

## REVIEW



# MicroRNAs in non-small cell lung cancer: Gene regulation, impact on cancer cellular processes, and therapeutic potential

Hannah Petrek | Ai-Ming Yu

Department of Biochemistry & Molecular Medicine, UC Davis School of Medicine, Sacramento, CA, USA

**Correspondence**

Ai-Ming Yu, Department of Biochemistry & Molecular Medicine, UC Davis School of Medicine, Sacramento, CA 95817, USA.  
Email: aimyu@ucdavis.edu

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**Abstract**

Lung cancer remains the most lethal cancer among men and women in the United States and worldwide. The majority of lung cancer cases are classified as non-small cell lung cancer (NSCLC). Developing new therapeutics on the basis of better understanding of NSCLC biology is critical to improve the treatment of NSCLC. MicroRNAs (miRNAs or miRs) are a superfamily of genome-derived, small noncoding RNAs that govern posttranscriptional gene expression in cells. Functional miRNAs are commonly dysregulated in NSCLC, caused by genomic deletion, methylation, or altered processing, which may lead to the changes of many cancer-related pathways and processes, such as growth and death signaling, metabolism, angiogenesis, cell cycle, and epithelial to mesenchymal transition, as well as sensitivity to current therapies. With the understanding of miRNA biology in NSCLC, there are growing interests in developing new therapeutic strategies, namely restoration of tumor suppressive miRNAs and inhibition of tumor promotive miRNAs, to combat against NSCLC. In this article, we provide an overview on the molecular features of NSCLC and current treatment options with a focus on pharmacotherapy and personalized medicine. By illustrating the roles of miRNAs in the control of NSCLC tumorigenesis and progression, we highlight the latest efforts in assessing miRNA-based therapies in animal models and discuss some critical challenges in developing RNA therapeutics.

**KEYWORDS**

Cancer, miRNA, NSCLC, regulation, therapy, tumorigenesis

**Abbreviations:** 3'UTR, 3'-untranslated region; ABC, ATP-binding cassette; ABCB9, ATP-binding cassette subfamily B member 9; AGO, Argonaute; ALK, anaplastic lymphoma kinase; Bak, BCL2 agonist/killer; Bax, BCL2 Associated X; BCL, B-cell leukemia/lymphoma; BCL2L2, BCL2-like 2; Bid, BH3 interacting domain death agonist; BRAF, v-Raf murine sarcoma viral oncogene homolog B; CCN, Cyclin; Cdc, cell division cycle; CDK, cyclin-dependent kinase; CTLA4, cytotoxic T-lymphocyte-associated protein 4; DISC, death induced signaling complex; E2F, E2 factor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EML4, echinoderm microtubule-associated protein-like 4; EMT, epithelial to mesenchymal transition; ERK, extracellular-signal-related kinase; FGF, fibroblast growth factor; FGFR, FGF receptor; FIH, factor inhibiting HIF-1; FOXM, forkhead box M; GLUT1, glucose transporter 1; GTP, guanosine triphosphate; HER, hormone epidermal growth factor receptor; HGF, hepatocyte growth factor; HGFR/MET, hepatocyte growth factor receptor; HIF-1, hypoxia-inducible-factor-1; HIF1AN, HIF-1 subunit alpha inhibitor; HK2, hexokinase 2; HUVEC, human umbilical vein endothelial cell; IFGR, insulin-like growth factor receptor; IGF, insulin-like growth factor; KRAS, Kirsten rat sarcoma; LDHA, lactate dehydrogenase A; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; miRNAs or miRs, MicroRNAs; MMP, matrix metalloproteinase; NDUFA4, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4; NRP, neuropilin; NSCLC, non-small cell lung cancer; PD-1, programmed cell death protein-1; PDK1, phosphoinositide-dependent protein kinase-1; PD-L1, PD-1 ligand; PI3K, phosphatidylinositol 3-kinase; PIGF, placenta growth factor; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit; pre-miRNA, precursor miRNA; pri-miRNA, primary micro-RNA; PTEN, phosphatase and tensin homolog; RAC, RAS-related C3 botulinum toxin substrate; RAF, v-Raf murine sarcoma; RAS, rat sarcoma; Rb, retinoblastoma tumor suppressor; RhoA, Ras homolog family protein A; RISC, RNA-induced silencing complex; ROCK, Rho-associated protein kinase; SDHD, dehydrogenase complex, subunit D; SMAD, mothers against decapentaplegic; SOX, Sry-related HMG box; STAT, signal transducer and activator of transcription; TGF $\beta$ , transforming growth factor beta; TGF $\beta$ R, TGF $\beta$  receptor; TIMP, tissue inhibitor of metalloproteinases 3; TNF, tumor necrosis factor; TNFR, TNF receptors; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; vHL, von Hippel Lindau; XPO5, Exportin-5; ZEB, zinc finger e-box-binding homeobox.

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## 1 | INTRODUCTION

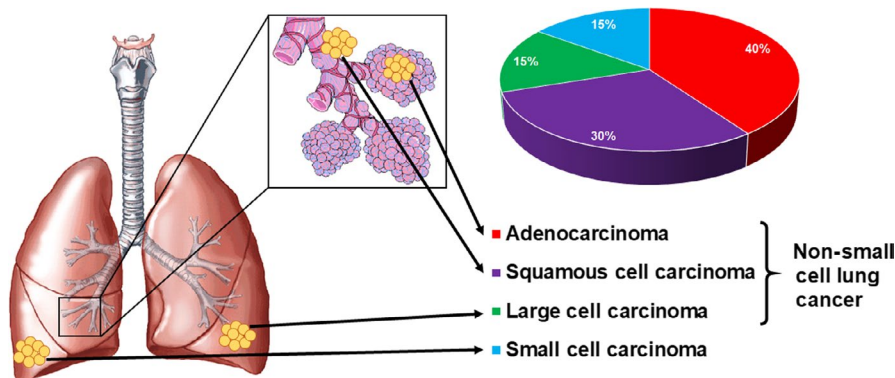
Lung and bronchus cancer is the second most commonly diagnosed cancer in the United States, with over 220,000 estimated new diagnoses in 2019, accounting for almost 13% of all cancer diagnoses.<sup>1</sup> One in 15 men and 1 in 17 women will be diagnosed with lung cancer during their lifetime and the average age at diagnosis is 70 years.<sup>1</sup> Lung cancer accounts for the highest cancer-related deaths in the United States, causing 23% of all cancer-related deaths which is more than colon, breast, and prostate cancers combined.<sup>1</sup> The five-year survival rate of all lung cancer diagnoses is 19%, which is lower than colon, breast, and prostate cancers. When the disease is detected while still localized to the lung, the five-year survival rate is 56%; however, only 16% of cases at diagnosed at that stage. By contrast, the five-year survival rate for metastatic lung cancer patients is only 5%. More than half of patients diagnosed with lung cancer will die within one year. It is also estimated that lung cancer care may be increased to 173 billion dollars in 2020 in the United States.<sup>2</sup>

The lung epithelium undergoes a series of morphological changes before becoming invasive, such as hyperplasia, metaplasia, and finally dysplasia and carcinoma in situ. Lung cancer is classified by the site of origin, and method of diagnosis, prognosis, and treatment. The two main types of lung cancer are small cell lung cancer, accounting for 15% of all cases, and non-small cell lung cancer (NSCLC) which is any type of epithelial lung cancer, and accounts for 80% to 85% of all cases (Figure 1).<sup>3</sup> The three most common histological forms of NSCLC are epidermoid or squamous cell carcinoma, large cell carcinoma, and adenoma; among them adenocarcinoma accounts for 40% of all lung cancer cases.<sup>4</sup> Squamous cell carcinoma occurs inside the airways, adenocarcinoma occurs in the cells lining the alveoli located in the outer part of the lungs, and large cell carcinoma is in any other part of the lung. A major risk factor for NCSLC is smoking;<sup>5</sup> other risks include secondhand smoke, radiation exposure, air pollution, family history, and human immunodeficiency virus infection. Lung cancer may present as a persistent cough, chest pain,

weight loss, malaise, difficulty breathing, pleural effusion, pneumonia, chronic obstructive pulmonary disease or pulmonary fibrosis.<sup>6</sup> Diagnostic procedures include sputum cytology, tissue biopsy and imaging tests such as bronchoscopy, X-ray, MRI, positron emission tomography, or computed tomography scan.<sup>7</sup> Lung cancer is staged based on the size of the primary tumor, the involvement of the lymph nodes, and the presence of distant metastasis.<sup>8</sup>

## 2 | MOLECULAR FEATURES OF NSCLC

Molecular features of NSCLC tumors may not only predict the prognosis and outcome the cancer but also serve as targets for therapies. The most frequent mutations in NSCLC occur in the *TP53* gene, occurring in about 50% of NSCLC cases (Table 1).<sup>9</sup> Mutations in *EGFR*, a tyrosine kinase receptor, account for 10%-35% of cases and can cause dysfunction of the *AKT* and *MAPK* signaling which enhances cell survival and stimulates proliferation.<sup>10</sup> The most common mutations of *EGFR* are in-frame deletions of exon 19, and the second most common *EGFR* mutation is single nucleotide substitutions L858R in exon 21.<sup>11</sup> The most common mutation detected after treatment with *EGFR* inhibitors is T790M in exon 20 which can confer drug resistance.<sup>12</sup> The third most frequent mutations occur in *KRAS*, accounting for 15%-25% of cases.<sup>13</sup> Usually mutations in *KRAS* and *EGFR* are mutually exclusive and non-overlapping. Another common molecular feature of NSCLC is the presence of *ALK* fusion gene, which encodes a receptor tyrosine kinase not normally expressed in the lung.<sup>13</sup> At least nine different variants of fusion of *ALK* with an upstream partner *EML4* have been identified causing constitutive activation of the kinase.<sup>13</sup> The *HER2* protein, a *HER* family receptor tyrosine kinase, is over-expressed in 20% of all NSCLC and gene amplification occurs in 2%.<sup>14,15</sup> These mutations commonly lead to constitutive activation of the *HER2* signaling pathway.<sup>16</sup> Mutations in the main catalytic subunit, *PIK3CA*, of phosphatidylinositol 3-kinase occur in about



**FIGURE 1** Lung cancer classifications and frequency of diagnosis. There are two main types of lung cancer: small-cell lung cancer and non-small cell lung cancer. Small-cell carcinoma occurs in the outer edges of the lungs and accounts for about 15% of all cases. Non-small cell lung cancer (NSCLC) makes up 85% of all lung cancer cases, and can be further classified into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Adenocarcinoma, the most common NSCLC subtype, occurs in the cells lining the alveoli, squamous cell carcinoma is generally found in the airways or bronchi, and large cell carcinoma is in the edges of the lungs

**TABLE 1** Common genetic alterations and their prevalence in NSCLC

Gene	Alteration	Prevalence in NSCLC
<i>TP53</i>	Mutation	50%
<i>EGFR</i>	Mutation	10%-35%
<i>KRAS</i>	Mutation	15%-27%
<i>ALK</i>	Fusion	5%-15%
<i>HER2</i>	Overexpression/ Gene amplification	20%/ 2%
<i>PIK3CA</i>	Mutation	2%-8%
<i>AKT1</i>	Mutation	1%
<i>BRAF</i>	Mutation	5%
<i>MET</i>	Mutation	5%

2% of NSCLC cases.<sup>17</sup> These tumors can activate the protein kinase B signaling pathway without growth factors. Protein kinase B is encoded by *AKT1*, which is mutated from a glutamate to a lysine at position 17 in 1% of NSCLC cases and causes PI3K-independent activation of protein kinase B.<sup>18,19</sup> *BRAF* is a member of the RAF kinase family which confers signaling of the MAPK family from the RAS GTPases to control cell proliferation.<sup>20</sup> *BRAF* mutations are mostly found in adenocarcinomas and lead to higher kinase activity and constitutively active MAPK2 and MAPK3<sup>21</sup>. MAPK2 and MAPK3 are downstream of *BRAF* and three mutations in the non-kinase portion of these proteins have been found in cancer.<sup>22</sup> Amplification of the gene *MET*, which codes hepatocyte growth factor receptor (HGFR) causes resistance to EGFR tyrosine kinase inhibitors.<sup>23</sup> The molecular heterogeneity of NSCLC tumors increases the complexity of treatment for NSCLC patients.

### 3 | CURRENT TREATMENTS FOR NSCLC PATIENTS

Lung cancer that is diagnosed at the early stages is commonly treated with resection surgery or lobectomy, chemotherapy, and radiation. Surgery may range from removing an entire lung to removing part of a lobe, depending on the size and location of the tumors. Radiation, alone or concurrent with surgery or chemotherapy is also commonly utilized. External beam radiation therapy, the most widely used form of radiation for NSCLC, consists of administering 1.5 to 2.5 Gray to the lungs usually 5 days a week for 5-7 weeks, while stereotactic radiotherapy consists of a larger dose, around 22 Gray, in usually fewer than 5 doses.<sup>24</sup> Brachytherapy, or internal radiation therapy, involves the inserting a radioactive pellet in or near the tumor for a short amount of time or permanently. The radiation stays localized and gets weaker over time. Radiofrequency ablation uses radio waves emitted from a probe guided by a computed tomography scan.

Molecular medicine or pharmacotherapy spans from chemotherapy to targeted therapy and the most recent immunotherapy, which utilize

small-molecule and protein or antibody drugs (Table 2). Commonly used chemotherapies for the treatment of NSCLC include cisplatin, carboplatin, docetaxel, paclitaxel, pemetrexed, and vinorelbine that usually interfere with DNA synthesis or replication to achieve the inhibition of cancer cell proliferation and growth (Table 2). Nevertheless, chemotherapy may not be effective for all patients and many cancers will eventually become resistant to the drugs. Furthermore, chemotherapy kills cancer cells less specifically and could cause some side effects like pain, nausea, vomiting, blood disorders, and hair-loss.

Pharmacotherapy for NSCLC has been benefited greatly by the development of targeted and personalized medications, either small molecules or antibodies, which act more selectively on particular molecular targets including transmembrane and cell surface proteins or receptors (eg, EGFR, PD-1, PD-L1, etc) as well as signal proteins (eg, cytokines, VEGF) and cytoplasmic kinases (eg, MEK) (Table 2). While all the therapies listed in Table 2 are approved in the United States, many are also approved in Europe and elsewhere. The response rate for most of the therapies is generally consistent across subtypes with higher rates for tumors with high mutational burden. The effectiveness of two antibody drugs, anti-VEGF bevacizumab and anti-VEGFR ramucirumab, is attributable to the inhibition of angiogenesis.<sup>25</sup> NSCLC patients with an overexpression of EGFR mRNA or increased copy number have a 70% or higher response rate to small-molecule EGFR inhibitors like gefitinib or EGFR antibodies like necitumumab.<sup>26</sup> Furthermore, some targeted therapies are approved for specific subsets of NSCLC patients. Osimertinib is used to treat NSCLC patients with T790M mutations of EGFR. The *EML4-ALK* tumors are mostly responsive to small-molecule tyrosine inhibitors of ALK like crizotinib. Dabrafenib and trametinib, which target *BRAF* and MEK1/2, respectively, are prescribed for patients with *BRAF* V600E mutations.<sup>27</sup> Immunotherapies such as PD-1 and PD-L1 antibodies (eg, nivolumab and atezolizumab) are also effective for the treatment of some NSCLC patients regardless of the subtype.<sup>28</sup> While targeted and immunotherapies are generally less toxic and personalized for particular patients, some patients do exhibit primary or acquires resistance<sup>29,30</sup> or show severe adverse effects such as diarrhea and pneumonitis.<sup>31-33</sup> In addition, targeted therapies have the greatest response rate for patients with the indicated mutation, therefore, due to the high heterogeneity of mutations within NSCLC, targeted therapies may not work in every patient. Large efforts are underway to advance the understanding of NSCLC biology and assess novel therapies.

### 4 | GENOME-DERIVED MICRORNAS ARE DYSREGULATED IN NSCLC

As less than 5% of the human genome is processed to functional proteins in cells, the majority is transcribed into enormous numbers of functional noncoding RNAs. Among them, microRNAs (miRNAs or miRs) are a superfamily of short RNAs that act on corresponding transcripts via complementary binding to achieve mRNA degradation or translation inhibition<sup>34</sup> (Figure 2). The biogenesis of miRNAs starts with the transcription of miRNA-coding genes into primary

**TABLE 2** List of drugs approved in the US for the treatment of NSCLC and their molecular targets or mechanistic actions

Treatment	Classification	Target or Action	Approval	Overall Response Rate
Bevacizumab	antibody/protein	VEGF	Non-squamous NSCLC	35% with carboplatin and paclitaxel <sup>150</sup>
Ramucirumab	antibody/protein	VEGFR	Metastatic non-squamous NSCLC	23% with docetaxel <sup>151</sup>
Erlotinib	small molecule	EGFR	EGFR L858R mutation, metastatic NSCLC	74.4% <sup>152</sup>
Necitumumab	antibody/protein	EGFR	Metastatic squamous NSCLC	48.1% with cisplatin and gemcitabine <sup>153</sup>
Gefitinib	small molecule	EGFR	Advanced or metastatic NSCLC with L858R EGFR mutations	76.9% <sup>152</sup>
Afatinib	small molecule	EGFR	Metastatic squamous NSCLC with non-resistant EGFR mutations	56% <sup>154</sup>
Osimertinibe	small molecule	EGFR T790M mutations	Advanced or metastatic NSCLC with T790M EGFR mutations	77% <sup>155</sup>
Crizotinib	small molecule	ALK/CD246, ROS	Advanced or metastatic ALK-positive NSCLC	74% <sup>156</sup>
Ceritinib	small molecule	ALK/CD246	Metastatic ALK-positive NSCLC	58% <sup>157</sup>
Brigatinib	small molecule	ALK/CD246	Metastatic ALK-positive NSCLC	71% <sup>158</sup>
Alectinib	small molecule	ALK/CD246, RET	Metastatic ALK-positive NSCLC	82.9% <sup>159</sup>
Dabrafenib	small molecule	B-Raf	Metastatic NSCLC with B-Raf V600E mutation	67% in combination with trametinib <sup>160</sup>
Trametinib	small molecule	MEK	Metastatic NSCLC with B-Raf V600E mutation	See dabrafenib
Entrectinib	small molecule	ROS1/NTRK fusion	Metastatic, ROS1/NTRK-positive NSCLC	78% <sup>161</sup>
Nivolumab	antibody/protein	PD-1/CD279	Metastatic squamous NSCLC	47% in patients with a high tumor-mutation burden <sup>162</sup>
Pembrolizumab	antibody/protein	PD-1/CD279	Advance or metastatic squamous NSCLC	44.8% <sup>163</sup>
Atezolizumab	antibody/protein	PD-L1/CD274/B7-H1	Metastatic non-squamous NSCLC	63.5% with bevacizumab, carboplatin, and paclitaxel in patients with no EGFR or ALK alterations <sup>164</sup>
Ipilimumab	antibody/protein	CTLA4/CD152	Metastatic NSCLC	45.3% with nivolumab <sup>165</sup>
Carboplatin & cisplatin	small molecule	Inhibition of DNA replication	Advanced or metastatic NSCLC	62% carboplatin with paclitaxel <sup>166</sup>
Irinotecan	small molecule	Topoisomerase I	Advanced NSCLC	43.7% with cisplatin <sup>167</sup>
Etoposide	small molecule	Topoisomerase II	Metastatic NSCLC	21.9% with cisplatin <sup>168</sup>
Docetaxel	small molecule	Microtubules; inhibition of mitosis	Advanced NSCLC	9% in patients previously treated with chemotherapy <sup>169</sup>
Paclitaxel	small molecule	Tubulin; inhibition of mitosis	Advanced or metastatic NSCLC	See carboplatin & cisplatin
Vinorelbine	small molecule	Tubulin; inhibition of mitosis	Advanced NSCLC	43% with cisplatin <sup>170</sup>

(Continues)

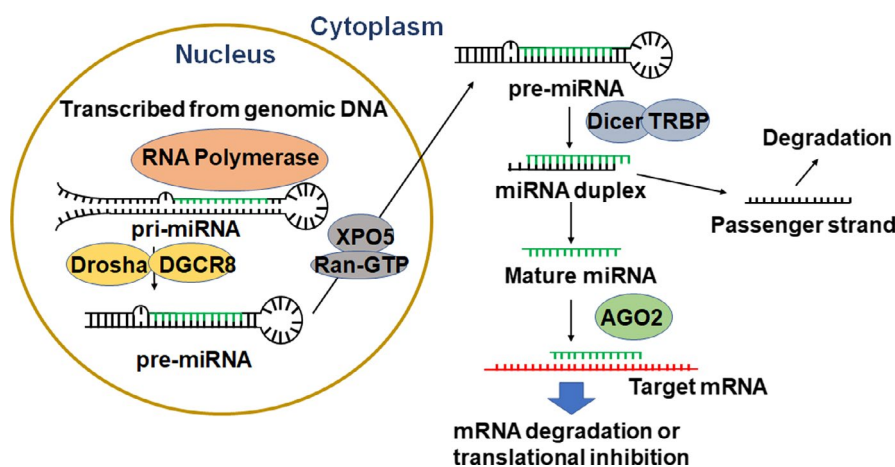
**TABLE 2** (Continued)

Treatment	Classification	Target or Action	Approval	Overall Response Rate
Vinblastine	small molecule	Microtubule; inhibition of mitosis	Advanced NSCLC	41% with cisplatin <sup>171</sup>
Pemetrexed	small molecule	Thymidylate synthase, dihydrofolate reductase	Advanced NSCLC	9.1 in patients previously treated with chemotherapy <sup>172</sup>
Gemcitabine	small molecule	Inhibition of DNA synthesis	Advanced or metastatic NSCLC	40.6% with cisplatin <sup>168</sup>

miRNA (pri-miRNA) transcripts. The pri-miRNA is thus processed by the Drosha-DGCR8 complex within the nucleus to produce a precursor miRNA (pre-miRNA) that can be exported into the cytoplasm by Ran-GTP-dependent Exportin-5 (XPO5). The pre-miRNA is cleaved into a miRNA duplex by the RNase Dicer in the cytoplasm<sup>35</sup> (Figure 2). The miRNA duplex is then unwound to offer two strands, among which the guide strand is preferably incorporated into the RNA-induced silencing complex (RISC) consisting of the Argonaute family of proteins while the passenger strand is readily degraded.<sup>36,37</sup> The RISC proteins stabilize and aid the mature miRNA in binding to the 3'-untranslated region (3'UTR) of a target transcript to accomplish the regulation of target gene expression (Figure 2).

Many miRNAs are involved in the control of target gene expression behind various cancer cellular processes (see the following section), exhibiting tumor suppressive or promotive activities. Specifically, a miRNA that reduces the expression of tumor suppressors acts as a tumor promotor, and a miRNA that degrades oncogene transcripts functions as a tumor suppressor. Interestingly, some miRNAs are dysregulated in NSCLC (Table 3) that may be indicative of disease status or therapeutic outcome. With some exceptions, generally there is a decrease of tumor suppressive miRNAs (eg, miR-34a-5p and miR-124-3p) and increase of tumor promotive

miRNAs (eg, miR-21-5p and miR-183-5p) in many human cancers including NSCLC (Table 3), as compared to normal tissues; however, the magnitude of dysregulation varies by case. Dysregulation of miRNA expression may be caused by different mechanisms such as chromosomal deletion or methylation, or dysregulation of their transcription factors, enzymes, or binding proteins involved in miRNA biogenesis. Dicer, the RNase responsible for the processing of pre-miRNAs, is essential for mouse development and stem cell maintenance.<sup>38</sup> Dicer was reported to be downregulated in some lung cancer patients, leading to a global decrease in miRNAs and associated with poor prognosis,<sup>39</sup> and conditional deletion of Dicer led to increased lung tumorigenesis in mice.<sup>40</sup> Actually, the role of Dicer in cancer remains contradictory.<sup>41</sup> It has been suggested that a partial loss or downregulation of Dicer is oncogenic, while a complete loss is tumor protective. In addition to Dicer, the expression of some miRNAs is also modulated by the tumor suppressive transcription factor p53.<sup>42</sup> During DNA damage, p53 associates with the Drosha/DGCR8 complex and facilitates processing of pri-miRNA to pre-miRNA. p53 is mutated and inactivated in many cancers, including lung,<sup>43</sup> reducing the total levels of pre-miRNAs. Taken together, a global decrease in miRNA levels, by either decreased Dicer expression or loss of p53, might be involved in tumor initiation and progression. Ultimately,



**FIGURE 2** MicroRNAs are derived from the genome to control target gene expression through their actions on mRNAs. Transcribed from the genomic DNA by RNA polymerase as primary miRNA (pri-miRNA) and subsequently processed by the Drosha/DGCR8 complex to a shorter form within the nucleus, the resultant precursor miRNA (pre-miRNA) is transported into the cytoplasm by Ran-GTP-dependent Exportin-5 (XPO5) and further processed by Dicer and TRBP to miRNA duplex. Unwinding of the duplex offers two strands, among which the passenger strand is readily degraded while the mature miRNA, guided by argonaut-2 (AGO2), acts on target transcript through complementary binding and leads to mRNA degradation or translational inhibition

miRNA	Expression	Direct Targets Verified	References
miR-124-3p	Decreased	STAT-3, MYO10, SMAD4	[173-176]
miR-126-3p	Decreased	PIK3R2, VEGF-A, Crk	[77,120,177-179]
miR-143-3p	Decreased	KRAS, NRAS, BCL2, HK2, PKC $\epsilon$ , Limk1, ATG2B	[112,180-186]
miR-34a-5p	Decreased	TGF $\beta$ R2, Cyclin E1, PEBP4, Notch-1, Axl	[187-192]
let-7c-5p	Decreased	N/K-RAS, ABCC2, Bcl-XL, ITGB3, MAP4K3, HOXA1	[122,193-196]
miR-101-3p	Decreased	ZEB1, ROCK2, MALAT-1, EZH2	[48,180,197-199]
miR-100-5p	Decreased	FGFR3, PLK1	[67,180,200,201]
miR-181a-5p	Decreased	BCL2, KRAS, VCAM-1, CDK1	[99,180,202-204]
miR-145-5p	Decreased	c-Myc, SMAD3, AEG/MTDH, OCT4, SOX2, Fascin1	[138,202,205-207]
miR-486-5p	Decreased	PIM-1, ARHGAP5, IGF1, IGFR, p85 $\alpha$ , CDK4	[62,63,208-210]
miR-451a-5p	Decreased	PSMB8, RAB14	[175,180,211]
miR-21-5p	Increased	PTEN	[180,212]
miR-210-3p	Increased	E2F3, NDUFA4, SDHD	[79,213]
miR-205-5p	Increased	PTEN, PHLPP2, ITG $\alpha$ 5	[131,214-216]
miR-31-5p	Increased	ABC9, hMLH1	[121,180,217]
miR-200b-5p	Increased	FOXF2, IL-8, CXCL1, FSCN1	[129,218-220]
miR-182-5p	Increased	PDCD4, RGS17	[213,221-223]
miR-183-5p	Increased	FOXO1, VIL2	[134,213,224]

**TABLE 3** List of miRNAs most commonly dysregulated in NSCLC and some of their corresponding targets validated by biological experiments

restoration of tumor suppressive miRNAs and inhibition of tumor promotive miRNAs represent new anti-cancer strategies.

## 5 | MICRORNAS ARE INVOLVED IN THE CONTROL OF MULTIPLE NSCLC CELLULAR PROCESSES

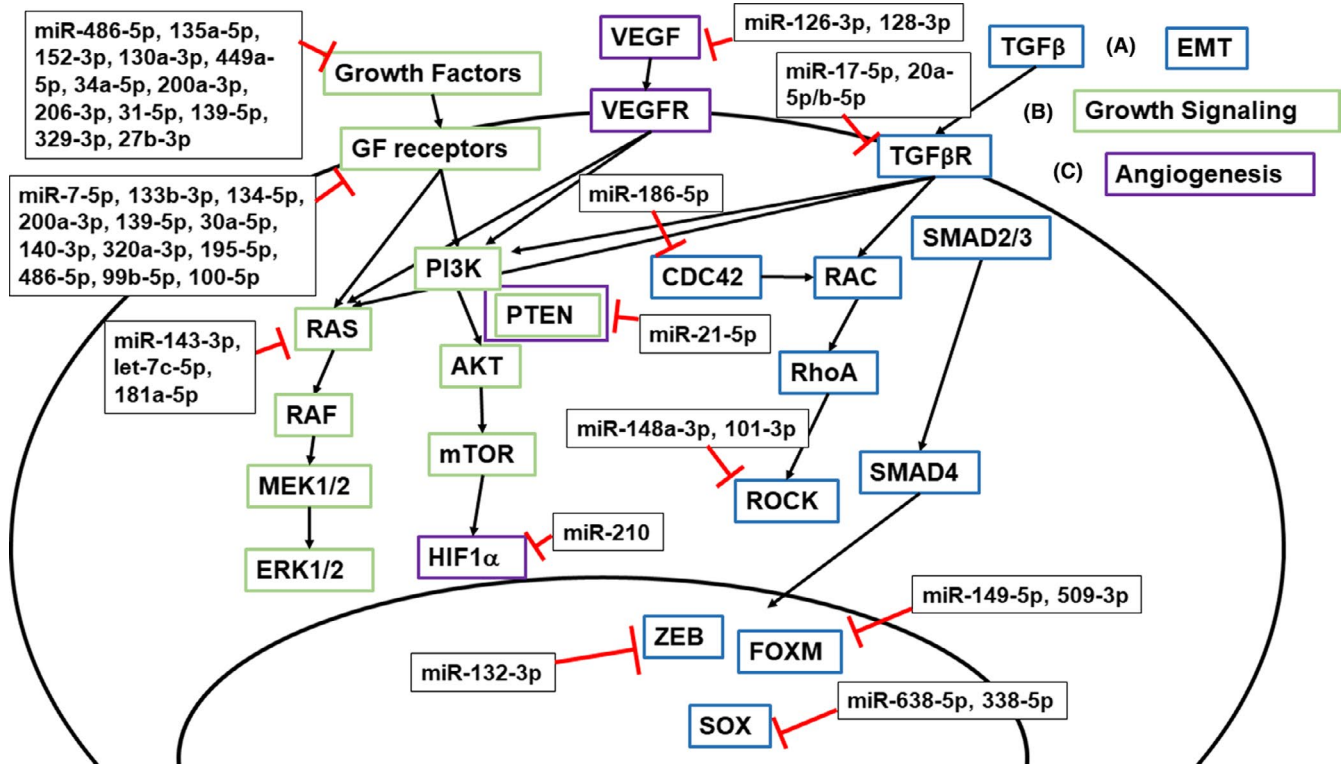
### 5.1 | Epithelial to Mesenchymal Transition

The epithelial to mesenchymal transition (EMT) is a process in which an epithelial-like cell loses its attachment to the basal membrane and assumes mesenchymal characteristics like greater motility and invasiveness. EMT allows for cancer cells to metastasize by migrating from the primary tumor through the blood stream and invading other organs. Comprehensive reviews of the process of EMT have recently been published.<sup>44</sup> Some miRNAs can affect cells' ability or likelihood to undergo EMT by regulating the expression of EMT-related genes. One important EMT signaling cascade involves TGF $\beta$ , is a signaling cytokine, that binds to its receptor, TGF $\beta$ R1/2 to transduce a signal through RAS, PI3K, RhoA/ROCK, or Smad2/3 and activate transcription factors, like ZEB1/2, Twist and Snail. This ultimately results in the loss of epithelial attachment proteins, such as E-cadherin, and gain of intermediate filament or cell-cell adhesion proteins, such

as vimentin and N-cadherin.<sup>45</sup> The EMT phenotype is reduced in NSCLC by the action of miR-17-5p, 20a-5p, and 20b-5p that directly target TGF $\beta$ R2,<sup>46</sup> miR-148a-3p and miR-101-3p that target ROCK1 and ROCK2 respectively,<sup>47,48</sup> and miR-132-3p, miR-638-5p, and miR-338-5p which target transcription factors ZEB2, SOX2, and SOX4, respectively<sup>49-51</sup> (Figure 3). Both miR-149-5p and miR-509-3p target the transcription factor FOXM1 to reduce invasion in H1299 cells as determined by Matrigel invasion assay.<sup>52</sup> In addition, miR-186-5p targets CDC42 leading to the inhibition of migration and related EMT processes.<sup>53</sup> Likewise, dysregulation of these miRNAs, as evident in NSCLC, leads to greater EMT and a more invasive, migratory, and potentially metastatic phenotype.

### 5.2 | Signal transduction in lung cancer survival and proliferation

Oncogenesis is driven by an over-expression or activation of growth signaling, such as growth factors, receptors, or downstream signaling molecules. Growth factor ligands bind to their corresponding receptors to relay a signal and induce proliferation. Cancer cells can hijack signaling by over-expressing or mutating growth factor receptors to increase proliferative signals and miRNAs target certain receptor to modulate signaling.



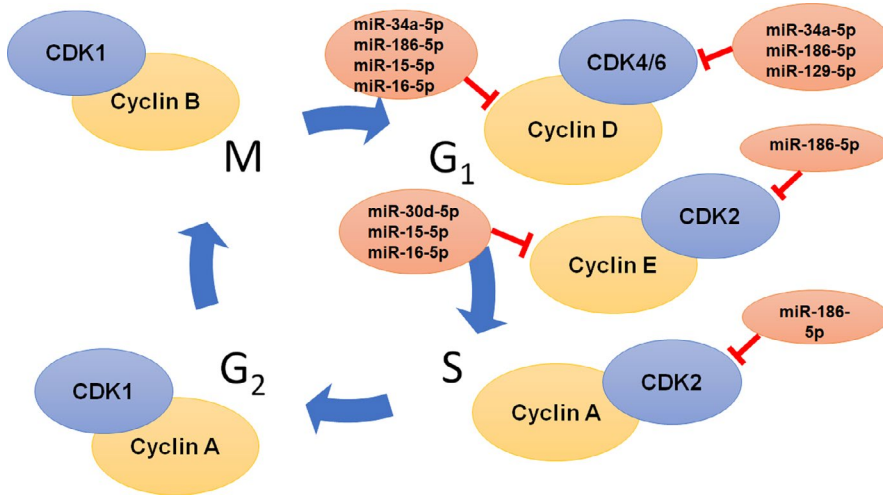
**FIGURE 3** MicroRNAs modulate many cancer cellular processes important in tumor initiation, progression, and metastasis. (A) The epithelial to mesenchymal transition (EMT) is driven in part by transforming Growth Factor β (TGFβ) binding to the TGFβ Receptor (TGFβR) and signaling to RAC and activating RhoA and ROCK. SMAD2/3 signaling activates transcription factors ZEB, FOXM, and SOX to turn on transcription of other genes necessary for EMT. Some miRNAs that target TGFβR, CDC42, ROCK, or the downstream transcription factors are dysregulated in NSCLC, which can increase EMT signaling and therefore enhance cancer cell invasion and metastasis. (B) Growth factors (GF), such as epidermal growth factor (EGF), bind to corresponding growth factor receptors, such as EGF receptor (EGFR), to activate RAS or PI3K. This leads to a series of signal transductions that eventually enhance cancer cell proliferation and growth. Inhibition of growth factors and their receptors by miRNAs may inhibit tumor progression. (C) One mechanism behind angiogenesis involves vascular endothelial growth factor (VEGF) binding to VEGF Receptor (VEGFR) and activating hypoxia-inducible factor-1α (HIF1α). Those miRNAs that target VEGF or HIF1α may reduce angiogenesis essential for tumor progression

miR-7-5p,<sup>54</sup> miR-133b-3p,<sup>55</sup> miR-134-5p,<sup>56</sup> and miR-200a-3p<sup>57</sup> target epidermal growth factor (EGFR) (Figure 3), which is commonly overexpressed in NSCLC (Table 1), to alter downstream signaling molecules such as AKT and ERK1/2 and decrease the growth phenotype. In addition, miR-139-5p,<sup>58</sup> miR-30a-5p,<sup>59</sup> miR-140-3p,<sup>60</sup> miR-320a-3p<sup>61</sup> and miR-195-5p<sup>62</sup> all target insulin-like growth factor 1 receptor (IGF1R), while miR-486-5p directly targets both IGF1R and its ligand insulin-like growth factor 1 (IGF1)<sup>63</sup> and miR-135a-5p targets only IGF1.<sup>64</sup> miRNA inhibition of the IGF pathway results in a lower proliferation as assayed by CCK-8 kit among others.<sup>60</sup> miR-152-3p targets fibroblast growth factor 2 (FGF2)<sup>65</sup> and miR-99b-5p<sup>66</sup> and miR-100-5p<sup>67</sup> target fibroblast growth factor receptor 3 (FGFR3). MET is a receptor for the hepatocyte growth factor (HGF) and is targeted by a number of miRNAs including miR-130a-3p,<sup>68</sup> miR-449a-5p<sup>69</sup> miR-34a-5p,<sup>70</sup> miR-200a-3p,<sup>57</sup> miR-206-3p,<sup>71</sup> miR-31-5p,<sup>72</sup> miR-139-5p,<sup>73</sup> miR-329-3p,<sup>74</sup> miR-27b-3p<sup>75</sup> to decrease growth and proliferation. EGF, IGF, FGF, and HGF signaling results in activation of RAS or PI3K pathways and downstream growth signaling. A decrease in the miRNAs that target growth factors and their receptors, as well

as downstream targets, as evident in NSCLC, results in an increase in growth signal transduction and an increase in cancer cell proliferation and growth.

### 5.3 | Angiogenesis

Angiogenesis is the process of building new blood vessels for nutrients and gas exchange which is essential for cancer cells to survive and proliferate. Dysregulation of miRNAs in cancer cells can lead to increased angiogenesis through multiple pathways, including vascular endothelial growth factor (VEGF) or placenta growth factor (PIGF) binding to VEGF receptor (VEGFR) or neuropilin (NRP).<sup>76</sup> During normal conditions, tumor suppressor von Hippel Lindau (vHL) mediates the degradation of hypoxia-inducible-factor-1 (HIF1) through the ubiquitin-proteasome pathway. By contrast, HIF-1α associates with HIF1β during hypoxia and thus increases VEGF transcription by binding to the promoter.<sup>76</sup> miRNAs regulate many important factors of angiogenesis (Figure 3). For example, miR-126-3p and miR-128-3p, both of which are commonly decreased in NSCLC, directly target VEGF-A and VEGF-C, respectively, to decrease



**FIGURE 4** MicroRNAs directly target core cell cycle regulators. Some miRNAs, such as miR-34a-5p, miR-186, and miR-15, which regulate the expression of cell cycle regulators, are downregulated in NSCLC. This causes dysregulation of the cell cycle and ultimately increases cancer cell proliferation. Restoration of such tumor suppressive miRNAs may lead to cell cycle arrest to achieve anticancer effects

angiogenesis and blood vessel formation, as measured by tube formation assay.<sup>77,78</sup> miR-210-3p is overexpressed in late-stage NSCLC and protects against hypoxia induced apoptosis by indirectly stabilizing HIF1 $\alpha$  to promote angiogenesis and increase glycolysis.<sup>79</sup> miR-21-5p directly targets PTEN and activates AKT and ERK1/2 which leads to higher levels of HIF1 $\alpha$  and VEGF expression.<sup>80</sup> miR-378-5p is over-expressed in NSCLC tumors in patients with brain metastasis and leads to increased VEGF expression and angiogenesis.<sup>81</sup> miR-206-3p directly suppresses the expression of protein 14-3-3 $\zeta$  which consequently decreases VEGF, HIF1 $\alpha$ , and phosphorylated STAT3 and results in a lower degree of angiogenesis as assayed by HUVECs recruitment as well as inhibition of intratumoral capillary tube formation in vivo.<sup>82</sup> In one study, coculture of NSCLC cell lines with vascular endothelial cells leads to higher levels of miR-494-3p in the vascular endothelial cells, in addition, a miR-494 antagomir decreases tumor vascularization, suggesting that miRNAs may be transferred to vascular endothelial cells to control angiogenesis.<sup>83</sup> Such miRNAs are important in the control and development of vascularization which is critical for tumorigenesis and metastasis.

## 5.4 | Cell Cycle

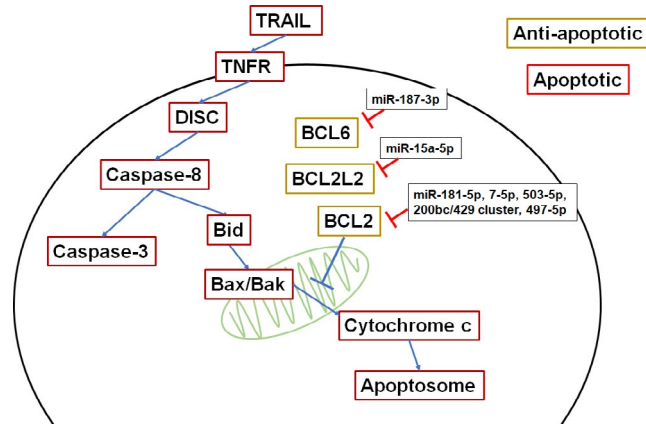
The cell cycle is altered among almost all cancer cells to allow for uncontrolled growth. Cyclins and cyclin-dependent kinases (CDKs) are partly responsible for entry into the different cell cycle stages. G<sub>1</sub> begins with cyclins D1, D2, and D3 associating with CDK4 and 6<sup>84</sup> to phosphorylate Rb and repress the E2F transcription factor.<sup>85</sup> G<sub>1</sub>/S transition is characterized by cyclin E complexing with CDK2<sup>86</sup> and cyclin A/CDK2 complex during S phase.<sup>87</sup> Cyclin A complexes with CDK1 to transition to M phase, then cyclin B and CDK1 are complexed during M phase.<sup>88</sup> Such proteins regulating G<sub>1</sub> and S phases of many types of cancers, including NSCLC, are dysregulated, and some are direct targets of particular miRNAs (Figure 4 and Table 3). Tumor suppressive miR-34a-5p directly targets CCND1 and CDK6, leading to the arrest of the cell cycle in G<sub>1</sub> phase.<sup>89</sup> Furthermore, miR-15a-5p and miR-16-5p, down-regulated in NSCLC, directly targets CCND1, CCND2, and CCNE1,

and arrests the cell cycle in G<sub>1</sub> to G<sub>0</sub> in an Rb-dependent manner.<sup>90</sup> Combination of miR-34a-5p and miR-15a/16 produces synergism in G<sub>1</sub> cell cycle arrest in an Rb-dependent manner due to an increase in miRNAs targeting more cell cycle related mRNAs.<sup>91</sup> In addition, miR-30d-5p targets CCNE2,<sup>92</sup> miR-186-5p targets CCND1, CDK2, and CDK6,<sup>93</sup> and miR-129-5p targets CDK6<sup>94</sup> to arrest the cells in G<sub>1</sub>. Generally, cell cycle-regulating miRNAs exhibit tumor suppressive actions by targeting cell cycle promotive genes to induce a cancer cell cycle arrest.

## 5.5 | Evading Apoptosis

Cell death mechanisms, including apoptosis or necrosis, are important for cells to maintain homeostasis, and dysregulation of these pathways leads to an alteration of cell proliferation, including NSCLC cells. Some miRNAs regulate certain proteins involved in cell death (Figure 5), and dysregulation of such miRNAs may make the cells evade death signals and continue to proliferate. In brief, apoptosis occurs when a death ligand, such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), binds to a death receptor, including TNF receptors 1 and 2 (TNFR1/2), causing receptor multimerization and activation of the death induced signaling complex (DISC).<sup>95</sup> This can result in direct activation of caspase-8 mediated cleavage of effector caspases, like caspase-3,<sup>96</sup> or caspase-8 cleavage of Bid which releases mitochondrial cytochrome c to associate with Bax and Bak and forms the apoptosome and cleaves effector caspases.<sup>97</sup> BCL2, an anti-apoptotic protein that mainly functions to inhibit release of cytochrome c from the mitochondria,<sup>98</sup> is targeted by miR-181-5p,<sup>99</sup> miR-7-5p,<sup>100</sup> miR-503-5p,<sup>101</sup> miR-200bc/429 cluster,<sup>102</sup> and miR-497-5p<sup>103</sup> (Figure 5). In addition, BCL2L2 and BCL6, also anti-apoptotic proteins, are directly targeted by miR-15a-5p and miR-187-3p, respectively, to enhance apoptosis.<sup>104,105</sup> TRAIL expression induces apoptosis; however, NSCLC can confer resistance to TRAIL-mediated apoptosis through many mechanisms including loss of PTEN and constitutive activation of AKT<sup>106</sup> or increased matrix metalloproteases.<sup>107</sup> Therefore, over-expression





**FIGURE 5** miRNAs affect the ability to evade of apoptosis. BCL6, BCL2L2, and BCL2 are anti-apoptotic as they inhibit cytochrome c release from the mitochondria. Dysregulation or malfunction of miRNAs in NSCLC that inhibit the anti-apoptotic cascade may reduce apoptotic capacity and enhance cancer progression. Therefore, restoration of such miRNA expression or function represents a novel therapeutic strategy

of miR-148a-3p can sensitize NSCLC to TRAIL by targeting MMP15.<sup>108</sup> miR-221-3p and miR-222-3p can confer TRAIL resistance by targeting tumor suppressors PTEN and tissue inhibitor of metalloproteases 3 (TIMP3),<sup>109</sup> while miR-130a-3p can reverse this effect by targeting miR-221-3p and miR-222-3p.<sup>68</sup> miR-21-5p also targets PTEN and results in the inhibition of apoptosis, which can be reversed by the transfection of anti-miR-21-5p.<sup>110</sup>

### 5.6 | Metabolism

Some miRNAs can alter the metabolic potential of cancer cells. A higher metabolic rate may enhance the tumorigenesis and growth of NSCLC cells. miR-155-5p promotes aerobic glycolysis by indirectly upregulating HK2, as determined by a hexokinase colorimetric assay as well as glucose and L-lactate test kits.<sup>111</sup> The increase in glycolysis leads to greater degree of cell viability. miR-143-3p directly targets HK2, the first rate-limiting enzyme in glycolysis, to decrease glycolysis and proliferation as well as tumorigenesis in vivo.<sup>112</sup> miR-124-3p overexpression decreases glucose consumption, lactate production, and ATP content by decreasing HK2 and glucose transporter 1 (GLUT1), leading to a lower extent of cell proliferation.<sup>113</sup> miR-449-5p directly targets lactate dehydrogenase A (LDHA) and suppresses glycolysis.<sup>114</sup> miR-182-5p and miR-31-5p target HIF1AN and FIH, respectively, both of which are HIF-1 $\alpha$  inhibitors and lead to enhanced glycolysis.<sup>115,116</sup> miR-210-3p directly targets two genes important in the electron transport chain, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4 (NDUFA4), and succinate dehydrogenase complex subunit D (SDHD), which leads to alteration of the physical structure of the mitochondria, visualized by electron microscopy as well as alterations of the mitochondrial membrane potential that are phenotypic of mitochondria dysfunction.<sup>79</sup> miR-145-5p and miR-138-5p directly target phosphoinositide-dependent protein kinase-1 (PDK1), an important enzyme in glucose and fatty acid metabolism.<sup>115,117</sup> Dysregulation or loss of those

miRNAs that inhibit the metabolic potential of NSCLC cells may alter tumor progression.

## 6 | MICRORNAS AFFECT THE SENSITIVITY OF NSCLC CELLS TO CURRENT THERAPIES

Dysregulation of miRNAs can confer the resistance to current therapies including chemotherapy and radiation therapy. For instance, upregulation of miR-21-5p leads to a reduction of apoptosis and decrease of sensitivity to two chemotherapeutics, docetaxel and cisplatin.<sup>110</sup> Induced by radiation, miR-155-5p does confer resistance to radiation therapy by indirectly increasing HK2 to promote aerobic glycolysis.<sup>111</sup> Furthermore, chronic treatment with EGFR inhibitor gefitinib reduces the expression of miR-155-5p and miR-200c-3p and may decrease the sensitivity to gefitinib.<sup>118</sup> Therefore, miRNA profiles from tissue or body fluids may also be used as predictive biomarkers for the sensitivity of NSCLC tumors to certain therapies and to determine optimal therapies for the treatment of NSCLC. As an example, miR-143-3p is downregulated in NSCLC and suppresses NSCLC cell proliferation, migration, and invasion by regulating EGFR expression.<sup>119</sup> NSCLC patients showing lower miR-143-3p levels and higher EGFR levels may benefit from anti-EGFR therapy like gefitinib. PI3K or VEGF inhibitors may be beneficial for patients with decreased expression of miR-126-3p because dysregulation of miR-126-3p may lead to an increase in PI3K and VEGF-A.<sup>77,120</sup> In addition, miRNAs can play an important role in drug uptake and efflux via direct targeting of drug transporters. For example, miR-31-5p is upregulated in cisplatin resistant cell lines and directly regulates the expression of ABCB9, a drug transporter, to confers cisplatin resistance.<sup>121</sup> Let-7c-5p modulates the expression of ABCC2 transporter to sensitize cisplatin resistant cells.<sup>122</sup> ABCC4 involved in the transport of many anti-cancer drugs such as methotrexate and topotecan and is directly targeted by miR-124-3p and miR-506-3p.<sup>123</sup> Excellent reviews on the potential of miRNAs as biomarkers in lung cancer have been recently published.<sup>124</sup> Understanding miRNA-controlled regulation may not only improve the understanding of multidrug resistance mechanisms but also offer clues to the development of new therapeutic strategies.<sup>125,126</sup>

## 7 | MICRORNAS IMPACT THE TUMORIGENESIS OF NSCLC CELLS

To determine the effect of a miRNA on tumorigenesis, investigators transiently or stably express the target miRNA in a cell line for implantation in vivo. While this does not necessarily model therapeutic potential of miRNAs, it does provide important information beyond cell-based findings regarding the importance of miRNAs in the control of tumor initiation and development or tumorigenesis (Table 4). For example, subcutaneously or tail vein injected A549 cells expressing miR-124-3p, miR-126-3p, miR-143-3p, miR-34a-5p, Let-7b-5p, or miR-182-5p showed reduced tumor growth and in some cases reduce lung metastasis

**TABLE 4** Some miRNAs shown to affect tumorigenesis of NSCLC cells in animal models

miRNA	Cell line	Mouse strain	Finding	Reference
miR-124-3p	A549	nude BALB/c	Reduced lung metastasis from tail vein injected cells	[176]
miR-126-3p	A549	nude BALB/c	Reduced tumor weight	[77]
miR-143-3p	A549	nude BALB/c	Reduced tumor weight	[225]
miR-34a-5p	A549	nude BALB/c	Reduced tumor weight, and lung tumor metastasis	[226]
let-7b-5p	A549, H460	nod/scid	Reduce tumor growth	[227]
miR-101-3p	LLC	C57BL/6	Reduced tumor weight, metastasis from IP injected cells	[127]
miR-100-5p	SPC-A1/DTX	nude	Reduced tumor volume in response to docetaxel	[201]
miR-145-5p	A549 CIC	nude	Reduced tumor volume	[128]
miR-486-5p	H460-luc2	athymic Swiss	Reduced lung metastasis from tail vein injected cells	[62]
miR-451-5p	A549	nude BALB/c	Reduced tumor volume in response to cisplatin	[228]
miR-21-5p		CAG-miR-21;K-ras <sup>LA2</sup>	Reduced tumor burden and increased survival	[130]
miR-205-5p	H460	BALB/c	Reduced tumor volume	[131]
miR-31-5p	H1993/ H1437/H460	nude	Reduced tumor volume	[132]
miR-200a/b	344SQ	nude	Reduced tumor volume	[129]
miR-182-5p	A549	nude	Reduced tumor volume and weight and increased survival	[229]

as compared to control cells. Lewis lung carcinoma cells transiently transfected with miR-101-3p displayed a smaller increase of tumor volume over time when subcutaneously injected into the flank of mice, as well as a reduction of metastasis to the lung when intraperitoneally injected.<sup>127</sup> Cells overexpressing miR-145-5p or miR-486-5p subcutaneously implanted or tail vein injected in mice displayed a slower rate of tumor growth.<sup>62,128</sup> Lung cancer cells 344SQ transfected with miR-200a-3p or miR-200b-5p or both showed smaller tumor volume with transfection of both miRNAs having the greatest impact.<sup>129</sup> By contrast, transgenic KRAS mutant mice with conditionally global overexpression of miR-21-5p showed much greater tumor burden as well as lower survival rate, as compared to KRAS mutant mice without miR-21-5p overexpression.<sup>130</sup> After subcutaneous implantation, H460 cells overexpressing miR-205-5p grew faster and led to greater tumor volume and vascularization, as compared to control cells.<sup>131</sup> Compared to corresponding controls, overexpression of miR-31-5p in three different lung cancer cell lines, H1993, H1437, and H460, led to an increase in subcutaneous tumor volume.<sup>132</sup> The same study also demonstrated that transgenic mice with a doxycycline-inducible miR-31-5p expression in the lung exhibited greater levels of hyperplasia and adenomas.<sup>132</sup> In conclusion, ectopic or overexpression of certain functional miRNAs can largely influence the tumorigenesis of NSCLC cells, which may provide insights into development of new miRNA-based therapies.

## 8 | THERAPEUTIC POTENTIAL OF MICRORNAS DEMONSTRATED IN NSCLC ANIMAL MODELS IN VIVO

Two miRNA-based therapeutic strategies have been established, aiming to restore tumor suppressive miRNAs and inhibit tumor promotive miRNAs, respectively. Many studies were thus conducted to define the effectiveness of specific miRNA therapeutics for the treatment of NSCLC in animal models in vivo (Table 5). AntagomiRs were employed for the inhibition of tumor promotive miR-21-5p, miR-183-5p, and miR-206-3p and shown to inhibit subcutaneous A549 tumors.<sup>82,133,134</sup> Whereas, tumor suppressive miRNAs let-7b-5p or miR-34a-5p reduced KRAS-activated tumor burden in vivo.<sup>135</sup> Let-7c-5p and let-7a-5p were both shown to decrease the progression of NSCLC in vivo.<sup>136</sup> Synthetic miR-34a-5p injected either intratumorally or through the tail vein was effective in inhibiting the growth of subcutaneous xenograft NSCLC,<sup>137</sup> and it did not show major impact on the cytokine profiles or liver or kidney functions. A specific type of chemical modification of miR-145-5p, namely locked nucleic acid, delivered with a polyurethane-short branched-polyethylenimine, led to significant inhibition of tumor growth and the effects were enhanced by radiation and cisplatin therapy.<sup>138</sup> Orthotopic NSCLC tumor growth, metastasis, and vascularization were decreased in mice treated with miR-200a/b.<sup>129</sup> miR-29b-3p decreased cell proliferation and increased apoptosis in subcutaneous NSCLC tumors.<sup>139</sup> Combination treatment with miR-34a-5p and

**TABLE 5** Some miRNA-based therapies for the treatment of NSCLC assessed in animal models in vivo

miR	Mouse model	Delivery	Findings	Reference
let-7b-5p and miR-34a-5p (synthetic)	Cre-Kras mutant	Neutral lipid emulsion (NLE)	Lower tumor burden, increased apoptosis, decreased proliferation	[135]
let-7b-5p (synthetic)	Subcutaneous H460 cell line	siPORTamine (lipid based)	Decreased proliferation	[136]
let-7a-5p (lenti-let-7)	Cre-Kras mutant	Lentiviral	Decreased proliferation	[136]
miR-34a-5p (synthetic)	Subcutaneous H460	MaxSuppressor in vivo RNALancerII (lipid based)	Decreased proliferation, increased apoptosis, minimal change in blood chemistry or cytokine profile	[137]
miR-145-5p (synthetic; LNA)	Subcutaneous, intra-bronchial or intravenous patient derived primary lung adenocarcinoma CD133+	Cationic polyurethane-short branched polyethylenimine (PU-PEI)	miR-145-5p alone showed moderate tumor inhibition, increased tumor inhibition and survival in combination with radiation and cisplatin	[138]
miR-200a/b (synthetic)	intrapulmonary 344SQ (murine) cell line	1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) nanoliposomes	Reduced proliferation, metastasis, and tumor vasculature permeabilization	[129]
miR-29b-3p (synthetic)	Subcutaneous A549 cell line	Lipoplex	Suppressed target expression, reduced proliferation, increased apoptosis	[139]
miR-34a-5p & miR-124-3p (biologic RNA)	Intravenous A549 cell line, (metastatic)	In vivo-jetPEI	Decreased lung lesions, minimal change on blood chemistry or cytokine release	[144]
miR-34a-5p (biologic RNA)	Subcutaneous A459 cell line	In vivo-jetPEI	Decreased tumor size, minimal change on blood chemistry or cytokine release	[145]
miR-34a-5p (synthetic)	Intramuscular H460 or H1299 cell line	NOV340 (Liposomal nanoparticle)	Sensitized tumor to irradiation	[146]
miR-34a-5p and let-7b-5p (synthetic)	Kras/p53 mutant, Cre-adenoviral activated	NOV340 (liposomal nanoparticle)	Combination of miR-34a-5p and let-7c-5p reduced tumor burden, decreased proliferation, and increased survival with minimal cytokine induction	[140]
anti-miR-21-5p (synthetic)	Subcutaneous A549 cell line	QTsome (cationic lipids)	Stable tumor growth or tumor regression after treatment, increased survival	[133]
anti-miR-183-5p (synthetic)	Subcutaneous A549-LUC-GFP	Adenovirus (intra-tumoral injection)	Decreased tumor growth as measured by luminescence	[134]
miR-206-3p-agomir (synthetic)	Subcutaneous A549 cell line	No vehicle mentioned (intra-tumoral injection)	Decreased tumor volume and formation of intra-tumoral capillary tubes, and increased apoptosis	[82]

let-7c-5p was effective in improving overall survival and reducing tumor burden in KRAS mutant mice.<sup>140</sup> Nevertheless, the majority of miRNAs used for in vivo therapies are made by chemical synthesis or in vitro transcription, or achieved through viral vectors or plasmids, where RNAs or mimics are often delivered with lipids or polymers. Very recently, a novel RNA bioengineering technology has been established for the production of new miRNA reagents for the assessment of miRNA therapies.<sup>141-145</sup> Biologic or recombinant miR-34a-5p or miR-124-3p molecules produced in bacteria and delivered with in vivo-jetPEI into tumor-bearing mouse models decreased the growth of both subcutaneous and metastatic NSCLC tumors, with minimal influence on blood chemistry or cytokine profiles, in two different studies.<sup>144,145</sup> A variety of different delivery methods have been tested in vivo to deliver the above-mentioned miRNAs. Many miRNAs were formulated with lipid-based technologies, such as

liposomes, lipoplex, siPORTamine, and MaxSuppressor, which surround the RNA and protect it from degradation.<sup>136,137,139,140,146</sup> Viral vectors, such as lentivirus or adenovirus, as well as positively charged polyethylenimine, which associates with the negatively charged RNA, were also used.<sup>134,136,138,144,145</sup> In most cases, miRNA therapeutics were administered through the tail vein or intra-tumoral injection. These in vivo findings demonstrate the promise of miRNA-based therapies for the treatment of NSCLC.

One tumor suppressive miRNA, namely MRX34 or liposomal miR-34a mimic, was also moved into first-in-human Phase I clinical trials.<sup>147</sup> It was evaluated as dose-escalating intravenous infusions under a regimen of twice a week in three-week cycles. This study consisted of 47 patients with advanced solid tumors, including one patient with NSCLC who had stable disease for 8 cycles of treatment. While efficacy of MRX34 was obvious among some patients, 96% of all patients

experienced immune-related adverse effects where multiple deaths also occurred with complex and uncertain causes. The most common adverse effects were fever, fatigue, nausea, diarrhea, and vomiting, and laboratory abnormalities included lymphopenia, neutropenia, and increased AST among others. The study does not distinguish whether the toxicity resulted from the RNA or the liposomal carrier, however, both components have shown immune toxicities in previous studies.<sup>148,149</sup> The immune-related toxicity suggests that more studies are warranted to understand the effect of miRNA therapies and the carriers on the immune system. The termination of this trial reiterates the importance of safety study in addition to efficacy during drug development.

## 9 | CONCLUSIONS AND PERSPECTIVES

Functional miRNAs derived from the human genome are critical factors in posttranscriptional regulation of target gene expression underlying many cellular processes, including metabolism, proliferation, apoptosis, and disease initiation and progression. Uncontrolled NSCLC cell growth and tumor development is associated with dysregulated miRNA expression in addition to the alterations of proteins and signaling pathways, among which tumor suppressive miRNAs are generally downregulated and tumor promotive miRNAs are commonly upregulated. With the improved understanding of miRNA biology in NSCLC, new miRNA-based therapies are under active investigations, in particular, the restoration of tumor suppressive miRNAs and inhibition of tumor promotive miRNAs. Nevertheless, many challenges remain for the development of new therapeutics. Although a number of RNA drugs have been approved for clinical practice,<sup>142</sup> the pharmacokinetic and pharmacodynamic properties of RNA molecules are still of concern since RNA molecules are generally susceptible to serum RNases and cannot pass freely through cell membrane barriers. Chemical modifications and formulation with biocompatible lipids or polymers have proven useful for improving the metabolic stability and delivery of RNA therapeutics. As chemical modifications undoubtedly lead to different RNA folding, stability, biologic activity, and safety profiles, there are growing interests in producing biologic or recombinant RNA molecules in living cells for RNA research and drug development,<sup>141,142</sup> similar as the success of protein-based therapeutic modalities. Improved formulations with lipid or polymer-based drug delivery systems may aid in protecting the RNA from degradation or recognition by the immune system. In any case, evidence is required to address two fundamental questions: whether the drug is effective against the disease and whether the drug is safe for the patients, which warrants more extensive studies.

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## DISCLOSURES

The authors declare that there is no conflict of interest.

## AUTHOR CONTRIBUTIONS

Research design/conducting experiments: n/a. Literature research & analysis: Petrek & Yu. Writing and revising the manuscript: Petrek & Yu.

## ORCID

Ai-Ming Yu  <https://orcid.org/0000-0003-1441-4012>

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