however, little has been known as to whether such alterations in metabolic systems at the side of cancer cells might influence on the host metabolic systems *in vivo*. Unraveling compensatory metabolic responses of cancer cells might benefit improvement of CD44-targeted chemotherapy. Of note is that shCD44 significantly increases the T/Pc ratio of glutamate (Fig. 4), suggesting that downregulation of CD44/xCT inhibits excretion of glutamate from tumors toward the host liver. Since mice are exposed to fasted conditions, such circumstances are likely to decrease availability of glutamate in the liver and may in turn suppress energy metabolism and/or glutathione synthesis that requires ATP. On the other hand, shCD44 treatment is known to accelerate glucose oxidation in mitochondria to generate reactive oxygen species and suppress GSH/GSSG in tumors. Furthermore, such impacts of oxidative stress might influence on extra-tumor regions that could secondarily decrease GSH/GSSG in the parenchyma. It is not unreasonable to suggest that exchange of cystine and glutamate between cancer cells and the host liver via xCT/CD44 system constitute bi-directionally coupled symbiotic systems that depend on each other for maintaining antioxidative capacity *in vivo*.

4.2. CD44 knockdown decreases methionine-derived metabolites in cancer cells

As seen in quantitative data in Fig. 3, CD44 knockdown in cancer cells did not suppress total amounts of glutathione (GSH+2GSSG) in culture and *in vivo*. These results suggest that GSH suppression in response to CD44 knockdown does not result from substrate shortage but from down-regulation of reducing capacity of the cancer cells. This notion was also supported by marked suppression of GSH/GSSG in tumors *in vivo*. In other words, even under suppression of extracellular supply of cysteine, the cancer cells were able to maintain total amounts of glutathione. Although the whole mechanisms remain unknown, the results shown in Fig. 5 suggest that CD44 knockdown triggers consumption of methionine and related metabolites (*e.g.* Σ(MS + RM + TS)) [19] to compensate glutathione synthesis in cancer cells. In order to examine which metabolites of methionine-metabolizing pathways play critical roles for cancer survival and development, we conducted experiments investigating sensitivity of cancer cells to methionine cessation and inhibition of transsulfuration pathway in culture. As seen in Fig. 7, the cancer cells treated with shCD44 but not those with control shRNA were sensitive to PPG, a potent inhibitor of CSE, suggesting that downstream metabolites such as cysteine and/or glutathione is necessary to maintain viability of CD44-KD cells. However, suppression of the cell viability by PPG was less than 10%. Of note is that the effects of methionine cessation is far greater than those of PPG for both the control and CD44-KD cells, suggesting greater roles of metabolites belonging to upstream pathways of methionine metabolism. The data shown in Fig. 2 indicating down-regulation of spermine and spermidine in response to CD44-KD support this concept. Considering the previous studies showing critical roles of these metabolites for cancer proliferation and development, marked decreases of the polyamines might play a critical role for inhibiting cancer development in CD44-KD cancer cells *in vitro* and *in vivo*. Other metabolites responding to CD44-KD in tumors *in vivo* involve taurine and aspartate (Fig. 4), while it is unchanged in culture. While mechanisms for these events *in vivo* require further investigation, the results suggest the presence of metabolic interplay between tumors and the host liver.

Another important finding in the current study is suppression of reactive cysteine persulfides and related persulfide metabolites (Hcy-SH and GSSH). Amounts of GSSH were not altered by CD44 knockdown in culture. This observation is in good agreement with the data collected by Q-IMS showing no alterations in $GSSO_3^-$ in tumors (Fig. 3). These reactive persulfides are nucleophiles that rapidly react with electrophiles such as many anti-cancer reagents, heme, lipid peroxides and aldehydes. Increased chemosensitivity of CD44-knocked down cancer cells [4,18,19] might result from decreases in these cystine-derived reactive persulfide species.

5. Conclusion

Impacts of CD44 knockdown on metabolic systems in cancer and host tissues were examined by Q-IMS combined with metabolomics approaches, suggesting that CD44 expressed in cancer accounts for a key regulator of metabolic interplay between tumor and the host tissue. Among key metabolites indicating alterations in response to CD44 knockdown, spermine, spermidine and reactive cystine persulfides appear to play an important role for cancer development. Further investigation is necessary to provide evidence that these CD44-sensitive metabolites might contribute to cancer proliferation and/or chemoresistance.

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Chapter 17
Challenges and Strategies for Pulmonary Delivery of MicroRNA-Based Therapeutics

Yu Fujita, Kazuyoshi Kuwano, and Takahiro Ochiya

Contents

Abstract Therapeutic options for various lung diseases, especially lung cancer, continue to expand with the development of novel therapeutic strategies. RNA interference (RNAi)-based approaches provide a promising modality for the treatment of lung diseases. One of the greatest challenges in RNAi-based therapy continues to be the method for delivering the therapeutic small interfering RNAs (siRNAs) and

Y. Fujita
Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

Division of Respiratory Diseases, Department of Internal Medicine, Jikei University School of Medicine, 3-19-18, Nishi-shinbashi, Minato-ku, Tokyo 105-8471, Japan

K. Kuwano
Division of Respiratory Diseases, Department of Internal Medicine, Jikei University School of Medicine, 3-19-18, Nishi-shinbashi, Minato-ku, Tokyo 105-8471, Japan

T. Ochiya (✉)
Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
e-mail: tochiya@ncc.go.jp

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microRNAs (miRNAs) to the target cells. The advance of pulmonary delivery systems into the clinic illustrates the notion that RNAi will be a valuable modality for the treatment of lung diseases. Currently, the development of miRNA-based therapies for lung cancer is rapidly advancing with the aid of new RNAi technologies. Given the important role of miRNAs in lung carcinogenesis, increasing effort is being dedicated to the research and development of miRNA-based therapies, including the restoration of tumor suppressive miRNA function and the inhibition of oncogenic miRNAs. In this chapter, we discuss the advantages of a pulmonary drug delivery system and the strategies for miRNA-based treatment of lung cancer.

Keywords RNA interference • Small interfering RNA • MicroRNA • Lung cancer Pulmonary delivery

1 Introduction

Lung cancer is the leading cause of cancer mortality worldwide. Lung cancer can be classified into two main subtypes: non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). Numerous differences are found between these two subtypes, including histological type, biological behavior, prevalence, prognosis and response to therapy. NSCLC accounts for more than 80 % of all lung cancer cases. Only a small percentage of patients with NSCLC present with early stage disease. In this circumstance, surgery remains the best therapeutic option for these patients. Approximately 70 % of all newly diagnosed patients present with locally advanced or metastatic disease and require systemic chemotherapy (Ramalingam et al. 2011). However, the commonly administered chemotherapeutics provide little benefit for patients with advanced stage disease and has reached a plateau in efficacy with a median survival of 8–10 months. The poor prognosis is due to late stage disease presentation, tumor heterogeneity within histological subtypes, and our relatively limited understanding of tumor biology. Furthermore, the high frequency of drug resistance is a key contributor to the poor survival rates of lung cancer patients; improvements in survival rely on continued elucidation of the molecular mechanisms underlying lung cancer tumorigenesis and drug response. Acquiring knowledge through genomic medicine raises the possibility of unraveling the remaining mysteries of lung cancer oncogenesis and opens the door to molecular classification and risk stratification based on gene expression profiles and microRNA (miRNA) signatures.

MiRNAs are short (19–23 nucleotides in length) non-coding RNAs found in multiple organisms that regulate gene expression primarily by decreasing the levels of their target mRNAs, through binding to specific target sites in the 3′ untranslated regions (3′UTRs) of these mRNAs (Winter et al. 2009). In the human genome, transcripts of approximately 60 % of all mRNAs are estimated to be targeted by miRNAs. Accumulating evidence shows that miRNAs are grossly dysregulated in human cancers, including NSCLC, and may serve as oncogenes

or tumor suppressors (Croce 2009; Babashah and Soleimani 2011). Recent studies have not only shown that miRNAs are useful in lung cancer diagnosis but that specific miRNA profiles may also predict prognosis, drug response and disease recurrence (Yanaihara et al. 2006; Yu et al. 2008). These findings suggest that miRNAs are a promising technology for therapeutic development. In fact, given the significant role of miRNAs in multiple pathways governing lung carcinogenesis, increasing efforts are dedicated to the research and development of miRNA-based therapies, including the restoration of tumor suppressive miRNA function and the inhibition of oncogenic miRNAs (Bader et al. 2010).

The critical problems impeding the development of RNAi-based therapeutics are effective delivery to target sites, therapeutic potency, and elimination of off-target effects (Boudreau et al. 2009). The success of miRNA-based therapeutic delivery is also dependent upon uncovering a delivery route that yields efficient outcomes, is convenient, and promotes patient compliance. For this reason, direct administration of miRNA-based therapeutics to target organs is a promising approach to overcome the problems of systemic administration. Pulmonary delivery offers a new method for the treatment of various lung diseases (Fujita et al. 2013). We believe that delivery of miRNA-based therapeutics using this approach will potentially be useful in clinical practice. Here, we provide an overview of miRNAs as therapeutic targets in lung cancer and discuss the promise and limitations of pulmonary delivery strategies for miRNA-based therapeutics.

2 Role of MicroRNAs in Lung Cancer

Lung cancer biology has traditionally focused on genomic and epigenomic deregulation of protein-coding genes to identify oncogenes and tumor suppressors that are useful as diagnostic and therapeutic targets. Recently, miRNAs were also shown to up-regulate target gene expression by either directly binding to the target mRNA or indirectly repressing nonsense-mediated RNA decay (Vasudevan et al. 2007; Bruno et al. 2011). MiRNAs play an essential role in various cellular processes, such as development, proliferation and apoptosis, to ensure the cellular homeostasis of human cells. Alterations in miRNA expression are increasingly noted in relation to pathophysiological changes in cancer cells, thereby making miRNAs one of the most currently analyzed molecule types in cancer research. Numerous miRNAs are dysregulated in lung cancers, and a single miRNA can have multiple targets that are involved in different oncogenic pathways. A large body of evidence reveals that the aberrant expression of miRNAs in cancer patients can be taken advantage of innumerous ways, such as for potential use as diagnostic, clinicopathological, and/or prognostic markers and as promising therapeutic targets in lung cancer. Aberrant miRNA expression profiles provide additional insight into the clinical application of miRNA-directed therapies in lung cancer (Leidinger et al. 2011). Here, we focus on reviewing the known roles of miRNAs as regulators of cancer cell survival, drug sensitivity and tumorigenesis. These miRNAs hold great potential as targets in the treatment of lung cancer.

2.1 MiRNAs Function as Oncogenes in Lung Cancer

Many oncogenes important in controlling lung cancer tumorigenesis are targets of miRNAs. The miRNAs found in the miR-17-92 cluster (miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a, miR-92-1) are oncogenic miRNAs (oncomiRs) that reside in the amplified chromosomal region 13q31.3 (He et al. 2005). These miRNAs cooperate with c-Myc to accelerate tumor development and promote tumor angiogenesis (Dews et al. 2006). It has been reported that the miR-17-92 cluster is over-expressed in SCLC (Hayashita et al. 2005). Moreover, Ebi et al. reported that miR-17-92 over-expression is associated with retinoblastoma (RB) inactivation (Ebi et al. 2009). Collectively, these results suggest that this miRNA cluster may be a potential therapeutic target in lung cancer.

The miR-21 gene is located on chromosome 17 and was one of the first miRNAs characterized as oncogenic, with its oncogenic function established in various types of cancers (Chan et al. 2005). MiR-21 has been suggested to be an independent negative prognostic factor for the overall survival of NSCLC patients (Markou et al. 2008). MiR-21 targets tumor suppressor genes such as programmed cell death 4 (PDCD4) and phosphatase and tensin homolog deleted from chromosome 10 (PTEN) (Lu et al. 2008; Zhang et al. 2010). Furthermore, miR-21 expression is up-regulated by epidermal growth factor receptor (EGFR) signaling in lung cancer. Antisense miR-21-enhanced EGFR tyrosine kinase inhibitors induce apoptosis of lung cancer cells (Seike et al. 2009). The critical function of miR-21 in regulating lung cancer tumorigenesis makes it a promising target for developing miRNA-based therapeutics and diagnostic tools. However, because miR-21 is also dysregulated in various type of cancer, it appears to be a general oncomiR without tissue specificity (Volinia et al. 2006).

MiR-31 is another miRNA with oncogenic properties in lung cancer. The host gene encoding miR-31 is located on chromosome 9. Liu et al. showed that miR-31 functions as an oncomiR by directly repressing large tumor suppressor 2 (LATS2) and Protein phosphatase 2, regulatory subunit B, Alpha isoform (PPP2R2A) and that knockdown of miR-31 represses lung cancer cell clonal growth and *in vivo* tumorigenicity (Liu et al. 2010).

2.2 MiRNAs Function as Tumor Suppressors in Lung Cancer

Among the numerous miRNAs that function as tumor suppressors, the let-7 family is one of the most studied. Let-7 was first identified in *C. elegans* as a regulator of the timing of cell fate determination (Reinhart et al. 2000). In humans, the let-7 family is a cluster of miRNAs whose encoding genes map to various chromosomal regions that are frequently deleted in lung cancer (Calin et al. 2004). Johnson et al. (2007) showed that let-7 over-expression in the A549 cell line inhibits cell growth and reduces cell-cycle progression. In mouse models of lung cancer, over-expression of let-7g reduces tumor growth (Kumar et al. 2008), and let-7a inhibits tumor growth via suppression of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)

and c-Myc (He et al. 2010). Furthermore, reduced let-7 gene expression in NSCLC patients correlates with poor prognosis (Yanaihara et al. 2006; Takamizawa et al. 2004). The 3′ UTR of members of the RAS GTPase family such as v-Ha-ras Harvey rat sarcoma viral oncogene homolog (HRAS), KRAS and neuroblastoma RAS viral oncogene homolog (NRAS) contains multiple putative let-7 binding sites. It has also been revealed that let-7 miRNAs negatively regulate multiple oncogenes, including MYC (Kumar et al. 2007), high mobility group AT-hook 2 (HMGA2) (Lee and Dutta 2007), B-cell leukemia/lymphoma 2 (BCL-2) (Xiong et al. 2011) and cell cycle proto-oncogenes such as cell division cycle 25A (CDC25A), cyclin-dependent kinase 6 (CDK6) and cyclin D2 (Johnson et al. 2007). These data show that let-7 miRNAs act as key tumor suppressors in regulating cell survival and proliferation in lung cancers.

The miR-34 family is another important group of miRNAs that function as tumor suppressors in many types of cancers (Hermeking 2010; Wong et al. 2011). The miR-34a gene is located on chromosome 1p36.22, and miR-34b/c are expressed from a polycistronic transcript encoded on chromosome 11q23.1. These genes are in chromosomal regions associated with fragile sites of the genome that are frequently altered in cancer (Calin et al. 2004). Structurally, miR-34 family members possess p53-binding sites, reflecting their function as tumor suppressors downstream of the p53 pathway. MiR-34a and miR-34b/c were found to be directly regulated by p53 to control apoptosis and cell cycle arrest in cancer cell lines, including lung cancer (Raver-Shapira et al. 2007; Wiggins et al. 2010). Subsequent studies demonstrated that the apoptotic function of miR-34a is mediated by the direct down-regulation of the expression of BCL-2 and sirtuin 1 (SIRT1) (Yamakuchi et al. 2008; Bommer et al. 2007). In addition, AXL (Mudduluru et al. 2011) and SNAIL1 (Kim et al. 2011) were identified as miR-34 direct targets in lung cancer cells; it is plausible that miR-34 expression inhibits lung cancer cell invasion and migration via repression of these genes. In various solid and hematological malignancies, including lung cancer, miR-34 antagonizes processes necessary for basic cancer cell viability as well as cancer stemness, metastasis and chemoresistance (Bader 2012). In the future, the utility of miR-34-directed therapeutics in the treatment of lung cancer will expand dramatically.

The anti-tumor activity of miR-143 and miR-145 in lung cancer is also well characterized. They are co-transcribed from a bicistronic gene cluster on chromosome 5 (Xin et al. 2009). MiR-143/145 have been identified as tumor suppressor in various types of cancer, including lung cancer. The restoration of miR-145 has been shown to inhibit cell growth in mouse and human lung cancer cells (Liu et al. 2009; Cho et al. 2009). It has also been reported that c-MYC, EGFR and nucleoside diphosphate linked moiety X-type motif 1 (NUDT1) are direct targets of miR-145 that regulate cell proliferation in lung cancer (Chen et al. 2010; Cho et al. 2011). Furthermore, miR-145 has also been shown to inhibit lung adenocarcinoma stem-like cell proliferation by targeting octamer-binding transcription factor 4 (OCT4) (Feng et al. 2011). Similarly, the expression of miR-143 was down-regulated in human lung tumor samples compared with normal tissues (Gao et al. 2010; Vosa et al. 2013).

Finally, miR-192 also might serve as a promising therapeutic target for lung cancer treatment. Retinoblastoma 1 (RB1) is a direct target of miR-192, and

over-expression of miR-192 results in decreased expression of RB1 mRNA and protein. Caspase-7 and poly ADP-ribose polymerase (PARP) protein were activated by miR-192 over-expression, suggesting that miR-192 induces cell apoptosis through the caspase pathway. In addition, the analysis of miRNA expression in clinical samples has revealed that miR-192 is significantly down-regulated in lung cancer tissues compared with adjacent, normal lung tissues (Feng et al. 2011).

3 MicroRNA-Based Therapies for Lung Cancer

The development of miRNA-based therapeutics represents a new strategy in cancer treatment and is growing rapidly with the help of new RNAi technologies. Compared to siRNA-based therapies, which are already in clinical trials, miRNAs are less toxic and have the potential to target multiple genes. As presented above, miRNAs are generally classified as oncomiRs or tumor suppressors, with different therapeutic approaches developed for each class. Generally, the up-regulation of miRNA expression is achieved through administration of synthetic miRNA mimics or miRNA-expressing vectors. The down-regulation of miRNA expression is achieved through administration of antisense nucleotides, often chemically modified to ensure stability and specificity. Although each approach shares similarities with other therapies, each is sufficiently distinct such that miRNA-inhibitory and replacement approaches should be viewed as separate therapeutic modalities. In view of cancer as a heterogenic disease that cannot be successfully treated via single gene targeting, miRNA-based strategies may hold the key to therapeutic success. Table 17.1 shows a summary of miRNA-based therapeutic strategies for *in vivo* models of lung cancer.

3.1 MiRNA Inhibitor-Based Therapeutics

To reduce endogenous miRNA levels, anti-miRs are typically employed. Targeting miRNAs for suppression through the use of anti-miRs is possibly the best-studied modality to date. This approach is conceptually similar to other

Table 17.1 MicroRNA-based therapeutic strategies for *in vivo* models of lung cancer

MicroRNA	Administration	Modulation strategy	Delivery technology	Reference
let-7a	Intranasal	Replacement	Adenoviruses	Esquela-Kerscher et al. (2008)
let-7b	Systemic	Replacement	Neutral liposomes	Trang et al. (2011)
let-7g	Intratracheal	Replacement	Lentiviruses	Kumar et al. (2008)
miR-7	Intratumoral	Replacement	Cationic liposomes	Rai et al. (2011)
miR-29b	Systemic	Replacement	Cationic liposomes	Wu et al. (2013)
miR-34a	Intratumoral	Replacement	Neutral liposomes	Wiggins et al. (2010)
miR-145	Intratumoral	Replacement	Polyethyleneimines	Chiou et al. (2012)
miR-150	Intratumoral	Inhibition	Cationic liposomes	Li et al. (2012)

inhibitory therapeutics that target a single gene product, such as small molecule inhibitors and siRNAs. Various methods have been employed to render anti-miR constructs more stable *in vivo* and ensure adequate tissue availability and specificity (Krutzfeldt et al. 2005). Constructs can be modified with a cholesterol-conjugated 2′-*O*-methyl group to inhibit degradation and hence improve stability. Locked nucleic acid (LNA) is an additional method of antisense oligonucleotide modification whereby the 2′ oxygen and 4′ carbon of the nucleotide is bridged with methylene to form a cyclic structure. LNA is more resistant to endogenous nucleases, less toxic, and possess a stronger affinity for the target nucleotide (Elmen et al. 2008; Wahlestedt et al. 2000). Relative to studies on miRNA mimics, studies with antisense oligonucleotides have demonstrated greater efficacy using naked oligonucleotides. Furthermore, the LNA-anti-miR compound was well tolerated in both mice and primates, as no acute or subchronic toxicities in the treated animals were detected (Elmen et al. 2008). Recent data from the first Phase IIa study in patients with chronic HCV infection treated with the LNA-modified anti-miR-122 revealed that this compound was well tolerated and provided continuing viral suppression (Janssen et al. 2013). With regard to lung cancer, anti-miR-150 delivered to lung tumor xenografts in mice caused tumor growth inhibition (Li et al. 2012). Although there are few reports using LNA-anti-miR therapeutics in lung cancer mouse models, their inhibition of miRNA function is an important and widely used approach. Currently, miRNA sponges are a novel approach to miRNA inhibition, and this technology works with multiple complementary 3′-UTR mRNA sites of a specific miRNA (Ebert et al. 2007). MiRNA sponges specifically inhibit miRNAs with a complementary heptameric seed; thus, a single sponge can inhibit an entire miRNA seed family. In fact, the development of lung metastasis in a murine breast cancer model was significantly reduced via inhibition of the MYC driven miR-9 using a miRNA sponge (Ma et al. 2010). Furthermore, the use of miRNA sponges to inhibit miR-31 in a breast cancer model resulted in a significant induction of lung metastasis (Valastyan et al. 2009). Of potential concern is the possibility that the antagonist might also non-specifically bind to other RNAs, resulting in unwanted side effects. Therefore, adequate assessment of the functional effects of miRNA inhibition is of key importance for miRNA inhibitor-based loss-of-function studies and development of miRNA therapeutics. The high potency and metabolic stability of chemically modified anti-miRs highlights the utility of anti-miRs in the development of novel RNAi therapeutic modalities based on lung cancer associated miRNAs.

3.2 MiRNA Mimic-Based Therapeutics

Tumor suppressor miRNAs are responsible for down-regulating oncogenes and are primarily expressed in cancer (Croce 2009). In this context, miRNA replacement strategies have been developed to restore normal cellular expression levels via

administration of tumor suppressor miRNA mimics (Bader et al. 2010). MiRNA mimics are synthetic RNA duplexes designed to imitate the endogenous functions of miRNAs. In addition, miRNAs may be unstable as a result of rapid degradation by endogenous nucleases or rapid elimination through renal and hepatic metabolism and extraction upon systemic administration (Bader et al. 2011). Local administration of RNAi-based therapeutics to the target cells is a promising approach to overcome the problems of systemic administration (see next section for details). Similarly, chemical modifications at specific positions or formulations with delivery vectors have been shown to improve stability. Lipid-based and polymer-based nanoparticles reduce the negative electrical charge of RNA nucleotides to promote cell uptake (Wu et al. 2011). Another strategy for efficient delivery of miRNA-based therapeutics is the use of viral vectors (Bonci et al. 2008). Indeed, adenoviral (Esquela-Kerscher et al. 2008) or lentiviral vectors (Kumar et al. 2008) can be used to transfer miRNAs to lung cancer cells. Successful delivery of miRNA-based therapeutics requires patient compliance with the intended delivery route and efficient delivery vectors. This approach has attracted much interest as it provides a novel opportunity to exploit tumor suppressors. The concept of miRNA replacement therapy is best exemplified by let-7 miRNA. Intranasal administration of a let-7 mimic into mouse models of lung cancer significantly reduced tumor growth, suggesting that miRNA replacement therapy is indeed promising (Trang et al. 2010). Based on these successful results, a clinical trial in non-small cell lung cancer using a let-7 based therapy will begin in the near future. As an additional example of the value of miRNA replacement strategies, miR-34a-based cancer therapies have powerful potential for clinical use. Both local and systemic delivery of a synthetic miR-34a mimic resulted in accumulation of miR-34a in the tumor tissue and inhibition of lung tumor growth. MiRNA therapeutics will initiate clinical trials of miR-34a mimics in 2013, making these mimics some of the first miRNA mimics to reach the clinic. Thus, the pharmacological delivery of miRNA mimics effectively inhibits tumor growth by targeting multiple genes. However, it is necessary to pay attention to any potential toxicities in normal tissues, given that therapeutic delivery of miRNA mimics can lead to an accumulation of exogenous miRNAs in normal cells. It will be important to investigate miRNA mimic-induced effects in normal cells and carefully assess the resultant toxicity before using such therapies in clinical practice.

4 Pulmonary Delivery of RNAi-Based Therapeutics

Despite the promise of miRNAs in cancer therapy, there are still hurdles to clear before clinical use, including safety, stability and successful delivery of therapeutic miRNAs to the appropriate tissue and into the appropriate cells. In general, the delivery of miRNAs can be achieved through systemic administration (via intravenous injection) or local administration (via a direct route). Conceptually, systemic delivery is an attractive option because it provides a simple route for miRNA administration to all tissues via the blood stream (Liu et al. 2007). Indeed, there have been some successful reports using systemic delivery of miRNAs in lung cancer models.

Nevertheless, this approach has more *in vivo* barriers to overcome, in addition to nuclease degradation. The delivery barriers are (i) renal clearance of molecules (<50 kDa), (ii) uptake by phagocytic immune cells, (iii) failure of molecules >5 nm in diameter to cross the capillary endothelium, (iv) limited passage through the extra-cellular matrix (polysaccharides and fibrous proteins), (v) inefficient endocytosis by target tumor cells, and (vi) inefficient endosomal release (Bader et al. 2011). Chemical modification and formulation with delivery vectors have been shown to improve stability and delivery to target tumor cells, but these alterations may attenuate the suppressive activity of oligonucleotides (Chernolovskaya and Zenkova 2010). In addition, systemic delivery of miRNAs may induce adverse events similar to those reported for other oligonucleotide-based therapies, such as aggregation and complement activation, liver toxicity and stimulation of the immune response (Kleinman et al. 2008). For these reasons, local administration of miRNAs to the target cancer cells is a promising approach to overcome the problems of systemic administration. Translation of locally administered modalities to the clinical setting is dependent upon the development of an efficient delivery system that is able to improve the pharmacokinetic and biodistribution properties of miRNAs. Thus far, locally administered modalities are available for ocular, transdermal, rectal and pulmonary delivery.

Dozens of RNAi-based therapeutics are being assessed in preclinical and clinical trials, and these studies provide further opportunities for successful results (Davidson and McCray 2011). Many of these studies are conducted using local administration to specific tissues. The lung is anatomically accessible to therapeutic drugs via the pulmonary route. Accessibility is a key requirement for successful RNAi-based *in vivo* and clinical studies, and this anatomical characteristic offers several important benefits over systemic delivery, including the use of lower doses of miRNAs, the reduction of undesirable systemic side effects, and improved miRNA stability due to reduced nuclease activity in the airways compared to serum. The local approach could potentially enhance the retention of RNAi-based therapeutics in the lungs. Because the delivery of siRNAs to the lungs is well studied using different routes and delivery strategies (Lam et al. 2012), many technologies developed for siRNAs may also be applicable to miRNAs. In most of the pulmonary RNAi-based therapy studies *in vivo*, agents were delivered intratracheally or intranasally. This approach has allowed remarkable progress in miRNA modulation in preclinical cancer models, bringing us closer to delivering on the promise of miRNAs as cancer therapeutics.

5 Strategies for Pulmonary Delivery of MicroRNA-Based Therapeutics

Pulmonary delivery approaches are very attractive because they tend to be non-invasive, locally restricted, and administered by the patient. With regard to siRNA-based therapeutics, Phase II clinical trials are underway for the treatment of respiratory syncytial virus (RSV) infection using an intranasal application of naked,

chemically modified siRNA molecules that target viral gene products (DeVincenzo et al. 2008, 2010). To date, two successful studies of pulmonary delivery of miRNA-based therapeutics for lung cancer mouse models have been reported (Kumar et al. 2008; Esquela-Kerscher et al. 2008). These studies show that pulmonary delivery of miRNA from the let-7 family reduces lung tumor formation in an orthotopic lung cancer mouse model without systemic side effects (Table 17.1). These data suggest that intranasal or intratracheal administration of miRNAs may be a potent strategy for treating lung cancer. Although there are no reports of pulmonary delivery of miRNA-inhibitors in lung cancer at present, we predict that this delivery strategy will become a valuable resource for implementing miRNA-based therapies *in vivo* and in humans.

We believe that pulmonary delivery of miRNAs has two primary advantages over systemic delivery for clinical use. First, several sophisticated inhalation devices for lung diseases are already in clinical use. Inhaled therapeutics are used routinely to treat a variety of pulmonary conditions, including asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis. Metered-dose inhalers (MDIs) and dry powder inhalers (DPIs) are the most common modes of inhaled delivery. The use of DPIs for the *in vivo* delivery of therapeutic macromolecules such as insulin (Mastrandrea and Quattrin 2006) and low-molecular-weight heparin (Bai et al. 2010) has yielded promising results. Currently, the use of spray-drying as a technique for engineering dry powder formulations of siRNA nanoparticles, which might allow the pulmonary delivery of biologically active siRNAs directly to the lung tissue, has been demonstrated (Jensen et al. 2010, 2012). Although a suitable carrier is also needed to protect miRNAs from degradation given the shear force and increased temperature of the drying process, these delivery technologies could open new avenues for pulmonary delivery of miRNAs and improve patient outcome. To make miRNA-based therapy practical in the treatment of lung cancer, we believe that the administration of inhaled miRNAs by DPIs is the best of choice of delivery strategy. Second, pulmonary delivery also offers the clinical benefit of a lower miRNA dose. The cost related to the development and application of a particular RNAi therapeutic delivery technology is undoubtedly an important factor (Dykxhoorn et al. 2006). Local administration is likely to be a more cost-efficient strategy for miRNA delivery *in vivo* and in the clinic than systemic administration. Furthermore, the advantage of pulmonary delivery is that it ensures high delivery efficiency with minimal drug loss. For this reason, pulmonary delivery of miRNAs has great potential for clinical use. However, the limitations of pulmonary delivery of miRNA-based therapeutics are important to consider. First, the pharmacokinetics of inhaled miRNAs in *in vivo* models and humans are estimated inaccurately. It is also unknown whether miRNA-based therapeutics delivered via the intrapulmonary route could also be delivered to other organs, such as the liver and kidneys. To prevent systemic side effects, the precise pharmacokinetics of miRNAs after intrapulmonary administration should be measured. Second, we also must pay attention to the pulmonary inflammatory and toxicological responses caused by the delivery vehicle. In fact, there are some reports that RNAi-based therapeutics with polyethyleneimine

(PEI) frequently cause inflammatory responses in the lungs (Beyerle et al. 2011). It has been reported that naked RNAi-therapeutic delivery possesses advantages over other delivery vectors, such as reduced toxicity and reduced inflammatory responses, as well as simple formulation (Heidel et al. 2004). However, the advantage of naked RNAi-therapeutics over delivery vectors in the treatment of lung diseases is controversial (Nielsen et al. 2010; Akinc et al. 2008). Therefore, we need to develop safer delivery technology for practical use in *in vivo* mouse models and humans.

6 Conclusions

During the past decade, miRNAs have quickly advanced from discovery to therapeutic development programs. This rapid progress reflects the importance of miRNA biology in cancer, leaving little doubt about the therapeutic potential of miRNAs in cancer treatment. Given the encouraging results of the profiled studies and preclinical testing, miRNAs are being integrated into human clinical trials. The first miRNA-targeted drug LNA-anti-miR-122 is successfully undergoing Phase II trials (Janssen et al. 2013). Accordingly, several companies are currently developing miRNA mimics or inhibitors for the treatment of cancer. The main focus in bringing miRNAs to cancer cells is the capacity of pharmacological drug delivery. The success of miRNA-based therapeutic delivery requires efficiency, convenience, and patient compliance using the delivery route. In this chapter, we showed that pulmonary delivery of miRNA-based therapeutics holds powerful potential for lung cancer treatment (Fig. 17.1). A realistic therapeutic intervention, such as inhalation, would

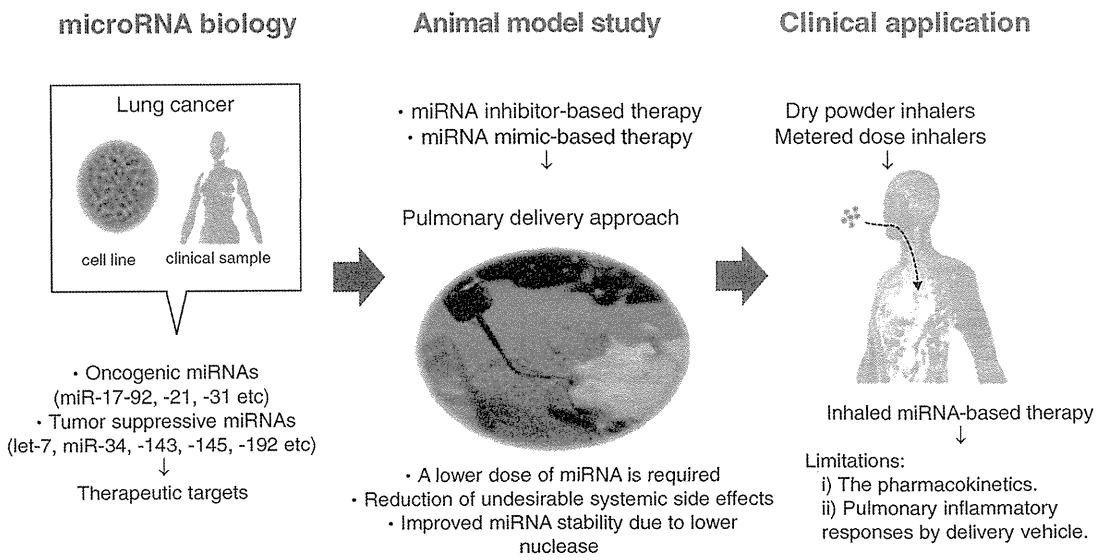

Fig. 17.1 Process for translating microRNA biology from bench to bedside in lung cancer

enhance drug delivery to the site of action and decrease systemic exposure, thereby reducing off-target effects. In the future, combining miRNA-based therapeutics with chemotherapy may potentiate the cancer treatment efficacy. Therefore, continued investigation on all fronts will be of equal importance to the eventual clinical application of miRNAs.

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MicroRNA binding to the HIV-1 Gag protein inhibits Gag assembly and virus production

Antony K. Chen[a,b], Prabuddha Sengupta[a], Kayoko Waki[c], Schuyler B. Van Engelenburg[a], Takahiro Ochiya[d], Sherimay D. Ablan[c], Eric O. Freed[c], and Jennifer Lippincott-Schwartz[a,1]

[a]Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; [b]Department of Biomedical Engineering, College of Engineering, Peking University, Beijing 100871, China; [c]Virus–Cell Interaction Section, HIV Drug Resistance Program, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702; and [d]Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Tokyo 104-0045, Japan

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MicroRNAs (miRNAs) are small, 18–22 nt long, noncoding RNAs that act as potent negative gene regulators in a variety of physiological and pathological processes. To repress gene expression, miRNAs are packaged into RNA-induced silencing complexes (RISCs) that target mRNAs for degradation and/or translational repression in a sequence-specific manner. Recently, miRNAs have been shown to also interact with proteins outside RISCs, impacting cellular processes through mechanisms not involving gene silencing. Here, we define a previously unappreciated activity of miRNAs in inhibiting RNA–protein interactions that in the context of HIV-1 biology blocks HIV virus budding and reduces virus infectivity. This occurs by miRNA binding to the nucleocapsid domain of the Gag protein, the main structural component of HIV-1 virions. The resulting miRNA–Gag complexes interfere with viral–RNA-mediated Gag assembly and viral budding at the plasma membrane, with imperfectly assembled Gag complexes endocytosed and delivered to lysosomes. The blockade of virus production by miRNA is reversed by adding the miRNA's target mRNA and stimulated by depleting Argonaute-2, suggesting that when miRNAs are not mediating gene silencing, they can block HIV-1 production through disruption of Gag assembly on membranes. Overall, our findings have significant implications for understanding how cells modulate HIV-1 infection by miRNA expression and raise the possibility that miRNAs can function to disrupt RNA-mediated protein assembly processes in other cellular contexts.

To trigger viral budding, Gag proteins must extensively multimerize at the plasma membrane (PM), forming a tightly packed lattice that remodels into a virus particle containing a few thousand Gag molecules (1). Whereas recruitment of the viral genome by Gag is driven by specific and highly efficient interactions between Gag and the genome's Psi-element (2, 3), increasing evidence suggests Gag multimerization can also be mediated by nonspecific Gag–RNA interactions. For example, long-stranded RNAs not possessing a Psi-element can drive Gag assembly, serving as a scaffold for concentrating Gag molecules (4–7). In in vitro systems, Gag can multimerize by binding nonspecifically to RNA through its nucleocapsid (NC) domain, with the degree of multimerization proportional to the length of the input RNA (4). Finally, in living cells, Gag binding to either HIV-1 viral RNA or cellular mRNA promotes viral particle assembly (5, 6). The proposed role of nonspecific Gag–RNA interactions in facilitating Gag multimerization and viral budding prompted us to investigate whether miRNAs could prevent viral budding by competing with viral RNA for Gag binding via an unconventional mechanism not involving gene silencing like others have shown (8–11).

Results and Discussion

To test whether miRNAs could block virus production without silencing HIV gene expression, we created a HEK293 cell line stably overexpressing an exogenous human microRNA (miRNA) (i.e., hsa-miR-146a) that is neither present in the parent cell line nor has target sites on HIV transcripts (12–14) (*SI Appendix*, Fig. S1). This cell line is referred to as miR$^+$. The parent cell line, HEK293, is referred to as wild type (WT). The viral RNA transcript and its encoded viral proteins were expressed in miR$^+$ or WT cells by transiently transfecting either a full-length HIV-1 proviral clone (HIV-1pNL43) or pNL43 derivatives in which the polymerase (*pol*) and envelope (*env*) genes were deleted and Gag was tagged with GFP (i.e., pNL43ΔPolΔEnvGag-GFP). The pNL43ΔPolΔEnvGag-GFP construct was always expressed in a 1:3 ratio with pNL43ΔPolΔEnvGag (lacking GFP) to ensure proper viral particle formation (15, 16) (*Materials and Methods* for details regarding derivative expression).

Compared with WT cells or empty-vector control cells, Gag in miR$^+$ cells showed reduced capacity to assemble into particles, assessed by the number of released Gag-GFP particles in the supernatant (Fig. 1 *A* and *B* and *SI Appendix*, Fig. S2). In addition, the viral supernatant from miR$^+$ cells contained fewer infectious virions (Fig. 1*C*). A reduction in released infectious viral particles was also observed in cells stably expressing a different exogenous miRNA (hsa-miR-888, referred to as miR$^+$888) after transfection of the viral constructs (*SI Appendix*, Fig. S3). Given the exogenous miRNAs expressed in miR$^+$ and miR$^+$888 cells were derived from different precursor families, with little sequence homology in the seed sequences (*SI Appendix*, Fig. S4) and no known target sites on the viral transcript (12–14), we concluded that overexpression of exogenous miRNAs can inhibit HIV-1 particle production within cells by a mechanism independent of RNA-interference silencing of HIV-1 protein

Significance

MicroRNAs normally function to regulate gene expression through RNA-interference–mediated gene silencing. Here, we demonstrate that microRNAs can inhibit HIV-1 virus production by a novel mechanism not involving RNAi-mediated interference. The new mechanism involves interactions between microRNA and HIV-1 Gag protein's RNA-binding (nucleocapsid) domain. These interactions prevent Gag proteins from effectively multimerizing into viral complexes at the plasma membrane and lead to inhibition of viral particle production. The microRNA–Gag interactions further result in Gag proteins being redirected into the endocytic pathway where they are degraded in lysosomes. These findings have significant implications for understanding how cells modulate HIV-1 infection and raise the possibility of manipulating total expression levels of host miRNAs to combat HIV-1 replication.

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The authors declare no conflict of interest.

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[1]To whom correspondence should be addressed. E-mail: lippincj@mail.nih.gov.

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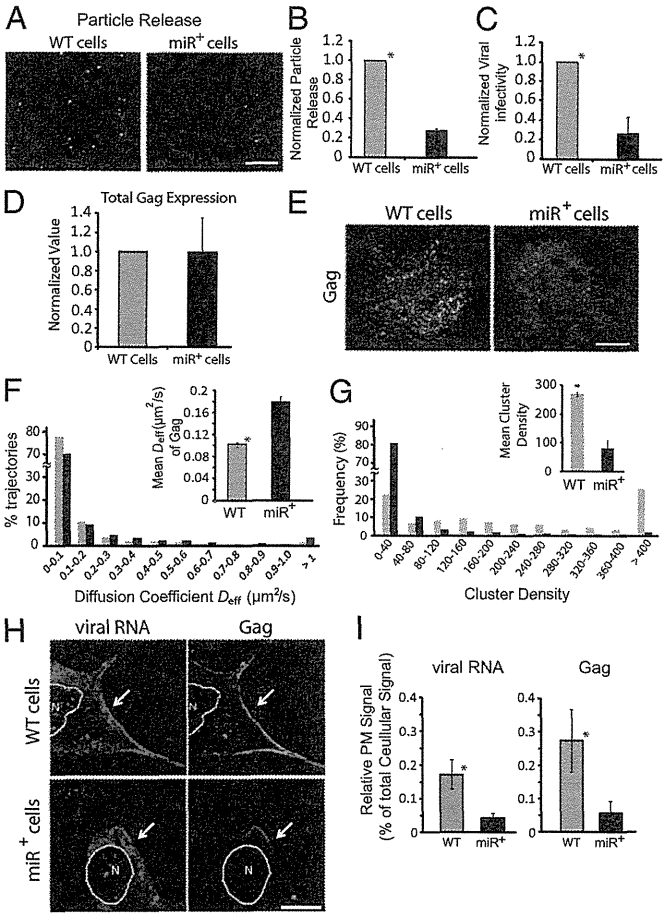

Fig. 1. Effect of miRNA overexpression on HIV-1 particle production and Gag dynamics at the PM. (*A*) Representative images of virus-like particles containing Gag-GFP released from WT or miR$^+$ cells transiently transfected with pNL43ΔPolΔEnvGag-GFP. Released viral particles from equivalent numbers of transfected cells were concentrated and absorbed onto poly-lysine-coated coverslips for imaging. Fewer viral particles were released from miR$^+$ cells. (*B*) Quantification of virus particle release from WT or miR$^+$ cells transiently transfected with pNL43ΔPolΔEnvGag-GFP. Plotted is the average number of particles per field in 50–60 different fields acquired from three different experiments, with particle counts normalized to those from WT cell supernatants. (*C*) Viral infectivity of supernatants from WT or miR$^+$ cells transfected with full-length HIV-1 proviral clone, normalized to WT cell supernatants. (*D*) Total Gag expression levels in WT or miR$^+$ cells after transfection with the full-length HIV-1 proviral clone, normalized to Gag expression in infected WT cells. (*E*) Representative TIRF images of Gag at the PM in WT or miR$^+$ cells transfected with pNL43ΔPolΔEnvGag-GFP. (*F*) spt-PALM measurements of single Gag molecules within Gag complexes diffusing across the PM in WT and miR$^+$ cells transfected with pNL43ΔPolΔEnvGag-mEOS2. The histogram shows the distribution of diffusion coefficients of single Gag complexes. Total trajectories of the WT cells (2,316 tracks) versus miR$^+$ cells (1,693 tracks) were acquired from >10 cells. *Inset* shows the mean ± SEM diffusion coefficients. (*G*) Cluster density distribution of Gag at the PM of WT and miR$^+$ cells. WT and miR$^+$ cells were transfected with pNL43ΔPolΔEnvGag-mEOS2 and imaged by PALM. For each cell, the cluster densities are normalized with respect to the mean density of Gag at the PM. Clusters are from more than four cells. Error bars, SEM (n = 1,252 for WT, n = 900 for miR$^+$). (*H*) Representative images of unspliced HIV-1 viral RNA (detected by FISH) and Gag (detected by Gag-GFP) in WT and miR$^+$ cells transfected with pNL43ΔPolΔEnvGag-GFP. Arrows point to the PM. Note the relative PM depletion of viral RNA and Gag from the PM in miR$^+$ cells compared with WT cells. (*I*) Quantification of HIV-1 viral RNA and Gag levels at the PM of cells from *H*. The fraction of PM versus total labeling per cell is depicted (n > 5). All data are represented as mean ± SD of at least three replicate experiments unless otherwise noted. (Scale bar, 10 μm.)

translation (12–14). Consistent with this, total Gag expression levels after transfection with the viral clones was similar in WT and miRNA-overexpressing cells (Fig. 1*D* and *SI Appendix*, Fig. S3).

To investigate how viral budding was inhibited in cells overexpressing exogenous miRNAs, we imaged Gag molecules at the PM in WT and miR$^+$ cells by total internal reflection fluorescence (TIRF) microscopy, which restricts the field of view to regions at or close to the PM (Fig. 1*E*). In WT cells, the majority of Gag molecules appeared within small punctate structures characteristic of viral buds. In miR$^+$ cells, by contrast, few Gag puncta were observed, with most of the PM pool of Gag widely distributed. This suggested that inhibition of viral particle budding in miRNA-overexpressing cells occurs by a mechanism involving disruption of Gag multimerization and coalescence into viral particles at the PM.

Hindrance of Gag multimerization and coalescence by exogenous miRNAs should alter the diffusional mobility of Gag at the PM. To test this, we used photoactivated localization microscopy (PALM), which can reveal protein dynamics and distribution patterns with tens of nanometer precision (17, 18). Gag was tagged with the photoactivable fluorescent protein mEOS2 (Gag-mEOS2) and expressed from the pNL4-3 derivative pNL43ΔPolΔEnvGag-mEOS2 in a 1:3 ratio with pNL43ΔPolΔEnvGag (*Materials and Methods*). Single-particle tracking PALM (spt-PALM) (18) was then used to measure the diffusional mobility of Gag. Gag's statistical distribution of diffusion coefficients (D_{eff}) shifted to more rapid diffusion in miRNA-overexpressing cells compared with WT cells (Fig. 1*F* and *SI Appendix*, Fig. S5). The mean D_{eff} of Gag at the PM of WT cells was 0.10 ± 0.00 μm^2/s (Fig. 1*F*, *Inset* and *SI Appendix*, Fig. S5, *Inset*), consistent with previous measurements (18). In miR$^+$ and miR$^+$888 cells, by contrast, Gag moved at the PM with an average diffusion coefficient of 0.18 ± 0.01 μm^2/s and 0.22 ± 0.02 μm^2/s, respectively. Hence, Gag molecules exhibit greater mobility at the PM of cells overexpressing miRNAs.

We next used PALM cluster analysis to characterize the relative distribution and density of Gag molecules at the PM of WT versus miRNA-overexpressing cells (Fig. 1*G*, *SI Appendix*, Fig. S6). The mean Gag cluster size in WT cells was 1.64 times greater than that in the miR$^+$ cells, with 75% of Gag molecules associated in clusters in WT cells compared with ~28% in miR$^+$ cells. Within each cluster, the average density of Gag was 3.3 times greater in WT cells than in miR$^+$ cells. Gag molecules at the PM in miR$^+$888 cells showed a similar distribution pattern as seen in miR$^+$ cells (*SI Appendix*, Fig. S6). Therefore, the extent that Gag clusters at the PM is reduced in cells overexpressing miRNAs.

The above results indicated that the organization and dynamics of Gag at the PM of miRNA-overexpressing cells is different from WT cells, with Gag less clustered and more highly mobile in miRNA-overexpressing cells. We speculated this was due to expressed miRNAs interfering with the binding of viral RNA to Gag at the PM. To test this possibility, we performed fluorescent in situ hybridization (FISH) to look for any changes in Gag–viral RNA interactions. Notably, less viral RNA and Gag were associated with the PM in miR$^+$ cells compared with WT cells (Fig. 1 *H* and *I*). In addition, fewer particles containing viral RNA or Gag-GFP were released by miR$^+$ cells compared with WT cells (*SI Appendix*, Fig. S7). These findings supported the view that exogenous miRNAs in miRNA-overexpressing cells disrupt viral RNA-mediated assembly of Gag into viral particles by competing with viral RNA for binding to Gag at the PM.

Previous studies have shown that deletion of the NC domain of Gag attenuates viral production (19). To investigate whether this arises from Gag's failure to undergo viral RNA-mediated clustering into viral-like particles, analogous to that occurring in miRNA-overexpressing cells, we generated an HIV-1 proviral clone containing a mutant Gag-GFP, whose main RNA binding domain (NC) was deleted (ΔNC-Gag-GFP) (see *SI Appendix*,