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REVIEW

Potential microRNA therapies targeting Ras, NFκB and p53 signaling

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MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression by binding to complementary sequences in mRNAs encoding downstream target genes. A large variety of cellular processes, including differentiation, development, apoptosis and cell cycle progression, are dependent on miRNA-mediated suppression of gene expression for their regulation. As such, it is unsurprising that these small RNA molecules are associated with signaling networks that are often altered in various diseases, including cancer. This review focuses on the function of miRNAs in three of the most well-documented signaling pathways that are dysregulated in tumors: the NFκB and Ras prosurvival signaling cascades and the tumor suppressor p53 pathway. Recent findings that connect these pathways through various miRNA families are reviewed, and support for using miRNA therapy as a novel method to counteract these tumor-promoting signaling events are presented.

Keywords Cancer, K-Ras, *let-7*, microRNA, NFκB, p53

Introduction

Developing therapeutics that target cell survival and death promoting signals is an intense area of focus in cancer research. Various studies conducted over multiple decades have demonstrated that tumors arise from misregulated signaling events that control the equilibrium between cell survival and death [1]. Alterations, which are frequently genomic mutations in the soma or germline, often lead to the activation of oncogenic proteins, such as Ras or NFκB, typically concomitantly with the inhibition of tumor suppressors, the most notable of which is p53. In this altered state, cells gain the ability to grow and proliferate irrepressibly, leading to tumorigenesis. Successful attempts have been made to reduce uncontrollable cell growth by interfering with the Ras [2-4] and NFκB [5-8] survival pathways, and/or introducing or reactivating p53 [4,9-15]. These preclinical and clinical studies suggest that targeting these pathways may represent one strategy toward the treatment of cancer. Nevertheless, additional approaches based on recent scientific developments need to be explored with the aim of identifying therapeutics with increased efficacy.

MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules that act as post-transcriptional regulators of gene expression, typically by binding to regions within the 3'-untranslated region (UTR) of target mRNA [16]; binding of miRNAs results in either translational repression [17,18] or degradation of the target mRNA [19]. NFκB, p53 and Ras have been identified as suppressors [20], activators [21] and/or targets [22,23] of miRNAs, and in many instances these signaling pathways are interconnected through miRNAs and their regulators. These innovative findings have expanded the knowledge of the post-transcriptional regulation of cancer genes and suggest miRNA therapies may be a novel approach that can be used to revert aberrant signaling events. Moreover, several miRNAs involved in these pathways have demonstrated therapeutic potential *in vivo*, including post-transcriptional suppression of the oncogene *KRAS* by the *let-7* tumor-suppressive family of miRNAs [24-26], warranting the continued research of these molecules and progression into the clinic. Based on molecular studies, other miRNAs such as miR-125b and the miR-34 family have demonstrated promise as antiproliferative agents, although this research is at an early stage.

let-7 miRNAs in cancer and cancer stem cell maintenance

The *let-7* gene was identified by genetic mutation as an essential regulator of temporal patterning in the nematode *Caenorhabditis elegans*, and was later cloned and categorized as one of the first-defined miRNAs [27-30]. Subsequent studies identified let-7 homologs in multiple species, including ten mature *let-7* family members in humans [31,32].

let-7 is important for stem cell differentiation in *C elegans* [27,33], and for both cellular differentiation and proliferation in humans [34,35]. In *C elegans*, *let-7* controls the fourth larval-to-adult transition [27,36]. During this transition, the self-renewing daughter cell ceases to proliferate and undergoes terminal differentiation.

In worms deficient for *let-7*, additional cellular divisions occur, leading to an increased number of seam cells and a bursting vulva phenotype that ultimately results in death [27]. Similar to *C elegans*, the mammalian homologs of *let-7* are important for regulating cellular proliferation and development, and have been implicated in diseases such as cancer. For example, reduced levels of multiple *let-7* family members are associated with tumorigenesis in tissues such as lung [23], breast [34], colon [37] and ovary [38]. This association has been attributed to the function of *let-7* as a tumor suppressor and suggests that *let-7* may be subject to modes of regulation that are altered in cancerous states.

Consistent with the idea that *let-7* functions as a tumor suppressor, mature *let-7* levels are reduced in multiple cancers through molecular events that can include deletion and mutation of *let-7* [39], epigenetic silencing of the *let-7* loci [40], and the regulation of *let-7* processing [41,42]. The processing of all miRNAs begins in the nucleus, where the primary transcript is microprocessed to a shorter (~ 70 nucleotide [nt]) precursor miRNA (pre-miR) through the action of the RNase III enzyme Drosha and its RNA-binding partner DGCR8 [43]. The shorter pre-miR is translocated into the cytoplasm by exportin 5 [44] and undergoes a final round of processing by Dicer to produce an ~ 22 nt dsRNA containing the mature miRNA [45]. Similar to the global miRNA processing enzymes Drosha, DGCR8 and Dicer, *let-7* miRNA processing is also regulated by an additional RNA-binding protein, Lin-28 [41,42]. Lin-28 binds to the loop structure of *let-7* in a sequence-specific manner, preventing pre-*let-7* from being processed to the mature miRNA form [42]. A recently identified cofactor involved in this process is the uridylyl transferase TUTase4, which is recruited by Lin-28 to add an oligouridine tail to pre-*let-7*, leading to a block in Dicer processing and *let-7* degradation [46-48]. The maturation of the *let-7* miRNA, which is directed by the action of Lin-28, contributes to the modulation of this miRNA and represents one of the many mechanisms by which *let-7* levels are reduced, leading to subsequent increases in the expression of *let-7* target genes.

Oncogenic Ras is a target of *let-7*

Ras as an oncogenic protein

The Ras proteins, which were identified in the rat genome in 1981 and have homologs in both mice and humans, belong to a large class of proteins with intrinsic guanosine triphosphate (GTP)ase activity [49-51]. Ras activity is suppressed when bound by guanosine diphosphate (GDP); a loss of GDP results in the high-affinity binding of GTP to Ras, leading to Ras activation [52]. Activated Ras triggers downstream effector pathways, including Raf/MAPK/ERK and PI3K pathways, that ultimately result in the activation or expression of genes involved in proliferation and survival or differentiation, depending on the cellular context (for a review of Ras signaling pathways in cancer, see reference [53]).

The first indications that Ras proteins may have oncogenic function were derived from a series of studies that identified an activated form of Ras in bladder and lung cancers [54-56]. Subsequent studies established an association between various Ras oncogenes and particular types of human cancer. For example, *KRAS* mutations occur frequently in pancreatic carcinomas [57] and lung cancers [58,59], while *HRAS* alterations are most often observed in salivary gland tumors [60]. Ras-induced GTP hydrolysis inactivates the protein. Altered Ras activity results from the loss of intrinsic GTPase activity [61-63]. Inactivation of the intrinsic GTPase activity of Ras is the most common genetic alteration of this protein, and is caused by a $Gly^{12}Val$ mutation in K-Ras [64]. This mutant has decreased GTP hydrolysis activity that favors the formation of the active oncogenic form, leading to persistent K-Ras signaling.

Because of the oncogenic nature of Ras, various approaches have been explored to suppress Ras activity. Unfortunately, attempts to develop therapies that directly block the function of the Ras oncoprotein have failed. As noted in the previous paragraph, Ras proteins have a high affinity for GTP, an observation that has discouraged attempts to identify GTP analogs as potential Ras inhibitors. Additionally, K-Ras, a Ras protein that plays a major role in human tumors, was demonstrated to be refractory to small-molecule inhibitors that target Ras processing enzymes [65]. Other therapeutics that have been developed to target downstream effectors of Ras signaling include Raf inhibitors and drugs that block MAPK/ERK activity and various steps in the PI3K pathway, the other effector arm of Ras activation [4]; however, multiple Ras effector pathways are required for cellular transformation [66-69]. For this reason, blocking oncogenic Ras could lead to the inhibition of multiple downstream Ras targets and decreased transforming potential. Similarly, suppression of upstream activators of Ras signaling also has clinical relevance, the most well-known of which is the EGFR. Small-molecule inhibitors of EGFR have demonstrated modest clinical success; however, mutations in Ras frequently rendered these therapeutics ineffective [70]. Furthermore, subsequent mutations in the EGFR act to abrogate the efficacy of these inhibitors [71,72]. Interestingly, mutations in Ras, EGFR and Raf are mutually exclusive of each other in a single tumor [73], suggesting that activation of the Ras pathway by any of these processes is sufficient to promote and sustain tumorigenesis.

let-7 in the regulation of Ras

Ras was also identified as a direct molecular target of the miRNA *let-7*, providing an additional mechanism to regulate Ras activity with therapeutic potential [23]. Multiple putative *let-7*-binding sites were discovered within 3'-UTRs of mRNAs encoding the three founding Ras oncogenes, *HRAS*, *NRAS* and *KRAS* [23]. Additionally, in lung cancer cells, overexpression of the *let-7* pre-miR reduced K-Ras protein levels [23], decreased cell

proliferation [24] and increased radiosensitivity [74]. Moreover, inverse relationships were observed between K-Ras protein levels and mature *let-7* levels in tissues from patients with NSCLC [23]. High K-Ras levels were associated with low *let-7* levels in tumor tissue, while *let-7* levels were elevated in normal adjacent tissue and correlated with low levels of K-Ras protein; these observations provided the first indication that Ras is controlled by *let-7* directly.

let-7 as an anticancer agent

The ability of *let-7* to act as both a cancer-preventative and cancer-therapeutic agent was established in subsequent *in vivo* studies using an autochthonous mouse model of NSCLC [24]. These animals possess the conditionally active $Gly^{12}Asp$ mutation of K-Ras under the control of a lox-STOP-lox cassette (a cassette that is used to control the expression of mutant *KRAS* in a Cre recombinase (Cre)-dependent manner). Following the intranasal administration of adenovirus expressing Cre (Ad-Cre), the K-Ras $Gly^{12}Asp$ mutant protein was expressed and, within 4 weeks, all of the mice had developed lung carcinomas [24]. When *let-7* was administered to the lungs of K-Ras $Gly^{12}Asp$ mutant animals concomitantly with Ad-Cre, a tumor preventive effect was observed [24]; tumor burden was decreased by 66% in the *let-7*-treated group compared with the control group. Additional studies have supported these initial observations. In one study, Jacks and colleagues observed a reduction in the number and size of lung tumors in the conditional mouse model K-Ras $Gly^{12}Asp/p53^{-/-}$ following the overexpression of *let-7* [26]. Additionally, in a recent study by Peng and colleagues, ectopic overexpression of *let-7* suppressed tumor formation in an A549 xenograph mouse model of NSCLC [75]. Tissue derived from the *let-7*-overexpressing group had decreased K-Ras and c-Myc protein levels, both of which are *let-7* target proteins [75]. These data suggest that the ectopic expression of *let-7* can prevent K-Ras tumor formation. Recently, the therapeutic efficacy of *let-7* in animals with preformed K-Ras $Gly^{12}Asp$ tumors was demonstrated [25]. The lungs of animals were first exposed to Ad-Cre to activate K-Ras and allow tumor development and growth. *let-7* was then administered and the therapeutic response to the miRNA was assessed at week 10 following K-Ras $Gly^{12}Asp$ expression. Intriguingly, not only did *let-7* suppress further tumor formation in these animals, the miRNA also reduced the size of tumors that were formed prior to treatment with *let-7* [25]. These data are highly supportive of a role for *let-7* in suppressing the formation of K-Ras $Gly^{12}Asp$-induced tumors *in vivo*. Moreover, in addition to a role in suppressing K-Ras, *let-7* itself is subject to multiple modes of regulation, including epigenetic silencing and post-transcriptional processing, and mature *let-7* miRNA levels are reduced in other cancers in addition to NSCLC, such as ovarian cancer [76], retinoblastoma [77], thyroid cancer [78], hepatocellular carcinoma [79] and melanoma [80]. Therefore, future research should focus on therapeutics that target *let-7* in more advanced cancers and cancers of various etiologies.

let-7 in cancer stem cells

A notable link between miRNA expression levels and stem cell maintenance has been demonstrated recently (for a review see reference [81]). Because of the self-renewing characteristics of cancer stem cells (CSCs), this cell type can sustain tumor populations. The first direct evidence in support of this tumor-sustaining function of CSCs was obtained in 1997, following the discovery that leukemia originates from hematopoietic stem cells [82]. Since this study, multiple CSC populations have been described, including those in breast [83], colon [84], brain [85], and head and neck cancers [86]. Moreover, maintenance and survival of CSCs are partially regulated by *let-7* [34]. For example, the overexpression of *let-7* in breast CSCs was demonstrated to reduce proliferation, mammosphere formation and the proportion of undifferentiated cells, all of which correlated with a decrease in the two *let-7* targets Ras and HMGA2 [34]. Conversely, antagonizing the activity of *let-7* with antisense oligonucleotides was demonstrated to enhance the self-renewal of CSCs *in vitro*.

The global loss of mature miRNAs results in the inability of stem cells to silence self-renewal. This phenomenon was described in detail in embryonic stem cells (ESCs) that were devoid of DGCR8 [87], a protein required for miRNA biogenesis. DGCR8-null ESCs were unable to silence self-renewal, leading to sustained ESC proliferation [87]. In support of the tumor suppressor role of *let-7*, the ectopic expression of *let-7* in $DGCR^{-/-}$ cells was demonstrated to suppress self-renewal [88], while in wild-type cells, the repression of *let-7* increased self-renewal. The ability of cells to self-renew in the absence of *let-7* is postulated to be the result of an increase in the *let-7* negative regulator Lin-28. Indeed, Lin-28 in combination with Oct-4, Nanog and Sox-2 have been demonstrated to reprogram human somatic fibroblasts to become pluripotent [89]. Furthermore, Lin-28 has been identified as a factor that promotes transformation and is associated with advanced human malignancies [90]. In epithelial ovarian cancer, a subpopulation of stem cell-like cells was observed to express high levels of both Lin-28 and Oct-4; the expression of both proteins correlated with advanced tumor grade, while siRNA-mediated knockdown of Lin-28 and Oct-4 resulted in diminished tumor growth and survival [89]. Moreover, the knockdown of both Lin-28 and TUTase4 resulted in a reduction of stem cell markers [46]. Conversely, Lin-28 levels were elevated following the transition of immortalized breast cells to a stably transformed line that formed self-renewing mammospheres containing CSCs [20]. These studies implicate Lin-28 as a central mediator in cellular transformation and stem cell maintenance, and suggest that Lin-28 represents an additional molecular target for cancer therapeutics.

NFκB directs suppression of *let-7* and is required for K-Ras-mediated oncogenesis

Since the discovery of NFκB in 1986 [91], it has been one of the most intensely studied transcription factors that is involved in immune system responses and in the deregulation of signal pathways observed in multiple diseases such as cancer. NFκB is believed to act as a link between inflammation and cancer. In fact, inflammation, particularly chronic inflammation, has been associated with cancer. Several well-established examples include ulcerative colitis and colorectal cancer [92], hepatitis and hepatocellular carcinoma [93], and gastritis and gastric cancers [94]. Moreover, multiple studies have determined an association between activated NFκB and tumorigenesis. For example, overrepresentation of nuclear NFκB has been observed in several cancers, including cervical and colorectal carcinomas [95,96], prostate [97,98], pancreatic [99], breast [100,101] and lung cancer [102,103], and head and neck tumors [104]. Furthermore, individuals with elevated NFκB levels are refractory to radiotherapies and many chemotherapies such as taxanes [105], gemcitabine [106], cisplatin [107] and etoposide [108]. These therapies themselves also result in further NFκB activation, triggering a positive feedback loop. Consequently, NFκB not only represents a promising single-agent molecular target for cancer intervention, but combinatorial treatments of NFκB inhibitors with traditional chemotherapies should enhance the efficacy of chemotherapeutics. Indeed, many non-steroidal anti-inflammatory drugs (NSAIDs) with cancer therapeutic and chemopreventive effects act through suppressing NFκB [109].

Although much is known regarding the biology of NFκB and its targets, the link between NFκB and K-Ras was identified only recently. Two research groups independently identified NFκB signaling as an essential component to K-Ras-mediated tumor formation [110,111]. In one study, a systematic RNAi approach was used by Hahn and colleagues to demonstrate that oncogenic K-Ras-expressing cancer cells require TANK-binding kinase 1 (TBK1) for survival [111]. TBK1 is a non-canonical inhibitor of IκB kinase that, upon activation, was demonstrated to cause the nuclear accumulation and subsequent activation of NFκB. Moreover, the suppression of TBK1 in cell lines with activated K-Ras increased apoptosis markedly, leading to the repression of K-Ras-mediated survival [111]. In a second study, the role of p53 in this survival process was elaborated [110]. In both cells and animals that were K-Ras $Gly^{12}Asp$/p53-null, NFκB was revealed to accumulate in the nucleus. Nuclear NFκB was demonstrated to bind to the promoter of target genes, many of which are involved in proliferation and survival, and induced their expression. Nuclear NFκB was not identified in cells with either the K-Ras $Gly^{12}Asp$ mutation or loss of p53; this phenomenon only persisted when both mutations were present [110]. As discussed in the following sections, one mechanism responsible for p53-mediated antiproliferative effects is the suppression of NFκB. It is likely that in a p53-null background, NFκB can promote survival through the activation of K-Ras (see Figure 1).

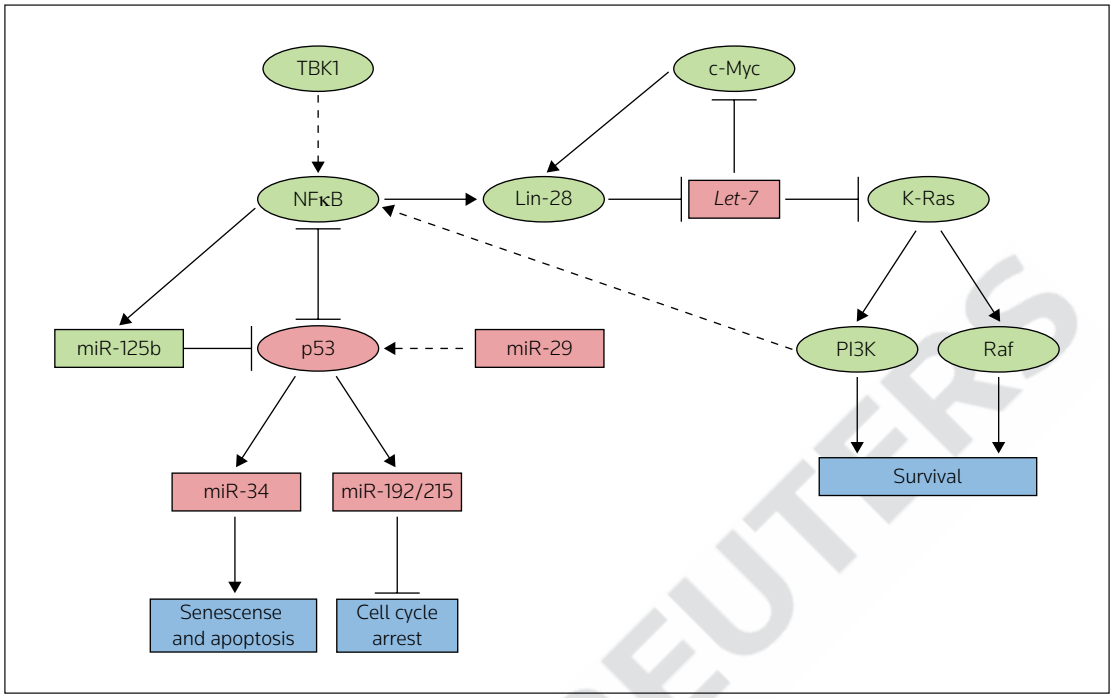
The link between NFκB and K-Ras-mediated tumor formation is under investigation. For example, Struhl and colleagues have suggested that *let-7* may provide a link between inflammation and cancer [20]. In this study, levels of *let-7* were reduced in response to active NFκB. The reduced levels of *let-7* were attributed to the direct transcriptional regulation of *LIN28B* by NFκB [20]. Binding elements for NFκB are present in the promoter for *LIN28B*, and chromatin immunoprecipitation studies have verified that the binding of NFκB to the element was enhanced following stimulation with tamoxifen, an activator of NFκB. *LIN28B* expression was also rapidly induced following stimulation with tamoxifen, leading to reduced levels of mature *let-7* miRNA. [20] Additionally, Myc, a *let-7* target, functions in a feedback loop to suppress the expression of *LET-7* through a direct association with the *LIN28B* promoter, resulting in the transcriptional upregulation of *LIN28B* [112] (Figure 1). Interestingly, Lin-28 proteins were overrepresented in primary human tumors, CSCs and human cancer cell lines, all of which have been linked to the repression of *let-7* miRNAs and derepression of *let-7* targets [90]. Whether these tumors and cell lines display constitutive NFκB activity would be of interest. Although not described in detail, based on the findings from the Jacks, Hahn and Struhl research groups and considering the role of *let-7* in suppressing K-Ras protein expression, it seems likely that NFκB may be necessary for the activation of the K-Ras $Gly^{12}Asp$ mutant protein via the suppression of *let-7* by Lin-28; *let-7* would therefore provide the link between NFκB and K-Ras (Figure 1). As such, mature *let-7* miRNA-based therapies could be used to treat tumors with oncogenic K-Ras and activated NFκB or to treat individuals receiving chemotherapy.

p53 as a target and transcription factor of miRNAs

Mutant p53 promotes tumorigenesis

It is almost impossible to study the molecular events leading to cancer without investigating one of the most intensely studied tumor suppressor proteins, p53. p53 is a stress-response transcription factor that induces target gene activation, which, in turn, suppresses cellular transformation, mainly by the induction of growth arrest, apoptosis, DNA repair and differentiation in damaged cells [113]. In the absence of wild-type p53, this checkpoint is removed and damaged cells are propagated, thereby promoting tumorigenesis. Mutations in the *TP53* gene encoding p53 are among the most common mutations observed in tumors of almost all origins. Somatic mutations in *TP53* occur in approximately half of all sporadic cancers [114]; for example, germline mutations cause a predisposition to a rare type of cancer known as Li-Fraumeni syndrome [114,115]. Regardless of whether a p53 mutation is in the germline or is somatic, a loss-of-heterozygosity follows, suggesting that a selective force acts to remove the remaining wild-type p53 allele [116,117].

Figure 1. MicroRNAs function in the regulation of signaling pathways that mediate tumor formation.



The *let-7* family of microRNAs (miRNAs) may represent a link between the NFκB inflammatory and K-Ras prosurvival signaling cascades. Additionally, a novel connection between NFκB and p53 may include miR-125b. The three additional miRNAs included in this pathway are miR-29 that regulate p53 and three that are downstream of p53 activation. The expression of miR-29 results in p53 activation through an indirect mechanism that targets two p53 suppressor proteins, p85α and Cdc42. Both the miR-34 family and miR-192/miR-215 are activated in response to p53 activation. These miRNAs lead to independent downstream cellular functions, including senescence and apoptosis or cell cycle arrest, respectively. Circles represent proteins and squares represent miRNAs. Molecules in green are involved in proliferation, growth or survival, while those in red are pro-apoptotic or growth inhibitors.
TBK1 TANK-binding kinase 1

Despite significant developments within the p53 research field, the translation of this knowledge into the clinic has not yet been accomplished. Several p53-based therapeutic approaches have been developed and their clinical use is under investigation, including compounds that reactivate specific p53 mutants, non-genotoxic p53-activating drugs and p53 gene-delivery approaches [118]. An insight into the future of potential p53-based therapeutics is gained when reviewing recently discovered miRNAs that are involved in regulating p53 expression and those that are direct p53 targets.

Regulation of p53 by miRNAs

The induction of p53 following genotoxic stress is dependent on both the inhibition of negative regulators of p53 and the increased translation of p53 mRNA, two processes that have been determined to have miRNA components. Multiple proteins act to suppress the activation of p53, including the recently elucidated proteins p85 and cell division cycle 42 (Cdc42) [119]. p85α is the regulatory subunit of PI3K [120] and Cdc42 is a Rho family GTPase [121]; both proteins were demonstrated to be regulated by miR-29 [119]. In a screen for miRNAs that modulate p53 activity, the miR-29 family members (miR-29a, miR-29b and miR-29c) were identified as enhancers of p53 levels. These same miRNAs also induced apoptosis in a p53-dependent manner [119]. The molecular events that contributed to apoptosis included direct transcriptional repression by miR-29 of the two p53 negative regulators p85α and Cdc42 [119]. Additionally, indirect regulation of p53 by miR-29 represents one possible explanation as to why some tumors without p53 mutations have decreased p53 activity. The *TP53* gene is mutated in more than 50% of tumors, and while mutations in the *TP53* locus have been excluded in the remaining 50%, alterations in p53 signaling have not [122-124]. In fact, many of these tumors have decreased p53 activity compared with normal tissue. As such, in a subset of tumors without mutant *TP53*, the modulation of p53 activity by miR-29 may be a contributing factor in tumor formation. An additional subgroup of tumors that are devoid of genomic *TP53* mutations may have altered p53 activity through the action of another miRNA, miR-125b. miR-125b was the first miRNA that was identified to bind directly to the 3'-UTR of p53 mRNA [22]. The binding of miR-125b repressed endogenous p53 protein levels and resulted in a marked decrease in p53-induced apoptosis. Moreover, miR-125b is not only required for correct embryonic development, but this miRNA is also altered in several cancers, including childhood leukemia [125] and breast carcinoma [126], and is required for the suppression

of glioma stem cell proliferation [127]. Interestingly, in addition to p53 being a novel miR-125b target, miR-125b was recently identified as a direct NFκB target following parasitic infection [128]. As such, miR-125b may represent an additional miRNA linking the p53, NFκB and K-Ras signaling pathways.

Regulation of miRNAs by p53

Not only is p53 subject to regulation by miRNAs, this protein also functions as a transcription factor to induce the expression of miRNAs with tumor suppressor activity. The first miRNAs identified as being regulated transcriptionally by p53 belong to the miR-34 family [21,129-133]. The miR-34 miRNAs are a family of three miRNAs, two of which are encoded by the same primary transcript [132]. Both primary transcripts have functional p53-binding sites within their promoters and are directly and positively transactivated by p53 [133]. Moreover, the expression of miR-34 family members suppresses genes that regulate apoptosis, cell cycle progression, DNA repair and angiogenesis post-transcriptionally [132]. As such, miR-34 miRNAs are postulated to be critical mediators that link p53 and several of the apoptotic roles this protein performs. Indeed, ectopic miR-34 expression in p53-null gastric cancer microspheres has been demonstrated to enhance programmed cell death and cell cycle arrest [134].

Additional miRNAs have been identified as participants in the p53 tumor-suppressor network, including the homologs miR-192 and miR-215, both of which are encoded by a single transcript [135-137]. The DNA sequence upstream of the *mir-192/mir-215* cluster binds p53 [136,137]. Although the exact location of the p53-binding site for this pri-miRNA transcript has not been confirmed experimentally, the binding of p53, which is enhanced by activators of p53, resulted in increased levels of both miR-192 and miR-215 [136,137]. Similar to miR-34, miR-192 and miR-215 are underrepresented in cancerous tissues, most notably in colorectal cancers [135], and levels of these miRNAs correlate with p53 status. Functionally, both miR-192 and miR-215 induce cell cycle arrest by repressing the expression of several transcripts that regulate the G_1 and G_2 cell cycle checkpoints, although cell cycle arrest mediated by miR-192 and miR-215 appears to be partially dependent on the presence of functional p53 [135,136]. Interestingly, the molecular events leading to suppression of cell colony formation by miR-192 and miR-215 are different to the events induced by miR-34a; miR-34a can induce cellular senescence and apoptosis [132], while one of the major phenotypes observed in cells that overexpress miR-192 or miR-215 was loss of adhesion [138]. Additionally, the treatment of cells with miR-192 or miR-215 induced cell detachment [137], which was not observed to the same extent in cells that overexpressed miR-34. Therefore, these two miRNA families, which are both regulated by p53, act to suppress growth and proliferation by different mechanisms. The miR-34 family acts mainly to induce senescence and apoptosis, while the miR-192/miR-215 cluster regulates p53-dependent cell cycle events (Figure 1). Therefore, a new therapeutic approach for p53-null or mutant tumors based on the molecular biology of p53 signaling could involve the restoration of p53-dependent miRNAs, including miR-34 and/or miR-192 and miR-215.

Additional miRNAs that are affected by p53 status include those that are regulated by p53 indirectly. As shown in Figure 1, p53 acts as a suppressor of NFκB. One explanation for this effect is that the p65 subunit of NFκB and p53 compete for a limited pool of the coactivators p300 and CBP, which are required for the transcriptional activities of both p53 and NFκB [139,140]. In the absence of p53, increased levels of p300 and CBP are freely available for binding to NFκB. Therefore, NFκB targets are transcribed more readily and proliferation occurs. Jacks and colleagues determined that a loss of p53 was necessary for the NFκB-mediated development of K-Ras $Gly^{12}Val$ mutant tumors [110]. This requirement for the loss of p53 in tumor formation may be caused by the suppression of NFκB activity in the presence of wild-type p53. In p53-null cells, NFκB would have increased activity and, although not tested in this background, may contribute to increased levels of Lin-28. Consequently, the levels of *let-7* would be reduced, oncogenic *KRAS* would no longer be controlled by the tumor suppressor *let-7*, and proliferation would proceed (see Figure 1). Accordingly, the addition of mature *let-7* to these cells would suppress the K-Ras $Gly^{12}Val/p53^{-/-}$ growth-promoting phenotype, while primary *let-7* processing may be blocked by NFκB-induced *LIN28* expression, although this remains to be tested.

The role of miR-34 in cancer stem cells

miR-34 family members have also been implicated in the maintenance and survival of cancer stem cells. The targets of miR-34 include Notch, c-Met and Bcl-2, all of which are important for the self-renewal of stem cells [21,133,141]. In 2009, Xu and colleagues presented findings suggesting that miR-34 may inhibit pancreatic CSC self-renewal [142]. In this study, the markers CD44 and CD133 were used to identify pancreatic CSCs from human tumor tissue. CD44+CD133+ MiaPaCa2 cells were selected and determined to have reduced miR-34a/b/c levels compared with CD44-CD133- cells. The CSCs were observed to develop tumorspheres that were sensitive to miR-34 overexpression, as demonstrated by the ability of ectopically expressed miR-34a to inhibit CD44+CD133+ tumorsphere-forming and tumor-initiating CSCs in p53-mutant pancreatic cancer cells [142]. Furthermore, miR-34 was demonstrated to induce apoptosis in glioma stem cells partly through the inhibition of Notch-1/Notch-2 [143], suggesting that the restoration of miR-34 may attenuate glioma CSC growth and survival.

Conclusion

While no single gene is altered in all cancers, the vast majority of tumors contain mutations in p53 and a significant number also harbor an activating *KRAS* allele. Additionally, it has long been established that tumors

with constitutively activated NFκB are refractory to many traditional treatments, and that these treatments alone can further activate NFκB [105-108]. As the biology of miRNAs is revealed, insight into the potential that these small molecules have in treating diseases with aberrant signaling such as cancer is gained. The use of radiotherapy to sensitize tumor cells may decrease *let-7* processing through activation of NFκB and subsequent induction of the pluripotent *let-7* repressor Lin-28. Blocking the expression of Lin-28 is important not only for inducing cancer regression, but also as a potential mechanism to overcome elevated Lin-28 levels in CSCs. These recent findings can guide the development of future miRNA-based therapeutics. The use of mature *let-7* miRNA (or pre-*let-7* mimics that lack the loop structure), which is not subject to suppression by Lin-28, may have increased efficacy in combination with radiation and chemotherapies that activate NFκB. Additionally, combining miRNA therapeutics with currently used chemotherapies may also act to enhance the efficacy of these traditional therapies. For example, the efficiency of methotrexate was enhanced when this agent was combined with miR-192, a p53-dependent miRNA [135]. In this study, the addition of miR-192 and methotrexate reduced proliferation by 60%, compared with cells treated with methotrexate only [135].

Although increasing the levels of individual tumor suppressor miRNAs has demonstrated considerable promise in experimental studies [24,25,144-146], therapy with a single miRNA will most likely not eradicate human cancers. While the vast majority of animal models harbor single mutations in well-documented oncogenes and tumor suppressor genes, human cancers have mutations in multiple genes. Studying these models provides insights into the molecular mechanisms of miRNA treatments; however, the cellular and mouse models described in this review are artificial, and while these models are currently the most representative of human carcinogenesis, they cannot be considered to be exact replicas [147]. Generating animals with mutations in multiple targets will more closely represent human cancers. For example, Jack and colleagues have generated a conditional mouse model that has both the K-Ras $Gly^{12}Val$ mutation and is p53-null [148]. Activating both mutant alleles in the lungs of these animals resulted in a median lifespan of 170 days, compared with K-Ras $Gly^{12}Val$ mutant animals that had a median survival of 266 days [148]. Intriguingly, *let-7* administered to the lungs of these animals as a preventive mechanism against tumor formation resulted in a reduction in tumor burden; however, lifespan remained unaltered [26]. The K-Ras $Gly^{12}Val$ mutant/p53-null animals may be more responsive to a dual treatment using both *let-7* and miR-34, although this remains to be determined. These studies should promote the use of dual miRNA therapy in human cancers that are dependent on one or both of these pathways. The notion that p53 suppresses NFκB suggests that expression of p53 or administration of NSAIDs in combination with *let-7* therapies may represent a new therapeutic approach in individuals with alterations in Ras, NFκB and p53 signaling.

Many of the studies relating to miRNA therapeutics are at an early stage and various questions remain. For example, the long-term reduction of tumors, perhaps correlating with increased survival, has not been demonstrated. However, these small RNA molecules may truly represent a 'magic bullet'. Potential exists for the use of miRNA-based therapeutics as combination therapies to sensitize tumors when combined with additional treatments, such as traditional chemotherapies and radiation. It will be interesting to determine if the reduction of Lin-28 levels by the combination treatment of *let-7* with NFκB inhibitors can enhance *let-7* processing. Based on the current understanding of these molecules and the exponential growth in the field, the future appears promising for miRNA therapeutics.

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•• of outstanding interest
• of special interest

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