. A more recent study demonstrates no ability of measurement of expression of a limited set of miRNAs to predict the prognosis of lung cancer, adding a cautionary note to such investigations [84]. In summary, several additional steps are necessary before the concept of miRNA-based prognostication can be used clinically. First, additional validation studies are crucial; preferably these should be conducted in a prospective fashion. Second, the studies should focus on prediction accuracy and not solely on time-to-recurrence or time-to-survival end-points. Third, the day-to-day variability of the assays used as well as the variability across several samples from the same tumor should be assessed; this has not yet been performed in a systematic manner. Fourth, it will be useful to assess the ability of these assays to prognosticate based on CT-guided biopsies as this may spur neoadjuvant trials for patients at a high risk for recurrence. As is shown in the list of issues, these assays are several years away from clinical application.

3.4 miRNAs in Lung Cancer Therapy

Since miRNAs act as oncomiRs or tumor suppressor miRs in lung cancer, they can be used as potential agents in cancer treatment [85]. In NSCLC cells, anti-miRNA

oligonucleotides (AMOs) show a inhibitory effect on cell growth [86]. A recent paradigm shift in the field of cancer is the concept of personalized therapy. This shift is based on the assumption that one can predict the response of an individual tumor to a particular drug. As with gene expression profiling, miRNA profiling is being considered for the prediction of response to therapy. Conceptually, miRNA expression has been demonstrated to be associated with cancer cell line sensitivity. Thus far, three different groups have published miRNA expression profiles of the NCI-60 cancer cell lines [87–89]. These expression profiles have a moderate correlation with cell line sensitivity to many drugs. An example of such a correlation is demonstrated in Fig. 3.2. In this figure, expression profiles performed using the Affymetrix (Affymetrix Inc., Calif) miRNA microarray profiling of the NCI-60 cancer cell lines (unpublished data) are used to predict sensitivity to oxaliplatin. Other investigators have demonstrated that altering the levels of specific miRNAs changes the sensitivity of cancer cell lines to therapeutic agents [8, 90]. A small body of literature also associates miRNA expression to chemosensitivity prediction in other cancer systems [91–93]. Of particular interest is the potential of such prediction in SCLC demonstrated by a preliminary study [94]. However, this field is still in its infancy and is faced with many hurdles. For one, *in vitro* sensitivity may not correlate with *in vivo* sensitivity, as cancer behavior is the result of interplay between cancer cells and their environment. Also, most treatment modalities use multiple agents and confounding is an issue. Some attempts at tackling these issues involve bioinformatics algorithms such as the COXEN algorithm [95]. Clinically, an evolution of platforms and bioinformatics is required to overcome these issues.

4 miRNAs in Lung Cancer Drug Resistance

Although the studies that miRNAs are involved in special signal transduction pathways and regulatory mechanisms in lung cancer drug resistance just begins, there are a large number of experimental and clinical studies have shown that miRNAs play critical roles in chemotherapy resistance.

4.1 miR-Polymorphisms and miR-Targeted mRNA Polymorphisms Alter Drug Metabolism

Polymorphisms in the miRNA regulatory pathway (miR-polymorphisms or single nucleotide polymorphisms [SNPs] that interfere with mRNA function [miRSNPs]) are a novel class of functional polymorphisms present in the human genome [96]. They include not only miR-polymorphisms, but also polymorphisms in miR-targeted mRNAs.

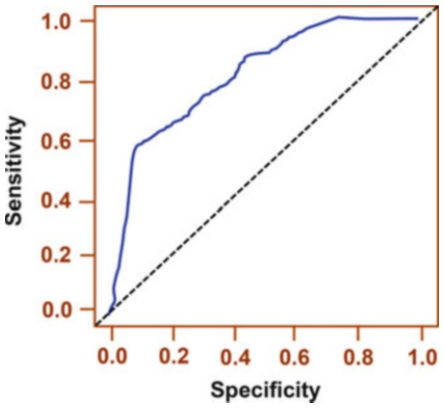

Fig. 3.2 Receiver operating characteristic curve demonstrating the ability of miRNA expression of the NCI-60 cancer cell line panel to predict chemosensitivity to oxaliplatin in an internal cross-validation analysis

In general, miRSNPs reside at or near a miRNA-binding site of a functional gene, influencing its expression by interfering with miRNA function [97–99]. In the clinical researches, it is found that the expression and polymorphisms in miR-targeted mRNAs are involved in drug responses. The expressions of multidrug resistance protein-1 (MRP1), breast cancer resistance protein (BCRP), lung resistance-related protein (LRP), and excision repair cross-complementing group-1 (ERCC1) in patients with advanced NSCLC are correlated with response to cisplatin-based chemotherapy [100]. These DNA repair gene polymorphisms are useful as predictors of clinical outcome to the platinum-based chemotherapy. Epidermal growth factor receptor (EGFR) kinase inhibitors induce dramatic clinical responses in NSCLC patients with advanced disease. EGFR gene polymorphism in intron 1 contains a polymorphic single sequence dinucleotide repeat (CA-SSR) shows a statistically significant correlation with the gefitinib response and is appeared to be a useful predictive marker of the development of clinical outcome containing skin rashes with gefitinib treatment. The other polymorphisms of EGFR are also associated with increased EGFR promoter activity. EGFR gene mutations and polymorphisms are also associated with EGFR kinase inhibitors response and toxicity [101]. Recent studies reveal that miRNAs affect drug metabolism by targeting ATP-binding cassette (ABC) genes, a class of drug transporters on cell membrane. Adenovirus-mediated RNA interference on endogenous miRNAs silences the ABC multidrug resistance protein 2 (ABCC2) expression in a mouse model [102]. Meanwhile, Zhu et al. reports that miR-27a and miR-451 are involved in activating the expression of P-glycoprotein, the MDR1 gene product that confers cancer cell resistance to a broad range of chemotherapeutics [103]. Expressions of miR-27a and miR-451 are upregulated in MDR cancer cell lines A2780DX5 and KB-V1, as compared with their parental lines A2780 and KB-3-1. Treatment of A2780DX5 cells with the antagomirs of miR-27a or miR-451 decreases the expression of P-glycoprotein and MDR1 mRNA. In contrast, the mimics of miR-27a and miR-451 increase MDR1 expression in the parental cells A2780. The sensitivity to and intracellular accumulation of cytotoxic drugs that are transported by P-glycoprotein are enhanced by the treatment with the antagomirs of

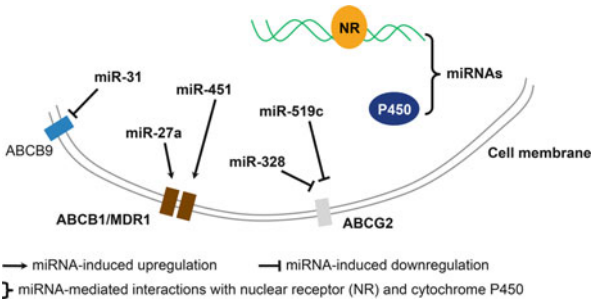

Fig. 3.3 Specific miRNAs regulate anticancer drug metabolism through targeting drug transporter (*DT*), nuclear receptor (*NR*) and human cytochrome P450

miR-27a or miR-451. Meanwhile, To et al. shows that miR-519c regulates ABCG2 expression at the 3′UTR of its mRNA through modulation of transcript stability and protein translation and then leads to drug resistance [104]. Similarly, miR-328-directed downregulation of ABCG2 expression in MCF-7/MX100 cells results in an increased mitoxantrone sensitivity [105]. Interestingly, our recent data indicates miR-31 targets ABCB9, an important member of ABC family, and inhibits cisplatin-induced cell apoptosis in NSCLC (accepted data by Cancer Letters). Using a bioinformatical tool, some miRNAs interact with drug transporter, nuclear receptor (NR) and human cytochrome P450 [106–108]. More and more studies show that miRNAs are involved in drug metabolism and distribution by regulating drug-metabolizing enzymes, drug transporters and/or NR genes (Fig. 3.3). These results demonstrate the dysregulated miRNA expression plays an important role in an abnormal drug metabolism.

4.2 miRNA Polymorphisms and Drug Resistance

MiR-polymorphisms potentially influence drug uptake, metabolism and distribution by regulating multiple gene expressions. As a result, polymorphisms affect the treatment efficiency or induce resistance to the special anticancer drug during this process.

Analysis of the publicly available SNP database reveals the presence of a relatively high level of variations in the 3′UTRs of miRNA target genes [109]. However, relatively low levels of variation are observed in the miRNA seed region of a functional miRNA. Approximately 250 SNPs are found to potentially create target sites for miRNAs [110]. Functional polymorphisms in the 3′UTRs of several genes have been reported to be associated with diseases by affecting gene expression [111]. Some of these polymorphisms may interfere with the function of miRNA and are potential miR-polymorphisms able to affect the expression of miRNA targets [112]. Therefore, miR-polymorphisms may result in gain or loss of a miRNA function. Based on the current knowledge of this field, miR-polymorphisms can be classified in the following three major categories [96]: (a) polymorphisms affecting miRNA biogenesis, (b) miR-polymorphisms in miRNA target sites and

(c) miR-polymorphisms altering epigenetic regulation of miRNA genes. Recently, miR-polymorphisms have been shown to affect drug response and have the potential to confer drug resistance [96]. It is demonstrated that a C to T SNP, identified in a case–control study of childhood leukemia patients, occurring with 14.2 % allelic frequency in the Japanese population, is present near the miR-24 binding site in the 3′UTR of the dihydrofolate reductase (*DHFR*) gene. The T allele of the SNP results in loss of miR-24-mediated regulation of DHFR, high DHFR protein levels and confers methotrexate (MTX) resistance [99]. This finding may also be useful in predicting the clinical outcome of MTX treatment.

4.3 miRNAs Altered in Drug Resistance

Recently, increasing studies have indicated that aberrant miRNA expression is strongly implicated in anticancer drug resistance phenotype. Their involvements in tumor cells response to chemotherapeutic agents are being confirmed by more and more reports.

4.3.1 miRNAs Affect Drug Sensitivity to EGFR Mutants

DNA sequencing of 623 genes with known or potential relationships to lung adenocarcinoma reveals more than 1,000 somatic mutations across the samples. It has been identified 26 genes that are mutated at significantly high frequencies and thus are probably involved in carcinogenesis. Notably, EGFR is one of the frequently mutated genes [113]. Interestingly, miRNAs may regulate EGFR mutation in NSCLC. Comparing paired lung cancer tissue with adjacent normal lung parenchyma, miR-126*, miR-145, miR-21, miR-182, miR-183 and miR-210 are found to be the most differentially expressed miRNAs. Most interestingly, an obvious inhibition of cell growth is observed in the EGFR mutant lung adenocarcinoma after transfection of pre-miR-145. These results also show that restoration of tumor suppressor miR-145 inhibits cancer cell growth in EGFR mutant lung adenocarcinoma [114]. Further study on these specific differentially expressed miRNAs may provide important information on peculiar tumorigenetic pathways and may identify useful biomarkers.

EGFR gene mutations, which are correlated with sensitivity to EGFR-tyrosine kinase inhibitors (EGFR-TKIs), are more frequent in never-smoker lung cancers. A miRNA expression profiling of 28 cases of never-smoker lung cancer identifies aberrantly expressed miRNAs, which are much fewer than in lung cancers of smokers and includes miRNAs previously identified (e.g., upregulated miR-21) and unidentified (e.g., downregulated miR-138) in those smoker cases. The changes in expression of some of these miRNAs, including miR-21, are more remarkable in cases with EGFR mutations than in those without these mutations. A significant correlation between phosphorylated-EGFR (p-EGFR) and miR-21 levels in lung

carcinoma cell lines and the suppression of miR-21 by an EGFR-TKI, AG1478, suggests that the EGFR signaling is a pathway positively regulating miR-21 expression. In the never-smoker-derived lung adenocarcinoma cell line H3255 with mutant EGFR and high levels of p-EGFR and miR-21, antisense inhibition of miR-21 enhances AG1478-induced apoptosis. In a never-smoker-derived adenocarcinoma cell line H441 with wild-type EGFR, the antisense miR-21 not only shows the additive effect with AG1478 but also induces apoptosis by itself [115]. These results suggest that aberrantly increased expression of miR-21, which is enhanced further by the activated EGFR signaling pathway, plays a significant role in lung carcinogenesis in never-smokers, as well as in smokers, and is a potential therapeutic target in both EGFR-mutant and wild-type cases.

To understand the role of miRNA in EGFR-TKI-resistant NSCLCs, Garofalo et al. examines the changes in miRNA that are mediated by tyrosine kinase receptors. They report that miR-30b/c, miR-221 and miR-222 are modulated by both EGF and MET receptors, whereas miR-103 and miR-203 are controlled only by MET. Importantly, they show that these miRNAs have important roles in gefitinib-induced apoptosis and epithelial-mesenchymal transition (EMT) of NSCLC cells *in vitro* and *in vivo* by inhibiting the expression of the genes encoding BCL2-like 11 (BIM), apoptotic peptidase activating factor 1 (APAF-1), protein kinase Cε (PKC-ε) and sarcoma viral oncogene homolog (SRC) [116]. These findings suggest that modulation of specific miRNAs may provide a therapeutic approach for the treatment of NSCLC.

A recent study in NSCLC cell lines demonstrates that the tumor microenvironment elicits transforming growth factor β1 (TGFβ1) and stimulates a miRNA gene expression program that induces resistance to anti-EGFR therapy and drives lung tumor cells to EMT, invasion, and metastasis [117]. Moreover, miR-214 regulates the acquired resistance to gefitinib via the PTEN/Akt pathway in EGFR-mutant cell lines [118]. To overcome this kind of resistance, Rai et al. focuses on EGFR suppression using miR-7, targeting multiple sites in the 3′UTR of EGFR mRNA. In this study, two EGFR-TKI-sensitive cell lines (PC-9 and H3255) and two EGFR-TKI-resistant cell lines harboring T790M (RPC-9 and H1975) are used. They construct miR-7-2 containing miR-7-expressing plasmid and the miR-7 expression level of the transfectants is approximately 30-fold higher, and the luciferase activity is ablated by 92 %. The results show miR-7 significantly inhibits cell growth not only in PC-9 and H3255 but also in RPC-9 and H1975. Expressions of insulin receptor substrate-1 (IRS-1), RAF-1, and EGFR are suppressed in these four cell lines. Injection of the miR-7-expressing plasmid reveals a remarkable tumor regression in a mouse xenograft model using RPC-9 and H1975. Moreover, EGFR, RAF-1, and IRS-1 are suppressed in the residual tumors [119]. These findings indicate promising therapeutic applications of miR-7-expressing plasmids against EGFR oncogene-addicted lung cancers including T790M resistance by liposomal delivery.

4.3.2 miRNAs Affect TRAIL Resistance

The TNF-related apoptosis inducing ligand (TRAIL) has gained much attention due to its specific anti-tumor potential without toxic side effects. Thus TRAIL is a promising new anticancer biotherapeutic [120]. As shown by many preclinical studies, TRAIL efficiently induces apoptosis in numerous tumor cell lines but not in the majority of normal cells. However, an increasing number of publications report on a predominance of TRAIL resistance in primary human tumor cells, which require sensitization for TRAIL-induced apoptosis. Sensitization of cancer cells by treatment with chemotherapeutic drugs and irradiation has been shown to restore TRAIL sensitivity in many TRAIL-resistant tumor cells [121]. How miRNAs regulate TRAIL resistance needs to be fully explored.

To define novel pathways that regulate susceptibility to TRAIL in NSCLC, a genome-wide expression profiling of miRNAs is performed by Garofalo and colleagues. They show that in TRAIL-resistant NSCLC cells, levels of different miRNAs are increased and in particular, miR-221 and -222. Then they demonstrate that these miRNAs impair TRAIL-dependent apoptosis by inhibiting the expression of key functional proteins. Indeed, transfection with anti-miR-221 and -222 renders CALU-1-resistant cells sensitive to TRAIL. Conversely, H460-sensitive cells treated with -221 and -222 pre-miRNAs become resistant to TRAIL. Both miR-221 and -222 target the 3′UTR of Kit and p27(kip1) mRNAs, but interfere with TRAIL signaling mainly through p27(kip1) [42]. The results show that high expression levels of miR-221 and -222 are needed to maintain the TRAIL-resistant phenotype, thus making these miRNAs as promising therapeutic targets or diagnostic tool for TRAIL resistance in NSCLC. Interestingly, these two miRNAs are upregulated by the MET proto-oncogene. A recent study shows that miR-130a, expresses at low level in lung cancer cell lines, by targeting MET was able to reduce TRAIL resistance in NSCLC cells through the c-Jun-mediated down-regulation of miR-221 and miR-222 [122]. Together, a better understanding of miR-221/-222 regulation in drug resistance is the key in developing new strategies in NSCLC therapy.

PED/PEA-15 (phosphoprotein enriched in diabetes, PED) is a death effector domain family member of 15 kDa with a broad antiapoptotic function found overexpressed in a number of different human tumors, including lung cancer. Incoronato et al. identifies miR-212 as a negative regulator of PED expression. Furthermore, they also show that ectopic expression of miR-212 increases TRAIL-induced cell death in NSCLC cells. In contrast, inhibition of endogenous miR-212 by use of antago-miR results in increase of PED protein expression and resistance to TRAIL treatment. Besides, in NSCLC, they show both *in vitro* and *in vivo* that PED and miR-212 expressions are inversely correlated, that is, PED is upregulated and miR-212 is rarely expressed [123]. These findings suggest that miR-212 should be considered as a tumor suppressor because it negatively regulates the antiapoptotic protein PED and regulates TRAIL sensitivity.

4.3.3 Ectopic Expressions of miRNAs Reverse Therapeutic Effects of Anti-cancer Drugs

It's well known that miRNAs are strongly implicated in drug resistance, cell survival and apoptosis. Therefore, it is likely that they can also modulate sensitivity and resistance to anticancer drugs in substantial ways. To test this hypothesis, Blower and colleagues investigate the pharmacologic roles of three microRNAs previously implicated in cancer biology (let-7i, miR-16, and miR-21) and also use *in silico* methods to test pharmacologic miRNA effects more broadly. In the experimental system, they increase the expression of individual miRNAs by transfecting their precursors (which are active) or suppress the expression by transfection of antisense oligomers. In three NCI-60 human cancer cell lines, a panel of 60 lines is used for anticancer drug discovery. They assess the growth-inhibitory potencies of 14 structurally diverse compounds with known anticancer activities. Changing the cellular levels of let-7i, miR-16, and miR-21 affect the potencies of a number of the anticancer agents by up to fourfold. The effect is the most prominent with mir-21, with 10 of 28 cell-compound pairs showing significant shifts in growth-inhibitory activity. Varying mir-21 levels change potencies in opposite directions depending on compound class; indicating that different mechanisms determine toxic and protective effects. *In silico* comparison of drug potencies with miRNA expression profiles across the entire NCI-60 panel reveal that approximately 30 miRNAs, including mir-21, show highly significant correlations with numerous anticancer agents [8]. There results support a substantial role for miRNAs in anticancer drug response, suggesting novel potential approaches to the improvement of chemotherapy.

The primary researches show miRNAs as biomarkers are associated with TKI resistance, such as up-regulations of miR-21 and miR-23b predict an increase of anticancer drug-sunitinib resistance, while down-regulation of miR-424 indicates an increase of resistance of erlotinib and vandetanib (data not published, but released on AACR 2010). The data from our own group demonstrate miR-31 is significantly upregulated in cisplatin-resistant cell line, as compared to that in cisplatin-sensitive cell line. As a result, miR-31 overexpression induces drug resistance in cisplatin-sensitive cell line and miR-31 knockdown rescues drug sensitivity in cisplatin-resistant cell line. MiR-31 exerts an anti-apoptotic effect probably through inhibition of ABCB9 and provides a novel strategy using miR-31 as a potential target in NSCLC chemotherapy (data accepted by Cancer Letters). Similarly, in A549 cell line, miR-1 is downregulated and exogenous miR-1 enhances their sensitization to doxorubicin-induced apoptosis [66].

Therefore, lung cancer resistance emerged in clinical treatments is closely related to some upregulated or downregulated miRNAs. To regulate the expressions of these miRNAs is an ideal approach to control therapeutic effects (Table 3.2). It is clear that more than one target or mechanism of drug resistance is activated in certain drugs. The targets or mechanisms that can be activated with more than one drug are more attractive for the broad therapeutic potential. miRNAs or

Table 3.2 Differentially expressed miRNAs associated with drug resistance in lung cancer

miRNA	Drugs	Protein targets
miR-1	Doxorubicin	Unknown
miR-145	TKI	EGFR
miR-21	TKI	EGFR
miR-31	Cisplatin	ABCB9
miR-221/222	TRAIL	P27kip1/PTEN/TIMP3
miR-130a	TRAIL	MET
miR-212	TRAIL	PED
miR-23b	Sunitinib	Unknown
miR-424	Erlotinib/Vandetanib	Unknown

ABCB9, ATP-binding cassette, sub-family B (MDR/TAP), member 9; EGFR, epidermal growth factor receptor; MET, a translational product of proto-oncogene c-met; PED, phosphoprotein enriched in diabetes; PTEN, phosphatase and tensin homolog; TIMP3, tissue inhibitor of metalloproteinase 3; TKI, tyrosine kinase inhibitor; TRAIL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand

antagomiRNAs might be more efficient in avoiding resistance or increasing the effectiveness of malignant tumors to chemotherapy. Furthermore, miRNAs might be used as a biomarker to predict the response to chemotherapy and the survival in patients with malignant tumors. In addition, miRNAs combined with traditional chemotherapy agents might provide a new strategy to treat malignant tumors in the future.

5 miRNAs and Lung Cancer Targeted Therapy: Small Molecules with Unlimited Potentials

As we have discussed above, miRNAs have fast become a field of interest, particularly for their discovered involvement in a number of different oncogenic pathways and the potential they bring for a deeper look into the mechanisms of chemoresistance and future therapies that can be used to circumvent this resistance. miRNAs are already showing their potential as biomarkers to predict treatment response in a number of different cancers, and continued clinical validation is needed to hone in on the most promising of these predictions. There has also been preliminary research showing the involvement of miRNAs in key pathways regulating cancer cell growth, proliferation, invasion, and so on, and this pushes them toward use as novel targets for new anticancer treatments. Again, further research is warranted to validate involvement in these and other pathways, and to continue pursuing miRNA delivery systems that can be translated to therapeutic treatments.

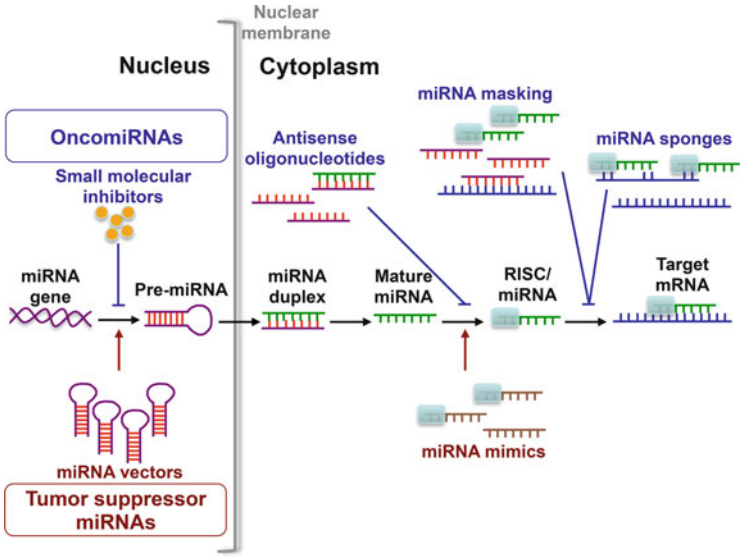

Fig. 3.4 Potential miRNA-based therapeutic strategies. The function of oncogenic miRNAs could be stopped by small-molecule inhibitors (regulation of miRNAs expression at the transcriptional level), antisense oligonucleotides (binding by complementarity miRNAs and inducing either duplex formation or miRNA degradation), miRNAs masking (molecules complementary to the 3′UTR of the target miRNA, resulting in competitive inhibition of the downstream target effects) or miRNAs sponges (oligonucleotide constructs with multiple complementary miRNA binding sites to the target miRNA). Tumor suppressor miRNAs function can be restored by introducing systemic miRNAs (miRNA mimics) or inserting genes coding for miRNAs into viral constructs

5.1 miRNAs as New Therapeutic Targets

Preclinical models have consistently underlined the feasibility and efficacy of miRNA-based therapies, either alone or in combination with current targeted therapies. The appealing strength of such therapeutic option dwells in miRNAs ability to concurrently target multiple genes, frequently in the context of a specific network/pathway, making miRNA-based therapy extremely efficient in regulating distinct biological processes relevant to normal and pathological cell homeostasis [124]. There are two main therapeutic strategies to target miRNA expression: miRNA reduction and miRNA replacement (Fig. 3.4) [6].

The use of oligonucleotides or virus-based constructs can either block the expression of an oncogenic miRNA or reintroduce the loss of expression of a tumor suppressor miRNA. A different approach is the use of drugs to modulate miRNA expression by targeting their transcription and their processing [124]. There are some fundamental issues, which have impeded development of miRNA-based treatments. First, we need to clearly demonstrate a tissue-specific delivery and develop a more efficient cellular uptake of synthetic oligonucleotides to achieve sustained target inhibition. This should result in significantly enhanced

patient benefits and reduced drug toxicity. In fact, the second and even more challenging problem to overcome is the biological instability of miRNAs in bodily fluids or tissues, as unmodified oligonucleotides are rapidly degraded by cellular and serum nucleases, requiring huge doses of drugs. As a result, various chemical modifications in oligonucleotides have been investigated, such as morpholinos, peptide nucleic acids, cholesterol conjugation and phosphorothioate backbone modifications. Among others, the locked nucleic acid (LNA) constructs provide the most promising results. LNA nucleosides are a class of nucleic acid analogues in which the ribose ring is "locked" by a methylene bridge connecting the 2′-O atom to the 4′-C atom. This feature confers to LNA oligonucleotides great advantages including: (a) High hybridization affinity towards complementary single-stranded RNA and complementary single-stranded or double-stranded DNA; (b) Excellent mismatch discrimination, and (c) High aqueous solubility. The so-called "LNA anti-miR" constructs have been successfully used in several *in vitro* and *in vivo* studies to knockdown the expression of specific miRNAs [125, 126]. This success has culminated in the first two miRNA-based clinical trials for the treatment of hepatitis C virus (HCV) infection by targeting miR-122 with an LNA-antimiR (miravirsen or SPC3649; Santaris Pharma, Denmark) [125, 126]. The phase IIa clinical trial [126] has shown a dose-dependent, long reduction in HCV RNA that continues to fall after completion of treatment without any recorded serious adverse effects.

The discovery of exosome-specific miRNA circulation among bodily fluids provided the "Trojan horse" for the forthcoming development of miRNA delivery vehicles for systemic gene therapy: exosomes, as natural cell-derived nano-carriers, are immunologically inert and possess an intrinsic ability to cross biological barriers [127]. On the other hand, exosome-released miRNAs represent a novel mechanism of cross-talk and genetic exchange between cells. Interestingly, cancer-released exosomes have been shown to carry oncogenic miRNAs, and the inhibition of cancer-related exosome secretion has been demonstrated to significantly reduce the metastatic potential of lung cancer cell lines [127]. The LNA and exosome data drew attention to the potential of miRNAs for cancer treatment. The development of safe and specific methods of delivery of miRNA-based treatments will allow modulation of miRNAs to become in the next few years a central feature of cancer treatment and management.

5.2 miRNA Therapeutics in Lung Cancer

Almost two decades have passed since the initial discovery of miRNAs, and many important biological roles have been attributed to this class of RNAs. Currently, the scientific community is pursuing different strategies to employ miRNA therapeutics [124]. Emerging miRNA antisense and mimic technologies, as well as other novel mechanisms of delivery, are being explored. In the very near future, we will be seeing more published studies focusing on assessing the effectiveness,

pharmacokinetics and toxicity of miRNA mimics and inhibitors. One major obstacle in advancing miRNA-based therapeutics lies in the very property of these complex molecules-their ability to act on multiple cellular targets could realize diverse side effects [128]. Evaluating off-target effects will be a necessity in early-phase human studies when arriving at the recommended dose and schedule for clinical efficacy trials. One example demonstrated a therapeutic use of miRNAs in a rodent model of NSCLC. Artificial let-7 is directly injected into already-established tumor mass, leading to tumor regression [129]. Similarly, *in vivo* application of miR-17-5p antagomir results in a reduction of therapy-resistant neuroblastoma in mice [130]. Various routes of delivery as well as formulations are also being investigated. Systemic delivery of the tumor suppressors let-7 and miR-34a complexed with a neutral lipid emulsion are shown to preferentially target lung tumors, resulting in up to 60 % tumor burden reduction in mouse models of lung cancer [131]. Intranasal delivery of let-7 also leads to tumor growth reduction in a mouse xenograft model [132]. Meanwhile, Wiggins et al. [10] develops a therapeutic formulation using chemically synthesized tumor suppressor miR-34a and a lipid-based delivery vehicle that blocks tumor growth in mouse models of NSCLC. This formulation is effective when administered locally or systemically, it is well tolerated and does not induce an immune response [133]. Taken together, these studies demonstrate the therapeutic potential of miRNAs in lung cancer.

5.3 MiRNA Based-Therapeutic Targets in Invasive and Metastatic Lung Cancer

The most deadly aspect of cancer is its ability to invade and metastasize. Garofalo et al. [41] shows that overexpressed miR-221 and miR-222, by targeting PTEN and TIMP3 tumor suppressors, induce TRAIL resistance and enhance cellular migration through the activation of the Akt pathway and metallopeptidases in aggressive NSCLC cells. They further demonstrate that the MET oncogene is involved in miR-221 and miR-222 activation through the c-Jun transcription factor. Muniyappa et al. [134] identifies that miR-29a has a significant anti-invasive and anti-proliferative effect on lung cancer cells *in vitro*. miR-29a functions as an antioncomir and this function is likely mediated through the post-transcriptional fine tuning of the cellular levels of several proteins, including RAN (a member of the RAS oncogene family). Gibbons et al. [135] demonstrates that forced expression of miR-200 abrogates the capacity of metastatic lung adenocarcinoma cells to undergo EMT, invade, and metastasize. Tumor cell metastasis is regulated by miR-200 expression that changes in response to contextual extracellular cues. Ma et al. [136] reports that silencing of miR-10b with antagomirs to mice bearing highly metastatic cells significantly increases the levels of Hoxd10 and markedly suppresses formation of lung metastases. miR-10b antagomir is well tolerated by normal animals and it appears to be a promising candidate for the development of

new anti-metastasis agents. These results indicate miRNAs can be used as therapeutic targets in invasive and metastatic lung cancers.

5.4 *Therapeutic Potential of miRNAs in Lung Cancer Chemotherapy*

Emerging evidence has also shown that some miRNAs could target genes related to drug sensitivity, resulting in the altered sensitivity of cancer cells to anti-cancer drugs [137]. Guo et al. [138] indicates that transfection of the drug resistant SCLC cells with the mimics of miR-134 greatly increases the sensitivity to anti-cancer drugs cisplatin, etoposide, and doxorubicin. miR-134 increases the cell survival by inducing G1 arrest and downregulates MRP1/ABCC1 protein in drug-resistant SCLC cells. Zhu et al. [139] demonstrates that enforced miR-181b expression reduces BCL2 protein level and sensitizes multidrug resistant lung cancer cells to cisplatin-induced apoptosis. Galluzzi et al. [10] also reports that pre-miR-181a and pre-miR-630 enhances and reduces cisplatin-triggered cell death in NSCLC cells, respectively. Pre-miR-181a and pre-miR-630 consistently modulated mitochondrial/postmitochondrial steps of the intrinsic pathway of apoptosis, including Bax oligomerization, mitochondrial transmembrane potential dissipation, and the proteolytic maturation of caspase-9 and caspase-3. Another two different groups show that both miR-98 and miR-34a regulates cisplatin-induced A549 cell death by inhibiting TP53 pathway [140, 141]. Similarly, miR-622 maybe function as a tumor suppressor by targeting K-RAS and enhancing the anticarcinogenic effect of resveratrol [142]. Therefore, miR-622 is potentially useful as a clinical therapy. miR-100 resensitizes docetaxel-resistant human lung adenocarcinoma cells (SPC-A1) to docetaxel by targeting Plk1 [143]. Thus, this suggests that downregulation of miR-100 could lead to Plk1 over-expression and eventually to docetaxel chemoresistance of human lung adenocarcinoma. miR-200b reverses chemoresistance of docetaxel-resistant human lung adenocarcinoma cells by targeting E2F3 [144]. The results suggest that downregulation of miR-200b could lead to E2F3 overexpression and in turn contribute to chemoresistance of lung adenocarcinoma cells to docetaxel. miR-126 enhances the sensitivity of NSCLC cells to anticancer agents LY294002, an inhibitor of the phosphoinositidyl-3 kinase (PI3K)/Akt signaling pathway, by targeting vascular endothelial growth factor (VEGF) A [145]. The identification of a miR-337-3p as a modulator of cellular response to taxanes, and STAT3 and RAP1A as regulatory targets which mediate that response, defines a novel regulatory pathway modulating paclitaxel sensitivity in lung cancer cells, which may provide novel adjuvant strategies along with paclitaxel in the treatment of lung cancer and may also provide biomarkers for predicting paclitaxel response in NSCLC [9]. miR-513a-3p sensitizes human lung adenocarcinoma cells to chemotherapy by targeting GSTP1 [146]. Interestingly, serum miR-125b can be used as a diagnostic or prognostic biomarker for advanced

NSCLC patients receiving cisplatin-based chemotherapy [147]. Recently, Franchina et al. showed that circulating miR-22, miR-24 and miR-34a act as novel predictive biomarkers to pemetrexed-based chemotherapy in advanced NSCLC patients [148].

5.5 *Therapeutic Potential of miRNAs in Lung Cancer Radiotherapy*

Another role for miRNAs that deserves mention is that of sensitizers to radiotherapy. This is of particular importance given that many tumors require combinations of chemotherapy and radiotherapy as optimal modes of treatment. miRNAs may modulate the DNA damage response, thus sensitizing tumor cells to both chemotherapy and radiotherapy.

The miRNA regulatory network may also be a potentially useful therapeutic target for overcoming the radioresistance of lung cancer. The overexpression of let-7a decreases expression of K-RAS and radiosensitizes A549 cells. Inhibition of Lin28, a repressor of let-7, attenuates K-RAS expression and radiosensitizes A549 cells. The Lin28-let7 regulatory network may be a potentially useful therapeutic target for overcoming the radioresistance of human cancers having activated K-RAS signaling [149]. Comparing with resistant NSCLC patients, five miRNAs (miR-126, miR-let-7a, miR-495, miR-451 and miR-128b) are significantly upregulated and seven miRNAs (miR-130a, miR-106b, miR-19b, miR-22, miR-15b, miR-17-5p and miR-21) are greatly downregulated in radiotherapy sensitive group. Overexpression of miR-126 inhibits the growth of SK-MES-1 cells and promotes its apoptosis induced by irradiation. The expression level of p-Akt decreases in the miR-126 overexpression group. After treating with PI3K constitutively activator (IGF-1) and inhibitor (LY294002), miR-126 overexpression has no significant effects on the apoptosis of SK-MES-1 cells. These results show miR-126 promotes NSCLC cells apoptosis induced by irradiation through the PI3K-Akt pathway [150]. The expression of miR-9 and let-7 g could enhance the efficiency of radiotherapy for lung cancer treatment through the inhibition of NF-κB1[151]. A small number of NSCLC cell lines have a high level of endogenous miR-101. The ectopic miR-101 is able to radiosensitize most NSCLC cells, except for the NSCLC cell lines that had a much higher endogenous miR-101 level [152]. In the p53 wild type, K-RAS mutated NSCLC cells, the overexpression of miR-34b increases radiosensitivity at low doses of radiation [153]. miR-214 is upregulated in radiotherapy-resistant NSCLC cells relative to radiosensitive counterparts and miR-214 modulates radiotherapy response of NSCLC cells through regulation of p38MAPK, apoptosis and senescence [154]. Overexpression of miR-449a in CL1-0 cells effectively increases irradiation-induced DNA damage and apoptosis, alters the cell cycle distribution and eventually leads to sensitization of CL1-0 to

irradiation [155]. Therefore, continued investigations will benefit the understanding of miRNA-based radiotherapy in lung cancer.

6 Conclusion

The discovery that noncoding regions of the genome harbored regulatory molecules that could regulate basic cellular functions is one of the most important discoveries in recent history. This discovery alone has provided insight into the mechanisms of tumor initiation and progression, identified potential biomarkers, and, finally, created the possibilities for novel small-molecule therapies. Each of these complex areas continues to be investigated. Among these noncoding RNAs, miRNAs are a short, noncoding class of RNAs that are involved in global regulation of protein expression, act on multiple targets and serve as physiological buffers in biological responses to internal and external stimuli.

It is becoming apparent that therapeutic inhibition or mimicking of a single miRNA can simultaneously target multiple genes within similar pathways and signaling cascades. miRNAs are now being studied for their potential as a new generation of therapeutics. The main challenges for miRNA therapeutics are stability, safety, and delivery to appropriate cells within a tissue or organ [156]. Technological advances have enabled important discoveries of molecular, cellular, clinical, and therapeutic cancer research findings in recent years [157]. There are various therapeutic tools that are currently being investigated to manipulate upregulated miRNAs, such as antagomir, small molecule, and miRNA sponge. Antagomir is the most widely used approach to regulate miRNA levels *in vivo*, including LNA antisense oligonucleotides. One study group finds that small molecule can also be used to modulate the functionality of a specific miRNA [158]. miRNA sponge is another technique that uses a vector expressing miRNA target sites to scavenge a miRNA and prevent it from regulating its natural targets [159]. On the other hand, there are also approaches to mimic or reexpress the downregulated miRNAs, such as lipid-formulated mimic and adeno-associated viruses' vector.

Nevertheless, considerable obstacles should be overcome before miRNA therapy becomes a real option for the management of cancer. The exciting therapeutic potential of miRNA is accompanied by the recognition of off-target effects. Integrative vectors allow life-long gene supplementation, but involve the risk of potential tumorigenicity resulting from activation of oncogenes located in the vicinity of the integration site [160]. The long terminal repeat enhancer element is removed and an internal promoter is added in lentiviral vector to solve this problem [161]. Although the development of these therapeutic tools are still in infancy, interdisciplinary helps from nanobiotechnology making research on miRNA therapeutics rapidly moving forward [162]. Several organizations already have active preclinical or clinical trials engaged in developing miRNA therapeutics in various cancers, including lung cancer, prostate cancer, liver cancer, esophageal

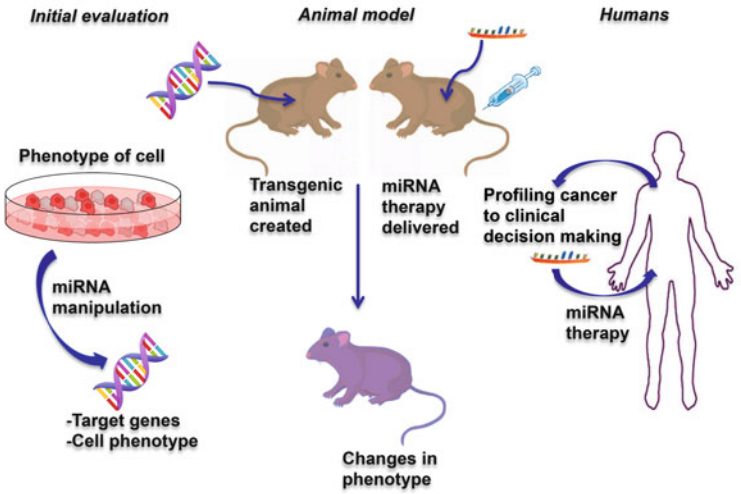

Fig. 3.5 Process for translating miRNA biology from the laboratory to the clinic

cancer, leukemia, skin cancer, and renal cell carcinoma (Fig. 3.5) [163, 164]. Recently, the therapeutic applicability of LNA-antimir technology has been reported in non-human primates. Treatment of chronically infected chimpanzees with a LNA-modified oligonucleotide complementary to miR-122 leads to long-lasting suppression of hepatitis C virus viremia, with no evidence of viral resistance or side effects in the treated animals [125]. This encouraging result highlights the scaling up of miRNAs from laboratory to translational research [165, 166]. Although there are still numerous hurdles in the development of miRNAs as a novel class of therapeutics, the available findings indicate the great potential of miRNAs in lung cancer therapy.

To date, a compelling body of evidence points to the direct involvement of miRNAs in lung carcinogenesis. Combined with high tissue specificity and stability in formalin-fixed paraffin-embedded (FFPE) tissues and bodily fluids, miRNA use in diagnosis, classification and prediction of disease course has become an appealing area of biomarker and therapeutic research. With more discoveries on the horizon, miRNA-based signatures could become prominent clinical tools for patient management and care.

7 Future Directions

As next-generation sequencing approaches become more affordable, they will begin to dominate the discovery field in miRNA research, uncovering novel miRNAs and their associations with lung oncopathology. Linking individual miRNAs to their respective targets within the context of complex pathway

networks that are commonly deregulated in cancer would be crucial in painting the 'big picture' underlying these cellular processes. While the miRNA signatures reported thus far provide evidence for the translational value of miRNAs and their utility as theranostics, much validation is still needed. In the future, we can expect to see standardization of sample collection techniques, discovery platforms and data analysis techniques to aid in cross-study comparison of results. Overall, miRNAs harbor immense potential in various areas of cancer biology, and realization of their potential as therapeutic targets is simply a matter of time.

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Chapter 4
The Potential Role of MicroRNA-Based Therapy for Lung Cancer Stem Cells

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1 Introduction

1.1 Lung Cancer and Lung Cancer Stem Cells

Lung cancer is the most common cause of cancer-related death worldwide [1]. It is a heterogeneous disease with two distinct pathological classes, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). SCLC is the more aggressive and potentially lethal form of the disease, whereas NSCLC is much more common and metastasizes at a slower rate [2]. Approximately 85 % of all lung cancers are categorized as NSCLC. The most common pathological types of NSCLC are adenocarcinoma (30–50 %) and squamous cell carcinoma (30 %). A number of therapeutic strategies including surgery, radiation therapy, chemotherapy and targeted therapies are commonly used to treat lung cancer, either alone or in combination. Despite the development of novel molecular therapies [3], the prognosis of lung cancer is still poor, and the median survival time is approximately 18 months in the operable stages. Patients who initially respond to treatment often

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relapse and succumb to chemotherapy-resistant tumors [4]. Therefore, novel treatments that specifically target chemotherapy-resistant cells are needed.

Recent evidence supports the presence of a small subset of self-renewing, stem-like cells within the tumor mass that possess the capacity to seed new tumors. These cells have been termed 'cancer stem cells,' (CSCs) and may be responsible for the poor outcome of lung cancer [5]. Similarly, the term 'tumor-initiating cell' has also been used to describe a cell with the potential to initiate a tumor. While CSCs were recognized several decades ago, only in the last 16 years have they been identified in acute myeloid leukemia [6]. Targeting CSCs is of great interest as CSCs are considered to be more resistant to radiotherapy and chemotherapy and are also thought to be responsible for the dissemination and growth of metastases [7]. Like normal pluripotent stem cells, CSCs are long-lived, display quiescent characteristics in a dormant state, and are responsible for angiogenic induction, apoptotic resistance, self-renewal, and differentiation. Embryonic stem cell (ESC) pathways such as the Hedgehog, Notch and Wnt signaling pathways might also be involved in driving CSC activity in a variety of cancers, including lung cancer. These characteristics suggest that CSCs themselves contribute to tumor development and progression. The CSC theory is attractive to both researchers and physicians because CSCs are central to cancer cell biology and cancer therapy. The identification of CSC-specific markers, the isolation and characterization of CSCs from malignant tissues, and the development of strategies for targeting CSCs represent important opportunities in cancer research.

Almost 30 years ago, Carney et al. first described a rare population of cells in lung cancer patient samples with the ability to form colonies in soft agar [8]. In 2005, Kim et al. identified bronchoalveolar stem cells from mouse bronchoalveolar ducts and discovered that these cells initiate the formation of lung cancer via K-Ras-driven oncogenesis [9]. Recently, several investigators isolated tumorigenic cells from lung cancers using different phenotypic cancer cell characteristics. A small subpopulation of lung CSCs appears to be responsible for the more aggressive lung cancer subtypes. This group expresses typical stem cell markers including CD133, CD44, aldehyde dehydrogenase (ALDH), Oct4 and Nanog [5, 10]. Isolated cancer cells need to be validated for CSC phenotypes by various experimental methods that include cell sorting based on cell surface markers and chemotherapy selection for drug-resistant cells [11]. For human lung CSCs, CD133 and CD44 appear to be the most appropriate markers identified thus far [12, 13]. In cell lines from both SCLC and NSCLC, CD133-positive cells generated long-term tumor spheres and differentiated into CD133-negative cells [5]. Chen et al. reported that CD133-positive NSCLC cell lines display self-renewal and chemo-radio-resistant properties [14]. CD44 is another transmembrane glycoprotein believed to be activated in a wide range of tumors, where it plays a critical role in a variety of cancer cell behaviors including adhesion, invasion and survival [15]. CD133-, CD44-positive lung CSCs display an increased capacity for self-renewal and produce unlimited, differentiated progeny of heterogeneous populations of NSCLC cells in comparison with cancer cells without these markers [5]. While CD133, CD44, and ALDH are common markers for both normal and cancer stem cells, there are

currently no selective markers specific for lung CSC populations. The phenotype of lung cancer propagating cells varies with the type of genetic change present in the tumor [16]. This may suggest that functional properties, rather than surface markers, represent better targets for specific therapies against lung CSCs. There remain a number of challenges in identifying lung CSC-specific and dynamic network biomarkers. Despite the potential clinical significance of CSCs, how intrinsic CSC properties are regulated at the molecular level remains poorly understood. Recently, an increasing number of reports have implicated a new class of small regulatory RNA molecules termed microRNAs (miRNAs) in lung cancer progression and stemness.

1.2 MicroRNA Biogenesis and Function in Lung Cancer

MiRNAs are endogenous single-stranded non-coding RNAs of 20–23 nucleotides (nt) in length that regulate translation through their interaction with messenger RNA (mRNA) transcripts [17]. MiRNAs post-transcriptionally inhibit gene expression by multiple mechanisms, all of which involve base-specific interactions with target mRNA transcripts. A single miRNA may target dozens of mRNAs, and one mRNA can be regulated by multiple miRNAs. Because of their huge impact in many cellular processes and in many pathological conditions including cancer, miRNAs have been extensively studied. Most miRNAs are derived from primary miRNA transcripts containing a cap and a poly (A) tail that are produced by RNA polymerase II from the miRNA genes. The primary miRNAs are further cleaved into ~22-nt mature miRNAs by the consecutive functions of RNAase III Drosha-DGCR8 and Dicer, which are present in the nucleus and cytoplasm, respectively. In animals, single-stranded miRNAs are assembled into an RNA-induced silencing complex (RISC) and primarily bind target mRNAs at specific sequence motifs predominantly found in the 3′-untranslated region of the transcripts. MiRNAs are key players in various critical cellular processes such as proliferation, cell cycle progression, apoptosis and differentiation. As a consequence, aberrant expression of miRNAs is frequently observed in many diseases, including cancer [18]. MiRNAs either promote tumor progression (oncomiRs) or suppress tumor growth (tumor suppressor miRNAs) [19]. Recent studies have shown not only that miRNAs are useful in lung cancer diagnosis but also that specific miRNA profiles may predict prognosis, drug response and disease recurrence [20, 21]. These findings suggest that miRNAs are a promising technology for the development of lung cancer therapeutics. In fact, given the significant role of miRNAs in multiple pathways governing lung carcinogenesis, increasing efforts are being dedicated to the research and development of miRNA-based therapies, including the restoration of tumor suppressor miRNA function and the inhibition of oncomiRs [22, 23]. Early studies linked miRNAs to controlling the self-renewal and differentiation of ESCs, and later, aberrant expression and functions of miRNAs were implicated in tumorigenesis [24]. More recent studies have also investigated the functional role

of miRNAs in CSCs, as they bear significance in tumor maintenance, progression, metastasis and poor prognosis [25–27]. In this review, we will describe recent advances in miRNAs and their regulatory roles in lung CSCs.

2 MicroRNAs and Their Role in Lung Cancer Stem Cells

2.1 MicroRNA Regulation of Cancer Stem Cells

Since the first CSCs were identified in leukemia, CSC populations have been isolated in various cancers including breast, prostate, brain, colon and lung cancers [28]. While the origins of CSCs are largely unknown [29], CSCs may be derived from somatic stem cells or differentiated cells. Somatic stem cells may require fewer transformational events than other cell types to become CSCs, including loss of the regulation of self-renewal pathways and/or loss of cell division control. Alternatively, differentiated cells may undergo de-differentiation with re-expression of self-renewal genes and loss of the regulation of cell division. Regardless of their origin, CSCs can be identified and isolated based on certain characteristics. Recent data suggest that poorly differentiated aggressive human tumors and CSCs possess human ESC-like gene expression signatures [30, 31]. In addition, human ESC markers such as Oct4, Sox2, Nanog and Lin28 are overexpressed in CSCs and can promote transformation [32]. Recent evidence suggests that miRNAs might be involved in tumor development by critically regulating CSCs [33]. MiRNAs play important roles in CSCs proliferation, differentiation and tumor formation. Therapies that target CSCs could potentially eliminate cancer and reduce the occurrence of relapse. A recent profiling study, performed in novel isolated CSCs, related the up- or down-regulation of miRNAs in CSCs to miRNA levels found in non-CSCs or normal stem populations [34]. Functional studies showed that miRNAs are major regulators of the acquisition and maintenance of the stemness of CSCs. For example, miRNAs such as *let-7*, miR-200, miR-34a, and miR-145 play key roles in CSC regulation via multiple signaling pathways that regulate cell growth and survival. In addition, these miRNAs can be differentially expressed in CSCs or CSC-like cells of various tumors. Due to these properties, these miRNAs are good potential therapeutic targets and may also be useful as CSC markers (Fig. 4.1).

Members of the *let-7* family act as tumor suppressors by targeting K-Ras and c-Myc. Interestingly, the expression of these miRNAs is repressed in CSCs of various tumors including those in the lungs [35]. As *let-7* is a common tumor suppressor and has anti-proliferative properties, it can regulate cell differentiation and apoptotic pathways. The down-regulation of *let-7* has been reported in various cancers, and the reconstitution of *let-7* expression has been shown to inhibit cancer growth [36, 37]. The *let-7* family stabilizes the differentiated cell fate by targeting the transcripts that are regulated by the pluripotency transcription factors Oct4,

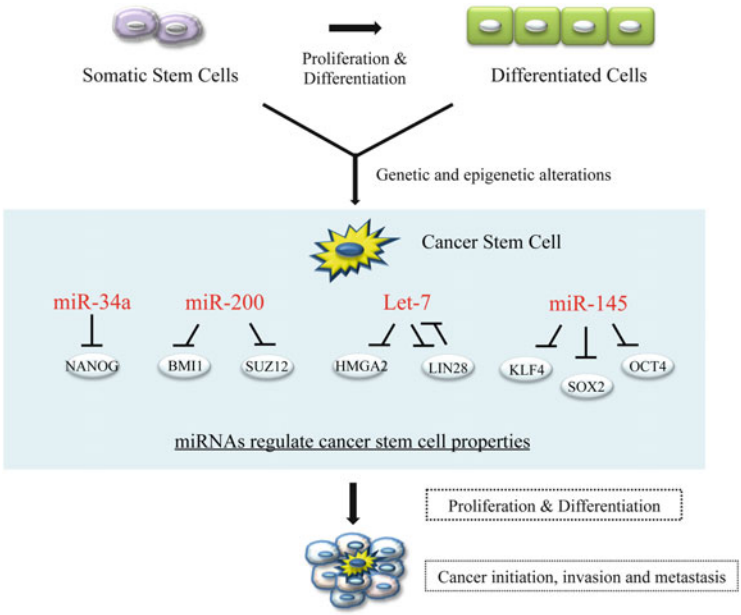

Fig. 4.1 MicroRNA-mediated regulation of the maintenance and function of cancer stem cells (CSCs). CSCs may be derived from either somatic stem cells or differentiated cells. Normal stem cells that have undergone mutational events leading to a loss of self-renewal control have been posited to be the source of CSCs. Alternatively, differentiated cells that have undergone de-differentiation, acquired a self-renewal capacity, and lost the ability to regulate cellular division could be the source of CSCs. The dysregulation of some miRNAs (e.g., miR-34a, miR-200, miR-145, and *let-7*) in CSCs provides evidence of their significance in maintaining self-renewal and pluripotency and in regulating differentiation by targeting some pluripotency marker genes

Sox2, Nanog, and Transcription factor 3 (Tcf3) [38]. Yu et al. showed that overexpression of *let-7* decreases the population of CSCs and reduces proliferation and mammosphere formation *in vitro,* as well as tumor formation and metastasis *in vivo* [25]. In addition, *let-7* miRNAs are also negative regulators of the epithelial-to-mesenchymal transition (EMT), a developmental event related to treatment resistance, metastasis, and recurrence [39]. In pancreatic and prostate cancer cells, the phenotypes are similar to those observed with CSCs, mediated through Phosphatase and Tensin Homolog Deleted from Chromosome 10 (PTEN) and CSC gene signature marker Lin28B [40–42]. Lin28 is an RNA-binding protein that regulates *let-7* family members, and the expression of Lin28 blocks the biogenesis of *let-7* [43]. These findings suggest that *let-7* is a potential molecular marker for CSCs and a next-generation therapeutic target in anti-cancer therapy [44].

The miR-200 family consists of five members: miR-200a, miR-200b, miR-200c, miR-141 and miR-429 [45]. Members of the miR-200 family are enriched in ESCs, play a role in induced-pluripotent stem cell (iPSC) induction [46] and are down-regulated in CSCs isolated from lung, ovarian, head and neck, liver, pancreatic and

breast cancer samples [47, 48]. Recent studies have also associated miR-200 family members and their target mRNAs with the establishment, maintenance and regulation of the CSC phenotype. The family inhibits the EMT phenotype by directly targeting mRNAs encoding the EMT regulators Zinc finger E-box-binding homeobox 1 (ZEB1) and ZEB2 [40]. As with CSCs, miR-200 decreases the expression of B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1), Suppressor of zeste 12 homolog (Suz12), and Notch-1, all of which regulate CSC and EMT phenotypes and function in various cancer cells [49, 50]. In breast CSCs, there have been significant studies associating the miR-200 family and their target mRNAs with the establishment, maintenance and regulation of CSC phenotype. Shimono et al. showed that the overexpression of miR-200c reduced the clonogenicity and tumor-initiation activity of breast CSCs and suppressed the formation of mammary ducts by normal mammary stem cells [27]. These data suggest that the miR-200 family might play critical roles in CSCs and normal stem cells via the regulation of multiple signaling pathways.

MiR-34a, one of the most prominent endogenous miRNAs involved in the genesis and progression of human cancers, functions as a tumor suppressor and is commonly down-regulated in many human cancers, including pancreatic, prostate, breast, and lung cancers [33, 51]. Enhanced expression of miR-34a in the lung inhibits tumor growth due to the down-regulation of proteins important for survival in the tumor [52]. The target mRNAs of miR-34a are involved in cell cycle progression, migration and the inhibition of apoptosis and include E2F transcription factor 3 (E2F3), Notch-1, Cyclin D, and Bcl-2 [53]. MiR-34a suppresses reprogramming through the repression of pluripotency genes, including Nanog, Sox2 and N-Myc [54]. In addition, the expression of miR-34a has been found to be significantly decreased in $CD133^+$ glioma CSC-like cells [55]. Recent data indicate that prostate CSCs enriched with surface markers CD44, CD133, or α2β1 prominently and commonly under-express miR-34a and *let-7b* [51]. Thus, these data suggest that the loss of miR-34a acts as a tumor suppressor in the regulation of CSC function and indicate the possibility of employing miR-34a as a CSC marker and therapeutic target.

Hence, the dysregulation of miRNAs has been intimately implicated in tumor development, and miRNAs may regulate tumorigenesis by modulating CSC properties. The novel findings discussed above better our understanding of CSC regulation and provide new insight into developing novel strategies to target therapy-resistant cancer cells. The use of miRNAs is potentially advantageous because they can simultaneously silence several molecules that regulate CSCs. In the next section, we present an emerging theme that several miRNAs might distinctively regulate the key biological properties of lung CSCs.

2.2 MicroRNAs Function in Lung Cancer Stem Cells

MiRNAs also play important roles in lung CSC proliferation, differentiation and drug resistance. However, the miRNA-dependent mechanisms that regulate tumor initiation and metastasis in lung CSCs remain unclear. Because little research has been carried out on miRNAs in lung CSCs, some novel miRNAs have been identified as attractive therapeutic targets for lung CSCs.

The tumor suppressive miRNA miR145 acts as a switch to modulate CSC properties in lung adenocarcinoma [56]. The expression of miR-145 was found to be decreased in metastatic lung adenocarcinoma and CSC-like tumor cells compared with primary lung adenocarcinoma and non-CSC cells. Additionally, low levels of miR-145 are correlated to poor prognosis. Further, *in vivo* studies have shown that miR-145 delivery to xenograft tumors reduced tumor growth and metastasis, sensitized tumors to chemoradiotherapies, and prolonged survival times. In Ewing sarcoma, the miR-145-mediated inhibition of Sox2 was discovered, which resulted in enhanced CSC phenotypes [57]. Remarkably, miR-145 was recently linked to ESC signatures, and endogenous levels of Oct4, Sox2, and Kruppel-like factor 4 (Klf4) were shown to be controlled post-transcriptionally by miR-145 in human ESCs [58]. This miRNA might be functionally regulated in de-differentiating or reprogramming processes in normal lung epithelial cells and lung cancer cells. These data suggest that miR-145 is a potential stemness-regulating factor as well as a novel miRNA-based treatment for lung CSCs.

Cheng et al. reported that miR-135a/b might play a pivotal role in modulating the effect of the single-nucleotide polymorphism (SNP) on the risk of lung cancer in cells expressing CD133 [59]. CD133 has been identified as a pleiotropic marker of CSCs in various human cancers. Further, CD133 is also implicated in the tumor initiation, maintenance, metastasis, and drug resistance of lung cancer [60]. Many previous studies have shown that CD133 could contribute to tumorigenesis and metastasis by virtue of CSC properties. Therefore, CD133 is an important surface marker of lung CSCs. Mimics of miR-135a/b could significantly suppress the mRNA expression of CD133 in peripheral blood lymphocytes containing the SNP. The SNP was inversely related to CD133 gene expression via the modulation of miR-135a/b. Recently, the modulation of miR-135b was shown to promote cancer invasion and metastasis via the down-regulation of multiple targets in the Hippo pathway as well as the Leucine zipper putative tumor suppressor 1 (LZTS1) [61]. The Hippo tumor suppressor pathway has emerged as a complex signaling network with a significant role in cancer development and stem cell biology. A miR-135b antagomir effectively reduced metastasis and tumor burden, which suggests the potential for the development of miR-135b antagonists for lung cancer therapy.

MiR-296 was recently reported to regulate the function of lung CSCs via Klf4-Numb-like (Numbl) signaling [62]. Decreased expression of miR-296 has been observed in several types of human cancers, sometimes correlating with disease progression [63, 64]. As a tumor suppressor, miR-296 actively repressed the

polarity protein Numbl [65]. One of the emerging functions of polarity proteins is to regulate stem cell-related phenotypes including CSCs [66]. In addition, Numbl inhibits Klf4-dependent transcription. Increased expression of Numbl was associated with multiple metastasis in lung cancer and enhanced cell invasion, resistance to therapy, maintenance of cancer initiation, progenitor-like cells, and metastatic competency *in vivo*. The pathway regulated by miR-296 might open novel therapeutic prospects for lung CSC treatment.

MiR-874 is located on chromosome 5q31.2, a well-known frequently fragile site in the human genome that is often deleted in cancers and genetic disorders and specifically correlated with chromosomal rearrangements in cancer [67, 68]. Recently, miR-874 was reported to have the potential to inhibit invasion, migration and CSC-phenotype by regulation of Matrix metallopeptidase 2 (MMP-2) and Urokinase plasminogen activator (uPA) proteins in lung cancer, both *in vitro* and *in vivo* [69]. The overexpression of miR-874 in the CD133-positive CSC population led to significant loss of the CSC phenotype and enhanced sphere de-differentiation into epithelial-like cells. In addition, miR-874 treatment decreased orthotopic tumor growth in a nude mouse model of lung cancer. These data suggest that miR-874 may play a tumor suppressor role in lung CSCs and may be a potential target in the treatment of NSCLC.

MiRNA expression profiles and functional studies explain their importance in stem cell biology. However, detailed investigation of the roles of these miRNAs is still required. Lung CSC-specific targeting has been introduced as an alternative because of its potential to kill tumor-initiating cancer cells. Knowing the functional role of miRNAs in lung cancer will allow for the development of therapies targeting lung CSCs to correct their aberrant expression levels [10].

3 Therapeutic Potential of Stemness-Related MicroRNAs in Lung Cancer

MiRNAs related to CSCs may significantly broaden the field of miRNA-based therapies and suggest that miRNAs can be potential tools to kill cancer cells associated with therapy resistance, recurrence, and metastasis [10]. CSCs can be targeted by agents that specifically kill them or that promote their differentiation into non-CSCs, which in turn will undergo apoptosis, senescence or terminal differentiation [70] (Fig. 4.2). Preclinical models have consistently underlined the feasibility and efficacy of miRNA-based therapies, either alone or in combination with current targeted therapies. The use of oligonucleotides or virus-based constructs can inhibit the expression of an oncogenic miRNA or reintroduce the expression of a tumor suppressor miRNA. As presented above, the involvement of some miRNAs in regulating lung CSC proliferation makes these miRNAs promising candidates for lung cancer treatment. Thus far, researchers have successfully used both miRNA mimics and anti-miRNAs to restore normal gene

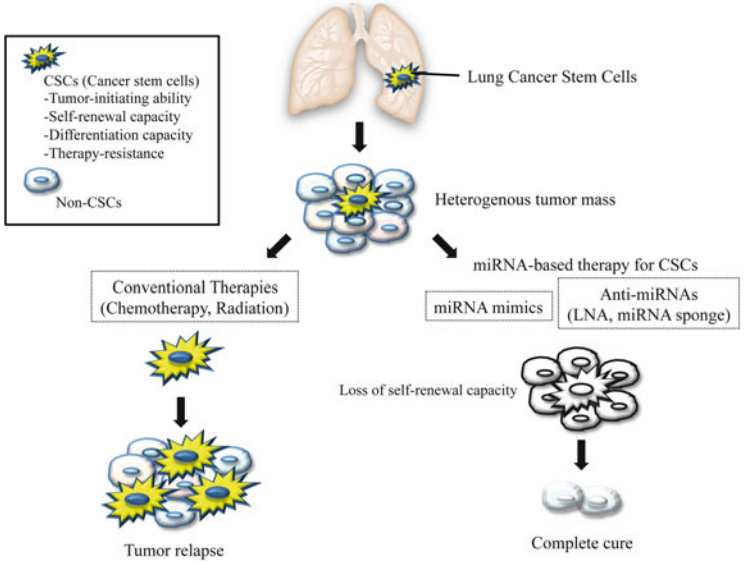

Fig. 4.2 Conventional therapy vs. CSC-targeted therapy. CSCs form and maintain a heterogeneous tumor mass. Conventional therapies are effective for differentiated cancer cells. However, the tumor ultimately recurs because the rare therapy-resistant CSCs have not been eliminated. In contrast, CSC-targeted therapy using miRNA mimics or anti-miRNA could degenerate a heterogeneous tumor due to the loss of the capacity to self-renew. The direct targeting of CSCs can result in their eradication, leaving only non-CSCs, which eventually will undergo apoptosis or terminal differentiation

networks in cancer cell lines and xenograft models [71]. Nevertheless, critical barriers for the development of this therapy exist, including effective delivery into target sites, the potency of the therapy, and the elimination of off-target effects [72]. MiRNA-based therapeutics are promptly degraded by nucleases when they are administered systemically, but chemical modifications at specific positions and formulation with delivery vectors have been shown to improve stability. Unfortunately, these modifications may attenuate the suppressive activity of the oligonucleotides [73]. Successful delivery of miRNA-based therapeutics also necessitates efficiency, convenience, and patient compliance of the delivery route. For this reason, direct administration of miRNA-based therapeutics into lung cancer cells is a promising approach for overcoming the problems of systemic administration. As a direct route to the lung, pulmonary delivery has offered a new method for the treatment of various lung diseases, including cancer [74–78]. This approach could potentially enhance the retention of miRNA-based therapeutics in the lungs and reduce systemic toxic effects. We believe that pulmonary delivery of miRNA-based therapeutics holds powerful potential for the treatment of lung cancer [23].

In general, miRNAs are classified as oncomiRs or tumor suppressor miRNAs, with different therapeutic approaches developed for each class. The up-regulation of miRNAs is achieved through the administration of synthetic miRNA mimics or

the administration of miRNA-expressing vectors. The down-regulation of miRNAs is achieved through the administration of antisense nucleotides that are often chemically modified to ensure stability and specificity.

3.1 MicroRNA Mimic-Based Therapeutics

One therapeutic strategy is miRNA replacement therapy, which involves the re-introduction of a tumor suppressor miRNA mimic to reverse the loss of miRNA function. MiRNA mimics are synthetic RNA duplexes with chemical modifications for stability and cellular uptake that are designed to mimic the endogenous functions of miRNAs. The concept of miRNA replacement therapy is best exemplified by the *let-7* miRNA. Intranasal administration of a *let-7* mimic into mouse models of lung cancer significantly reduced tumor growth, indicating the promise of miRNA replacement therapy [79–81]. Based on this successful evidence, a clinical trial in NSCLC patients using a *let-7a-1*-based therapy has been initiated. Remarkably, Shi et al. reported that systemic delivery of miR-34a induced the apoptosis of tumor cells by regulating CD44 expression and the migratory, invasive, and metastatic properties of CSC-like cells [82]. These data suggest that using miRNAs for lung CSC therapy has powerful potential for clinical use. Indeed, Mirna Therapeutics, Inc., will initiate clinical trials for miR-34 in 2013, making this one of the first miRNA mimics to reach the clinic. The pharmacological delivery of miRNA mimics effectively inhibits tumor growth by attacking multiple genes. However, it is necessary to pay attention to any potential toxicity in normal tissues. Under conditions in which the therapeutic delivery of miRNA mimics will lead to an accumulation of exogenous miRNAs in normal cells, monitoring miRNA mimic-induced effects in normal cells and carefully assessing toxicity will be necessary before using them in clinical practice.

3.2 MicroRNA Inhibitor-Based Therapeutics

The second strategy for miRNA therapeutics aims to cause a gain of function by inhibiting oncomiRs with anti-miRNAs. Chemical modifications such as the 2′-O-methyl-group and locked nucleic acid (LNA) would increase oligo stability against nucleases [83]. LNA nucleosides are a class of nucleic acid analogs in which the ribose ring is ‘locked’ by a methylene bridge connecting the 2′-O atom to the 4′-C atom. Because LNAs are locked by this bridge, no conformational transition occurs, in contrast to naked nucleotide sequences [84, 85]. Although there have been no reports of LNA anti-miRNA-based therapies for lung CSCs, this technology has culminated in the first two miRNA-based clinical trials for treating hepatitis C virus infection by targeting miR-122 with an LNA anti-miRNA [84, 85]. This LNA anti-miRNA data calls attention to the potential of miRNAs for CSC treatment. With

regard to lung cancer, anti-miR-150 has been reported to inhibit tumor growth when delivered into lung tumor xenograft models [86]. Relative to studies on miRNA mimics, studies with antisense oligonucleotides have shown more effective evidence with naked oligonucleotides. This illustrates the potential for using chemical modifications to improve oligonucleotide stability, RNase resistance and pharmacologic properties. Currently, miRNA sponges are being developed as a novel approach to inhibit miRNAs. This technology works with multiple complementary 3′-UTR mRNA sites for a specific miRNA [87, 88]. These sponges specifically inhibit miRNAs with a complementary heptameric seed, such that a single sponge can be used to block an entire miRNA seed family. In fact, in a murine breast cancer model, the development of lung metastases was significantly reduced by inhibiting the MYC-driven miR-9 using an miRNA sponge [89]. Furthermore, the inhibition of miR-31 in a breast cancer model by miRNA sponges resulted in a significant induction of lung metastasis [90]. The development of efficient methods of miRNA sponge delivery might allow the modulation of miRNAs to become a central feature of CSC treatment in the near future.

4 Future Perspectives

It is now well accepted that CSCs are master regulators of cancer initiation, development and therapy resistance and therefore represent novel translational targets for cancer therapy. Here, we discussed the emergence of miRNAs as the key micromanagers of malignant progression through the control of several genes and pathways associated with stemness of CSCs. For example, miRNAs such as *let-7*, miR-200, and miR-34a are possible therapeutic targets, as they play important roles in CSC regulation via multiple signaling pathways that regulate cell growth and survival. Understanding the role of miRNAs in the biology of CSCs can provide promising advances for cancer treatment and might be helpful to improve cancer diagnosis. These findings have provided strong support for the translational relevance of miRNAs in cancer to improve clinical outcomes. There are also a number of challenges to translate the research and understanding of lung CSCs to clinical applications and therapies. However, the data on miRNA-dependent mechanisms regulating tumor initiation and metastasis in lung CSCs are insufficient.

Recently, several *in vitro* and *in vivo* studies have provided experimental support for the effectiveness of miRNA-based therapies against CSCs. For example, the overexpression of miR-7 in highly metastatic breast CSCs was recently reported to suppress the metastatic capacity of CSCs in xenograft models [91]. In another recent study, anti-miR-143 was shown to suppress the differentiation and metastatic ability of prostate cancer stem cells *in vivo* [92]. Such therapeutic approaches have targeted both OncomiR and anti-miRNAs and have yielded promising data. Although there are still many hurdles to overcome before clinical setting of miRNA therapeutics will be possible, including delivery and chemical modification of miRNA modulators, we expect that miRNAs and miRNA-targeting

oligonucleotides might become promising tools for cancer treatment in the near future. In fact, an efficient RNA decoys achieve the long-term suppression of specific microRNA activity in mammalian cells is currently developed [93, 94]. This "Tough Decoy (TuD)" miRNA Inhibitor along with a stem-loop stabilized secondary structure, resists cellular nuclease degradation and facilitates sustained miRNA inhibition for longer than 1 month. In addition, both strands of a TuD RNA contain an miRNA binding site for more efficient sequestration of target miRNAs at lower, nanomolar concentrations, providing a strong platform for miRNA medicine.

There are powerful indications that miRNAs may serve as promising targets for lung CSC management. Anti-cancer therapy with miRNAs has the potential to eliminate the self-renewal capacity and anti-apoptotic phenotype of lung CSCs, thereby improving the development of resistance against current lung cancer treatments. Designing effective therapies to target CSC biomarkers and signaling pathways, reverse drug resistance and induce differentiation of lung CSCs remains a necessity. For these reasons, future research should address the therapeutic potential of miRNAs to prevent cancer growth, relapse and metastasis through the control of CSCs.

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Chapter 5
miRNA Targeted Therapy in Lung Cancer

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1 Introduction

Lung cancer is the leading cause of cancer-associated deaths [1] with non-small-cell lung cancer (NSCLC) being the most prevalent histological cancer subtype worldwide [2]. Lung cancer is often associated with poor prognosis because many patients are diagnosed at an advanced stage when surgery is no longer an option. In order to reduce the high mortality rate, new predictive and prognostic biomarkers need to be validated in lung cancer models. The individual components of various signaling pathways have long been tested for their use as biomarkers and/or therapeutic targets with limited success. Research in recent years has proposed microRNAs (miRNAs) as molecules with enormous potential as therapeutic targets.

The miRNAs are small (19–24 nucleotides) non-coding RNA molecules that down-regulate gene expression by interacting with sequences located mostly in the 3′ untranslated region (UTR) region of multiple target mRNAs, resulting in either translational repression or degradation of mRNAs. The miRNA-mediated regulation of oncogenes/tumor suppressor genes is now widely accepted as a key step in

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the progression of human malignancies. The miRNAs are believed to play a regulatory role in almost every aspect of tumor progression. They are known to regulate proliferation, migration, invasion, angiogenesis, metastasis as well as relapse of human cancers. As such they offer an attractive target for therapy of human cancers. While the importance of miRNA-mediated regulation has been demonstrated in virtually every known cancer type, we will limit our discussion in this chapter to lung cancer. Without going into the details of individual miRNAs that have been associated with lung cancer cells' proliferation and other *in vitro* characteristics, we will discuss the most recent literature that has demonstrated the possible use of miRNAs in lung cancer therapy. The focus of this chapter will be on the studies with clinical implications. We will start with a discussion of reports on the regulation of resistance phenotype by miRNAs and then look at numerous recent reports that have tied miRNAs with some diagnostic/prognostic importance in clinical lung cancers.

2 Resistance to Therapies

Despite significant progress made in cancer research in recent years, the mortality rate for lung cancer has largely remained unchanged. A major factor that has contributed to this is acquired resistance to conventional and targeted therapies [3]. Simply speaking, this refers to the ability of lung cancers to turn refractory/resistant to very therapeutic regimes to which they responded initially. Accumulating evidence has connected miRNAs to the phenomenon of drug resistance [4]. In the context of targeted therapy, the topic of resistance is very important. Any future targeted therapy needs to carefully evaluate the possible mechanisms that can lead to resistance against it. In the next few sub-sections, we will detail the role of miRNAs in acquired resistance against chemotherapy as well as radiation therapy, the two major types of therapies used for treatment of cancers.

2.1 Drug Resistance

We start our discussion on resistance to therapies with resistance against chemotherapy. A number of chemotherapeutic drugs have been approved for the treatment of lung cancers. These drugs often meet the same fate when used for prolonged periods and the tumors that acquire resistance against chemotherapy are most often very aggressive and very difficult to manage.

2.1.1 Cisplatin

Cisplatin belongs to the class of platinum-containing anticancer drugs. The miRNAs are now well known to influence the phenomenon of drug resistance against cisplatin. In one study that focused on understanding the mechanism of cisplatin resistance, a role of let-7c was discovered [5]. It was observed that the expression of let-7c miRNA is reduced in A549-derived cells that are resistant to cisplatin. Re-expression of let-7c levels led to alterations in the sensitivity of cells to cisplatin, suggesting a specific role of this miRNA in determining resistance to cisplatin. In this context, ABCC2 and Bcl-XL were identified as targets of this miRNA. It might be important to point out that let-7 family of miRNAs are known regulators of EMT (epithelial-to-mesenchymal transition) wherein their high expression is largely associated with a less aggressive and epithelial phenotype. This study reported lower levels of let-7c in cisplatin resistant cells which means that the resistant cells might be exhibiting a mesenchymal phenotype. These results, therefore, indicate a possible role of EMT in drug resistance. Although there are numerous reports that connect EMT to drug resistance, this report was one of the first connecting let-7c to cisplatin resistance in a lung cancer model. Similar to the effects of let-7c, another report [6] implicated a similar activity of miR-503. This miRNA was also observed to be down-regulated in cisplatin resistant A549 cells. Though this study did not identify a single target of miR-503 leading to cisplatin resistance mediated effects, a number of drug resistance related factors such as MDR1, MRP1, ERCC1, survivin and bcl-2 were reported to be down-regulated significantly with the over-expression of miR-503. Qiu et al. also reported a down-regulated miR-503 in A549 cells that were resistant to cisplatin [7]. They identified Bcl-2 as a target of miR-503. Ectopic expression of miR-503 reduced the levels of its target Bcl-2 and resulted in re-sensitization of cisplatin-resistant A549 cells to cisplatin. Recently, miR-101 over-expression has also been shown to sensitize A549 cells to cisplatin with increased apoptosis through activation of caspase 3 [8].

Another miRNA involved in cisplatin resistance in lung cancer cells is miR-135 [9]. The miR-135a/b were observed to be expressed at relatively low levels in A549 cells that were resistant to cisplatin. To confirm the role of miR-135a/b in cisplatin resistance, these miRNAs were over-expressed in cisplatin resistant cells which led to the reversal of cisplatin resistance. This study identified MCL1 as a direct target of miR-135a/b and, therefore, levels of MCL1 were high in the cisplatin resistance cells which went down with the over-expression of miR-135a/b. The miR-98 is yet another miRNA implicated in cisplatin resistance [10]. This miRNA was identified for its role in cisplatin resistance in a study that looked at differential expression of miRNAs in cisplatin resistant A549-derived cells vs. cisplatin sensitive parental A549 cells. Based on whether the expression was increased at least two-folds or halved, 14 miRNAs were listed to be up-regulated and 8 miRNAs were down-regulated in cisplatin resistance cells, compared to parental cells. The miR-98 was found to be down-regulated three-folds in the resistant cells while its

target gene HMGA2 was up-regulated. Increased expression of miR-98 in cells led to increase sensitivity to cisplatin, thus confirming its role in cisplatin resistance.

With the knowledge that copper-transporting p-type adenosine triphosphatase A (ATP7A) is involved in the resistance to cisplatin, Song et al. [11] looked for miRNA(s) that can target ATP7A which, in turn, may play a role in cisplatin resistance. The miR-495 was identified as one such miRNA that could target ATP7A. ATP7A is induced in cisplatin-resistant cells while miR-495 was found to be down-regulated in the same cells. Up-regulation of miR-495 reduced the levels of ATP7A and sensitized cells to cisplatin. Increased miR-495 levels resulted in accumulation of drug inside the cells which can explain enhanced sensitivity to cisplatin. Conversely, low levels of miR-495 (and high levels of ATP7A) resulted in lower intracellular levels of cisplatin. A role of miR-31 has also been suggested in resistance to cisplatin [12]. Endogenous expression of miR-31 was observed to be up-regulated in cells resistant to cisplatin and its down-regulation led to the sensitization of cells to cisplatin. Interestingly, ABCB9, the drug-resistance associated gene, was identified as a target gene.

2.1.2 Docetaxel (Taxotere)

Docetaxel is a chemotherapeutic agent that interferes with mitosis, and as such targets the relatively fast dividing cancer cells. Similar to its involvement in resistance to cisplatin, let-7 has been shown to be involved in resistance to docetaxel as well [13]. The lung cancer cells, SPC-A1 that were engineered to be resistant to docetaxel, were found to express low endogenous levels of let-7c. Ectopic over-expression of let-7c once again sensitized these cells to docetaxel. Interestingly, resistance to docetaxel was accompanied by resistance to radiations as well, and thus ectopic over-expression of let-7c sensitized the cells to radiation therapy. As expected, increased expression of let-7c reversed EMT and the metastatic potential of cells as well. This study identified Bcl-xL as a molecular target of let-7c and deregulations of Bcl-xL levels were shown to be enough to abrogate let-7c effects. This study successfully demonstrated a similar underlying molecular mechanism for chemo- and radio-resistance that involved modulation by a miRNA.

2.1.3 EGFR-TKI

Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) are routinely used in clinical setting for the treatment of lung cancer. However, like most of the other conventional drugs, lung cancer patients often develop resistance to EGFR-TKIs which remains a major concern. EMT, in particular, has been linked to the development of resistance to EGFR-TKIs. Kitamura and coworkers [14] looked for a correlation between EMT-inducing miRNAs and resistance to EGFR-TKI gefitinib. TGF-β1 was used as an inducer of EMT and it was observed to induce the expression of several miRNAs. Mechanistic studies revealed an

important role of miR-134 and miR-487b in the induction of EMT and resistance to gefitinib. Knockdown of these two miRNAs reversed EMT as well as TGF-β1-induced resistance to gefitinib.

Investigations in our own laboratory have revealed an important role of hedgehog signaling in TGF-β1-induced EMT, and resistance of NSCLC cells to EGFR-TKI erlotinib [15]. We observed reduced levels of miR-200 and let-7 families of miRNAs in TGF-β1-treated A549 cells that were resistant to erlotinib. From the two families of miRNAs, we chose to focus on miR-200b and let-7c because these were the two most differentially expressed miRNAs in our study. Over-expression of these two miRNAs significantly reversed EMT and also re-sensitized cells to erlotinib. Inhibition of hedgehog signaling pathway, by use of pharmacological inhibitor GDC-0449 as well as the use of specific siRNA, also had similar effects, i.e. reversal of EMT and sensitization to erlotinib. Our results, thus, confirmed a complex relationship between hedgehog signaling, EMT and erlotinib resistance, which involved regulation by EMT-modulatory miRNAs.

2.1.4 Pemetrexed

Pemetrexed is another drug used for the treatment of NSCLC. Its structure is similar to folic acid and, therefore, it belongs to the class of anticancer drugs called "folate antimetabolites". It has been approved for use against locally advanced and metastatic NSCLC in combination with cisplatin. Franchina et al. [16] evaluated the expression levels of miR-22, miR-24 and miR-34a in blood samples of NSCLC patients treated with pemetrexed for a possible correlation with clinical outcome. Of the three miRNAs tested, miR-22 was reported as the most promising miRNA because its levels were significantly high in patients that developed progressive disease. Thus, miR-22 can be a marker for predicting progressive disease in patients being treated with pemetrexed.

2.2 Radioresistance

Resistance to radiotherapy is a major clinical problem. Advanced stage tumors often exhibit resistance to radiation therapy. To understand the molecular basis of such radioresistance, Zhang and coworkers [17] developed a radioresistant derivative of NSCLC cell line A549. This was achieved by subjecting A549 cells to 2Gy/day X-rays amounting to a total of 68 Gy. Western blot analysis revealed an up-regulated TRIB2 in the cells that were consistent with resistance to radiation. As a regulator, miR-511 was observed to be down-regulated in the resistant cells. Based on these observations, it was hypothesized that miR-511 expression is lost in cells that acquire resistance to radiation. To prove this hypothesis, miR-511 was

over-expressed in the resistant cells which led to a decrease in the levels of TRIB2 with simultaneous inhibition of cell growth and also increased apoptosis. An involvement of Bax was also suggested based on the observation that resistant cells had low levels of Bax and these levels were restored with over-expression of miR-511. This study suggested that miR-511 can be targeted in lung cancer patients for the reversal of radioresistance. Another study by Grosso et al. [18] found a similar role of miR-201 in the radioresistance. The logic behind the study on miR-201 was based on the connection of this miRNA with hypoxia-inducible factor (HIF)-1. In this work, A549 cells were stably transfected with miR-210 and it was observed that these cells had significantly stabilized HIF-1. As a result, miR-210-expressing cells were similarly radioresistant under normoxic conditions as the un-transfected cells under hypoxia. Furthermore, under hypoxic conditions, miR-210 overexpressing cells were able to tolerate radiations up to 10 Gy and showed significantly reduced apoptosis upon exposure to radiation. It was suggested that specific targeting of miR-210 might be an option for sensitization of radioresistant lung cancers to radiation treatment.

Liu and co-workers [19] exposed isogenic lung cancer cells CL1-0 and CL1-5 to 10 Gy radiation and looked at the differential expression of miRNAs in an attempt to list the miRNAs that play a role in sensitivity to radiation. Of the 26 miRNAs, miR-449a was found to be down-regulated in cells after 24 h of exposure to radiation. Based on this observation, this miRNA was further evaluated for testing the hypothesis that down-regulation of this miRNA might be a mechanism for resistance to radiation. To prove this hypothesis, miR-449a was over-expressed in CL1-0 cells which resulted in increased apoptosis leading to sensitization of cells to radiation, thus confirming a role of miR-449a in radiosensitization of lung cancer cells. Wang et al. [20] observed increased levels of miR-21 in NSCLC samples which was correlated with increased metastasis and poor prognosis. With the down-regulation of miR-21, sensitization to radiation was observed and there was clear evidence of increased degree of apoptosis. These observations connected miR-21 as a molecular determinant of sensitivity of lung cancer cells to radiation therapy.

The process of EMT has been linked to resistance to chemotherapy as discussed earlier. In the sections above, we have discussed the role of EMT-influencing let-7 family of miRNAs in determining resistance to chemotherapy. We pointed out that let-7 miRNAs, being negative regulators of EMT, are down-regulated in resistant cells. Another family of miRNAs which functions similarly to negatively regulate EMT is the miR-200 family. In a study [21] that tried to connect miR-200c with resistance to radiation therapy, it was observed that ectopic miR-200c over-expression resulted in sensitization of lung cancer cells (A549 cells) to radiation. Over-expression of miR-200c would normally result in the reversal of EMT and can explain the reversal of resistance to therapy. This work also evaluated VEGF-VEGFR2 pathway as a target of miR-200c. Down-regulation of miR-200c in resistant cells abrogated the repression of pro-oncogenic VEGFR2 and activated pro-survival and angiogenic mechanisms.

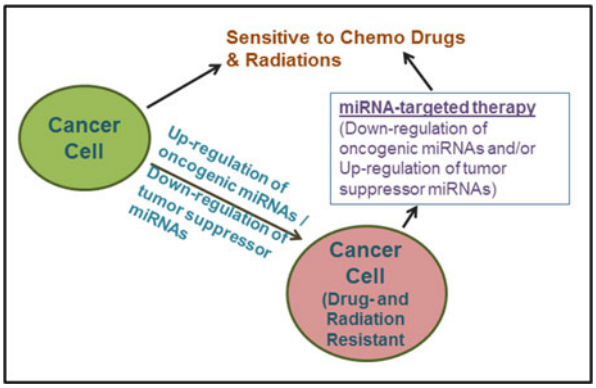

Fig. 5.1 miRNAs in Drug and Radiation Resistance. Oncogenic miRNAs are up-regulated while tumor suppressor miRNAs are frequently down-regulated leading to resistance against chemotherapeutic drugs and/or radiations. Emerging evidence has suggested that a miRNA-based therapy that involves reversal of such miRNA events can reverse resistance against drugs and radiations leading to re-sensitization of cancer cells

In the last few decades, there has been a realization that non-toxic natural agents, the 'nutraceuticals', can be potent anticancer agents by virtue of their pleiotropic roles, including their ability to modulate resistance to radiation therapy [22]. Such activity of flavonoid compounds rhamnetin and cirsiliol was recently described in NSCLC cells H1299 and H460 [23]. It was determined that the resistance of NSCLC cells to radiation involved up-regulation of Notch signaling. Since rhamnetin and cirsiliol could down-regulate Notch-1, it was suggested that such inhibition of Notch-1 by these natural compounds could result in re-sensitization of cells to radiation. This was found to be true and the underlying mechanism was found to be an up-regulation of miR-34a. Notch-1 is a target of miR-34a and up-regulation of miR-34a resulted in the down-regulation of Notch-1, resulting in the reversal of resistance to radiation therapy.

Based on the reports discussed so far, it is evident that miRNAs play interesting roles in determining resistance to therapies (Fig. 5.1). These effects are summarized in Table 5.1. It is interesting to note that many studies describe the same cell type, for example, A549 NSCLC cells that have been made resistant to a specific chemotherapeutic drug. In spite of working with essentially the same model system, individual reports have mostly implicated a novel miRNA for the observed effect, which suggests the complexity with miRNAs research. It appears that multiple miRNAs might be involved in the regulation of similar patho-physiological responses, and thus more detailed studies will be needed to better understand these regulatory mechanisms.

Table 5.1 miRNAs involved in regulation of resistance to chemotherapy/radiation therapy

miRNA	Expression	Effect	References
miR-21	High	Radioresistance	[20]
miR-31	High	Cisplatin Resistance	[12]
miR-34a	Low	Radioresistance	[23]
miR-92b	High	Cisplatin Resistance	[24]
miR-98	Down	Cisplatin Resistance	[10]
miR-134	High	EGFR-TKI Resistance	[14]
miR-135a/b	Down	Cisplatin Resistance	[9]
miR-200b	Down	EGFR-TKI Resistance	[15]
miR-200c	Down	Radioresistance	[21]
miR-210	High	Radioresistance	[18]
miR-449a	Down	Radioresistance	[19]
miR-487b	High	EGFR-TKI Resistance	[14]
miR-495	Down	Cisplatin Resistance	[11]
miR-503	Down	Cisplatin Resistance	[6, 7]
miR-511	Down	Radioresistance	[17]
Let-7c	Down	Cisplatin Resistance	[5]
Let-7c	Down	Docetaxel Resistance	[13]
Let-7c	Down	Radioresistance	[13]
Let-7c	Down	EGFR-TKI Resistance	[15]

3 miRNA in Diagnosis and Prognosis of Lung Cancer

The miRNAs have largely been studied for their therapeutic potential. In addition to possible use of miRNAs in the therapy of lung cancer, their use as diagnostic, prognostic and predictive biomarkers has also been advocated [24–29]. For example, miR-34c was found to be inversely correlated with histology and has been suggested as a quantitative biomarker which parallels histologic response in formalin-fixed biopsies [30]. The miRNAs can also distinguish between current and former smokers, and miR-375 has been found to be up-regulated in the current smokers [30]. The advantage of using miRNAs for prediction, diagnosis and prognosis is that this approach is non-invasive. Many cancers are now believed to have a "miRNA fingerprint", which, in simple terms, refers to the expression pattern (up-regulation/down-regulation) of certain specific miRNAs in the biological fluids within the physiological systems which can be exploited for diagnosis and/or prognosis in the clinical setting. In addition to the traditional screening of blood/serum/plasma/tissue specimens described in the following subsections, it is worth mentioning that recent literature has pointed to a possible use of exosome-derived miRNAs as biomarkers of lung cancer diagnosis [31].

3.1 miRNAs in Serum/Plasma/Circulation

Most of the procedures in practice for the detection of human cancers, including lung cancer, are invasive. However, recent work with miRNAs has opened up the

possibility of using these tiny molecules as non-invasive diagnostic tools for reliable detection of lung cancer. Tang et al. explored three miRNAs – miR-21, miR-145 and miR-155 for their possible role in predicting onset of lung cancer [32]. The expression levels of these miRNAs were evaluated in 62 lung cancer patients and 60 smokers. Of the three miRNAs, plasma levels of miR-145 were down-regulated while those of miR-21 and miR-155 were up-regulated in lung cancer patients, compared to the healthy smokers. In a study in Egyptian population [33] which looked at serum levels of some of these miRNAs and a few additional miRNAs (miR-21, miR-155, miR-182 and miR-197), the findings on miR-21 and miR-155 were confirmed i.e. these miRNAs were found to be expressed at higher levels in lung cancer patients' sera. Additionally, miR-182 and miR-197 levels were also found to be high in lung cancer patients [33]. The prognostic value of miR-21 and miR-155 has been confirmed in a meta-analysis by Wang et al. [34]. This meta-analysis of 19 studies concluded that high levels of miR-21 and miR-155 are indeed reliable prognostic biomarkers for NSCLC progression as well as increased risk of lymphoid cells infiltration.

The recovery of miRNAs from circulation has opened a new field with the possibility of the use of blood-based miRNAs in diagnosis and/or assessing progression of lung cancer [35, 36]. In one such study [37], expression of 12 plasma miRNAs, miR-20a-5p, miR-24-3p, miR-25-3p, miR-126-3p, miR-145-5p, miR-152-3p, miR-155-5p, miR-191-5p, miR-223-3p, miR-199a-5p, miR-296-5p, and let-7f-5p allowed significant discrimination between controls and NSCLC patients with an accuracy of 82.1 %. A six-plasma miRNA panel was found to distinguish between NSCLC patients and chronic obstructive pulmonary disease patients. A three-miRNA plasma signature was found to be significantly associated with a higher risk for progression in adenocarcinoma patients and a separate three-miRNA plasma panel significantly predicted survival of squamous cell carcinoma patients. In a study [38] that aimed at identifying diagnostically relevant miRNAs which can distinguish between benign pleural effusion and lung carcinoma-associated malignant pleural effusion, miR-198 was reported to be significantly down-regulated in lung carcinoma-associated malignant pleural effusions.

Clinical management of patients with lung cancer offers a lot of challenges, one of which is the metastatic and recurrent disease. It is desirable that the physicians have an access to a 'molecular signature' which can predict the high vs low risk of disease recurrence [39]. A number of molecular factors have been evaluated for their ability to predict the recurrence of lung cancer and in recent years miRNAs have been the target of many such investigations. One such study reported high levels of circulating miR-142-3p and miR-29b in serum of patients with early relapse of lung adenocarcinoma [40]. This study compared patients with and without recurrence 24 months post-surgical intervention. With the identification of these two miRNAs at high levels in the serum of patients with recurrence, it might be possible for the physicians to put a patient at high risk of cancer relapse incase the levels of these miRNAs are found to be elevated in regular follow-up studies after surgery. In a report by Markou et al. [41], worst disease free interval was correlated with low tissue levels of miR-10a while high expression of miR-30e-

5p was correlated with shortened overall survival. There is evidence supporting an influence of single-nucleotide polymorphism within miRNA processing on the prognosis of lung cancer patients [42, 43]. Such genetic variations can lead to altered regulation of target genes by miRNAs although this field need more in-depth investigations.

3.2 miRNAs in Tissue Specimens

Formalin-fixed paraffin-embedded tissue specimens are invaluable sources for investigation. Lin et al. found high expression of miR-19a in NSCLC tissues, compared to non-malignant cancerous tissues [44]. These high levels were also confirmed in serum of the patients. High miR-19a levels were correlated with TNM stage, increased metastases and poor survival. In another study aimed at identifying miRNA that might be involved in lymph node metastasis, miR-31 was found to be up-regulated in lung cancer tissue, compared to normal tissue [45]. This miRNA was distinctly up-regulated in patients with lymph node metastases, compared to those without lymph node metastases. It was no surprise that reduced expression of miR-31 was found to be correlated with excellent survival. In a very similar study [46], miR-9 was found to be up-regulated in lung cancer tissues, compared to adjacent normal tissues. Furthermore, up-regulation of miR-9 was correlated with advanced stage tumors and lymph node metastases. The miR-92b is another miRNA that is expressed at higher levels in lung cancer tissues, compared to matched adjacent normal tissues [47]. Through the regulation of its well-known tumor suppressor target PTEN, miR-92b was shown to regulate response to cisplatin. High levels of miR-92b and concomitant low PTEN levels resulted in cisplatin resistance.

Epigenetic events can also help predict prognosis. As an example, hypermethylation of miR-886 promoter leading to the loss of miR-886-3p expression has been linked to poor outcome prediction in small cell lung cancer [48]. Similarly, hypermethylation of miR-148a coding region resulting in the reduced expression of this miRNA has been linked to poor prognosis of NSCLC with increased metastasis, shortened disease-free survival and reduced overall survival [49].

3.3 miRNAs in Sputum

Shen et al. [50] performed an analysis for the possible use of miRNAs as biomarkers in the sputum. The analyses were done using qRT-PCR in 64 lung cancer patients and 73 cancer-free smokers. Of the short-listed 12 miRNAs, miR-31 and miR-210 were found to be the best predictors of lung cancer. When combined, these two miRNAs resulted in 65.2 % sensitivity and 89.7 % specificity. When combined

Table 5.2 miRNAs implicated in diagnosis and prognosis of lung cancers

miRNA	Source	Diagnosis/ prognosis	Observation	References
miR-9	Tissue	Prognosis	High levels correlate with poor prognosis	[47]
miR-10a	Tissue	Prognosis	Low levels correlate with worst disease free interval	[42]
miR-19a	Serum, Tissue	Prognosis	High levels correlate with poor survival	[45]
miR-21	Plasma, Serum	Diagnosis	High in lung cancer patients	[33–35]
miR-22	Blood	Prognosis	High levels in patients with progressive disease	[16]
miR-29b	Serum	Prognosis	High in recurrent disease	[41]
miR-30e-5p	Tissue	Prognosis	High levels correlate with poor overall survival	[42]
miR-31	Tissue	Prognosis	High in lymph node metastases	[46]
miR-142-3p	Serum	Prognosis	High in recurrent disease	[41]
miR-145	Plasma	Diagnosis	Low in lung cancer patients	[33]
miR-148a	Tissue	Prognosis	Low levels correlate with poor survival	[49]
miR-155	Plasma, Serum	Diagnosis	High in lung cancer patients	[33–35]
miR-182	Serum	Diagnosis	High in lung cancer patients	[34]
miR-197	Serum	Diagnosis	High in lung cancer patients	[34]
miR-198	Cell-free circulation	Diagnosis	Low in malignant pleural effusion	[39]
miR-886	Tissue	Prognosis	Low levels associated with poor survival	[48]

with the standard diagnostic tool – computed tomography, these two miRNAs increased the sensitivity of computed tomography.

The literature discussed in this section outlined the remarkable progress with regards to the use of miRNAs as biomarkers for prediction, diagnosis and prognosis. The information is summarized in Table 5.2. This information suggests that miRNAs can be invaluable tool for monitoring the onset and progression of lung cancer. While this information is encouraging, it is to be noted that all this information has emerged within the last 1 year and novel information is trickling in very fast. It might be some time before a complete picture emerges and when it does many of these important miRNAs will become the target for lung cancer therapy.

4 Targeted Delivery of miRNAs – Challenges and Progress

The discussion so far attests to the fact that miRNAs have great potential as regulators of cellular signaling affecting key physiological functions that determine cancer cells' behavior such as drug resistance, metastasis and tumor aggressiveness.

Therefore, targeting of miRNAs for therapeutic purposes sounds a reasonable approach moving forward although the proof-of-concept has been documented in pre-clinical setting. However, accomplishing such targeting *in vivo* is very challenging. One of the first challenges associated with *in vivo* injecting of miRNAs is that these tiny molecules are highly unstable or not bioavailable especially in circulation for executing their function. They are degraded very rapidly in the systemic circulation and it is highly unlikely they can reach their target tumor cells under normal conditions which is in part due to their binding to large molecular weight proteins. Another challenge is that they are negatively charged which leads to a poor uptake by cancer cells. Therefore, to target miRNAs to their intended targets, the very first step is to afford them protection. In a study performed in breast cancer model [51], we achieved an *in vivo* effect of miRNA through the use of locked nucleic acid (LNA)-modified oligonucleotide (miR-200b in this study) that was delivered intravenously to mice three times a week for a total of 5 weeks. Such persistent dosing was suitable to observe the effects of miRNA regulation on its target genes and the phenotype *in vivo* in an experimental assay of pulmonary metastases. Prior to our study, Chen et al. [52], reported a liposome-polycation-hyaluronic acid nanoparticle formulation which was modified with tumor-targeting single-chain antibody fragment. This was used for systemic delivery of not only miRNAs but also siRNA. Similar to our study where we studied experimental pulmonary metastases of breast cancer cells [51], this study also looked into pulmonary metastasis where the tumor was induced by murine B16F10 melanoma cells.

Not much progress has been made in the targeted delivery of miRNAs but there are some preliminary reports which are encouraging. As discussed above, let-7 miRNA is a promising target for therapy of lung cancer. It is involved in EMT and the drug resistance phenotype. Also, since it is a tumor suppressor miRNA and it is usually down-regulated in metastatic, drug resistant cancers, its delivery to tumor cells *in vivo* can possibly be a good strategy to inhibit metastasis and/or sensitize drug resistant cells to conventional therapeutics. Recently, let-7a was packaged in a novel liposomal preparation – DOTAP (N-(1-(2.3-dioleoyloxy)propyl)-N,N, N-trimethyl ammonium)/Cholesterol/DSPE (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(cyanur(polyethylene glycol) – 2000)) – PEG (polyethylene glycol)-cyanur liposomal nanoparticles (LNP) [53]. DOTAP and DSPE-PEG are cationic lipids that contain cyanuric groups. The ligand of EphA2, ephrin A1, was conjugated on surface of liposomal nanoparticles to specifically target lung cancer cells that express EphA2 receptor. Such transfections in lung cancer cells were found to be very effective and the expected effects on cellular functions and expression of target genes were observed. This study only looked at lung cancer cells-based assays. The next important step would be to demonstrate the efficacy of formulation *in vivo*. Another important tool that can help translation studies is the development of more appropriate *in vivo* model systems. Towards this end, a metastatic fluorescent Lewis lung carcinoma mouse model has been described [54] that can potentially help identify/screen novel miRNAs involved in malignant progression.

5 Conclusions

The research on miRNAs in lung cancer has come a long way in recent years, more so in the past 1 year that we have discussed here. A lot of information is rapidly emerging which will take some time for more robust analysis and independent verifications. Meanwhile, the treatment of lung cancer in the clinical setting is steadily moving towards using approaches that are much more 'individualized'. This approach is based on the realization that each and every tumor presented in the clinical setting has its individuality. While there are major classes for grouping most cancers, including lung cancers, it is also evident that every single tumor also has its unique characteristics even though a tumor mass is composed of highly heterogeneous population of cancer cells. This heterogeneity is in part responsible for treatment failure using conventional therapeutics including targeted therapy. Personalized treatment of human lung cancers is an emerging field and it is envisioned that miRNAs have their own unique niche in such personalized therapy for the management of lung cancer patients [55]. On a similar note, understanding the epigenetic changes that accompany the onset of carcinogenesis is crucial [56]. In recent years, a number of agents that can target epigenetic changes have been investigated in translation studies. It is interesting to note an important role that miRNAs are increasingly being realized to play in the epigenetic regulation of lung cancer [48, 57–60]. Based on these evidences, it appears that miRNAs hold a lot of promise in advancing the research that focuses on finding a cure for lung cancer.

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Chapter 6
miRNA-Based Ovarian Cancer Diagnosis and Therapy

Rong Guo, Cheryl Sherman-Baust, and Kotb Abdelmohsen

1 Introduction

Ovarian cancer is the fifth most common cancer among women and is the leading cause of death from gynecological cancers. In the United States, ovarian cancer causes 5 % of all cancer deaths with an estimated 14,030 deaths and 22,240 new cases in 2013 according to http://www.cancer.gov/cancertopics/types/ovarian of the National Cancer Institute [1]. The high mortality rate associated with ovarian cancer is due mostly to diagnosis at late stage and the high prevalence of drug resistance among patients [2, 3]. Thus, new markers for early diagnosis and targeted therapy may permit more effective ways to combat this malignancy. MicroRNAs (miRNAs) are emerging as new therapeutic targets in a number of diseases including cancer [4]. miRNAs have a high degree of pleiotropy since one can impact hundreds of targets and multiple miRNAs can affect a single target [5, 6]. These small noncoding RNA molecules, 22 nucleotides in length, have been implicated in several cellular processes relevant to cancer development and progression including cell differentiation, extracellular matrix remodeling, angiogenesis, proliferation, and apoptosis [7, 8]. To date, more than 1,200 miRNAs have been identified and validated in humans according to the miRbase database (http://www.mirbase.org/). Through binding to their targets, miRNAs post-transcriptionally regulate mRNA translation and/or decay [9, 10]. Several studies revealed that miRNAs are altered in neurodegenerative, cardiovascular, hepatic diseases, as well as cancer [11–14]. Alteration of the normal miRNA signature in the ovary provides a perfect opportunity for early detection, diagnosis, and therapy [15]. In addition, miRNAs

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can enhance the efficacy of chemotherapeutic drugs and reduce drug resistance, suggesting promising outcomes for miRNA-drug combinations. Additionally, the discovery of circulating miRNAs may offer an early detection method using whole blood or other biofluids. Indeed, studies indicate that circulating miRNAs can be used as biomarkers for early detection of various cancers [16–18]. In this chapter, we will focus on the recent advances in the applications of miRNAs in early diagnosis and treatments of ovarian cancer.

2 miRNA Biogenesis and Function

Primary miRNAs (pri-miRNAs) are transcribed in the nucleus by RNA polymerase II. These long transcripts are processed by a microprocessor complex, which includes an RNase III enzyme Drosha and its partner DGCR8 (DiGeorge critical syndrome region gene 8, also known as PASHA), into approximately 70 nucleotide sequences called miRNA precursors (pre-miRNAs). Pre-miRNAs are exported to the cytoplasm by the nuclear export protein Exportin 5 and cleaved by another RNase III enzyme, Dicer, into the functional, mature miRNA. Mature miRNAs bind Argonaute proteins to form the RNA-induced silencing complex which regulates mRNA translation or stability [9, 19, 20]. miRNA function is regulated by RNA binding proteins (RBPs) and noncoding RNAs. For example, the RBP HuR is believed to exist in a functional interplay with miRNAs [21]. Indeed, HuR was found to promote the dissociation of the miRNA complex from target RNAs bearing let-7 sites [22]. Recent studies have shown different types of interplay between miRNAs and long noncoding RNAs (lncRNAs), allowing cross-regulation of abundance and functions. For example, lncRNAs can sponge, compete with, or generate miRNAs [23–26]. Newly discovered circular RNAs, another class of noncoding RNAs, were reported to sponge miRNAs and regulate their function. Circular RNA ciRS-7 was found to harbor more than 70 miRNA target sites for miR-7 indicating that they can function as efficient miRNA sponges [27]. Future studies may reveal other examples of such naturally existing miRNA sponges.

3 miRNAs and Hallmarks of Cancer

It is very well established that miRNAs are altered in cancer cells. For a cell to become malignant, certain characteristics need to be acquired including enhanced cell survival, proliferation, angiogenesis and invasion, as well as evasion of immune recognition and apoptosis. Cancer cells may avoid elimination by driving up or down the levels of certain miRNAs [28]. miRNAs are generally involved in several processes that are essential for cancer progression as reviewed in Ruan et al. [29]. The general scheme in cancer cells is that downregulated miRNAs could act as tumor suppressors while upregulated miRNAs could function as tumor

enhancers (knows as oncomiRs). Indeed, several miRNAs including miR-34a, miR-125a, miR-519, miR-28, miR-296 were reported to act as tumor suppressors through the disruption of essential processes required for cancer progression [7]. miRNAs such as miR-214, miR-155; miR-146a, miR-224, miR-18, miR-21, and others enhance tumorigenesis by promoting processes required for cancer progression [30]. Together these studies and several others have indicated that miRNAs can promote or suppress carcinogenesis.

4 miRNA Regulation in Cancer

Altered miRNA expression is commonly observed in human cancers. Mechanisms involved in the aberrant expression of miRNAs include chromosomal alterations, DNA methylation, abnormal transcription and post-transcriptional events. Several miRNA loci in the human genome including four out of the nine distinct let-7 loci are located in segments that are frequently deleted in human cancers [31]. Hypermethylation of miR-148a, miR-34b/c, and miR-9 was found to contribute in the silencing of these miRNAs [32]. Several transcription factors (TF) such as c-Myc, p53, and NF-κB have been implicated in the regulation of miRNA abundance. For example, the oncogenic TF c-Myc binds various enhancers of a let-7 cluster and can activate or inhibit promoter activity [33–35]. The tumor suppressor protein p53 regulates transcription of several miRNAs like let-7a and let-7b through binding to the gene enhancer to repress their expression in HCT116 colon cancer cells in response to radiation and oxidative stress [36]. In contrast, glycogen synthase kinase 3 beta (GSK3β) was found to suppress let-7 expression through the down regulation of p53 in the ovarian cancer cell line BG1 [37]. Other miRNAs such as miR-34, one of the well-established tumor suppressor miRNAs, is also regulated by p53 [38]. These findings demonstrate that the effect of p53 on miRNA could be dependent on stress or cancer cell type.

Post-transcriptional modulation of miRNAs is mediated by RNA-binding proteins involved in miRNA processing. One of the best characterized post-transcriptional repressors of let-7 is the RNA-binding protein Lin28, which has two paralogs in mammalian cells: Lin28A and Lin28B. Lin28A is present in the cytoplasm and blocks Dicer processing of pre-let-7, while nuclear Lin28B sequesters pri-let-7 to block its nuclear processing [39–41]. Other microprocessors such as Drosha, DGCR8 and Dicer are also altered in cancer cells. For instance, low Dicer expression has been associated with lung, advanced breast, skin, and ovarian cancers [42–45]. Low Dicer expression was also found in tissue arrays containing pairs of normal and cancer tissues including colon, stomach, breast, kidney, liver and pancreas. It is suggested that low Dicer expression in these cancer tissues could be due to enhanced expression of the RNA binding protein AUF1 that negatively regulates Dicer expression [46]. Drosha is also downregulated in skin and ovarian cancers [45, 47]. Other regulators in the miRNA biogenesis pathway such as p68, p72, Ago3, Ago4 and Piwil4 are expressed less in primary hepatocellular carcinoma

compared to normal liver [48]. In contrast, Dicer expression was found to be highly expressed in prostate adenocarcinoma [49]. These findings suggest that the miRNA changes in ovarian cancer are at least partially due to altered expression of the regulators that govern their transcription or processing.

5 miRNA Signature in Cancer

The miRNA expression profile is altered in cancer cells and provides a cancer-specific signature. Global miRNA microarray analysis of six tumor types (lung, breast, stomach, prostate, colon, and pancreatic) identified a solid cancer miRNA signature in which miRNAs like miR-17-5p, miR-20a, miR-21, miR-92, miR-106a, and miR-155 are altered [50]. A comparison of miRNA expression of human pancreatic adenocarcinoma, benign tissue, normal pancreas, chronic pancreatitis and nine pancreatic cancer cell lines revealed that at least 100 miRNA precursors including miR-155, miR-21, miR-221, miR-222, miR-376a, and miR-301 are aberrantly expressed in pancreatic cancer [51]. Profiling of miRNAs in clear cell renal cell carcinoma (RCC) indicated distinct differences between malignant and non-malignant tissues. In these tissues miRNAs like miR-16, miR-452*, miR-224, miR-155 and miR-210 are increased, while miR-200b, miR-363, miR-429, miR-200c, miR-514 and miR-141 are decreased in cancer tissues [52].

Several miRNAs are aberrantly expressed in human ovarian cancer tissues and cell lines. While miR-221 is highly expressed in ovarian cancer, miR-21 and several members of the let-7 family are downregulated [53]. miRNA signature in cancer cells can be further utilized to classify tumor molecular diversity, subtypes and overall survival. Unique miRNA signatures were developed to distinguish different renal cell carcinoma RCC subtypes [54]. Computational analysis of ovarian cancer transcriptional profiles supported by miRNA expression profiles indicated angiogenesis signature associated with overall survival [55]. miRNA microarray analysis of 62 advanced ovarian cancer cases identified three miRNAs (miR-337, miR-410, and miR-645) associated with patients survival rates. This study suggested that miR-410 and miR-645 are negatively associated with overall survival in advanced serous ovarian cancer [56]. Together these findings indicate that miRNAs are altered in cancer cells, which provide a signature to distinguish normal and cancer tissues.

6 Circulating miRNA in Ovarian Cancer

Screening or profiling miRNAs in ovarian cancer is mostly performed using tissues obtained from patients or cell lines. This however may not be helpful for early diagnosis. Detection of abnormal expression of miRNAs in blood or other biofluids may provide a useful tool for early diagnosis in a non-invasive fashion. Circulating

miRNAs are present in blood exosomes, which are small membrane-bound particles released from normal and neoplastic cells [57]. Several studies were conducted to assess the presence or differential enrichment of miRNAs in the blood of ovarian cancer patients. miRNA profiling of high grade serous epithelial ovarian cancer patient sera revealed elevated levels of miR-182, miR-200a, miR-200b and miR-200c relative to normal human samples [58]. Profiling miRNAs using microarrays showed low levels of 5 miRNAs (miR-132, miR-26a, let-7b, miR-145, and miR-143) in the serum, tissue and ascites of ovarian cancer patients [59]. The levels of miR-21 were investigated in the serum of 94 epithelial ovarian cancer (EOC) patients and 40 healthy age-matched samples. In ovarian cancer patients, the levels of miR-21 were significantly higher and correlated with advanced tumor stage, high tumor grade and shortened overall survival [60]. Similarly, miR-221 was studied in serum samples from 96 patients with primary EOC and 35 healthy age-matched volunteers. Serum miR-221 was elevated in patients with EOC and correlated with high tumor stage, high tumor grade, and shortened overall survival [61]. In another study, the levels of miR-92 were higher in the serum of 50 ovarian cancer patients compared to 50 controls [62]. Plasma miRNA profiling of patients with endometriosis, endometriosis-associated ovarian cancer (EAOC), and healthy individuals revealed distinct serum miRNA signature for each group. While endometriosis and EAOC showed common miRNAs such as miR-16, miR-195, miR-191, miR-1974, miR-4284, miR-15b and miR-1973 elevated in serum, miR-1978, miR-1979, and miR-362-5p were specifically elevated in endometriosis and miR-21, miR-1977, and miR-1979 were elevated in EAOC [63]. Additionally, PCR arrays indicated that miR-21, miR-92 and miR-93 were elevated in serum of ovarian cancer patients [64].

In addition to blood, miRNAs were also reported to be secreted in other biofluids such as urine, saliva, breast milk, tears, bronchial lavage, peritoneal, seminal, cerebrospinal, and amniotic fluids [65]. A study of miRNAs in urine samples of bladder cancer patients indicated differential presence of some miRNAs like miR-200a. These findings suggest that miRNAs provide a non-invasive diagnostic of the recurrence of bladder cancer [66]. Thus, miRNAs in biofluids may be potential diagnostic biomarkers for early detection of ovarian cancer. Based on these findings, we conclude that miRNAs are secreted into biofluids and differentially enriched in pathological conditions of ovarian cancer. Thus, assessing miRNAs in biofluids, particularly in serum, may provide an early diagnostic and therapeutic approach for ovarian cancer treatment.

7 miRNAs and Ovarian Cancer Chemoresistance

Surgery followed by chemotherapy is the standard regimen for the treatment of most ovarian cancer. While surgery is performed to optimally debulk the existing tumors, chemotherapy is used to both kill any remaining cancer and to prevent the recurrence of ovarian cancer. Cisplatin, paclitaxel, and their derivatives are

approved by the Food and Drug Administration as the standard chemotherapeutics to treat women with advanced epithelial ovarian cancer. Many patients initially respond to chemotherapy, but eventually relapse with drug-resistant disease presenting a major obstacle to lasting, successful treatment. Identifying the correlation between miRNA expression and drug response in ovarian cancer patients would allow clinicians to take advantage of miRNAs as biomarkers to predict chemoresistance.

Dicer expression is low in cisplatin resistant ovarian cancer A2780 cells (A2780/DDP) compared to parental A2780 cells indicating a possible impact of miRNAs on drug sensitivity in EOC [67]. Indeed, analysis of cisplatin sensitive and resistant ovarian cancer cell lines indicated differential expression of 11 miRNAs that may provide a unique signature for ovarian cancer treatment. In this study both microarrays and real time PCR analysis showed that miR-625 and let-7c are downregulated, while miR-193b and miR-642 are upregulated in A2780/CP70 cells as compared to A2780 cells [68]. Similarly, miR-106a was found to be downregulated in cisplatin resistant A2780/DDP cells compared to A2780 cells. Thus, antagonizing miR-106a in A2780 cells decreased the antiproliferative and apoptotic effects induced by cisplatin in A2780 cells. Restoration or overexpression of miR-106a in A2780/DDP enhanced cisplatin-induced antiproliferative effects and apoptosis. The authors concluded that the influence of miR-106a could be mediated by its regulatory effect on the expression of the oncogene Mcl-1 [69]. Array analysis of miRNA expression in patient tumor samples indicated a signature associated with chemoresistance. Three miRNAs (miR-484, miR-642, and miR-217) were downregulated in nonresponder ovarian cancer patients and were able to predict chemoresistance of a highly aggressive epithelial ovarian cancer. The study also indicated that miR-484 regulates the expression VEGFR2 in tumor-associated endothelial cells upon its secretion into the local tumor microenvironment and circulation [70]. In another study using paclitaxel resistant SKOV3-TR30 and paclitaxel sensitive SKOV3 ovarian cancer cell lines, several miRNAs were found to be differentially expressed. While miRNAs such as miR-320a, miR-22, and miR-129-5p were upregulated, other miRNAs such as miR-9, miR-155 and miR-640 were downregulated in the paclitaxel resistant cell line [71]. A similar approach was performed in 4 paclitaxel resistant SKpac sublines and parental SKOV3 ovarian cancer cell line. Data indicated that increased miR-106a and decreased miR-591 expression is associated with paclitaxel resistance. Antagonizing miR-106a or restoration of miR-591 re-sensitized paclitaxel resistant SKpac cells, enhanced apoptosis, inhibited their cell migration and proliferation [72]. Patients without complete response to paclitaxel–carboplatin treatments showed lower levels of miR-200c compared to complete responders. The incomplete responders also had higher levels of β-tubulin class III (TUBB3), a factor associated with resistance to taxanes [73, 74]. Similar results were also observed in two other independent studies. These findings support the loss of miR-200c as a marker of chemoresistance and aggressiveness in ovarian cancer [75]. Additionally, low miR-200c expression enhances binding of the RNA binding protein HuR to TUBB3 mRNA to increase mRNA translation. Repression of

TUBB3 expression by overexpression of miR-200c restored sensitivity to paclitaxel and cisplatin [76]. The miR-31 is downregulated in paclitaxel resistant KFr13Tx cells and its overexpression restored paclitaxel response in resistant ovarian cancer cells. Low levels of miR-31 leads to increased expression of receptor tyrosine kinase MET, which contributes to paclitaxel resistance in ovarian cancer [77]. Downregulation of let-7i in late stage ovarian cancer patients was found to be associated with drug resistance and shorter survival rates [78]. This data suggested that restoration of let-7i in ovarian cancer cells may enhance drug sensitivity. Indeed, successful delivery of let-7i with MUC1 aptamer into paclitaxel/cisplatin resistant OVCAR-3 ovary tumor cells significantly re-sensitized cells to paclitaxel and inhibited cell proliferation [79]. Several other miRNAs such as miR-125b and miR-182 were reported to contribute to chemoresistance in ovarian cancer by reducing cisplatin induced cytotoxicity [80, 81]. Overexpression of miR-29 increased cisplatin sensitivity in CP70, HeyC2, SKOV3, and A2780 ovarian cancer cells [82].

These findings suggest that delivery of specific miRNAs into tumor cells is a strategy for reversing chemoresistance of ovarian cancer. Assessing miRNA changes may provide a cancer-specific signature that can be associated with tumor stage, resistance to chemotherapy and patient survival rates. However, these signatures may not be specific to ovarian cancer but could also be associated with drug resistance in other cancer types. Additionally, early diagnosis remains a major obstacle in the battle against ovarian cancer. It is important to note that these analyses were performed on patient samples with existing tumors and thus, future studies are needed to test if such signature provides a tool for early diagnosis of ovarian cancer.

8 miRNA-Based Therapy

Although miRNA delivery to a specific tissue could present limitations, several delivery systems can be employed to knockdown or overexpress miRNAs. These systems include viral vector-based systems (adenoviral, retroviral, and lentiviral), nanoparticles, and lipid-based emulsions [83–86].

The involvement of miRNAs in many biological processes and diseases prompted the idea of miRNA-based therapeutic applications. As mentioned above, miRNAs can function as oncomiRs or tumor suppressors depending on the impact on cancer cells. Several experiments have been undertaken to provide evidence that miRNAs can be used in cancer therapy. These studies were based on replacing the downregulated miRNA by overexpression of miRNA mimic or inhibiting the upregulated miRNA using miRNA antagomiRs. For example, miRNAs like miR-519, miR-15a, miR-16, and let-7 were found downregulated in cancer cells and restoring the expression of these miRNAs suppressed tumorigenesis [7]. On the other hand, inhibition of cancer-upregulated miRNAs like miR-20a, miR-21, miR-106a, or miR-155 were also found to suppress tumorigenesis

[87]. These and other studies have shown that miRNAs can be used in cancer treatments. For ovarian cancer treatments, miRNAs can be used either alone or in a combination with conventional therapy including both surgery and chemotherapy. Using miRNAs in clinical trials for cancer treatment has begun. Companies such as Rosetta Genomics and Mirna Therapeutics have recently initiated miRNA-based cancer treatments. Rosetta Genomics is using a modified oligonucleotide anti-miR-222 for treating hepatocellular carcinoma (HCC). Mirna Therapeutics has initiated a Phase 1 clinical study of miR-34, which is the first miRNA to advance into a human clinical trial for cancer. Let-7, miR-16, and other miRNAs are in Mirna's pipeline. Although these studies may benefit ovarian cancer patients, there is a need for ovarian cancer specific prevention and treatment trials. Since miRNAs can enhance the sensitivity of ovarian cancer cells to chemotherapy and also suppress acquired drug resistance, a combination of chemotherapy agent and miRNA could render a more effective therapeutic approach. Also certain drugs may specifically enhance the expression of a downregulated miRNA in ovarian cancer to suppress tumorigenesis. Thus, a wide screen of these drugs may result in identification of a new class of drugs that function by reversing the expression of a specific miRNA in ovarian cancer cells. This approach may also overcome the limitation of miRNA delivery, since the drug will enhance its expression. miRNA-based therapy of ovarian cancer can also be used during, before, or after surgery to limit or even inhibit disease progression caused by the remaining or circulating tumor cells. Although the presence of miRNAs in biofluids such as blood is used as biomarkers, the impact of these miRNAs on tumor development, recurrence, or metastasis is not well understood. Since some miRNAs are abnormally altered in the blood of ovarian cancer patients, manipulation of such miRNAs may provide a new approach of miRNA-based therapy. One goal would be to establish the ratio of up/down miRNA in plasma from patients, which may have pathological implications for cancer treatments. Strategies for miRNA-based cancer diagnosis and therapy are summarized in Fig. 6.1.

9 Concluding Remarks and Perspectives

The discovery that miRNAs can modulate gene expression and cellular processes created a new dimension for understanding the mechanisms of tumorigenesis. In the past decade we have gained a great deal of knowledge about miRNA biology and their applications as potential biomarkers and novel cancer therapy adjuvants. The existing challenge is to translate that knowledge into clinical approaches that can be used in the treatment of pathological conditions such as ovarian cancer. These challenges add to the standing challenges in ovarian cancer treatment, in particular early detection and drug resistance. Although several miRNAs have been identified as tumor suppressor or enhancers, very few miRNAs are tested in clinical trials. This indicates that a greater understanding is required and that additional modifications to miRNA mimics and antagomiRs are needed to make them more effective

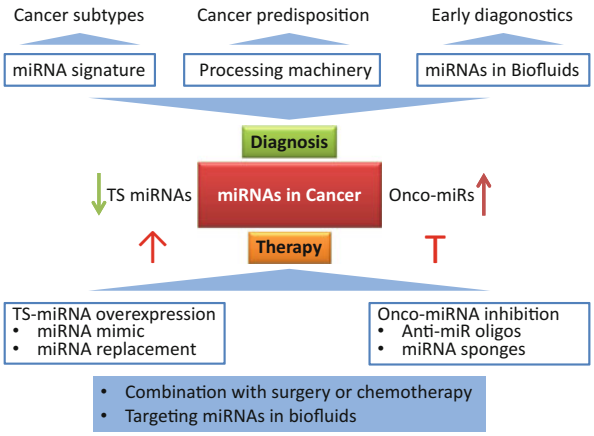

Fig. 6.1 A schematic representation of possible strategies for miRNA-based cancer diagnosis and therapy. Diagnosis *(top)*, profiling miRNA signature in cancer is important to identify cancer subtypes. Studying miRNA processing pathway in cancer tissues may predict cancer predisposition. Identification of miRNA levels in biofluids (such as circulating miRNAs in blood) is useful for early diagnosis. Therapy *(Bottom)*, tumor suppressor miRNAs (TS-miRNA) can be expressed in cancer cells as individual miRNA mimic or several miRNA mimics as replacement for the downregulated miRNAs. Conversely, highly expressed miRNAs can be inhibited by anti-miRNA oligos or sponges. Other approaches include combination of miRNA mimics or inhibition with surgery and/or chemotherapy. Finally, targeting miRNAs in biofluids may provide a novel approach for cancer therapy

or to facilitate their delivery. Combination of miRNAs with surgery or chemotherapeutic approaches for ovarian cancer treatments have not been initiated in clinical trials. Targeting the circulating miRNAs in human patients may provide an additional avenue for miRNA-based ovarian cancer treatment. Thus, animal studies should be instituted to understand the impact of targeting circulating miRNAs on tumorigenesis. These perspectives will improve the understanding of the involvement of miRNAs in cancer biology and provide novel therapeutic approaches, particularly for ovarian cancer.

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Chapter 7
Application of MicroRNA in the Treatment and Diagnosis of Cervical Cancer

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1 Introduction

MicroRNAs (miRNAs) are small non-coding RNA molecules of 20–23 nucleotides that bind to complementary mRNA sequences and regulate gene expression at the transcriptional, post-transcriptional and post-translational levels [1–3]. Since miRNAs have a regulatory role in the human genome, aberrant expression of miRNAs can enhance tumorigenesis and malignant cell transformation [4]. Cervical cancer is a primary cancer of the uterine cervix, that is the second most prevalent female cancer worldwide and the fifth leading cause of cancer-related death among women [5]. It is associated with the infection of high-risk human papillomavirus (HR-HPV), which is detected in more than 90 % of squamous cell carcinoma and adenocarcinoma [6]. The number of cases of cervical cancer in developed countries has declined [7], but diagnostic and treatment methods are still needed. A recent study showed that expression patterns of miRNAs differ among cancers [6] and this may allow application of miRNAs as clinical biomarkers, including in cervical cancer.

2 Mechanism of Action of MicroRNA

The miRNA genes are estimated to account for 2–5 % of all human genes. Many are concentrated at introns of protein-coding genes. Each miRNA regulates the expression of hundreds of target mRNAs, and miRNAs as a whole regulate about 30 % of

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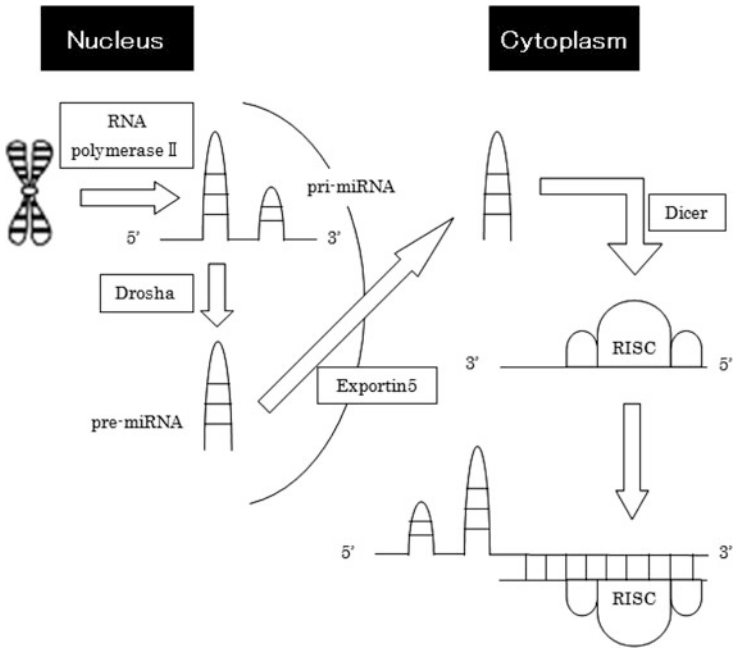

Fig. 7.1 Gene expression mechanism of microRNAs

expression of protein-coding genes [1, 2]. The miRNAs are processed in the nucleus and cytoplasm (Fig. 7.1). First, miRNA is transcribed by RNA polymerase II to a primary miRNA (pri-miRNA) [8]. Subsequently, pri-miRNA is processed to precursor miRNA (pre-miRNA) with a short hairpin loop by Drosha, a RNase III, and is transferred to the cytoplasm by Exportin-5 [9]. The pre-miRNA is transformed in to mature single-stranded miRNA (ss miRNA) by Dicer, a RNase III, and is finally incorporated into the RNA-induced silencing complex (RISC) (Fig. 7.1). The miRNAs have bioactivity when incorporated into the RISC, forming the microRNA-induced silencing complex (miRISC). miRNAs with bioactivity interact with the 3′ untranslated region (3′ UTR) of the target mRNA and decrease mRNA expression [10].

3 Relationship Between MicroRNA and Cancer

The miRNAs are involved in many biological phenomena, including apoptosis, proliferation, differentiation, angiogenesis and immunoreactivity [1]. Many studies have investigated the relationship between miRNAs and carcinogenesis, since Calin et al. first showed that aberrant expression of miR-15a and miR-16-1 was implicated in the pathogenic mechanism of chronic lymphocytic leukemia in 2002 [11]. These studies have shown that miRNAs have tissue-dependent expression and

that miRNA expression can change significantly in cancer tissues in comparison with normal tissues, leading to involvement in carcinogenesis as oncogenic or tumor suppressor miRNAs [12].

3.1 Oncogenic MicroRNA

Oncogenic miRNA is upregulated in tumor cells and acts as an oncogene, whereas tumor suppressor miRNA is downregulated in tumor cells and has the effect of a tumor suppressor. The miR-21 is a typical oncogenic miRNA that is located on chromosome 17 and partially overlaps with the terminal sequence of VMP1, a protein-coding gene. Cell proliferation, migration and infiltration are increased in cancers with overexpressed miR-21 [13], whereas miR-21 silencing induces apoptosis [14]. A clinical trial in 540 patients with breast, colon, lung, pancreatic, prostate or gastric cancer suggested a relationship between carcinogenesis and overexpression of miR-21 alone among miRNAs [15]. Similar miR-21 overexpression occurs in hepatocellular carcinoma, leukemia, and ovarian, cervical, head and neck, and thyroid cancer.

3.2 Tumor Suppressor MicroRNA

The miR-34 is a tumor suppressor miRNA that occurs as miR-34a located on chromosome 1 and miR-34b/c on chromosome 11. The miR-34a induces cell apoptosis and is located in the second exon of the transcription product, EF570049. The miR-34a also has a p53-binding domain and a CpG island in the promoter region. Downregulation of miR-34a induced by CpG island methylation is found in bladder, lung, breast, renal, pancreatic and colon cancer [16]. The miR-34b/c expression is regulated by p53 binding to the promoter region of the transcription product, BC021736. A CpG island is located in the promoter region of BC021736, upstream of the miRNA coding sequence. Therefore, miR-34b/c expression is reduced if this CpG island is hypermethylated. Downregulation of miR-34b/c is a common finding in leukemia, lymphoma and solid tumors, including gastric cancer [16].

4 MicroRNA as a Biomarker

A biomarker refers to a measured or evaluable characteristic that reflects a normal biological or pathogenic process or a response to a chemosensitivity [17]. Current clinical practice for cancer diagnosis uses X-ray, CT, MRI and PET imaging and

tumor markers as biomarkers based on this definition. These biomarkers are easily measured with only a small burden on patients, but many have low specificity.

Application of miRNAs as biomarkers is based on differences in their expression patterns in normal and cancer tissues. The miR-21, an oncogenic miRNA, is overexpressed in many cancers and may be useful as a diagnostic biomarker. The blood concentration of miR-21 in patients with breast, esophageal, gastric and lung cancer differs significantly from that in healthy subjects, and miR-21 has high sensitivity and specificity as a biomarker for cancer diagnosis [18]. However, miR-21 has aberrant expression in many different cancers. Consequently, diagnosis using miR-21 alone is difficult and diagnostic imaging is also required. Diagnosis of a particular cancer in a tissue-specific manner may require a combination of multiple miRNA expression patterns.

The miRNA level may also be a predictor of cancer stage and prognosis. A meta-analysis of miR-21 expression in patients with head and neck squamous cell carcinoma; lung, breast, pancreatic, gastric, esophageal and colon cancer; and leukemia and lymphoma indicated a significant decrease in overall survival in those with overexpression of miR-21 [17]. Other miRNAs are also implicated in the prognosis of cancer patients. Downregulation of miR-34a is positively correlated with malignancy of bladder cancer and tumor size. In patients with lung cancer, downregulation of let-7 is related to decrease in overall survival regardless of the disease stage, and overexpression of miR-155 has similarly been implicated in a decrease in postoperative survival in lung cancer [19]. In esophageal cancer, miR-133a, a tumor suppressor miRNA, is an indicator of stage and prognosis [20].

5 MicroRNA as a Therapeutic Target

The possible involvement of aberrant expression of miRNA in the onset and progression of cancer suggests the possibility of developing new therapy targeting miRNAs. Potential therapies involve targeting and downregulation of oncogenic miRNAs using antisense miRNA oligonucleotides (AMOs) and supplementation of tumor suppressor miRNAs to reinforce their effects [21]. An in vivo study in mice showed that AMOs delivered with lipid molecules for cellular transport induced silencing of miRNAs expressed in many organs, suggesting an antitumor effect [22]. Other applications of miRNA in cancer treatment include increasing the drug-sensitivity of cancer cells by regulating miRNA expression. For example, downregulation of miR-21 enhances the sensitivity of cholangiocarcinoma to gemcitabine [23] and knockdown of miR-203, which is related to cisplatin resistance, induces cisplatin-sensitive apoptosis in MCF-7 breast cancer cells [24]. Thus, various applications of miRNAs are in development for cancer treatment. However, most studies have examined exogenous miRNAs in vitro or in vivo and it is unclear if these miRNAs act similarly to endogenous miRNAs [25]. This may limit the utility of miRNAs in cancer therapy.

6 Cervical Cancer and MicroRNA

Infection of high-risk human papillomavirus (HR-HPV) is the main cause of cervical cancer. HR-HPV includes HPV-16, HPV-18 and HPV-31, which are detected at rates of 99.7 % and 94–100 % in squamous cell carcinoma and adenocarcinoma of the cervix, respectively [26]. The E6 and E7 genes of HPV DNA are implicated in the onset of cervical cancer. E6 downregulates p53, a tumor suppressor gene, which binds to respective gene promoters and act as a transcription factor [27], via an E6AP ubiquitin-mediated degradation pathway. Overexpression of miR-23a, miR-26a and miR-34a is involved in regulation of this activity, and p53-induced silencing of an miRNA cluster including miR-196b/miR-93/miR-25, miR-17-5p/18a/19a/20a/19b-1/92-1 and miR-106a/18b/20b/19b-2/92-2 has been described [28]. E7 induces downregulation of pRB, which acts as a tumor suppressor gene similarly to p53, and E2F is then released from the pRB-E2F complex. Binding sites for E2F are located in many miRNA promoter regions and E2F binding at these regions causes overexpression of miRNAs including miR-17-92, let-7a-d, let-7i, miR-15b/16-2 and miR-106b-25 [29]. The p53 and pRB expression is silenced due to HR-HPV infection and resistance to apoptosis and proangiogenesis are enhanced, resulting in progression of cancer [30].

These findings indicate that aberrant expression of miRNAs is strongly associated with carcinogenesis of cervical cancer because HR-HPV infection influences miRNA expression [3]. An analysis of cervical cancer tissues in 11 patients using Northern blotting and miRNA arrays by Wang et al. [31] and a similar analysis by Li et al. [32] showed overexpression of 12 miRNAs: miR-15b, miR-16, miR-17-5p, miR-20a, miR-20b, miR-21, miR-93, miR-106a, miR-155, miR-182, miR-185 and miR-224; and downregulation of 9 miRNAs: miR-29a, miR-34a, miR-126, miR-127, miR-145, miR-218, miR-424, miR-450 and miR-455 (Table 7.1). The findings of overexpression of miR-20a, miR-20b, miR-93 and miR-224 and downregulation of miR-127, miR-145 and miR-218 are supported by comparison of cervical cancer tissues and adjacent normal tissues [33].

7 Clinical Application of MicroRNAs in Cervical Cancer

7.1 Diagnosis of Cervical Cancer Based on MicroRNAs

Secretion of miRNA from exosomes was discovered in 2007 and is implicated in intercellular communication [34, 35]. Reagents that extract miRNAs from patient plasma and serum are available and such miRNAs can be used in diagnosis. This approach is useful in cervical cancer through application of miRNAs as biomarkers for diagnosis. Aberrant expression patterns of downregulated miR-126 and overexpressed miR-21 are found in plasma of patients with cervical cancer, and detection of these patterns may permit early diagnosis. Some miRNAs involved in

Table 7.1 Expression of microRNAs in cervical cancer

Upregulated	Downregulated
miR-15b	miR-29a
miR-16	miR-34a
miR-17-5p	miR-126
miR-20a	miR-127
miR-20b	miR-145
miR-21	miR-218
miR-93	miR-424
miR-106a	miR-450
miR-155	miR-455
miR-182	
miR-185	
miR-224	

Data are taken from Wang et al. [31], Li et al. [32], and Rao et al. [33]

carcinogenesis are downregulated by epigenetic mutation, including DNA methylation, which can be analyzed by methylation-specific PCR (MSP) and bisulfite restriction analysis (COBRA). For example, miR-124 is an aberrantly methylated miRNA that is silenced in patients with cervical cancer [36].

7.2 Treatment of Cervical Cancer Using MicroRNAs

HPV infection is the major cause of cervical cancer and glucocorticoids may be involved in activating tumor-related viruses, including HPV. Glucocorticoids also enhance cancer cell growth via the glucocorticoid receptor (GR) and play a significant role in malignancy [37]. A glucocorticoid response element (GRE) is present in several upstream regulatory regions of HPV, including HPV16 and HPV18, and GR binding with GRE has effects on the viral cell cycle [38]. The miR-145 is downregulated by cortisol in HPV-positive cervical cancer cells and significant differences in mitomycin resistance have been found in cervical cancer cells [39]. Cell proliferation, infiltration, migration and drug-resistance in cervical cancer progress with miR-145 downregulation due to increased p53 silencing by the E6 gene. Therefore, targeting of miR-145 as a strategy for stopping the E6-p53-miR-145 cycle may be of therapeutic value.

Treatment for functional recovery by supplementation of miRNAs is also under development for cervical cancer and other cancers. The efficacy of this treatment was shown by inhibitory effects on lung metastasis from osteosarcoma in 2007. The miR-143 has no effect on primary lesion growth, but inhibits human osteosarcoma cell infiltration in vitro and lung metastasis in vivo through a mechanism that involves a decrease in matrix metalloprotease 13 (MMP13) upon addition of miR-143 to osteosarcoma cells [40]. The miR-143 is also downregulated in cervical

cancer and supplementation of miR-143 may be an effective therapeutic strategy for cervical cancer.

8 Conclusion

The miRNAs are useful biomarkers for diagnosis of various cancers and as treatment targets in cholangiocarcinoma and breast cancer. In cervical cancer, aberrant expression patterns of miR-126 and miR-21 are used as biomarkers and miR-145 and miR-143 may serve as treatment targets. However, current therapeutic strategies using miRNAs are based on administration of exogenous miRNAs, of which the effects in humans remain unknown. At present, commercialization of miRNAs is ongoing and application of exogenous miRNAs may expand. If endogenous miRNAs are also used, clinical application can be diversified. Thus, large-scale studies in vivo are required to determine the effects of miRNAs on carcinogenesis and progression of cancer.

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Chapter 8
Overcoming Drug Resistance in Colorectal Cancer by MicroRNAs

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1 Introduction

The state of our knowledge on the role of microRNAs (miRNAs) in therapeutic resistance of colon cancer is discussed in the context of cancer stem cells (CSCs) in this chapter.

2 MicroRNAs (miRNAs) Definition and Function

The miRNAs are a class of 18–24 nucleotide long endogenous noncoding RNAs that control gene expression through binding to the seed sequence at the 3′-UTR of target mRNAs, resulting in translational repression or mRNA degradation [1]. A given

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species of miR can perfectly or imperfectly base pair with multiple targets, allowing it to potentially regulate the translation of several mRNAs. It has been predicted that over 30 % of the human protein coding genes are post-transcriptionally regulated by this mechanism [2]. The miRNAs have emerged as critical gene regulators, which modulate a variety of cellular events that among others include growth, differentiation and apoptosis. Several hundred (940, http://www.mirbase.org/) miRNAs have been identified in human cells and the list is growing [3].

Based on miRNA's localization in the genome, miRNAs can be intergenic, intronic, or exonic and can be transcribed as a single miRNA from its own promoter (monocistronic) or several miRNAs as a cluster from a shared promoter (polycistronic). Intergenic miRNAs are found in between genes in distinct transcription units. The miRNAs can be intronic of coding or noncoding genes, where they may be transcribed from the same promoter as the host gene. The exonic miRNAs are rare and are mainly found in exons of coding or noncoding genes [4]. Sevignani et al. have demonstrated that in the mouse, miRNA genes are frequently located near cancer susceptibility loci, which are often subjected to genomic alterations leading to activation by translocations or amplifications, or loss of function due to deletions, insertions, or mutations [5].

The miRNAs are transcribed by RNA polymerase II as long primary transcripts of variable sizes (pri-miRNA), which are processed into ~70 nucleotide long precursor miRNAs (pre-miR) by an RNAse-III-like enzyme, Drosha, together with DGCR8 (DiGeorge syndrome critical region gene 8), an RNA binding protein in the nucleus [6, 7]. Export of pre-miR to cytoplasm is mediated by Exportin-5 via GTP-dependent export, where it is further cleaved by another RNAse-III enzyme, called Dicer, into a mature dsRNA duplex. After strand-selection, mature miRNA is assembled into the RNA-induced silencing complex (RISC), which ultimately performs regulatory functions [8].

The miRNA-mRNA interactions are characterized by binding of the perfect or nearly perfect miRNA seed region (typically 2–8 bases) to the target mRNA. Each miRNA can control hundreds of target mRNAs just as a single mRNA can be targeted by multiple miRNAs. The targets of specific miRNA can be predicted by bioinformatics' algorithms (Targetscan), but validation must be achieved through luciferase reporter assays, quantitative real-time PCR, and immunoblotting studies.

In addition to the canonical mechanisms of miRNA gene regulation through 3′ UTR interactions, other "noncanonical" miRNA-mediated mechanisms of modulation of mRNA expression are emerging [9]. Some miRNAs have been shown to bind to the open reading frame or to the 5′ UTR of the target genes and, in some cases, activate rather than inhibit gene expression. Garzon et al. have recently reported that miRNAs exhibit decoy activity and bind to ribonucleoproteins in a seed sequence in an RISC-independent manner and interfere with their RNA binding functions [9]. Few studies have reported that miRNAs can also regulate gene expression at the transcriptional level by binding directly to the DNA [9]. Overall, these data show the complexity and widespread regulation of gene expression by miRNAs that should be taken into consideration when developing miRNA-based therapies.

It is not surprising that miRNAs are involved in diverse biological processes, including cell differentiation, proliferation, and apoptosis, presumably through a myriad of targets. Deregulation of miRNAs contributes to human pathogenesis [3]. Aberrant expression of miRNAs, including *miR-21, miR-17-92, miR-15, miR-16*, and *let-7*, has been reported in cancer [10]. Furthermore, a substantial number of miRNA genes are located in the fragile sites in the genomic regions that are frequently amplified, deleted, or rearranged in cancer, providing plausible mechanisms of deregulated expression [11]. A miRNA can act as a tumor suppressor or an oncogene depending on its targets in different tissues and cell types [12].

Dysregulated miRNA expression is reported in various human diseases including colorectal cancer, suggesting an important role in its pathogenesis. Various studies have established that a subset of miRNAs plays a role in the initiation and progression of colorectal cancer. The purpose of this review article is to summarize the available data on miRNA profiling in light of developing therapeutic strategies specific for drug resistant colorectal cancer.

3 Cause of Colorectal Cancer: Multi-genetic Mutations

Human colorectal cancer, the third most common cancer in the US, is considered the end result of stepwise accumulation of genetic and epigenetic alterations (mutations) in oncogenes and tumor suppressor gene [13]. The process of tumor progression starting from its initiation to the development of malignant lesions as a result of acquiring a series of mutations over time, has been particularly well studied [14, 15] (Fig. 8.1).

The first, gatekeeping mutations occur in APC gene that makes normal epithelial cells outgrow and develop into a small adenoma. The APC protein acts as a "brake" on the accumulation of β-catenin protein. Without APC, β-catenin accumulates to high levels and translocates into the nucleus, binds to DNA, and activates the transcription of genes that are important for stem cell renewal and differentiation. While APC is mutated in most colon cancers, some studies have shown that mutations in β-catenin block its degradation and result in its increased levels [16]. Mutation(s) in other genes with functions analogous to APC such as AXIN1, AXIN2, TCF7L2, or NKD1 have also been reported to regulate β-catenin levels [17].

The second important mutation identified in colorectal cancer is in *K-Ras gene*, which unleashes an expansion of cell number[14]. K-Ras acts as a molecular on/off switch. Once it is turned on, it recruits and activates proteins necessary for the propagation of growth factors and other receptor signals such as c-Raf and PI3-kinase. Activation of EGFR signaling pathway is also involved at this step. The cells with mutation only in *APC* gene may persist, but their cell numbers are small compared to those that have mutations in both the genes. This process of mutation followed by clonal expansion continues, and mutations in genes such as *PIK3CA*, *SMAD4*, and *TP53*. Mutation in *TP53* gene transforms the tissue from an

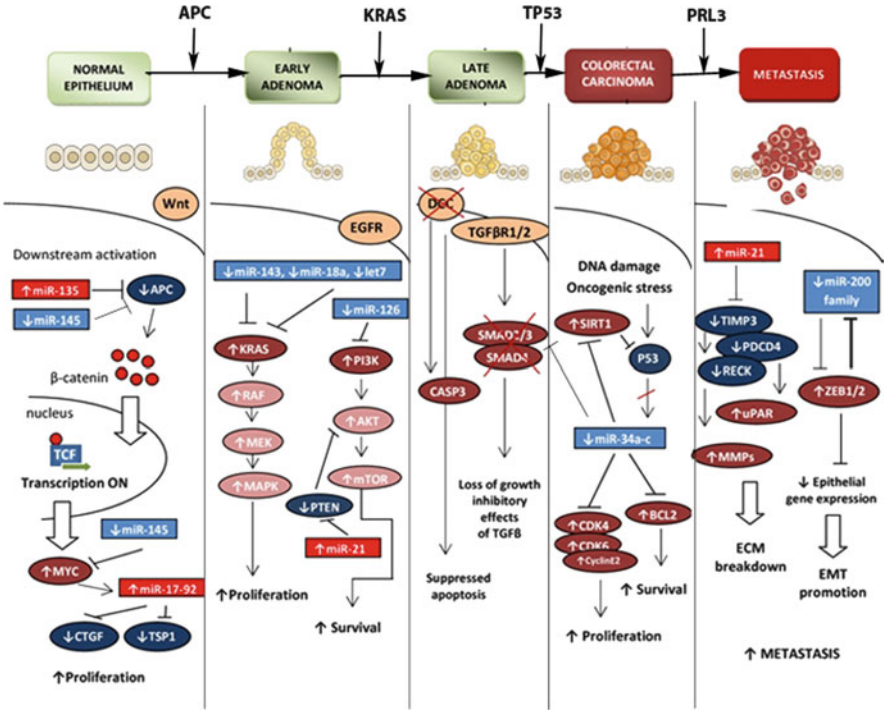

Fig. 8.1 Role of various mutations and miRNAs in colorectal cancer progression and pathogenesis. Colorectal cancer is the end result of stepwise accumulation of genetic and epigenetic alterations (mutations) in oncogenes and tumor suppressor gene affecting various signaling pathways. Selected miRNAs and their target genes are indicated in the scheme (This scheme is adapted from the genetic model for colorectal cancer highlighted by Fearon and Vogelstein [79] and Lao and Grady [80]). Abbreviations: *APC* adenomatous polyposis coli, *CASP3* caspase 3, *CTGF* connective tissue growth factor, *TSP1* thrombospondin1, *EGFR* epidermal growth factor receptor, *P13K* phosphoinositide-3 kinase, *mTOR* mammalian target of rapamycin, *PTEN* phosphatase and tensin homolog, *DCC* deleted in colorectal carcinoma, *TGFβRI/II* transforming growth factor β receptor I and II, *SMAD2,3,4* mothers against decapentaplegic homolog 2,3,4, *SIRT1* sirutin1, *CDK4,6* cyclin dependent kinase 4,6, *TIMP3* tissue inhibitor of metalloproteinase 3, *PDCD4* programmed cell death 4, *RECK* reversion-inducing cysteine-rich protein with kazal motifs, *uPAR* plasminogen activator urokinase receptor, *MMPs* matrix metalloproteases, *ECM* extra cellular matrix, *ZEB1/2* zinc finger E-box binding homeobox 1, *EMT* epithelial to mesenchymal transition, *PRL3* Phosphatase of regenerating liver 3

adenoma into an invasive carcinoma and metastasis to lymph nodes and distant organs [18]. The tumor suppressor p53, produced by the *TP53* gene, normally monitors cell division and selectively kills cells that have Wnt or EGFR pathway defects.

The mutations that confer a selective growth advantage to the tumor cell are called "driver" mutations. It has been estimated that each driver mutation provides only a small selective growth advantage to the cell, to the order of a 0.4 % increase between cell birth and cell death [19]. Over many years, however, this slight

increase, compounded once or twice per week, can result in a large mass, containing billions of cells [18]. The mutations can be inherited or are acquired and most probably occur in the intestinal crypt stem cell. However, only about 4 % colorectal cancer cases show a family history, most of the mutations are acquired during the life span of an individual.

4 Colon Cancer Stem Cells (CSC): Adult Stem Cells with Accumulated Mutations

As mentioned above, colorectal cancer is generally a result of accumulated genetic and epigenetic mutations. Only long-lived cells may serve as reservoirs for such precancerous mutations. Colonic mucosa is a highly dynamic tissue. Mucosal surface epithelium cells are constantly replaced with cells derived from stem cells that are located at the base of the crypt. Although the origin of t colon cancer stem or stem-like cells (CSCs) is not fully known, they are thought to originate from stem cells that have acquired mutations. Considering that the appearance of CSCs might be one of the initial events in neoplastic transformation in solid tumors as well as in intestinal neoplasia, we investigated the status of CSCs in normal appearing colonic mucosa during aging in patients with adenomatous polyps. Colon CSCs, as evidenced by the expression of CSC markers (CD44, CD166 and Ep-CAM) were observed not only in premalignant adenomatous polyps, but also in normal appearing colonic mucosa, where expression increased with advancing age indicating increased risk of developing colorectal cancer during aging [20]. Additionally, we found the age-related increase in adenomatous polyps in the colon was associated with increased expression of colon CSC markers [38].

Over the last decade, the cancer stem cell model has become increasingly accepted as an explanation for cancer development, spread and recurrence. This model posits that a small subpopulation of tumor cells, termed cancer stem cells (CSCs), which are distinct from the bulk of the cells in the tumor, can self-renew, differentiate into multiple lineages, and drive tumor growth, metastasis and recurrence. Currently, most CSCs are identified by the use of a variety of cell surface markers, including CD44, CD166 and Ep-CAM. They can be isolated using FACS technology, and then tested by propagation in immune deficient mice [21].

5 Cancer Stem Cells (CSCs) and Drug Resistance

Although surgery and subsequent chemotherapy can cure over 75 % of colon cancer patients, more than 30 % of these patients develop new neoplastic polyps, and 10 % progress to frank second malignancy, underscoring the need for a better understanding of the underlying mechanisms of recurrence [22, 23].

The standard therapy for colorectal cancer includes surgery followed by chemotherapy or other effective therapeutic regimen to eliminate any cancer cells. However, most chemotherapy drugs only targets the rapidly dividing cells that form bulk of the tumor. The effectiveness of cancer therapeutics is evaluated by the reduction in tumor mass, resulting in elimination/killing of dividing differentiated or undifferentiated cells that form the bulk of the tumor. However, the slow growing CSCs/CSLCs remains untouched and may even be enriched resulting in relapse of the disease. Recently, we have shown that exposure of colon cancer HCT-116 or HT-29 cells to the combination of 5-FluoroUracil (FU) and Oxaliplatin (OX) [FUOX] inhibited their growth but led to the enrichment of CSC phenotype [24]. We have now generated FUOX-resistant HCT116 and HT29 cells that exhibit both enrichment of CSCs/CSLCs and elevated levels miR-21. We have further demonstrated that, miR-21 plays a determinant role in inducing stemness in colon cancer cells [25, 26].

CSCs/CSLCs show resistance to a number of conventional chemotherapies [27]. These include therapies targeting drug-efflux capabilities, anti-apoptotic mechanisms, and induction of differentiation as well as to other stem cell pathways resulting in chemotherapy-refractory tumors. Changes in these properties may explain why it is difficult to completely eradicate cancer and why recurrence is an ever-present threat. Thus, therapeutic strategies that specifically target colon CSCs are likely to be effective in eradicating tumors and in reducing the risk of relapse and metastasis.

6 Micro-RNAs Regulate Cancer Stem Cell Proliferation and Differentiation

As previously stated, miRNAs bind to target mRNA resulting in either its degradation or inhibition of translation. The miRNAs that normally down-regulate the expression of an oncogene and is often lost in tumor cells can be defined as a tumor suppressor. The lost expression of this miRNA by mutation, deletion, promoter methylation or by any other factor(s) that might result in an abnormal expression of the target oncogene, which subsequently contributes to tumor formation by inducing cell proliferation, invasion, angiogenesis or/and decreased cell death.

Alternatively, miRNAs that down-regulate tumor suppressor gene expression or other important genes are involved in differentiation, and could contribute to tumor formation by stimulating proliferation, angiogenesis and invasion. These miRNAs are defined as oncogenic miRNA or “oncomiR”, and are normally up-regulated in distinct types of human neoplasia and also often associated with distinct cytogenetic abnormalities. The miRNAs that function as tumor suppressors or oncogenes in colorectal cancer are listed in Table 8.1.

Two of the most notorious miRNAs, an oncomiR miR-21 and tumor suppressor miR-145 are discussed in detail in the following section, since these miRNAs play a critical role in recurring colorectal cancer by regulating stem cell growth and differentiation.

Table 8.1 Expression of various miRNAs in colorectal carcinoma tissues and their targets

microRNA	Genomi location	Expression in CRC tissues	Targets	Expression after chemotherapy	Function
miR-9-1	1q22	Down	REST a-Catenin	Up [62]	Tumor suppressor
miR-10b	2q31.1	Up	BIM	Up [63]	Oncogene
miR-139	11q13.4	Down [64]	IGF-IR [65]		Tumor suppressor
miR-143	5q32	Down	ERK5 [66]		Tumor suppressor
miR-145		Down	SOX2, APC		
miR-195	17p13.1	Down	Bcl-2 [67]		Tumor suppressor
miR-135a	3p21.1	Up	APC [68]		Oncogene
miR-135b	1q32.1	Up	APC	Up [69]	Oncogene
miR-21	17q23.1	Up	PDCD4 TGFBRII	Up [70]	Oncogene
miR-17	13q31.3	Up	Bim [71]		Oncogene
miR-18a	13q31.3	Up	hnRNP A1		
miR-19b	13q31.3	Up	MYBL2	Up [70]	
miR-92a	13q31.3	Up	BIM [72]	Down [62]	
miR-182	7q32.2	Up	TSP-1 [73]	Up	Oncogene
miR-96	7q32.	Up [43]	K-RAS	Up [70]	
miR-31	9p21.3	Up [74]	Fzd3, RhoA [75]	Up [70]	Oncogene
miR-34a	1p36.22	Down [76]	SIRT1 [77]	Up [62]	Tumor suppressor
miR-34b	11q23.1	Down [76]	Smad3 [78]		Tumor suppressor
miR-200a	1p36.33	Up	MSH2		
miR-200b	1p36.33	Up	MLH1	Up [62]	?
miR-200c	12p13.31	Up	MLH1, MAD2		?
miR-155	21q21.3	Up			Oncogene
miR-205	1q32.2	Up [43]	MAD4, PTEN		Oncogene
miR-210	11p15.5	Up			Oncogene

6.1 miR-21

The miRNA-21 is encoded by the *MIR21* gene [28]. The human microRNA-21 gene is located on chromosome 17q23.2 within a coding gene TMEM49 (also called vacuole membrane protein). The stem–loop precursor of miR-21(pre-miR-21) resides between nucleotides 2445 and 2516 of pri-miR-21. Despite being located in intronic regions of a coding gene in the direction of transcription, it has its own promoter regions and forms a ~3433-nt long primary transcript of miR-21 (pri-miR-21), which is independently transcribed. The pri-miR-21 promoter has been partially characterized [29]. Fujita et al. described a promoter mapping −3,770 to −3,337 upstream to the miR-21 hairpin. This has several conserved enhancer elements including binding sites for activation protein 1 (AP-1; composed of Fos and Jun family proteins), Ets/PU.1, SRF, p53 and STAT3. Talotta et al. [30] have reported that the miR-21 is induced by AP-1 in response to Ras, and the tumor suppressors

PTEN and PDCD4 are down-regulated by Ras in an AP-1- and miR-21-dependent fashion. They have demonstrated that PDCD4 is a negative regulator of AP-1. The miR-21-mediated down-regulation of PDCD4 is essential for the maximal induction of AP-1 activity in response to Ras. The data reveal a mechanism of positive auto-regulation of the AP-1 complex in Ras transformation and disclose the function of oncomiRs as critical targets and regulators of AP-1 in tumorigenesis [31].

In addition to the positive regulators of miR-21 transcription, several transcriptional suppressors have been reported. For example, miR-21 transcription was found to be repressed by NFI, C/EBPα [32]. In addition, Gfi1[33] and estrogen receptor[34] were also shown to negatively regulate miR-21 promoter activity.

The miR-21 expression is regulated at multiple levels, including transcription and post-transcriptional processing. Kern and colleagues showed that EGF/Ras efficiently induced the miR-21 primary transcript, but this does not rapidly and simply translate into higher mature miR-21 levels. Rather, induction of mature miR-21 by constitutive activation of this pathway is slow, is associated with only minimal activation of mitogen-activated protein kinase (MAPK), and may involve stimulation of post-transcriptional processing by mechanisms other than Dicer stabilization. Further, they identified Ets transcription factors as modifiers of miR-21 expression in colorectal cancer [35]. The effects of Ets factors on miR-21 expression involve both direct and indirect mechanisms and are cell context-dependent. The Ets factor Pea3 emerges from these studies as a consistent repressor of miR-21 transcription [35].

A number of targets for microRNA-21 have been experimentally validated and most of them are tumor suppressors. Notable targets include PTEN, PDCD4, Tropomyosin, Sprouty 1, Sprouty 2, RECK, TGFβRII, MEF2C, ANP32A, SMARCA4, RhoB, and hMSH2 [36, 37].

The miR-21 has been found to be overexpressed in most epithelial cancers such as lung, breast, stomach, prostate, colon, brain, head and neck, esophagus and pancreatic cancers. Data from own laboratory have demonstrated that miR-21 levels are markedly elevated in FUOX-resistant colon cancer cells [26] that are highly enriched in CSLCs/CSCs and exhibit increased drug-efflux property [38]. Studies show that knockdown of miR-21 impairs growth, induces apoptosis and reduces the migration and invasion of various cancer cells including those of colon cancer [32]. Induced expression of miR-21 leads to increased β-catenin activity, augmentation of c-Myc and Cyclin-D expression, increase in the number of cancer stem cells, and is accompanied by increased colonosphere forming ability *in vitro* and tumor formation in SCID mice [26]. Therefore, miR-21 is believed to play a pivotal role in the progression of many malignancies and has been called an “oncomiR”.

6.2 miR-145

The miR-145 is located on chromosome 5 (5q32-33) within a 4.09 kb region, and is co-transcribed with miR-143 by RNA polymerase II into pri-miRNA, which is

processed to ~88 bp long pre-miRNA involving RNA splicing and exporting, and finally to mature miR-145.

The miR-145 is a p53-regulated gene. Several reports suggest that miR-145 can be induced transcriptionally by p53 in response to stress such as serum starvation or anticancer drugs [39, 40]. The tumor suppressor, p53, also enhances the post-transcriptional maturation of miR-143 and miR-145 in response to DNA damage by interacting with the Drosha processing complex [41]. A recent study has demonstrated that activated Ras can suppress miR-143/145 cluster through Ras-responsive element-binding protein (RREB1), which represses the miR-143/145 promoter [42].

Down-regulation of miR-145 has been found in multiple tumors including colon, breast, prostate, pancreas etc. [39, 43]. In fact, miR-145 has been well documented as a tumor suppressor gene because it negatively regulates multiple oncogenes such as Myc, K-Ras, IRS-1, ERK5[39, 44]. Moreover, miR-145 negatively regulates junctional cell adhesion molecule (JAM-A), fascin and MUC1 and suppresses breast cancer cell motility and invasiveness [45, 46]. miR-145 also inhibits colon cancer cells' proliferation and sensitizes them to 5-fluorouracil by targeting oncogenic FLI1 [47]. We have recently observed FUOX-resistant colon cancer HCT-116 cells which have been stably transfected with miR-145 loses their ability to form colonospheres *in vitro* or tumor in SCID mice (unpublished observations). In FUOX-resistant cells, over-expression of miR-145 causes a marked increase in CK-20, indicating induction of differentiation (unpublished observation). Whether the latter renders them more susceptible to chemotherapy or other types of therapeutics remains to be determined.

Several other targets of miR-145 that participate in stem cell growth and dedifferentiation have also been identified. Xu et al. have shown that miR-145 is induced during differentiation, and it directly silences the stem cell self-renewal and pluripotency by suppressing multiple pluripotent genes such as OCT4, SOX2 and KLF4 [48]. Moreover, clinical studies show that down-regulation of miR-145 is frequently associated with cancers and in smaller adenomas, suggesting a negative role for miR-145 in the initiation of tumor development by regulating cancer stem cell proliferation and differentiation [49]. Kamatani et al. recently reported that the decreased expression of miR-143 and -145 frequently occurred before APC gene aberrations [50]. The down-regulation of miR-143 and -145 is thus an important genetic event for the initiation step in colorectal tumor development.

7 miRNA and Chemotherapy Resistance in Colorectal Cancer

In cancer, as a result of multiple genetic and epigenetic alteration events, multiple genes, protein and their network are abnormally expressed. The current chemotherapy regimen such as 5-FU based FOLFOX (5-FU plus Oxaliplatin and Folinic acid), the backbone of colorectal cancer chemotherapeutics, interferes with and halts the growth of rapidly dividing cancer cells, but with limited success. While

differentiated or differentiating cells that form the bulk of the tumor, are sensitive to chemo or other therapeutic agent(s), CSCs/CSLCs are resistant to conventional chemotherapy. Therefore, targeting CSC directly and/or inducing their differentiation rendering them susceptible to therapy could be a novel strategy to overcome drug resistance in colorectal cancer. Indeed, we have demonstrated that induction of differentiation of FUOX-resistant colon CSLCs/CSCs by Schlafen-3, a gene that induces differentiation, renders them highly susceptible to the combination of 5-FU and Oxaliplatin [38, 51].

Dysregulation of miRNA profiles has been reported in cancer cells and tissues. As mentioned above, a single miRNA regulates many different signaling pathways and orchestrates integrated responses in normal cells and tissues. It is reasonable to think that miRNAs play key roles in coordinating cancerous networks and cause their diversity and complexity. Developing therapeutic strategies to restore homeostasis by modifying miRNA expression may prove to be more comprehensive and successful than targeting individual genes or proteins, since only a few specific deregulated miRNAs modulate large target gene expression and multiple signaling pathways in cancer cells. For example, expression of miR-21 is higher in colon adenocarcinomas than normal mucosa and is associated with decreased overall survival [52]. A number of genes, including *PTEN, TPM1, PDCD4, Spry1*, and *Spry2*, have been reported to be targets of *miR-21*, suggesting its potential function in regulating cell proliferation, apoptosis, and invasion [36, 37]. Furthermore, over-expression of miR-21 has been shown to dramatically reduce the therapeutic efficacy of 5-FU [53]. In addition, increased Ras signaling activity by miR-21 mediated by Ras-responsive element-binding protein (RREB1) represses expression of the miR-143/145 cluster [42, 54], which induces differentiation of CSCs. Thus, targeting miRNAs may be a worthwhile therapeutic strategy for CSCs enriched recurrent cancer.

8 miRNA-Based Therapeutic Strategies

There are two main strategies to target miRNA expression in cancer: (a) block the expression of an oncogenic miRNA or (b) restore the expression of a tumour suppressor miRNA. The methods include (a) direct use of oligonucleotides or virus-based constructs to either block the expression of an oncogenic miRNA or to substitute for the loss of expression of a tumour suppressor miRNA, (b) indirect use of the drugs to modulate miRNA expression by targeting their transcription and their processing.

8.1 Blocking Oncogenic miRNAs

This can be achieved by the use of antisense oligonucleotides, miRNA sponges, and small RNA inhibitors. Antisense oligonucleotides work as competitive inhibitors of

miRNAs, presumably by annealing to the mature miRNA guide strand and inducing degradation or stoichiometric duplex formation. We have reported that transfection of FUOX-resistant colon cancer cells with anti-sense miR-21 induces differentiation, as evidenced by the stimulation of alkaline phosphates activity and increased expression of CK-20 [55]. Additionally, we reported that these differentiating/differentiated cells become highly susceptible to difluorinated curcumin (CDF), a synthetic analog of curcumin, which we have shown to induce apoptosis of FUOX-resistant cells [55, 56].

Chemically modified antisense oligonucleotides such as locked nucleic acid (LNA), 2′–O-methyl and phosphorothioate show increased stability, binding affinity and specificity [9]. Krutzfeldt et al. reported the synthesis of 2′–O-methyl-modified cholesterol-conjugated single-stranded RNA analogues, with phosphorothiolate (phosphor-orthothiolate) linkages, named "antagomirs" complementary to miR-122 and miR-16. When these antagomirs were injected into mice, silencing of endogenous miR-122 and miR-16 was observed; the antagomirs were stable and the effects were still observed 23 days after injections [57]. We have observed that the growth of xenograft of FUOX-resistant colon cancer cells in SCID mice could be greatly inhibited by administration of antagomir-21 (unpublished observation). Residual colon tumors from antagomir-21-treated SCID mice showed suppression of colon CSC marker CD44, indicating decrease in CSCs in the tumor (unpublished observation).

Locked nucleic acids (LNA) are a class of high-affinity RNA analogs, in which the ribose ring is "locked" by a methylene bridge between the 2′–O and 4′–C atoms. As a result, LNA oligonucleotides display unprecedented thermal stability when hybridized to a complementary RNA or DNA strand. In addition, they display excellent mismatch discrimination and high aqueous solubility. Elmen and colleagues reported that combining LNA anti-miR with phosphorothiolate modifications markedly improved delivery of the compounds and silenced target miR-122 more efficiently compared with the antagomirs [58]. Ebert and colleagues reported another miRNA inhibitor: miRNA sponges [59]. These competitive inhibitors are transcripts expressed from strong promoters, containing multiple, tandem binding sites to a miRNA of interest. When vectors encoding these sponges are transfected into cultured cells, sponges de-repress miRNA targets at least as strongly as chemically modified antisense oligonucleotides. They specifically inhibit miRNAs with a complementary heptameric seed, such that a single sponge can be used to block an entire miRNA seed family.

8.2 Restoring Expression of Tumour-Suppressor miRNAs in Cancer

The loss or down-regulation of a tumor-suppressor miRNAs could be overcome by introducing synthetic oligonucleotides, known as miRNA mimics or miRNA

precursor (pre-miRNA). The miRNA mimics are usually small, double stranded and may be chemically modified (2′–O-methyl with phosphorothioate modifications). However, Ibrahim and colleagues reported that when unmodified miR-145 and miR-33a were delivered into mouse xenograft tumors, it caused profound antitumor effects [44]. As stated above, we also noted a similar phenomenon. miR-145 delivery reduced tumor proliferation and increased apoptosis, with concomitant repression of c-Myc and ERK5 as novel regulatory target of miR-145 [40]. Similarly, antitumor effects of miR-33a were validated in this model. miRNA with chemical modifications have been demonstrated to enhance stability and delivery to tissues. However, care has to be taken to avoid off-target effects due to extensive modifications.

As for the *in vivo* delivery strategies, a number of approaches have been attempted such as the use of adeno-associated virus (AAV) vectors. Kota and colleagues first reported the use of adenovirus-associated (AAV) vectors to deliver miR-26a in a mouse model of hepatocellular carcinoma [60]. The authors cloned mir-26 into an AAV vector and viral particles were tested in an established Myc-dependent liver cancer mouse model. Intravenous injection of viral miRNA26a resulted in the suppression of tumorigenicity by repressing cell growth and by inducing tumour apoptosis without signs of toxicity.

Compared with a number of approaches for the *in vivo* delivery, the advantage in AAV vector is its small size and self-complementary genome, which enhances therapeutic gene expression. In addition, they are eliminated efficiently with minimal toxicity, as shown in Phase I and Phase II clinical trials[61]. Another advantage of AAV vectors is the efficient transduction of target cells. The development of self-complementary genome and non-human primate AAV serotype allows more than 90 % transduction efficiency of hepatocytes and long-term gene expression without toxicity, following a single systemic administration of recombinant virus [61].

Many factors contribute to colorectal cancer specific chemo-resistance mechanism. The miRNAs and mRNA regulated network have been shown to play major roles in cancer stem cell acquired ability of self-renewal and resistance to chemotherapeutics agents. We are now in the middle of a critical and exciting time in miRNA-CSC based cancer research and anticancer therapeutic development. The miRNA-based therapy targeting cancer stem cells shows the potential to overcome drug resistance and be highly beneficial to patients.

9 Conclusion

Many factors contribute to colorectal cancer specific chemo-resistance mechanisms. Based on the available data it can be concluded that miRNAs and miRNA regulated networks play major roles in cancer stem cells' acquired ability of self-renewal and resistance to chemotherapeutic agents. oncomiRNA21 and tumor suppressor miR145 have emerged as critical gene regulators, which modulate

multiple signaling pathways. Up-regulation of miRNA21, down-regulation of miR145 and their positive feedback in chemo-resistant colon cancer cells suggest that they are important players in the process of chemo-resistance. Targeted therapies that can modulate these and other miRNAs involved in regulating chemoresistance in colorectal cancer will be highly beneficial in overcoming drug-resistance in patients with recurrent colorectal cancer.

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Chapter 9
MicroRNAs as Novel Targets in Liver Cancer: Facing the Clinical Challenge

Jens U. Marquardt and Peter R. Galle

1 Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related mortality accounting for more than 600,000 yearly deaths worldwide [1]. The major etiologic factors underlying chronic liver disease, cirrhosis and, ultimately HCC are well characterized. Among those, chronic viral hepatitis (e.g., hepatitis B (HBV) and C viruses (HCV)) as well as aflatoxin B exposure and ethanol abuse are the most common causes of hepatocarcinogenesis worldwide [2]. Other predisposing factors include nonalcoholic fatty liver disease (NAFLD) and metabolic as well as hereditary disorders. Highest incidences are traditionally seen in third-world regions such as Southeast Asia and sub-Sahara Africa. However, over the last decades generalized vaccination programs for HBV and improved preservation of food led to a reduction in two major etiological factors (i.e. HBV and aflatoxin B) resulting in stabilization of prevalence and declining incidences in these regions. However, due to a steady increase in HCV infections as well as obesity the incidence and, concomitantly, also mortality rates of HCC have almost doubled in the United States and Europe over the past four decades and are predicted to continue rising. Although several confounding factors (e.g. immigration from high incidence countries) contribute to these high numbers in the western world, HCC ranks among the fastest growing causes of cancer related deaths in the USA. Curative therapeutic options include resection or ablation of small HCCs and/or liver transplantation. However, these approaches are frequently limited by the underlying liver disease and at the time of diagnosis less than 20 % of the patients are eligible for curative treatment strategies [3]. These observations clearly indicate that liver cancer has become a major health problem in western countries and underline the importance for a better understanding of the pathophysiology to

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improve the outcome of this deadly disease. Major progress in unraveling the molecular mechanisms in liver diseases associated with increased risk of HCC as well as several cellular alterations that precede HCC have been made over the last 10 years [4, 5]. A major focus of translational research is the inter-relationship of abnormal genomics, epigenomics, proteomics and downstream alterations in molecular signaling pathways. The overall aim of these efforts is to integrate the generated data with clinicopathological features of HCC in order to uncover new diagnostic classes, improve treatment options, and implement effective prevention strategies [6].

Integrity of the epigenome is a key component of organ homeostasis. Growing evidence suggests that disruption of epigenetic regulation is one of the fundamental mechanisms underlying many human diseases including cancer [7, 8]. This epigenetic landscape of alterations adds further complexity to the pathogenesis of solid tumors. Changes in the epigenome are believed to be early events in carcinogenesis preceding allelic imbalances and ultimately lead to cancer progression [9]. Not surprisingly, epigenetic alterations, in particular aberrant expression of microRNAs with subsequent dysregulation of gene expression have been linked to the pathogenesis of chronic liver diseases as well as hepatocarcinogenesis [10]. In this context, the identification of these small regulatory RNAs have greatly advanced our understanding of liver cancer development and aberrant expression of microRNAs is significantly associated with liver cancer initiation, propagation and progression [11]. Emerging evidence further indicates that certain microRNAs directly contribute to cell proliferation, apoptosis, and metastasis of HCC and correlate with several clinicopathological features [12]. Hereby, number of microRNAs have been identified to be involved in the regulation of key protein-coding genes associated with hepatocarcinogenesis such as WNT/β-Catenin, MYC and TGFβ [13, 14]. Therefore, the investigation of abnormal microRNA patterns is a rapidly emerging field of translational science in liver cancer and has great potential to transform current approaches for both diagnosis and therapy, thereby providing the foundation for predictive and preventive personalized medicine (Fig. 9.1).

In this chapter we will review current knowledge of microRNA regulation in the context of liver development, diseases and hepatocarcinogenesis as well their implications for clinical and translational efforts. We will further highlight challenges and limitations for the application of microRNA-based treatment strategies in HCC and underline the implications of next-generation technologies for improving our understanding of the role of this interesting class of molecules in hepatocarcinogenesis [15].

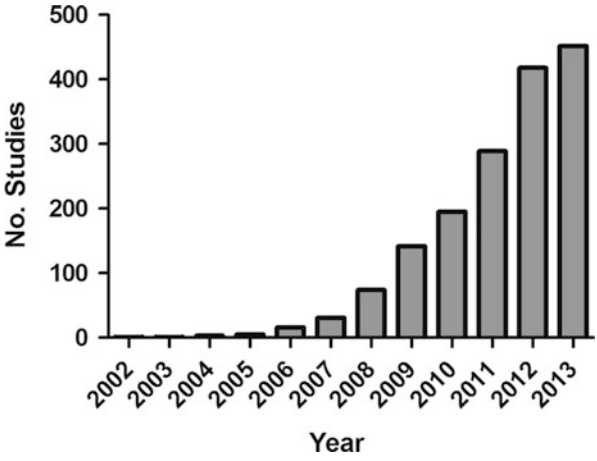

Fig. 9.1 Studies of microRNAs in liver diseases. Timeline for publications of miRNAs in liver diseases was generated using a PubMed query with the keywords "microRNA and liver". From the 1,627 studies included in the query 832 studies (51 %) focused on liver cancer

2 MicroRNAs in Liver Development, Regeneration and Disease

MicroRNAs play a diverse role for liver biology and dysregulation of microRNAs has been implicated in virtually all pathophysiological conditions in the liver [16]. During liver development microRNAs modulate a variety of physiological processes thereby significantly contributing to proper organ homeostasis. Mechanistic analyses of mice with deletion of Dicer1 showed increase hepatocyte turnover at 3 weeks after birth resulting in increased steatosis as well as depletion of glycogen storage underlining the key role of mature microRNAs for liver development. Notably, over time the phenotype was gradually rescued by expansion of Dicer1 competent hepatocytes. Regardless, two thirds of the Dicer1-deficient animals developed hepatocellular carcinomas originating from residual Dicer1 deficient hepatocytes [17]. Interestingly, one of the most striking findings of this study was the almost complete absence of miR-122 in Dicer1-deficient animals. miR-122 is a highly abundant liver specific microRNA accounting for ~70 % of all expressed microRNAs in the liver whilst undetectable in the majority of other organs [18]. Furthermore, miR-122 drives hepatocyte differentiation in a HNF6 dependent manner [19]. The prominent role of miR-122 for hepatocyte metabolism was confirmed in a study by Esau et al. using in vivo antisense knockdown. The inhibition in normal mice resulted in disruption of plasma cholesterol, increased hepatic fatty-acid oxidation, and decreased hepatic fatty-acid and cholesterol synthesis [20]. Conversely, miR-122 inhibition in a diet-induced obesity model resulted in significant attenuation of liver steatosis, as well as a downregulation of several genes involved in lipid homeostasis. A global analyses of 42 microRNAs between human fetal and adult liver tissue identified a number of differentially expressed microRNAs including miR-122, miR-148a, miR-192, miR-194, miR-451, miR-21 and let-7a with a progressive downregulation from fetal to adult livers

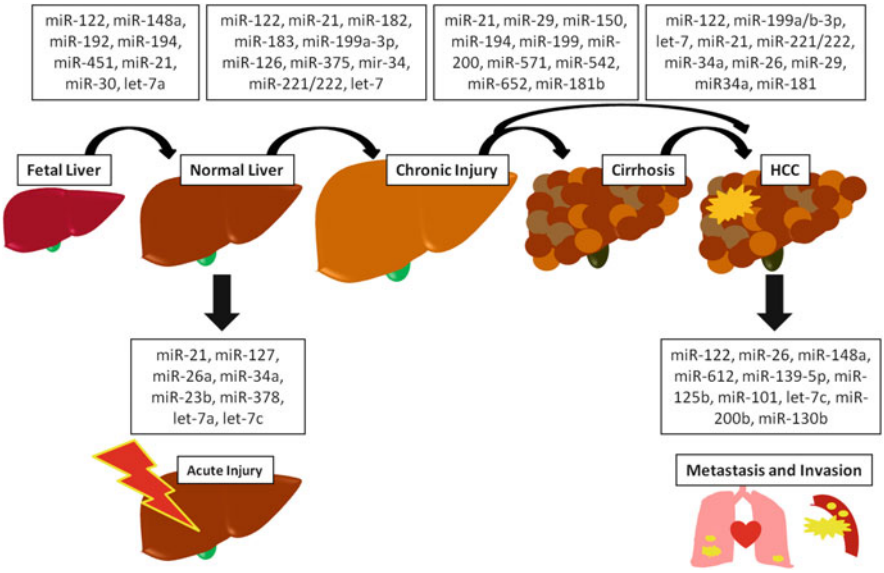

Fig. 9.2 MicroRNAs in liver development and diseases. The figure shows (up to 10) key microRNAs associated with fetal liver development, liver diseases and progression of liver cancer. A striking redundancy of several microRNAs (e.g. miR-122, miR-21, miR-26) is obvious indicating the potential of these microRNAs as targets for therapeutic and/or preventive strategies

(Fig. 9.2) [21]. Little is known about microRNAs that specifically regulate biliary differentiation. A recent study showed that two microRNAs (miR-30a and miR-30c) are critically involved in the development of ductal plate and bile ducts [22]. Consistently, knockdown of miR-30a in zebrafish resulted in defective biliary morphogenesis.

The role of microRNAs in response to acute liver injury has been addressed in several studies. Hereby, deregulation of several microRNAs (e.g. miR-21, miR-127, miR-26a, miR-34a, miR-23b, let-7a and let-7c) could be associated with proper initiation and termination of proliferative stimuli following a 2/3 partial hepatectomy (PH) in mice [23–26]. Song et al. performed a global analysis of changes in microRNA profiles in response to PH. To specifically address the role of microRNAs for hepatocyte proliferation they utilized a mouse model with hepatocyte-specific inactivation of DiGeorge syndrome critical region gene 8 (DGCR8), an essential component of the microRNA processing pathway [27]. In this model, hepatocytes were microRNA-deficient which caused a significant delay in cell cycle progression particularly in the G(1) to S phase transition. Among the most prominent differentially expressed microRNAs were mir-21 and miR-378. They further showed that miR-21 suppressed DNA synthesis via activation of FoxM1 by directly targeting Btg2, a strong inhibitor of the cell cycle. In continuation of the study, the same group more closely dissected the role of miR-21 in liver injury by using an in vivo antisense oligonucleotide in the PH model [28]. As a result of this

investigations, the authors established a regulatory feedback between mir-21 and cyclin D1 translation that caused the observed delay in G(1) to S phase transition by activating the Akt/mTOR complex (and thus eIF-4 F-mediated translation initiation) in the early phase of liver regeneration.

The majority of HCCs develop on the basis of a chronic inflammation caused by an underlying liver disease and, in more than 80 % of the cases, a pre-existing liver cirrhosis. To gain a complete understanding of the molecular mechanisms of hepatocarcinogenesis the etiology of the liver disease needs to be considered [29]. The major etiologies associated with the evolution of liver cancer are well characterized (e.g., infections with hepatitis B (HBV) and C viruses (HCV) as well as ethanol abuse and aflatoxin B exposure). Other common etiological factors include nonalcoholic fatty liver disease (NAFLD) and metabolic disorders that have become particularly relevant in Western countries due to a sharp increase in prevalence and increasing numbers of HCCs [14].

Already in 2008 a global microRNA analysis showed that HCCs associated with alcoholic liver disease (ALD) display a decrease in miR-126 as well as miR-107 and miR-375 which were specifically associated with HNF1α and β-catenin gene mutations in these patients [30]. Another study performed microRNA expression profiles in two murine models of ALD and NAFLD. In both models the development of steatohepatitis was associated with altered microRNA expression patterns (e.g. miR-705, miR-1224, miR-182, miR-183, miR-199a-3p) [31]. Notably, several of these microRNAs showed a differential regulation between ALD and NAFLD indicating that etiological differences might contribute to activation of different microRNAs. Another common microRNA associated with steatohepatitis in both ALD and NALFD is mir-34. Recently, Min et al. revealed mechanistic insights into the role of miR-34a in NAFLD. They demonstrated that mir-34 down-regulates sirtuin-1 and causes dephosphorylation of AMP kinase and HMGCR, a key regulator of cholesterol synthesis (Fig. 9.1).

Over the last years several studies demonstrated that microRNAs significantly contribute to the regulation of HCV and HBV infections [32]. Several studies addressed the role of microRNAs for HCV infections [10]. HCV infects hepatocytes and replication of the virus is exerted by a unique interaction between host miR-122 and HCV 5′UTR along with proteins of RISC (Ago2, GW182 and HSPs), which results in enhanced replication of HCV virus and mediates stability of the viral RNA [16, 33]. HCV infection leads to hepatocyte damage and subsequent release of pro-inflammatory molecules, which in turn activate immune cells that induce systemic inflammation, fibrogenesis and, ultimately, carcinogenesis. Marquez et al. investigated the role of miR-21 and miR-122 for HCV infection in pre-cirrhotic human patients and in cell culture [34]. Both microRNAs showed deregulated expression in HCV-infected liver tissues when compared to control livers without viral infections. Further, miR-21 induction was positively correlated with fibrotic stage, viral load and serum transaminases levels. Conversely, miR-122 expression inversely correlated with fibrosis and biochemical markers of hepatocyte damage. Besides miR-122 and mir-21 other host microRNAs such as miR-34a, miR-21, miR-146a and miR-125b are increased in patients infected with HCV

infection and might contribute to progression of the disease [16]. Additionally, miR-155 and miR-499 in hepatocytes, are associated with the progression from inflammation to cancer via Notch and Wnt signaling [35]. However, due to the prominent role of miR-122, this microRNA is believed to have the greatest potential as a (complementary) target for antiviral therapies and preliminary results from ongoing clinical trials are very promising [36]. miR-122 was also found to be involved in the pathogenesis of HBV infections. A significant down-regulation of mir-122 was observed in chronic HBV infected patients, inversely correlating with viral load. Hereby, miR-122 stimulated HBV replication by up-regulation of cyclin G1 thereby preventing p53-mediated repression of a HBV enhancer element [37]. Several other microRNAs associated with HBV infection promote inflammation induced hepatocarcinogenesis (Fig. 9.2) [32]. Ura et al. further demonstrated that microRNAs associated pathways related to cell death, DNA damage, recombination and signal transduction were activated in HBV-infected livers [38]. However, despite considerable commonalities in the clinical and molecular phenotype (e.g. upregulation of miR-21 and miR-221/222 as well as down-regulation of miR-122, miR-199 and miR-200) there are fundamental differences in the regulation of microRNAs between HBV and HCV during the progression from inflammation over fibrosis to HCC development (e.g. miR-145, miR-9-2, miR-138-1 and miR-2, miR-320, miR-33, miR-10a and 10b, miR-146, miR-220, let-7) [38, 39]. In the context of hepatocarcinogenesis, the let-7 family should be particularly highlighted, since the observed differences might contribute to specific HB-x related oncogenic effects in liver cancer.

Up to now, there are no studies to directly address the effect of aflatoxin B1 on microRNA expression. Due to the interaction with p53, in particular at codon 249, it is reasonable to conceive that aflatoxin could modulate p53 induced microRNAs [32].

Liver fibrosis is the common final path of chronic liver diseases regardless of the etiological differences. It is orchestrated by different resident (e.g. hepatocytes, hepatic stellate cells and Kuppfer cells) and non-resident (e.g. immune cells) cell types and develops on the basis of complex alterations in pro-fibrotic signaling pathways such as TGFβ, SMADs, and MYC (e.g. miR-21, miR-150, miR-194, miR-199, miR-200) that cause a dysbalance between the disposition of extracellular matrix proteins and matrix metalloproteinases [40]. Therefore, microRNAs are involved in regulation of liver fibrosis at multiple levels, i.e. cell type and context dependent [16]. Number of microRNAs have been identified to regulate hepatic stellate cell activation during liver fibrogenesis [41]. Recently, miR-29 was identified to induce fibrogenesis by activating TGF-β as well as NF-κB in hepatic stellate cells [42]. Further, members of the miR-29 family were significantly repressed following CCl4 induction of fibrosis in mice and in livers of patients with advanced fibrosis [43]. Importantly, several microRNAs could also be detected in sera of patients with progressed liver diseases (e.g. miR-571, miR-542, miR-652, miR-181b) suggesting that microRNAs might possess potential as biomarkers during liver fibrogenesis [44, 45].

3 MicroRNAs in Hepatocellular Carcinoma

3.1 MicroRNAs in Hepatocarcinogenesis

Hepatocarcinogenesis is considered a multi-step process that results from sequential alterations of epigenetic and genetic mechanisms leading to a disruption of three core cellular processes i.e. cell fate, cell survival, and genome maintenance, that can promote or "drive" tumorigenesis in the majority of human cancers [46]. During this sequence an activation/inhibition of at least 12 key signaling pathways and downstream molecules such as p53, WNT, β-Catenin, MYC, ErbB family as well as chromatin modifications is observed. MicroRNAs are involved in the regulation of virtually all of these processes and pathways [47]. Given the prominent role of miR-122 for liver homeostasis, several studies demonstrated its relevance also for hepatocarcinogenesis [48, 49]. Mechanistic proof for the tumor suppressive role of miR-122 has been recently revealed by two independent studies. Hsu et al.demonstrated that deletion of mouse miR-122 not only leads to hepatic steatosis and inflammation but also to HCC development. On the molecular level this phenotype was associated with hyperactivity of oncogenic pathways as well as increased infiltration of inflammatory cells that produce pro-tumorigenic cytokines, including IL-6 and TNF [50]. Furthermore, Tsai et al. revealed that loss of miR-122 resulted in phenotypic similarities to common human liver diseases and leads to the activation of several key oncogenic pathways associated with HCC, e.g., TGFβ, MAPK and PTEN [51]. Interestingly, a crucial clinical and functional relevance of miR-122 for human HCC was already established years before these two compelling mechanistic studies [52, 53]. Consistently, miR-122 was preferentially downregulated in a subset of primary tumors that display a particular poor prognosis and showed enrichment of gene sets linked to cancer progression [52]. The authors further identified that miR-122 cooperates with liver-enriched transcription factors such as HNF1A, HNF3A and HNF3B. Functionally, loss of miR-122 resulted in an increase of cell migration and invasion indicating that miR-122 is a marker of hepatocyte-specific differentiation and an important determinant in the control of cell migration and invasion. An epigenetic switch between inflammation and cancer was initiated by a feedback regulation of miR-124, IL6R, STAT3, miR-24, and miR-629 causing sustained oncogenic signaling and downregulation of HNF4α [54]. Furthermore, systemic administration of miR-124, prevented hepatocarcinogenesis by inducing tumor-specific apoptosis without overt liver toxicity indicating the therapeutic potential of this microRNA feedback-inflammatory loop in HCC. Global analyses of microRNAs by next-generation sequencing with differential expression in human normal liver, hepatitis and HCC identified nine microRNAs (miR-122, miR-99a, miR-101, miR-192, miR-199a/b-3p and several let-7 family members) accounting for ∼88.2 % of the "miRNome" in human liver [55]. Further, decreased miR-199a/b-3p expression significantly correlated with survival of HCC patients. Moreover, targeting of miR-199a/b-3p using adeno-associated virus (AAV) 8 inhibited tumor growth via interacting with PAK4/Raf/MEK/ERK pathway. Fornari et al. further

showed an inverse correlation between miR-199a-3p with mTOR and c-Met associated with a shorter time to recurrence after HCC resection [56]. Another global microRNA analysis in 104 HCC, 90 adjacent cirrhotic livers, 21 normal livers as well as in 35 HCC cell lines detected a set of 12 microRNAs (including miR-21, miR-221/222, miR-34a, miR-519a, miR-93, miR-96, and let-7c) associated with malignant progression in liver cancer. Hereby, miR-221/222 were the most upregulated microRNAs in HCC and identified to target the CDK inhibitor p27 as well as DNA damage-inducible transcript 4 (DDIT4), a modulator of mTOR pathway, to enhance cell growth in vitro [57, 58]. Another microRNA with high expression in HCC targeting the PTEN/mTOR pathway is miR-21 [59]. During hepatocarcinogenesis, miR-21 functionally confers to malignant properties such as proliferation, migration, and invasion. Other microRNAs involved in the activation/repression of key signaling pathways and oncogenic molecules in HCC such as c-MYC, c-MET and Hippo are members of the let-7 family, miR-1 as well as miR-375 [60]. Besides the above mentioned studies several other investigations revealed microRNAs with both tumor suppressive (e.g. miR-1, miR-26, miR-29, miR34a, miR-195, miR-223) and oncogenic activity (e.g. Mir-224, Mir-9 and Mir-181) [10, 60]. For some of the mentioned microRNAs (e.g. miR-221, miR-125B, miR-26, miR-122) a prognostic relevance and prediction of drug sensitivity (e.g. interferon) could be demonstrated [11, 61–63].

Besides the malignant transformation and promotion of HCC microRNAs have been implicated to promote or repress the generation of metastatic disease [13]. Not surprisingly, the prominent role of miR-122 was also confirmed in this process. Loss of miR-122 induced the generation of intrahepatic metastasis by promoting angiogenesis via regulation of ADAM17 [53]. A recent study further demonstrated that downregulation of the prognostic microRNA miR-26a also correlated with HCC recurrence and metastasis and was functionally associated with cell proliferation, migration, and invasion [64]. Xu et al. recently showed that miR-148a is repressed by the HBx protein in a p53-dependent manner thereby promoting cancer growth and metastasis through targeting hematopoietic pre-B cell leukemia transcription factor-interacting protein (HPIP) [65]. Inhibition of HPIP expression by miR-148a, reduced the levels of AKT, ERK as well as mTOR through the AKT/ERK/FOXO4/ATF5 pathway. The authors conclude that miR-148a activation or HPIP inhibition may be a useful strategy for cancer treatment. Another recently described tumor suppressive microRNA with anti-metastatic properties is miR-612 [66]. The authors showed that this function was exerted by regulation AKT2 during epithelial-mesenchymal transition (EMT) and metastasis. Consistently, miR-612 levels in HCC patients inversely correlated with tumor size, stage, EMT, and metastasis. Furthermore, miR-612 not only affected local invasion but also intravasation at distant sites indicating that the microRNA is involved in the complete sequence of the metastatic cascade. Two large scale analyses have been conducted to identify metastasis-related microRNAs in HCC [62, 67]. Budhu et al. investigated microRNA profiles in 241 HCC patients and generated a 20-microRNA signature that efficiently predicted the occurrence of venous metastases

and was associated with the patients' outcome [62]. Wong et al. compared microRNA profiles of primary HCC and venous metastasis within 20 matched patients [67]. Interestingly, although non-tumorous livers showed distinct profiles from primary HCCs as well as venous metastases, no apparent differences in the expression pattern of primary HCCs and venous metastases could be detected. However, microRNA expression levels were markedly reduced in venous metastases compared to primary HCCs suggesting that microRNA deregulation occurs early in hepatocarcinogenesis and that the generation of metastasis is aggravated by a stepwise disruption of the deregulated microRNAs.

3.2 MicroRNAs for Diagnostic and Prognostic Classification

Prognostic classification of HCC using expression profiles has a long standing history in HCC [14]. In the last years, the power of microRNA profiling for classification of liver cancers has been demonstrated. Profiling of microRNA patterns by microarray revealed subclasses associated with clinico-pathological features as well as mutations in several oncogenic pathways such as β-Catenin and HNF1A [12]. Recently, microRNA profiling of 89 HCC samples using a ligation-mediated amplification method revealed three distinct clusters of HCCs that reflected the clinical behavior of the tumors. The functional role of different identified microRNAs in particular of the miR-517 family was further investigated in cell lines and in an orthotopic mouse model of liver cancer. As a result the authors could associate these microRNAs with increased proliferation, migration, and invasion of HCC cells in vitro and in vivo, indicating the therapeutic potential of microRNA based treatment modalities [68]. Sato et al. examined the microRNA expression profiling in paired tumor and non-tumor liver tissues from 73 HCC patients and constructed prediction models of recurrence-free survival using the Cox proportional hazard model and principal component analysis [69]. As a result, the authors identified 13 and 56 recurrence-related microRNAs in the tumor and non-tumor tissues, respectively. While the number of recurrence-related microRNAs was significantly larger in the non-tumor-derived microRNAs and predicted late recurrence, the tumor-derived microRNAs were superior to predict early recurrence.

MicroRNAs are good biomarkers since they are released through vesicles (microvesicles or exosomes) into the circulation and can be detected in almost all body fluids. The diagnostic power of microRNAs has been addressed in numerous studies [70]. Li et al. performed a microRNA screen in serum of 513 patients (210 controls and 135 HBV-, 48 hepatitis C virus (HCV)-, and 120 HCC-affected individuals) by Solexa sequencing followed by validation with TaqMan probe-based quantitative reverse transcription-PCR [71]. They identified six microRNAs that were significantly upregulated in HCC samples vs control samples. Among those, two microRNAs (miR-375 and miR-92a), were also enriched in patients with HBV infections. Further, a combination of three of these microRNAs (miR-25, miR-375,

and let-7f) accurately classified HCC and control patients. Zhou et al. examined the role of microRNAs for diagnosing HBV-related HCC in plasma of 934 patients [72]. The authors identified microRNAs with high diagnostic accuracy for HCC irrespective of disease status. However, it was particularly useful for the diagnosis of early-stage HBV-related HCCs and could discriminate HCC from healthy, chronic HBV and cirrhosis. Other microRNAs associated with HCC were miR-199a, miR-222, miR-223, miR-21 as well as miR-122 [73–75]. Liu et al. further aimed to investigate if circulating microRNAs could outperform AFP for HCC detection in 96 HCCs [76]. A combined use of miR-15b and miR-130 yielded 98.2 % sensitivity and 91.5 % specificity and detection sensitivity was even higher (96.7 %) in a subgroup of HCCs with low AFP (<20 ng/mL) and identified AFP negative early-stage HCC cases. Altogether, these data provide compelling evidence for the feasibility of circulating miRNAs as biomarkers for HCC diagnosis.

3.3 MicroRNAs as Therapeutic Targets

Due to the striking aberration of several microRNAs in liver diseases as well as liver cancer, microRNAs emerged to attractive molecular targets in liver cancer and great promise rests on these microRNA-based therapeutic approaches to improve the dismal outcome of HCC patients [77, 78]. The first evidence for the efficiency of a microRNA-based treatment strategy stems from Kota et al. [79]. Already in 2009 the authors demonstrate that induction of miR-26a expression in hepatoma cells in vitro induces cell-cycle arrest associated with direct targeting of cyclins D2 and E2. Systemic administration of this microRNA in a mouse model of HCC using AAVs resulted in reduced proliferation and induction of apoptosis in tumor cells without overt cellular toxicity in normal cells, a side-effect that is commonly feared in this context. Two recent studies established the therapeutic potential of targeting miR-221 [80, 81]. Park et al. demonstrated that anti-miR-221 effectively reduced in vivo miR-221 levels and led to subsequent inhibition of tumor cell proliferation as well as increased apoptosis, ultimately leading to improved survival [81]. Callegari et al. utilized a transgenic miR-221 mouse model that exhibits spontaneous nodular liver lesions with further increase in miR-221 expression and a concomitant inhibition of its target protein-coding genes (i.e., cyclin-dependent kinase inhibitor [Cdkn]1b/p27, Cdkn1c/p57, and B-cell lymphoma 2-modifying factor) [80]. Consistently, in vivo delivery of anti-miR-221 oligonucleotides lead to a significant reduction of the number and size of tumor nodules in this model. Ji and colleagues confirmed the therapeutic potential of microRNA based treatment modalities in HCC [63]. In a large series combining different cohorts including 445 HCC patients, they could demonstrate that expression patterns of microRNAs in liver tissue are vastly different between men and women with HCC. Furthermore, the authors identified that miR-26 levels are associated with response to adjuvant therapy with interferon alfa and, more recently, developed a simple and reliable

companion diagnostic (MIR26-DX) to select HCC patients for adjuvant interferon-alpha therapy as a first step to successfully translate information from large scale analyses into the clinics [82].

3.4 Epigenetic Crosstalk in Liver Cancer

The interaction of different epigenetic layers such as convergence of microRNAs and DNA methylation as well as chromatin modifications is a field of growing scientific interest in translational HCC studies. Takata et al. investigated whether components of microRNA machinery and subsequent functional impairment of microRNAs are involved in hepatocarcinogenesis and identified DDX20 as frequently down-regulated in human hepatocellular carcinomas [83]. Mechanistically, this disruption led to reduced levels of miR-140 resulting in enhanced nuclear factor-κB (NF-κB) activity. Furthermore, the authors identified DNA methyltransferase 1 (Dnmt1) as a bona fide target of miRNA-140. Genetic loss of DDX20 caused increased Dnmt1 expression and hypermethylation of metallothionein gene promoters that enhanced NF-κB activity. In agreement with this finding, miR-140 deficient animals were prone to hepatocarcinogenesis and displayed an overlapping phenotype to that of DDX20 deficiency, suggesting that miRNA-140 plays a central role in DDX20 deficiency-related pathogenesis.

Several studies addressed the cross-talk between microRNA and histone deacetylases [84–86]. Au et al. recently demonstrated that EZH2, a member of the polycomb repressive complex 2 (PRC2) that catalyses histone H3 lysine 27 (H3K27) tri-methylation, epigenetically silenced multiple miRNAs that negatively regulate HCC metastasis [84]. They compared the expression of 90 epigenetic regulators in 38 primary HCC and paired non-tumorous livers and identified that EZH2 was upregulated in more than two thirds of the investigated HCC. The alterations in EZH2 were further associated with HCC progression and multiple HCC metastatic features, including venous and intrahepatic invasion as well as the absence of tumor encapsulation. They further identified a subset of microRNAs that were epigenetically suppressed by EZH2 in human HCC. These included well-characterized tumor-suppressor microRNAs, such as miR-139-5p, miR-125b, miR-101, let-7c, and miR-200b. Another study revealed a regulatory network between HDAC4/Sp1/miR-200a that conferred to aberrant histone acetylation in HCC patients and could potentially be targeted in therapeutic approaches [86]. Buurman et al. further demonstrated that up-regulation of HDAC1-3 in HCC cells reduces the activity of miR-449. They further established that miR-449 binds to the well known oncogene c-MET leading to subsequent down-regulation with downstream activation of apoptosis and reduced proliferation [85]. Another interesting study demonstrated the regulatory cross-talk between MATA1 and microRNAs during hepatocarcinogenesis [87]. The authors identified three novel microRNAs (miR-664, miR-485-3p, and miR-495) that negatively regulate MAT1A expression thereby contributing to a better understanding how

decreased MAT1A levels contribute to liver cancer development. This study has several important mechanistic, technical and clinical implications [88]. The study nicely demonstrates that a tight interaction of different epigenetic layers is an important driver for the development and progression of liver cancer. Consistently, tumors with low microRNA miR-664, miR-485-3p, and miR-495 activity showed higher DNA methylation, increased repressive H3K27me3 levels, lower Let7 expression (via promoter methylation of Lin28B) and vice versa. Additionally, the study confirms that microRNA-based therapy is an effective therapeutic approach for HCC.

3.5 MicroRNAs and Hepatic Cancer Stem Cells

The two dominant models of carcinogenesis postulate stochastic (clonal evolution) or hierarchic organization of tumors (cancer stem cell model) [89]. The latter places a cancer stem cell (CSC) with functional properties similar to untransformed adult stem cells at the germinal center of tumor evolution. Over the past few years, compelling evidence has emerged in support of this hierarchic cancer model for many solid tumors including hepatocellular cancers [61]. The CSCs are held responsible not only for tumor initiation but also for the generation of distant metastasis and relapse after therapy [90]. These characteristics are particularly relevant for a multi-resistant tumor entity like human hepatocellular carcinoma and may herald a paradigm shift in the management of this deadly disease. Accordingly, intense research focused on the identification of microRNAs with functional consequences for hepatic CSCs to identify novel therapeutic targets [91]. Ji et al. performed a global microarray-based microRNA profiling in EpCAM-positive putative liver CSCs and demonstrated that the highly conserved miR-181 family members were activated in the isolated liver CSCs [92]. They further demonstrated that miR-181 was highly expressed in fetal livers as well as in isolated hepatic stem cells. Importantly, inhibition of miR-181 led to a reduction in frequency of CSCs. Mechanistically, miR-181 could directly target hepatic transcriptional regulators of differentiation such as caudal type homeobox transcription factor 2 (CDX2) and GATA binding protein 6 (GATA6) as well as the inhibitor of Wnt/β-catenin signaling nemo-like kinase (NLK). Wu et al. further suggested that hepatic CSCs may originate from hepatic progenitor cells that are continuously exposed to TGF-β stimulation in cirrhotic liver [93]. They then demonstrated that inhibition of microRNA-216a/PTEN/Akt signaling could be a novel strategy for HCC prevention and therapy by targeting of hepatic CSCs. In extension of their previous investigations on CD133 as a marker of liver CSCs [94], Ma et al. identified differential microRNA expression profiles in CD133+ and $CD133^-$ cells from human HCC clinical specimens and cell lines [95]. As a result of this investigation they demonstrated that miR-130b is significantly activated in CSCs. Functional studies on miR-130b lentiviral-transduced $CD133^-$ cells further demonstrated superior chemoresistance, enhanced tumorigenicity in vivo, and a greater

potential for self renewal. Conversely, inhibition of miR-130b in CD133+ CSCs yielded an opposing effect. The authors further established TP53INP1, a known target of miR-130b, as a crucial regulator of self renewal and tumorigenicity. Furthermore, a recent study conducted on 65 hepatoblastomas (HBs) showed that undifferentiated aggressive HBs overexpressed the miR-371-3 cluster with concomitant down-regulation of the miR-100/let-7a-2/miR-125b-1 cluster, which caused an activation of gene sets enriched in embryonic stem cells [96]. ChIP and Myc inhibition assays in hepatoma cells demonstrated that both microRNA clusters are regulated by Myc. A four-miR signature representative of these clusters efficiently stratified HB as well as HCC according to their prognosis. These data suggests that Myc-driven reprogramming of microRNA expression patterns contributes to the aggressive phenotype of liver tumors originated from hepatic progenitor cells.

4 Outlook and Conclusions

Over the last years several microRNAs with differential expression during the development of liver diseases and hepatocarcinogenesis have been identified. Compelling evidence from integrative and mechanistic microRNA profiling studies supports a role for microRNAs for almost all essential processes in liver cancer progression by directly targeting large number of key pathways and molecules. Consequently, microRNAs emerged to promising diagnostic and therapeutic targets. The advent of novel technological platforms further enabled a feasible and safe use of miRNA mimics or "antagomirs" as therapeutics and has already contributed to the development of miRNA-based therapies for HCC. However, despite this growing translational interest in the field microRNAs and microRNA-based therapies, there remain several conceptual and technical issues and challenges [60]. Computational target prediction software are unsensitive and unspecific which underlines a need for thorough experimental validation in authentic tumors [97]. Many of the known microRNAs are believed to regulate multiple target genes. Similarly, microRNA-based gene regulation is supposed to be overlapping with multiple microRNAs contributing to gene expression of one target gene. Therefore, inhibition of a single microRNA might only lead to slight changes in the gene expression of its targets and, therefore, might not be very efficient [8]. Additionally, the regulatory effect of microRNA, with very few exceptions (e.g. miR-122), is unspecific and affects number of cellular downstream targets. Therefore, therapeutic strategies might cause off-target effects and cellular toxicity due to the lack of cell type specificity. In case of the liver where a variety of resident and non-resident cells with opposing effects on hepatocarcinogenesis orchestrate organ homeostasis, this could cause substantial additional problems. Another unanswered but critical question relates to the systemic delivery of microRNA-based therapies for authentic liver tumors. Although results from recent studies indicate that systemic administration of "antagomirs" and miRNA mimics can be safely

performed, considerably more efforts are needed before a broad clinical translation is plausible [10]. Furthermore, the majority of studies are performed on progressed HCC rather than the early phase of malignant transformation. Due to the clinical difficulty of diagnosing and obtaining these samples, better animal models of hepatocarcinogenesis, in which distinct lesions at different stages of progression can be characterized, are urgently needed. Most importantly, up to now, none of the experimental findings have been translated into clinical practice [10]. To serve this unmet need and fully unravel the clinical potential of regulatory microRNAs, several critical technical burdens, such as the best detections method and the ideal sample type as well as the isolation method, have to be overcome.

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Chapter 10
MicroRNA Based Therapeutic Strategies for Cancer: Emphasis on Advances in Renal Cell Carcinoma

Shahana Majid and Rajvir Dahiya

1 Introduction

In recent years, it has become increasingly apparent that the non-protein-coding portion of the genome is of crucial functional importance for normal development, physiology and disease [1]. The functional relevance of the non-protein-coding genome is particularly evident for a class of small non-coding RNAs (ncRNAs) called microRNAs (miRNAs) [2, 3]. miRNAs are small ncRNAs of ~22 nucleotides that mediate post-transcriptional gene silencing by controlling the translation of mRNA into proteins [2, 3]. miRNAs are estimated to regulate the translation of more than 60 % of protein-coding genes. They are involved in regulating many processes, including proliferation, differentiation, apoptosis and development. The frequent aberrant expression and functional implication of miRNAs in many human diseases have highlighted their potential as preferred drug targets [4, 5]. Notably in cancer, certain miRNAs meet the stringent criteria for being ideal therapeutic targets since they by function as bona fide oncogenes and tumor suppressors. 'Oncogene addiction', a term previously reserved for protein-encoding oncogenes, has been extended to miRNAs [6]. Since mammalian microRNAs do not require perfect complementarity for target recognition, a single microRNA is able to regulate multiple, perhaps hundreds of, messenger RNAs [7, 8]. MicroRNAs may impact a given phenotype through regulation of a single key target or through concomitant regulation of a subset of targets. In some instances a phenotypic change can be explained by partial suppression of a single target, as illustrated by the ability of miR-150 to control lymphocyte development by regulating the expression of the seed matched target c-Myb [9]. For other microRNAs the story is more complex, with the phenotype being controlled by the coordinated suppression of multiple targets [10]. MicroRNAs modestly down-regulate individual

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mRNAs (30–50 % down-regulation), yet this degree of target silencing, is sufficient to induce phenotypic changes. Since cancer is a heterogenic disease that cannot be successfully treated by targeting a single gene of interest [11–13], the ability of miRNAs to regulate multiple pathways deregulated in cancer may be key to therapeutic success. Here, we focus on targeting these miRNAs using novel therapeutic strategies for cancer with emphasis on advances in renal cell carcinoma.

2 Disruption of miRNAs in Cancer

In human cancer, miRNA expression profiles differ between normal tissues and the tumors that are derived from them and also between tumor types. miRNAs can act as oncogenes or tumor suppressor genes and can play key roles in tumorigenesis [5, 14]. Transcriptional regulation of miRNAs by oncogenes and tumor suppressor genes provided initial evidence that miRNAs are linked to cancer. In parallel, strong genetic evidence has emerged showing that miRNA genes could be amplified, deleted, or epigenetically regulated in cancer in the same way that canonical cancer genes are affected by somatic or germline mutations. Furthermore, dysregulation of miRNAs in cancer can occur through epigenetic changes such as promoter CpG island hypermethylation. For example, in the case of the miR-200 family [15], genetic alterations can affect the production of the primary miRNA transcript, their processing to mature miRNAs and/or interactions with mRNA targets [16]. From a genetic standpoint, one of the first associations to be observed between miRNAs and cancer development was miR-15 and miR-16 dysregulation in most B cell chronic lymphocytic leukemias as a result of chromosome 13q14 deletion40. Finally, over-expression/down-regulation of certain miRNAs in advanced versus early-stage cancers lead to the idea that some miRNAs might confer metastatic properties and function as metastamiRs in cancer [17].

3 Therapies Targeting miRNAs

Depending on miRNA function and its status in diseased tissues, there are two approaches to developing miRNA-based therapies: antagonists and mimics. MicroRNA antagonists are generated to inhibit miRNAs that acquire a gain of function in human disease. In contrast, miRNA mimics are used to restore miRNAs that show a loss of function. This approach, also known as miRNA replacement therapy, has attracted much interest as it provides a new opportunity to therapeutically exploit dysregulated miRNAs [18] (Fig. 10.1).

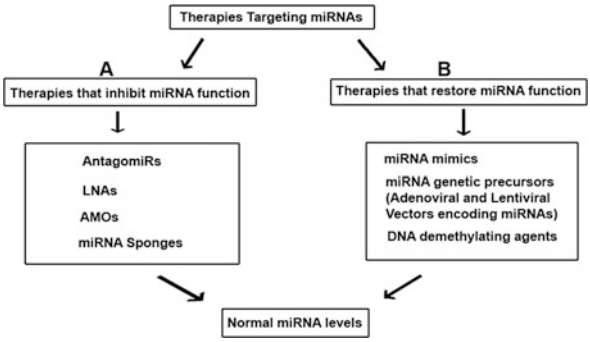

Fig. 10.1 Therapies targeting miRNAs in cancer. (**a**) Strategies that inhibit oncogenic miRNAs include synthetic antisense oligonucleotides (ASOs) or modified ASOs that incorporate chemical groups to improve the stability and efficacy, such as anti-miRNA oligonucleotides (AMOs), antagomirs or locked nucleic acids (LNAs), miRNA sponges that contain multiple artificial miRNA binding sites that act as sponges for the cognate miRNA, preventing its association with endogenous targets. (**b**) Strategies that restore miRNAs include miRNA mimics, virus delivery systems: for example, adenovirus and lentivirus-associated vectors that code for downregulated miRNAs and epigenetic drug treatments for reactivating the transcription of silenced miRNA using DNA demethylating agents

3.1 Therapies that Inhibit miRNA Function

The knowledge that miRNAs regulate their targets through base pairing has led to the use of antisense oligonucleotides (ASOs) to inhibit miRNA function therapeutically. ASOs inhibit miRNA targets based on base pair complementarity. Three main classes of ASOs that have been developed are locked nucleic acids (LNAs), anti-miRNA oligonucleotides (AMOs) and antagomirs that incorporate different chemical modifications to increase stability and efficacy [19–21]. MiRNAs with oncogenic capacity can be deactivated or silenced by various strategies given below.

3.1.1 Anti-miRNA Oligonucleotides (AMOs)

AMOs are single-stranded molecules that form direct complementarity and thus inhibit a specific miRNA [22].

3.1.2 Antagomirs

Antagomirs are 21–23 long ribonucleotide chains wherein 2′-hydroxyl on the ribose is replaced with a 2′-O-methyl group. The backbone is also modified by replacing some of the phosphodiester linkages by phosphothioate ones. These two modifications greatly improve biostability yielding oligonucleotides more resistant to

degradation. To enhance cell penetration, the single-stranded modified RNA is conjugated to a molecule of cholesterol. In addition, antagomir sequences are not perfectly complementary to their targeted miRNA and have base modifications and mispairings that inhibit cleavage by Ago proteins. This results in a near-irreversible binding between the miRNA and the antagomir, which prevents miRNA binding to its cognate mRNA target. The first *in vivo* delivery of antagomirs was via intravenous injection of antagomirs against miR-16, miR-122, miR-192 and miR-194 that resulted in long-lasting reduction of these miRNAs in several organs [19].

3.1.3 Locked Nucleic Acids (LNAs)

LNAs have a methylene bridge that functionally locks ribose conformation. This change results in increased binding affinity and stability [22]. LNAs can be mixed with regular RNA and DNA nucleotides yielding more stable oligomers [23].

3.1.4 Multiple-Target Anti-miRNA Antisense Oligodeoxyribonucleotide (MTg-AMO)

Silencing of a single miRNA might not be sufficient in all cases owing to the pleiotropic and multifaceted biology of cancer cells. Recent research in this field suggests that several miRNAs can be simultaneously inhibited using single ASOs that are targeted against multiple miRNAs. In this approach, multiple antisense units are engineered into a single unit called a multiple-target anti-miRNA antisense oligodeoxyribonucleotide (MTg-AMO) [24]. One MTg-AMO was designed to target three oncogenic miRNAs, miR-21, miR-155 and miR-17-5p, that are overexpressed in many tumors. Use of this MTg-AMO resulted in increased inhibition of cancer growth [24].

3.1.5 miRNA Sponges

Another innovative strategy involves expressing competitive inhibitors of miRNA function. These ‘miRNA sponges’ are vectors containing multiple artificial miRNA binding sites that are placed under the control of strong promoters to produce large quantities of transcript. They act as sponges for cognate miRNAs, preventing their association with natural targets [25, 26]. This strategy has been used, for example, to inhibit miR-9 in highly malignant cells, demonstrating the role of miR-9 in metastasis [26].

3.1.6 Nanoparticles

Nanoparticle formulations have been used primarily *for in vitro* delivery of siRNAs. Few studies to date have used this technology for miRNA delivery. Systemic delivery of miR-34a in a lipid-based vehicle by intravenous injection was used to block proliferation of grafted subcutaneous human lung cancer cells, inducing tumor cell death without triggering toxicity in liver, kidney or heart, as shown in a study from Mirna Therapeutics, Inc. Furthermore, the formulated miR-34a didn't trigger an immune response [27]. Chen et al. demonstrated that by using liposome-polycation-hyaluronic acid (LPH) particles as a carrier for miRNA modified with a tumor targeting monoclonal antibody (GC4 single-chain variable fragment), they could target lung metastases in a murine model of metastatic melanoma [28].

3.2 Therapies that Restore miRNA Function

With regard to tumor suppressor miRNAs or those with decreased expression in benign disease states, the fundamental principle in miRNA-based treatment strategies is to restore their expression level to normal. This can be achieved through miRNA mimicry, viral vector-encoded miRNA replacement or by DNA demethylating agents.

3.2.1 miRNA Mimics

MiRNA mimics are small chemically altered double-stranded RNA molecules that imitate endogenous miRNAs [29], or precursor premiRNA molecules. Liu and coauthors observed that systemic delivery of miR-34a inhibited prostate cancer metastasis and extended survival of tumor-bearing mice, at least in part by targeting CD44 [30]. The delivery was achieved using intratumoral and, notably, intravenous injection and newly emerging delivery reagents such RNALancerII (BIOO Scientific) and siPORT™ amine (Ambion).

3.2.2 miRNA Genetic Precursors

Gene therapy in the form of viral vectors is another approach for the therapeutic replacement of miRNAs. Adenoviral and lentiviral vectors encoding miRNAs have been investigated as miRNA delivery vehicles in this context, with encouraging results [31, 32]. In fact, adenoviral vector-encoded miRNA replacement strategies have already been studied in vivo [33] and have attracted interest from miRNA therapeutics companies such as Mirna Therapeutics and Asuragen. These studies

reported transduction efficiency and minimal toxicity. However, Grimm et al. [34] highlighted the potential for serious toxicity to occur with this miRNA replacement strategy. Systemic administration of short RNAs was achieved in adult mice using a delivery vector based on duplex-DNA-containing adeno-associated virus type 8 (AAV8), resulting in down-regulation of critical liver derived miRNAs, resulting in morbidity and even fatality [34]. The authors postulated that mortality in this instance was consequent to oversaturation of endogenous miRNA pathways. Their experience is important to consider in bringing this strategy from bench to bedside. In parallel, another study demonstrated that systemic delivery of a miR-26a-expressing-adenoviral vector by intravenous injection resulted in impaired Myc-induced hepatic cancer progression by inducing tumor-specific cell cycle arrest and apoptosis [35].

A large body of evidence shows that most human tumors are characterized by defects in miRNA production that lead to global miRNA downregulation. It is therefore tempting to speculate that restoring the global miRNAome could have beneficial therapeutic effects. Global miRNA repression triggers cellular transformation and tumorigenesis in both *in vitro* and *in vivo* models [36–38]. As a result of these findings, a new 'miRNAome-based' strategy has been suggested. The small-molecule drug enoxacin enhances RNAi and promotes miRNA processing by binding to TARBP2 (Ref. [39]). Proof-of-principle studies in human cancer cell lines and xenografted primary tumors have shown that through global reconstitution of downregulated miRNAs to a more 'normal' miRNA expression pattern following enoxacin treatment, the malignant phenotype can be blocked [40]. The drug did not affect normal cells and was not associated with toxicity in mouse models [40].

3.2.3 DNA Demethylating Agents

Another approach for restoring the global miRNAome is the use of DNA demethylating agents and histone deacetylase inhibitors. These compounds reverse epigenetic silencing of tumor suppressor miRNAs and T-UCRs, thereby stopping tumor growth and ultimately resulting in the programmed cell death of the transformed cells [41–43]. These agents, even without any target specificity, have shown themselves to have therapeutic benefits and have received clinical approval for the treatment of certain haematological malignancies [44].

4 Delivery of Therapeutic MicroRNAs

Increasing evidence demonstrates that miRNAs are promising agents in cancer therapy. However, similar to other therapeutic oligonucleotides, the main challenge remains the successful delivery of therapeutic miRNAs to target tissues without compromising the integrity of the miRNA [45–47]. Naked ribonucleic acids are

subject to rapid nuclease dependent degradation and are therefore inherently unstable in biofluids. Thus, many therapeutic RNAi applications are limited to local administration where potential RNAi degradation is limited. However, local administration is only applicable to a short list of target tissues and frequently does not facilitate exposure of all diseased cells to the drug. Systemic delivery is therefore a better route of administration because—in theory—it provides a much more efficient dissemination of the therapeutic to target tissues. However, the miRNA will have to overcome many obstacles before it reaches the target. In addition, systemic delivery of miRNAs may induce similar adverse events that have been reported for other oligonucleotide-based therapies, such as aggregation and complement activation, liver toxicity and stimulation of an immune response by the nonspecific activation of toll-like receptors [48]. Criteria critical in the evaluation process are- (i) sufficient delivery to induce a therapeutic effect in disease models and (ii) a significant safety margin at therapeutic levels. Nevertheless, several strategies described above have had some measure of success, albeit in preclinical settings.

5 Dose Response and MicroRNA Therapeutics

For microRNA targeting therapeutics, there have been clear demonstrations of dose–response relationships in various animal models and microRNA targets. Using anti-miRs or antagomiRs to inhibit miR-122, multiple laboratories have demonstrated dose-dependent pharmacologic effects in species ranging from mouse to man [19, 20, 49, 50]. Thorough characterization of the dose response relationships *in vivo* may be complicated by the biology of microRNA/RNA induced silencing complex (RISC)-mediated effects in which the bottom portion of the typical sigmoidal curve may be very shallow owing to the relatively small changes in target de-repression observed even at maximal inhibition. Thus, dose response curves may take on the appearance of an all-or-none type of response simply because of the subtlety of the responses.

At the higher end of the dose response curve, like the more typical pharmacology of small molecule drugs, the pharmacologic effects of microRNA-targeting drugs should be saturable as the receptors become fully occupied. In the case of microRNA inhibitors, activity is dependent on the binding and sequestration of the target microRNA in an inactive microRNA/anti-miR heteroduplex. The receptor being in this case targeted microRNA. When all of the target microRNA in the cell is sequestered, addition of more anti-miR will not induce additional pharmacological effects but can induce non-specific ones. In any case, determining the saturating concentration or the dose of anti-miR required to produce saturation is critical in clinical trials where the desire is to produce the pharmacologic effect of interest at the lowest dose possible to avoid non-specific effects. For microRNA mimetics, in which the mature microRNA is replaced or expressed in a cell, the dose response relationships will be similar to those observed for exogenous siRNAs. Typical dose response relationships for exogenous siRNAs have been

reported in cell culture [51, 52] and in vivo in species from mouse to man [53]. The pharmacology of microRNA mimetics would be expected to be limited by the available RISC. Once these complexes have been completely occupied with the microRNA mimetic, the pharmacology would be expected to saturate. Additionally, there is the potential to induce unwanted effects when novel microRNAs are introduced into a cell. This problem can be avoided by administration of mimetics only for microRNAs that are ubiquitously expressed, thereby avoiding unwanted and unanticipated effects in a cell that has never expressed that microRNA, and only replacing activity needed to produce the pharmacology of interest in a target cell type that has lost the function of that microRNA. The field of microRNA mimetic toxicity is generally unexplored at this time. The potentially adverse effects of full saturation of RISC and other related complexes were demonstrated by the overexpression of an shRNA in mice [34] with lethal consequences. Whether exogenous microRNA mimetics can be delivered at concentrations that achieve those resulting from shRNA overexpression [34] has yet to be established, but these results suggest the nature of the effects of RISC saturation and other micro-RNA processing activities that could be extreme. A study by Khan et al. [54] might provide some insight into unexpected effects associated with RISC saturation. Khan et al. performed meta-analyses to conclude that siRNAs transfected into cells could compete with endogenous microRNAs for association with RISC, resulting in de-repression of micro-RNA targets [54]. Bioinformatic analyses of the transcriptome of tissues treated with miRNA mimetics will ultimately address these issues.

6 miRNAs in Renal Cancer

Renal cell carcinoma (RCC) is genetically and histopathologically a heterogeneous disorder. The most common subtype of RCC is clear cell RCC (ccRCC; approximately 75 %) and the next most frequent subtype is papillary RCC (pRCC; approximately 15 %) [55]. The most frequent genetic abnormality in ccRCC is inactivation of the von Hippel-Lindau (*VHL*) tumor suppressor gene [56] and promoter methylation of tumor suppressor genes (TSGs) is common in both RCC subtypes. Dysregulation of miRNA expression is also pivotal for RCC development and progression. A number of miRNA expression and functional studies have been carried out in RCC. Table 10.1 presents a list of miRNAs that are either tumor suppressors or oncogenic in RCC. Our group has reported that several tumor suppressor and oncogenic miRNAs such as miR-1826 [57], miR-708 [58], miR-205 [59], miR-584 [60], miR-21 [61] are potential therapeutic targets in RCC. We observed that the expression of miR-205 was significantly suppressed in renal cancer cell lines and RCC samples [59]. This miRNA suppresses potential gene targets encoding Src, Lyn, Yes and Lck which are involved in cell migration invasion and cell proliferation. Therefore, its downregulation may facilitate RCC proliferation and diffusion. This study demonstrated that local administration of

Table 10.1 MicroRNAs and their targets in renal cell carcinoma

MicroRNAs	Function	Target gene	References
miR-99a	Tumor suppressor	mTOR	[64]
miR-138	Tumor suppressor	Vimentin	[65]
miR-204	Tumor suppressor	MAP1LC3B	[66]
miR-708	Tumor suppressor	Survivin	[58]
miR-1	Tumor suppressor	transgelin-2	[67]
miR-133a	Tumor suppressor	transgelin-2	[67]
miR-1826	Tumor suppressor	CTNNB1, MAP2K1	[57]
miR-34a	Tumor suppressor	c-Myc	[68]
miR-205	Tumor suppressor	Src kinase	[59]
miR-584	Tumor suppressor	ROCK1	[60]
miR-23b	Oncogenic	Proline oxidase	[69]
miR-21	Oncogenic	PTEN	[70]
miR-122	Oncogenic	PI3K/Akt	[71]
miR-30c	tumor suppressor	Slug	[72]
miR-590-5p	Oncogenic	PBRM1	[73]
miR-143/145	Tumor suppressor	hexokinase-2	[74]
miR-187	Tumor suppressor	B7 homolog	[75]
miR-1291	Tumor suppressor	Glucose Transportar 1	[76]
miR-1260b	Oncogenic	sFRP1, Dkk2, Smad4	[77]
miR-218	Tumor suppressor	CAV2	[78]
miR-30d	Tumor suppressor	MTDH	[79]
miR-21	Oncogenic	TCF21	[80]
miR-23b-3p	Oncogenic	PTEN	[81]
miR-135a	Tumor suppressor	c-myc	[82]
miR-99a	Tumor suppressor	Rapamycin	[64]
miR-210	Oncogenic	HIF1a	[83]
miR-101	Tumor suppressor	EZH2	[84]

miR-205 mimic complexed with SiPortAmine delivery reagent in established tumors induced a dramatic regression of tumor growth and hence miR-205 may be a potential therapeutic target for the treatment of RCC [59]. Another study by our group reported that miR-708 expression was widely attenuated in human RCC specimens. Restoration of miR-708 expression by miR-708 mimics in RCC cell lines decreased cell growth, clonability, invasion, and migration and elicited a dramatic increase in apoptosis. Moreover, intratumoral delivery of miR-708 mimic was sufficient to trigger in vivo regression of established tumors in murine xenograft models of human RCC. This report highlighted a major tumor suppressive role for miR-708 that may be an attractive target for therapeutic intervention in RCC [58]. Similarly, expression of miR-584 was found to be downregulated in RCC tissue samples [60]. It was correlated with high expression of R0CK-1 protein which modulates cell motility. This promotes tumor cell diffusion; therefore, targeting miR-584 may inhibit RCC progression and patient survival. Other laboratories have also studied numerous miRNAs as listed in Table 10.1, that might be therapeutic targets for the treatment of RCC. Aberrant expression of miR-1285 was

found to inhibit cancer cell proliferation, invasion and migration [62]. Downregulation of this miRNA, which targets oncogenic genes, might contribute to RCC development. This novel miRNA target may provide new insights into the potential mechanisms of RCC oncogenesis. Low levels of miR-508-3p and miR-509-3p have been found in plasma and renal cancer tissue samples of patients with RCC [63]. The over-expression of these miRNAs *in vitro* suppressed the proliferation of RCC cells, induced cell apoptosis and inhibited cell migration. These findings suggest that miR-508-3p and 509-3p play an important role as tumor formation modulators and may be novel RCC therapeutic targets. All these studies have reported gain or loss of function of individual miRNAs with pathological roles in tumor cell proliferation, progression and cancer development. These proof-of-principle studies in human cancer cell lines and primary tumor xenografts have demonstrated the therapeutic potential of targeting miRNAs for the treatment of renal cancer. Translation from an *in vitro*/local delivery to systemic *in vivo* delivery systems; however, remains a work in progress for RCC.

7 Closing Remarks and Conclusions

The global dysregulation of miRNAs has been described in several malignancies including cancers and it is clear that miRNAs can alter biological processes fundamental to tumor initiation and progression. miRNAs have quickly moved from discovery into therapeutic development programs. This rapid progress reflects the importance of miRNAs in cancer and is based on the thorough validation of key miRNAs as ideal candidates for therapeutic intervention. Although there is little doubt about their therapeutic potential, the challenge is to translate this potential into readily available drugs. The main focus in using miRNAs for cancer treatment is the problem of pharmacological delivery of miRNA, a task that has hampered the progress of related antisense and siRNA therapeutics. Yet, the recent clinical successes using existing delivery technologies and the continuous emergence of new delivery systems suggests that miRNA therapeutics for cancer is within the realm of possibility. As many current delivery systems show distinct biodistribution profiles, the type of cancer treated may largely depend on the performance of the underlying delivery system. Establishing ideal delivery systems with organ specificity while minimizing toxicity and off target effects will be essential to moving the field forward. It is envisioned that in the coming years there will be important advances in the field of miRNA-based therapeutics, and it remains to be seen whether these will result in effective miRNA-based-drugs.

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Chapter 11
Modulating MicroRNA Expression for the Therapy of Pancreatic Cancer

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1 Introduction

Pancreatic cancer is the fourth leading cause of death by cancer worldwide with an increasing incidence and a very poor prognosis [1]. The estimated 5-year survival is lower than 5 %. Patients' median survival following diagnosis is approximately 6 months. Nowadays, there is no curative treatment excepting surgery for 15 % of patients. Nevertheless palliative chemotherapy (gemcitabine) can be applied. Development of pancreatic cancer is very slow and involves many actors from which microRNAs.

MicroRNAs (miRNAs, miRs) derive from endogenous genes (from intergenic or intragenic genomic regions) transcribed for the most part by RNA polymerase II. They follow a complex maturation process implicating key enzymes such as DROSHA, DGCR8 and DICER [2]. They are small non coding RNA that functions as translation inhibitors of messenger RNA mainly following binding to 3′-untranslated region [3–5]. This mechanism is conserved from plants to humans.

Because they regulate more than 30 % of mammalian gene products, microRNAs are tightly involved in the regulation of many physiological processes including development, proliferation, cell signaling and apoptosis. In addition, microRNAs play important roles in many diseases, including cancer, cardiovascular disease, and immune disorders. In oncology, two main families of microRNAs can be defined: oncomiRs (such as miR-21 and miR-155) which target messenger RNAs from tumor suppressor genes and tumor suppressor microRNAs (tsmiR) (let7, miR-34a and miR-146a) which target oncogenic mRNAs. More recently, another class of microRNA implicated in cell metastasis has been described

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(MetastmiR). Indeed, Ma et al. described that the over expression of miR-10b in non invasive breast cancer cell line alone confer metastatic potential. MicroRNAs are involved in many oncogenic pathways [2] for example miR-34a and miR-146a are induced by p53 and NF-kB, respectively [6] while miR-21 which inhibits the p53 network [7] is induced by many oncogenic pathways including activated KRAS and EGF receptor among other [8].

The alteration of microRNA expression in cancer has been described for the first time by Calin and colleagues in 2002 [9]. Several mechanisms are implicated in this deregulation such chromosomal aberrations, transcriptional control by oncogenic transcription factors (such as MYC) [10], environmental factors, polymorphisms [11], epigenetics [12] and altered expression or function of proteins involved in microRNAs maturation [13]. Recently, Dicer and Drosha were found decreased in 60 % and 51 % of ovarian-cancer specimens, respectively [14]. As a consequence, microRNA profiling permits the differential diagnosis between normal *vs* cancerous tissue and to indentify tissues of origin for metastases [2]. In pancreatic cancer, Bloomston et al. originally published that 21 upregulated and 4 downregulated microRNAs could differentiate pancreatic tumors from benign pancreatic tissue in 90 % of their samples [15].

2 MicroRNAs as Emerging Therapeutic Targets

Single microRNA are demonstrated to control the expression of hundreds of genes, and represent a new class of therapeutic targets to modulate many pathways simultaneously and to reduce the emergence of resistant cellular clones that remains a major concern in oncology [16]. In addition, recent publications demonstrate that altering the level of expression of the entire population of cellular microRNAs by targeting microRNA processing alters tumor progression in a disease-specific manner [17].

MicroRNAs are also established as key players in cancer cell resistance to treatment. MiR-21, one of the most cited oncomiR, is implicated in the resistance to chemotherapy of many types of cancer including breast and pancreatic cancer among others [18, 19]. In the later example, miR-21 targeting in combination with gemcitabine treatment induces tumor regression. Other microRNAs are implicated in pancreatic cancer cells chemoresistance such as miR-17-5p [20] and miR-181b [21]. MicroRNAs are also implicated in cancer cell resistance to radiotherapy. Indeed, Di Francesco and colleagues demonstrated that DNA damage response is affected by miR-27a in lung adenocarcinoma-derived cell lines by a direct interaction between miR-27a and the 3′UTR region of the ATM kinase (Ataxia-Telangiectasia Mutated) [22]. ATM regulates H2AX phosphorylation and the activation of check point and cell cycle arrest following DNA damages. Taken together, these studies demonstrate the importance of microRNA in carcinogenesis but also in response to treatment making microRNAs very appealing therapeutic targets.

3 MicroRNA Targeting in Cancer

microRNA can be considered as emerging targets for the treatment of cancer including pancreatic cancer either following restoration of the expression of tumor suppressor microRNAs (let-7, miR-143-145, miR-34) or the targeting of pro-oncogenic microRNA (miR-21, miR-155, miR-27). Many strategies have been developed to achieve this goal (antisens, microRNA decoys. . .). Interestingly, some approaches allow the synchronized targeting of several microRNAs by using so called 'Tough Decoys' (TuDs) [23]. Consequently, many microRNAs carriers are needed to deliver these moieties and to avoid the different biological barriers [24]. Later in the chapter we will suggest which vector could be used for the specific targeting of a diseased cell.

3.1 In the Absence of Carriers

Nowadays, microRNAs upregulation (tsmiR) is done by the use of microRNA mimics contrary to downregulation of oncomiR that is achieved using antisense oligonucleotide (ASO or antagomiR) or microRNA sponges (with repeated miRNA antisense sequence). These strategies take advantage of small RNAs (19–22 nt) that are by definition very sensitive to nuclease degradation. Consequently, it is mandatory to conjugate cholesterol with 2′-O-methyl (2′-O-Me), 2′-O-methoxyethyl (2′-O-MOE) or 2′-fluoro substitutions. These substitutions improve microRNA modulators stability and effectiveness of microRNA inhibition *in vivo* [25].

3.2 Non Viral Nanovectors

MicroRNA modulators have a small size (7–20 kDa) so they undergo kidney filtration [2]. In addition, these non endogenous modulators should avoid phagocytic immune cells (macrophages and monocytes) in the bloodstream. So it is necessary to combine them with a carrier. There are different nanovectors which can be used to protect microRNA modulators, to improve targeting and to improve the cellular uptake of the modulator. Lipid-based nanovectors (liposomes) can be toxic for cells, are non specific and can induce immune response [26]. Accordingly, they must be modified to serve as microRNA carriers. Pramanik and colleagues demonstrated that the systemic injection of miR-34a and the miR-143/145 clusters (two main tsmiR lost in pancreatic cancer) using lipid-based nanovectors in orthotopic xenografts model induce tumor growth inhibition with increasing apoptosis and decreasing proliferation [27]. Interestingly, tumor cells can be targeted with modified liposomes. Polycationic liposome-hyaluronic acid (LPH) are used because hyaluronic acid is a targeting agent due to its cell surface receptor CD44

which is overexpressed on various tumors. LPH could be combined with the tumor targeting GC4 single-chain antibody fragment (scFv-LPH) or with an integrin-binding tripeptide (cRGD-LPH) for targeting integrin receptors on tumor vasculature. Many other possibilities of lipid-based nanovectors combination are described by Dr Leone's group [28]. Polyethyleneimines (PEI) is commonly used due to its global positive charge which ensures a strong interaction with the negatively charged plasma membrane. Polyurethane-short branch polyethylenimine (PU-PEI) is not cytotoxic and has high transfection efficiency as described by Chiou *and al* for the delivery of miR-145 to treat lung adenocarcinoma *in vivo* [29]. Nowadays it is possible to modify PEI nanovectors with rabies virus glycoprotein (RVG) to allow PEI-microRNA modulator system to cross through the blood–brain barrier. For example miR-124a (neuron specific microRNA) delivery in brain promotes neurogenesis [30]. Atelocollagen that derives from type I collagen can also be used as a microRNA carrier. MicroRNA modulators-atelocollagen complex have a high delivery efficiency and limited immunogenicity. Matsuyama and colleagues described that the local administration of miR-135b inhibitors with atelocollagen suppressed the growth of subcutaneous Karpas 299 tumors in a xenograft model [31]. Last, Calin's team has recently described nanovector inspired from endogenous intracellular transport of microRNA. Indeed, microRNA-protein complex composed by Argonaute 2 protein or lipoproteins (HDL) are actively secreted or can be part of cell-derived membrane vesicles such as exosomes or apoptotic bodies [32]. Recently, Ohno et al., demonstrated the feasibly of targeting EGFR-expressing cancerous tissues after systemic injection in a RAG 2−/− mice of let-7a microRNA in a modified exosomes by the GE11 peptide (specific ligand of EGFR less mitogenic than EGF). Their results suggest that exosomes can be used therapeutically as a nanovector delivery system for microRNAs [33].

3.3 Viral Vectors

Viral vectors are very efficient for gene transfer and can be easily targeted to diseased cells. MicroRNA replacement or inhibition using lentivectors, adenovectors or adeno-associated vectors (AAV) have been shown to inhibit tumor growth in experimental models of lung, prostate, breast and liver cancer. Pr Tyler Jacks's team demonstrated that let-7 g overexpression using lentiviral vector in both murine and human non-small cell lung tumors induced significant growth reduction [34]. In another study, miR-145 overexpression using adenoviral vector in combination with 5-FU treatment in orthotopic breast cancer mice *in vivo* significantly showed anti-tumor effects as compared to chemotherapy alone [35]. Last, Dr Mendell's team described that the systemic injection of miR-26a in a mouse model of hepatocellular carcinoma (HCC) using AAV, inhibits cancer cell proliferation and induces tumor-specific apoptosis without toxicity. This study is a proof of concept that expression of a microRNA lost in cancer using a dedicated delivery system is well tolerated [36].

3.4 Route of Administration

There are three main routes of administration depending on the type of microRNA delivery systems used [2]. MicroRNA can be injected systemically in the absence of carrier (antagomiR, LNA and modified oligos), while non viral and viral vectors permit systemic, locally or intranasal delivery. Importantly, local injection can help minimize microRNA modulators exposure to nuclease degradation in body fluids and decrease unspecific uptake in non target tissues. Accordingly, Dr Slack's group described that the intranasal injection of *let-7*-encoding adenovector reduces tumor growth in mouse models of lung cancer due to the capability of this class of vector to have a unique cell surface receptor and to transduce epithelial cells [37]. To finish, systemic or local injection can also be done for nanoparticles carrying microRNAs [2].

4 MicroRNA Targeting in Pancreatic Cancer

Concerning pancreatic cancer, there are several studies of microRNA targeting using different carriers. The most recent studies of microRNA targeting are described below. First of all, intravenous injections of miR-34 or miR143/145 lipid-based nanoparticules in pancreatic cancer xenografts induced tumor growth reduction and apoptosis [27]. However, while this strategy may permit the targeting of distant metastasis, the transfection efficacy of this approach was not mentioned. In a similar work, Hu and colleagues developed a nanovector-based miR-34a delivery system combined with CC9 peptide that increases the targeting and penetrating capability in pancreatic cancer-derived cells. Interestingly, systemic administration of this complex inhibits tumor growth and induces pancreatic cancer cell apoptosis in a murine model of PANC-1 subcutaneous xenografts [38]. Again, in vivo transfection efficacy of this approach is not quantified. In addition, both strategies used subcutaneaous models of pancreatic tumor growth that greatly diverges from orthotopic tumors. On the other hand, miR-21 is barely expressed in normal cells and participates in many oncogenic pathways. This particular miRNA is most frequently associated with poor outcome in cancer including pancreatic neoplasia. Our group recently asked whether targeting miR-21 could impair tumor growth and sensitize pancreatic tumors to chemotherapy. We used lentiviral vectors encoding for miR-21 decoys that efficiently silence miR-21 in cancer cells. Intratumoral injection of miR-21 decoys in an orthotopic human pancreatic cancer xenograft model inhibits tumor progression. We next combined miR-21 targeting vector with repeated intra peritoneal gemcitabine injection. We demonstrate that miR-21 alone is more efficient than the standard of care chemotherapy to inhibit tumor progression and, more importantly, that combining miR-21 targeting with chemotherapy induced tumor regression in a very aggressive model

of pancreatic cancer [19]. Thus, microRNAs such as miR-21, are promising targets for pancreatic cancer therapy.

Nevertheless, few reports demonstrated that microRNAs modulators are ineffective to inhibit cancer growth. In these studies; the delivery systems do not appear to be faulty, but the enforced expression of the candidate microRNA may not result in the antitumoral effect expected. For instance, Delpu and colleagues analyzed the potential role of miR-148a over-expression in PDAC using lentiviral vector carriers. While this microRNA is lost during pancreatic carcinogenesis [12], they demonstrated that miR-148a expression *in vivo* using lentiviral vectors does not impede tumor growth [39]. In another example, restoring Let-7 expression using lentiviral vectors in pancreatic cancer derived cell lines strongly inhibits cell proliferation but fails to impede tumor growth [40]. Thus, it is mandatory to perform *in vivo* studies to demonstrate the antitumoral activity of microRNA-based therapeutics before further (pre)clinical evaluation.

5 MicroRNA and Clinical Trials for Cancer

Nowadays, microRNAs are commonly associated with clinical trials and can be used as robust and reliable biomarkers for different diseases. Nevertheless there is no clinical trial to date using microRNA as a therapeutic target in cancer. Indeed, MiR-122 is the only microRNA that has been implicated in clinical trials (phase 2 with 36 patients) for patients with chronic hepatitis C viral infection. This microRNA is liver-specific and *de rigueur* for hepatitis C virus replication. Repeated weekly subcutaneously injection of different doses of miravirsen (LNA-antimiR-122) have been performed. Miravirsen efficiently inhibits miR-122 in HCV patients. Interestingly, miravirsen is safe and well tolerated and provoke a dose dependent reduction in HCV RNA levels [41].

6 Conclusion

Despite these very encouraging results, it is important to question why microRNAs are not widely used in cancer clinical trials. Importantly, most of the studies have been performed in immunosuppressed experimental models and in very few immune-competent animals. As microRNAs have been associated with the regulation of TLRs [42], further experiments are needed to demonstrate the safety of such approach. In addition long term studies in model organisms must be performed, to identify unexpected serious adverse events linked to microRNA modulators administration. Along with, targeting tumor cells using delivery vehicles remains a challenge in the gene therapy field of research. Last but not least, the specificity of microRNA modulators must be scrutinized because lead off-target effects (i.e. silencing of non targeted genes) have been already described for other RNA

interference strategies using siRNA [43]. In conclusion, the potential benefits for basic cancer research, medicine and public health of using microRNAs as therapeutic targets are numerous. As existing treatment offer little benefit, targeting microRNAs may give therapeutic perspectives for the treatment of pancreatic cancer or other human solid tumors. Such challenges notwithstanding, this strategy represents a welcome and refreshing set of new considerations to ponder in a disease that has too often been met with frustration and nihilism in the past.

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Chapter 12
MicroRNA Targeted Therapy for Overcoming Drug Resistance, Reversal of EMT and Elimination of Cancer Stem Cells in Prostate and Pancreatic Cancer

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1 Introduction

In recent years, microRNAs (miRNAs), epithelial-to-mesenchymal transition (EMT) phenotypic cells, and cancer stem cells (CSCs) have emerged as hot topics in the area of cancer research. The numbers of studies on miRNAs, EMT, and CSCs have been significantly increased in recent 5 years. Mechanistic understanding of these cellular processes may lead to overcome therapeutic resistance because drug resistance is one of the main reasons for the treatment failure in cancer chemotherapy and radiotherapy. Studies are underway to better understand the molecular mechanism(s) underlying drug resistance in order to find drugs to block or reverse the development of drug resistance. Unfortunately, no efficient drugs that are targeted for overcoming drug resistance have been discovered thus far. Emerging evidences have demonstrated that miRNAs could directly or indirectly regulate drug resistance through modulation of the biology of EMT and CSCs. Therefore, targeting miRNA could become a promising therapeutic approach for overcoming drug resistance, which could in part be due to the reversal of EMT phenotype and elimination of CSCs that would likely lead to the successful treatment of cancers especially those that are highly resistant to conventional therapeutics.

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The miRNAs are small non-coding RNAs and commonly consists of 19–25 nucleotides. Although miRNA does not code for any proteins or peptides, it plays important roles in the control of a number of biological processes under normal and disease conditions through the regulation of target gene expression. The mature miRNAs bind to their target mRNAs and subsequently cause the degradation of target mRNAs or inhibition of the translational ability of target mRNAs. In this way, miRNAs are key players in the regulation of gene expression of multiple genes that are involved in the regulation of many biological processes such as cell development, proliferation, apoptosis, etc. Importantly, recent studies have demonstrated that miRNAs also play critical roles in the development and progression of various cancers. By genome wide screening of miRNA expression, the aberrant expression of miRNAs has been found in different types of tumors. Based on their role and expression level, miRNAs in tumors could be categorized into two groups: tumor suppressive miRNAs and oncogenic miRNAs. Commonly, let-7, miR-15, miR-16, miR-29a, miR-34, etc. have been believed to be tumor suppressors while miR-21, miR-17-92, miR-155, miR-221, and miR-222 have oncogenic feature in different types of cancers. By regulation of cellular signaling, these miRNAs control cancer cell development, proliferation, drug sensitivity, invasion, tumor recurrence and metastasis (Fig. 12.1). More importantly, it has been shown that miRNAs regulates differentiation and self-renewal of normal stem cells; therefore, miRNAs could also contribute to the formation, renewal, and differentiation of CSCs [1, 2], which have been linked to drug resistance, tumor cell aggressiveness and recurrence that contribute to the demise of patients diagnosed with cancers.

CSCs are undifferentiated cancer cells which possess the ability of self-renewal, multi-lineage differentiation, and have the propensity for increased proliferation, leading to oncogenesis. It is becoming clear that CSCs are present in almost all kinds of hematopoietic and solid malignancies. CSCs are critically involved in drug resistance, tumor progression and recurrence of cancers. The *in vitro* and *in vivo* studies have demonstrated that CSCs are not sensitive to conventional chemotherapeutic agents or radiotherapy. CSCs also show their high capacity of migration, invasion and metastasis, which are responsible for cancer progression. Moreover, it has been found that cancer recurrence commonly occurs due to persistence of CSCs which survive after conventional anti-cancer therapy, resulting in self-renewal and proliferation of CSCs for tumor recurrence and metastasis. Two critical properties of CSCs including their capacity for self-renewal and their potential to differentiate into unlimited heterogeneous populations of cancer cells contribute to the functional role of CSCs in the establishment of tumors and cancer recurrence [3]. Therefore, CSCs are the major culprits of cancer drug resistance, tumor progression and recurrence, suggesting that novel therapeutics must be discovered for the elimination of CSCs in order to eradicate human malignancies.

Reminiscent of CSCs, EMT is a biological process in which well-differentiated and polarized epithelial cells converse to become motile and un-polarized mesenchymal phenotypic cells. EMT plays important roles during biological and pathological events such as embryogenesis, organ development, implantation, tissue regeneration, and organ fibrosis. Importantly, it has also been found that the cancer

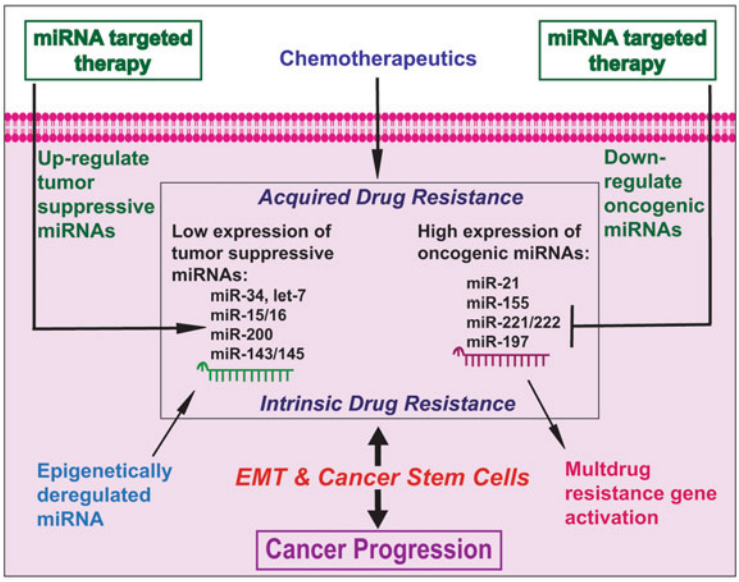

Fig. 12.1 Targeting miRNA regulated EMT and CSCs to induce drug sensitivity for the treatment of cancers with drug resistance

cells consisting EMT phenotypic cells exert higher drug resistance and higher capacities for tumor cell migration, invasion and metastasis, which is also consistent with the characteristics of CSCs. Molecular studies have shown that CSCs and EMT-type cancer cells share molecular signatures, which could be responsible for the similarities of drug resistance and aggressiveness in CSCs and EMT-type cancer cells. Importantly, studies have demonstrated that miRNAs could control the development, self-renewal, and differentiation of CSCs and the processes of EMT and the reversal of EMT to MET (mesenchymal-to-epithelial transition) phenotype [4]. Therefore, targeting miRNAs could become a novel approach for the elimination of CSCs and reversal of EMT phenotype of cancer cells, which would eventually lead to induce drug sensitivity and suppression of tumor progression and recurrence, resulting in better treatment outcome of patients (Fig. 12.1).

2 The miRNAs in the Regulation of Drug Resistance, EMT and CSCs in Pancreatic Cancer

Pancreatic cancer is an aggressive tumor with characteristics of rapid invasion, early metastasis, and resistance to standard chemotherapy or radiotherapy. The resistance of pancreatic cancer cells to therapy is largely due to the existence of pancreatic CSCs and EMT phenotype status of cancer cells. Pancreatic CSCs are

not only capable of self-renewal and differentiation, but also cause multi-drug resistance which is one of many important characteristics of pancreatic cancer. However, the CSC mediated drug resistance could be critically controlled by miRNAs [5] as discussed later.

2.1 *miRNA Mediated Regulation of Drug Resistance in Pancreatic Cancer*

Since miRNAs play important role in drug resistance in pancreatic cancers, profiling of miRNA expression in tumor tissues is beneficial for diagnostic, prognostic and for assessing therapeutic activity. Studies have shown that the levels of certain tissue-specific miRNAs including miR-216 and miR-217 were significantly down-regulated in pancreatic tumor tissues compared to matched normal controls [6]. Conversely, up-regulation of miR196a, miR-190, miR-186, miR-221, miR-222, and miR-95 have been found in the pancreatic cancer tissues [6]. By profiling of miRNA in pancreatic invasive ductal adenocarcinoma (IDA), it has been found that miR-21 is highly expressed whereas the expression of miR-126 is reduced. Mechanistic studies have shown that ADAM9 (disintegrin and metalloproteinase domain-containing protein 9) highly expressed in IDA is a target of miR-126. Re-expression of miR-126 or knockdown of ADAM9 in IDA cells led to the induction of E-cadherin and reduction of invasion and drug resistance [7]. These results suggest that the miRNA profiling of tumor tissues could be crucial for the treatment of pancreatic cancer. However, in pancreatic cancer, getting optimal amount of pancreatic cancer tissue is a problem for early testing of miRNA profile. Some investigators have profiled miRNAs in blood as markers to predict drug resistance and prognosis. They found that miR-21, miR-210, and miR-155 regulated p53, TGF-β, $p16^{INK4A}$, BRCA1/2, and Ki-ras signaling, resulting in drug resistance and aggressiveness of pancreatic cancer [8–10].

Chemotherapy for pancreatic cancer often fails to kill all cancer cells because of drug resistance. It has been reported that RAD51 protein is an important molecule in the process of DNA repair and that its overexpression is implicated in acquired drug resistance. Experimental studies have shown that overexpression of RAD51 leads to the development, progression and drug resistance of pancreatic cancer cells through the regulation of EMT and pancreatic CSCs [11]. Importantly, the overexpression of RAD51 has been found to be due to the deregulation of several miRNAs. It has been found that miR-103 and miR-107 could inhibit FANCD2/RAD51 foci formation and sensitize cancer cells to cisplatin. Furthermore, miR-103 and miR-107 could inhibit the expression of RAD51 and RAD51 paralog RAD51D probably through the bindings of miR-103 and miR-107 to the 3′-UTR sequences of RAD51 mRNA [11].

The epidermal growth factor receptor (EGFR) inhibitors have been used for the treatment of pancreatic ductal adenocarcinoma (PDAC). The amplification of

EGFR and the mutation status of EGFR and Ki-ras have influenced the sensitivity of cancer cells to EGFR inhibition in lung cancer. By conducting miRNA analysis, the expression levels of several miRNAs have been selected to predict the response of pancreatic cancer cells to EGFR inhibitor erlotinib. These miRNAs include miR-140, miR-628, miR-141, miR-200b, miR-200c, miR-135b, miR-301a, miR-224, miR-197, miR-34c, miR-205, miR-518f, and miR-636 with up-regulated or down-regulated levels [12]. The targeted proteins by these miRNAs are mostly for EMT. The EMT transcription factor, ZEB1, was significantly up-regulated in erlotinib-resistant PDAC. The treatment with TGF-β up-regulated ZEB1 while ectopic expression of miR-200c decreased the level of ZEB1 and increased sensitivity to erlotinib [12], suggesting that TGF-β induces resistance to anti-EGFR therapy through the regulation of miR-200c mediated by regulating the expression of its target.

In addition, overexpression of ribonucleotide reductase subunit M2 (RRM2) which regulates deoxyribonucleotide synthesis could promote chemoresistance of pancreatic cancer to gemcitabine. It was found that tumor suppressive miRNA let-7 could bind to the 3′UTR of RRM2 and inhibit the expression of RRM2 protein [13]. In gemcitabine-resistant pancreatic cancer cell lines, decreased expression of let-7 family and increased expression of RRM2 have been observed, suggesting the inhibitory effects of let-7 on gemcitabine resistance. Indeed, re-expression of let-7 miRNA induced chemosensitivity of pancreatic cancer cells to gemcitabine [13]. Therefore, silencing RRM2 by introduction of let-7 could be a promising strategy for reducing chemoresistance of pancreatic cancer; however, such a strategy awaits human application.

2.2 miRNA Mediated Regulation of Pancreatic CSCs and EMT in Drug Resistance

It is known that CSCs reside within the cancer cell populations capable of forming holoclones continuously. To identify pancreatic CSCs from the population of pancreatic cancer cells, BxPC3 pancreatic cancer cells were subjected to monoclonal cultivation to generate colonies. It was found that holoclones of BxPC3 cells exhibited higher capacities of long-term survival, tumor initiation, and chemoresistance. These cells showed high expressions of CSC related marker CXCR4 and BMI1, Hedgehog signaling molecules such as GLI1 and GLI2, and several miRNAs including miR-21, miR-221, miR-222 and miR-155 [14]. These results demonstrate that pancreatic CSCs with high level of certain oncogenic miRNAs and chemoresistance characteristics are enriched in holoclones from pancreatic cancer cells.

Growing evidences have demonstrated that EMT contributes to pancreatic cancer progression and drug resistance. We have also investigated the relationship between drug resistance and miRNA mediated regulation of EMT in pancreatic

cancer cells. We found that the levels of miR-200 and let-7 families were significantly lower in gemcitabine-resistant cells with EMT characteristics. Moreover, introduction of miR-200 reversed EMT phenotype, inhibited the expression of mesenchymal makers, and increased drug sensitivity of pancreatic cancer cells to gemcitabine [15], suggesting the crucial roles of certain miRNAs in the control of drug sensitivity of pancreatic cancer. Such knowledge would be useful for the development of miRNA-targeted therapeutic strategy in the future.

ZEB1 is a crucial promoter of EMT and metastasis in cancer [16]. ZEB1 inhibits the expression of miR-200 family, which is an inducer of epithelial differentiation, suggesting the effects of ZEB1 in EMT. Moreover, experimental studies have shown that ZEB1 could also suppress the expression of CSC-related miR-203 and that miR-200c, miR-203 and miR-183 cooperate to inhibit CSC markers in cancer cells [16]. In addition, ZEB1 could also directly inhibit the expression of miR-141 which strongly activates epithelial differentiation in pancreatic cancer cells [17]. These findings suggest that ZEB1 could regulate the miRNA mediated EMT, resulting in drug resistance of pancreatic cancer as we have reported earlier [15].

DCAMKL-1 has been known to be a marker of stem cells. DCAMKL-1 regulated miRNAs have been found to play crucial roles in the process of pancreatic cancer EMT, leading to drug resistance in pancreatic cancer. The *in vivo* studies have found that DCAMKL-1 was significantly up-regulated in an established Ki-ras transgenic mouse model of pancreatic cancer and in human pancreatic cancer tissues [18]. Colocalization of DCAMKL-1 with mesenchymal marker vimentin was observed within premalignant PanIN lesions, suggesting its role in EMT. Down-regulation of DCAMKL-1 in human pancreatic cancer cells induced the expression of miR-200a and decreased the expression of ZEB1, ZEB2, Snail, Slug, and Twist [18]. Moreover, inhibition of DCAMKL-1 caused down-regulation of c-Myc and Ki-ras through the regulation of let-7a. Furthermore, the down-regulation of DCAMKL-1 suppressed Notch-1 signaling through a miR-144 dependent mechanism, leading to the inhibition of pancreatic cancer progression and drug resistance [18]. These results demonstrate a strong correlation between DCAMKL-1 regulated miRNA, EMT, and drug resistance in pancreatic cancer, and such knowledge would be helpful in designing novel therapeutics in the future.

FoxM1 plays a crucial role in the control of carcinogenesis and aggressiveness of pancreatic cancer. We have previously shown that over-expression of FoxM1 induced EMT in pancreatic cancer cells with high levels of ZEB1, ZEB2, Snail2, and vimentin expression, and caused pancreatosphere formation with high levels of CSC markers such as CD44 and EpCAM [19]. Forced expression of FoxM1 led to the down-regulation of let-7a, let-7b, let-7c, miR-200b, and miR-200c whereas introduction of miR-200b decreased the levels of ZEB1, ZEB2, and vimentin [19]. These results suggest that FoxM1 is involved in the miRNA regulated EMT and pancreatic CSCs, which could be one of the molecular mechanisms underlying pancreatic cancer drug resistance.

Similar to FoxM1, the activation of Notch-1 also contributes to the miRNA regulated acquisition of EMT and induction of drug resistance. We found that

over-expression of Notch-1 induced EMT phenotype in AsPC-1 pancreatic cancer cells with high levels of EMT and CSC marker expression such as ZEB1, CD44, EpCAM, and Hes-1 [20]. Moreover, the increased expression of Notch-1 led to the up-regulation of miR-21 and down-regulation of miR-200b, miR-200c, let-7a, let-7b, and let-7c, suggesting that miRNAs play important roles in Notch-1 regulated EMT and pancreatic CSCs which are critically involved in chemoresistance of pancreatic cancers [20]. Other investigators also reported that miR-200 inhibited the molecules in the Notch signaling pathway including Jagged1, mastermind-like coactivators Maml2 and Maml3, leading to abrogation of Notch activation stimulated by ZEB1 [21]. These results suggest the importance of miRNAs in Notch regulated drug resistance of pancreatic cancer.

Another signaling pathway involved in the regulation of chemoresistance is hypoxia-inducible factor (HIF) signaling. Emerging evidences suggest that hypoxia and HIF signaling critically contribute to the acquisition of EMT and the maintenance of CSCs, leading to drug resistance of cancer cells. We have found that hypoxia increased the expression of miR-21, miR-210, VEGF, IL-6, and CSC signature gene Nanog, Oct4 and EZH2, leading to increased invasion, angiogenesis, and pancreatosphere formation, all of which are related to drug resistance [22].

The miR-34a has been found to be down-regulated in pancreatic cancer [23]. It has been reported that the treatment of pancreatic cancer cells with de-methylating agent 5-Aza-2′-deoxycytidine (5-Aza-dC) and HDAC inhibitor Vorinostat (SAHA) significantly increased the level of miR-34a, leading to the inhibition of cell proliferation, self-renewal, EMT, and invasion [24]. Moreover, treatment of pancreatic CSCs with SAHA or 5-Aza-dC caused down-regulation of Bcl-2, CDK6 and SIRT1, all of which are targets of miR-34a. The up-regulation of miR-34a in pancreatic CSCs by these agents also induced acetylated p53, $p21^{WAF1}$, $p27^{KIP1}$ and PUMA [24], which could reduce drug resistance of pancreatic CSCs. Moreover, SAHA inhibited Notch pathway and increased E-cadherin expression in pancreatic CSCs, resulting in the inhibition of self-renewal capacity and EMT [24]. These findings suggest that the restoration of epigenetically deregulated miR-34a by SAHA or 5-Aza-dC could eliminate pancreatic CSCs and could also reverse EMT, leading to decreased drug resistance. In addition, pancreatic CSCs with $CD44^+/CD133^+$ could be enriched and isolated from sphere-forming MiaPaCa2 pancreatic cancer cells [25]. These pancreatic CSCs showed loss of miR-34 expression and had high expression of Notch1 and Bcl-2. Re-expression of miR-34 in these pancreatic CSCs significantly decreased sphere formation capacity and tumorigenesis through the inhibition of Notch1 and Bcl-2 expression, and further led to sensitization of $CD44^+/CD133^+$ MiaPaCa2 cells to chemotherapy and radiation [25]. These results demonstrate the inhibitory effect of miR-34 on pancreatic CSCs and EMT, suggesting its roles in the inhibition of drug resistance.

Other miRNAs also contribute to drug sensitivity through the regulation of EMT and CSCs in pancreatic cancers. Recent studies have shown that miR-655 is a novel EMT-suppressive miRNA. Overexpression of miR-655 induced the expression of E-cadherin and directly inhibited the expression of ZEB1 and TGFBR2, leading to the suppression of EMT [26]. The invasive ductal adenocarcinoma (IDA) of

pancreas exhibits poor prognosis because of early invasion, distant metastasis, and drug resistance. It has been found that miR-197 was significantly up-regulated in IDA. Overexpression of miR-197 in pancreatic cancer led to EMT through decreased expression of p120 catenin which is a target of miR-197 [27]. By miRNA profiling analysis of CSCs enriched from spheres, several miRNAs including miR-99a, miR-100, miR-125b, miR-192, and miR-429 have been found to be differentially expressed in pancreatic CSCs [28], suggesting the roles of these miRNAs in drug resistance. Therefore, targeting miRNAs and related signaling could inhibit pancreatic CSCs and EMT, resulting in the sensitization of pancreatic cancer cells to chemotherapy.

3 The miRNAs in the Regulation of Drug Resistance, EMT and CSCs in Prostate Cancer

Prostate cancer is the second leading cause of cancer related death in men in the United States. Patients with advanced prostate cancer are commonly treated with taxane such as paclitaxel and docetaxel; however, most patients eventually become drug resistant. Therefore, elucidating the molecular mechanisms underlying taxane resistance of advanced prostate cancer is important for designing new therapeutic strategy for the successful treatment of prostate cancer.

3.1 miRNA Mediated Regulation of Drug Resistance in Prostate Cancer Cells

Prostate cancer cell metastasis to bone is the major cause for high mortality of prostate cancer patients' after treatment which in part due to acquired drug resistance. The miRNAs are known to play crucial roles in bone metastasis of prostate cancer. It has been found that the expression of miR-143 and miR-145 are significantly down-regulated in metastasis samples. The down-regulation of miR-143 and miR-145 was negatively correlated with bone metastasis, Gleason score, and PSA level of prostate cancer. Re-expression miR-143 and miR-145 in PC3 prostate cancer cells increased E-cadherin expression and decreased the expression of fibronectin and the ability of PC3 cancer cells for migration and invasion [29], suggesting that miR-143 and miR-145 could inhibit EMT and drug resistance as discussed below.

In an experimental study, the paclitaxel-resistant cells (PC3PR) have been developed from parental PC3 prostate cancer cells by long exposure of PC3 cells with low dose of paclitaxel. The study showed that miR-34a was significantly down-regulated in PC3PR cells compared with PC3 cells and that the expression of HuR, Bcl2, and SIRT1 were significantly increased in PC3PR cells [30]. Further

studies showed that the activity of SIRT1 3′-UTR and promoter was up-regulated in PC3PR cells compared to parental cells and that forced expression of miR-34a inhibited the activity of SIRT1 3′-UTR, and the expression of HuR, Bcl2, and SIRT1 [30]. These results demonstrate that miR-34a could target and down-regulate SIRT1, HuR, and Bcl2, leading to increased sensitivity of prostate cancer cells to paclitaxel treatment.

To reveal the reliable markers for drug resistance of prostate cancer, circulating miRNAs in prostate cancer have been tested and evaluated *in vitro*. The studies have shown that several prostate cancer secretory miRNAs (PCS-miRNAs) were spontaneously secreted into the growth medium from DU-145 prostate cancer cells and that the levels of PCS-miRNAs including miR-21 and miR-100 were increased upon treatment of cells with the cytotoxic drug fludarabine [31]. Moreover, studies have shown that PCS-miRNAs were associated with exosomes, suggesting that the secreted miRNAs were released from exosomes. Furthermore, in fludarabine treated cells, the secreted miR-485-3p and its association with exosomes were down-regulated, suggesting that miR-485-3p was retained by surviving cells. Further studies have demonstrated that the molecular regulation in the fludarabine resistance could be mediated through the expression of miR-485-3p mediated regulation in the activation of topoisomerase II alpha, multidrug resistance gene 1 and cyclin B2 [31].

An interesting study has been conducted to investigate whether miR-21 contributes to drug resistance of prostate cancer cells to docetaxel which is a commonly used drug for the treatment of prostate cancer. For this study, a docetaxel-resistant prostate cancer cell line (PC3R) has been developed [32]. By microarray and RT-PCR analysis, the authors have found a significantly higher expression of miR-21 in PC3R cells. Forced expression of miR-21 led to enhanced resistance of parental cells to docetaxel while knockdown of miR-21 in PC3R cells significantly induced sensitivity of PC3R cells to docetaxel [32]. Further experiments have shown that PDCD4 is a direct target of miR-21 and that the up-regulation of PDCD4 by silencing miR-21 increased sensitivity of PC3R to docetaxel [32]. Therefore, targeting miR-21 could be a novel therapeutic strategy for sensitizing cancer cells to docetaxel in the clinical setting. In addition, several miRNAs that could be repressed by EZH2, a regulator of CSCs, have been identified [33]. These miRNAs includes tumor suppressive let-7, miR-15, miR-16, miR-34, and miR-200 which could regulate the expression of BMI1 and RING2, and leading to the inhibition of prostate CSCs characteristics, cell growth and invasiveness [33], suggesting their inhibitory effects on drug resistance.

In addition, aberrant lipid and cholesterol metabolism was found to be involved in prostate cancer development, progression, and drug resistance. It was found that sterol regulatory element-binding protein-1 (SREBP-1) could induce fatty acid and lipid accumulation and AR transcriptional activity, leading to increased prostate cancer cell growth and castration resistance. SREBP-1 was overexpressed in the specimens of castration-resistant prostate cancer patients. However, miR-185 and miR-342 which control lipogenesis and cholesterogenesis found to inhibit the

expression of SREBP-1, resulting in the inhibition of tumorigenicity, cell migration, and drug resistance [34].

Moreover, several clinical trials have been conducted to determine the value of specific miRNA expression in the diagnosis, drug sensitivity screen, and prognosis of prostate cancers (clinicaltrials.gov). Patients with clinically localized high risk prostate cancer have been involved in a clinical trial for assessing the relationship between specific miRNA expression profiles and prostate cancer outcome. The molecular signatures of treatment-sensitive and treatment-resistant prostate cancers were tested in another clinical trial. The differences in DNA sequence pattern, mRNA expression pattern, miRNAs/noncoding RNA pattern, protein expressions, and metabolic products are being investigated between the patients with different drug sensitivities. In addition, the specific miRNA expression profiles in prostate cancer patients treated with abiraterone acetate will be assessed in a clinical trial to investigate the potential mechanisms of resistance to abiraterone acetate (clinicaltrials.gov). The ongoing clinical trials will fulfill the molecular mechanisms underlying the miRNA mediated regulation of drug resistance *in vivo* in prostate cancer.

3.2 miRNA Mediated Regulation of Prostate CSCs and EMT in Drug Resistance

Emerging evidences have indicated that the recurrence and the drug resistance of prostate cancer are linked with prostate CSCs which are reminiscent with the acquisition of EMT phenotype. We have found that prostate cancer cells with EMT phenotype displayed stem-like cell features characterized by up-regulated expression of Sox2, Nanog, Oct4, Lin28B, and Notch1. These cells exhibited increased capacities of prostasphere formation *in vitro* and tumorigenecity in mice *in vivo* [35]. We also found that the expression of miR-200 and let-7 families was down-regulated and that forced expression of miR-200 suppressed the prostasphere formation and the expression of Lin28B and Notch. In addition, down-regulation of Lin28B led to increased let-7 expression and decreased self-renewal capacity [35]. These findings suggest that prostate CSCs and EMT-type cells share similar biological phenotype and that both cells could contribute to prostate cancer drug resistance and recurrence mediated through the regulation of miRNAs.

Prostate cancers often relapse due to the occurrence of drug resistance to taxane which is the first line drug for chemotherapy. A study has been conducted to investigate the role of miRNAs and related cellular signaling in prostate cancer with drug resistance and evaluated the effects of combination treatment with paclitaxel and Hh inhibitor cyclopamine [36]. The study found that the paclitaxel resistant cell lines and human prostate cancer tissues possessed a distinct CSC like populations [36]. These CSC like cells had higher expression of stem cell markers.

Combination treatment with paclitaxel and cyclopamine showed significantly improved cytotoxicity and also significantly reduced CSC like cells, suggesting the effectiveness of combination therapy. Combination treatment also decreased the level of P-gp efflux protein in resistant cells, suggesting increased drug sensitivity. The miRNA profiles of DU145 and PC3 drug-resistant cells and prostate cancer tissue showed significantly lower levels of tumor suppressive miRNAs including miR-34a and miR-200c. Moreover, combination treatment increased the expression levels of miR-200c and miR-34a [36]. These results suggest that miRNAs are important and regulate the functions of CSCs in prostate cancer associated with paclitaxel resistance and that the combination treatment could increase drug sensitivity through the up-regulation of tumor suppressive miRNAs.

It is known that CD44 is a marker of CSCs and the direct target of miR-34a. In prostate CSCs with $CD44^+$ (positive) cells, the expression of miR-34a was significantly down-regulated. Transfection of miR-34a mimic into the prostate CSCs significantly reduced tumor regeneration and metastasis [37]. Moreover, introduction of miR-34a antagomirs into $CD44^-$ (negative) prostate cancer cells enhanced tumor development and metastasis. Furthermore, systemic delivery of miR-34a inhibited cancer metastasis, suggesting that miR-34a could inhibit prostate CSCs through down-regulation of its target CD44. Similar to miR-34a, the level of miR-320 was found to be significantly lower in prostate CSCs in $CD44^+$ cells [38]. Re-expression of miR-320 into prostate CSCs inhibited CSC phenotypes and suppressed tumorigenesis *in vivo*. Moreover, the expression of miR-708 was also found to be down-regulated in prostate CSCs with $CD44^+$ cells [39]. Forced expression of miR-708 in the prostate CSCs was found to inhibit tumorigenicity by targeting CD44 and AKT2 [39]. In addition, miR-101 is known to regulate EZH2 expression, which plays an important role in the regulation of drug resistance in prostate CSCs. EZH2 has been found to be up-regulated and miR-101 was down-regulated in prostate CSCs [40]. Inhibition of EZH2 significantly suppressed cell growth and promoted apoptosis [40], suggesting that high level of EZH2 in prostate CSCs contributes to the maintenance of prostate CSC survival and drug resistance. These results together demonstrate that miR-34a, miR-320, miR-708, and miR-101 have inhibitory effects on prostate CSCs, suggesting that they could enhance drug sensitivity by eliminating prostate CSCs; however, further pre-clinical and clinical studies are warranted in this exciting area.

Several molecules have been found to regulate epithelial or mesenchymal cell makers, and thereby controlling the processes of EMT. These molecules are known to cross-talk with deregulated expression of miRNAs. Slug is a major regulator of mesenchymal differentiation. It has been found that down-regulation of Slug inhibits EMT by targeting miR-1 and miR-200 during tumorigenesis whereas forced expression of miR-1 or miR-200 inhibited both EMT and tumorigenesis in prostate cancer [41]. Basal protein B-cell translocation gene 2 (BTG2) is an epithelial regulator. Down-regulation of BTG2 promoted normal prostate basal cells to express luminal markers, which is a known phenotype in EMT. It has been found that the down-regulation of BTG2 in prostate cancer was in part due to high expression of miR-21 [42], suggesting the roles of miR-21 regulated BTG2 in

EMT of prostate cancer. We also found that overexpression of PDGF-D in prostate cancer cells (PC3 PDGF-D cells) could lead to the acquisition of EMT phenotype. Furthermore, we showed that forced expression of miR-200b in PC3 PDGF-D cells led to the reversal of EMT phenotype mediated through the down-regulation of ZEB1, ZEB2, and Snail2 expression. Moreover, transfection of PC3 PDGF-D cells with miR-200b led to the suppression of cell migration and invasion [43], suggesting that miR-200b could regulate the processes of EMT which could be responsible for controlling drug sensitivity in prostate cancer. In addition, p63 could also inhibit EMT and metastasis of prostate cancer cells through the up-regulation of miR-205 [44] which has inhibitory effects on the markers of EMT including ZEB1 and vimentin [45]. The miR-23b is another miRNA which is known to inhibit EMT. High expression of miR-23b has been found to be positively correlated with higher overall and recurrence-free survival in patients with prostate cancer. It was found that miR-23b directly inhibited proto-oncogene Src kinase and Akt, suggesting the tumor suppressive role of miR-23b. Overexpression of miR-23b inhibited EMT through up-regulation of E-cadherin and down-regulation of Vimentin and Snail, leading to the inhibition of tumor growth [46]. All of these results suggest that miRNAs play important role in the regulation of EMT and CSC in prostate cancer drug resistance. Therefore, targeting miRNAs could become a promising strategy for the treatment of drug resistant prostate cancers.

4 Targeting miRNA for Increasing Drug Sensitivity, Reversing EMT, and Eliminating CSCs in Pancreatic and Prostate Cancers

Because miRNAs critically regulate EMT and CSCs which are the major culprits for drug resistance, targeting deregulated miRNAs in EMT-type cells and CSCs could be a promising approach for increasing drug sensitivity toward the treatment of pancreatic and prostate cancers. The therapeutic strategies for targeting deregulated miRNAs in EMT-type cells and CSCs include antisense oligonucleotide delivery to inhibit oncogenic miRNAs, sense oligonucleotide delivery to introduce tumor suppressive miRNAs, and regulation of drug resistant-related miRNAs by synthetic or natural agent administration. The better treatment outcome could be achieved for cancer therapy using these strategies through eliminating EMT-type cells and CSCs. The synthetic oligonucleotides are more specific while synthetic small molecule or natural agents have pleiotropic effects on miRNAs and cellular signaling, which may be a good attributes for treating cancer because of their heterogeneous molecular and cellular characteristics.

4.1 Synthetic Small Molecule or Natural Agents for Targeting miRNA

In 2009, the first synthesized small molecule, salinomycin was investigated for the inhibition of CSCs function. It was found that salinomycin exhibited CSC-specific toxicity with 100 times more effective compared to anti-cancer drug paclitaxel [47]. Salinomycin significantly suppressed tumor growth and induced epithelial differentiation of cancer cells in mice [47], suggesting that salinomycin could be used for the reversal of EMT. In pancreatic cancer, salinomycin combined with gemcitabine significantly inhibited cell growth of pancreatic cancer cells by targeting both pancreatic CSCs and non-CSCs [48], suggesting that salinomycin could be used for increasing drug sensitivity. However, clinical studies incorporating salinomycin would be required for the treatment of pancreas and other human malignancies.

Natural agents are also known as nutraceuticals which could be used for the killing of CSCs and EMT-type cells in order to overcome drug resistance. We have found that natural agents including isoflavone and 3,3′-diindolylmethane (DIM) could inhibit CSCs and EMT-type cells through the regulation of miRNAs and related cell signaling pathways [15] in pancreatic and prostate cancer cells. Through the up-regulation of miR-200 and let-7 expression which could inhibit the growth of EMT-type cells and CSCs, isoflavone and DIM significantly increased sensitivity of pancreatic cancer cells to gemcitabine, and consequently suppressed invasion of pancreatic cancer cells [15]. We also found that isoflavone suppressed pancreatic cancer cell proliferation, migration, invasion, EMT phenotype, and pancreatosphere formation through the up-regulation of miR-200 and the down-regulation of FoxM1, CD44 and EpCAM which are CSC makers [19], suggesting that miRNA regulated FoxM1 over-expression in EMT type cells and CSCs could be inhibited by natural agents [19]. Moreover, we have also found that DIM could increase the levels of let-7 and miR-34a, resulting in decreased expression of CSC signatures, and led to the suppression of growth of prostate cancer cells [49, 50]. Furthermore, we found that the treatment of prostate cancer cells with BR-DIM (formulated DIM with greater bioavailability) up-regulated let-7 and down-regulated EZH2 expression, leading to the inhibition of self-renewal and clonogenic capacity of prostate cancer cells [50]. Importantly, BR-DIM intervention in patients with prostate cancer prior to radical prostatectomy caused increased level of let-7 and the decreased level of EZH2 in prostate cancer tissue specimens. These results suggest that the loss of let-7 and the subsequent increased expression of EZH2 contribute to the emergence of prostate CSCs and drug resistance, which were inhibited by BR-DIM [50]. These results further suggest that future therapeutic trials of BR-DIM in combination with conventional therapeutics are warranted.

In our effort for finding novel synthetic small molecule, we found that CDF which is a curcumin analogue, showed greater bioavailability in animal model. We have found that CDF was very effective in causing down-regulation in the expression of oncogenic miR-21 and also up-regulated the expression of several tumor

suppressive miRNAs such as miR-26a, miR-101, miR-146a, miR-200, and let-7 [51, 52]. Most of these tumor suppressive miRNAs are known to contribute to the regulation of molecular markers of pancreatic CSCs and EMT-type cells. Further investigation showed that CDF increased drug sensitivity and suppressed tumor growth and aggressiveness of pancreatic cancer through the regulation of miRNA-mediated EZH2, VEGF, IL-6, and Akt signaling [45, 51–54]. Moreover, CDF treatment was found to down-regulate the expression of Notch-1, CD44, EpCAM, and Nanog which are the markers of CSCs [52]. These results suggest the beneficial effects of CDF on the inhibition of pancreatic CSCs and drug resistance, and thus further development of CDF for clinical studies are warranted.

Metformin has been found to have the ability in killing CSCs. We have found that metformin significantly inhibited the capacities of cell proliferation, clonogenicity, wound-healing, and sphere-forming in both gemcitabine-sensitive and gemcitabine-resistant pancreatic cancer cells. Metformin was also found to down-regulate the expression of CSC markers such as CD44, EpCAM, EZH2, Notch-1, Nanog and Oct4. Moreover, metformin was able to significantly up-regulate the expression of EMT suppressive miRNAs such as let-7a, let-7b, miR-26a, miR-101, miR-200b, and miR-200c. All of these miRNAs are typically lost in pancreatic cancer and especially in pancreatic CSCs. Therefore, these results clearly demonstrate that the effects of metformin are mediated through miRNA regulated CSC signaling in pancreatic CSCs [55], which could be exploited for therapeutic intervention of patients diagnosed with pancreatic cancer.

All the findings described above suggest that synthetic small molecule or natural agents could induce sensitivity of pancreatic or prostate cancer cells to chemotherapeutics and that the combination treatment with nutraceuticals and commonly used chemotherapeutics could be a promising strategy for overcoming drug resistance by targeting EMT-type cells, CSCs, and cancer cells via the modulation of miRNAs.

4.2 Oligonucleotide Delivery

Synthesized miRNA oligonucleotides could be delivered by lipid-based formulations and nanoparticles [56, 57]. The oncogenic miRNAs are commonly up-regulated in CSCs or EMT-type cells in cancers with drug resistance. Experimental studies have shown that intravenous administration of chemically engineered anti-sense oligonucleotides against oncogenic miRNAs into mice could significantly lower the levels of oncogenic miRNAs [58], suggesting that silencing oncogenic miRNAs could be achieved by anti-sense oligonucleotide delivery.

The introduction of tumor suppressive miRNAs by sense oligonucleotide delivery is another important strategy for enhancing drug sensitivity through eliminating EMT-type cells and CSCs. To further investigate the therapeutic strategy using specific miRNA for the treatment of pancreatic cancer, a nanoparticle for delivery

of vectors (nanovector) which express miRNA has been developed [59]. The nanovectors had been used for the delivery of miR-34a and miR-143/145 which are down-regulated in the majority of pancreatic cancers. The miR-34a is known to regulate CSC survival and the miR-143/145 cluster down-regulates the expressions of KRAS2 and Ras-responsive element binding protein-1 (RREB1). It was found that the delivery of miR-34a or miR-143/145 suppressed tumor growth of subcutaneous and orthotopic xenografts of MiaPaCa-2 cells with increased apoptosis and decreased proliferation [59]. The miRNA delivery led to significantly increased corresponding miRNA and decreased miRNA targets including SIRT1, CD44, aldehyde dehydrogenase, KRAS2, and RREB1 [59]. These results suggest that the nanovector is useful for the systemic miRNA delivery for increasing drug sensitivity by targeting CSCs, and such a strategy could be useful in patients.

The miR-199b-5p has been shown to regulate Hes-1, a downstream effector of Notch and Hedgehog singling. A stable nucleic acid lipid particle (SNALP) that encapsulates miR-199b-5p has been developed and tested for the efficacy of delivery in prostate cancer. It was found that treatment with SNALP miR-199b-5p significantly decreased the levels of Hes-1 and CSC markers, leading to the inhibition of cell proliferation [60]. These findings demonstrate the proof-of-concept for the efficacy of SNALP for miRNA delivery and down-regulation of targets in prostate cancer; however, this pre-clinical knowledge must be translated in the clinical setting. In another experimental study, a new delivery system was developed to deliver tumor suppressive miR-15 and miR-16 into the prostate cancer cells which express prostate-specific membrane antigen (PSMA) [61]. The new system was a second-generation RNA aptamer A10-3.2 designed as a PSMA-targeting ligand conjugated to a polyamidoamine (PAMAM)-based miRNA vector for the delivery of miR-15 and miR-16. This delivery system was able to effectively deliver miR-15 and miR-16 into prostate cancer cells overexpressing PSMA, which led to significant inhibition of growth of prostate cancer cells [61]. In addition, no toxicity of this delivery system was observed, suggesting that using nanocarrier-based targeted delivery of miRNAs could be a novel approach for the treatment of prostate cancer, which must be tested clinically.

5 Conclusions

Emerging evidences have shown that EMT-type cells and CSCs contribute to *de novo* and acquired drug resistance and that certain miRNAs are critically involved in the regulation of EMT, CSCs, and drug resistance characteristics of cancer cells (Fig. 12.1). Therefore, targeting deregulated miRNAs in EMT-type cells and CSCs toward reversal of EMT and elimination of CSCs would be a promising strategy for overcoming drug resistance for the treatment of pancreatic and prostate cancers. It is believed that the strategies for up-regulation of let-7, miR-15/16, miR-200, miR-143/145, and miR-34a or down-regulation of miR-21, miR-155, miR-197, and miR-221/222 would be able to increase drug sensitivity by reversal of EMT

and elimination of CSCs. Therefore such novel strategies would likely improve the treatment outcome. To that end, sense or antisense oligonucleotide delivery system could be useful for targeting specific miRNAs that are aberrantly expressed in drug-resistant cancer cells. However, the poor stability, immune system stimulation, and off-target effects of oligonucleotides delivered *in vivo* limit the use of these oligonucleotides in miRNA targeted therapy at present, suggesting that further innovative research in this area is urgently needed. Moreover, synthetic small molecule or nutraceuticals could provide more pleiotropic effects on miRNAs and related cellular signaling; however, more studies are needed to uncover the specific targets of miRNAs within the biological context associated with ultimate biological consequence after treatment in pre-clinical models and ultimately in the clinical setting. We believe that overcoming drug resistance by miRNA targeted therapy could be achievable by optimizing the strategies of miRNA targeted therapy in combination with conventional therapy. However, more in-depth pre-clinical experimental studies followed by well-designed clinical trials are needed in order to enjoy the benefit of miRNA targeted therapy for overcoming therapeutic resistance (by targeting drug-resistant cells such as EMT phenotypic cells and CSCs) toward successful treatment of pancreatic and prostate cancers.

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Chapter 13
Modulation of Deregulated MicroRNAs for Target Therapy in Thyroid Cancer

Cesar Seigi Fuziwara and Edna Teruko Kimura

1 Introduction

Thyroid cancer is the most frequent endocrine malignancy, the incidence of which has doubled in the last decade in the USA [1, 2]; and is the fifth leading cause of cancer in women, in line with the increase in global thyroid cancer incidence [3].

The thyroid gland is composed of two different cellular types that give rise to two major groups of cancer: follicular cell-derived thyroid cancer (PTC/FTC/ATC) that corresponds to 97 % of cases, and C-cell-derived thyroid cancer (medullary thyroid cancer) corresponding to 3 % of cases. Follicular cell-derived thyroid cancer histotypes are described in Table 13.1 and predominantly comprise Papillary Thyroid Cancer (PTC), followed by Follicular Thyroid Cancer (FTC), and less frequently Anaplastic Thyroid Cancer (ATC). Follicular-derived thyroid cancer can be further divided in three subgroups according to the differentiation level of the thyroid follicular cells, or their similarity to the original thyroid gland, namely well-differentiated (PTC + FTC), poorly-differentiated (PDTC) and undifferentiated (ATC) thyroid cancer [4].

The overall outcome of thyroid cancer is generally favorable. However, poorly-differentiated thyroid carcinoma, which includes a subset of papillary thyroid carcinoma and follicular thyroid carcinoma, exhibits aggressive behavior that does not respond to the conventional therapy of thyroidectomy and radio-iodine ablation treatment of remaining thyroid tissue, leading to tumor recurrence and death in the subsequent 5 years [5–7]. Moreover, anaplastic thyroid carcinoma, the

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Table 13.1 Classification of thyroid cancer of follicular origin and prevalence

Classification of follicular cell differentiation status		
Well-differentiated	***Poorly-differentiated***	***Undifferentiated***
PTC (80 %)	**PTC + FTC (5 %)**	**ATC (2 %)**
FTC (10 %)		
good-outcome		**poor-outcome**

PTC papillary thyroid cancer, *FTC* follicular thyroid cancer, *ATC* anaplastic thyroid cancer

most aggressive and fast-growing histotype of thyroid cancer, is unresponsive to therapy and leads to patient death within 6 months [8]. The lack of therapy for aggressive thyroid carcinoma urges a faster bench-to-bed translation of cancer molecular knowledge such as genetic, epigenetic and gene expression modifications in cancer, in order to develop new treatment strategies.

2 Thyroid Cancer Oncogenes

Genetic alterations promote papillary thyroid carcinoma, and are aligned in genes of the mitogen-activated protein kinase (MAPK) signaling pathway namely: *RAS*, *BRAF* and *RET* (Table 13.2). The $BRAF^{T1799A}$ mutation is frequently detected in papillary thyroid cancer (~40 % of cases), but is also found in anaplastic thyroid cancer patients [9–11]. The $BRAF^{T1799A}$ mutation and *RET*/*PTC* rearrangements (RET tyrosine kinase domain fused to a heterologous gene) are markers of malignancy. *RET*/*PTC* and *RAS* mutations at codons 12, 13 and 61 are prevalent in classic and follicular variants of papillary thyroid cancers, respectively [12, 13]. Moreover, *RAS* mutations are also detected in other types of thyroid cancer, such as follicular and anaplastic thyroid cancer. Among the distinct molecular effects caused by MAPK oncogenes, the most significant is the constitutive activation of ERK signaling. The *RET*/*PTC*, *RAS* and *BRAF* oncogenes drive thyroid tumorigenesis, demonstrated by transgenic animal models that harbor these genetic alterations, which develop thyroid cancer with characteristic histopathology [14–16].

A plethora of studies have analyzed the correlation of the $BRAF^{T1799A}$ mutation with prognostic characteristics of papillary thyroid cancer. The presence of the $BRAF^{T1799A}$ mutation is associated with characteristics of poor prognosis in patients, such as extra-thyroidal invasion, lymph node metastases, recurrence, and progression to a more aggressive disease [11, 19–21]. However, a conclusive understanding of the influence of BRAF in clinical characteristics remains to be elucidated, as some studies have observed that there was no clear association [22, 23]. Thyroid follicular cell oncogenesis can be experimentally induced *in vivo*. A transgenic mouse model for thyroid gland-restricted $BRAF^{T1799A}$ activation showed that the BRAF oncogene induces proliferation of thyroid follicular cells, leading to papillary thyroid cancer.

Table 13.2 Genetic alterations in thyroid cancer of follicular origin

Histopathological classification		Primary genetic alteration				Secondary genetic alteration				
		RET/PTC	RAS	BRAF	PAX8-PPARγ	TP53	AKT	PIK3CA	PTEN	CTNNB1
PTC	Classic	x		x					x	
	Follicular variant		x	x						
	Tall-cell			x			x	x		
FTC			x		x			x	x	
PDTC		x	x	x		x	x	x		x
ATC			x	x		x	x	x	x	x

PTC papillary thyroid cancer, *FTC* follicular thyroid cancer, *PDTC* poorly-differentiated thyroid cancer, *ATC* anaplastic thyroid cancer [6, 17, 18]

Moreover, this papillary thyroid cancer undergoes de-differentiation through activation of the epithelial-to-mesenchymal transition (EMT) [16], recapitulating thyroid cancer progression to more aggressive histotypes.

As MAPK signaling is the main oncogenic pathway in thyroid cancer, molecular targeting of this pathway, at multiple points of the signal cascade, would be an imperative therapeutical approach to block this oncogenic signaling. To this end, an array of specific inhibitors of the MAPK signaling pathway was developed. Sorafenib (Nexavar®), which blocks tyrosine kinase receptor and RAF kinase [24] activation has been recently approved by the U.S. Food and Drug Administration (FDA) for the treatment of radio-iodine refractory recurrent or metastatic thyroid cancer. Other inhibitors, such as Selumetinib, which blocks MEK1 and MEK2 [25], along with Vemurafenib, which blocks $BRAF^{V600E}$ [26], are currently under clinical trial in patients. However, despite exhibiting promising suppressive effects in the culture of cancer cells [27, 28], many of these inhibitors provoke pronounced side effects and fail to completely block tumor growth and progression.

Intense efforts have been made in order to understand the influence of genetic alterations in cancer. In the last decade, the discovery of the regulatory function of non-coding RNAs has expanded their significance, pointing to their key participation in the oncogenic process. Of particular importance is the family of microRNAs (*miRNAs*), which are a class of small non-coding RNAs (~22 nt) that blocks target mRNA translation via post-transcriptional regulation. In cancer, the alteration of *miRNA* expression potentiates oncogenic processes, activated by a driver oncogene, by two mechanisms (Fig.13.1): (1) up-regulation of a specific *miRNA* that may decrease translation of tumor suppressor genes or (2) down-regulation of a specific *miRNA* that may increase translation of a proto-oncogene [29]. Thus, *miRNA* deregulation is able to modulate tumorigenesis, acting in confluence with oncogenes and tumor suppressor deregulation [30].

The role of *miRNA* in cancer has been investigated by several high-throughput *miRNA* gene expression studies that have identified *miRNA* expression patterns for cancer. Analysis of a set of tumors including prostate, breast, lung, pancreas, colon and stomach, has led to the discovery of a common signature of up-regulated

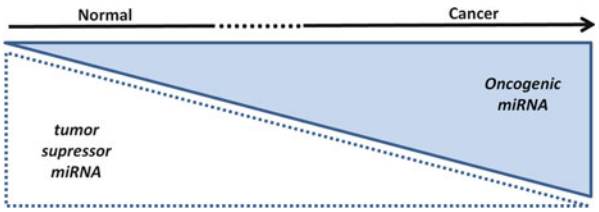

Fig. 13.1 Tumor suppressor *miRNAs* are highly expressed in normal thyroid gland. However, during thyroid malignant transformation, activated by driver mutations, expression of oncogenic *miRNAs* is enhanced, while tumor suppressor *miRNA* expression is inhibited

miRNA in malignant tissues, which comprised the *miRNAs: miR-21, miR-17-5p, miR-191, miR-146* and *miR-20a* [31]. Of particular interest, this deregulated *miRNA* signature in solid tumors is consistent and highly specific for malignancy distinction, and even more accurate than any mRNA array signature [32].

3 Targeting *miRNA* in Thyroid Cancer

The *miRNA* expression profile shows distinctive *miRNA* deregulation according to tumor stage and histotype of thyroid cancer. Interestingly, in the well-differentiated thyroid cancers, an overall increase in *miRNA* expression prevails, while in undifferentiated thyroid cancers, an overall reduction in *miRNA* is observed [33–35].

In a seminal study, He et al. showed a panel of deregulated *miRNAs* by microarray analysis, which included pronounced over-expression of *miR-221*, *miR-222* and *miR-146b* in a set of papillary thyroid cancers, that clearly distinguished this type of cancer from normal thyroid tissue [36]. Subsequently, Pallante et al. revealed another potential papillary thyroid cancer signature, composed of over-expression of *miR-221, miR-222* and *miR-181b*, and down-regulation of *let-7f* [33]. As papillary thyroid cancer oncogenesis is triggered by different MAPK driver oncogenes, distinctive *miRNA* expression profiles are predicted according to each oncogene. Indeed, activation of either $BRAF^{T1799A}$ or *RET*/*PTC* in normal human thyroid follicular cells generates a profile of specifically deregulated *miRNA*, but maintains a common set of deregulated *miRNAs,* irrespective of mutational background [37, 38]. Particular *miRNA* deregulation occurs in the other well-differentiated thyroid cancer histotype. In follicular thyroid carcinoma, a limited set of *miRNAs* was deregulated, with over-expression of *miR-197* and *miR-346* [35].

Based on consistent *miRNA* expression changes observed in thyroid cancer, *miRNA* research is being used as a cutting-edge application in thyroid translational medicine, for molecularly screening and discrimination of malignant thyroid tumors from benign ones [39, 40]. A common *miRNA* signature associated with a papillary thyroid cancer malignant phenotype involves *miR-146b*, *miR-221*, *miR-222* and *miR-21* [40–42], and the detection of these *miRNAs* is suggested as an

additional molecular investigation, by means of Fine Needle Aspiration Biopsy (FNAB). Moreover, recent studies have indicated that *miRNA* deregulation is also associated with aggressive thyroid cancer and a poor outcome, showing the potential of *miRNAs* as molecular markers of prognosis [43]. Aggressive thyroid cancer comprises a set of histotypes that often leads to relapse after surgery, and the loss of the ability to concentrate radioiodine. Analysis of *miRNA* expression in anaplastic thyroid carcinoma (the most aggressive histotype) demonstrated a decreased expression of *miR-30*, *miR-26*, *miR-125, miR-92* and *let-7*, together with an increase in *miR-21, miR-146, miR-221, miR-22, miR-17* and *miR-19* [34, 40, 44, 45]. Therefore, patients with a poor prognosis would benefit from a *miRNA*-directed molecular therapy as these small RNAs interfere simultaneously in different oncogenic signaling pathways.

Expression studies have uncovered deregulated panels of *miRNAs* in distinct histotypes of thyroid cancer, as well as expression signatures associated with a poor outcome. However, less is known about deregulated *miRNA* biology in cancer. In the following section, we will explore molecular and functional aspects of certain thyroid cancer-related *miRNA*s.

3.1 MiR-146

miR-146b has become a new marker in papillary thyroid cancer outcome, associated with a malignant phenotype as its deregulation occurs almost exclusively in thyroid cancer [33, 36, 46]. *miR-146b* firstly appeared as the strongest up-regulated *miRNA* in a set of papillary thyroid cancer samples analyzed by He et al. [36]. Since then, a number of studies have substantiated this finding, and *miR-146b* has become a selective thyroid cancer marker, being predominantly over-expressed in papillary and anaplastic thyroid cancer [40, 44].

Clinical-pathological studies have indicated that *miR-146b* is associated with a more aggressive phenotype of papillary thyroid cancer, and a poorer prognosis. Analysis of *miR-146b* expression in a set of thyroid cancer samples revealed that aggressive papillary thyroid cancer displayed over-expression of *miR-146b* compared to non-aggressive cancer [43]. Moreover, *miR-146b* is an independent risk factor for thyroid cancer, as patients with higher levels of *miR-146b* exhibited larger tumors, extra-thyroid invasion, a higher tumor node metastasis stage, and had a significantly poorer overall survival [47–49]. It is also important to mention that in lung squamous-cell carcinoma, higher levels of *miR-146b* are an indication of poorer overall survival [46].

Considering a BRAF-mutated set of papillary thyroid cancers, higher expression levels of *miR-146b* were detected compared to non-BRAF mutated samples [47]. Moreover, within the group of BRAF–mutated tumors, those that expressed the highest levels of *miR-146b* exhibited the most aggressive behavior [43]. Interestingly, increased plasma circulating levels of *miR-146b* can be detected in papillary thyroid cancer pre-surgery, which also correlates with tumor aggressiveness [50].

Regulation of *miR-146b* gene expression by oncogenic signaling is still under investigation. Isoforms of *miR-146* are transcribed by two independent genes in the human genome: *miR-146a* at chromosome 5 and *miR-146b* at chromosome 10. These isoforms differ by two nucleotides in the 3′ region, but maintain the seed region and, therefore, share common targets. Analysis of the *miRNA* promoter region indicated that both *miR-146a* and *miR-146b* genes exhibit NFκB binding sites, demonstrating regulatory feedback of this signaling pathway, which is usually deregulated in thyroid cancer. NFκB signaling was strongly activated in anaplastic thyroid cancer, which showed robust nuclear staining for RelA (p65), the subunit of the NFκB dimer, while no stain was observed in normal thyroid cells, and faint staining was observed in papillary thyroid cancer [51]. Indeed, in anaplastic thyroid cancer, *miR-146a* up-regulation was found to be associated with activation of NFkB signaling [52]. Interestingly, when analyzing a set of aggressive papillary thyroid cancer samples with local invasion, over-expression of NFκB signaling component genes in the invasive front of the tumors was detected, compared to the central regions [53]. Moreover, oncogenic BRAF activation in human thyroid cell lines resulted in increased NFκB binding activity in specific sites, up-regulation of anti-apoptotic proteins, and activation of metalloproteinases involved cell migration and invasion [54]. Interestingly, ectopic over-expression of *miR-146b* in human papillary thyroid cancer cell lines harboring $BRAF^{T1799A}$ and *RET*/*PTC1* mutations increases cell migration and invasion [48], indicating a molecular role for *miR-146b* in thyroid cancer progression.

Fewer studies have explored the functional role of *miR-146b* in the regulation of specific signaling pathway targets. In this context, a recent study elucidated the influence of *miR-146b* targeting upon SMAD4, an important transducer of TGFβ signaling to the nucleus Using an *anti-miR* approach to inhibit endogenous high levels of *miR-146b-5p* in human papillary thyroid cancer cell lines, Geraldo *el al.* observed that transient *anti-miR-146b-5p* incubation in the papillary thyroid cancer cell line, BCPAP, restored SMAD4 protein levels. Moreover, papillary thyroid cancer cell responsiveness to TGFβ signal was enhanced, leading to an increased SMAD4 accumulation in the nucleus, and transcription of its target genes such as *CDKN1A* (p21) [55]. TGFβ signaling exerts an important anti-mitogenic effect in normal thyroid follicular cells [56, 57]. Recent studies have shown that there is a deregulated expression of TGFβ signaling components in human thyroid tumors [58–61], implicating an unbalanced responsiveness to the TGFβ anti-mitogenic effect, and an epithelial-to-mesenchymal transition (EMT) in more aggressive and invasive thyroid tumors [53]. Other important validated targets of *miR-146* are shown in Table 13.3, such as the specific proteins related to NFκB signaling in the immune response, IRAK1 and TRAF6 [62].

Interestingly, few types of cancer show *miR-146b* deregulation. Unlike thyroid cancer, *miR-146b* is down-regulated in breast cancer, resulting in increased EGFR and NFκB signaling [64, 65], while in glioma, *miR-146b* influences cell migration and invasion by targeting the metalloproteinase MMP-16 [66].

In thyroid cancer, *miR-146b* plays a crucial role in oncogenesis and progression. Therefore, targeted inhibition of *miR-146b* would be a promising novel approach in

Table 13.3 *miR-146b* validated targets

	NFkB signaling	RTK signaling	TGFβ signaling	Cell cycle	Invasion
miR-146b	TRAF6 IRAK1 NFKB1	KIT EGFR	SMAD4	CDKN1A	MMP16

Source: TarBase [63]

the treatment of thyroid cancer. *In vitro* studies using thyroid cancer cell lines have shown that inhibition of *miR-146b* via *antagomiR* (*antimiR*), an antisense oligonucleotide with LNA (locked nucleic acid) modification, reverts specific *miR-146b*-mediated effects in papillary thyroid cancer cells. Moreover, targeted blockage of *miR-146b* in aggressive refractory thyroid cancer is expected to translate into important clinical implications, as *miR-146b* is associated with a poor outcome.

3.2 Let-7 *Family*

Although *miRNA* was initially discovered in 1991 in *C. elegans*, as a small RNA, *lin-4*, that regulated lin-14 protein levels, via interaction with 3-UTR, its importance remained unrecognized until 2004, when it was first associated with human cancer [67]. Loss of *let-7a* expression was associated with a poor prognosis in lung cancer, and *let-7* was the first *miRNA* to have a validated target involved in tumorigenesis described in mammals, namely the proto-oncogene *RAS* mRNA, which is associated with a poor prognosis of lung cancer [67, 68]. Since then, several studies have reported *miRNA* deregulation in many different human tumors, but few have pointed to the functional role of *miRNA* in cancer or the mechanism of transcriptional activation.

Let-7 family *miRNAs* are abundantly expressed in normal thyroid gland (*let-7a, let-7b, let-7c, let-7d, let-7e, let-7f* and *let-7 g*) [69]. Thyroid gland exhibits restricted proliferative capacity that is mainly controlled by pituitary TSH (Thyroid stimulating hormone) in response to fluctuations in thyroid hormone serum levels. However, oncogene activation may bypass this physiological regulation, and promote malignant transformation of thyroid cells into cancer.

The first evidence of *let-7* involvement in thyroid tumorigenesis was described by a *miRNA*-microarray approach study that showed *let-7f* down-regulation in a set of human papillary thyroid cancer samples [33]. Moreover, an overall reduction in the expression of *let-7* isoforms in papillary thyroid cancer has recently been shown through next-generation deep sequencing [70]. Interestingly, oncogenic *RET*/*PTC3* activation in normal thyroid cells *in vitro* has been shown to strongly repress *let-7f* expression. The reintroduction of *let-7f* in a human papillary thyroid cancer cell line harboring *RET*/*PTC* rearrangement induced proliferation arrest via blockage of

ERK phosphorylation, reduction of *CCND1* levels, and induction of P21 expression [71]. In addition, reintroduction of *let-7f* in the thyroid cancer cell line, TPC-1, restored expression levels of *TTF-1* (thyroid transcription factor-1), an important transcription factor involved in thyroid gland differentiation and function, which influences iodine trapping capacity that is usually impaired in aggressive thyroid cancer.

Another isoform of the *let-7* family, *let-7a*, is down-regulated in follicular thyroid carcinoma, a histotype that frequently displays RAS mutations. Indeed, induction of *Hras* in normal rat cells strongly reduced *let-7a* expression. Reintroduction of *let-7a* in a follicular carcinoma cell line, WRO, rescued the epithelial phenotype, characterized by a flattened and adherent morphology, and reduced cell migration via targeting of *FXYD5* (dysadherin) [72].

Deregulation of *let-7* also occurs in aggressive thyroid cancer. Poorly differentiated and anaplastic thyroid cancer samples were shown to exhibit reduced expression of *let-7c,* with a slightly increased expression of *let-7a* and *let-7f* [34, 44]. Interestingly, in a lung adenocarcinoma model, reduced expression of *let-7c* is associated with chemo- and radio-therapy resistance *in vitro* [73]. Indeed, ectopic reintroduction of *let-7c* led to restoration of chemo- and radio-sensitivity, and blocked the EMT in lung cancer. In Table 13.4, some validated *let7* family targets are listed.

3.3 Cluster miR-17-92

A group of seven different *miRNAs*, known as the *miR-17-92* cluster (also known as oncomiR-1), was described to be over-expressed in B-cell lymphoma and lung cancer, and contributed to oncogenesis [74, 75]. *MIR17HG* [miR-17-92 cluster host gene (non-protein coding)] is located at chromosome 13 (13q31.3) and is the host gene for the *miR-17-92* cluster. The *miR-17-92* cluster is transcribed as a polycistronic primary *miRNA* that, after processing, yields seven mature *miRNAs*: *miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a*.

Over-expression of the *miR-17-92* cluster *miRNAs* is observed in several types of solid cancers such as breast, lung, colon, prostate and pancreas [31]. The shared *miRNA* signature of different cancers includes *miR-17-5p*, *miR-20a* and *miR-92*, indicating the participation of these *miRNAs* in the process of tumorigenesis. Moreover, *miR-17-92* cluster components may contribute to elicit poor outcome as aggressive lung cancer with a poor prognosis exhibits an incremental expression of the cluster [74]. In addition, combined expression of *miR-17-3p* and *miR-19b-1* interacts with c-Myc expression to accelerate tumor development in a mouse B-cell lymphoma model [75].

In thyroid cancer, several studies have shown *miR-17-92* deregulation. Using a high-throughput *miRNA* sequencing methodology, over-expression of *miR-17* in papillary thyroid cancer was recently determined [70]. Moreover, studies showed that aggressive undifferentiated anaplastic thyroid cancer exhibits high levels of the

Table 13.4 *Let-7f* validated targets

	Pro-oncogenic signaling		Cell cycle	
Let-7 family	NRAS	LIN28B	E2F1	CDC25A
	HRAS	NKFB1	E2F2	CCND2
	KRAS	BCL2	P27	CCNJ
	HMGA2	MYC	CDKN2A	CDK6

Source: TarBase [63]

different components of the *miR-17-92* cluster. Clinical outcome of anaplastic thyroid cancer patients is poor, and patients often die after 6 months of disease detection, due to rapid tumor growth in the cervical area, and metastatic spread to bones and lungs. *miRNA*-targeted therapy against *miR-17-92* results in important modulation of anaplastic thyroid cancer cell biology in cell culture. Selective treatment with *anti-miR* against *miR-17-5p*, *miR-17-3p* and *miR-19a* resulted in strong growth inhibition and apoptosis in anaplastic thyroid cancer cell lines. Moreover, *anti-miR* restored expression of PTEN and RB proteins *in vitro* [45], indicating a potential role for *miR-17-92* inhibition in aggressive thyroid cancer. Furthermore, in follicular thyroid cancer, *miRNA* expression analysis revealed high expression levels of *miR-92a* in highly metastatic samples, compared to non-metastatic ones [76]. In addition, a recent in-depth large-scale *miRNA* study showed high levels of *miR-17*-92 in BRAF-mutated papillary thyroid cancer patients [70]. Of particular interest, an experimental study using conditional induction of the $BRAF^{T1799A}$ oncogene in normal thyroid follicular cells showed a robust increase in the expression of all components of the *miR-17-92* cluster in response to BRAF activation [77].

The *miR-17-92* cluster influences several cellular processes associated with tumor aggressiveness. The modulation of metalloproteinase expression plays a key role in the metastatic spread and invasiveness of tumor cells. In this context, *miR-17-5p* and *miR-17-3p* target TIMP3, an important inhibitor of metalloproteinase activation. Functional validation of *miR-17-92* targets not only revealed several mRNAs that code for proteins involved in inhibitory signaling pathways and tumor suppressor genes, but also for proteins considered promoters of cancer (Table 13.5). Therefore, the overall role of the cluster is to promote a complex balance between suppressor and promoter effects of its components.

In silico analyses revealed that several predicted targets of the *miR-17-92* cluster belong to the transforming growth factor β (TGFβ) signaling pathway [78], indicating that modulation of this signal cascade is an important event in cancer progression. As previously mentioned, *miR-146b*, which is over-expressed in papillary and anaplastic thyroid cancer, also influences a downstream target of TGFβ signaling, indicating that collaborative *miRNA*-directed ablation of TGFβ inhibitory signaling is a crucial step in thyroid oncogenesis. TGFβ signaling exerts a key regulatory influence in thyroid follicular cell proliferation. A recent functional study showed that *miR-19a* and *miR-19b* target SMAD4 in response to BRAF oncogene induction, and impair the inhibitory TGFβ signal in thyroid cancer cells. Moreover, the blockage of *miR-19* function by means of *anti-miR-19*, led to

Table 13.5 *miR-17-92* cluster validated targets

	Cell cycle	TGFβ signaling	Invasion	Angiogenesis	Apoptosis	PI3K signaling
Cluster *miR-17-92*	E2F1/2/3	SMAD2	MMP2	VEGFA	BCL2	PTEN
	CDKN1A	SMAD3	TIMP3	HIF1α	BIM	
	CCND1	SMAD4				
	MYCN	TGFBR2				

Source: TarBase [63]

restoration of SMAD4 levels and recovery of responsiveness to TGFβ-induced G1-cell cycle arrest [77]. Reduced SMAD4 expression has been observed in papillary thyroid cancer cell lines, which limits the response to TGFβ anti-proliferative signaling. Moreover, SMAD4 rescue experiments have demonstrated that overexpression of SMAD4 blocks cell proliferation and reduces invasion in thyroid cancer cells [79]. In addition, TGFβ signaling is also regulated by the *miR-17-92 cluster* in different types of cancer. In another example, in neuroblastoma, *miR-17* and *miR-20* target *TGFBR2,* while *miR-18* targets SMAD2 and SMAD4 [80], which when down-regulated could ablate TGFβ inhibitory signaling and contribute to tumorigenesis.

One important molecular mechanism underlying *miR-17-92* cluster activation during BRAF-mediated thyroid follicular cell oncogenesis is associated with the cross-talk of MAPK and NOTCH signaling, frequently deregulated in human thyroid cancer. Indeed, papillary thyroid cancer samples showed high expression of the receptor NOTCH1, indicating activation of this signaling pathway in this type of cancer [81]. Notch signaling regulates *miR-17-92* transcription. Genomic analysis of the *miR-17-92* putative promoter region revealed predicted binding sites for HES1, the effector of Notch signaling, along with Myc [82]. Indeed, complete inhibition of Notch signaling via *Notch1* siRNA suppressed BRAF-induced *miR-19* activation in normal thyroid cells. Moreover, in the anaplastic thyroid cancer cell line, KTC2, harboring the BRAF mutation, specific inhibition of $BRAF^{V600E}$, using PLX4032 or *NOTCH1* siRNA, reduced endogenous expression of the *miR-17-92* cluster. Moreover, human, mouse and rat *miR-17-92* putative promoter regions contain multiple predicted consensus binding sites for cMyc. Additionally, in HeLa cells, Myc activation induced *miR-17-92* expression, while Myc deletion reduced the cluster expression [83]. One elegant study dissected the role of each component of the *miR-17-92* cluster in lymphoma, by selective deletion of *miRNAs*, and observed a loss of the oncogenic effect without *miR-19a* and *miR-19b*. In this study, it was demonstrated that *miR-19* is the main oncogenic component of the cluster, as this *miRNA* alone is sufficient for promotion of c-Myc-induced lymphomagenesis, via repression of apoptosis and activation of the AKT-mTOR pathway [84]. On the other hand, another study indicated *miR-92a* as the key oncogenic *miRNA* in colon cancer, as it is highly expressed and targets the anti-apoptotic gene *BIM*, while its antisense inhibition induces massive apoptosis [85].

3.4 MiR-221 *and* miR-222

miR-221/*miR-222* is a cluster of *miRNAs* located at chromosome X, the deregulation of which was originally reported by seminal studies of He et al. and Pallante et al. [33, 36]. Since then, cumulative literature has consolidated *miR-221*/*miR-222* as being the most over-expressed *miRNAs* in papillary thyroid, follicular, and also in undifferentiated anaplastic thyroid cancer patients [40, 44, 86]. The presence of high levels of *miR-221*/*miR-222* in the different follicular cell-derived thyroid cancers indicates that this cluster is essential for maintaining tumorigenesis.

Among the clinical implications of elevated levels of *miR-221* and *miR-222* are increased extra-thyroidal invasion, tumor size, higher tumor node metastasis stage, and papillary thyroid cancer recurrence [47, 49, 50]. Moreover, detection of increased circulating plasma levels of *miR-222* is associated with the presence of the BRAF mutation and recurrent papillary thyroid cancer [50]. Interestingly, these findings are somewhat similar to those regarding *miR-146b*, another highly over-expressed *miRNA* in papillary thyroid cancer.

Furthermore, follicular thyroid cancer exhibits high levels of *miR-221* and *miR-222* [40], which also correlates with clinical-pathological characteristics. Analysis of *miRNA* expression in highly metastatic non-invasive follicular thyroid cancer revealed increased levels of *miR-221* and *miR-222* compared to non-metastatic ones [76], indicating the participation of these *miRNAs* in cell migration and metastasis. Indeed, blockage of *miR-221* using *anti-miR* resulted in reduced cell migration and invasion in a colorectal cell line, and metastases formation *in vivo* by targeting RECK, an inhibitor of metalloproteinases [87]. Moreover, *RECK* gene expression levels are inversely correlated with *miR-221* in colorectal cancer tissue.

Another important validated target of *miR-221* and *miR-222* is the receptor tyrosine kinase KIT, involved in cell growth and differentiation [88]. KIT has been found to be an oncogene in different types of cancer [89]; however, in papillary thyroid cancer cell lines, KIT expression is low [90, 91]. Moreover, over-expression of *miR-221* and *miR-222* is associated with reduced levels of KIT in papillary thyroid cancer [36].

In vitro modulation of *miR-221* in thyroid cancer cells results in important effects in cancer cell growth. Ectopic over-expression of *miR-221* in cancer cells results in a robust increase in the number of colonies in a soft-agar medium [33]. The blockage of *miR-221* with antisense oligonucleotide transfection significantly decreases cell proliferation, indicating that these *miRNAs* are important modulators of papillary thyroid cancer cell growth. Indeed, over-expression of *miR-221* and *miR-222* in papillary thyroid cancer cell lines significantly increases the transition from G1 to S phase, as these *miRNAs* target the $p27^{kip1}$ (CDKN1B), a key regulator of cell cycle progression [92]. Moreover, in papillary thyroid cancer samples, reduction of p27 protein levels is also observed. In Table 13.6, some important validated targets of *miR-221*/*miR-222* are shown.

Table 13.6 *miR-221/222* validated targets

	Cell cycle	Invasion	RTK signaling	PI3K signaling	Apoptosis
miR-221/222	CDKN1B	ZEB2	KIT	PTEN	CASP3
	CDKN1C	MMP1	ER1a		
	E2F3	RECK			

Source: TarBase [63]

4 Conclusion and Future Perspectives

In thyroid cancer, despite efforts to develop specific small molecules for inhibition of oncogenic MAPK signaling, treating aggressive thyroid cancer remains a challenge due to refractoriness to cancer therapy. Therefore, intervention using a molecular approach, especially via *miRNA* modulation, emerges as a potent adjuvant strategy for amelioration of responsiveness to conventional treatments. Molecular interference in *miRNA* expression can be a central target for aggressive cancers, as the pleiotropic mechanism of action of *miRNAs* can simultaneously influence several signaling pathways that promote refractoriness.

A specific *miRNA* signature composed of *miR-146b*, *miR-221*, *miR-222* and *miR-17-92* is associated with an aggressive thyroid cancer phenotype. The collective influence of these *miRNAs* in different signaling pathways cooperates with the acquisition of aggressive characteristics and a poor outcome in some thyroid cancer patients. Recent studies have shown the potential of using circulating *miRNA* levels to detect and predict cancer prognosis. Indeed, *miR-146b, miR-221* and *miR-222* plasma levels are indicators of thyroid cancer, and higher levels are predictors of more aggressive thyroid carcinomas [50]. Thus, combined modulation of *miRNAs* that play a role in several signaling pathways involved not only in aggressive behavior, such as TGFβ, Notch or NFkB, but also in signaling related to apoptosis, cell survival, invasion and metabolism, is expected to yield a more complete response for adjuvant treatment of aggressive and refractory thyroid cancer.

The development of a safe and specific method to implement *miRNA*-targeted therapy would help improving the treatment of unresponsive thyroid cancer. Efficient *miRNA* modulation was produced *in vitro* and xenograft mouse model for *miRNA* targeting in different types of cancer cells has demonstrated tumor growth suppression. Different molecular approaches have been developed to modulate *miRNA* expression. Among the inhibitory methodologies, antisense oligonucleotide transfection, usually carrying special ribose modifications (e.g. LNA) in the *anti-miR* sequence is applied to block *miRNA* interaction with its targets by preferential interaction and highly stable binding to *anti-miR*. Moreover, another validated technology is the use of *miRNA* sponges, that are involved in "*miRNA de-targeting*", based on the over-expression of specific RNA sequences that interact (sequestrate) with mature *miRNA* and inhibit its function [93–95]. A current study has demonstrated the efficacy of *miRNA*-de-targeting *in vivo*. Using this approach, tail injections of a de-targeting plasmid over-expressing *miRNA*-binding sequences significantly inhibited endogenous levels of *miR-122* in a mouse hepatocellular

carcinoma model [96]. Conversely, in order to increase a suppressed *miRNA* in a given cell, plasmidial or viral transduction of the primary or precursor *miRNA* sequence is the most used approach.

A recent innovative study into molecular-targeted therapy has shown the promising potential of *miRNA* targeting as a new therapeutical approach. A pioneer treatment (*Miravisen*) for the Hepatitis C virus (HCV) [97] was developed, based on the interference in *miR-122* function that mediates HCV pathogenesis. *Miravisen* is an LNA antisense 15-nucleotide DNA sequence complimentary to the 5' region of *miR-122* that blocks *miR-122* function by forming a highly stable *miRNA*-DNA duplex. Current trials in patients with HCV have shown promising results following *Miravisen* injection that resulted in a significant and long-lasting decrease in the HCV viral load.

Thus, *miRNA*-targeted therapy opens a new field of perspective in cancer therapeutics. The actual challenge in thyroid cancer research is to adapt methodologies of *miRNA* modulation in order to turn *miRNA*-targeted therapy into a feasible methodology for use in patients with aggressive thyroid cancer.

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Chapter 14
miRNA-Targeted Therapies in the Most Prevalent Pediatric Solid Tumors

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1 Hallmarks of Pediatric Tumors

Childhood cancers have specific characteristics that define them as entities that differ strongly from adult malignancies owing to their different etiology, biology, response to treatment and outcome. In adults, epithelial cancers are the most common and quite often linked to a sustained exposure to environmental carcinogenic agents. Conversely, pediatric malignancies tend to be of hematologic, mesenchymal or nervous system origin and their etiology is often unknown. Furthermore, the incidence of cancer in childhood is clearly lower than in adulthood. In the United States, only 2 % of patients with a malignancy are children, with almost 8,500 under the age of 15 presenting with a malignancy every year [1]. However, despite the small number of cases of childhood cancers compared to the numbers in the adult population, great advances in treatment protocols have been made in the last three decades. In addition, knowledge on the molecular biology of pediatric tumors has been growing considerably, mainly owing to advances in cancer genetics and better molecular characterization of cancers. Advances in treatments have improved the overall survival in all childhood cancers from less than 30 % in 1960 to over 70 % today. Even with this improved overall survival, cancer still remains the second cause of childhood mortality.

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2 MicroRNAs: A Novel Approach to Treat Pediatric Tumors

Treatment of pediatric solid tumors, which fall into the category of rare diseases, even the most prevalent ones, has evolved at a much slower pace than adult cancers, possibly because fewer resources have been channeled into research on low-incidence tumors. Since the patients are children, some treatments cannot be used at the same doses or schedules as adults, thereby hindering the success of therapy. Targeted therapies are an emerging alternative for cancer treatment. Specific monoclonal antibodies (e.g. Trastuzumab) or small molecules (e.g. Gleevec) against breast cancer and chronic myelogenous leukemia, respectively, have yielded excellent clinical results for certain subsets of adult patients [2, 3]. Considerable efforts are being made to engage targeted therapies clinical trials for pediatric solid tumors, such as neuroblastoma. One of the therapies with encouraging initial results is the use of ALK inhibitors [4]. ALK is a tyrosine kinase receptor that has been shown to be amplified in 5 % of neuroblastomas and ALK mutations were found in 11 % [5]. This trend towards personalized medicine will clearly have an impact on outcome, owing to the selection of patients who are more likely to respond. However, it is not clear whether this patient stratification will be economically viable.

An emerging alternative is the use of epigenetic therapies, i.e., therapies designed against modulators of gene expression which, in turn, modulate several genes, pathways or cellular processes. A clear paradigm of this modality will be the use of microRNAs as therapeutic targets. MicroRNAs are small non-coding RNAs that regulate gene expression, generally by direct binding to the 3′UTR of their target genes. MiRNAs have been shown to participate in almost all cellular processes and be deregulated in several diseases including cancer. They can have oncogenic or tumor suppressor functions if they target classical tumor suppressors or oncogenes, respectively. One single miRNA may target multiple genes of the same or different pathways, thereby minimizing the risk of resistance mechanisms development.

MiRNA function can be restored using miRNA mimetics, or inhibited using antagomirs. Several strategies are being developed to ensure stable and safe delivery to patients (reviewed in Soriano et al. [6]). In fact, a miR-34-based therapy has recently entered Phase I trials [7].

This chapter reviews the knowledge gathered on miRNA expression and function in the most prevalent pediatric solid tumors (see Fig. 14.1) and summarizes the most interesting candidates for the development of miRNA-targeted therapies (Table 14.1).

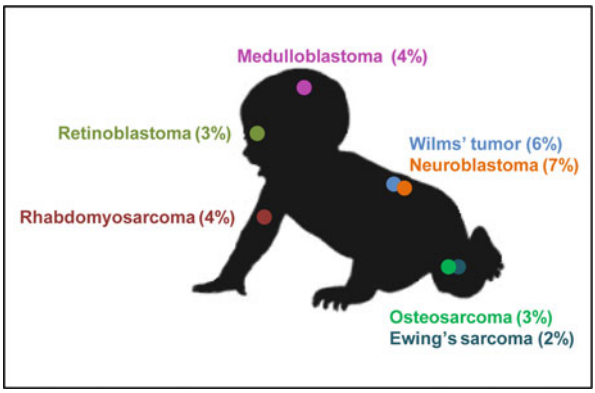

Fig. 14.1 Illustrative localization and incidence of the most prevalent pediatric solid tumors [113]

2.1 Pediatric Brain Tumors

Pediatric tumors of the central nervous system (CNS) are the most common solid malignancies of childhood cancer and second only to hematologic malignancies [8]. They are the leading cause of cancer-related deaths in children. The therapies currently used have improved patient outcome but continue to carry a high risk of side effects [9]. In fact, most of these therapies are only intended to improve patient survival by minimizing long-term morbidities [10]. Therefore, alternative therapies must be developed. The possibilities of using miRNAs as an alternative therapeutic approach in the main CNS tumor are emerging. MicroRNA profiling of pediatric brain tumors samples show promising new therapeutic candidates in the most prevalent pediatric brain tumors such as medulloblastoma, astrocytoma [11–13], ependymoma [14] or brain stem gliomas [15]. However, the functional impact of these findings has only been addressed in medulloblastoma which is reviewed below.

2.1.1 Medulloblastoma

Medulloblastoma (MB) is the most frequent brain tumor in children, representing ~20 % of all pediatric brain tumors [16]. This tumor arises from primitive neuroectodermal cells located in the external granular layer of the cerebellum, which grow and invade the ependyma and brainstem [17]. Currently, there are two main risk groups, depending on patient age, extent of surgical resection, histology of the primary tumor and presence of metastasis: standard-risk patients with an overall 5-year survival around 75 % and high-risk patients with 5-year overall survival between 55 and 80 % [18]. Large scale genomic analyses classify MB in four major groups with unique molecular profiles and clinical behavior: SHH, WNT, Group 3 and Group 4 [19]. Classical treatment starts by surgical resection of the primary tumor, followed by cranio-spinal radiotherapy. Owing to the devastating side effects of radiation, the amount of radiation has been reduced

Table 14.1 MicroRNAs with therapeutic potential in preclinical mouse models

Tumor	Name	↑/↓	Targets	Restoration causes	Ref.
Medulloblastoma	miR-124a	Down	CDK6	↓ proliferation	[23]
	miR-199b-5p	Down	HES1	↓ proliferation, depletion CSCs	[29]
	miR-34	Down	DLL1	↓ proliferation, ↑ apoptosis, ↑ differentiation	[34]
	miR-33b	Down	c-MYC	↓ proliferation	[36]
	miR-17-92	Up	BMPR2	↓ proliferation	[39]
	miR-182	Up	*ND*	↓ migration and invasion	[40]
Neuroblastomas	miR-34a	Down	TIMP2	↓ proliferation, ↑ apoptosis ↓ vascularization	[56]
	miR-542-5p	Down	*ND*	↓ proliferation, ↓ metastasis	[59]
	miR-27b	Down	PPARγ	↓ proliferation, ↓ inflammation	[69]
	miR-9	Down	MMP14	↓ proliferation, ↓ metastasis	[70]
	miR-335	Down	SOX4, TNC	↓ colony growth, ↓ metastasis	[72]
	miR-363	Down	AMDM15, MYO1B	↓ colony growthg, ↓ metastasis	[72]
	miR-183	Down	*ND*	↓ proliferation, ↑ apoptosis	[81]
	miR-380-5p	Up	TP53	↓ proliferation, ↑ apoptosis	[82]
Wilm's tumor	miR-483-3p	Up	PUMA	↓ proliferation, ↑ apoptosis	[96]
Rhabdomyosarcoma	miR-1/206	Down	c-MET	↓ proliferation, ↓ migration	[108]
Retinoblastoma	miR-17-92	Up	ND	↓ tumor formation	[123]
	miR-365b-3p	Down	PAX6	↓ proliferation, ↑ apoptosis	[128]
Osteosarcoma	miR-34a	Down	EAG1, c-MET	↓ proliferation, ↓ metastasis	[133, 134]
	miR-20a	Up	FAS	↓ metastasis	[136]
	miR-143	Down	MMP13	↓ metastasis	[138]
	miR-125b	Down	STAT3	↓ proliferation, ↓ migration	[140]
	miR-340	Down	ROCK1	↓ proliferation, ↓ migration and invasion	[147]
	miR-16	Down	IFG1R	↓ proliferation	[153]
	miR-133a	Down	Mcl-1, Bcl-XL	↓ proliferation, ↑ apoptosis	[155]
	miR-24	Down	LPAATβ	↓ proliferation	[156]
	miR-223	Down	HSP90B1	↓ proliferation, ↑ apoptosis	[180]
Ewing's sarcoma	miR-145	Down	EWS-FLI1	↓ proliferation, ↓ clonogenicity CSCs	[172, 175, 181]
	Let-7a	Down	HMGA2	↓ proliferation	[175]

CSC cancer stem cells, *ND* not determined

over the years and chemotherapy has been introduced as standard of care [9]. It is tempting to speculate that the different molecular profiles of MB subgroups will lead to different patient stratification and customized targeted therapies.

Numerous reports support the deregulation of miRNAs in MB and, consequently, these alterations can be exploited as new therapeutic targets. The first example was reported by Pierson and collaborators. CDK6 was previously reported to be overexpressed in MB [20]. Those authors found that CDK6 levels can be directly modulated by miR-124a, the restoration of which resulted in decreased cell proliferation [21]. Further studies confirmed their findings *in vitro* [22] and *in vivo* [23].

Analysis of miRNA profiles generated in different mouse models and human tumor samples yielded a significant number of deregulated miRNAs in MBs compared to cerebellar tissues. Two of these miRNAs were miR-9 and miR-125a. Restoration of both miRNAs individually led to reduced MB proliferation and increased cell death, possibly by targeting a pro-proliferative truncated form of TRKC [24]. A reduction in cell proliferation was also observed when miR-128a, a miRNA found to be underexpressed in primary MB cell lines compared to adult or pediatric cerebellum, was restored. The effects of miR-128a were mediated by targeting the polycomb repressor complex I, BMI-1 [25]. Similar results were found when the same authors restored the levels of miR-218. For target analysis, they used the HITS-CLIP technique and identified CDK6 and RICTOR as critical targets for the tumor suppressive functions of miR-218 [26]. Shi and collaborators reported that miR-218 restoration also reduced migration and invasion mediated by the downregulation of SH3GL1 [27].

Another broadly downregulated miRNA is miR-383. Restoration of miR-383 causes a reduction in cell proliferation and increased cell death through downregulation of PRDX3 [15, 28]. Similar effects were also observed when miR-199b-5p was overexpressed in MB cell lines. This miRNA was found to be downregulated in MB patients with advanced disease and poor prognosis [29]. The levels of miR-199b-5p seemed to be repressed by HES1 [30], a well-known mediator of Notch and SHH signaling in MB [31, 32]. The restoration of miR-199b-5p also impacted on *HES1* levels and reduced proliferation, invasion and growth of MB cells in the cerebellum [29, 30].

Genetic aberrations are an alternative cause of miRNA deregulation. Genomic deletion of 1p36, which contains the miR-34a gene, has been observed in a subset of MB [33]. Restoration of miR-34a levels led to a reduction in MB proliferation *in vitro* and enhanced sensitivity to chemotherapeutic drugs through repression of MAGE-A [33]. Furthermore, miR-34 has been shown to reduce tumor growth *in vivo* by targeting DLL1 and enhancing MB differentiation [34]. Another region frequently lost in MB is chromosome 17p [35]. MiR-33b is located in intron 17 of the sterol regulatory element binding transcription factor 1 (SREBF1) gene. Restoration of miR-33b reduced proliferation, invasion and neural-stem cell properties of MB cell lines *in vitro* by repression of c-myc. Moreover, miR-33b can be therapeutically upregulated using lovastatin, an FDA-approved drug, the *in vivo*

administration of which is able to reduce brain tumour burden in an orthotopic MB model [36].

While most miRNAs with functional impact have been found to be downregulated in MB, few miRNAs have been found to be upregulated. Among these, several members of the miR-17-92 cluster were found to be overexpressed only in the SHH subgroup [37]. These results have been validated in human tissues from different institutions [24, 38]. MicroRNAs belonging to the same family can be inhibited using 8-mer LNA-antimiRs (named Tiny LNAs). Inhibition of miR-17 and miR-19 families with tiny LNAs reduced tumor growth in flank and brain MB allografts and extended the survival of mice with intracranial tumors [39].

Other miRNAs have been found to be specifically upregulated in non-SHH MB. The overexpression of miR-182-96-183 seems to confer enhanced migration and invasion properties on MB cells that lead to leptomeningeal spread [40]. Inhibition of the miR-182-96-183 cluster causes cell cycle arrest and apoptosis, by possibly regulating a myriad of genes involved in response to growth factors, DNA repair, apoptosis, senescence, and migration or invasion [41].

The subgroup of WNT signaling-associated MB also displays differential expression of miRNAs. Patients belonging to the WNT subgroup are above median age for all those with MBs [42] and have prolonged survival [43]. The better clinical behavior of these tumors could be explained, at least in part, by to the expression of miRNAs with tumor suppressive functions. Two of the most expressed miRNAs in this subgroup are miR-193a and miR-224 [44]. The overexpression of these two miRNAs causes a reduction in cell proliferation and the colony-formation capacity of MB cell lines [44].

Despite the specific miRNA signatures of each MB subgroup, other miRNAs have been found to be generally upregulated. This is the case of miR-21, reported to be amplified in almost all cell lines and tissues compared to normal cerebellum. The inhibition of miR-21 *in vitro* resulted in upregulation of the metastasis suppressor PDCD4, migration and invasion repression, but did not alter the viability or proliferation of MB cell lines [45].

2.2 *Neuroblastoma*

Neuroblastoma (NBL) is an extracranial neoplasm originated in the neural crest lineage of the sympathetic nervous system. It is considered an embryonal cancer and occurs mainly in children and adolescents. NBL represents 15 % of all cancer deaths in children [46] and is the embryonal tumor with less five-year relative survival [47]. NBL is frequently localized in one of the adrenal glands, but also appears in nerve tissues in the cervical area, thorax, abdomen, or pelvis. NBL patients are assigned to three different risk groups according to clinicopathological variables such as age at diagnosis, MYCN amplification, tumor histology and DNA ploidy. While prognosis is good for low (>95 % survival) or intermediate-risk (70–90 % survival), high-risk patients, however, have poor prognosis (30–40 %

survival) and need intense therapeutic regimens [48]. The treatment sequence for this group consists of at least four components: induction, local control, consolidation and treatment of minimal disease with biologic agents [48, 49]. Despite the intense multimodal therapy, high-risk patients relapse and present progression of the disease. Therefore alternative therapeutic approaches must be considered.

MiRNAs have been found to be deregulated in NBL, and the over- or under-expression of specific miRNAs correlates with stage, progression and patient outcome [50, 51].

In particular, Lin and collaborators found a general downregulation of miRNA expression in advanced neuroblastoma and identified 27 miRNAs that can clearly distinguish low- from high-risk patients. In addition, they observed low expression levels of miRNA processing machinery components (i.e. DICER and DROSHA) in high-risk neuroblastoma tumors, which could explain for the overall down-regulation of miRNAs in high-risk cases. Functionally, they demonstrated that the *in vitro* knockdown of DICER or DROSHA promoted the growth of neuroblastoma cell lines [52]. These results in NBL concur with previous observations of an association with low levels of miRNAs and the formation of several types of cancer [53]. However, studies that link deregulated miRNAs with their direct effects on NBL formation or progression are ongoing. One of the first examples was miR-34a. This miRNA locus is mapped to the chromosome 1p36 region that is commonly deleted in NBL [54]. Mir-34a has been found to act as a tumor suppressor through the targeting of numerous genes associated with cell proliferation and apoptosis. The ectopic expression of miR-34a in NBL cell lines resulted in significant cell growth inhibition *in vitro* [55] and *in vivo* [56].

As we mentioned previously, some studies reported an association between miRNA levels and NBL survival [50, 57, 58]. In those studies, miR-542-5p appeared to be one of the miRNAs with the lowest expression and the highest correlation with poor survival. Bray and collaborators demonstrated that miR-542-5p acts as a tumor suppressor in NBL by blocking cellular invasiveness and reducing primary tumor growth and metastasis in an orthotopic mouse xenograft model [59]. In this case, the mediators of miR-542-5p effects also remain unidentified. The same authors also found miR-204 to be downregulated in aggressive NBL. The restoration of miR-204 by ectopic overexpression in NBL cell lines did not result in major changes in proliferation and viability; however, it did increase the sensitivity to cisplatin in NBL by targeting BCL2 [60]. Continuing with the microRNAs associated with survival, the same group also determined the role of miR-497. Creevey et al. found miR-497 to be lowly expressed in stage 4 and MYCN-amplified tumors. Functional characterization revealed that miR-497 restoration reduced proliferation and induced apoptotic cell death only in MYCN-amplified NBL cell lines. These effects could be mediated by targeting of the tyrosine kinase WEE1 by miR-497 [61].

In addition to the miRNA expression affected by the lower expression of the miRNA-processing machinery, some miRNAs can be regulated by epigenetic mechanisms. This is the case of miR-340, one of the miRNAs identified when methylated miRNA promoters were sought in NBL. The restoration of miR-340

induced either differentiation or apoptosis in a cell context dependent manner, thereby indicating a tumor suppressive function for this miRNA. Intriguingly, it was determined that miR-340 is upregulated by demethylation of an upstream genomic region that occurs during the process of neuroblastoma cell differentiation induced by all-trans retinoic acid (ATRA). Further biological studies on miR-340 revealed that the pro-differentiation phenotype depended on the direct repression of SOX2 [62]. A further miRNA involved in NBL differentiation is miR-124. This miRNA is one of the most abundant miRNAs in the nervous system. Inhibition of miR-124 resulted in NBL differentiation, cell cycle arrest and apoptosis in the SK-N-SH NBL cell line [63]. The proposed mediator of these effects was the aryl hydrocarbon receptor (AHR).

Another strategy to identify miRNAs that might play a role in NBL is to analyze the potential miRNA binding sites in the 3′UTR of known NBL oncogenes. This was the case of Buechner and others who studied the proto-oncogene MYCN as a target for miRNA regulation. The results of that study showed that MYCN was targeted by several miRNAs. The study reported that mir-34a/c, mir-449, mir-19a/b, mir-29a/b/c, mir-101 and let-7e/mir-202 targeted MYCN directly. These miRNAs were able to suppress endogenous MYCN protein in a MYCN-amplified neuroblastoma cell line. Moreover, the mir-101 and the let-7 family miRNAs let-7e and mir-202 inhibited proliferation and clonogenic growth when overexpressed in NBL cells [64]. Furthermore, Molenaar et al. elegantly showed that LIN28B-mediated repression of let-7 resulted in elevated MYCN levels, blocked differentiation of normal neuroblasts and induced NBL formation [65]. Another protein found to be strongly expressed in NBL was KDM1A. This protein is strongly expressed in undifferentiated NBL, and overexpression correlates with poor patient prognosis [66]. Analysis of *KDM1*-3′UTR revealed potential miR-137 binding sites. Althoff et al. reported that miR-137 directly regulates KDM1 expression. Moreover, miR-137 overexpression recapitulates the phenotypic effects of inhibiting KDM1 such as a reduction in cell proliferation and increased apoptosis [67].

PPARγ is another abundantly expressed protein in NBL [68]. Although most reports show that PPARγ is a tumor suppressor, examples also exist of its pro-oncogenic role. PPARγ levels can be modulated by miR-27 in several tumor types, including NBL. In fact, miR-27 levels were decreased in NBL tissues, and miR-27 treatment reduced cell proliferation and tumor growth *in vivo* [69].

An analogous approach focused on the regulation of MMP-14, a matrix metalloprotease implicated in invasion, metastasis and angiogenesis. Since metastasis is the leading cause of death in NBL, Zhang et al. analyzed the regulation of MMP-14 in NBL. They found that *MMP-14* levels inversely correlated with miR-9 and reported direct binding of miR-9 to the 3′UTR of *MMP-14*. Overexpression of miR-9 resulted in reduced tumor growth and metastasis *in vitro* and *in vivo* [70].

The other most characterized miRNA implicated in NBL metastasis is miR-335. This miRNA was shown to be directly repressed by MYCN and have tumor suppressive functions through inhibiting several components of the TGFβ pathway such as ROCK1, MAPK1 and LRG1 [71]. Similar results were obtained by Qiao

et al. who observed that the overexpression of miR-335 inhibited NBL metastasis *in vivo* [72]. In the same study, the authors reported that miR-363 can also inhibit metastasis by targeting ADAM15 or MYO1B [72]. Other recent *in vitro*-based experiments revealed promising miRNA participants in NBL metastasis such as miR-15a [73] and miR-338-3p [74].

Conversely, overexpression of certain miRNAs may participate in the origin, growth and progression of NBL. It has been reported that MYCN participates directly in the regulation of miRNA promoters [75] and induce the expression of certain miRNAs. One of the first examples was miR-421 which regulates ATM, a high-molecular-weight protein serine/threonine kinase that plays a central role in the maintenance of genomic integrity by activating cell cycle checkpoints and promoting repair of DNA double-strand breaks. Paradoxically, the overexpression of miR-421 sensitizes NBL cells to ionizing radiation [76]. MYCN also directly regulates miR-558. The overexpression of this miRNA increased proliferation, colony formation, and growth of xenografted tumors [75]. The mediators of miR-558 remain to be elucidated.

A further example is the miR-17-92 cluster that is also induced by NMYC. Expression of the members of this cluster enhanced the aggressive behavior of NBL through downregulation of components of the TGF-beta pathway [77]. In addition, it has been shown that miR-92a can target DKK3, a tumor suppressor gene that inhibits the proliferation of several cancers including neuroblastoma [78, 79]. Another MYCN-regulated miRNA is miR-21, the expression of which is further increased in cisplatin-resistant cell lines. Functional studies revealed that miR-21 alone is sufficient to reduce sensitivity to cisplatin and, conversely, inhibition of miR-21 is able to render cisplatin-resistant cells vulnerable to apoptotic cell death [80].

MYCN can also function as a transcriptional repressor; it cooperates with HDAC2 to repress the expression of miR-183. When NBL cells were treated with HDAC inhibitors, miR-183 was the most overexpressed miRNA. The ectopic expression of miR-183 alone was sufficient to induce cell death *in vitro* and a reduction in tumor growth *in vivo* [81].

In line with the above-mentioned strategy to identify miRNAs targeting NBL oncogenes, Swarbrick et al. decided to investigate the regulation of tumor suppressors by miRNAs. They focused on TP53 (p53). Inactivation of the p53 pathway permits cell survival in times of stress and occurs in many human cancers; however, primary NBL tumors often maintain wild-type human TP53. On analyzing the 3′UTR of *TP53*, the authors found potential miR-380-5p binding sites. Although miR-380-5p is highly expressed in mouse embryonic stem cells and NBLs, this high expression correlates with poor outcome in neuroblastomas with MYCN amplification. MiR-380-5p overexpression cooperates with activated HRAS oncoprotein to transform primary cells, block oncogene-induced senescence and form tumors in mice. Conversely, the inhibition of endogenous miR-380-5p in embryonic stem or neuroblastoma cells results in the induction of p53 and extensive apoptotic cell death [82].

2.3 Wilms' Tumor

Nephroblastoma, most commonly known as Wilms' Tumor (WT), represents 6–8 % of all pediatric cancers. Besides being the most common renal malignancy in children and the second in the case of extracranial solid tumors, it remains a rare disease affecting 1 in 100,000 children [83]. WT can be heritable [84] but are mostly sporadic, and frequently associated with several syndromes such as WAGR (Wilms' tumor, aniridia, genitourinary abnormalities and mental retardation), Simpson-Golabi-Behmel or Beck-with-Wide [85].

Like other sarcomas, Wilms' tumor is believed to originate from aberrant differentiation of mesenchymal cells, particularly into nephrons [86]. Genetic events that may participate in the origin and/or progression of the disease include somatic mutations or loss of function in several tumor suppressor genes such as the *WT1* gene [87], *CTNNB1* (β-catenin), *WTX* (Wilms' tumor on the X) gene [88], *IFG2* [89], *TP53* [90], *DIS3L2* [91], *FWXB7* and *MYCN* [92].

From the early 1960s, the treatment of Wilms' tumor has been based on surgical excision of the tumor, chemotherapy and radiotherapy. When these three therapeutic approaches are combined, the cure rate currently nears 90 % [93]. However, prognostic molecular markers and alternative therapies for the remaining 10 % of patients who suffer relapse and progression of their disease are lacking.

Recent research on miRNA and cancer has yielded potential new therapeutic targets. The first example was reported by Kort et al. Combined analysis of mRNA and miRNA revealed a strong E2F3 signature in all Wilms' tumors analyzed. E2F3 is a member of the E2F family of transcription factors and is upregulated in advanced stages of WT compared to normal tissues. Those authors reported that E2F3 directly regulates transcription of the miR-17-92 cluster [94], which has been shown to be one of the most characterized groups of oncogenic miRNAs in both adult and pediatric malignancies. These findings suggest that inhibition of miR-17-92 cluster function may represent an attractive novel therapeutic approach.

Another broadly overexpressed miRNA in WT is miR-483-3p. This miRNA is located within the *IGF2* locus at chromosome 11p15. Aberrant IGF2 expression has been involved in the Beckwith-Wiedemann syndrome which predisposes to the development of nephroblastoma, hepatoblastoma and rhabdomyosarcoma [95]. It was thought that amplification of *IGF2* may result in impaired differentiation capabilities, increased proliferation and tumor formation [96]. However, *Igf2* overexpression transgenic mouse models developed some of the features associated with the Beckwith-Wiedemann syndrome but not tumors [97], thereby suggesting that other genes present in the same locus could be responsible for the cancer phenotype. In fact, inhibition of miR-483-3p with antisense strategies led to decreased cell viability *in vitro* and tumor progression *in vivo* due, at least in part, to upregulation of the proapoptotic BH3-only protein PUMA [96]. These results place miR-483-3p as the strongest therapeutic candidate for WT.

Recently, another transcription factor has been implicated in the oncogenic properties of WT cells. STAT3 is a protein that integrates the signaling of cytokines

and growth factors through the JAK/STAT pathway. The group of Dr Wang in China identified activation of STAT3 in WT samples and a pro-proliferative role *in vitro*, and found miR-370 to be strongly upregulated by STAT3, which, in turn, inhibits the expression of WTX, a well-established tumor suppressor in WT [98]. The inhibition of miR-370 caused strong upregulation of WTX and a reduction in cell growth, associated with decreased Cyclin D1 expression and upregulation of the cell cycle inhibitors p21 and p27 [99].

Loss of miRNA may also be implicated in the origin and progression of WT and can be exploited therapeutically. Loss of *DICER1* function has been associated with pleiotropic tumor predisposition syndrome which occasionally gives rise to WT. Biallelic mutations of *DICER1* have been found in a subset of WT patients [100]. These mutations could compromise the miRNA processing activity of DICER and subsequently lead to an overall reduction in miRNA levels.

Other miRNAs have been found to be underexpressed due to genomic loss. A small subset of WT patients presents a deletion of the 2q37.1 locus, which contains miR-562. Patients with 2q37 deletion have lower miR-562 levels in comparison to normal kidney, with a concomitant upregulation of EYA1 [101], a tyrosine phosphatase that participates in processes of DNA repair. However, the potential therapeutic effect of restoring miR-562 levels in patients with 2q37 deletion remains to be elucidated.

2.4 Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) belongs to the family of soft tissue sarcomas, a highly heterogeneous group of tumors with respect to their histogenesis, molecular genetics and clinical behavior. RMS is the most common type of soft tissue sarcoma in children and is considered one of the most prevalent extracranial solid tumors in children. RMS cells are thought to arise from myogenic precursor cells or alternatively, from adult muscle progenitor cells. In both cases cells could not complete their normal differentiation program thereby retaining migrative and proliferative status owing to an oncogenic genetic event.

RMS can be divided into two major histopathologic subtypes: embryonal and alveolar (eRMS and aRMS, respectively). From a molecular point of view, the majority of aRMS (80–85 %) contain one of the reciprocal chromosomal translocations: either t(2;13) (q35;q14) or t(1;13)(p36;q14). These translocations generate the anomalous fusion genes *PAX3-FOXO1* and *PAX7-FOXO1*, respectively [102, 103]. The resulting chimerical proteins have potent transforming effects and are thought to inhibit myogenic differentiation. However, no characteristic translocations have been described in eRMS. The embryonal subtype is characterized by the loss of heterozygosity in the short arm of chromosome 11 (11p15.5) [104] and gains in chromosomes 2, 7, 8, 11, 12, 13 and 17 [105]. Although the majority of RMS cases are thought to be sporadic, patients with some genetic syndromes such

as Gorlin's, Li-Fraumeni's, Wiedemann-Beckwith's and neurofibromatosis type I are at increased risk of developing RMS.

Current RMS treatments include surgery, radiotherapy and chemotherapy. Surgery is first indicated in patients with completely resectable tumors; however, inoperable tumors or those for which mutilating surgery is the only option, chemotherapy should be the treatment of choice and surgery will be delayed until tumor has regressed. Radiotherapy is indicated in cases where radical surgery is not feasible or in cases with microscopic or macroscopic tumor remains.

The prognosis of RMS patients has improved in recent decades, from 60 to 70 %. However, despite the application of these treatments, metastatic RMS continues to have very poor prognosis with 5-year survival below 30 % [106].

MiRNAs have been shown to regulate diverse cell functions in rhabdomyosarcoma such as proliferation, differentiation and cell migration. Consequently, several miRNAs have been associated with cell cycle regulation. Thus, the downregulation of miR-1, miR-206 and miR-29 stabilizes the expression of PAX3 and the cell cycle protein CCND2 in aRMS and eRMS subtypes. Furthermore, miR-29 is also able to activate E2F7, another cell cycle regulator [107] and an expression decrease in miR-1 and miR-206 has been demonstrated to promote a c-Met-induced proliferation increase in RMS models *in vivo* [108]. Recently, a novel mechanism for blocking myogenic differentiation based on TGF-β suppression of miR-450b-5p has been described [109].

The cell's ability to invade can also be influenced by some miRNAs. Both miR-1 and miR-206 act as tumor suppressors owing to their ability to inhibit c-Met, particularly in c-Met-overexpressing tumors. Thus, miR-1/206 inhibition has been suggested as a contributor to aberrant cell proliferation and migration [108]. In some pediatric tumors, including rhabdomyosarcoma, miR-183 has been shown to behave as an oncogene since it targets the transcription factor EGR1, thereby promoting tumor cell migration [110].

2.5 Retinoblastoma

Retinoblastoma is the most common malignant ocular tumor in children, with a relative incidence of 3 % of all pediatric tumors and affecting 1 in 20,000 children worldwide [111]. Tumors originate from retinoblasts located in the internal nuclear layer, nerve fiber layer, ganglion cell layer or external nuclear layer [112]. Tumors growing from external layers are known as exophytic type and those from inner layers as endophytic type. Most cases (~66 %) appear only in one eye (unilateral), while the remaining 33 % are bilateral [113]. Retinoblastoma can occur sporadically (~60 %) or be hereditary (40 %) [114]; however, both are associated with loss of function of the retinoblastoma gene (*RB*), the mutation of which and loss of heterozygosity predispose to the development of retinoblastoma [115]. Retinoblastoma treatment is focused on saving the child's life rather than on preserving vision. When there is no possibility of restoring vision, enucleation of the eye is

usually applied, particularly in unilateral tumors. Photocoagulation or cryotherapy can be used in children with limited unilateral or bilateral tumors [116]. Eternal Beam Radiation therapy (EBRT) was the treatment of choice in children bilateral disease and/or active or recurrent disease [117]. However, ERBT had acute and long-term complications such as the appearance of secondary tumors, especially in patients with hereditary disease [118]. Radiation-associated toxicities led to systemic chemotherapy becoming the primary treatment modality. The use of chemotherapy and focal laser consolidation results yields high survival rates (~99 %). Despite the therapeutic success, secondary effects such as vision loss, secondary malignancies or enucleation of the eye may occur. Therefore, safe alternative treatments must be sought.

Like other tumors, miRNA have been found to be differentially expressed between retinoblastoma and normal retinas [119–121]. The six members of the miR-17-92 cluster have been found to be overexpressed in human retinoblastoma samples. Interestingly, mouse models overexpressing this cluster showed retinoblastoma formation in mouse models with deletion of RB and p107 [122]. Further studies confirmed that inhibition of the miR-17-92 cluster may be therapeutically effective. Nittner et al. demonstrated that miR-17-92 gene inactivation does indeed suppress retinoblastoma formation [123], and inhibition of all cluster members reduced cell viability and induced apoptosis of retinoblastoma cell lines [124].

One miRNA with lower expression in retinoblastoma tumors is miR-34. The exogenous administration of miR-34 in retinoblastoma cell lines reduced cell proliferation and induced apoptosis [125]. Another miRNA with tumor suppressive functions is miR-22. This miRNA was identified as differentially expressed in retinoblastoma cells treated with curcumin, a natural product with well-established anticancer properties. MiR-22 overexpression was sufficient to reduce the proliferation and invasion of retinoblastoma cells [126]. MiR-365b-3p was identified as a potential modulator of PAX6, a transcription factor that correlates with the development and progression of retinoblastoma [127]. The ectopic expression of miR-365b-3p inhibited the growth of retinoblastoma cell lines *in vitro* and *in vivo* through direct modulation of PAX6 levels [128].

2.6 Osteosarcoma

Osteosarcoma (OS) is the most common malignant bone tumor, representing 2–3 % of all pediatric malignancies and 20 % of all primary bone tumors [129], with an incidence of ~5 cases per million in the United States [113]. OS is thought to originate from primitive cells of mesenchymal origin. It usually appears in areas of active bone growth such as the lower part of the femur or upper part of the tibia. OS might also appear in the skull, jaw or pelvis [129]. The most frequent OS are classified as conventional (i.e. osteoblastic, fibroblastic or chondroblastic); however up to eight other less frequent subtypes also exist. The tumors are locally destructive and metastasis is present in ~20 % of patients at the time of diagnosis

[113]. Historically, despite amputation of the affected limbs, patients continued to die of pulmonary metastasis [130]. Surgical techniques have improved and most patients now undergo limb-salvage procedures. The combination of surgery and neoadjuvant and adjuvant chemotherapy has considerably extended the 5-year survival of patients; however, OS continues to be one of the pediatric cancers with low cure rates (below 60 %) [131].

Two of the genes that play a role in the development of OS are the tumor suppressors RB and p53, the mutation of which (Li-Fraumeni syndrome) predisposes to OS development. Some of the p53 functions are mediated by miRNAs, particularly the miR-34 family. MiR-34 was found to be downregulated in primary OS samples [132]. Forced expression of miR-34 in OS cell lines induced cell cycle arrest and apoptosis through the direct modulation of genes such as *E2F3*, *Cyclin E2*, *EAG1* [132, 133]. Moreover, miR-34 overexpression is also able to inhibit migration and invasion *in vitro* and *in vivo* by direct targeting of c-met [134].

Owing to the role of metastasis in OS patient prognosis, migration and invasion related-miRNAs have been studied extensively. Thus, miR-21[135], miR-20a [136] and miR-93 [137] have been identified as metastasis-promoter miRNAs whereas miR-143 [138], miR-199a-3p [139], miR-125b [140], miR-145 [141], miR-195 [142], miR-183 [143, 144], miR-376c [145], miR-335 [146] and miR-340 [147] have been demonstrated to be negative regulators of invasion and metastasis through the modulation of many different targets.

Another feature of OS is its resistance to chemotherapy. MiRNA profiling of OS xenografts treated with different chemotherapeutic agents (i.e. doxorubicin, cisplatin, and/or ifosfamide) found miR-140 to be commonly upregulated in the drug-treated tumors. The blockade of miR-140 with antimiR strategies partially reversed the chemoresistance [148]. The inhibition of miR-215 [149] or miR-221 [150] was also shown to increase chemosensitivity of OS cell lines.

A different approach to identifying potential therapeutic targets has been to study miRNAs found to be tumor-suppressive in other malignancies. The ectopic expression of miR-143 [151], miR-15a [152], miR-16-1 [153], miR-29 [154], miR-133a [155], miR-24 [156] and miR-302b [157] was able to induce cell cycle arrest and/or apoptosis in OS cell lines, thus providing a good rationale for further developing this miRNA-based strategy for the treatment of OS.

2.7 Ewing's Sarcoma

Ewing's sarcoma (ES) comprises ~2 % of all pediatric malignancies and is the second most prevalent primary bone tumor in children and adolescents, with the first being osteosarcoma. The annual incidence of ES is approximately 3 cases per million with a peak incidence in the second decade of life [158]. The majority of ES occur in bone, especially in the pelvis, the diaphyseal regions of the long bones and bones of the chest wall [159]. ES is an aggressive childhood cancer with a tendency to recur and metastasize [160]. Patients with localized ES have 5-year event-free survival around 70 %, whereas patients with metastatic or recurrent disease have an

overall survival rate <20 % [161]. At present, the favorite candidate cell of origin is a multipotent mesenchymal precursor cell (MPCs) [162]. MPCs can differentiate into numerous mesodermal cell types (including chondrocytes, osteoblasts and adipocytes) and their distribution mimics that of ES tumors [163].

Almost all ES tumors are characterized by the presence of the chromosomal translocation EWS-ETS which fuses the 5′ end of the EWS gene in chromosome 22 to the 3′ portion of a gene of the ETS transcription factor family [164], of which *EWS-FLI1* is the most frequent (~85 %) [162]. Several findings suggest that the simple presence of this translocation suffices to cause oncogenic transformation [165–167].

There is currently no internationally recognized risk classification scheme for ES. The most useful prognostic indicators in clinical practice are the presence of metastatic disease at diagnosis and other variables such as tumor volume, primary tumor site or presence of the *EWS-FLI1* type 1 translocation [168–170].

Current treatment protocols are a combination of chemotherapy, surgery and radiation. The objective of primary chemotherapy prior to surgery is to achieve tumor reduction to facilitate limb-salvage surgery [159].

Since EWS-FLI1 plays a central role in the pathogenesis of ES, several works have aimed at identifying downstream effectors including miRNAs. Silencing of EWS-FLI in ES cells followed by miRNA profiling has been the most common procedure for altered miRNAs identification. MiR-145 has been found to be the top EWS-FLI1-repressed miRNA, and forced mir-145 expression resulted in EWS-FLI1 repression and halted ES cell lines growth *in vitro* [171] and *in vivo* [172]. Other miRNAs strongly repressed by EWS-FLI1 are: miR-100, miR-125b, miR-22, miR-221/222, miR-27a and miR-29a. When overexpressed, these miRNAs manifest growth inhibitory properties in ES cell lines, through the negative modulation of several members of the IGF-signalling pathway (i.e. IGF-1, IGF-1 receptor, mammalian/mechanistic target of rapamycin and ribosomal protein S6 kinase A1). EWS-FLI also mediates upregulation of EYA3 via the repression of miRNA-708, resulting in increased cell survival and chemoresistance [173].

CD99 is a critical biomarker of ES that contributes to tumor progression, and has been shown to be regulated by EWS-FLI1. The slight reduction in the CD99 transcript and the dramatic depletion at protein level after EWS-FLI1 silencing suggest the post-transcriptional regulation of CD99 by miRNAs. Franzetti et al. placed miR-30a-5p between EWS-FLI1 and the upregulation of CD99; miR-30a-5p which is repressed by EWS-FLI1 has the ability to interact with the 3′ UTR region of CD99 and reduce its expression. The restoration of miR-30a-5p in ES cell lines reduced proliferation and invasion [174].

The first EWS-FLI1-regulated miRNA studied *in vivo* was let-7a. Systemic delivery of synthetic let-7a to ES tumor-bearing mice resulted in growth inhibition *in vivo* and upregulation of its target gene *HMGA2*, pointing to let-7a as a promising new therapeutic target for ES [175].

Comparison of general miRNA expression profiles between human mesenchymal stem cells and ES cell lines showed an induction of the oncogenic miR-17-92, miR-106b-25 and miR-106a-363 clusters [176]. Inhibition of these clusters using

miRNA-sponge methodology reduced clonogenic growth of ES cell lines, with inhibition of the miR-106a-36 cluster being the most potent [177]. The repression of tumor-suppressive miRNAs such as the let-7 family [175] or miR-34a [178] has also been observed. A reduction in cell proliferation and sensitization to doxorubicin and vincristine was observed in ES cells transfected with miR-34a mimics [178]. Mir-125b was found to be overexpressed in doxorubicin-resistant ES cell lines by miRNA profiling. Inhibition of miR-125b also resulted in increased sensitivity to doxorubicin through upregulation of p53 and the proapoptotic protein BAK [179].

3 Concluding Remarks

The growing number of reports in the literature on miRNA and pediatric cancer supports the rationale of using miRNAs for the treatment of childhood malignancies. Current therapeutic approaches comprise surgery of the primary tumor when possible, cell cycle or DNA synthesis-interfering chemotherapeutic agents, and/or radiotherapy. Although the combination of these elements in sophisticated multimodal treatments has increased patient survival, a significant proportion of cases remain incurable; however, when cured, patients suffer severe side effects associated with these forms of treatment. Therefore, it is imperative to participate in the development of therapies with increased efficacy and less toxicity. In this chapter, we review a considerable number of miRNAs that have been shown to have therapeutic potential both *in vitro* and in preclinical mouse models. Some miRNAs (e.g. miR-34) are currently used in ongoing phase I clinical trials on adult malignancies and evidence show they could also be beneficial for a good representation of pediatric solid tumors. However, the progress of miRNA research into pediatric cancers must not be restricted to miRNAs that have been shown to be effective in adult tumors. Given the different origin, etiology and behavior of pediatric cancers, attention should be focused on their specific miRNA alterations and identifying their potential therapeutic targets. Otherwise, the success of pediatric cancer treatments will always play second fiddle to adult cancer and critical therapeutic targets might be missed.

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Chapter 15
Targeting Immune System Through Targeting miRNA for Cancer Therapy

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1 Introduction

MicroRNAs (miRNAs) are a group of small non-coding RNA molecules that play a central role in a number of biological processes through the post-transcriptional regulation of gene expression. This class of molecules provides the relevant regulation epigenetically in addition to the major categories of DNA methylation, histone deacetylation, chromatin remodelling, gene imprinting, and noncoding RNA regulation [1].

The miRNAs have been shown to be critical contributors in the pathogenesis of many diseases including cancer. On one hand, miRNAs present as potential future diagnostic and prognostic markers and as viable therapeutic targets for cancer treatment [2–4]. On the other hand, miRNAs are also important regulators of the development and functions of diverse immunologically important cell types and the related complex cytokine network.

There are two major arms of the immune system, known as the innate and adaptive immune responses, which work in a complementary manner to help the body maintain its healthy status. The major players of the innate immune system that provide the first line of defence are natural killer (NK) cells, γδ T cells and macrophages. These cells, together with some inflammatory cytokines, critically defend the barriers at the mucosal and cutaneous levels. In the adaptive immune system, specificity and memory are the two key characteristics that are absent in the innate immune system. Dendritic cells (DC), B cells and T cells are the three cooperating major cell types that constitute the adaptive immune system.

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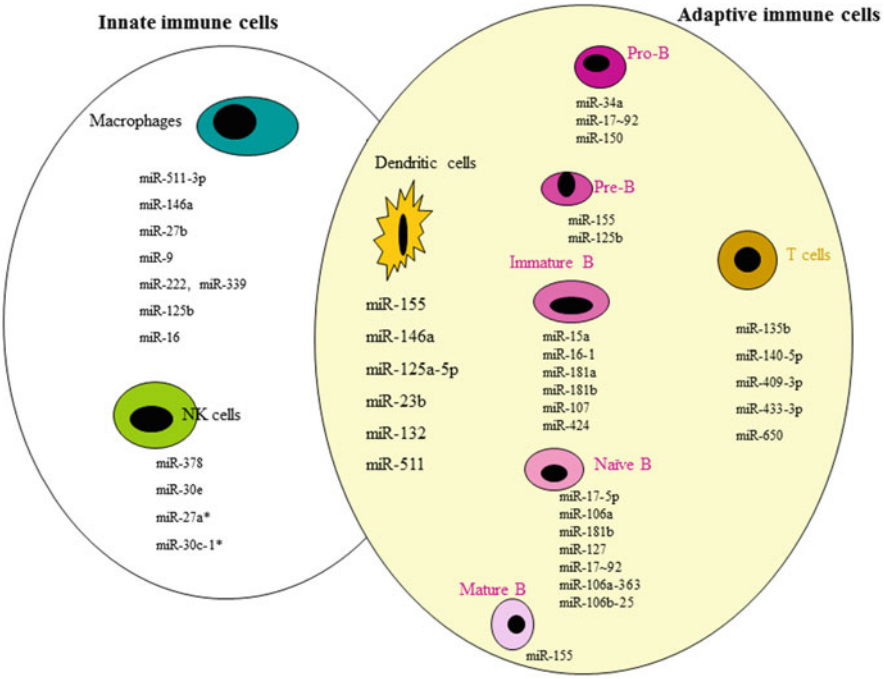

Fig. 15.1 miRNAs Involvement in immune cells [9]

Some lineage-specific miRNAs have been found to play a key role in regulating various developmental stages of the lineage development of the two major arms and thus affect different cell types in the mature state and their progenitor counterparts [5]. miRNAs are also involved in the activation of various immune cell components and thus regulate various cancer-related inflammatory responses and cytokine signalling [6, 7]. Furthermore, certain miRNAs display the important modulatory capabilities on the life span, migration and immunogenicity of immune cells. For example, research showing that harnessing these features of miRNA targeting of DCs may lead to possible DC vaccine development for future clinical trials [8]. Figure 15.1 forms a visible representation of the involved miRNAs in immune cells.

2 miRNAs and Innate Immunity

One of the important cell types for the first line of defence of the immune system is the epithelial cell population that expresses some pathogen pattern recognition receptors, including the Toll-like receptors (TLRs), which recognise pathogen-associated molecular patterns and can induce strong pro-inflammatory responses

[10]. In the recognition of pathogens, TLRs recruit adaptor proteins to facilitate the activation of downstream signalling cascades, such as the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase pathways. The activation process induces the expression of adhesion molecules, inflammatory mediators of cytokines or chemokines, and antimicrobial peptides that initiate innate immune responses and anti-tumour involvement, which are regulated by a number of complex networks. miRNAs are the essential portion of these complex regulatory networks for the cellular processes, differentiation and final fate of these innate immune cells [11].

Another major cell population of the innate immune response is the macrophages. Tissue macrophages detect pathogens through TLRs and after phagocytosing these pathogens, initiate the innate immune responses [12]. Macrophages, in response to microbes, produce cytokines that stimulate inflammation via leukocyte recruitment. Subsequently, NK cells are activated and produce the macrophage-activating cytokine IFN-γ, which is now known to have a central role in the regulation of inflammation. Additionally, miR-19 has been found to be involved in modulating this NF-κB-based inflammatory activity [13]. Table 15.1 provides a summary of a list of miRNAs and their corresponding target genes in the activation of macrophages and NK cells.

2.1 Macrophages

Macrophage subsets include the 'classically activated' pro-inflammatory (M1) and 'alternatively activated' anti-inflammatory (M2) cells [24]. The transcriptional activation of macrophage mannose receptor 1 (MRC1) in M2-polarised tumour-associated macrophages (TAMs) involves miR-511-3p regulation to establish the threshold for inflammatory cell activation in tumours in which the induced expression of miR-511-3p downregulates the pro-tumour gene signature of MRC1(+) TAMs and inhibits tumour growth [14]. The transcription of the miR-146a gene in human THP-1 cells has been reported in response to TLR-4 signalling, and miR-146a acts on the target genes TNF-receptor associated factor 6 (TRAF6) and IL-1 receptor associated kinase 1 (IRAK1) through the NF-κB-dependent cascade [15]. Similarly, miR-27b expression can also be induced directly by lipopolysaccharide (LPS) [16]. In addition, LPS will regulate miR-9 expression via the myeloid differentiation primary response gene 88 through the NF-κB pathway in human macrophages and neutrophils [17].

The miRNAs, such as miR-222 and miR-339, have also been implicated in the expression of adhesion and co-stimulatory molecules, including the intercellular adhesion molecule 1 (ICAM1), which is essential in the interactions of innate immune cells [18]. The miR-125b has also been reported to regulate the expression of cytokines and chemokines by targeting and suppressing tumour necrosis factor α (TNF-α) transcription in mouse Raw 264.7 macrophages [19]. Furthermore, miR-16 has been demonstrated to be one of the critical regulators of inflammatory

Table 15.1 miRNAs involved in innate immunity

		miRNAs	Target gene	Reference
Innate				
	Macrophages	miR-511-3p	MRC1	[14]
		miR-146a	TRAF6, IRAK1	[15]
		miR-27b	–	[16]
		miR-9	NF-κB1	[17]
		miR-222, miR-339	ICAM-1	[18]
		miR-125b	TNF-α	[19]
		miR-16	–	[20]
	NK cells	miR-378	GZMB	[21]
		miR-30e	PRF	[21]
		miR-27a*	PRF1 & GZMB	[22]
		miR-30c-1*	HMBOX1	[23]

*indicates an miRNA expressed at *a low level* relative to the miRNA in the opposite arm of a hairpin

responses, with a high level of miR-16 in inflammatory cells that inhibit the production of inflammatory mediators [20].

2.2 NK Cells

NK cells are essential innate immune components with potent cytotoxicity and type I IFN (INF-α) initiation capability. Wang et al. [21] reported that the expression of miR-378 and miR-30e is markedly decreased in IFN-α-activated NK cells that are labelled as granzyme B (GZMB)- and perforin (PRF)-positive. In contrast, downregulated miR-378 and miR-30e are the negative regulators of NK cell cytotoxicity during activation in the subset of sorted CD16(+)CD56(dim)CD69(+) human NK cells.

Another important cytokine produced by NK cells is TNF-α. The up-regulation of miR-30c-1* is now known to trigger the overexpression of transmembrane TNF-α, which in turn enhances NK cell cytotoxicity against the hepatoma cell lines SMMC-7721 and HepG2 by targeting the transcriptional repressor gene HMBOX1 [23, 25]. In contrast, miR-27a* is able to negatively regulate NK cell cytotoxicity by silencing the genes PRF1 and GZMB, as shown by the knockdown of miR-27a* in NK cells, which leads to decreased tumour growth in a human tumour xenograft model [22].

3 miRNAs and Adaptive Immunity

miRNAs play an important role in the early differentiation of B cells and act as regulators of the immune response by T cells and DCs in adaptive immunity. Great efforts have been made to demonstrate the role of miRNAs in adaptive immune

Table 15.2 miRNAs involved in adaptive immunity

		miRNAs	Target gene	References
Adaptive				
B cells	Pro B cells	miR-34a	FOXP1	[26]
		miR-17-92	BIM	[27, 28]
		miR-150	c-MYB	[29–31]
	Pre B cells	miR-155	BIC	[32, 33]
		miR-155	SHIP & C/EBPβ	[34]
		miR-125b	LIN28A	[35]
	Immature B cells	miR-15a, miR-16-1		[36]
		miR-181a, miR-181b, miR-107, miR-424	PLAG1	[37, 38]
	Naïve B cells	miR-17-5p, miR-106a, miR-181b		[39]
		miR-17-5p, miR-127		[40]
		miR-17-92, miR-106a-363, miR-106b-25		[41]
	Mature B cells	miR-155	PU.1	[42]
T cells		miR-135b	FOXO1, STAT6 & GATA3	[43]
		miR-140-5p, miR-409-3p, miR-433-3p, miR-650	ULBP1	[44]
DCs		miR-155, miR-146a, miR-125a-5p		[8]
		miR-155	AGO2, AGO4	[45]
		miR-23b	NOTCH1	[46]
		miR-146a, miR-155, miR-132		[47, 48]
		miR-511		[49]

cells in recent years. Table 15.2 summarises the miRNAs involved in adaptive immunity and their respective target genes, with the third column showing those miRNAs participating in these cell types at various developmental stages.

3.1 B Cells

The expression of the transcription factors for B-cell development has been found to be precise and time-specific under the influence of a few miRNAs on B cells at various maturation stages. It is evident that the alteration of miRNA expression may lead to important functions in cellular differentiation and be associated with the activation status of the mature B cells in the immune system [50]. The role of miRNAs in B-cell development and B-cell lymphogenesis is largely unknown. The stage-specific expression of various miRNAs has suggested highly specialised regulatory functions in B-cell biology, which would reveal the cell type-specificity of miRNAs in B lymphocytes [51].

The expression of miR-150 was shown to block the transition from the pro-B to pre-B stage, likely through the down-regulation of c-MYB [29–31]. miR-17-92 is also essential for B-cell development, in which the absence of miR-17-92 will lead to the elevated expression of the pro-apoptotic protein BIM, which in turn inhibits B-cell development at the pro-B to pre-B transition. A link between the oncogenic properties of miR-17-92 and its physiological functions during B lymphopoiesis has been suggested [27]. Accordingly, B-cell development could be partially rescued by the ablation of BIM through the suppression of miR-17-92 [28]. In addition to miR-150 and miR-17-92, miR-34a was also found to block B-cell development at the transition from pro-B to pre-B cells via the target gene forkhead box transcription factor (FOXP1), which is a transcription factor gene; this process leads to a reduction in mature B cells. Accordingly, the knockdown of miR-34a resulted in the elevation of FOXP1 expression and an increase in mature B cells [26].

The association of miR-155 with the primary transcript of the host gene BIC was observed in preleukaemic pre–B-cell proliferation in the spleen and bone marrow of transgenic mice [32, 33]. The high expression of miR-155 was also observed in murine B-cell precursors of acute lymphoblastic leukaemia or high-grade lymphoma, which were preceded by polyclonal pre-B cell proliferation. miR-155 directly targeted SRC homology 2 domain-containing inositol-5-phosphatase (SHIP) and CCAAT enhancer-binding protein beta (C/EBPβ), which are both regulators of the IL-6 signalling pathway. miR-155 was hypothesised to cause accumulation of large pre-B cells and acute lymphoblastic leukaemia by down-regulating SHIP and C/EBPβ [34].

The over-expression of miR-21 has been found in a number of tumour types. Medina et al. [52] demonstrated that the over-expression of miR-21 leads to a pre-B malignant lymphoid-like phenotype and that the tumours regressed completely in a few days after miR-21 was inactivated. miR-125b has been found to up-regulate a number of common myeloid progenitors and to inhibit the development of pre-B cells by acting on some candidate targets, including the induced pluripotent stem cell gene LIN28A [35]. The miR-125b physiologically regulates haematopoietic development. LIN28A was saliently suppressed in mouse haematopoietic stem cells and progenitor cells, and the knockdown of LIN28A can lead to haematopoietic lineage skewing with an increase in myeloid cells but decrease in B cells.

The deregulation of miR-181a, miR-181b, miR-107 and miR-424 was found to lead to the subsequent overexpression of the oncogenic transcription factor pleomorphic adenoma gene 1 (PLAG1) in a number of chronic lymphocytic leukaemia (CLL) cases [37, 38]. CLL is characterised by the clonal expansion of immature CD5-positive B cells; up to 20 % of the patients with CLL are not controlled with standard therapies using cytotoxic agents. The 13q14.3 chromosomal region often found in patients with CLL contains miR-15a and miR-16-1 [36]. Tan et al. [39] characterised the miRNA expression profile of normal B cell subsets that included naïve, germinal centre (GC) B cells and memory B cells with a miRNA *in situ* hybridisation technique. Several miRNAs were elevated in GC B cells, such as miR-17-5p, miR-106a and miR-181b, whereas the gradual decrease in the staining

intensity of these three miRNAs from the dark to light zone was observed in GC. miR-150 was the most abundant in all three B-cell subsets.

In a study of the expression of a panel of 15 miRNAs in some DLBCL cases, the expression of miR-17-5p was significantly higher in central nervous system diffuse large B cell lymphoma (DLBCL) than in testicular DLBCL, and miR-127 was found to be highly expressed in testicular DLBCL compared with central nervous system DLBCL [40].

Iqbal et al. [41] identified a 19-miRNA classifier including 6 up-regulated miRNAs and 13 down-regulated miRNAs that helped distinguish mantle cell lymphoma (MCL) from other aggressive lymphomas, and some of the up-regulated miRNAs were highly expressed in naïve B cells. The high expression of miR-17-92, miR-106a-363 and miR-106b-25 was observed in some patients with MCL in their studies. For example, miR-155 is encoded in the B cell integration cluster, and its knockdown leads to B cell defects and a failure of immunoglobulin-switched plasma cells. The transcription factor PU.1 is the validated target of miR-155 [42].

3.1.1 Related Signalling Pathways in Lymphoma

The PI3K/PTEN/AKT pathway is one of the key signalling pathways involved in the regulation of cell growth. The frequent dysregulation of the PI3K/PTEN pathway in human cancer demonstrates that this pathway is an appropriate target for cancer therapeutics [53]. Hafsi et al. [54] suggested that the dysregulated signalling of this pathway might be associated with activating mutations in PI3K-related genes. An increased PI3K signal will stimulate downstream AKT signalling, promote growth factor-independent growth and facilitate cell invasion and metastasis, which account for 50 % of all human malignancies [55]. A common secondary genomic alteration detected in MCL is chromosome 13q31-q32 gain or amplification, which targets the miR-17-92 cluster. Rao et al. [56] demonstrated that the protein phosphatase PHLPP2, an important negative regulator of the PI3K/AKT pathway, was a direct target of miR-17-92 which also targeted PTEN and BIM. The inhibition of miR-17-92 suppressed the PI3K/AKT pathway, which in turn inhibited tumour growth in the xenograft MCL mouse model. Hence, targeting the miR-17-92 cluster may provide a novel therapeutic approach for patients with MCL.

3.2 T Cells

miRNAs have been shown to be involved in the regulation of T-cell responses, with a dynamic expression pattern relative to the various stages of T cell development. miR-135b was reported to mediate nucleophosmin-anaplastic lymphoma kinase (NPM-ALK)-driven oncogenicity and induce the immunophenotype of IL-17 in

anaplastic large cell lymphoma (ALCL). Oncogene NPM-ALK strongly promoted miR-135b expression through the activation of transducer and activator of transcription (STAT) 3, and the elevated miR-135b targets FOXO1, a transcription factor regulating gluconeogenesis and glyconeogenesis via insulin signalling in ALCL cells. Chemosensitivity in Jurkat cell line was found to be decreased by miR-135b [43]. Furthermore, miR-135b suppresses the T-helper 2 regulator gene STAT6 and GATA3, whereas antisense-based miR-135b inhibition induces tumour angiogenesis *in vivo*.

NKG2D, encoded by the KLRK1 gene, is one of the activating immunoreceptors found on CD8 T cells and NK cells, and its ligands are stress-inducible proteins, including ULBP1, that enable the recognition and lysis of tumour cells. Some miRNAs were shown to be involved in the post-transcriptional regulation of ULBP1 expression in Jurkat and HeLa cells. Among these miRNAs, miR-140-5p, miR-409-3p, miR-433-3p and miR-650 are involved in the regulation of ULBP1 expression [44].

3.3 Dendritic Cells

DCs are found in almost all peripheral tissues and in primary and secondary lymphoid organs. The antigen presentation of DC controls immunity and tolerance, is linked with almost all types of immune cells and plays major roles in regulation of immune responses [57].

Cubillos-Ruiz et al. [45] took advantage of the spontaneous enhanced endocytic activity of ovarian cancer-associated DCs to selectively supplement the immunostimulatory miR-155. Modulating the activity of miRNAs may lead to cancer interventions. miR-155 that has been processed endogenously would favour Argonaute 2 (AGO2) and AGO4 loading, resulting in transcriptional changes that might silence multiple immunosuppressive mediators [58]. Thus, tumour-infiltrating DCs were transformed into highly immunostimulatory cells, triggering potent anti-tumour responses to abrogate the progression of ovarian cancer.

Concerning the tolerogenic property of DCs, miR-23b may be one of the entry points in targeting the therapeutic management of allergies. The upregulation of miR-23b could be observed in bone marrow DCs (BMDCs) by ovalbumin in a murine model. Increased IL-10 levels, decreased IL-12 levels and an enhancement of the $FOXP3^+$ $CD4^+$ T regulatory cell differentiation were shown in BMDCs by the transfection of miR-23b, likely through the inhibition of the transmembrane protein family member NOTCH1 and the NF-κB signalling pathway [46]. Similar results were also obtained in human monocyte-derived DCs.

Some miRNAs, such as miR-146a and miR-155, may act as checkpoints in the cellular differentiation aspect of the immune system [47]. Using a miRNA array, Holmstrom et al. [8] demonstrated a significant induction of miR-155, miR-146a and miR-125a-5p in some donor DCs treated with LPS. In addition to miR-146a and miR-155, miR-132 is a TLR ligand-induced regulator of inflammatory

mediators, which may modulate TLR pathway activation and may be used to develop relevant therapeutics for inflammatory diseases [48]. miR-511 has been identified as a novel potent modulator of the human immune response through the validation of CD80 expression and inhibition of miR-511 in HEK293 cells. Similarly, DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) was found to be reduced, thereby revealing that miR-511 may act as a positive regulator of TLR-4 [49].

4 miRNAs Related to Anti-tumour Immunity

Cancer, as a result of a complicated multi-step process, involves the accumulation of sequential genomic alterations, including those of miRNAs, and can be characterised by uncontrolled proliferation, invasion and metastasis [59]. Increasing evidence shows an association with the aberrant miRNA expression patterns in human cancers [60, 61]. miRNAs play crucial roles in the initiation and progression of human cancer [62], which in turn suggests their potential diagnostic and therapeutic roles in anti-tumour immunity. Table 15.3 summarises the altered expression of miRNAs with their respective target genes in various types of cancer.

Cancers are the major causes of mortality and morbidity in industrialised countries. Some concepts related to the mechanisms of the modulation of both innate and adaptive immune cells leading to anti-tumour effects will contribute to the regulation of carcinogenic processes and associated inflammatory effects. The strong balance between anti-tumour immunity and proinflammatory activity caused by the tumour in the tumour microenvironment niche may weaken anti-tumour immunity. The modulation of immune cells targeted for therapeutic intervention in malignant diseases may restore the sensitivity of cancer cells to chemotherapies. miRNAs should be analysed with nuclear factors, such as NF-κB, that serve as molecular links between inflammation and tumour progression [97].

With an increasing number of studies showing that miRNAs could function as oncosuppressors, the exploration of miRNA-based anticancer therapies was conducted to improve the response to current targeted cancer treatments. This improvement will provide the essential enhancement of the capability of targeting multiple effectors in pathways involving cancer cell proliferation and survival [98].

Some malignant diseases are developed in association with tumour viruses, and a number of studies have illustrated miRNAs as critical regulators of tumour pathways in which the dysregulation of cellular miRNA expression could promote tumour formation. Tumour viruses encode their own miRNAs, which manipulate the expression of cellular miRNAs to modulate the host cellular environment and in turn facilitate their respective infection cycles. The modulation of miRNA expression might influence the signal transduction cascades that favour tumourigenesis [99].

Table 15.3 Tumour types with miRNA expression against gene targets

Tumour type	Up-regulated	Down-regulated	Brief description of miRNAs	Target genes	References
CNS involvement					
CNS glioblastoma		miR-222, miR-339	Possible therapeutic targets	ICAM1	[18, 63]
Blood component					
ALL	miR-128a		Possible diagnostic markers		[64]
	miR-128b				
	miR-19		Oncomir	PTEN, BIM	[65]
	miR-15a			BCL2	[66]
	miR-155				[67]
AML	miR-223, let-7b		Possible diagnostic markers		[64]
APL	miR-125b		Potential therapeutic target	BAK1	[68]
Gynaecological					
Breast cancer	miR-125b				[69, 70]
Cervical		miR-424	Tumour suppressor	CHK1, p-CHK1	[71]
		miR-375		SP1	[72]
		miR-214		PLEXIN-B1	[73]
Gastrointestinal					
Laryngeal squamous cell carcinoma		miR-206	Tumour suppressor miRNA	VEGF	[74]
Oesophagus squamous cell carcinoma	miR-92			CDH1	[75]
	miR-25			CDH1	[76]
	miR-142-3p		Potential prognostic marker		[77]
	miR-21		Oncomir		[78]
		miR-145, miR-133a, miR-133b	Tumour suppressor miRNAs	FSCN1	[79]
Gastric cancer	miR-21			PTEN	[80]
	miR-223			FBXW7/hCDC4	[81]
		miR-335	Metastasis suppressor miRNA	SP1, BCL-W	[82]
		miR-409-3p		RDX	[83]
		miR-409-3p		PHF10	[84]

		miR-155	Tumour suppressor miRNA	SMAD2	[85]
		miR-148a		ROCK1	[86]
		miR-148a		P27 or CDKN1B	[87]
		miR-182	Tumour suppressor miRNA	CREB1	[88]
Hepatocellular cancer (HCC)					
HCC	miR-519d		Oncomir	PTEN, AKT3, TIMP2	[89]
	miR-373			PPP6C	[90]
	miR-199a			HIF-1α	[91]
	miR-124			ROCK2, EZH2	[92]
HCC cell line QGY-7703		miR-519d	Tumour suppressor miRNA	MKI67	[93]
Other cancers					
Lung cancer		miR-330		DCK	[94]
Prostate cancer		miR-222	Tumour suppressor miRNAs		[95]
		miR-31			
Colon cancer	miR-155		Oncomir		[96]

Abbreviations: *CNS* central nervous system, *ALL* acute lymphoblastic leukemia, *AML* acute myeloid leukemia, *APL* acute promyelocytic leukemia

4.1 CNS Involvement

Dicer-mediated expression of miR-222 and miR-339 demonstrated the promoting effect on the immunoresistance of cancer cells through the regulation of the intercellular adhesion molecule ICAM1. The miR-222 and miR-339 were found to be specifically down-regulated by the alteration of dicer expression in both colorectal and glioblastoma cell lines [18], suggesting novel methods to develop miRNA-targeted therapies to enhance the cytolysis of tumour cells in a variety of malignancies, including glioblastoma [63].

4.2 Cancer in Blood Components

The miRNA expression has been found to be associated with the clinical outcome of patients and with drug resistance, which was described in B-CLL, lung cancer [100] and B-ALL [101]. The potential anti-tumour activity of IL-23 in paediatric B-acute lymphoblastic leukaemia (B-ALL) cells was investigated. The up-regulation of IL-23 dampened the tumour growth *in vitro* and *in vivo* through the inhibition of tumour cell proliferation and the induction of apoptosis, which was found to be associated with the IL-23-induced up-regulation of miR-15a expression and the consequent down-regulation of BCL-2 protein expression in paediatric B-ALL cells [66]. A similar study showed the anti-tumour effect of IL-27 against paediatric B-ALL cells with the down-regulated expression of miR-155 [67].

ALL and AML were demonstrated to have different miRNA expression patterns [64]. Moreover, miR-128a and miR-128b were highly expressed in ALL cells, whereas miR-223 and let-7b were detected in AML, which seemed to be significant and discriminatory for these leukaemias. Notably, 50 % of known human miRNAs were found to be located at fragile sites and genomic regions involved in cancer, and these miRNAs might function as tumour suppressors or oncogenes [102]. The deregulation of miRNAs was observed in B-ALL, which appears to be one of the key mechanisms involved in B-ALL leukaemogenesis, which led to the suggestion that miRNAs represent new potential therapeutic targets [103]. In addition, miR-19 promotes leukaemogenesis in NOTCH1-induced T cell acute lymphoblastic leukaemia *in vivo* by regulating PTEN and BIM [65].

4.3 Gynaecological Cancers

The miRNAs are involved in gynaecological disorders affecting the ovary or uterus, frequently affected by endometriosis, which is classified as a tumour lesion, and in malignant gynaecological diseases including endometrial, cervical and ovarian cancers. Emerging evidence has shown that deregulated miRNA expression

might be involved in the multifactorial and polygenic diseases of endometriosis and that miRNAs appear to be potent regulators of gene expression in endometriosis, resulting in the prospect of using miRNAs as biomarkers or therapeutic measures for these cancers [104].

A significant decrease in the expression of miR-424 was observed in a number of cervical cancer tissue samples, which was positively correlated with poor prognostic clinicopathological parameters. The tumour suppressive role of miR-424 in the progression of cervical cancer via the up-regulated expression of CHK1 and p-CHK1 suggested miR-424 as a probable anticancer therapeutic target for cervical cancer patients [71]. Similarly, down-regulated miR-375 might contribute to the progression of cervical cancer based on the findings that miR-375 promoted cell malignant transformation via its target gene, SP1 [72]. Qiang et al. [73] also found that undermining miR214 expression in cervical cancer tissue and HeLa cells would insufficiently inhibit the probable oncogene plexin-B1, which might contribute to cervical tumour metastasis and invasion.

4.4 Cancers of the GI Tract

The miR-206 was found to be significantly down-regulated in laryngeal squamous cell carcinoma (LSCC) tissue, and its transfection decreases the expression of vascular endothelial growth factor (VEGF) in LSCC cells, contributing to the tumour suppression function of miR-206 [74].

The high level of expression of miR-92 [75] and overexpression of miR-25 [76] were demonstrated in oesophageal squamous cell carcinoma (ESCC) tissues, and both miRNAs modulate the migration and invasion of ESCC cells. The expression of miR-92 and miR-25 was correlated with the status of lymph node metastasis, most likely through the repression of the CD324-induced expression of the tumour suppressor CDH1 gene but not with the apoptosis and proliferation of ESCC cells. Another study showed the high expression of miR-142-3p in ESCC cells, which was well correlated with the poor prognosis of ESCC patients [77]. miR-21, which had been recognised as an oncogenic miRNA in various malignancies, might also be one of the oncogenic miRNAs involved in ESCC [78]. miR-145, miR-133a and miR-133b were also revealed to have tumour suppressive effects inhibiting ESCC cell proliferation and invasion via repression of the FSCN1 gene, whereas FSCN1 was known to be involved in the metastasis of multiple tumour types and regulated by a number of miRNAs [79].

A relatively higher expression of miR-21 was exhibited in gastric cancer tissue. The miR-21 might be involved in the initiation and development of gastric cancer by regulating the PTEN expression level [80]. The miR-223 was found to be highly expressed in gastric cancer tissues and the corresponding gastric mucosal tissues, specifically in patients with lymph-node metastasis or metastatic disease at the advanced pathological M1 stage, in which the expression of the target FBXW7/hCDC4, a general tumour suppressor gene, was inversely correlated in the study

[81]. The significantly down-regulated expression of miR-335 was confirmed in 4 gastric cell lines and 70 gastric cancer tissues, and elevated miR-335 was verified to suppress gastric cancer invasion and metastasis *in vitro* and *in vivo*, most likely via targeting the transcription factor gene SP1 directly and the apoptosis regulator gene BCL-W indirectly [82]. miR-409-3p was found to be down-regulated in human gastric tumours, and its expression was significantly associated with the tumour node metastasis stage. An increase in miR-409-3p reduced the migration and invasion of cancer cells *in vitro*, most likely via the pro-metastatic gene radixin (RDX) [83] and the PHF10 gene [84].

Down-regulation of miR-155 was observed in gastric cancer cell lines. The miR-155 might act as a tumour suppressor through the repression of its target gene SMAD2, which encodes a signal transducer and transcription modulator protein involved in multiple signalling pathways [85]. Similarly, the down-regulation of miR-148a was observed in gastric tumour tissues, with miR-148a acting as a tumour metastasis suppressor in gastric cancer through the repression of the gene ROCK1, which encodes a serine/threonine kinase involved in the regulation of cell proliferation and programmed cell death [86], and through the repression of the p27 or CDKN1B gene, which encodes an inhibitor protein of the cell division cycle [87]. miR-182 was also found to be significantly down-regulated in human gastric adenocarcinoma tissues, and the over-expression of miR-182 would suppress the proliferation and colony formation of gastric tumours by targeting the oncogene cAMP-responsive element binding protein 1 (CREB1) [88].

The exploration of deregulated miRNAs in gastrointestinal tumours could contribute essential data for the possible development of novel cancer gene therapies [105].

4.5 Hepatocellular Cancers

The over-expression of miR-519d was found to have an oncogenic role in hepatocellular carcinoma (HCC) with promoting effects on cell proliferation, invasion and apoptotic impairment by directly targeting the tumour suppressor gene PTEN, insulin regulating gene AKT3 and endothelial suppressing gene TIMP2 [89]. In addition, the down-regulation of miR-519d was demonstrated to have a tumour-suppressive role in the human HCC cell line QGY-7703 through its action on the targetgene MKI67, which is associated with the ribosomal RNA transcription necessary for cellular proliferation [93]. miR-373 was up-regulated in human HCC tissue, and protein phosphatase 6 catalytic subunit (PPP6C), acting as a negative cell cycle regulator, was identified as the target gene of miR-373 [90]. In addition, miR-199a was significantly down-regulated in HCC tissues and a few HCC cell lines, including SMMC-7721, BEL-7402 and HepG2, most likely through its effect on the target gene HIF-1α, which is associated with cell growth under low oxygen conditions [91]. Down-regulated miR-124 was reported in HCC tissues and was found to be associated with more aggressive HCC in patients with a poor prognostic phenotype. The miR-124 likely targets ROCK2 and EZH2, which

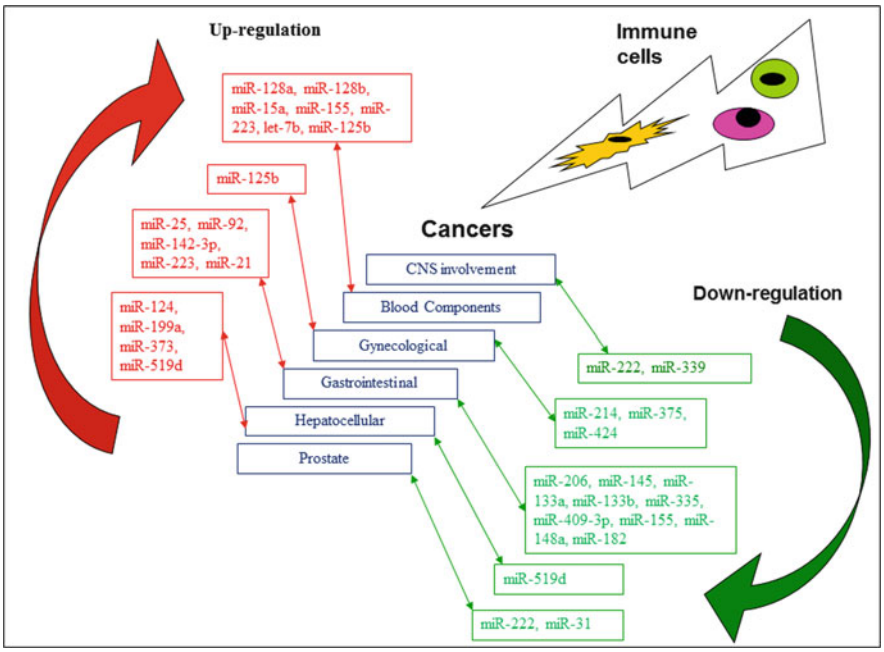

Fig. 15.2 Tumour types and their miRNA expression [9]

are key modulators of cytoskeletal rearrangement and the maintenance of the transcriptional repressive state over successive cell generations, respectively [92].

4.6 Other Cancers

The miR-330 was found to act post-transcriptionally by regulating deoxycytidine kinase (DCK) mRNA expression, which is essential for the phosphorylation of natural deoxyribonucleosides and their nucleoside analogues, which are widely used as anticancer compounds, including gemcitabine and cytarabine [94]. Elevated miR-330 would suppress DCK expression, leading to the undermined sensitivity to gemcitabine.

The miRNAs are involved in the pathogenesis of prostate cancer, and their roles as oncogenes, tumour suppressors and metastasis regulators lead to the hope that they might be molecularly targeted as diagnostic or prognostic markers for the treatment of prostate cancer [106]. Fuse et al. [95] demonstrated that miR-222 and miR-31 inhibited cell proliferation, invasion and migration in the prostate cancer cell lines PC3 and DU145, indicating that these two miRNAs might act as tumour suppressors in prostate cancer. miR-155 is a member of oncomir class of miRNAs and implicated in a wide variety of tumours including colon cancer [96]. Figure 15.2 signifies the regulated miRNA expressions in various tumour types which might be therapeutically instituted in the arms of immune system.

5 Discussion

Emerging evidence has shown that miRNAs are important modulators in cancer pathogenesis within the bigger picture of how cells are transformed into malignant cells and multiply in an uncontrolled manner, followed by tissue invasion and metastasis. Equally, research has also indicated that miRNAs can be effective inhibitors as anti-tumour agents. In this regard, an example such as the antisense-based inhibition of a specific miRNA has been found to be useful due to the enhancement of the corresponding anti-tumour immunity [19].

The development of plasma-circulating miRNA detection and expression profiles, which have been found in association with a range of tumour types, can also be used in therapeutic strategies [104, 107] and in various clinical settings of cancer management [108]. As shown in Table 15.3, a variety of miRNAs are either up-regulated or down-regulated in different tumour types. Examples include the following: oesophageal squamous cell carcinoma identified with the elevated expression of miR-21, miR-25, miR-92, and miR-142-3p; gastric cancer with the down-regulated expression of miR-155, miR-148a, miR-182 and miR-409-3p; and hepatic cellular cancer with the up-regulated expression of miR-124, miR-199a, miR-373 and miR-519d.

6 Current Studies Targeting miRNAs for Therapeutic Purposes

Mechanisms of resistance in anticancer treatment have been postulated to be associated with the altered expression of the ATP-binding cassette family of transporters involved in cell membrane transportation. Thus, the emerging role of miRNAs as key gene expression regulators in drug resistance can be the specific mechanism involved in combating the resistance to tyrosine kinase inhibitors in chronic myeloid leukaemia [109]. An understanding of how the involved miRNAs influence the phosphatidylinositol 3 kinase PI3K/AKT signalling pathway in modulating the function of the breast cancer-resistant protein can lead to a therapeutic benefit in breast cancer [110, 111].

Techniques that restore the activity of tumour suppressor miRNAs by the inhibition of oncogenic miRNAs using single-stranded antisense oligonucleotides or antimiR have been recently employed for the development of miRNA-based cancer therapeutics [112–114]. In recent studies, certain cancers exhibited dependence on the expression of a single oncogenic miRNA or oncomir [115]. The possible programming of the balance between the expression of oncogenic miRNAs and tumour suppressor miRNAs may result in the specific anti-tumour effect [98].

To target miRNAs in cancer, one strategy involves hindering the oncomir from expression or rebuilding the corresponding tumour suppressor miRNA that might

have lost in the cancer. The apoptosis of leukaemic MEG01 could be induced through the reintroduction of miR-15a and miR-16-1, which were shown to inhibit tumour growth *in vivo* in a xenograft model [116], and silencing the oncogenic miR-21 via antisense oligonucleotides has generated an anti-proliferative response *in vitro* in a number of cellular models [117]. Although chemically modified anti-miRNA oligonucleotides have been developed [118, 119], nevertheless, their effective delivery into target tissues remains a limitation and needs to be further evaluated for a more specific delivery method with fewer side effects. In addition, the modulation of the miRNA expression via drugs or other agents during their transcription might show the potential of miRNAs as therapeutic adjuvant tools to improve the response and overcome resistance [120].

7 Future Challenges and Conclusions

The quantification of extracellular miRNAs in the blood circulation of both healthy and diseased patients was discovered to be confined to the lipid or lipoprotein complexes, such as microvesicles, exosomes or apoptotic bodies, which were highly stable [121]. These circulating miRNAs in cancer patients may serve as a novel diagnostic marker, although their logistic mechanism and the meaning of the quantified signatories of these extracellular miRNAs remain unclear. Hopefully, such identified molecular markers for the prediction of treatment outcome will be very useful, with the expression of circulating miRNAs being used to determine the clinical outcome in cancer patients treated with adjuvant chemotherapy [70].

Different studies have reported conflicting findings or inconsistencies regarding miRNAs from the same tumour, as shown in Table 15.3; for example, miR-519d has been found to be up-regulated in HCC [89] but down-regulated in a HCC cell line [93]. These findings show that there is a strong need to establish endogenous miRNA controls for normalisation in various methodologies during extraction and quantification. Furthermore, designing a well-planned and controlled analysis of miRNAs in a large cohort of both patients and healthy subjects may be necessary to provide more meaningful evidence for the quantification of miRNA expression and an insightful understanding concerning immunity and tumour biology. Such a trial will eventually lead to clinical advancements in cancer therapy and the significant enhancement of the management of various malignancies.

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Chapter 16
miRNA Regulation of DNA Damage Repair Proteins in Cancer Cells: Interplay of ATM, TRAIL and miRNA

Ammad Ahmad Farooqi

1 Introduction

Ever increasing evidence from both cell culture and animal models is substantiating the fact that high-fidelity transmission of genetic information is a critical step and to ensure that, cells have evolved mechanisms and sophisticated nanomachinery to monitor genome integrity which is constantly under threat, even in perfectly healthy cells. Phosphoinositide-3-kinase-related protein kinase (PIKK) family members have emerged as master regulators in modulating DNA damage repair signaling and use many different mechanisms to regulate their downstream effectors through phosphorylation to maintain the specificity that is observed in non-homologous end joining (NHEJ) and homologous recombination (HR) signaling pathways. Repair of damaged DNA depends on convergence on either of the two DNA damage signaling cascades. The complicated molecular protein network that cooperatively forms the DNA damage response (DDR) machinery is further categorized into two hierarchically ordered and mutually coordinated pathways essentially NHEJ and homologous recombination [1, 2].

DNA damage signaling networks are highly divergent in spatio-temporally and decision-making process upon DNA damage is currently being broadly investigated and it is now known that in case of HR, DSB is recognized by MRN, a heterotrimeric complex formed by assembly of MRE11, RAD50 and NBS1. This DNA damage during S and G2 phases preferentially activate ATM, through the MRN. The resection of damage DNA promotes binding of RPA, RAD51 and RAD52 to the ssDNA tails. ssDNA invades the homologous region with the courtesy of RAD54 in the duplex thus forming a DNA joint, known as the displacement (D)-loop. The pathway is shown in Fig. 16.1.

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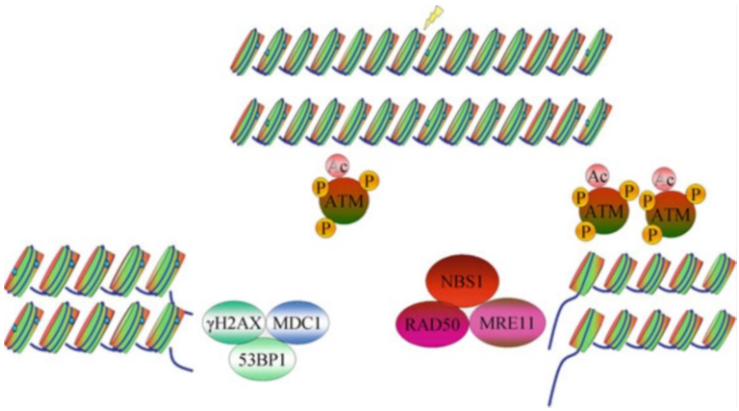

Fig. 16.1 Shows DNA damage repair signaling. ATM is a member of PIKK family and is activated to transduce the signal to downstream effectors. MRN is hetero-trimeric complex that is recruited at double stranded breaks

In case of NHEJ that occurs during G1 phase DSB is recognized by the Ku dimer (Ku70–Ku80) and DNA-PKcs. NHEJ is a multistep process that is triggered by different proteins and as a result two DNA ends are synapsed. DNA-PKcs and Artemis are phosphorylated, and consequently, DNA-PK increases the recruitment of XRCC4, DNA ligase IV and XRCC4-like complex (XLF), which finalize rejoining reaction.

Research over the years has added very important pieces of information to puzzle of DNA damage signaling. Interestingly, among many proteins reported to be involved in repair of DNA, ATM incontrovertibly occupies an important slot because of its multifaceted roles in normal and cancer cells. There is a progressive increase in the documentations related to ATM, addressing regulation of new proteins, context dependent and cell type specific responses of ATM to various external and internal stimuli. Although tremendous expansions are made in unraveling various facets of ATM biology, it will be even more important to sequentially gather and re-interpret the ideas derived from continuously upgrading research. High throughput technologies have undeniably broadened the knowledge related to regulation of regulators in DNA damage repair and how ATM itself is regulated at post transcriptional level. In this chapter, I will outline the current trends and paradigms related to ATM regulation by miRNAs. Moreover, increasingly it is being realized that genomic rearrangements act as a trigger for various miRNAs. This particular aspect will also be discussed independently.

RNA interference technology and advanced proteomics have revolutionized existing comprehension of multifaceted roles of ATM together with exceptional broadening of landscape of DNA damage response over the past few years. Here I will re-visit current developments and enrichments in the miRNA regulation of ATM, with a particular emphasis on how regulation of ATM mRNA by miRNA subsets influences cellular survival.

It is now understandable that recruitment/retention of ATM at DSBs requires its kinase activity because transient transfection of kinase-dead mutant of ATM in ATM deficient cells was unable to form damage-induced foci. But before I start discussion regarding miRNA mediated control of DNA damage regulators particularly ATM, ATR, BRCA, it will be even more important to give an overview of miRNA.

2 miRNA: Master Regulators of DNA Damage Response

The miRNA processing is a multistep mechanism that includes the RNA polymerase II or III mediated generation of the primary miRNA transcript (pri-miRNA). pri-miRNA is later cleaved by molecular machinery consisting of Drosha–DGCR8 in the nucleus. Cleaved transcript is known as pre-miRNA and has a hairpin like structure. It is transported from the nucleus to cytoplasm by Exportin-5-Ran-GTP. A recent report indicated ATM mediated transportation of miRNA from nucleus to cytoplasm. However this new aspect will be discussed in the relevant paragraph. In the cytoplasm pre-miRNA hairpin by Dicer that results in the conversion of miRNA into its biologically functional form. The functional strand of the mature miRNA works synchronously with Argonaute (Ago2) proteins and both proteins are loaded into the RNA-induced silencing complex (RISC). RISC is a multi-component machinery and loaded functional strand of miRNA is used as a template to silence target mRNAs [3, 4]. The Fig. 16.2 describes the pathway. The next section focuses on a recent report that highlights role of ATM in promoting the transfer of pre-miRNA from nucleus to cytoplasm. Particularly, rapidly expanding list of miRNAs reported to quantitatively control the expression of ATM is discussed and illustrated. Moreover, miRNA regulation of DNA damage repair regulators including ATR and BRCA1 is also described.

3 ATM Is Involved in Facilitating the Transportation of Pre-miRNA from Nucleus to Cytoplasm

Rapidly escalating evidence has started to disentangle the complicated web of miRNA regulation of ATM and ATM regulation of miRNA processing. It is intriguing to note that DNA damage signaling speeds up the cytoplasmic processing of pre-miRNAs. This damage induced enhanced transportation of pre-miRNAs occurs via ATM. ATM has been shown to phosphorylated its downstream substrate AKT that in turn activates Nup153, a structural component of the nuclear pore complex. Activated Nup153 efficiently interacts with Exportin-5 (XPO5) and promotes nuclear export of pre-miRNAs [5]. In the following section, I describe how ATM regulates the expression of miRNAs through its downstream effectors including p53 and ΔNp63α.

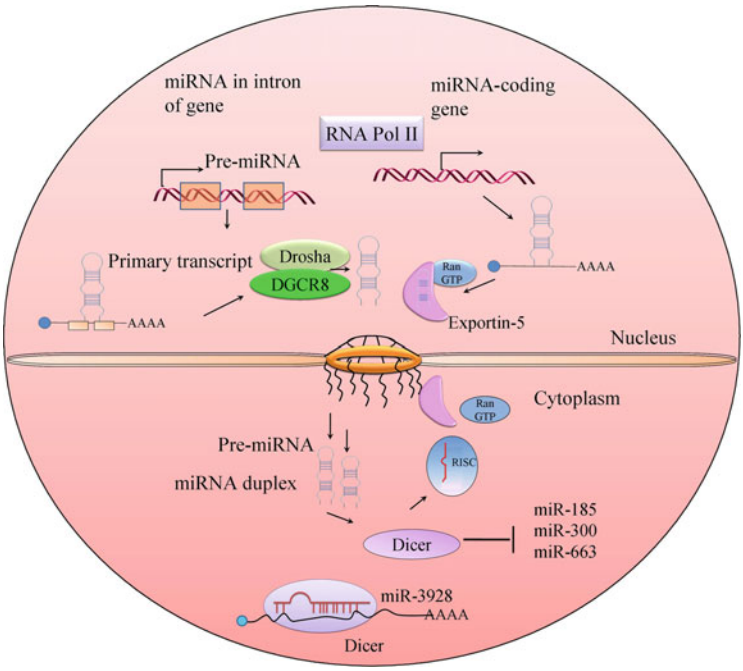

Fig. 16.2 Describes miRNA biogenesis pathway

4 MiRNA Regulation by ATM

ATM has been shown to phosphorylate ΔNp63α and consequently phosphorylated ΔNp63α triggered the expression of miR-885-3p [6]. Moreover, head and neck squamous cell carcinoma (HNSCC) cells upon treatment with cisplatin displayed pΔNp63α mediated downregulation of miR-181a, miR-519a, and miR-374a and over expression of miR-630 [7]. let-7a and let-7b are triggered by ATM/p53 signaling axis and it is appropriate to mention that there was no detectable rise in expression of let-7a and let-7b either in p53 deficient colon cancer cell line (HCT116) or ATM deficient human fibroblasts [8].

4.1 ATM Ensures Genomic Stability via HR by Repressing miR-335

ATM also represses the expression of miRNAs by inactivating components of transcriptional machinery involved in mediating expression of miR-335 in HeLa cells. Shown in Fig. 16.3. CHIP assays have shown that CREB binds to the

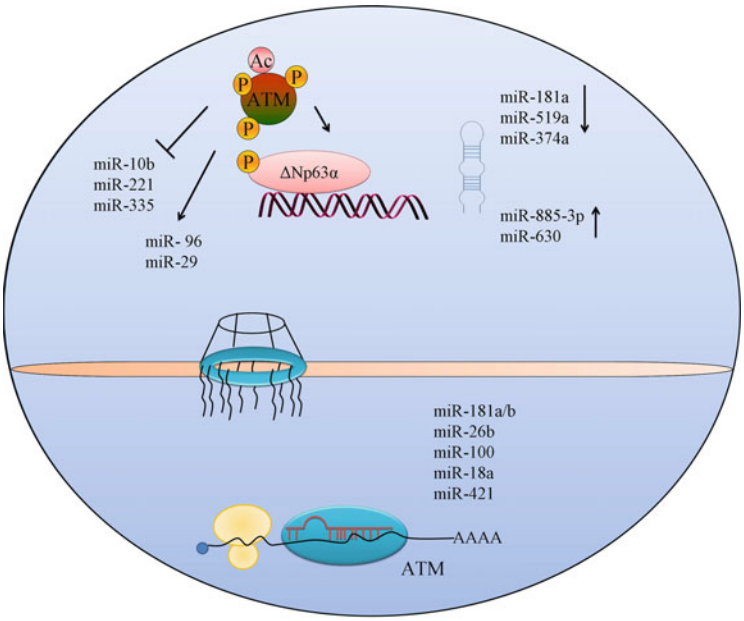

Fig. 16.3 Shows ATM mediated regulation of miRNAs. ATM inhibits the expression of miR-10b, miR-221, miR-335. miR-96 and miR-29 are activated by ATM. ATM also regulates expression of miRNAs via phosphorylation of ΔNp63α. pΔNp63α represses the expression of miR-181a, miR-519a and miR-374. However miR-885-3p and miR-630 are upregulated. ATM is quantitatively controlled by miR-181a, miR-18a, miR-421, miR-26b and miR-100

promoter region of miR-335 thus stimulating the expression of its target genes. Expression of miR-335 impairs an important step of DNA end resection in HRR by targeting CtIP. Therefore repression of miR-335 by ATM rescues the expression of CtIP [9].

4.2 ATM Sufficient Cells Have Over-Expressed Tumor Suppressor miRNA

It is noteworthy that ATM is involved in regulation of tumor suppressor and oncogenic miRNA subsets. Detailed analysis of ATM deficient cells displayed down-regulated tumor suppressor miR- 96, miR-29 family, which included miR-29b-1, miR-29b-2, and miR-29c. Shown in Fig. 16.3. However certain oncogenic miRNA subsets (miR-10b and miR-221) were over-expressed [10].

5 miRNA Subsets Mediated Control of ATM and ATR

5.1 miR-181a/b

miR-181a/b were found to be overexpressed in aggressive breast cancer cells and negatively regulated ATM [11]. Moreover it has previously been shown that breast cancer cells treated with Transforming growth factor (TGF)-β displayed an increase in the expression of miR-181. Overexpressed miR-181 post-transcriptionally controlled ATM and induced sphere-initiating stem cell-like feature in breast cancer [12]. Shown in Fig. 16.3.

5.2 miR-26b and miR-100

miR-26b negatively regulated ATM and it was noted that there was an increase in the number of DNA breaks that consequently resulted in apoptosis [13]. Shown in Fig. 16.3. M059J and M059K cells are developed from different portions of the same human malignant glioma. ATM is down regulated in M059J cells particularly because of targeting by miR-100 [14]. miR-24 is upregulated in CD8(+) CD28(−) T cells and has been noted to effectively target and suppress H2AX formation. Astonishingly, because of DNA repair deficiency, CD8+CD28− T cells revealed greater accumulation of DNA damage [15].

5.3 miR-18a and miR-421

Surprisingly, ATM regulation by miRNA is controlled by different signaling cascades. It has been shown that mTOR signaling negatively regulates ATM at transcriptional and Translational level. Interfering with mTORC1 and mTORC2 provided convincing evidence that there was a considerably increased protein level of ATM. More importantly, mTOR signaling triggered expression of ATM targeting miRNAs. The expression of miRNAs was controlled through downstream effector of mTOR signaling, S6K. Gene silencing strategies revealed that targeted inhibition of S6K resulted in upregulation of ATM mRNA. Pathway that resulted in suppression of ATM mRNA was regulated through an increase in expression of miR421 and miR-18a. Shown in Fig. 16.3. These miRNAs were direct targets of MYCN and it was shown that MYCN deficient cells had downregulated miR-421 and miR-18a. However cell reconstituted with MYCN displayed dramatic rise in expression of miR-421 and miR-18a [16].

miR-18a is also studied in detail in colorectal cancer cells and it has been shown that miR-18a is over-expressed in cancer cells and negatively modulates ATM mRNA transcript. Negative regulation of ATM by miR-18a resulted in persistent

DNA damage foci and consequent induction of apoptosis upon treatment with etoposide [17]. It has lately been reported that ER negative breast cancer cells displayed an over-expressed ATM. ERα negatively regulated ATM by enhancing the expression of miRNA- 18a and 106a [18]. Shown in Fig. 16.3.

5.4 miRNA Mediated Control of Dicer

It is surprising to note that cancer cells exposed to X-rays displayed an increase in miR-3928 expression. Results provided convincing evidence that Dicer mediated processing of miR-185, miR-300, and miR-663 was severely impaired because of miR-3928 mediated negative regulation of Dicer mRNA. Therefore, miR-3928 over-expressing cells indicated notably reduced dicer expression and marked decrease in maturation of miR-185, miR-300, and miR-663. Shown in Fig. 16.2. More significantly, miR-3928 activates ATR mediated signaling pathway by targeting Dicer [19].

5.5 ATR Is Regulated by miR-185

ATM- and Rad3-related (ATR) kinase is negatively regulated by miR-185. It had been shown that miR-185 over-expressing renal cell carcinoma cells upon exposure to X-rays were predisposed to undergo apoptosis [20]. Shown in Fig. 16.4.

6 miRNA Control of BRCA1

miR-146a and miR-146b-5p are over-expressed in basal-like mammary tumour epithelial cell lines. Triple negative breast tumours have also been studied and there is a notable overexpression of miR-146a and miR-146b-5p. miR-146a and miR-146b-5p have been shown to target BRCA1 [21]. Shown in Fig. 16.4.

miR-182-5p is highly expressed breast cancer samples and has recently been reported to disrupt homologous recombination by targeting modulators of DNA damage signaling [22]. miR-96 has been observed to target RAD51 and trans-lesion synthesis DNA polymerase REV1 thus compromising HR [23]. Shown in Fig. 16.4. Stable transfection miR-138 in osteosarcoma cell line, U2OS resulted in considerably enhanced genomic instability because of impaired HR [24].

miR-99 family has recently been shown to target chromatin remodeling factor SNF2H/SMARCA5. Depletion of SNF2H has been observed to markedly reduce BRCA1 localization to sites of DNA damage. Increasingly it is being acknowledged that transfection of the miR-99 family into cells considerably impairs HR and NHEJ mediated DNA repair [25].

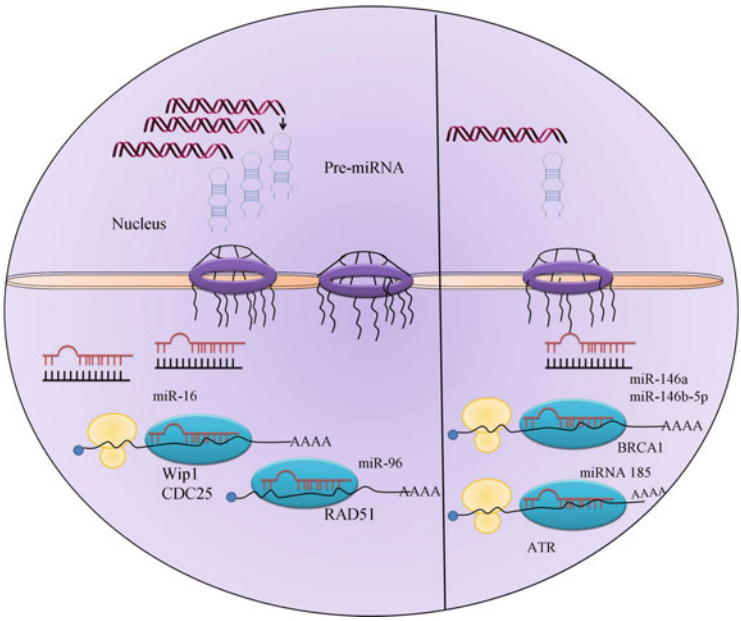

Fig. 16.4 Shows negative regulation of DNA damage regulators by miRNAs. BRCA1, ATR, RAD51 are controlled by miR-146a/miR-146b-5p, miR-185 and miR-96 respectively. Wip1 and CDC25 are post-transcriptionally regulated by miR-16

7 Wip1 and CDC25 Are Controlled by miR-16

Wild-type p53-induced phosphatase 1 (Wip1) and CDC25are phosphatases which are documented to be involved in antagonizing DNA damage response. It is apparent that these negative regulators of DNA damage repair signaling are negatively controlled by miRNA subsets to initiate repair mechanism. There is a direct piece of evidence that suggests that although Wip1 is transactivated transcriptionally by p53 however its protein levels are not detectable in early stages of DNA damage repair signaling primarily through negative regulation by miR-16 and miR-29 [26].

Exposure of p53 deficient colon cancer cells to hypoxic conditions induced replication stress that resulted in activation of checkpoints and a decline in protein level of Cdc25A. It was hypothesized that Cdc25A was under direct control of ATM-ATR/Chk1/2 DNA damage checkpoint response pathway. Chk1 mediated phosphorylation of cdc25 resulted in degradation of cdc25 however cancer cells placed in hypoxic conditions did not display Chk1 mediated inhibition of cdc25 as, targeted inhibition of Chk1 and Chk2 did not influence levels of Cdc25A. Later it was shown that Cdc25A was regulated by miR-21 that was induced in cancer cells

exposed to hypoxic conditions. miR-21 mediated negative regulation of Cdc25 was confirmed by evaluating the expression of cdc25 in miR-21 deficient cells which displayed an enhanced expression of cdc25 [27]. CDC25a is also controlled by miR-16 in UV exposed cells [28]. Shown in Fig. 16.4.

8 miR-34c

It has been shown that p53 is activated by its upstream modulators ATM/ATR and p38 MAPK however p53 deficient cells also display DNA damage response. It is surprising to note that p53 deficient cells respond to DNA damage by inhibition of c-Myc that is known to initiate S-phase by promoting DNA replication. This replication upon DNA damage generates replication stress however it is now known that c-Myc is negatively modulated by miR-34c in p53 deficient cells. Therefore for arrest of cells in S-phase following DNA damage targeted inhibition and miR-34c mediated regulation of c-Myc prevented replication stress [29].

A better understanding of the cellular response to DNA damage is not only informing our knowledge addressing the mechanism opted by cellular machinery to maintain genome stability but also uncovers intricate feedback and feedforward mechanisms that exist between miRNA subsets and DNA damage repair regulators. Therefore, perhaps, while we wait to understand more about the miRNA regulation of ATM, identification of miRNA subsets involved in regulation of genomic instability and apoptosis will be essential.

9 Genomic Rearrangements Mediated Control of miRNA

There is a paradigm shift in the understanding of cancer after rapidly accumulating information related to chromosomal rearrangements. Genomic rearrangements are cell type specific for example BCR-ABL is found in leukemia and TMPRSS2-ERG is solely identified in prostate cancer cells [30, 31]. These are important types of mutations initiated by two double-strand DNA breaks (DSBs) and considerable evidence highlights the implications of these complex layers of regulation in cancer initiation and progression. Confluence of information suggests that there are wide ranging molecular mechanisms including NHEJ, microhomology-mediated replication-dependent recombination (MMRDR), which underpin genesis of genomic rearrangements. Other biological mechanisms documented to be contributory consist of homologous recombination including non-allelic homologous recombination (NAHR), gene conversion, single strand annealing (SSA) and break-induced replication (BIR) [32].

It is becoming increasingly clear that genomic rearrangements contribute to transcriptional regulation of different miRNA subsets. In the forth coming section, I will discuss recently emerging data with emphasis on how genomic

rearrangements in different cancer positively or negatively regulate the expression of miRNA subsets.

EWS/FLI1 fusion protein has been shown to suppress miR-708 thus rescuing the expression of EYA3. Remarkably, A673 Ewing sarcoma cells reconstituted with miR-708 displayed considerably enhanced apoptosis [33]. miRNA subsets including 100, 125b, 22, 221/222, 27a and 29a are also strongly repressed by EWS/Fli1 [34].

There is a direct piece of evidence that suggests that miR-495 is significantly repressed in MLL-rearranged AML samples. It was shown that cells reconstructed with miR-495 displayed marked increase in apoptosis [35]. let-7b has also been studied to be considerably suppressed in MLL-rearranged AML samples. Cell culture based experimental data revealed that regulatory region for let-7b expression was found to be hypermethylated. Moreover, azacitidine (demethylating agent) treated cells displayed partially restored let-7b expression [36]. Similarly it has lately been shown that because of CpG hypermethylation in t(4;11)-positive infant ALL 11 miRNAs are transcriptionally repressed. Treatment of these cells with a demethylating agent Zebularine, resulted in restoration of seven of these downregulated miRNAs. miR-152 was noted to be significantly suppressed in these cancer cells expressing MLL or MLL fusions. Moreover, DNA methyltransferase 1 (DNMT1) was observed to be a potential target of miR-152 [37]. It is obvious that there is a negative feedback regulation that exists in case of miR-152. miR-152 negatively regulates DNMT1 and suppression of miR-152 restores the protein level of DNMT1 that consequently methylates miR-152.

Diffuse large B-cell lymphoma (DLBCL) cells positive for ITPR2-BCL6 fusion gene rearrangement demonstrated that miR-17-92 members were upregulated ~15-fold [38]. It has also been persuasively reported that cell reconstructed with AML1-ETO indicated over-expression of endogenous miR-126 [39]. miR-196b has been shown to be upregulated in CALM-AF10, SET-NUP214 carrying cells [40]. Cell lines derived from thyroid adenomas with 19q13.4 rearrangements and primary adenomas positive for 19q13.4 rearrangements showed an overexpression of C19MC and miR-371-3 cluster compared to chromosome abnormality deficient cells [41]. Surprisingly, locus for immunoglobulin genes encodes a miRNA miR-650 and it is regulated by coupled expression with its host gene for IgLλ [42].

There is a direct evidence that suggests loss of 3′-UTR of FGFR3 results in escape from quantitative regulation by miR-99a. In line with this approach, it has been shown that 3′-UTR is lost in FGFR3-TACC3 gene fusion. It was noted that cells transfected with miR-99a mimics displayed a decrease in FGFGR3 expression. To verify if loss of 3′-UTR underlies overexpression of FGFR3-TACC3, cells were re-constructed with 3′-UTR containing FGFR3 and FGFR3-TACC3. Interestingly, there was remarkable decline in the expression of FGFR3 and its fusion transcript upon miR-99a transfection [43].

CEBPA encodes the C/EBPα transcription factor and increasingly it is being recognized that N-terminal mutant CEBPA gene or differential translation of wild-type CEBPA mRNA results in formation of truncated C/EBPα-p30 isoform. This truncated C/EBPα-p30 isoform has been shown to upregulate the expression of miR-181a-1 via binding to its promoter. Lenalidomide is a drug approved for

Table 16.1 Showing list of miRNAs misrepresented (downregulated and upregulated) in genomically rearranged cancer cells

Genomic	miRNA ↓	miRNA ↑
EWS/FLII	miR-708, miR-100, miR-125b, miR-22, miR-221/222, miR-27a and 29a	
MLL-rearranged AML samples	miR-495, let-7b, miR-152	
ITPR2-BCL6		miR-17-92
AML1-ETO		miR-126
CALM-AF10, SET-NUP214		miR-196b
19q13.4 rearrangements		miR-371-3 cluster

myelodysplastic syndromes and multiple myeloma and there is sufficient experimental evidence that lenalidomide treated cells displayed notably enhanced translation of the C/EBPα-p30 isoform. Additionally there was markedly increased expression of miR-181a-1 [44]. It is becoming more understandable that miRNAs negatively regulate different fusion transcripts. However it is frequently observed in fusion positive cancer cells that fusion transcript targeting miRNAs are often downregulated. In accordance with this approach, it was found that miR-128b controlled MLL, AF4, and both MLL-AF4 and AF4-MLL fusion genes. Cells reconstituted with miR-128b and miR-221 were re-sensitized to glucocorticoids [45].

There is emerging evidence of loss of apoptosis in fusion positive cancer cells. Cellular studies suggest that TEL-AML1 positive cancer cells have over-expressed survivin and a downregulated miR-494 and miR-320a. Cells reconstituted with miR-494 and miR-320a indicated noteworthy decrease in expression of survivin [46]. Fenretinide (N-(4-hydroxyphenyl) retinamide) is a synthetic vitamin A analogue and it is effective in repressing the expression of PAX3/FOXO1 in rhabdomyosarcoma cells. Moreover there was an increase in the expression of miR-9. In addition has been reported for inducing apoptosis in cancer cells via downregulation of Bcl-2 and activation of caspase-9 [47].

There is another contemporary study that suggests that In the absence of the ALK fusion protein, miR-29a level is significantly upregulated by 1.6-fold in transgenic mice. In-depth analysis of regulatory mechanism of regulation of miR-29a by ALK fusion protein revealed phosphorylated STAT mediated increase in DNMT expression. Epigenetic repression of miR-29a was modulated by DNMT3b. miR-29a has been shown to induce apoptosis by negative regulation of Mcl-1 [48]. However it is also surprising to note that miRNA is also involved in simultaneous targeting of tumor suppressor and oncogene. In line with this notion it had been noted that miR-196b quantitatively controlled HOXA9/MEIS1 and FAS [49]. miR-486 expression was considerably downregulated in TLS-CHOP-positive NIH3T3 fibroblasts [50]. Table 16.1 shows list of miRNAs upregulated or downregulated in genomically rearranged cancer cells.

It is becoming systematically more understandable that there is a layered regulation of miRNA. Genomic rearrangements induced dysregulation of miRNA subsets resultantly promotes cancer progression. Fusion transcript encoded proteins use various mechanisms to suppress the expression of tumor suppressor miRNAs. It has been shown that epigenetic machinery is recruited that severely impairs the transcription of miRNAs. However, fusion protein positive cancer cells treated with demethylating agents displayed restoration of expression of tumor suppressor miRNAs. Moreover, apoptosis is inhibited by oncogenic fusion proteins. These proteins suppress apoptosis inducing miRNAs and laboratory methodologies have shown that reintroduction of apoptosis inducing miRNAs in the oncogenic fusion protein positive cancer cells induced apoptosis.

10 Diametrically Opposed Role of ATM in Regulation of TRAIL Mediated Apoptosis

TRAIL mediated signaling has emerged as an important signaling cascade reported to induce apoptosis in cancer cells while leaving normal cells intact. This tremendous potential of TRAIL compelled scientists to pursue details of TRAIL induced signaling. There is a progressive expansion in the proteome involved in induction of apoptosis and it has been shown that caspase activity is essential and hence, a multifaceted regulatory system triggers apoptosis. Biologically, caspases are categorized into two sub-divisions including initiator caspases and effector caspases. There is sufficient experimental evidence that suggests that effector caspases are constitutively expressed as dimers however, initiator caspases mediated activation of effector caspases is necessary. However initiator caspases are produced as monomers and death-inducing signaling complex (DISC) acts as protein assembly platform for recruitment of caspase-8. Increasingly it is being recognized that apoptotic pathway is further classified into extrinsic and intrinsic pathways. In the receptor mediated pathway, extracellular ligands (FasL, TRAIL) modulate receptor oligomerization and DISC assembly. Procaspase-8 is activated and consequently activates its downstream effector caspase-3. Discordantly, intrinsic pathway is triggered by proteins from the Bcl-2 family which controls the release of proteins from mitochondria which contribute to apoptosome formation [51, 52]. Caspase-9 and -3 are negatively regulated by inhibitor of apoptosis proteins (IAPs) however it had previously been established that translocation of truncated Bid into mitochondria mediated release of SMAC/DIABLO and HtrA2/Omi inhibited IAPs. There is a rapidly emerging list of negative regulators of apoptosis and it is worth mentioning that cellular FLICE inhibitory protein (c-FLIP) is a catalytically inactive homologue of caspase-8 and has been noted to inhibit their activation by interfering with binding sites on the DISC. In the upcoming section I will sequentially discuss the steps and protein network which is targeted to restore TRAIL mediated apoptosis in resistant cancer cells [53, 54].

This section is aimed at critically discussing these new developments, which are based on the emerging realization that ATM mediated signaling is involved in regulation of death receptors. Moreover in this section exciting segments of information highlighting dual role of ATM in regulation of TRAIL mediated signaling are discussed.

There is also a direct piece of evidence that suggests that disruption of ATM/p53 axis severely compromises expression of DR5. There is a significant role of ATM-p53 signaling cascade in transcriptional regulation of DR5 after DNA damage [55]. Subsequent studies using synthetic and natural compounds verified the fact that in cancer cells death receptors are regulated by ATM/p53 signaling axis. Pemetrexed is a new-generation antifolate and A549 cells displayed remarkably enhanced apoptosis upon treatment with pemetrexed. Pemetrexed treated cancer cells exhibited upregulated expression of DR4 and DR5. In addition there was an activated ATM mediated signaling cascade that activated one of its downstream effector p53 to trigger p53 dependent and -independent signaling pathways [56].

Retigeric acid B is a pentacyclic triterpenoid from the lichen species Lobaria kurokawae and is effective anticancer agent. Prostate cancer cells treated with Retigeric acid B (RB) demonstrated ATM and ATR mediated activation of Chk1/2 and Cdc25. Surprisingly there was a notable expression of a stress-responsive gene activating transcription factor 3 (ATF3) in RB treated prostate cancer cells. It is also relevant to mention that interfering with ATF3 considerably reduced expression of DR5, CDC25A and GADD45 [57].

Although ATM mediated signaling has been shown to regulate the expression of death receptors, but the available literature is insufficient and we do not have broader information about cell type specific regulation of death receptor by ATM. An outstanding question that has yet to be reconciled with a mechanistic hypothesis is how miRNA mediated quantitative control of ATM influences expression of DR.

Diametrically Opposed Role of ATM in Regulation of TRAIL Mediated Signaling

cFLIP has emerged as an important anti-apoptotic protein that severely compromises TRAIL mediated signaling in different cancer types. However it has been compellingly revealed that hepatocellular carcinoma (HCC) cell lines upon treatment with DNA damaging drugs demonstrated activated ATM that modulated cFLIP to induce TRAIL mediated apoptosis in cancer cells [58]. However this particular mechanism of ATM regulation of TRAIL mediated apoptosis was in contradiction to the diametrically opposed role of ATM in regulation of apoptosis. It was shown that targeted inhibition of ATM substantially reduced cFLIP protein content in TRAIL resistant melanoma cells. Furthermore, there was an up-regulation of DR5 in gamma irradiated melanoma cells [59]. Therefore, although an exciting first step has been made in improving our concept regarding

divergent roles of ATM in TRAIL mediated signaling, substantial work remains to be done before these findings can be translated into pharmacologically tractable species. Therefore, more comprehensive knowledge regarding effects of kinase inhibitors and kinase knockouts on these crosstalk mechanisms between ATM signaling and TRAIL mediated signaling is warranted.

Circumstantial studies provide interesting evidence that colon carcinoma cells and cervical cancer cells treated with TRAIL displayed rapid phosphorylation of Chk2, ATM, H2AX, and DNA-PK, that initiated immediately after 1 h upon TRAIL treatment. Besides, important information obtained was regulation of ATM by DNA-PK in TRAIL treated cancer cells. It was shown that targeted inhibition of DNA-PK resulted in reduction of the phosphorylation of ATM on S1981 after TRAIL treatment [60].

11 Therapeutic Targets

Thymidylate synthase (TYMS) is an important folate-dependent enzyme in DNA synthesis and its over-expression is associated with resistance to 5-fluorouracil in HeLa cells. Emerging evidence suggests that miR-433 negatively regulates the expression of TYMS thus restoring the sensitivity of HeLa cells to 5-fluorouracil [61]. Cisplatin resistance is a major problem that confounds standardization of therapy. However it has recently been revealed that cell-cycle kinases WEE1 and CHK1 over-expression induces cisplatin resistance in cancer cells. However, WEE1 and CHK1 were found to be negatively regulated by miR-15/16/195/424/497 family and gene silencing strategies confirmed that WEE1 and CHK1 inhibition resulted in restoration of cisplatin sensitivity [62].

12 Conclusion

Substantial fraction of information has successively been added and there is an ever- increasing list of miRNA subsets which are important for regulation of DNA damage repair signaling by modulating expression of ATM. Moreover, there is a push and pull between TRAIL mediated signaling and DNA damage signaling to generate apoptotic signals. It is undeniable that there is fragmented information about how both of these signalings regulate apoptosis in cancer cells. Recently emerging information has started to dissect pathways opted by ATM to suppress miRNA expression. However cell type specific miRNA regulation of DNA damage signaling is incompletely understood. We have several hints obtained through in-vitro studies about miRNA regulation of ATM, ATR and DNA PK, however how different subsets of miRNA control ATM mediated signaling in a specific cancer type needs detailed research. In line with the aspect, it is also well established that transcriptional control of Death receptors and TRAIL is modulated

by p53, however there are missing links with reference to post-transcriptional and post-translational regulation of TRAIL and DRs by ATM/p53 axis. As discussed previously, there is a paradigm in parallax related to role of ATM in regulation of TRAIL mediated signaling. As convergent literature exists suggesting positive and negative regulation of anti-apoptotic proteins of TRAIL mediated signaling. There is a report that indicates that TRAIL mediated signaling activates ATM thus adding another level of regulation of ATM other than DNA damage signaling. However it needs to be seen if TRAIL triggered activation of ATM is contributory to regulation of anti-apoptotic proteins to induce apoptosis. Cell type specific research is necessary to enhance our understanding of ATM regulation of pro-apoptotic and ant-apoptotic proteins. Moreover genomic rearrangements in leukemia and prostate cancer also offer new horizons for research in the area of TRAIL mediated signaling in BCR-ABL positive leukemic cells and TMPRSS2-ERG positive prostate cancer cells. How inactivation of ATM leads to genomic rearrangements and influences TRAIL mediated apoptosis in cancer cells still needs to be connected to miRNA regulation of ATM, ATR and DNA-PK.

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Chapter 17
miRNA Regulation of VEGF/VEGFR Signaling

Ammad Ahmad Farooqi and Ilhan Yaylim

1 Introduction

Tumor angiogenesis is a multifaceted molecular mechanism and depends on the release of angiogenic factors/growth factors by neoplastic cells specific for endothelial cells and development of microvasculature. There is a mutational activation of oncogenes and signaling cascades that promote endothelial cell migration and invasion of the surrounding extracellular matrix (ECM) [1].

Vascular endothelial growth factor (VEGF) gene is an extensively investigated regulator reported to be involved in angiogenesis and lymphangiogenesis thus promoting growth and metastasis of neoplasms. VEGF encodes five polypeptide growth factors, VEGF-A, -B, -C, -D, and -E. VEGF signals through VEGFR-1 (Flt-1) and VEGFR-2 (KDR) thus transducing signals intracellularly [2]. Ligands and receptors are shown in Fig. 17.1. In the upcoming sections we will discuss regulation of VEGF and VEGFR by miRNA. However, before discussing intricate network of miRNA regulation of VEGF/VEGFR signaling axis, we provide an overview of miRNA biogenesis.

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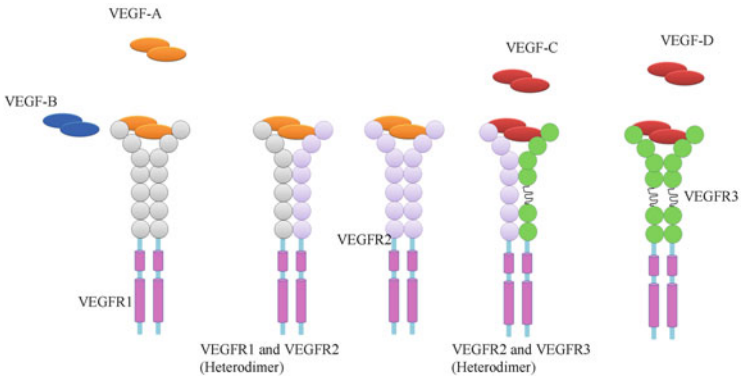

Fig. 17.1 Shows ligands and receptors. Homodimerization and heterodimerization of receptors is also shown

2 miRNA Biogenesis

The miRNA biogenesis is a well orchestrated mechanism and tremendously mounting scientific information is improving our understanding of this biological phenomenon that includes the RNA polymerase II or III mediated generation of the primary miRNA transcript (pri-miRNA). Another important mechanism that occurs in the nucleus is Drosha–DGCR8 complex mediated processing of pri-miRNA into ~70-nucleotide precursor hairpin. The pre-miRNA hairpin is transported from the nucleus to cytoplasm by Exportin-5-Ran-GTP [3]. After its export from nucleus to the cytoplasm, pre-miRNA hairpin is processed by Dicer that yields ~20-bp miRNA/miRNA* duplex. This step is necessary for loading of one strand of the miRNA/miRNA* duplex (the guide strand) into miRNA-induced silencing complex (miRISC) and binding of target mRNAs to miRNAs in RISC is followed by translational inhibition or RISC mediated mRNA degradation. Passenger strand is degraded [4]. In the next section miRNA regulation of VEGFR1 and VEGFR2 will be discussed more extensively. VEGFR3 is insufficiently studied and needs more experimental evidence for a better understanding and information regarding the miRNA subsets which regulate VEGFR3 expression.

3 VEGFR1

Extracellular ligand-binding region of full-length VEGFR1 (FLT1) has signal peptide at N terminal followed by 7 immunoglobulin-like domains [5]. FLT1 domains 2 and 3 are necessary and sufficient for binding VEGF [6]. VEGFR1 has been shown to be targeted by different miRNAs particularly, miR-10 and miR-200.

3.1 miR-10

There is a recent report suggesting miR-10 mediated quantitative control of VEGFR1 and it was shown that miR-10 depleted HUVECs represented reduced phosphorylation of VEGFR2 upon treatment with low dose VEGF. It was indicated that VEGF/VEGFR1 interaction reduced VEGF mediated activation of VEGFR2 [7].

3.2 miR-200

Cancer-associated fibroblasts (CAFs) are isolated from murine lung adenocarcinomas. In-vitro assays have shown that CAFs secrete VEGF thus enhancing tumor cell invasion. Tumor cells are observed to be more invasive in coculture with CAFs as compared to normal fibroblasts (LFs) in the culture. CAFs mediated invasive potential of 344SQ cells was notably reduced upon using neutralizing antibody against VEGF-A. Moreover, enforced expression of miR-200 resulted in downregulation of VEGFR1 thus suppressing CAF mediated invasion promoting effects in 344SQ cells. Role of VEGFR1 in transducing VEGF mediated signals intracellularly was further confirmed by subcutaneously injecting VEGFR1 expressing 344SQ cells into syngeneic mice. Syngeneic mice bearing VEGFR1 silenced 344SQ cells did not show metastasis [8].

4 VEGFR2

VEGFR2 is also expressed on the surface of blood endothelial cells and accumulating research is underscoring wide ranging VEGFR2 mediated responses in endothelial cells including proliferation, regulation of survival, migration, and vascular tube formation. VEGFR via phosphotyrosine residues located in the carboxy-terminal region activates downstream effectors, for example pTyr1175 results in activation of PKC and ERK pathway [9]. The pTyr1214 consequently activates tyrosine kinase Fyn which further activates Cdc42 and p38MAPK thus modulating reorganization of the actin cytoskeleton [10]. The following section will provide an overview of regulation of VEGFR2 by miR-200b and miR-200c.

4.1 miR-200b and miR-200c

The miR-200b has been shown to negatively regulate GATA2 and VEGFR2 and intriguingly, miR-200b mimic inhibited the angiogenic tube-forming ability of

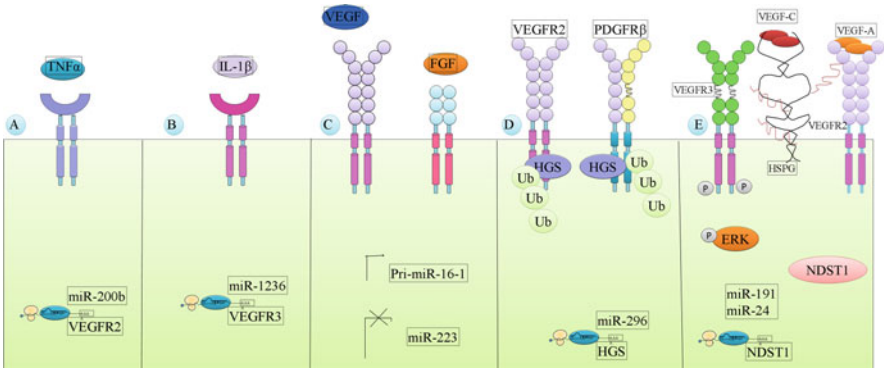

Fig. 17.2 (*A*) TNFα induced miR-200b negatively regulated VEGFR2. (*B*) IL-1β induced miR-1236 controlled expression of VEGFR3. (*C*) VEGF and FGF induced signals repressed expression of miR-223. (*D*) Ubiquitylated VEGFR2 and PDGFRβ are recognized by the endocytosis machinery and targeted through the endosomes and the multivesicular body for degradation. Hepatocyte growth-factor-regulated tyrosine kinase substrate (HRS) is an adaptor molecule required for the intracellular trafficking. HGS is a target of miR-296. (*E*) Heparan Sulphate Proteoglycan (HSPG) is post-translationally modified by NDST1. HSPG bound VEGF transduces signals through VEGFRs. miRNA mediated targeting of NDST1 resulted in suppression of VEGF induced intracellular signaling through VEGFR

endothelial cells. Experimental data provided convincing evidence that treating human dermal microvascular endothelial cells (HDMECs) with TNF-α considerably reduced angiogenic response. Detailed mechanistic insights suggested TNF-α mediated upregulation of miR-200b, shown in Fig. 17.2. The anti-miR-200b treated HDMECs had substantially enhanced GATA2 and VEGFR2 protein levels [11]. Non-Small-Cell Lung Cancer Cell Line A549 was radiosensitized by enforced expression of miR-200c. It was shown that miR-200c negatively regulated VEGFR2 in A549 cancer cells [12].

4.2 miR-15

VEGFR2 has also been shown to be regulated by miR-15. HUVECs overexpressing miR-15 displayed significant decrease in migration and tubulogenesis however, ginsenoside-Rg1 (Rg1) mediated repression of miR-15 enhanced VEGFR2 expression. Moreover, in-vivo analysis verified that injecting pre-miR-15b precursor into zebrafish embryos substantially inhibited subintestinal vessels formation [13].

Mice bearing Lewis lung carcinoma (LLC) or B16.F10 melanoma were treated with vandetanib (ZD6474). The results revealed that vandetanib significantly suppressed protein expression of VEGFR2 thus retarding the tumor growth in mice. Moreover, miR-296 mediated negative regulation of VEGFR2 was not observed [14].

5 VEGFR3

Lymphatic endothelial cells upon treatment with IL-1β revealed considerably reduced protein expression of VEGFR3 however mRNA level was not altered. Mechanistically it was shown that IL-1β exerted its inhibitory effects on VEGFR3 protein expression through miR-1236, shown in Fig. 17.2. Using antagomirs against miR-1236 verified the fact that VEGFR3 protein expression was under direct control of miR-1236 [15].

6 VEGF and bFGF Mediated Repression of miRNAs

VEGF- and bFGF have been shown to transcriptionally upregulate the expression of pri-miR-16-1. However, miR-424 was not under transcriptional control of VEGF- and bFGF. However, post-transcriptional processing of miR-424 was noted to be triggered by these cytokines. It has been shown that enforced expression of miR-16 or miR-424 considerably inhibited basal migration, as well as VEGF- or bFGF-induced migration, in bovine aortic ECs. The miR-16 or miR-424 transfected HUVECs upon treatment with VEGF or bFGF demonstrated significant impairment of cord formation as well as under basal conditions. Transfecting HUVECs with VEGFR2 or FGFR1 cDNA lacking respective 3′UTR rescued migration in miR-16 or miR-424 transfected ECs. Akt and ERK1/2 are downstream effectors of growth factor mediated signaling. It was noted that pAkt and pERK1/2 were remarkably reduced in miR-16 transfected ECs upon treatment with VEGF or bFGF [16]. In line with this mechanism, another recent study indicated VEGF- and bFGF mediated repression of miR-223, shown in Fig. 17.2. Interestingly, cells reconstituted with miR-223 displayed dramatically reduced phosphorylation of receptor and Akt upon treatment with VEGF- and bFGF. The β1 integrin was noted to be negatively regulated by miR-223 and miR-223 overexpressing cells reconstructed with β1 integrin rescued growth factor mediated signaling and angiogenesis [17].

7 HSPG, NDST-1 and VEGF: Companionship During Signaling

Increasingly it is being realized that heparan sulfate proteoglycans (HSPG) act as co-receptors thus regulating angiogenesis. HS proteoglycan is post-translationally modified by NDST-1 and later binds with VEGF. HS expressed by primary lymphatic endothelium binds to VEGF, thus transducing signals intracellularly via phosphorylation of VEGFR and downstream effectors ERK1/2. Heparinase treated cells displayed dramatically reduced VEGFR3 phosphorylation in response to VEGF-C. RNA interference strategies against N-deacetylase/N-sulfotransferase-

1 (Ndst1) have shown that VEGF-C-mediated Erk1/2 phosphorylation was reduced markedly [18]. Circumstantial evidence substantiates the fact that Ndst1 is negatively regulated by miR-191 and miR-24 [19, 20]. Overexpressing miR-24 in cells remarkably suppressed Ndst1, thus inhibiting VEGF-A mediated activation of VEGFR2 [20], shown in Fig. 17.2. It is concluded that targeting of NDST-1 is an effective strategy to suppress VEGF/VEGFR signaling axis.

8 DCLK1 Suppresses miRNA Expression

DCLK1 is an intestinal and pancreatic stem cell marker. It is frequently upregulated in the stroma and epithelium of pancreatic ductal adenocarcinoma. It is appropriate to mention that ablation of Dclk1 expressing cells in $Apc^{min/+}$ mice resulted in regression of polyps. Use of nanoparticle-encapsulated siRNA against DCLK1 (NPsiDCLK1) in tumor xenografted mice has been noted to upregulate expression of miRNA subsets including miR-143/145 cluster, let-7a and miR-200a, b and c. NPsiDCLK1 treated tumors had remarkably reduced mRNA and protein expression of VEGFR1 and VEGFR2 [21].

9 miRNAs Enter into HUVECs to Regulate VEGFR

It has recently been persuasively revealed that miRNA produced from epithelial ovarian cancer cells (EOC) is secreted into the local microenvironment and enters HUVEC cells within 24 h. The findings were substantiated by co-culturing HUVECs with control and miR-484–overexpressing SKOV-3 cells. It was found that VEGFR2 protein on endothelial cells was considerably reduced in HUVECs co-cultured with miR-484–overexpressing SKOV-3 cells [22].

10 Dicer Expression Is Suppressed in Hypoxic HUVECs

It seems intriguing to note that hypoxia represses mRNA and protein expression of Dicer in HUVECs treated with hypoxia mimetic desferrioxamine. Enforced expression of dicer in HUVECs remarkably reduced the expression of HIF-2α via miR-185. It was noted that there was an accumulation of miR-185 precursors instead of mature miR-185 in hypoxic HUVECs [23].

11 miRNA Regulation of VEGF-A

VEGF-A has emerged as a potent mitogen that underlies physiological and pathological angiogenesis. Interestingly, intricacy and multiplicity of regulatory mechanisms involved in VEGF-A expression are deeply studied and significant breakthroughs have been made in identification of miRNAs involved in regulation of VEGF-A.

11.1 miR-26a

Migration and tube forming of HUVECs is remarkably reduced upon co-culturing with HepG2 cells reconstituted with miR-26a. VEGF-A was noted to be targeted by miR-26a in HepG2 cells. The results obtained from in-vivo studies were encouraging as evidenced by miR-26a mediated suppression of ectopic and orthotopic tumor growth and vascularity in nude mice. PIK3C2α belongs to class II PI3Ks and is a known target of miR-26a. pAkt and HIF-α was substantially reduced in miR-26a transfected cells. To verify that PI3K/Akt/HIF-α signaling axis is involved in mediating VEGF-A, cells were treated with PI3K inhibitor and HIF-1α inhibitor that resulted in suppression of VEGF-A expression [24].

11.2 miR-29a/b

DNA methyltransferase is a direct target of miR-29b and it has been shown that transfection of miR-29b in multiple myeloma cells restored expression of SOCS-1 via promoter demethylation. SOCS-1 expression notably suppressed pSTAT3 levels in miR-29b transfected cells. Shown in Fig. 17.3. VEGF-A mRNA was also repressed in miR-29b transfected multiple myeloma cells [25]. VEGF-A is also negatively regulated by miR-29a in gastric cancer cells. Ectopic expression of miR-29a in low expressing gastric cancer cells dramatically reduced VEGF-A expression [26].

11.3 miR-125a and miR-126

MMP11 and VEGF-A are regulated by miR-125a in HCC cells. Proliferation and metastasis of HCC cells was suppressed upon overexpression of miR-125a [27]. The miR-126 gene is embedded in intron7 of EGF-like domain 7 (EGFL7). The miR-126 was notably repressed in breast cancer cells MCF-7 and reconstitution strategies effectively reduced VEGF/PI3K/AKT signaling activity [28]. Enforced

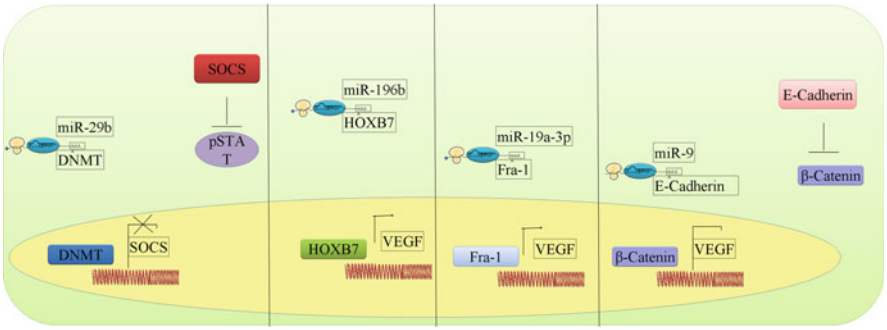

Fig. 17.3 Shows miRNA regulation of regulators involved in modulation of VEGF. DNMT is targeted by miR-29b. Suppressor of Cytokine Signaling (SOCS) is epigenetically repressed and targeted inhibition of DNMT restored expression of SOCS that inhibited STAT. HOXB7 is also involved in regulation of VEGF and targeted by miR-196b. Fra-1 is involved in regulation of VEGF and targeted by miR-19a-3p. β-Catenin modulates the expression of VEGF. βCatenin is negatively regulated by E-Cadherin. However, E-Cadherin is targeted by miR-9

expression of miR-126 resulted in an improved response of drug resistant NSCLC cancer cells to adriamycin and vincristine. The miR-126 transfected cancer cells remarkably reduced tumor formation in xenografted mice via negative regulation of VEGF-A [29]. VEGF is a known target of miR-126 reported to be epigenetically repressed in colorectal cancer cells. Colorectal cancer cells treated with 5-aza-CdR displayed an increase in miR-126 expression and consequently VEGF was downregulated [30].

11.4 miR-185

Leucine-rich repeat C4 (LRRC4) belongs to LRR protein superfamily and specifically expressed in brain tissue and regulates the expression of miR-185. CDC42 and RhoA are direct targets of miR-185 in glioma cells and downregulation of miR-185 restores their expression. Enforced expression of miR-185 resulted in suppression of CDC42 and RhoA. Additionally, VEGF-A was also found to be indirectly regulated by miR-185 [31].

11.5 miR-203 and miR-205

VEGF-A is negatively regulated by miR-203 and enforced expression of miR-203 resulted in inhibition of tumor growth and angiogenesis in nude mice [32]. miR-205 is suppressed in glioblastoma cells and cells reconstituted with miR-205 resulted in induction of apoptosis and cell cycle arrest [33], shown in Table 17.1.

Table 17.1 Shows list of miRNAs regulating VEGF-A and VEGF-C

VEGF-A	miR-26a, miR-29a, miR-29b, miR-126, miR-203, miR-361-5p, miR-503
VEGF-C	miR-27b, miR-1826

11.6 miR-361-5p and miR-503

The miR-361-5p targets VEGF-A [34]. Overexpression of miR-503 in hepatocellular carcinoma cells inhibited VEGF-A [35], shown in Table 17.1.

11.7 miR-196b

The miR-196b suppressed expression of VEGF via targeting of HOXB7 in cervical cancer cells. VEGF was noted to be reduced in HOXB7 silenced cancer cells. Overexpression miR-196b or gene silencing of HOXB7 resulted in suppression of VEGF in cervical cancer cells [36], shown in Fig. 17.3.

11.8 miR-378

5-aza-dC treated gastric cancer cells presented enhanced expression of miR-195 and miR-378 and consequent suppression of VEGF [37]. It is surprising to note that there is a report indicating that stable miR-378 overexpression in NSCLC NCI-H292 cells dramatically enhanced the expression of VEGF. It was concluded that miR-378 promoted non-small cell lung carcinoma growth, vascularization, and metastasis [38].

11.9 miR-20b

The miR-20b negatively regulates HIF-1α and VEGF. HIF-1α transfected normoxic H22 cells showed downregulation of miR-20b [39].

12 miRNA Regulation of VEGF-C

There is a recent report that suggests dual targeting of VEGF-A and VEGF-C in gastric cancer cells. In-vitro analysis provided evidence that Lentivirus-mediated RNAi suppressed mRNA and protein expression of VEGF-A and VEGF-C in the SGC7901 cells [40]. Data obtained through 3′UTR luciferase assay has revealed

that VEGF-C mRNA has complimentary binding site with miR-1826 within its 3′UTR. Interestingly, VEGF-C protein expression is suppressed in miR-1826-transfected bladder cancer cells [41]. VEGF-C has also been reported to be targeted by miR-27b. miR-27b is downregulated in colorectal cancer cells because of hypermethylation of CpG islands [42]. Shown in Table 17.1.

13 VEGF Regulation

Substantial fraction of information has been added into the modes underlying expression of VEGF. There are various proteins which are involved in regulation of expression of VEGF. In this segment we will discuss regulatory mechanisms of VEGF by β-catenin, N-RAS, IRS, NF-κB1, Fra-1 and p70S6K1.

13.1 β-Catenin

Surprisingly, miR-9 induced activation of β-catenin that consequently triggered expression of VEGF. miR-9 modulated targeting of E-cadherin promoted nuclear translocation of β-catenin thus stimulating expression of VEGF. miR-9 was reported to be triggered by MYC/MYCN in breast cancer cells [43], shown in Fig. 17.3.

13.2 N-RAS, IRS1 and NF-κB1

N-RAS and IRS1 mediated expression of VEGF is also reduced in miR-145 overexpressing colorectal cancer cells. Phosphorylated AKT and ERK1/2 levels were reduced notably in miR-145 overexpressing cells [44]. Similarly, VEGFA, MMP2 and MMP9 are transcriptional targets of NF-κB1. NF-κB1 itself is under direct control of miR-9 in uveal melanoma cells [45].

13.3 Fra-1

It is getting increasingly clear that tissue associated macrophages (TAMs) overexpress Fra-1, Stat3 and c-Jun. It is noteworthy that RAW mouse macrophages displayed enhanced expressions of Stat3 and p-Stat3 which was dependent on cytokines primarily released from tumor cells. TAMs co-cultured with 4T1 tumor cells indicated activated intracellular JAK/Stat3 signaling pathway and an increased expression of VEGF. Certain clues have emerged which point towards

miR-19a-3p mediated negative regulation of Fra-1 and transfecting RAW264.7 macrophages with miR-19a-3p mimic resulted in considerably reduced expression of Fra-1 and its target gene VEGF. Shown in Fig. 17.3. The strategy was found to be effective upon intratumoral injection of miR-19a-3p in tumor bearing mice which inhibited growth of 4T1 breast tumor cells [46].

13.4 mTOR/p70S6K1

The mTOR/p70S6K1 regulates tumor angiogenesis and tumorigenesis and p70S6K1 is a direct target of miR-128. Reintroduction of p70S6K1 cDNA or ectopic expression of p70S6K1 in U87 and U251 cells resulted in upregulation of VEGF [47].

13.5 Specificity Proteins Regulate Expression of VEGF and VEGFR

VEGF and VEGFR are triggered by specificity proteins. It has been experimentally verified that targeting of specificity proteins using natural and synthetic agents effectively reduced expression of VEGF and VEGFR.

Methyl 2-cyano-3,11-dioxo-18beta-olean-1,12-dien-30-oate (CDODA-Me) is isolated from licorice extracts. RKO colon cancer cells treated with CDODA-Me displayed remarkably reduced expression of Sp1, Sp3 and Sp4 mRNA levels. Surprisingly, Sp-target genes including VEGFR1 (Flt-1), and VEGF were also downregulated. CDODA-Me effectively induced regression of tumor load in athymic nude mice inoculated with RKO cells [48], shown in Table 17.2.

GT-094 is a novel nitric oxide (NO) chimera containing an NSAID and NO moieties. RKO and SW480 cancer cells treated with GT-094 demonstrated gradual reduction in Sp1, Sp3, and Sp4 proteins with increasing concentration of the drug. Additionally, protein expression of VEGF and VEGFR reduced significantly in a concentration dependent manner [49], shown in Table 17.2.

ER-negative MDA-MB-231 breast cancer cells displayed an increase in zinc finger ZBTB10 gene upon treatment with antisense miR-27a. Furthermore, ZBTB10 mediated repression of specificity proteins (Sp), Sp1, Sp3, and Sp4 was associated with reduced expression of target genes including VEGF and VEGFR1 as evidenced by RT-PCR and western blot assays [50].

Table 17.2 Shows CDODA-Me and GT-094 mediated regulation of specificity proteins, VEGF and VEGFR

Agent	Sp1	Sp3	Sp4	VEGF	VEGFRI
CDODA-Me	↓	↓	↓	↓	↓
GT-094	↓	↓	↓	↓	↓

13.6 HER2 and HER3 Mediated Up-Regulation of VEGF

Mounting evidence suggested that conditioned medium from miR-199a or miR-125b overexpressing OVCAR-3 and A2780 cells induced substantially reduced tube formation by HUVEC. Furthermore HER2 and HER3 were also noted to be targeted by miR-199a and miR-125b. pAkt and VEGF mRNA were suppressed in miR-199a or miR-125b transfected ovarian cancer cells. However, reintroduction of HER2 or HER3 in miR-199a or miR-125b transfected ovarian cancer cells resulted in restoration of pAkt levels and upregulated VEGF mRNA expression [51], shown in Table 17.3.

14 Therapeutic Interventions

The miRNA research has revolutionized molecular oncology and there is a rapidly increasing interest in developing strategies particularly, plasmids containing anti-VEGF miRNA clusters. These plasmids have shown gene silencing effect exerted by miRNA clusters composed of multiple miRNA-mimicked RNA interference effectors. Combinatorial approach using different miRNAs against VEGF revealed that delivery of miR-5,10,7 resulted in a knockdown of VEGF by approximately 75 %. This strategy was further tested in-vivo and noted to be effective as injection of scAAV2/8 vectors expressing miR-5,10,7 into murine hindlimb muscles, resulted in a 44 % suppression of VEGF [52].

Lentivirus-mediated expression of miR-20a precursor has been shown to inhibit endothelial cell migration upon treatment with VEGF. Astonishingly miR-20a reconstituted cells displayed reduced phosphorylated p38 protein levels. Results indicated miR-20a mediated negative regulation of MKK3. MKK3 is a modulator situated upstream to p38 MAPK in protein hierarchy [53]. Monitoring of the expression of VEGFR is of sufficient importance during evaluation of therapeutic strategies in vivo. In accordance with this approach, VEGFR2-luc transgenic mice have been used to monitor the VEGFR2 expression using bioluminescent imaging (BLI). VEGFR2-luc transgenic mice implanted with 4T1 murine breast cancer cells were treated with antagomir-21. Tumor volumes of control group and scramble treatment group were notably larger as compared to antagomir-21 treated group of mice [54].

There is a direct piece of evidence highlighting hepatocyte growth factor-regulated tyrosine kinase substrate (HGS) mediated degradation of VEGFR2 and

Table 17.3 Shows list of miRNAs positively and negatively regulating VEGF

VEGF	Tumor suppressor miRNAs	Oncomir
VEGF	miR-199a miR-125b	miR-378

PDGFRβ. miR-296 was found to be upregulated in human brain microvascular endothelial cells and targeting of miR-296 restored expression of HGS, shown in Fig. 17.2. Nude mice bearing U87 glioma cells injected with synthetic cholesterol-conjugated antagomir-296 displayed marked regression of tumor growth [55].

Confluence of information suggested Notch signaling mediated expression of truncated intracellular isoform transcribed from intron 21 (i21 Flt1). Breast cancer cells MDA-MB-231 were treated with γ-secretase inhibitor DAPT and i21 Flt1 was notably repressed. Similar results were obtained in Notch-1 and Notch-3 silenced MDA-MB-231 cells. Retinoic acid mediated inhibitory effects on i21 Flt1 expression were achieved via miR-200 upregulation [56].

14.1 Natural Agents Mediated Inhibition of VEGFR

Barbigerone is an isoflavone recently reported to inhibit VEGF induced phosphorylation of VEGFR2 and downstream effectors including ERK, p38, AKT. Moreover, Barbigerone effectively inhibited tumor growth in A549 and SPC-A1 bearing mice [57]. VEGF mediated VEGFR2 phosphorylation was also noted to be inhibited by quercetin-4′-O-β-D-glucopyranoside (QODG), a flavonoid isolated from *Hypericum attenuatum Choisy* [58].

14.2 Natural Agents Mediated Regulation of miRNA

Increasingly it is being realized that hypoxia-induced expression of miR-21 and miR-210 in pancreatic cancer cells. However, synthetic derivative of curcumin (CDF) inhibited miR-21 and miR-210 expression. Moreover, cancer stem cell (CSC) markers CD44 and EpCAM in CSC-like cells derived from pancreatic cancer cells were remarkably reduced in MiaPaCa-2 tumor sphere cells under hypoxic conditions. VEGF production by MiaPaCa-2 tumor sphere cells was suppressed significantly upon CDF treatment [59]. Garcinol is a polyisoprenylated benzophenone derivative isolated from Garcinia indica. Garcinol works synergistically with gemcitabine in pancreatic adenocarcinoma cells thus controlling different miRNAs including miR-21, miR-196a, miR-495, miR-605, miR-638, and miR-453 [60].

15 Conclusion

In this chapter we discussed recent advances in miRNA regulation of VEGF/VEGFR signaling axis. Moreover, it is evident that VEGF and VEGFR controlling miRNAs are frequently suppressed, therefore, modulation of miRNA levels via either antagomirs or miRNA mimicry seems to be a promising target for future therapeutics.

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Chapter 18
Systems Biology Approaches in the Design of Effective miRNA-Targeted Therapeutics

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1 Introduction

Originally discovered in *C. elegans* development biology, microRNAs (miRNAs) are short (~22 nucleotides) non-coding RNAs that carry out complex regulatory functions through post-transcriptional inhibition of target mRNAs [1]. MiRNAs are synthesized in the cell nucleus and exported via specialized transporters [majority by exportins (exportin 5/Xpo5) and some by exportin 1/Xpo1] from the nucleus and undergo processing from larger to smaller segments via various endonucleases [2]. Right after the discovery miRNAs, their functional roles remained largely unknown. However, over the last decade with increasing analyses of sequence complementarity, mounting evidences suggested that miRNAs functions in gene regulation post-transcriptionally [3].

Given the critical placement of miRNAs in the cells regulatory signaling, it is quite logical that their expression may be context dependent and also affected by the pathological state in a cell, tissue or an organ. This is certainly true as various

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specific types of miRNAs are exclusively found in certain cellular systems in a context dependent manner. For example certain mir-122 and mir-223 are found to be highly concentrated in the liver and bone marrow granulocytes and macrophages in mice respectively [4]. Such differences in expression levels could also be seen in stem vs. differentiated cells in animal models. Recently, it was shown that the expression of miR-291-3p, miR-294, and miR-295 resulted in the enhancement of induced pluripotency, highlighting the valuable role these molecules play in normal development [5]. It is expected that, given the delicate placement of miRNAs within the post-transcriptional regulatory pathway; disease development and progression could be closely correlated to aberrant miRNA function. Indeed this is the case because erroneous or deregulated expression or depletion of miRNAs results in a concurrent defect in cellular function ranging from contrasting phenotypic changes such as developmental aberrations to physiological abnormalities especially those commonly found in degenerative conditions linked to malignancies [6].

The list of target gene(s) regulated by each miRNA in normal and disease conditions are slowly being deciphered. Nevertheless, there is a daunting realization that the scientific field is still a long way from fully understanding extent of effects that a single miRNA can induce in a given cell and the resulting impact it can exert of the phenotype [7]. Similarly with each passing year, there are newer partners emerging in the small non-coding RNA spectrum that are recognized to work in tandem with miRNAs. Each component within this spectrum is further regulated epigenetically as well and can influence not one, but many different geno- and phenotypes simultaneously in normal and disease situations. The enormous number of permutation combinations arising from these complex interactions cannot be fully deciphered using reductionism alone (Fig. 18.1 depicts the various tier of miRNA regulatory mechanisms). This chapter thus intends on exploring the significance of miRNAs in disease and the various facets surrounding their relevance clinically and experimentally as well as describes the strategies to target them through powerful and ever-evolving systems biology paradigms.

2 Systems Analysis to Predict miRNA-mRNA Interactions

A number of web-based tools are available for the prediction and identification of target miRNA targets. The choice, availability, validity and selection of an optimal yet appropriate tool are a challenge for the design of high throughput assays with promising miRNA targets and therefore, the prediction of miRNA targets remains a daunting task [8]. Most of the target prediction tools identify sequence complementarity of mirs to a specific mRNA. However, a one-miRNA-one-target identification molecular approach may not yield the complete picture of the extent of the influence of a single miRNA in a given system. There is emerging evidence that a large body of mirs can also regulate within the coding sequence of mRNAs that are distinct from the 3′UTRs [9]. Therefore, in order to fully understand the targets of miRNAs a systems approach is needed.

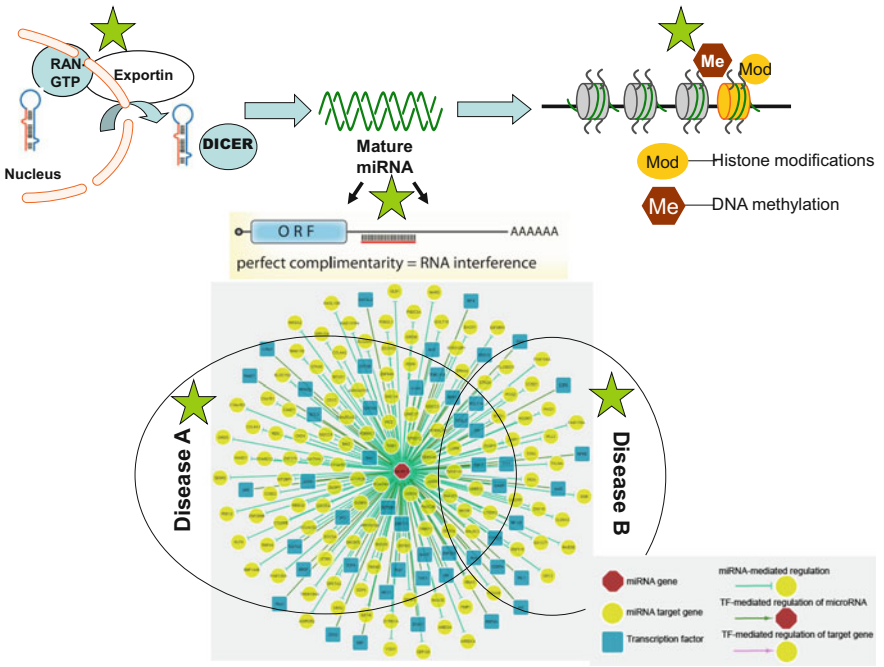

Fig. 18.1 The complexity of MiRNAs. MiRNAs are regulated at multiple levels (depicted by *green stars*). Precursor miRNAs are generated in the nucleus and exported to the cytoplasm for maturity through specialized exporter called exportin. Once matured, miRNAs are recognized to be regulated epigenetically either through methylation or acetylation processes. Due in part to the promiscuous binding to target mRNAs, a single miRNAs can target more than one gene. Often times the target genes are may have distinct roles in two completely different diseases (*hollow circles*). miRNA developed from CHIPBase database Nucleic Acid Res 2013; 41:D177–D187

Systems biology is an emerging field of biology-based interdisciplinary research that focuses on evaluating the complex interactions within biological systems, using a more holistic perspective [10]. The seeds of systems biology were sown in the late twentieth century; however, it was only from year 2000 onwards that the concept has been used widely in the biosciences in a variety of contexts. The technology is heavily dependent on mathematical and computational models and most often provides new mathematical theories. Given its scope and power, systems biology serves as an attractive field of research for studying miRNA complexity especially in diseases such as cancer [11]. Hence, in the last 8–10 years there has been a great deal of work done on miRNAs that have utilized systems sciences. A PubMed search using key words *MiRNA* and *Systems Biology* returns ~600 hits/publications. These papers cover multiple different aspects such as the application of systems sciences in miRNA diagnostics, target identification and as presented in later passages of this chapter, in therapeutics as well. Earlier studies were performed to prioritize specific miRNAs for differential expression in normal and cancer cell. For example, Landgraf and colleagues provided a miRNA expression

atlas based on small RNA library sequencing [12]. This study utilized 250 small RNA libraries from 26 different organ systems and cell types of human and rodents that were enriched in neuronal as well as normal and malignant hematopoietic tissues. The study presented expression profiles derived from clone count data and provide computational tools for their analyses. Unexpectedly, a relatively small set of miRNAs, many of which are ubiquitously expressed, were found to account for most of the differences in miRNA profiles between cell lineages and tissues. These studies in a way prove that targeting a limited set of miRNAs may lead to desirable outcome. However, given that multiple miRNAs co-express in complex diseases especially cancer, the selection of the most critical miRNA in a specific disease remains an elusive task for which prediction algorithms such as that developed from systems sciences become critical. Applications of systems sciences to miRNA research have resulted in the development of several prediction algorithms. Earlier studies in this field focused on identification of miRNA targets only and in this direction, Yoon and De-Michelis were among the first group to apply such computational tools [13].

More recently, databases of predicted targets were used in a miRNA target enrichment analysis, thereby enhancing the capacity to extract functional information from gene lists. However, following the lines discussed above, available tools in target prediction field analyze gene sets one by one limiting their use in a meta-analysis. To overcome this problem, recently, Guruceaga and Segura utilized a systems based approach to enhance the functional interpretation of miRNA-mRNA association in biological systems [14]. In this study they utilized an R system for miRNA enrichment analysis that is suitable from a systems biology perspective. Their collection of R scripts and embedded data allowed for using predicted targets of public databases or a custom integration of them. As a proof-of-principle, they successfully performed analysis of 2,158 tumor samples at a time and summarized their result in a network where each cancer disease is linked to enriched miRNAs and over-represented functions. These network connections proved to be a resource for the study of biological and pathological causes and effects of the expression of miRNAs.

Another important issue is the analysis of biological functions of pathways collectively targeted by co-expressed miRNAs in diseases. Addressing this problem, Gusev and colleagues reported results of computational analysis of five datasets of aberrantly expressed miRNAs in five human cancers [15]. Using the combinatorial target prediction algorithm miRgate and a two-step data reduction procedure they determined Gene Ontology categories as well as biological functions, disease categories, toxicological categories and signaling pathways that are (a) targeted by multiple miRNAs (b) statistically significantly enriched with target genes and (c) known to be affected in specific cancers. Their global analysis of predicted miRNA targets demonstrated that co-expressed miRNAs collectively provide systemic compensatory response to the abnormal phenotypic changes in cancer cells by targeting a broad range of functional categories and signaling pathways known to be affected in a specific cancer.

3 Identifying Therapy Resistance Sustaining miRNAs Using Systems Approaches

A number of miRNAs have been linked to cancer drug resistance which contributes to the low prognosis and represents the biggest barrier to effective treatment [16]. Thus, improving our understanding of the miRNA based mechanisms underlying drug-resistance in cancer may contribute to new strategies in combating the disease. As multiple miRNAs work in tandem to give rise to the drug resistance pathways, a systems level analysis looking at the holistic interactions among resistance sustaining pathways is needed. A number of laboratories have attempted holistic evaluations of drug resistance miRNAs networks. Using a colorectal cancer model, Ragusa and colleagues evaluated the specific alterations of miRNA transcriptome and global network structure post cetuximab treatment [17]. Their investigation evaluated 25 transcription factors putatively controlling the differentially expression miRNAs between sensitive and resistant cell lines. Interestingly, 11 of the transcription factors were previously reported to be involved in colorectal cancer. On the basis of these data, they suggested that the downregulation of let-7b and let-7e, that are recognized to target and the upregulation of miR-17* (a CRC marker) could be considered as candidate molecular markers of cetuximab resistance. More importantly, global network functional analysis (based on miRNA targets) showed a significant over-representation of cancer-related biological processes and networks centered on critical nodes involved in EGFR internalization and ubiquitin-mediated degradation. Such identification of miRNAs that are linked to the efficacy of a therapy was proposed to allow the ability to predict the responses of patients to treatment and possibly lead to a better understanding of the molecular mechanisms of drug responses. In another study Vera and colleagues utilized network biology and kinetic modeling to evaluate miRNA driven cell drug resistance [18]. This study used a regulatory map developed to summarize knowledge on E2F1 and its interplay with p73/DNp73 and miR-205 in cancer drug responses. The authors derived a kinetic model that represents the network response to different genotoxic and cytostatic anticancer drugs. By perturbing the model parameters, they heterogeneous cell configurations that represented actual cell lines. The primary goal was to detect genetic signatures that are characteristic for single or two drug treatment resistance. The study identified a signature composed of high E2F1 and low miR-205 expression that promotes resistance to genotoxic drugs. Interestingly, in this signature, downregulation of miR-205, can be mediated by an imbalance in the p73/DNp73 ratio or by dysregulation of other cancer-related regulators of miR-205 expression such as TGFβ-1 or TWIST1. This model simulations also suggested that conventional genotoxic drug treatment favors selection of chemoresistant cells in genetically heterogeneous tumors, in a manner requiring dysregulation of incoherent feed-forward loops that involve E2F1, p73/DNp73, and miR-205. In another such study, Uboldi and colleagues utilized a systems approach to characterize the regulatory network leading to trabectedin resistance in an in vitro model of myxoid liposarcoma [19]. Their study identified 3,083 genes,

47 miRNAs and 336 proteins differentially expressed between 402-91 (trabectedin sensitive) and 402-91/ET (trabectedin resistant) cell lines. Interestingly three miRNAs among those differentially expressed, miR-130a, miR-21 and miR-7, harbored CHOP binding sites in their promoter region. The authors also used computational approaches to integrate the three regulatory layers and to generate a molecular map describing the altered circuits in sensitive and resistant cell lines. By combining transcriptomic and proteomic data, they reconstructed two different networks, i.e. apoptosis and cell cycle regulation that were proposed to a key role in modulating trabectedin resistance. This approach highlights the central role of genes such as CCDN1, RB1, E2F4, TNF, CDKN1C and ABL1 in both pre- and post-transcriptional regulatory network. Nevertheless, the study did not perform wet lab experiments to evaluate the consequence of modulating the identified mirs in restoring sensitivity to trabectedin (discussed for another model in the forthcoming passage). In conclusion, such systems biology based approach provides new avenues for biological interpretation of miRNA profiling data and generation of experimentally testable hypotheses regarding collective regulatory functions of miRNA in cancer drug resistance.

4 Prioritizing miRNAs in Cancer Specimens Using Systems Biology and Pathway Tools

In order to identify/prioritize miRNAs in pancreatic cancer (PC) models, our group has earlier compared the expression profile of mirs in the plasma of patients diagnosed with PC (n = 50) with healthy volunteers (n = 10) [20]. In this study, 37 miRNAs were down-regulated and 54 were up-regulated in plasma. The expression of miR-21 was significantly higher, and the expression of let-7 family (especially let-7d) and miR-146a was significantly lower in PC. Most interestingly, the expression of miR-21 was correlated with poor overall survival, and the expression of let-7 was inversely correlated with survival in these studies. Moreover, we found that miR-21 family was markedly over-expressed in chemo-resistant PC cell lines, which was consistent with the plasma data from PC patients. In this direction, our previous studies have shown increased expression of miR-21 with concomitant loss of PTEN expression in PC cells, which is consistent with our patient findings showing the loss of three additional targets of miR-21 i.e. PDCD4, Maspin and TPM1. Therefore, these results suggest that identifying and validating the expression of miRNAs in newly diagnosed patients could serve as potential biomarker for tumor aggressiveness, and such miRNAs could be useful for the screening of high-risk patients, and may also serve as targets for future miRNA based development strategies. In a follow up study we investigated the expression of miRNAs in pancreas tissues obtained from PC transgenic mouse models of K-Ras (K), Pdx1-Cre (C), K-Ras;Pdx1-Cre (KC), and K-Ras;Pdx1-Cre;INK4a/Arf (KCI), initially from pooled RNA samples using miRNA profiling, and further confirmed in

individual specimens by quantitative RT-PCR [21]. We found over-expression of certain miRNAs (miR-21, miR-221, miR-27a, miR-27b, and miR-155), and down-regulation of another specific set (miR-216a, miR-216b, miR-217, and miR-146a) expression in tumors derived from KC and KCI mouse model, which was consistent with data from KCI-derived RInk-1 cells (cells derived from mice with KCI genotype). Mechanistic investigations revealed a significant induction of EGFR, K-Ras, and MT1-MMP protein expression in tissues from both KC and KCI mouse compared to tissues from K or C, and these results were consistent with similar findings in RInk-1 cells compared to human MiaPaCa-2 cells. Furthermore, miR-155 knock-down in RInk-1 cells resulted in the inhibition of cell growth and colony formation consistent with down-regulation of EGFR, MT1-MMP, and K-Ras expression. In addition, miR-216b which target Ras, and forced re-expression of miR-216b in RInk-1 cells showed inhibition of cell proliferation and colony formation, which was correlated with reduced expression of Ras, EGFR, and MT1-MMP. Our findings suggest that transgenic models would be useful for preclinical evaluation of novel miRNA-targeted agents for designing personalized therapy for PC.

In another study, an attempt was made to prioritize druggable PC promoting miRNAs. In this study we utilizing serum and paraffin embedded tissue (PFE) from fine needle aspirate (FNA) biopsies, we were able to selectively pinpoint the differential expression of a number of miRNAs in tumor vs. normal tissue [22]. Among the major miRNAs discovered, the levels of mirR-21, miR-155 and miR-205 were found to be higher in tumors compared to normal tissue**.** On the other hand, let-7b mir-146a, let7b, miR-185 were consistently lower in patient tumor samples compared to normal counterparts. Evaluation of the differentially expressed miRNAs using pathway analysis demonstrated that the lower expression of mir-146 led to the activation of pro-survival signaling NF-κB and related pathway. Interestingly, targeting mir-146 using chemical inhibition strategies resulted in suppression of PC cell proliferation and aggressiveness. These studies provide insights into how pathway analysis can help prioritize PDAC related miRNAs that can further be incorporated in therapeutic strategies.

5 Identifying and Targeting Cancer Stem Cell Sustaining miRNAs Using Pathway Analysis Tools

Although much of the mechanism is still under investigation, there has been increasing evidence over the past decade which points to the critical role of tumor plasticity arising from cancer stem cells in regulating drug-resistance [23]. Cancer stem cells (CSCs), similar to normal stem cells, have the ability to self-regenerate, replicate heterogeneously, and resist the activation of cell death signaling especially the pathways related to apoptosis [24]. Originally discovered in leukemia cells in the late 1990s, several investigations have identified CSCs in

numerous solid tumors including breast, brain, colon, prostate, lung, and PC tumors [25, 26]. Even though CSCs represent only a small sub-population of the tumor cells (<1 %), current evidence suggests that they are responsible for producing the malignant tumor sustaining differentiated tumor cell lineage mass. CSCs share many characteristics with epithelial-to-mesenchymal (EMT) cells including CD44 and CD24 cell surface marker expression, enhanced expression of vimentin, and suppression of cadherin 1 [27]. There is strong evidence that the presence of CSCs contributes to the low efficacy of chemo-radiation therapy. Generally chemo-radiation may reduce tumor mass by killing differentiated cell progenies, the continued existence of CSCs has been suggested to result in recurring tumors. The molecular mechanism of CSC's tumorigenic regulation and signaling pathways are currently being investigated in order to find more effective therapies for patients. miRNAs have been shown to support the pathways that maintain gastric CSCs making the study of mirs in these resistant cells highly relevant to the successful design of therapeutic strategies against cancer [28, 29]. Therefore, we applied a systems approach to evaluate the role of miRNAs in sustaining the cancer stem cell signaling in PC derived CSC models. The CSCs were obtained by flow sorting MiaPaCa-2 PC cells for markers triple-positive for CD44, CD133 and EpCAM (CD44+CD133+EpCAM+). Microarray analysis was performed to examine the differential expression of miRNAs of these triple positive CSCs, compared to either the parental MiaPaCa-2 cells or triple negative cells. The Log2 fold change miRNAs from microarrays of flow sorted PC stem cells triple positive for CD133/CD24/CD44 were subjected to systems and pathway analysis by using the web-based bioinformatics tool IPA (Ingenuity Systems, Redwood, CA) for predicting functional networks. Our results showed ~400 miRNAs to be differentially expressed in triple positive CSCs vs. triple negative cells. Among these miRNAs, ~200 miRNAs were up-regulated and ~250 were down-regulated in CSCs, compared to triple negative cells. Moreover, we found a little less than 500 miRNAs that were differentially expressed between triple positive CSCs and the parental MiaPaCa-2 cells. Among those miRNAs, 243 miRNAs were up-regulated and 243 miRNAs were down-regulated. However, there were only 180 differentially expressed miRNAs between triple negative cells and the parental MiaPaCa-2 cells. Among those miRNAs, 108 miRNAs were up-regulated and 72 miRNAs were down-regulated.

We further subjected the differentially expressed miRNAs to IPA analyses to better understand the miRNA target pathways that are involved and how they influence their target genes which were generated based on their pathway connectivity. Our pathway enrichment analysis of CSCs (triple positive) vs. triple negative cells showed ten biological functional groups with connectivity including cancer, gastrointestinal (GI) disease, and genetic disorder (Table 18.1). These results suggest that differential expression of miRNAs in CSCs (triple positive cells) is highly associated with the development and progression of tumors including GI tumors (note that here the model used is PC). The network analysis of selected miRNAs in CSCs (triple positive) vs. triple negative cells is similar to that of CSCs (triple positive) vs. MiaPaCa-2 cells. Furthermore, our network analysis showed

Table 18.1 The pathway enrichment analysis showing differential expression of selected genes that involved in 21 (top eleven shown here) of the major biological functional groups

List of major biological functional groups regulated by differentially expressed genes between CSC+++ vs. parent PC cells
1. Antigen presentation
2. Cell cycle
3. Mitotic role of Polo Like Kinase
4. Tight junction signaling
5. OX40 signaling
6. Aryl hydrocarbon receptor signaling
7. Virus entry via endocytic pathway
8. Sertoli cell junction signaling
9. PI3/AKT signaling
10. HMGB1 signaling
11. VEGF signaling

that many miRNAs were intricately regulated by each other either directly or indirectly, which were also further regulated by several target genes indicating a very complex interaction system. We found changes in a number of miRNAs in CSCs (triple positive cells), compared to MiaPaCa-2 and triple negative cells including let-7f,i, miR-30a,b, miR-125b, and miR-335, compared to its parental MiaPaCa-2 cells and triple negative cells. Most importantly, the knockdown of miR-125b by transfecting its siRNA inhibitor reduced spheroid forming ability, clonogenicity, cell migration, and self-renewal capacity. These experiments validated their role in sustaining CSCs networks. Collectively, these systems studies identified key miRNAs that promote CSCs signaling that could not have been pinpointed using traditional molecular biology.

6 Conclusion

Malignancies results from aberration in the entire system of regulation and control and there is increasing acceptance of cancer being a systems biology disease. The cancer system is intricately connected through multiple layers of both genetic and epigenetic de-regulation mechanisms, which require sophisticated analyses to provide insight into their dynamic actions. The complexity of signaling emanating from cancer associated miRNAs and their relevant target genes cannot be understood through the reductionist biochemical principles. The reality is that the entire miRNA system contains layer upon layer of fine control that can at times be redundant and seemingly unrelated to the disease in question. As presented in this chapter, systems and network biology can help in the identification of miRNAs responsible for drug resistance as well as in targeting critical mirs that drive cancer stemness. By bridging the vast science of computational analyses with the various basic science disciplines as well as with the clinical application of basic science

tools, an incredible detailed and versatile picture of cancer related miRNAs can be ascertained. This exercise can ascertain successful targeting and manipulation of miRNAs in order to better fight the disease. Through this very same mechanism, truly personalized treatments can be created that both more efficiently target a patients' cancer cells as well as limit the harm caused to their healthy cells. As presented here, it is thus only logical to increase our knowledge of miRNA function through systems biology, its relationship to the larger physiological system and its greater implication in patient health and treatment outcome.

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