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miR-221&222 Regulate TRAIL Resistance and Enhance Tumorigenicity through PTEN and TIMP3 Downregulation

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SUMMARY

Lung and liver cancers are among the most deadly types of cancer. Despite improvements in treatment over the past few decades, patient survival remains poor, underlining the need for development of targeted therapies. MicroRNAs represent a class of small RNAs frequently deregulated in human malignancies. We now report that *miR-221&222* are overexpressed in aggressive non-small cell lung cancer and hepatocarcinoma cells, as compared with less invasive and/or normal lung and liver cells. We show that *miR-221&222*, by targeting PTEN and TIMP3 tumor suppressors, induce TRAIL resistance and enhance cellular migration through the activation of the AKT pathway and metallopeptidases. Finally, we demonstrate that the *MET* oncogene is involved in *miR-221&222* activation through the c-Jun transcription factor.

INTRODUCTION

Despite advances in early detection and standard treatment, non-small cell lung cancer (NSCLC) and hepatocellular carcinoma (HCC) are often diagnosed at an advanced stage and have poor prognoses. The development of innovative, targeted therapies may represent an alternative for the treatment of these cancers. Promoting apoptosis is a possible goal for drug development (Fesik, 2005). TNF-related apoptosis-inducing ligand (TRAIL) is currently being tested in clinical trials; however, the resistance of many tumors, including NSCLC and HCC, to TRAIL represents obstacles to its clinical application. Recently, the discovery of microRNAs (miRNAs) has expanded our knowledge regarding the complex control of gene expression; miRNAs are small non-coding RNAs of 19–25 nt that can block mRNA translation and/or negatively regulate its stability (Ambros, 2004). At this time, over 500 different miRNAs have been identified in human cells (Griffiths-Jones et al., 2006) and evidence indicates that regulation of miRNA levels is very important for proper growth and differentiation of many cell types and tissues (Bartel, 2004; Krichevsky et al., 2003). Dysregulated miRNA expression is a common feature of solid and hematopoietic malignancies (Calin et al., 2002; Ruvkun, 2006), and there is strong evidence that they function as a class of oncogenes or tumor suppressor genes (Calin and Croce, 2006). PTEN is one of the most commonly altered tumor suppressors in human cancers and a key regulator of cell growth and apoptosis (Di Cristofano and Pandolfi, 2000). Functionally, PTEN converts phosphatidylinositol-3,4,5-trisphosphate in the cytoplasm to phosphatidylinositol-4,5-bisphosphate, thereby directly antagonizing the activity of

SIGNIFICANCE

Drug resistance and tumor metastasis represent the main obstacles to successful cancer treatment. MicroRNAs are small noncoding RNAs that show expression loss or gain in most cancers, and there is growing evidence that they play substantial roles in the pathogenesis and prognosis of human malignancies. In this study we found that MET, through Jun transcriptional activation, upregulates *miR-221&222* expression, which, in turn, by targeting *PTEN* and *TIMP3*, confers resistance to TRAIL-induced cell death and enhances tumorigenicity of lung and liver cancer cells. The results suggest that therapeutic intervention, involving the use of microRNAs, should not only sensitize tumor cells to drug-inducing apoptosis but also inhibit their survival, proliferation, and invasive capabilities.

phosphatidylinositol 3-OH kinase (PI3K) (Leevers, 1999). Its inactivation results in constitutive activation of the PI3K/AKT pathway and in subsequent increase in protein synthesis, cell-cycle progression, migration, and survival (Li and Sun, 1998). In addition, various studies have demonstrated that the protein phosphatase activity of PTEN inhibits activation of mitogen-activated protein kinase via several pathways (Lee et al., 2005; Saito et al., 2003). The matrix metalloproteinases (MMPs) are a family of zinc proteases involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, tissue and bone remodeling, wound healing, and angiogenesis. (Nagase et al., 2006; Chakraborti et al., 2003). Within the extracellular matrix, the tissue inhibitors of metalloproteinases (TIMPs), of which there are four family members (TIMP1–4) (Cruz-Munoz and Khokha, 2008), inhibit the activity of MMPs by binding with a 1:1 stoichiometry to the active site (Bode et al., 1994). Previous studies have shown that overexpression of TIMP3 in vascular smooth muscle cells and melanoma cell lines inhibits invasion and promotes apoptotic cell death (Ahonen et al., 1998; Baker et al., 1998). MET, also known as c-Met, is a membrane receptor for the hepatocyte growth factor (HGF)/scatter factor. MET is normally expressed by cells of epithelial origin, while expression of HGF is restricted to cells of mesenchymal origin. Upon HGF stimulation, MET stimulates the invasive growth of cancer cells and increases their metastatic potential, principally through increased phosphorylation of ERK1/2 and JNK (Song et al., 2007). Phosphorylated JNKs activate the oncoprotein c-Jun, which is known to form the activator protein-1 (AP-1) transcription factor as a homodimer or heterodimer with its partner c-Fos. Aberrant expression of HGF/scatter factor and its receptor MET often correlates with poor prognosis in a variety of human malignancies. In this study, we investigated, by in vitro and in vivo experiments, the role of *miR-221&222* in TRAIL resistance and tumorigenesis of NSCLC and HCC and their regulation through *c-Met* oncogene.

RESULTS

MiR-221 and *miR-222* Directly Target *PTEN* and *TIMP3* 3′UTRs

In a previous study, to identify mechanisms implicated in TRAIL resistance, we determined the miRNA expression profile in four NSCLC cell lines. We found that *miR-221* and *miR-222* are markedly upregulated in TRAIL-resistant (Calu-1) and semi-resistant (A459 and A549), versus TRAIL-sensitive, NSCLC cells (H460). The results indicated that *miR-221&222* modulate TRAIL sensitivity in lung cancer cells mainly by interfering with $p27^{kip1}$ expression and TRAIL-induced caspase machinery (Garofalo et al., 2008a). To identify *miR-221* and *miR-222* targets, we performed a bioinformatics search (Targetscan, Pictar, RNhybrid) for putative mRNA targets of both miRNAs. Among the candidate targets, 3′UTRs of human *PTEN* (nucleotides 200–207; NM_000314) and human *TIMP3* (nucleotides 2443–2449; NM_000362) contained regions that matched the seed sequences of *hsa-miR-221* and *miR-222* (Figure 1A). To verify that *PTEN* and *TIMP3* are direct targets of *miR-221&222*, *PTEN* and *TIMP3* 3′UTR, containing the *miR-221&222* binding sites, were cloned downstream of the luciferase open reading frame. These reporter constructs were used to transfect MEG01 cells, which express very low levels of *miR-221&222* (Figure 1B) and are highly transfectable (Freson et al., 2005). Increased expression of these miRs upon transfection, confirmed by qRT-PCR (Figure 1B), significantly affected luciferase expression, measured as relative luciferase activity (Figure 1C). Conversely, when we performed luciferase assays using a plasmid harboring the 3′UTR of *PTEN* and *TIMP3* mRNAs, where the binding sites for *miR-221* and *miR-222* were inactivated by site-directed mutagenesis, we observed a consistent reduction in *miR-221&222* inhibitory effect (Figure 1C). To determine if these miRNAs affect PTEN and TIMP3 expression in the H460 cellular environment, we analyzed the consequences of the ectopic expression of *miR-221&222* in H460 cells. Increased expression of these miRs upon transfection was confirmed by qRT-PCR (Figure 1D), and then the effects on endogenous levels of PTEN and TIMP3 were analyzed by western blot (Figure 1E). *MiR-221&222* overexpression significantly reduced the endogenous levels of PTEN and TIMP3 compared to H460 cells transfected with scrambled pre-miR. Conversely, knockdown of *miR-221&222* by 2′-O-me-anti-*miR-221* and 2′-O-me-anti- *miR-222*, confirmed by quantitative RT-PCR (qRT-PCR) (Figure 1F) in Calu-1 lung-derived cells with high levels of endogenous *miR-221&222*, increased the protein levels of PTEN and TIMP3 (Figure 1G). Intriguingly, by qRT-PCR, we found that *PTEN*, but not *TIMP3*, mRNA levels were strongly reduced in the *miR-221&222* transfected cells (Figure 1H), indicating that *miR-221&222* induce the degradation of *PTEN* mRNA while *TIMP3* is regulated by these miRNAs only at the translational level. In summary, these results supported the bioinformatics predictions indicating *PTEN* and *TIMP3* 3′UTRs as direct targets of *miR-221* and *miR-222*.

MiR-221 and *miR-222* Are Inversely Correlated with PTEN and TIMP3 Expression in NSCLC and HCC

Since PTEN is a major tumor suppressor, and since *miR-221&222* are upregulated in human hepatocarcinoma cells (Fornari et al., 2008), we decided to evaluate the endogenous levels of *miR-221* and *miR-222* by northern blot in large panels of primary NSCLCs and HCCs, compared with the normal counterpart. *MiR-221&222* expression was almost undetectable in normal lung and liver cells but highly expressed in the majority of tumor cell lines. Moreover, we found, as assessed by western blot, an inverse correlation between *miR-221&222* RNA expression and PTEN and TIMP3 protein expression in most cell lines analyzed (Figure 2A), confirmed also by qRT-PCR (Figure 2B). We did not check by qRT-PCR TIMP3 mRNA expression levels because we did not observe downregulation of TIMP3 mRNA after enforced *miR-221&222* expression (Figure 1H). These results suggested that high expression of *miR-221* and *miR-222* might be one of the mechanisms acting to negatively regulate PTEN and TIMP3 in NSCLC and HCC. To verify whether these miRNAs affected PTEN and TIMP3 endogenous levels also in HCC, we analyzed the effects of the ectopic expression of *miR-221* and *miR-222* in the Sk-Hep1 cell line, which expresses low levels of *miR-221&222*. As shown in Figure 3A, PTEN and TIMP3 proteins were clearly reduced in Sk-Hep1 cells upon *miR-221* and *miR-222* overexpression. Conversely, knockdown of *miR-221&222* by 2′-O-me-anti-*miR-221* and 2′-O-me-anti-*miR-222* in Snu-387 cells, which expressed high levels of

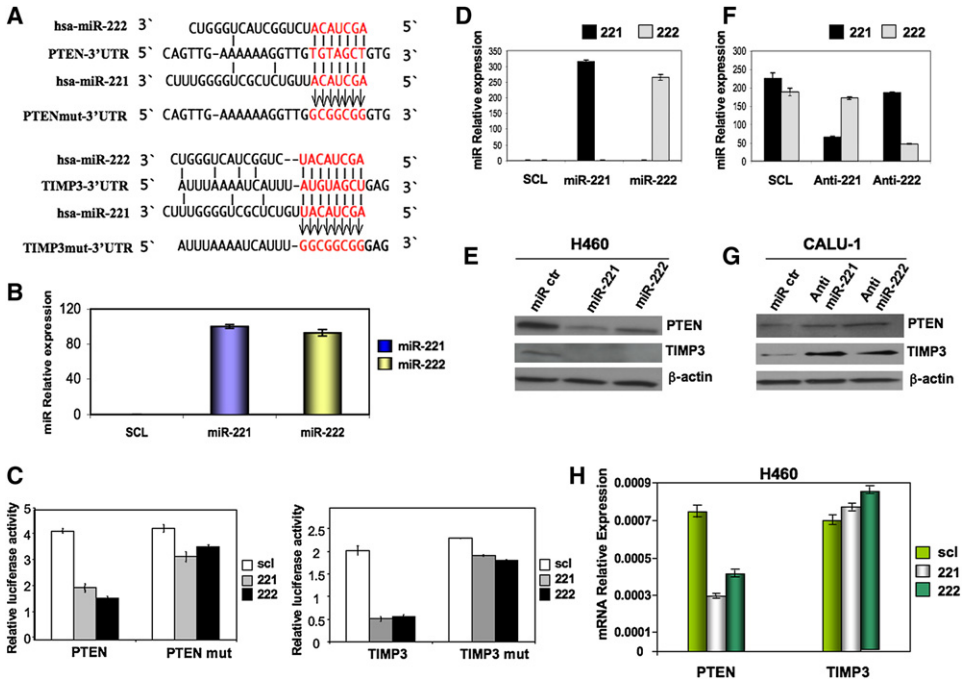


Figure 1. *PTEN* and *TIMP3* Are Targets of *miR-221&222*

(A) *PTEN* and *TIMP3* 3′UTRs contain one predicted *miR-221* and *miR-222* binding site. In the figure the alignment of the seed regions of *miR-221&222* with *PTEN* and *TIMP3* 3′UTRs is shown. The sites of target mutagenesis are indicated in red.

(B) qRT-PCR in MEG01 cells after enforced expression of *miR-221* and *miR-222*.

(C) *PTEN* and *TIMP3* 3′UTRs are targets of *miR-221&222*. pGL3-PTEN and pGL3-TIMP3 luciferase constructs, containing a wild-type (left side of the histograms) or mutated (right side of the histograms) *PTEN* and *TIMP3* 3′UTRs, were transfected into MEG01 cells. Relative repression of firefly luciferase expression was standardized to a transfection control. The reporter assays were performed three times with essentially identical results.

(D) qRT-PCR in H460 cells after enforced expression of *miR-221* and *miR-222*.

(E) *miR-221&222*-enforced expression decreases endogenous levels of PTEN and TIMP3 proteins in H460 NSCLC. H460 cells were transfected with either scrambled, *miR-221*, or *miR-222* for 72 hr. PTEN and TIMP3 expression was assessed by western blot. Loading control was obtained using anti-β-actin antibody.

(F) qRT-PCR showing *miR-221&222* downmodulation in Calu-1 cells after anti-miRs transfection.

(G) Western blot showing PTEN and TIMP3 expression after *miR-221&222* downregulation using anti-*miR-221&222*. Anti-*miR-221* and anti-*miR-222* were able to increase PTEN and TIMP3 expression in Calu-1 cell line.

(H) qRT-PCR of *PTEN* and *TIMP3* mRNA after *miR-221* and *miR-222* forced expression in H460 cells. *PTEN* but not *TIMP3* mRNA was downregulated by *miR-221* and *miR-222*. Data are presented as ± SD.

endogenous *miR-221&222*, increased the protein level of PTEN and TIMP3 (Figure 3A). Having noted that *miR-221&222* downregulate PTEN and TIMP3 expression in both NSCLC and HCC-derived cells in culture, we wondered if this regulation also occurs in vivo. To answer this question, we investigated *PTEN* mRNA and *miR-221&222* expression by qRT-PCR in primary lung tumor specimens, in comparison with normal human lung tissue samples. *MiR-221* and *miR-222* were almost undetectable in normal human lung samples and highly expressed in all the tumor samples analyzed. Of the 22 primary lung tumors examined, in fact, all of them exhibited downregulation of *PTEN* and overexpression of *miR-221&222* (Figure 3B). These data further support the finding that *PTEN* is a direct target of *miR-221&222* also in vivo. To corroborate these findings, in situ hybridization analysis was performed using 5′-dig-labeled LNA probes on hepatocarcinoma and normal liver tissues, followed by immunohistochemistry for PTEN and TIMP3 (Figure 3C). *MiR-221&222* and PTEN/TIMP3 expressions were inversely related in liver cancers and the adjacent normal/cirrhotic liver tissues. Liver cancer cells showed high expression of *miR-221&222* and rarely expressed PTEN or TIMP3 (Figure 3Cg, 3Ch, 3Ck, and 3Cl), whereas the adjacent non-malignant liver expressed PTEN and TIMP3 abundantly and rarely showed detectable *miR-221&222* signal (Figure 3C, a, b, e, and f). *MiR-221&222* and PTEN/TIMP3 expression were also inversely related in lung cancers and the adjacent normal lung tissues (see Figure S1 available online). The majority of cancer cells were positive for *miR-221* and *miR-222* and negative for PTEN (Figure S1F and S1G) and TIMP3 (Figure S1I and S1J). In Figure S1I and S1J, miRNA expression was evident in the cancer cells and TIMP3 expression in the surrounding cells. A strong *miR-222* signal (Figure S1, large arrow) was found in the nests of tumor cells that are infiltrating the adjacent fibrotic lung tissue (Figure S1K and S1L).

MiR-221&222 Induce TRAIL Resistance in NSCLC and HCC by Targeting *PTEN* and *TIMP3*

Since PTEN regulates the PI3K/AKT pathway, which plays a key role in multiple drug resistance (Garofalo et al., 2008b), including TRAIL (Kandasamy and Srivastava, 2002), and since TIMP3 is

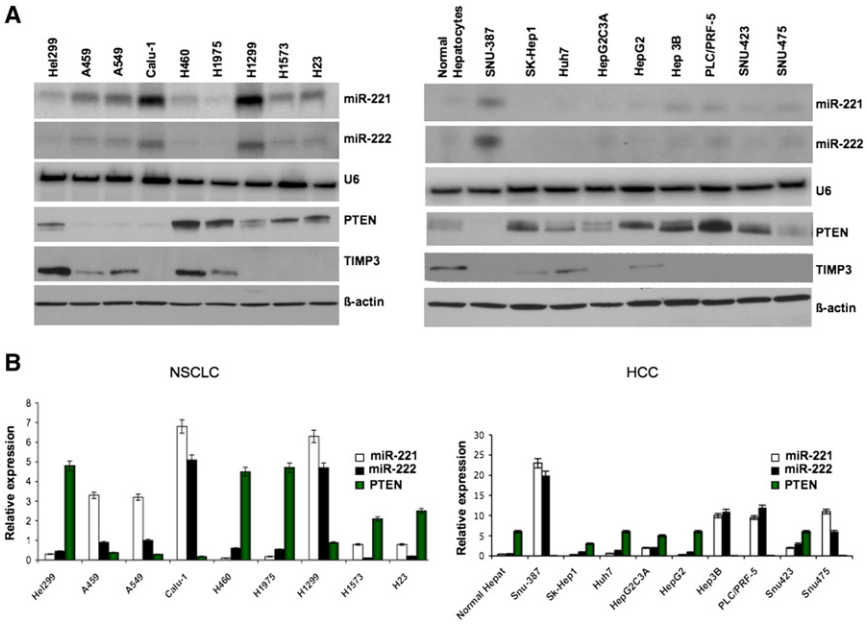


Figure 2. PTEN and TIMP3 Expression Is Inversely Related to that of *miR-221&222* in NSCLC and HCC

(A) *miR-221* and *miR-222* expression levels were assessed by northern blot analysis using 15 μg of total RNA for NSCLC and 10 μg of total RNA for HCC cells. Western blots of anti-PTEN and TIMP3 were performed using total protein extract (50 μg) isolated from the different NSCLCs and HCCs.

(B) qRT-PCR of *miR-221&222* and PTEN mRNA was performed by extracting RNA from the different NSCLC and HCC cells as described in the Supplemental Experimental Procedures. *miR-221&222* were inversely related to PTEN mRNA expression in all the different NSCLC and HCC cells. Data are presented as ± SD.

involved in apoptosis through activation of caspases (Lee et al., 2008a), we next examined the effects of *miR-221&222* and/or *PTEN-TIMP3* silencing on cell survival and TRAIL resistance in both NSCLC and HCC. First we performed a proliferation assay on five HCC-derived cell lines, three of them (HepG2, Sk-Hep1, and Huh 7) with low *miR-221&222* expression and two (PLC/PRF-5 and Snu-387) with high *miR-221&222* expression level (Figure 4A). Cells were exposed to TRAIL for 24 hr and then cell proliferation was assessed using an MTT assay. Interestingly, cells expressing low levels of *miR-221&222* underwent TRAIL-induced cell death, showing a very low proliferation rate, whereas cells overexpressing *miR-221&222* did not display sensitivity when exposed to soluble TRAIL (Figure 4A). Moreover, Annexin-FITC and caspase 3/7 assays on TRAIL-sensitive cell lines Sk-Hep1 cells, (Figures 4B and 4C), HepG2, and Huh7 (Figures S2A and S2B) revealed an increase of about 30%–40% in TRAIL resistance after *miR-221&222* overexpression, as well as after *PTEN* and *TIMP3* silencing by *PTEN* and *TIMP3* siRNAs. TRAIL-sensitive H460 cells also became more resistant to TRAIL-inducing apoptosis after PTEN and TIMP3 knockdown, as determined by caspase 3/7 activity (Figure 4D) and Annexin-FITC assay (Figure 4E), although PTEN silencing was more effective than TIMP3. Moreover, to further evaluate the contribution of these targets on TRAIL-inducing apoptosis, *PTEN* and *TIMP3* sequences were cloned in pCruz-HA plasmid (Santa Cruz Biotechnology, Inc.) and used to transfect Calu-1 TRAIL-resistant cells. Calu-1 cells became more sensitive to TRAIL-inducing apoptosis after PTEN and TIMP3 restoration, alone or in combination, as determined by caspase 3/7 activity (Figure 4D) and Annexin-FITC staining (Figures S3A and S3B). To further investigate the role of TIMP3 in TRAIL-inducing apoptosis the expression of caspase-3, -8, and -9, poly-ADP-ribose polymerase, and some of the molecules involved in the TRAIL-signaling pathway were tested by western blot after TIMP3 overexpression in the Calu-1 cell line (Figure S3C). Interestingly, the activation of poly-ADP-ribose polymerase and the caspase cascade were observed, as assessed by the appearance of the cleaved fragments. Moreover, Mcl-1 expression was downregulated while cytochrome *c* expression increased (Figure S3C). All together these results suggest an involvement of TIMP3 in both the extrinsic and intrinsic apoptotic pathways and highlight its role in TRAIL-inducing apoptosis. The same results were obtained after TIMP3 restoration in Snu-387 cells (data not shown). Because AKT is regulated by PI3K signaling and has been shown to be hyperactivated through the loss of PTEN, we investigated by immunostaining the expression and/or the activation of some of the proteins involved in the PI3K/AKT pathway after *miR-221&222* enforced expression in H460 cells or after *miR-221&222* silencing in Snu-387 cells. As shown in Figure 5A, the expression levels of PI3K, AKT, and its phosphorylated substrate, phospho-glycogen synthase kinase 3β, were elevated by ectopic expression of *miR-221&222* and, in contrast, were decreased by knockdown of *miR-221&222* in Snu-387 cells, suggesting that *miR-221&222* target the PTEN/AKT pathway (Figure 5B). Since *miR-221&222* has been suggested to induce tumorigenesis (Felicetti et al., 2008) and PTEN downregulation of phosphorylation influences the activation of proteins, such as ERKs and PAK1, involved in cellular migration and invasion (Lee et al., 2008b; Chan et al., 2008), we investigated the activation and expression levels of these proteins. We found an increase in ERK phosphorylation and PAK1 expression, as compared with H460 cells transfected with the control miR (Figure 5C). Interestingly, increased expression of metallopeptidase 3 and metallopeptidase 9 was also found, possibly a result of TIMP3 downregulation (Figures 5A–5C). To test if the activation of the previous proteins was PTEN and/or TIMP3 dependent, we silenced *PTEN* and *TIMP3* in H460 cells. As shown in Figures 5D and 5E, the activation of the ERKs and PAK1 is both PTEN and TIMP3 dependent, while AKT phosphorylation is PTEN dependent and MMP3 and MMP9 are upregulated after TIMP3 knockdown. Finally, because ectopic expression of *miR-221&222* reduces PTEN levels, leading to activation of the AKT pathway and inhibition of TRAIL-induced cell death, we wondered if AKT inhibition could override *miR-221&222*-induced cell survival and TRAIL resistance. Calu-1 and Snu-387 were

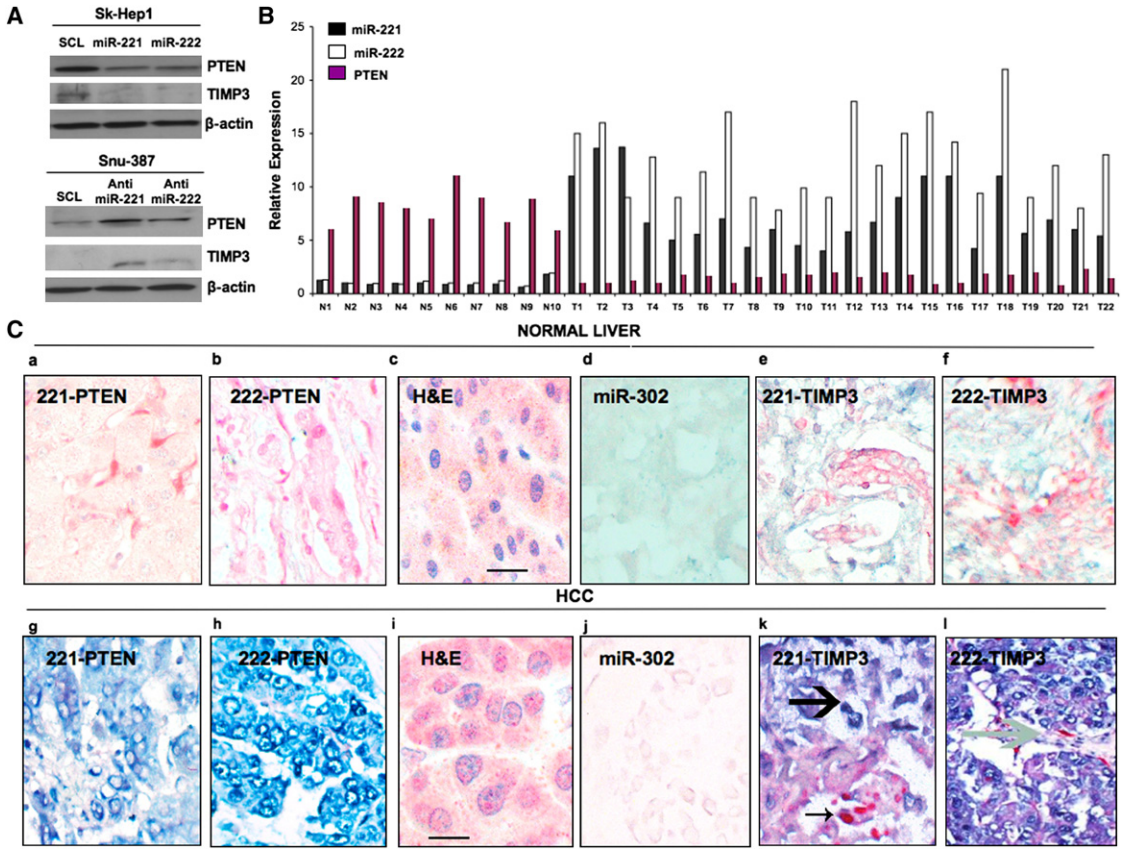


Figure 3. *PTEN* and *TIMP3* Are Direct Targets of *miR-221&222* in HCC In Vitro and In Vivo

(A) Western blot showing PTEN and TIMP3 expression in Sk-Hep1 and Snu-387 cells after *miR-221&222* overexpression or downregulation. *MiR-221* and *miR-222* were able to downregulate PTEN and TIMP3 expression in Sk-Hep1; conversely, anti-*miR-221&222* were able to increase PTEN and TIMP3 expression in Snu-387 cells.

(B) qRT-PCR on 22 lung cancer patients and 10 normal lung tissues. The association between *miR-221&222* and *PTEN* mRNA for the 10 subjects in the normal class and for the 22 subjects in the tumor class was calculated statistically using the Pearson correlation coefficient (r) and the respective p value, all significant at $p < 0.05$. The Pearson correlation indicated an inverse relation between *miR-221*, *miR-222*, and *PTEN* mRNA in the normal and tumor samples.

(C) Immunohistochemistry and in situ hybridization on hepatocarcinoma and normal liver tissues samples. *miR-221&222* (blue) and PTEN/TIMP3 (red) expression were inversely related in liver cancers and the adjacent normal/cirrhotic liver tissues. These tissues were analyzed for *miR-221* and *miR-222* expression by in situ hybridization, followed by immunohistochemistry for PTEN and TIMP3. Liver cancer cells abundantly expressed *miR-221&222* and rarely expressed PTEN or TIMP3 (Cg, Ch, Ck, and Cl), whereas the adjacent non-malignant liver abundantly expressed PTEN or TIMP3 and rarely had detectable *miR-221&222* (Ca, Cb, Ce, and Cf). In the cases of HCC, where both *miR-221&222* and TIMP3 expression were noted, the cancer cells expressing *miR-221* (Ck, large arrow; TIMP3 is depicted by arrow in l) were distinct from those cells expressing TIMP3 (Ck, small arrow). (Cc–Ci) H&E. (Cd–Cj) MiR-302, which is not express in liver, was used as negative control. 80 human HCCs were analyzed. Scale bars indicate 25 μm.

transfected with 2′-O-methyl (2′-O-me)-anti-*miR-221&222* oligoribonucleotides. Cells transfected with 2′-O-me-scrambled miR were used as control. Blocking *miR-221&222* expression considerably sensitized these cells to TRAIL-induced apoptosis, as assessed by caspase 3/7 assay (Figures 5F and 5G). Moreover, Calu-1 and Snu-387 cells were treated with the specific AKT inhibitor, API-2/triciribine (Yang et al., 2004), with or without TRAIL. As shown in Figures 5F and 5G, API-2 abrogated *miR-221&222*-activated AKT and significantly inhibited *miR-221&222*-induced cell survival and TRAIL resistance. Next, to directly compare the growth of tumors with and without PTEN and TIMP3, we used short hairpin RNA (shRNA) constructs designed to knockdown gene expression to silence *PTEN* and *TIMP3* in H460 cells. An shRNA plasmid, encoding a scrambled shRNA sequence that does not lead to the specific degradation of any known cellular mRNA, was used as control. We assessed the consequences of PTEN and TIMP3 disruption on tumor growth and TRAIL resistance in vivo by implanting H460 PTEN and TIMP3 knockdown cells into the right dorsal sides of nude mice. TRAIL treatment was initiated 5 days afterwards, when lung carcinoma had been established. PTEN and TIMP3 loss (Figure S4A) conferred not only a significant tumor growth advantage but also resistance to TRAIL-inducing apoptosis over control tumors (Figures S4B–S4G). These findings led us to conclude that PTEN and TIMP3 are important targets in TRAIL resistance and could play an important role in tumorigenicity of NSCLC and HCC cells.

PTEN and TIMP3 Downregulation by *miR-221&222* Induces Migration and Invasiveness in NSCLC and HCC Cells

To directly test the functional role of *miR-221&222* in tumorigenesis, we overexpressed these two miRNAs or silenced *PTEN* and

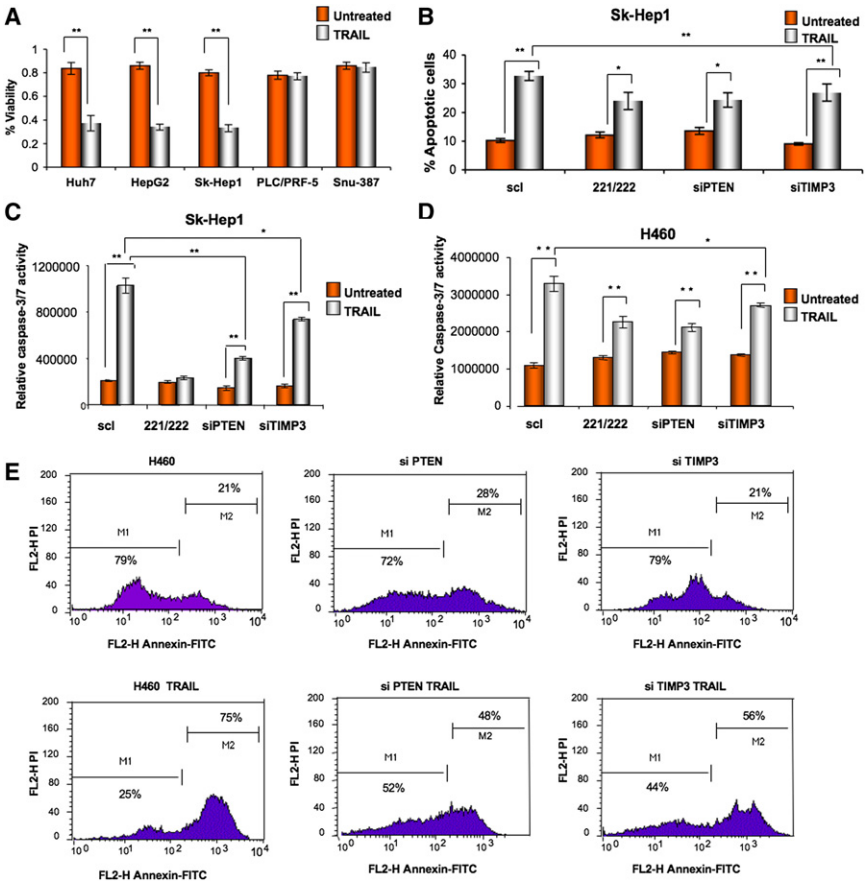


Figure 4. *MiR-221&222* Induce TRAIL Resistance in NSCLC and HCC by Targeting PTEN and TIMP3

(A) Proliferation assay on five different HCCs. Cells were incubated with Super-Killer TRAIL (400 ng/ml) for 24 hr and viability was evaluated as described in the Supplemental Experimental Procedures. Huh7, HepG2, and Sk-Hep1 with low *miR-221* and *miR-222* expression were more sensitive to TRAIL-induced apoptosis compared to PLC/PLF-5 and Snu-387, highly expressing *miR-221&222*. Mean ± SD of four independent experiments repeated in triplicate.

(B) Cell death effects in Sk-Hep1 cells after *miR-221&222* forced expression and PTEN or TIMP3 downregulation. Cells were transfected either with control miR or with *pre-miR-221&222*. Twenty-four hours after transfection, cells were treated with Super-Killer TRAIL for 24 hr. Apoptosis was evaluated either with Annexin-FITC or with caspase-Glo 3/7 kit.

(C) TRAIL resistance increased after *miR-221&222* overexpression or PTEN and TIMP3 downmodulation.

(D) Effects of *miR-221&222* on cell death. H460 cells were transfected either with control siRNA or control miR or with 100 nmol of *PTEN* and *TIMP3* siRNA. After 48 hr from the transfection, cells were treated with Super-Killer TRAIL for 24 hr.

(E) Apoptosis was evaluated by caspase 3/7 activity or Annexin-V.

The percentage of apoptotic cells decreased after PTEN and TIMP3 downregulation. Error bars indicate ± SD. *$p < 0.05$ and **$p < 0.001$ by Student's t test.

TIMP3 in H460 and Sk-Hep1 cells. Then, by cell-cycle analysis, *miR-221&222* and *PTEN* siRNA H460 transfected cells showed a decrease of G1 and a corresponding increase of the S and G2-M phases (Figure 6A). After 72 hr of transfection, the analysis revealed an earlier onset of DNA synthesis induced by *miR-221* and *miR-222* or PTEN knockdown, paralleled by a faster reduction of G1 cells, contributing to the proliferative advantage (Figure 6A). The same change was observed in Sk-Hep1 cells (Figure S5A). Next, we analyzed the effects of *miR-221* and *miR-222* overexpression on cellular migration and invasion of NSCLC and HCC cells. Interestingly, we observed a significant increase on the migratory (Figures 6B and 6C) and invasive (Figure 6D) capabilities of H460 and Sk-Hep1 (Figure S5B) cells after *miR-221&222* overexpression as well after PTEN and TIMP3 downregulation. Conversely, when we downregulated *miR-221&222* by transfection with 2′-O-me-anti-*miR-221&222*, we observed a decrease in cell migration and invasion in both Calu-1 and Snu-387 cells (Figures S6A and S6B).

MET Controls *miR-221&222* Activation through AP-1 Transcription Factor

Activation of MET signaling is a frequent event observed in many types of cancers, including lung and liver. MET receptor promotes a complex biological program designated ''invasive growth'' that results from stimulation of cell motility, invasion, and protection from apoptosis. Several studies have shown that MET induces tumor cell migration/invasion through the activation of the PI3K/AKT pathway (Segarra et al., 2006; Zhou et al., 2008), so we determined if MET could induce PTEN downregulation and AKT activation through *miR-221&222*. To test this hypothesis, we silenced *MET*, by using siRNA, in Calu-1 and Snu-387 cells and in a gastric cell line (GTL16) previously reported to overexpress *MET* oncogene because of DNA amplification (Giordano et al., 1989). First, *miR-221&222* expression levels were evaluated by qRT-PCR. After *MET* knockdown, *miR-221&222* expression was downregulated in all cell lines analyzed (Figures 7A–7C). The same result was obtained by treating GTL16 cells with a MET inhibitor, SU11274 (Figure S7A). Second, by immunostaining, we observed increased PTEN and TIMP3 expression levels after MET downregulation or inhibition, a further hint that MET is involved in *miR-221&222* activation (Figures 7D–7F). Next, we were interested in determining how MET could be involved in *miR-221&222* regulation. By bioinformatics search (TESS database: http://www.cbil.upenn.edu/cgi-bin/tess/tess), we found that the only transcription factor involved in the MET pathway predicted to bind and transcriptionally activate *miR-221&222* promoter was AP-1. AP-1 is a dimeric basic region leucine zipper protein that belongs to the Jun and Fos subfamilies. c-Jun is the most potent transcriptional activator in its group (Ryseck and Bravo, 1991). To define which factor, belonging to the AP-1 family, was involved in *miR-221&222* transcriptional activation, we began with analysis of c-Jun and c-Fos. First, we investigated the correlation between *miR-221&222* expression and c-Jun and c-Fos protein levels in

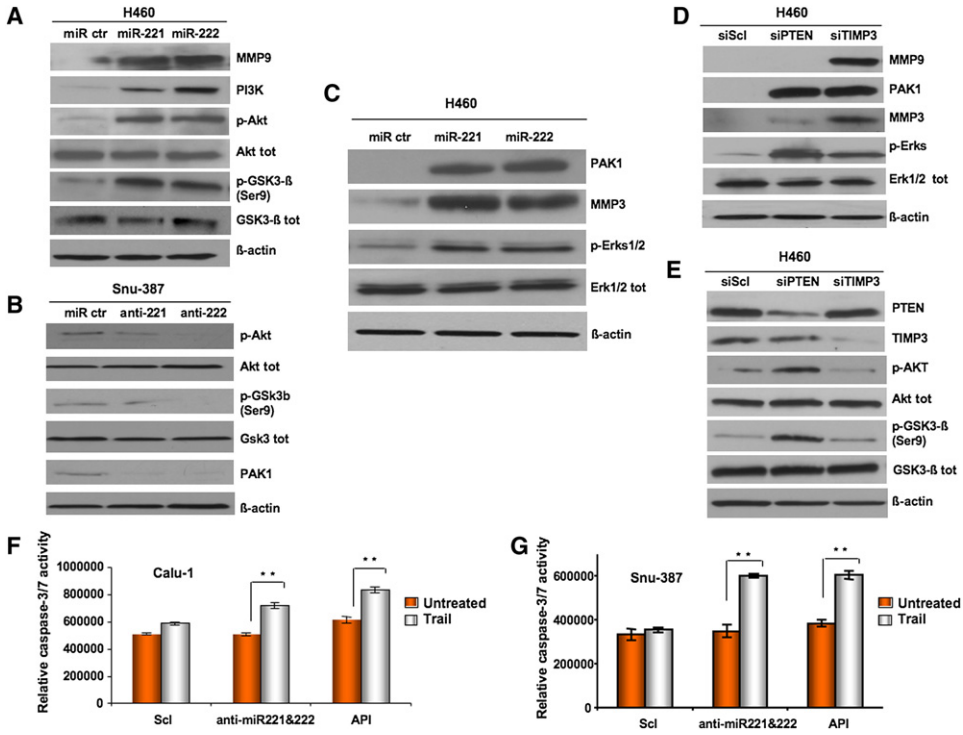


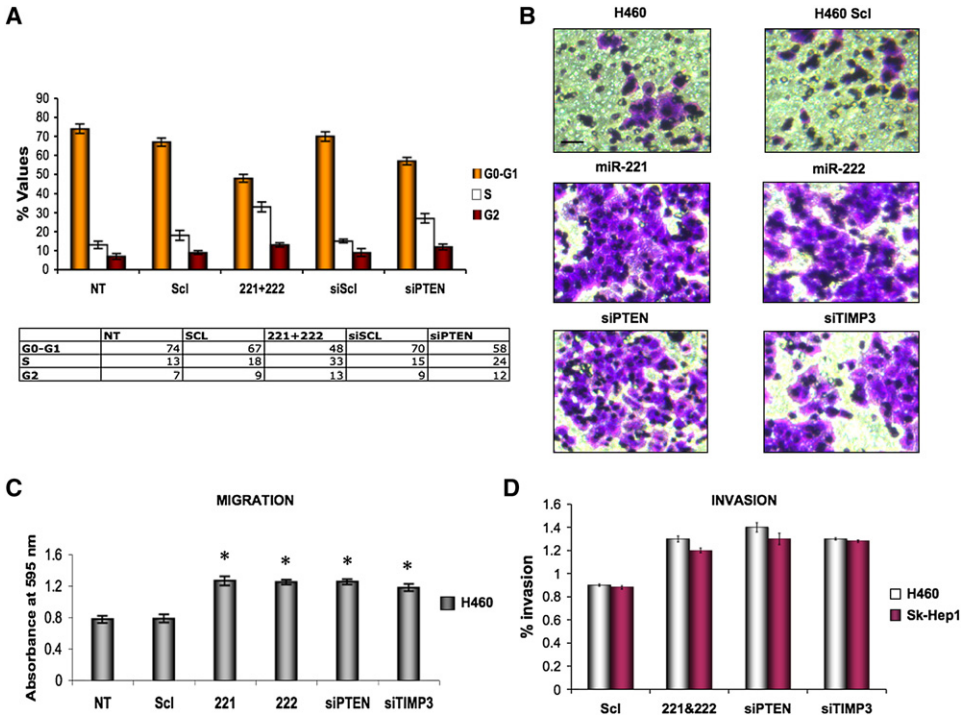
Figure 5. Anti-*miR-221&222* Override TRAIL Resistance in NSCLC and HCC through the Inhibition of the AKT Pathway

(A–C) Western blots in H460 cells after *miR-221&222* forced expression. *MiR-221&222* forced expression induces the activation of the AKT/ERKs pathways and metallopeptidases. (B) Western blots in Snu-387 cells after *miR-221&222* knockdown by anti-*miR-221&222*. The inhibition of the AKT pathway is shown as a result of *miR-221&222* downregulation.

(D and E) Western blots after PTEN or TIMP3 knockdown. Erks phosphorylation and PAK1 activation are both PTEN and TIMP3 dependent. The activation of the AKT pathway is PTEN dependent, while TIMP3 silencing induces the expression of metallopeptidases.

(F and G) Effects of anti-miRs and AKT pathway inhibition by API2/triciribine on cell death. Calu-1 and Snu-387 cells were transfected with anti-*miR-221&222* for 72 hr or treated with API2/triciribine for 48 hr. *MiR-221&222* downmodulation and/or the inhibition of the Akt pathway induced an increase in apoptosis percentage in both Calu-1 and Snu-387 cell lines, as assessed by caspase 3/7 activity. Error bars indicate ± SD. **p < 0.001 by Student's t test.

four different cell lines (H460, Calu-1, Huh7, and Snu-387) (Figure S7B). Calu-1 cells, highly expressing c-Jun and c-Fos, were cotransfected with *MET* siRNA, *c-Jun* siRNA, or *c-Fos* siRNA. Subsequent qRT-PCR amplification showed that *MET* and *c-Jun* downregulation, but not *c-Fos* knockdown, gave rise to a reduction of ~45%–50% in *miR-221&222* expression levels, as compared with the negative control (Figure S7C). To further confirm these results we carried out luciferase assays. In previous work, we found that *miR-221&222* are transcribed into a single species of 2.1 kb RNA and the transcription is regulated by the upstream sequence located at −150 bp/−50 bp from the 5′ end of *miR-222* hairpin structure (Di Leva et al., 2009). To determine if the previously identified *miR-221&222* promoter region was affected by MET/AP-1, we performed luciferase assay using the reporter plasmids containing the fragments spanning +3 to ~−150, +3 to ~−600, and +3 to ~−1000 (+1 position corresponds to the 5′ terminus of *miR-222* hairpin) (Figure 7G) into the pGL3basic vector, which harbors the promoterless luciferase gene (Di Leva et al., 2009). The pGL3b, −150, −600, and −1000 pGL3b were cotransfected with *MET* siRNA, *c-Jun* siRNA, or *c-Fos* siRNA into Calu-1 cells (Figures S7D and S7E). Subsequent luciferase assays showed that *MET* and *c-Jun* downregulation gave rise to a reduction of ~45% in luciferase activity, as compared to the basal activity determined by transfection with pGL3b empty vector; we did not observe a reduction of luciferase activity after *c-Fos* siRNA transfection (Figures S7D and S7E). Together these data led us to conclude that c-Jun and not c-Fos is the transcription factor involved in the MET pathway responsible for *miR-221&222* activation in NSCLC and HCC cells. Since we noticed that the promoter region was responsive to c-Jun modulation, to verify a direct binding of c-Jun on *miR-221&222* promoter, we carried out chromatin immunoprecipitation (ChIP) assays. First, by bioinformatics analysis, we found only one AP-1 putative binding site located ~130 bp upstream of the *pre-miR-222* 5′ end. Taking into account the predicted AP-1 binding site, a total of two chromatin regions were analyzed (Figure 7G): one spanning the AP-1 binding site and the second, as negative control, ~1700 nt upstream of the *pre-miR-222* 5′ end, where we did not find any predicted binding site for AP-1. As expected, ChIP assay of c-Jun-positive Calu-1 and Snu-387 cells showed remarkable AP-1 binding at ChIP analyzed region 2, proximal to the promoter (Figures 7H and 7I). No chromatin enrichment by c-Jun ChIP was observed in c-Jun-negative H460 cells, verifying the specificity of the ChIP assay. Finally, because the induction of AP-1 is mostly mediated by the JNK-MAP kinase cascades we treated



	NT	SCL	221+222	siSCL	siPTEN
G0-G1	74	67	48	70	58
S	13	18	33	15	24
G2	7	9	13	9	12

Figure 6. Ectopic Expression of *miR-221* and *miR-222* Affects the Cell-Cycle Distribution and Migration/Invasion Capabilities of H460 Cells
(A) Flow cytometric distributions of H460 cells transfected with pre-miR scrambled, *miR-221&222*, siRNA scrambled, and siRNA *PTEN*. H460 transfected cells showed a decrease of G1 and a corresponding increase of the S and G2-M phases, as a consequence of PTEN downregulation.
(B and C) *MiR-221&222* regulate cell migration ability in H460 cells. Migration assay was performed as described in the Experimental Procedures.
(D) *miR-221&222* influences H460 and Sk-Hep1 cell invasion ability. Histogram reports the percentage of cells that invaded through Matrigel-coated membrane after transfection with negative control miRNA, *miR-221*, *miR-222*, si*PTEN*, and si*TIMP3*. One-way analysis of variance was performed to test the differences among means of invasion values. The Scheffe multiple-comparison method was used to test the differences between each pair of means. Significant differences were found between the scrambled versus *miR-221&222*, *PTEN*, and *TIMP3* H460 transfected cells ($p < 0.001$). The same results were obtained using the Bonferroni and Sidak methods. Error bars indicate ± SD. *$p < 0.001$ by Student's t test. Scale bar indicates 25 μm. The magnification is the same for all panels.

Huh7 cells, which show low levels of *miR-221&222*, with anisomycin, an antibiotic able to activate JNK kinases and, thus, AP-1, and we checked *miR-221&222* and PTEN-TIMP3 expression levels. After c-Jun activation (Figure 7K) by anisomycin, *miR-221* and *miR-222* expression increased (*miR-221* = 80% and *miR-222* = 40%) as confirmed by qRT-PCR (Figure 7J), while PTEN and TIMP3 expression levels decreased drastically (Figure 7K). To further prove that JNK is the intermediate signaling factor between c-Met and c-Jun and that c-Jun knockdown leads to increased PTEN and TIMP3 expression, we silenced *c-Met* and *c-Jun* in Calu-1 cells and analyzed the JNK1/2 phosphorylation and PTEN and TIMP3 expression, respectively. As shown in Figure S7F, MET knockdown reduces JNK1/2 phosphorylation while c-Jun silencing increases PTEN/TIMP3 expression as a result of *miR-221&222* downmodulation. To corroborate the direct relation between MET and PTEN/TIMP3 also in vivo, immunohistochemistry analysis was performed on lung and liver cancer and normal samples. The colabeling MET/PTEN and MET/TIMP3 clearly shows that PTEN and TIMP3 are abundantly expressed only in the normal cells, where MET is not present, whereas c-Met is expressed exclusively in the cancer cells (Figure S8). These data confirm that MET is implicated in *miR-221* and *miR-222* regulation, at least in part through JNK, AP-1, and in particular c-Jun transcription factor.

DISCUSSION

Lung cancer is the leading cause of cancer death in both men and women worldwide. It is becoming apparent, through candidate gene and genome-wide approaches, that clinically evident lung cancers accumulate numerous clonal genetic and epigenetic alterations during a multistep process. These alterations include tumor suppressor gene inactivation and activation of growth or survival promoting oncogenes (Sekido et al., 2003). HCC is one of the most common causes of cancer-related death worldwide and the principal cause of death among cirrhotic patients (Sangiovanni et al., 2004) with an annual occurrence of one million cases. The prognosis of HCC is poor, due to frequent intrahepatic metastasis and tumor recurrence. One of the most important factors that affect survival rate is resistance to therapeutic drugs. Thus development of effective therapeutic approaches is necessary for the management of these common cancers. Due to their specific toxicity for malignant cells, recombinant forms of TRAIL are among the most promising apoptosis-based antitumor agents (Walczak et al., 1999). However, many human cancer cells remain resistant to TRAIL-induced apoptosis, but the mechanism of such resistance is not clear. MiRNAs are attractive drug targets since they regulate expression of many cellular proteins and are differentially

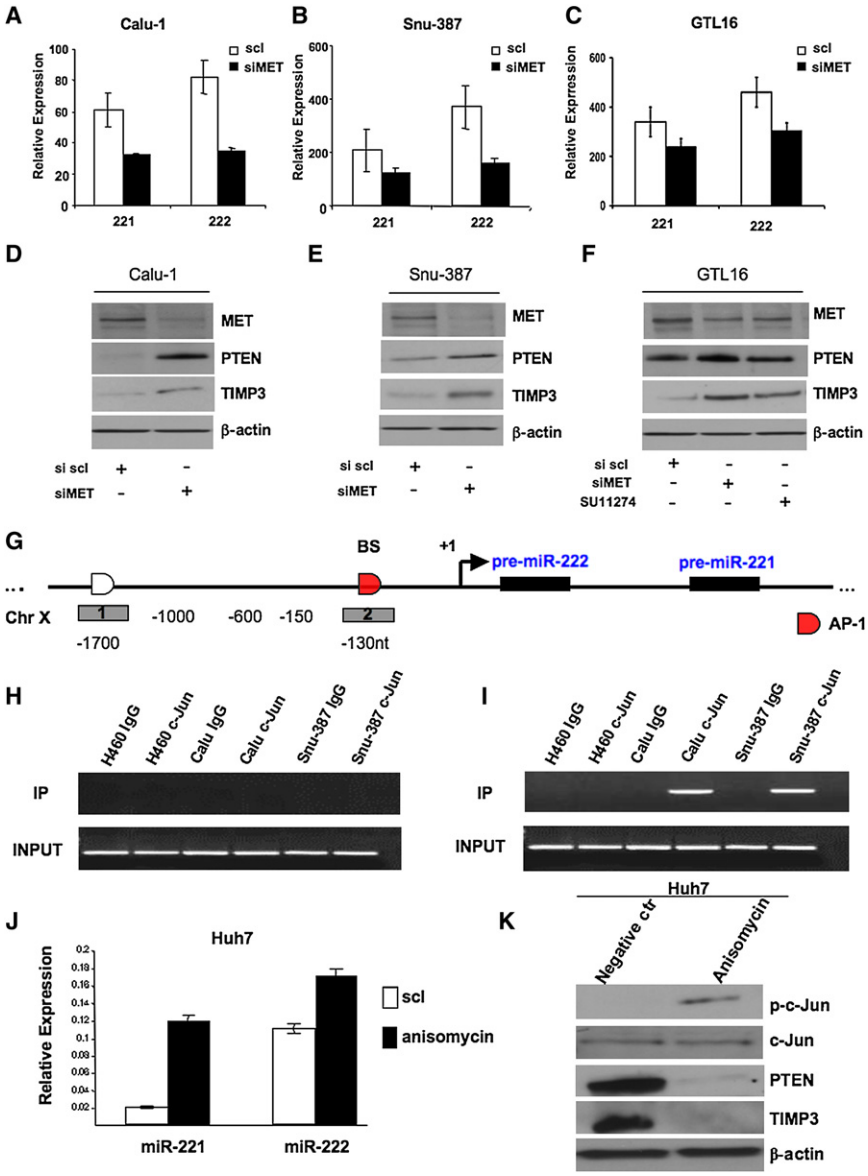


Figure 7. *MET* Oncogene Regulates *miR-221&222* Activation

(A–C) Relative expression levels of *miR-221&222* in Calu-1, Snu-387, and GTL16 after transfection with miR control and siRNA *MET*. *MiR-221&222* expression decreased after MET knockdown.

(D–F) Western blots after siRNA *MET* transfection in Calu-1, Snu-387, and GTL16 cells. MET knockdown decreased *miR-221&222* expression levels, giving rise to PTEN and TIMP3 upregulation in all the different cell lines. Moreover, GTL16 cells were treated for 24 hr with 4 μM of the MET inhibitor SU11274. MET inhibition increased *miR-221&222* target expression levels.

(G–I) Identification of c-Jun (AP-1) interacting region using two different amplicons across the *miR-221&222* transcription start site. ChIP analysis was performed with chromatin from H460 c-Jun-negative cells and Calu-1 and Snu-387 c-Jun-positive cells. BS, binding site.

(J) qRT-PCR of *miR-221&222* in Huh7 cells after treatment with anisomycin (10 μM) for 30 min. Anisomycin induced *miR-221&222* upregulation.

(K) Anisomycin induced c-Jun activation and PTEN and TIMP3 downregulation in Huh7 cells. Total lysate was analyzed by western blot using anti-PTEN and anti-TIMP3 antibody. Error bars indicate ± SD.

expressed in malignant versus normal cells. In previous work we focused on two highly related miRs, *miR-221* and *miR-222*, differentially expressed in TRAIL-resistant and in TRAIL-sensitive NSCLCs. Our experiments indicated that *miR-221&222* modulated TRAIL sensitivity in lung cancer cells mainly by interfering with $p27^{kip1}$ expression and TRAIL-induced caspase machinery. However, it seemed plausible that silencing of additional targets of *miR-221* and *miR-222* contributed to TRAIL resistance in NSCLC cells. In the present study, we attempted to identify major mRNA targets and signaling pathways that mediate *miR-221&222* regulation in a wide panel of NSCLC and HCC-derived cell lines. In vitro and in vivo experiments revealed that elevated levels of *miR-221&222* in NSCLCs and HCCs correlate with PTEN and TIMP3 downregulation, indicating that these two miRNAs could be a causal factor in the downregulation of PTEN and TIMP3 in these types of cancers. The tumor suppressor PTEN regulates the PI3K/AKT pathway, a major cell survival pathway, playing a key role in the development of multiple drug resistance, including that to TRAIL (Kandasamy and Srivastava, 2002). TIMP3 has been reported to induce the activation of both initiator caspases-8 and -9 (Lee et al., 2008a). Therefore, we examined the effects of *miR-221&222* and their targets on cell survival and TRAIL resistance. Interestingly, we found that after *miR-221&222* enforced expression, or PTEN and TIMP3 downregulation, TRAIL-sensitive NSCLC and HCC cells became resistant to TRAIL-inducing apoptosis, although PTEN downregulation was slightly more effective than that of TIMP3. Our results clearly indicate that *miR-221&222* overexpression is a "prerequisite" of TRAIL-resistant NSCLC and HCC cells, corroborating previous indications that the expression levels of few miRNAs could sensitize cancer cells to drug-inducing cell death. Importantly, tumor stratification, on the basis of *miR-221&222* expression levels, could be used as prognostic tool to predict TRAIL sensitivity or resistance in the treatment of NSCLCs and HCCs. Although many data already exist, establishing an important role for miRNAs in the pathogenesis of cancer, the molecular mechanisms by which miRNAs can regulate tumor growth, invasion, or metastasis are still in their infancy. It has been well documented that constitutive activation of AKT contributes to cell migration and invasion in different types of tumors, including lung (Fong et al., 2008) and liver carcinoma. Our data indicate that *miR-221&222* block PTEN expression leading to activation of the AKT pathway,

showing that *miR-221&222* could play an important role in cell growth and invasiveness by targeting the PTEN/AKT pathway. In this regard cell-cycle analysis showed an increase in cell growth tightly linked to the G1 to S shift, which is in agreement with modulation of PTEN and also of $p27^{kip1}$, a known regulator of the G1/S cell-cycle checkpoint and a downstream effector of PTEN. We report here that NSCLC and HCC cells overexpressing *miR-221&222* are not only TRAIL resistant but they also show an increase in migration and invasion capabilities, compared to cells expressing lower levels of *miR-221&222* cells. Our experiments demonstrate that *miR-221&222* promote cell migration, invasion, and growth via direct repression of PTEN and TIMP3 expression and of downstream pathways involving AKT and ERKs phosphorylation, and the activation of MMP-3 and MMP-9. Moreover, PTEN and TIMP3 loss in H460 tumor xenograft conferred not only a significant tumor growth advantage but also a resistance to TRAIL-inducing apoptosis over control tumors also in vivo. Interestingly, the TIMP3 knockdown tumors were more vascularized than the control tumors, highlighting its role in angiogenesis and tumor formation (Janssen et al., 2008). A recent study has shown that miR-21 regulates multiple genes associated with glioma migration and invasiveness, including *TIMP3*, although indirectly (Gabriely et al., 2008). In fact, the *TIMP3* 3′UTR construct did not consistently respond to changes in miR-21 levels. The identification of *miR-221&222* as important regulators of tumor cell proliferation, migration, and invasion of NSCLC and HCC, in vitro and in vivo, provides insights into the role of these miRNAs in hepatic and lung oncogenesis and tumor behavior. *miR-221&222* are among the most deregulated miRNAs implicated in cancer (Volinia et al., 2006). Their expression is highly upregulated in a variety of solid tumors, including thyroid cancer (Pallante et al., 2006), hepatocarcinoma (Fornari et al., 2008), and melanoma (Felicetti et al., 2008) cells. Elevated *miR-221&222* expression has been causally linked to proliferation (le Sage et al., 2007), apoptosis, (Garofalo et al., 2008a), and migration (Felicetti et al., 2008) of several cancer cell lines. However, our knowledge of the molecular mechanisms mediating *miR-221&222* function in cancer generally, and in NSCLC and HCC specifically, has been limited. Here, we provide evidence that the activation of *miR-221&222* is regulated, at least in part, by the *MET* oncogene and the c-Jun transcription factor. Activation of MET signaling is a frequent genetic event observed in liver and lung cancer development (Patil et al., 2009). AP-1 is a complex of dimeric basic region leucine zipper proteins that belong to the Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra-1, and Fra-2), Maf, and ATF subfamilies. c-Jun is the most potent transcriptional activator in its group (Ryseck and Bravo, 1991) whose transcriptional activity is attenuated and sometimes antagonized by JunB (Chiu et al., 1989). The Fos proteins, which cannot homodimerize, form stable heterodimers with Jun proteins and thereby enhance their DNA binding activity. We focused on these two AP-1 subfamilies, and in particular on c-Jun and c-Fos, although we found by bioinformatics search (TESS database) that ATF-1 and JunD also could be potential transcription factors involved in *miR-221&222* activation. Our experiments demonstrate that c-Jun and not c-Fos is involved in *miR-221&222* activation and that c-Jun has one binding site in the *miR-221&222* promoter region. The induction of AP-1 is mostly mediated by the JNK cascades. By using anisomycin, an antibiotic that activates the JNK cascade, we found an increase of *miR-221&222* expression in Huh7 hepatocarcinoma cells, as a consequence of c-Jun phosphorylation. Intriguingly, when we grew Huh7 cells in serum-free medium, we did not observe any variation in the expression level of *miR-221&222* or PTEN and TIMP3 (data not shown), suggesting that MET activation is important for *miR-221&222* transcription regulation and subsequent cellular migration. To address this issue we investigated Calu-1 and Snu-387 cell migration and invasion after *MET* silencing. As expected, migratory and invasive capabilities of both cell lines were clearly reduced after *MET* oncogene silencing (Figures S9A and S9B). Furthermore, a xenograft model of Calu-1 cells in which *c-Met* was silenced using an shMET plasmid (Figure S9C) clearly showed that mice injected with Calu-1 shMET cells are more sensitive to TRAIL-inducing apoptosis compared to the mice injected with the sh control (Figures S9D and S9E). Thus, MET confers not only a tumor growth advantage but also resistance to TRAIL-inducing apoptosis over control tumors in vivo. Therefore, *MET* oncogene regulates *miR-221&222* levels and, accordingly, cellular invasion and migration through c-Jun transcription factor and JNK activation (Figure 8). Taken together, these data highlight a mechanism, involving MET, through which *miR-221&222* could promote tumorigenesis and metastasis. Thus, approaches targeting MET receptor and/or *miR-221&222* could be used not only to sensitize NSCLC and HCC to TRAIL-inducing apoptosis but also in the prevention and inhibition of lung cancer and HCC.

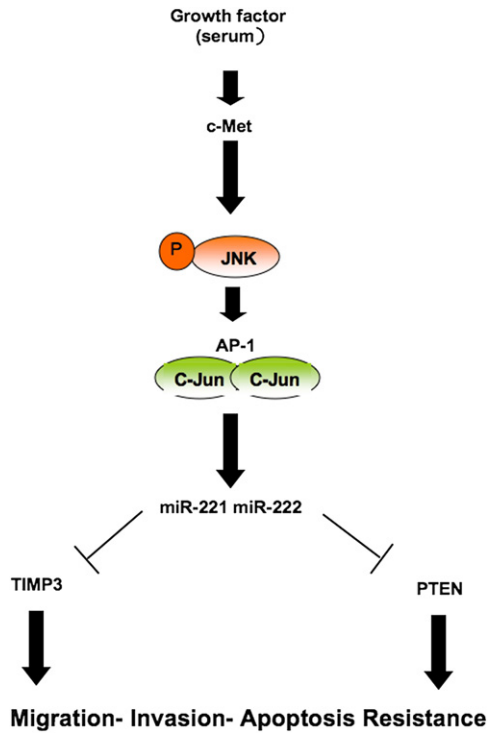


Figure 8. MET Induces *miR-221&222* Activation through AP-1 (c-Jun) Transcription Factor

A model is reported in which growth factors determine c-Met activation, which, in turn, through AP-1 and accordingly *miR-221&222* upregulation, gives rise to PTEN and TIMP3 downregulation and subsequent apoptosis resistance, cellular migration, and invasion.

EXPERIMENTAL PROCEDURES

Luciferase Assay

The 3′UTR of the human *PTEN* and *TIMP3* genes were PCR amplified using the following primers: PTEN forward (Fw), 5′-TCTAGAGACTCTGATCCAGAGAATGAACC-3′, and PTEN reverse (Rw), 5′-TCTAGAGTTGCCACAAGTGCAAAGGGGTAGGATGTG-3′; TIMP3 Fw, 5′-TCTAGACTGGGCAAAGAAGGGTCTTTCGCAAAGC-3′, and TIMP3 Rw, 5′-TCTAGATTCCAATAGGGAGGAGGCTGGAGGAGTCTC-3′. They were then cloned downstream of the Renilla luciferase stop codon in pGL3 control vector (Promega), giving rise to the p3′UTR-PTEN and p3′UTR-TIMP3 plasmids. These constructs were used to generate, by inverse PCR, the p3′UTRmut-*PTEN* plasmid (primers: Fw, 5′-GTTGAAAAAAGGTTGGGGGCGGGTGTCATGTATATAC-3; Rw, 5′-GTATATACATGACACCCGCCCCCAACCTTTTTTCAAC-3′) and p3′UTRmut-*TIMP3* plasmid (primers: Fw, 5′-GTATAATTTAAAATCATTGGGCGGCGGGAGACACTTCTGTATTTC-3′; Rw, 5′-GAAATACAGAAGTGTCTCCCGCCGCCCAATGATTTTAAATTATAC-3′). MeG01 cells were cotransfected with 1 μg of p3′UTR-*PTEN* or p3′UTR-*TIMP3* and with p3′UTRmut-*PTEN* or p3′UTR-*TIMP3* plasmids and 1 μg of a Renilla luciferase expression construct, pRL-TK (Promega), using Lipofectamine 2000 (Invitrogen). Cells were harvested 24 hr posttransfection and assayed with Dual Luciferase Assay (Promega) according to the manufacturer's instructions. Three independent experiments were performed in triplicate.

Lung and Liver Cancer Samples and Cell Lines

A total of 32 snap-frozen normal and malignant lung tissues (19 men and 13 women, median age: 70.0, range: 55–82) and 60 snap-frozen normal and 60 malignant liver tissues were collected at the Ohio State University Medical Center (Columbus, OH). Other 72 cancer and normal (24) lung tissues were purchased from US Biomax, Inc. The human tissues were obtained and studied in strict adherence to the Ohio State University Institutional Review Board-approved protocol. This protocol, since biopsy material already obtained and embedded in paraffin was used, was exempt from informed consent. As per the policy of the associated HIPAA waiver, all patient health information was kept strictly confidential. Finally, tissue microarrays from an outside source were also used and, since these did not contain any patient health information, Institutional Review Board protocols and informed consent were not applicable.

In Vivo Experiments

Animal studies were performed according to institutional guidelines. NCI-H460 cells were stable transfected using shPTEN and TIMP3 plasmids (Santa Cruz Biotechnology, Inc.); Calu-1 cells were stable transfected with shMET. After the selection in puromycin for 10 days, 5×10^6 (H460) or 7×10^6 (Calu-1) viable cells were injected subcutaneously into the right flanks of 6-wk-old male nude mice (Charles RiverBreeding Laboratories). Treatment started 5 days (H460 xenograft) or 10 days (Calu-1 xenograft) from tumor cell inoculation by daily i.p. injections of TRAIL/Apo2 (10 mg/kg/day) or vehicle (PBS) for two cycles of 5 days. Tumor size was assessed every 5 days by a digital caliper. The tumor volumes were determined by measuring the length (l) and the width (w) and calculating the volume ($V = lw^2/2$). Thirty-five days after injection, mice were sacrificed and tumor samples were analyzed by western blot for PTEN, TIMP3, and MET expression. Statistical significance between control and treated animals was evaluated using Student's t test. Animal experiments were conducted after approval of the Institutional animal care and use committee, Ohio State University.

Statistical Analysis

Student's t test and one-way analysis of variance was used to determine significance. All error bars represent the standard error of the mean. Pearson correlation coefficient was calculated to test the association between *miR-221&222* and *PTEN* in the classes normal versus tumor. Statistical significance for all the tests, assessed by calculating p value, was <0.05.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and nine figures and can be found with this article online at http://www.cell.com/cancer-cell/supplemental/S1535-6108(09)00383-3.

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