

MicroRNAs Impair MET-Mediated Invasive Growth

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Abstract

MicroRNAs (miRNA) are a recently identified class of non-coding, endogenous, small RNAs that regulate gene expression, mainly at the translational level. These molecules play critical roles in several biological processes, such as cell proliferation and differentiation, development, and aging. It is also known that miRNAs play a role in human cancers where they can act either as oncogenes, down-regulating tumor suppressor genes, or as onco-suppressors, targeting molecules critically involved in promotion of tumor growth. One of such molecules is the tyrosine kinase receptor for hepatocyte growth factor, encoded by the *MET* oncogene. The *MET* receptor promotes a complex biological program named “invasive growth” that results from stimulation of cell motility, invasion, and protection from apoptosis. This oncogene is deregulated in many human tumors, where its most frequent alteration is overexpression. In this work, we have identified three miRNAs (miR-34b, miR-34c, and miR-199a*) that negatively regulate *MET* expression. Inhibition of these endogenous miRNAs, by use of antagomirRs, resulted in increased expression of *MET* protein, whereas their exogenous expression in cancer cells blocked *MET*-induced signal transduction and the execution of the invasive growth program, both in cells expressing normal levels of *MET* and in cancer cells overexpressing a constitutively active *MET*. Moreover, we show that these same miRNAs play a role in regulating the *MET*-induced migratory ability of melanoma-derived primary cells. In conclusion, we have identified miRNAs that behave as oncosuppressors by negatively targeting *MET* and might thus provide an additional option to inhibit this oncogene in tumors displaying its deregulation.

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Introduction

During recent years, many efforts have been made to untangle the pathways involved in tumor progression, to identify oncogenes and tumor suppressor genes, and to understand their interactions and mutual regulation. In the last two decades, it has been consistently proven that one of the players of the intricate scenario leading to tumorigenesis is MET, the tyrosine kinase receptor for hepatocyte growth factor (HGF). The proof of principle of the

direct involvement of MET signaling in tumorigenesis was given by the identification of germ line activating mutations in patients with hereditary papillary renal carcinoma (1). MET activation as a consequence of ligand binding, receptor overexpression or interaction with other membrane receptors (2, 3), evokes pleiotropic biological responses, often defined as “invasive growth”: this is a genetic program consisting of rate-limiting steps that takes place physiologically during embryogenesis and tissue repair, and pathologically in oncogenesis (4). This process occurs in three phases: first, cells acquire the ability to dissociate from their neighbors, breaking cell-cell junctions (scatter); then they leave their original environment degrading the basal membrane and reaching the circulation (directional migration and invasion); and, finally, they extravasate, proliferate, and eventually, undergo terminal differentiation (5). Understanding when, how, and why this process takes place has acquired a primary importance in the perspective of developing tools capable to counteract MET aberrant activation.

Many studies show that the activation of the invasive growth program crucially depends on the control of *MET* expression (6): in fact, it has been widely proven that the overexpression of the receptor—the commonest *MET* alteration in human cancers—causes the constitutive activation of down-stream pathways (7). For the time being, all the mechanisms found to modulate the levels of *MET* in human tumors depend on control of gene transcription. In fact, an increase of *MET* transcription can be induced as follows: (a) by stimulation of cells with HGF (8); (b) by hypoxia, through binding of Hypoxia Inducible Factor-1α (HIF-1α) to the *MET* promoter (9); and (c) by constitutive activation of oncogenes such as *Ras* (10), *Ets* (11), and *Ret* (12).

Recently, a new mechanism to regulate protein expression has been discovered, which involves microRNAs (miRNA), an abundant class of small nonprotein-coding RNAs that function as negative gene regulators (13). MiRNAs recognize target mRNAs by imperfect base pairing to the 3' untranslated region (UTR) of RNA transcripts and induce either degradation of the target mRNA or translational repression. This imperfect base pairing enables miRNAs to control hundreds of target genes, underscoring the potential influence of these noncoding RNAs on almost any biological process. Loss or amplification of miRNA genes in a variety of cancers alters the pattern of miRNA expression, affecting cell cycle and survival programs (14). Recently, some reports have highlighted that miRNAs can influence signaling pathways and affect different levels of the signal transduction cascade (15–20).

Here, we show that a novel mechanism based on miRNAs contributes to control MET expression and activity. We show, in fact, that miR-34b, miR-34c, and miR-199a* negatively modulate MET expression and block the biological responses to HGF stimulation. Moreover, these miRNAs impair invasive growth also

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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in tumor cells overexpressing a constitutively active form of the MET receptor.

Materials and Methods

Cell culture and transfection. EKVX and HOP62 cells (lung adenocarcinomas) were obtained from NCI-60 collection; the following cell lines were obtained from American Type Culture Collection: A549 and NCI-H1299 (lung carcinomas); HEK-293 (Embryonic Kidney cells); MDA-MB-435 (melanoma cells); HT-29 (colon adenocarcinoma) NCI-SNU-5, AGS, NCI-N87, and Hs-746T (gastric carcinomas); SK-BR-3, BT-474, and T-47D (breast carcinomas); and MCF 10A (normal breast), A431 (squamous cell carcinoma), COS-7 (kidney fibroblast like), and WI-38 (lung fibroblasts); RT112/84 cell line (bladder carcinoma was obtained from European Collection of Cell Culture); A2780 cell line (ovarian carcinoma) was obtained from European Collection of Cell Cultures; and MKN-7 and MKN-45 were described by Motoyama and colleagues (21); and GTL16 was described by Giordano and colleagues (22). Cells were cultured in DMEM or RPMI, both supplemented with 10% fetal bovine serum (FBS), at 37°C in 5% CO₂.

Primary melanoma cells were achieved as follows. Tumor samples were obtained in accordance with consent procedures approved by the Internal Review Board of Department of Laboratory Medicine and Pathology, Sant'Andrea Hospital, University La Sapienza, Rome. Tumor dissociation was carried out mechanically and by enzymatic digestion, using 1.5 mg/mL collagenase II (Life Technologies-Invitrogen) and 20 µg/mL DNase I (Roche Diagnostics GMB) for 2 h at 37°C. Recovered cells were cultured in serum-free medium (Neural Progenitor Maintenance Medium; CC-3209; Clonetics), which allowed the formation of melanoma spheroids. The medium was replaced or supplemented with fresh growth factors twice a week. Cells were used before passage six.

For transient transfection, cells at 50% confluence were transfected using Lipofectamine 2000 (Invitrogen) with 0.2 µmol/mL of synthetic pre-miRNAs (Applied Biosystem) or 0.1 µmol/mL of synthetic MET/Ctrl siRNAs (23), according to standard protocols. Twenty-four hours after transfection, cells were seeded at the appropriate density for biochemical or biological assays.

For TPR-MET (24) and MET transduction, viruses were produced as described elsewhere (25).

Quantitative analysis of miRNAs and mRNA. Total RNAs were extracted from cultured cells using TriReagent lysis buffer (Applied Biosystem) according to the manufacturer protocols. Quantitative analysis of miRNAs was performed starting from equal amounts of total RNA/sample (0.5 µg) by real-time PCR, using the specific Taqman miRNA assay kits (Applied Biosystem); quantitative analysis of MET and of β-actin (as internal reference) expression was done as follows: 1 µg of total RNA was used as a template for synthesis of random-primed double stranded cDNA, using Moloney murine leukemia virus reverse transcription (Promega); cDNA was subjected to quantitative PCR, using Sybr green Master MIX (Applied Biosystem). Primer sequences were designed using Primer Express Software (Version 1.5). Sequences are available from the authors.

Luciferase assay. The full 3' UTR of MET was amplified by PCR from GTL16 total RNA and cloned in the pGL3 Luciferase vector (Promega), in the unique XbaI site, just downstream the luciferase stop codon. The obtained construct was transfected in COS-7 cells with the DEAE-dextran method. The day after, cells were split in 48-well plates and, 10 h later, transfected with miRNAs, as previously described. After additional 48 h, luciferase activity was determined as the average of triplicates, using the Luciferase assay system (Promega) and normalized on the basis of the total protein amount.

Protein extraction and Western blot. For Western blot analysis, cells were lysed in LB buffer [2% SDS, 0.5 mol/L Tris-HCl (pH 6.8)]. Protein concentration of whole-cell lysates was evaluated with the BCA Protein Assay kit (Pierce) and equal amounts of total proteins were analyzed by SDS-PAGE and Western blotting. Western blots were performed according to standard methods. The antibodies used were as follows: anti-MET antibody DL21 (26) and h-MET (C-12; Santa Cruz Biotechnology, Inc.), anti-phospho MET Tyr1234/1235 (Cell Signaling Technology), anti-vinculin (SIGMA), anti-Hsp70 (Stressgen), anti-actin (I-19; Santa Cruz Biotechnology,

Inc.); anti-phospho Akt Ser473, anti-Akt, anti-phospho Map Kinase Thr202/Thr204, and anti-Map Kinase were all purchased from Cell Signaling Technology, Inc. Final detection was done with enhanced chemiluminescence system (Amersham).

In vitro biological assays. For viability/3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 3 to 5 × 10³ cells were seeded in 96-well plates and grown in DMEM/RPMI supplemented with 10% FBS; when indicated, 20 ng/mL of HGF were added. After 3 d, cell viability was evaluated using the MTT assay; briefly, the culture medium was replaced with fresh DMEM containing 10% MTT and incubated for 45 min at 37°C, to allow the incorporation of the dye into the cells, which were then solubilized with DMSO. The colorimetric assay was read at 570 nm in a Microplate Reader (Model 3550 Bio-Rad).

To evaluate growth ability, 3 d after plating, cells were fixed with glutaraldehyde 11%, stained with 0.1% crystal violet, and then solubilized with 10% acetic acid. The colorimetric assay was read at 595 nm in a Microplate Reader (Model 3550 Bio-Rad).

The scatter assay was performed as follows: A549/HT-29 cells were seeded at low density in 6-well plates, in the presence of DMEM/RPMI supplemented with 10% FBS. After formation of islets, cells were stimulated with 20 ng/mL of HGF for 16 h. Pictures were taken at ×20 or ×42 magnification, with a Leica DMIL microscope connected to a Leica DFC320 camera. Fields shown in the figures are representative of two experiments performed in duplicate.

To evaluate migration ability, 3 × 10⁴ cells were seeded on the upper side of a Transwell chamber (Corning), on a porous polycarbonate membrane (8.0-µm pore size). To study the invasive ability, the polycarbonate filters were previously coated with Matrigel (Becton Dickinson; ref. 27). The lower chamber of the Transwell was filled with DMEM/RPMI containing 10% FBS, either in the absence or in the presence of HGF (20 ng/mL) or of epidermal growth factor (100 ng/mL). After 16 h of incubation, cells on the upper side of the filters were mechanically removed and cells migrated to the lower side were fixed, stained, and counted.

Statistics. Comparisons were made using two-tailed Student's *t* test.

Results

Identification of miRNA binding elements in the 3' UTR of MET gene. To identify miRNAs capable to negatively regulate MET mRNA, we performed a bioinformatics analysis, using different algorithms available in the web (the Sanger miRNA Sequence Data Base,³ PicTar,⁴ Target Scan,⁵ and the Sloan Kettering mirnaviewer site).⁶

When we compared the results obtained from the different searches, two members of the miR-34 family—namely miR-34b and miR-34c—and miR-199a* consistently showed the highest score of probability for targeting MET mRNA. In the 3' UTR of MET, we identified the potential binding sites for miR-34b at nt 2121, for miR-34c at nt 2149, and for miR-199a* at nt 1414 (Fig. 1A).⁷

Interest in the miR-34 family was bolstered by recent works showing that miRNAs of this family are widely expressed and are p53 targets that most likely contribute to the execution of p53-dependent biological activities (28); moreover, these observations shed light on past works showing that MET expression is up-regulated in cells where p53 is lost (29). On the other hand, miR-199a* was found to be present at high levels in normal tissues, mainly of mesenchymal origin (30), although little is known of its expression in human tumors, with the exception of ovarian

³ <http://microrna.sanger.ac.uk/sequences>

⁴ <http://pictar.org>

⁵ <http://www.targetscan.org>

⁶ <http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl>

⁷ <http://www.microrna.org/microrna/getMrna>

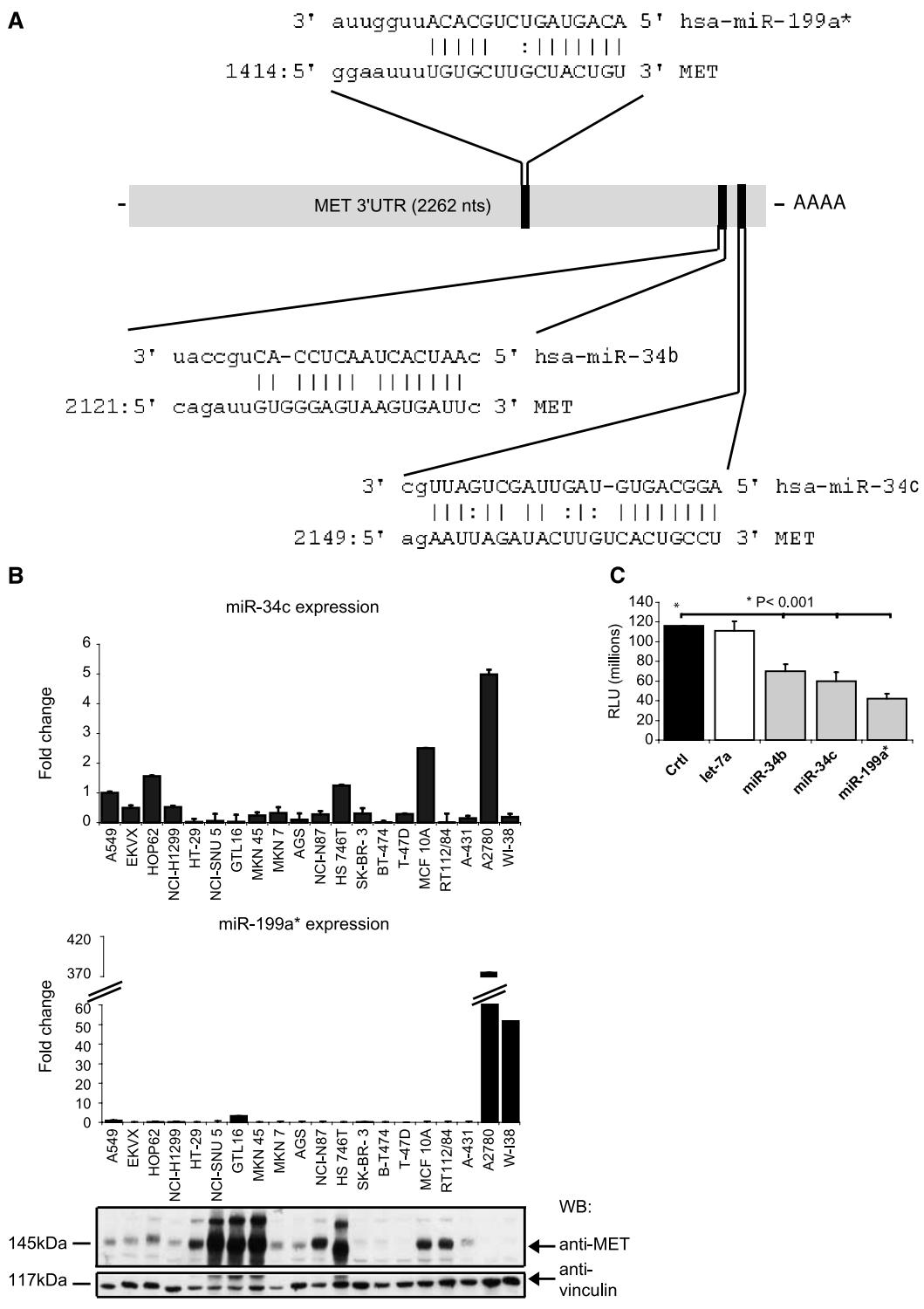
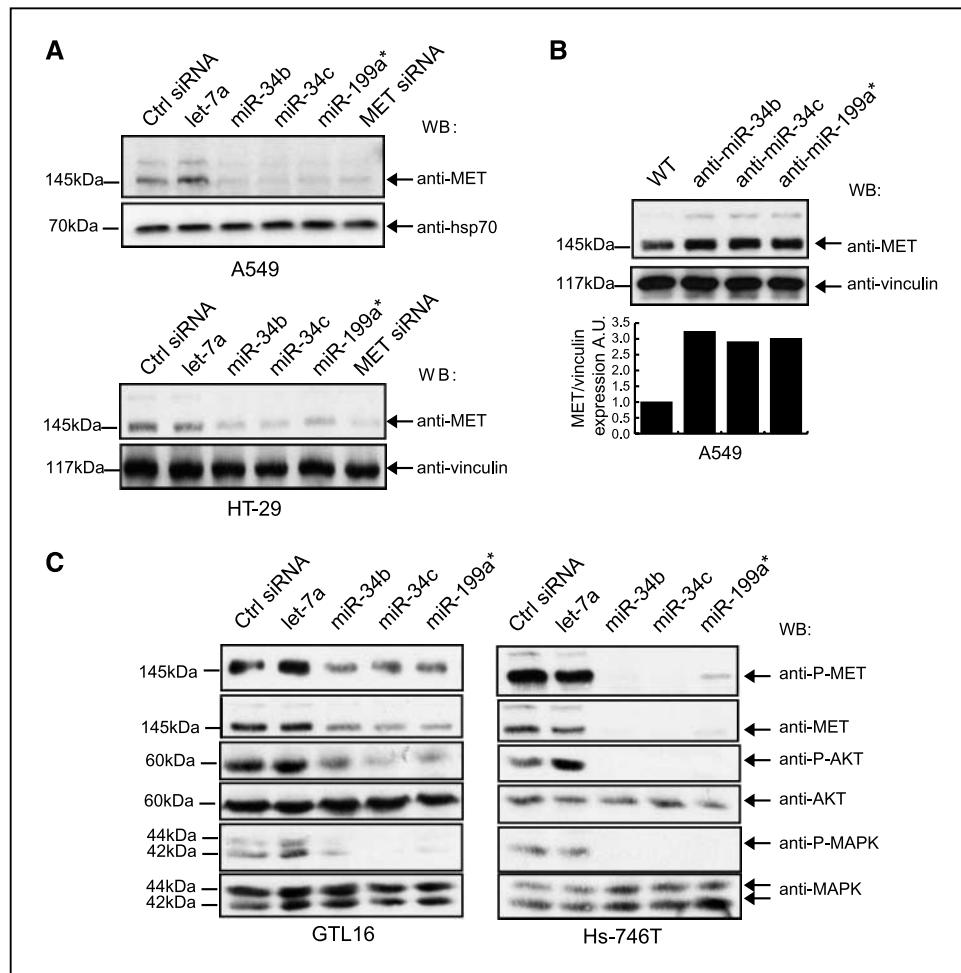


Figure 1. MiR-34b, miR-34c, and miR-199a* directly bind the 3'UTR of the *MET* mRNA. **A**, schematic representation of miRNA binding sites in the *MET* 3'UTR. **B**, expression profiles of miR-34c (which is transcribed as a polycistronic unit with miR-34b) and miR-199a* in different cancer cell lines (*top*). RNA (0.5 µg) was reverse transcribed and amplified with miRNA-specific primers and Taqman probes. Relative expression was calculated using the comparative cycle threshold method; miRNA amount was normalized relative to human RNU48. Results are expressed as fold changes relative to miRNA levels observed in A549 cells, taken as standard. As shown, miR-199a* is expressed at very low levels in almost all the epithelial cancer cell lines examined, with the notable exception of the ovarian cell line A2780; in agreement with data from the literature, the level of expression is high in WI-38 fibroblasts (30). MiR-34c is uniformly expressed at higher levels, compared with miR-199a*. **Bottom**, Western blot (WB) analysis of total cell lysates (40 µg per lane) probed with anti-MET (*top line*) or anti-vinculin (as a loading control, *bottom line*) antibodies. **C**, COS-7 cells were transiently transfected with the luciferase reporter vector and, 24 h later, transfected with the indicated miRNAs. Luciferase activity was evaluated 48 h after transfection and normalized for total proteins. As shown, the luciferase activity was significantly decreased ($P < 0.001$) in cells transfected with miR-34b, miR-34c, and miR-199a* but not in cells containing let-7a. *RLU*, relative luciferase units.

Figure 2. MiR-34b, miR-34c, and miR-199a* regulate the level of MET expression. *A*, A549 lung carcinoma cells and HT-29 colon carcinoma cells expressing the MET tyrosine kinase were transfected with the indicated miRNAs, with a specific MET siRNA (23), or with a control mismatched siRNA (*ctrl siRNA*). Expression of the MET protein was evaluated 72 h later by Western blot. As shown, the levels of MET were significantly decreased in the presence of miR-34b, miR-34c, miR-199a*, and MET siRNA but not in the presence of let-7a. The same blots were also probed for hsp70 (top blot) or vinculin (bottom blot), as loading controls. *B*, A549 lung carcinoma cells were transfected with the indicated antagomir (*anti-miRs*) to evaluate the effect of blocking the endogenous miRNAs. *Top*, Western blot of a total cell lysate probed with either anti-MET or anti-vinculin (bottom) antibodies. Bands were scanned and quantified. *Columns*, ratio between MET and vinculin expression. As shown, abrogation of the function of each of the three miRNAs resulted in 3-fold increased MET expression, further demonstrating a physiologic role of these noncoding RNAs in regulating MET expression. *C*, Western blot analysis of total cell lysates of GTL16 and Hs-746T gastric carcinoma cells (both displaying amplification of the MET gene and overexpression of MET protein) transfected with the indicated miRNAs. As shown, transfection with miR-34b, miR-34c, and miR-199a* induced efficient MET decrease, abrogation of MET tyrosine phosphorylation, and impairment of AKT and p42/44 MAPK activation. Activation of downstream transducers was not curbed by expression of let-7a.



carcinomas, where it is up-regulated compared with the normal tissue (31).

We evaluated the expression of these miRNAs by Real-time PCR in several cancer cell lines expressing different levels of MET protein (Fig. 1B, bottom), and we found that although miR-34c is widely expressed, miR-199a* is expressed at low levels, with the notable exception, as expected, of ovarian cancer cells (Fig. 1B, top).

miR-34b, miR-34c, and miR-199a* bind to the 3'UTR of MET mRNA. To confirm the *in silico* predictions, we tested if miR-34b, miR-34c, and miR-199a* can bind to the 3' UTR of the *MET* mRNA and inhibit protein translation. To this end, we cloned the full-length 3'-UTR sequence of human *MET* into the luciferase expressing vector pGL3, just downstream of the luciferase stop codon. We then transiently expressed this construct in COS-7 cells, in the presence or absence of the different miRNAs. As shown in Fig. 1C, in the presence of miR-34b, miR-34c, or miR-199a*, we observed a significant 40% to 50% decrease of luciferase activity, compared with control cells (not expressing the miRNAs; $P < 0.001$) or to cells transfected with the unrelated miRNA let-7a (known to target the Ras oncogene; ref. 17). These data suggest that all the three miRNAs bind to the 3'UTR of *MET* and impair *MET* mRNA translation.

Expression of miR-34b, miR-34c, and miR-199a* leads to decrease of MET mRNA and protein levels. To evaluate if miR-34b, miR-34c, and miR-199a* can modulate the levels of MET protein, we transfected different human carcinoma cell lines with

chemically synthesized miRNA precursors. As shown in Fig. 2A, 72 hours after miRNA transfection, the cellular amount of MET protein was significantly reduced at levels comparable with those observed in cells transfected with a specific *MET* siRNA (23). As a control, cells were transfected with the unrelated miRNA let-7a or with a mismatched *MET* siRNA (Ctrl siRNA). Notably, miR-34b, miR-34c, and miR-199a* can regulate MET levels in a tissue independent manner, as similar results were obtained upon expression of these miRNAs in tumor cell lines of different histologic origin (Supplementary Fig. S1; Fig. 2A and C). In the analyzed cell lines, the decrease of MET protein was paralleled by a decrease in *MET* mRNA (Supplementary Fig. S2).

To verify if miR-34b, miR-34c, and miR-199a* play a physiologic role in controlling MET expression, we transfected A549 lung carcinoma cells (that express moderate levels of all these miRNAs; Fig. 1B) with miR-34b, miR-34c, and miR-199a* specific antagomir, molecules that are able to suppress the function of the complementary endogenous miRNAs (32). As shown in Fig. 2B, an increase in MET expression was observed upon antagomir transfection, suggesting that the identified miRNAs physiologically regulate MET expression.

To assess if the identified miRNAs could modulate the levels of MET even in carcinoma cells displaying overexpression and constitutive activation of this oncogenic receptor (such as the gastric carcinoma cells GTL16 and Hs-746T; refs. 23, 33), we transfected miR-34b, miR-34c, and miR-199a* in these cells. As

shown in Fig. 2C, also in this cellular context, the amount of MET protein was significantly reduced upon miRNAs transfection.

In conclusion, we have found that miR-34b, miR-34c, and miR-199a* can negatively regulate MET expression in carcinoma cells of different histopathologic origin, including cells carrying *MET* gene amplification and overexpression.

To evaluate if the observed decrease of MET protein was paralleled by a reduction of MET tyrosine phosphorylation and activation of downstream targets, we assessed the phosphorylation status of MET and the activation of mitogen-activated protein kinase (MAPK) and AKT (known to be critical mediators of proliferation and resistance to apoptosis, respectively) upon

exogenous miRNA expression. As shown in Fig. 2C, GTL16 and Hs-746T cells expressing exogenous miR-34b, miR-34c, or miR-199a* displayed not only a reduction of total MET tyrosine phosphorylation but also a decreased level of activated MAPK and AKT. This was not observed upon transfection with the unrelated miRNA let-7a.

MiRNA-mediated MET decrease impairs the accomplishment of the invasive program. MET activation contributes to several important steps in tumor progression and metastatic dissemination (7). To verify if the expression of the identified miRNAs could impair the execution of the invasive growth program in cancer cells, we transfected A549 lung carcinoma cells

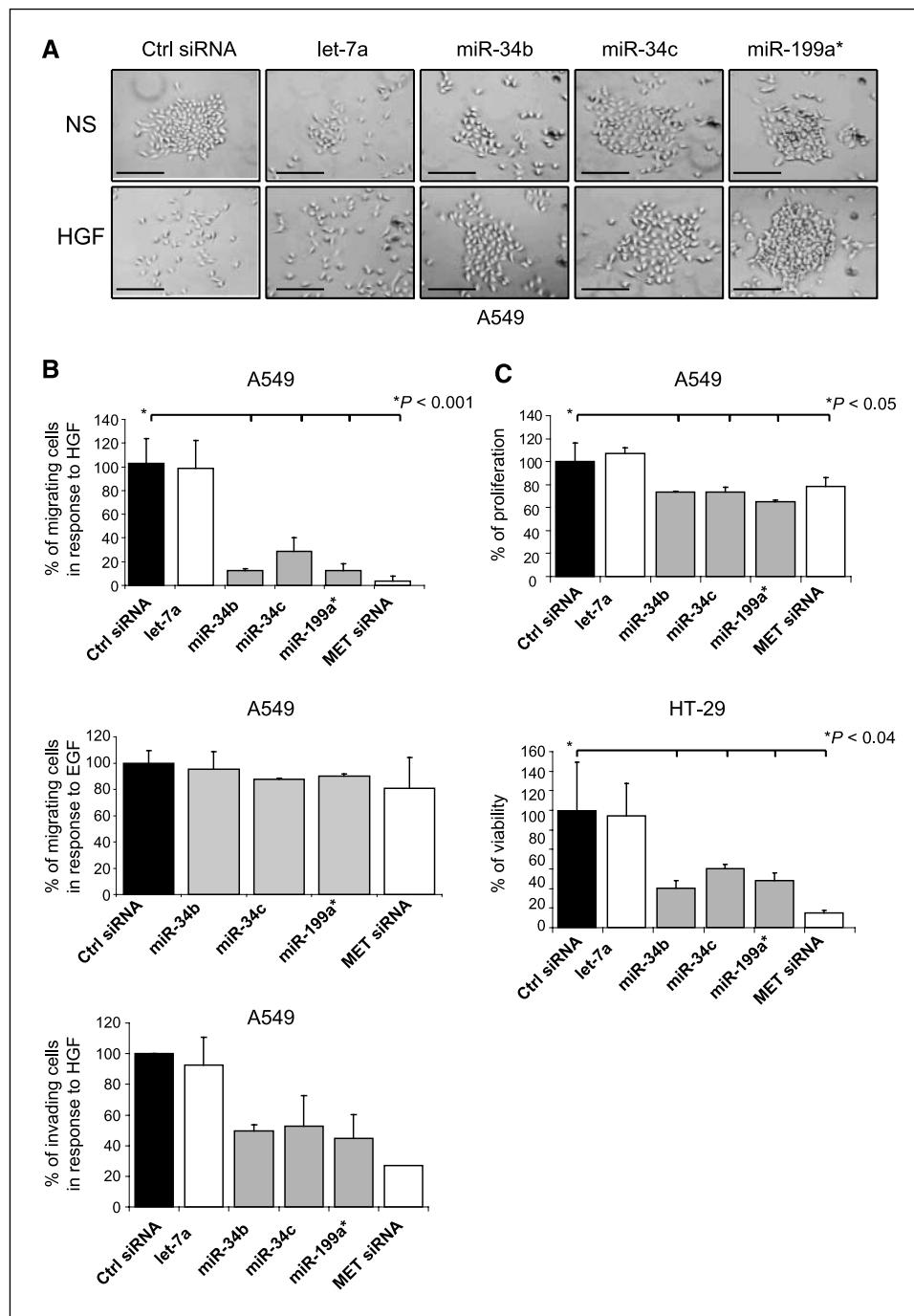


Figure 3. MiR-34b, miR-34c, and miR-199a* impair the ability of tumor cells to activate the invasive growth program. *A*, scatter assay performed on A549 lung carcinoma cells transfected with the indicated miRNAs. Cells were seeded at low density in the presence of DMEM supplemented with 10% FBS. After formation of the islets, cells were left untreated (NS) or stimulated for 16 h with 20 ng/ml of HGF. As shown, cells transfected with miR-34b, miR-34c, or miR-199a* were almost unable to scatter in response to HGF. Bars, 0.25 mm. Fields shown in the figure are representative of two experiments performed in duplicate. *B*, A549 cells expressing the different miRNAs were seeded on the upper face of Transwell filters, coated (*bottom graph*) or not (*top and middle graphs*) with Matrigel, and exposed to a gradient of either HGF (*top and bottom graphs*) or EGF (*middle graph*). As shown, cells transfected with miR-34b, miR-34c, or miR-199a* were severely impaired in their ability to migrate or to invade in response to HGF, although they did not show significant alterations in their migration in response to EGF. Columns, percentage of migrating or invading cells relative to control cells, considered as 100%. *C*, A549 lung carcinoma cells and HT-29 colon carcinoma cells transfected with the different miRNAs were evaluated for their growth ability (A549) or viability (HT-29). Columns, percentage of proliferating or viable cells relative to cells transfected with control siRNA, considered as 100%.

and HT-29 colon carcinoma cells with *MET*-targeting miRNAs and evaluated different biological properties contributing to invasive growth. These cells were chosen for their ability to activate *in vitro* a motogenic and invasive response to HGF. Motility and invasive ability were assessed both in scatter and in Boyden chamber assays. As shown in Fig. 3A and in the Supplementary Fig. S3A, upon miRNA transfection, cells were unable to scatter (i.e., to break intercellular junctions and to undergo radial migration) in response to HGF; moreover, we also found that the ability of cancer cells to migrate and invade an *in vitro* reconstituted basal membrane, in response to an HGF gradient, was strongly impaired ($P < 0.001$; Supplementary Fig. 3B; Fig. 3B, top and bottom). The specificity of the response was shown by the fact that the ability to migrate toward an endothelial growth factor (EGF) gradient was almost unaffected, thus suggesting that the basic machinery mediating directional migration is not altered in the presence of these miRNAs (Fig. 3B, middle). In cells expressing exogenous miRNAs, we also observed decreased cell growth (Fig. 3C), which was more evident in HT-29 cells ($P < 0.005$), that display a moderate level of constitutive MET phosphorylation, independent from the presence of HGF (34).

It is known that each miRNA can negatively control the expression of a panel of RNA transcripts. To prove that the biological effects promoted by the studied miRNAs are mainly mediated by down-regulation of MET, we transduced A549 cells with a *MET* cDNA that lacks the 3' UTR region recognized by miR-34b, miR-34c, and miR-199a*. *MET*-transduced cells were then

transfected with the miRNA precursors and analyzed for their ability to migrate in response to HGF. As shown in Fig. 4, the expression of MET allowed the recovery of the migratory ability of A549 cells, suggesting that the effects of these miRNAs on genes other than MET are not critical to prevent the accomplishment of the invasive growth program.

We also investigated if miRNA-mediated control of MET expression is active not only in established cell lines but also in primary cancer cells, a cellular system more closely reflecting tumoral features. To this end, we evaluated the biological effect of exogenous expression of miRNAs in primary melanoma cells that endogenously express limited amounts of the investigated miRNAs (Supplementary Fig. S4A): also in these cells, miR-34b, miR-34c, and miR-199a* led to a consistent decrease of MET protein levels that in turn resulted in the impairment of MET-mediated motility (Supplementary Fig. S4B).

Exogenous expression of miR-34b, miR-34c, and miR-199a* impairs survival of cells overexpressing a constitutively active MET. As we and others have recently proven that some cancer cell lines displaying MET gene amplification and overexpression (leading to the presence of high levels of constitutively active receptor) are “addicted” to the expression of this oncogene (23, 33), we wondered if we could impair their viability and invasive ability by inhibiting *MET* expression through transfection of exogenous miRNAs. GTL16 and Hs-746T cells were transfected with the aforementioned miRNAs; as shown in Fig. 5A, B, and C, we found that the viability and invasive ability of MET-addicted cells were severely impaired in the presence of these miRNAs. The percentage of viable and migrating cells was similar to that observed for cells transfected with the *MET* siRNA. Migratory ability and viability of cells transfected with control siRNA or miRNA let-7a remained unchanged.

To prove that also in cells overexpressing a constitutively active MET, the biological effects promoted by the studied miRNAs are mainly mediated by down-regulation of MET, we transduced GTL16 cells with a constitutively active form of MET (TPR-MET), lacking the 3' UTR region (Fig. 5C; ref. 24). TPR-MET-transduced cells were then transfected with miRNAs and analyzed for their ability to grow and to invade. As shown in Fig. 5C (middle), the expression of TPR-MET allowed an almost complete recovery of the invasive ability of GTL16 cells, whereas viability (bottom) was only partially recovered, indicating the involvement of other miRNA-regulated genes for this biological property.

Discussion

Tumor progression is a complex, multistep process involving genetic and epigenetic events that activate oncogenes and inactivate tumor suppressor genes, which are linked to the acquisition of distinct malignant features, affecting cell growth, differentiation, and survival. In recent years, it has become evident that genes whose expression is critical for the tight control of these basic processes can be deregulated not only as consequence of genetic or transcriptional alterations but also because of impaired posttranscriptional control exerted by miRNAs (35). miRNAs are small (19–25 nucleotides long) noncoding RNA molecules, capable of negatively controlling gene expression, mainly at translational level. It has been calculated that the human genome contains ~1,000 miRNAs, with the potential of regulating around 30% of human genes (36). In fact, each miRNA can potentially interact with several targets via perfect or imperfect base pairing, primarily

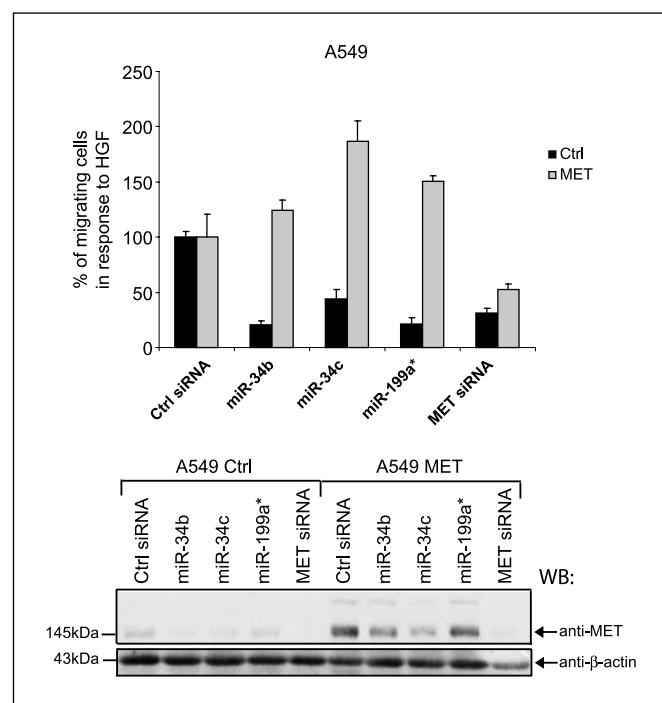


Figure 4. Reexpression of MET in miRNA-transfected A549 cells leads to recovery of invasive growth properties. A549 lung carcinoma cells transduced with either an empty lentiviral vector (A549 Ctrl) or a vector containing a *MET* cDNA devoid of the 3' UTR (A549 MET) were transfected with the indicated miRNAs or siRNAs. Forty-eight hours after transfection, 40,000 cells were plated on the upper sides of transwell filters. After 24 h, the number of cells migrated to the lower side of the filters was evaluated (top). Columns, percentage of migrated cells relative to cells transfected with control siRNA, considered as 100%. As shown, when MET was reexpressed in cells (bottom), the different miRNAs were unable to inhibit HGF-induced cell motility.

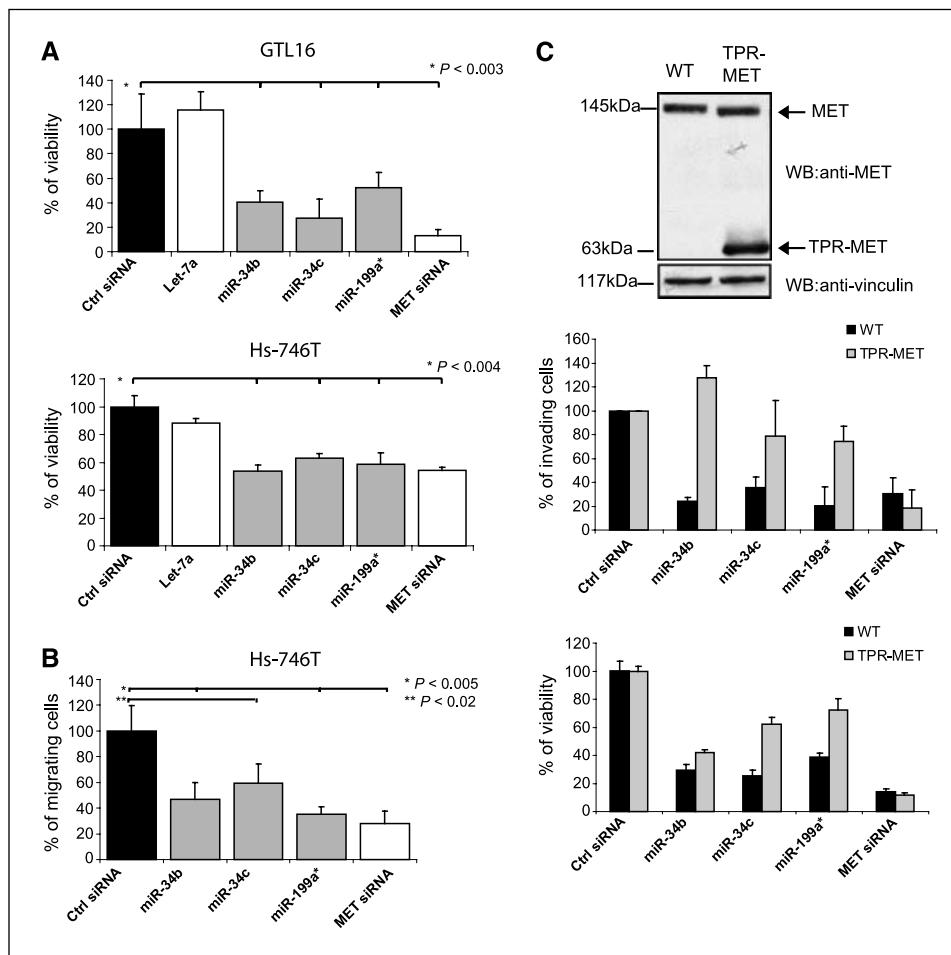


Figure 5. MiR-34b, miR-34c, and miR-199a* impair viability and invasive properties of MET-addicted cells. *A*, viability of GTL16 and Hs-746T gastric carcinoma cells transfected with the indicated small RNAs. Columns, percentage of viable cells relative to cells transfected with control siRNA, considered as 100%. As shown, viability of miRNA-transfected cells was severely impaired, compared with controls. *B*, migration of Hs-746T cells transfected with the indicated miRNAs. Columns, percentage of migrated cells relative to cells transfected with control siRNA, considered as 100%. As shown, cell motility was significantly impaired upon miRNA transfection. *C*, GTL16 cells were transduced with TPR-MET, a constitutively active form of MET that cannot be targeted by miRNAs, as it lacks the 3'UTR sequences. Top, Western blot that shows the expression of TPR-MET; the same blot was probed for vinculin, as loading control. TPR-MET-GTL16 and wt-GTL16 cells were transfected with miR-34b, miR-34c, or miR-199a*, and their invasive ability and viability were evaluated. Columns, percentage of invading (middle graph) and viable (bottom graph) cells relative to cells transfected with control siRNA, considered as 100%. As shown, expression of TPR-MET allowed an almost complete recovery of the invasive ability of GTL16 cells, suggesting that genes other than MET that may be coregulated by these miRNAs are not critical to allow the accomplishment of the invasive growth program. Viability was only partially recovered in the presence of TPR-MET, indicating the possible involvement of other miRNA-regulated genes.

in the 3'-UTR portion (37). Because direct experimental methods for discovering miRNA targets are lacking, a number of target prediction algorithms, relying on specific-base pairing rules and conservational analysis, have been developed to score possible recognition sites and identify putative gene targets. However, these predictions usually yield a large number of false-positive candidates and experimental validation is thus strictly required.

In our work, by *in silico* analysis, we have identified miR-34b, miR-34c, and miR-199a* as potential candidates capable of targeting the *MET* oncogene. The most widely used approach for experimentally validating miRNA targets is to clone the predicted miRNA binding sequence downstream to a luciferase reporter construct and to cotransfect it with the miRNA of interest. By using this technique, we have shown that indeed *MET* is a true target for the above mentioned miRNAs. Moreover, exogenous expression of each miRNA resulted in a strong decrease of the amount of MET protein, whereas blockade of endogenous miRNAs (by means of antagomirRs) led to a 3-fold increase of MET expression; these data suggest that miR-34b, miR-34c, and miR-199a* play a physiologic role in the regulation of the expression of this potentially oncogenic receptor. In agreement with our data, Kim and colleagues (38) have recently shown that miR-199a* directly binds to *MET* promoter.

We also provide evidence that exogenous expression of miR-34b, miR-34c, and miR-199a* decreased the level of *MET* mRNA and protein in tumor cells as well as in primary tumor cells and thus precluded the activation of the biological programs stimulated by this tyrosine kinase receptor. Moreover, the identified miRNAs

were also effective in impairing the invasive growth ability of "MET-addicted" cancer cells that express high levels of a constitutively activated receptor. The observed miRNA-induced impairment of the invasive growth program is mainly due to *MET* silencing as its exogenous reexpression rescued the ability to perform this biological program. Although it is very likely that many other genes are coregulated with *MET* by these specific miRNAs, none of them seems to be absolutely required for the execution of this program.

It has been shown that in many tumors the MET protein is expressed at levels much higher than in the normal counterpart.⁸ Some factors contributing to increased gene transcription, such as activation of other oncogenes (11, 12), inactivation of tumor suppressor genes—for instance p53 (29)—or hypoxia (9) have been identified. However, also an altered posttranscriptional control of the *MET* mRNA by miRNAs might be implicated in the up-regulation of MET expression. These two levels of control of gene expression can be intermingled. In fact, these factors can act either directly, by controlling the transcription of *MET*, or indirectly, by decreasing the expression of miRNAs that negatively regulates the level of MET protein. For example, a feedback mechanism involving miR-34 family has been shown between p53 and MET. MET-triggered activation of PI3K/Akt increases mammalian target of

⁸ <http://www.vai.org/met>

rapamycin activation that, in turn, inhibits p53 by promoting the translation of Mdm2, a negative regulator of p53 (39). MiR-34 family is a direct transcriptional target of p53 (40); thus, inhibition of p53 results in decreased transcription of these miRNAs, which cannot any longer down-regulate MET expression. These observations can contribute an explanation to the data available in the literature, showing increased levels of MET expression in cells devoid of p53 and in patients affected by Li-Fraumeni syndrome (29). Also MET regulation by hypoxia can happen through a dual mechanism. The transcription factor HIF-1 α , which levels are increased in hypoxic conditions as a consequence of protein stabilization, directly binds the MET promoter, inducing the transcription of this gene (9). At the same time, HIF-1 α controls the expression of many hypoxia-regulated miRNAs (41). One of them, miR-15b, which decreases in hypoxic conditions, negatively regulates MET (42); hypoxia, therefore, reduces the amount of the negative regulator of MET miR-15b, thus further enhancing MET expression.

These miRNA-mediated crossregulations between oncogenes and tumor suppressor genes are very intriguing as they introduce a further level of complexity in the control of cellular biology, providing the cell with a more immediate and accurate system to modulate protein expression, as it does not require transcriptional activation and subsequent mRNA processing steps. However, the interplay between miRNAs and genes controlling critical biological processes not only introduces elements of versatility but also aspects of weakness. It is interesting to note that many of the miRNA coding genes are indeed located in fragile sites, often lost, or rearranged in tumors (43). A notable example is the loss of miR-15a and miR-16 in hematopoietic malignancies and in prostate

cancers (44, 45). Loss of these miRNAs leads to up-regulation of normally repressed targets, such as the antiapoptotic protein BCL2 (45), which can contribute to the tumorigenic process. In the same line of thinking, we can hypothesize that expression of miR-34b, miR-34c, and miR-199a* can be lost for yet unknown reasons in some tumors, thus contributing to increase MET expression. Mir-199a* is indeed expressed at lower levels in tumor cells compared with normal tissues (30). Understanding the physiologic regulation of miRNAs is also a very interesting field of investigation; results obtained by these studies could help to shed light on the different mechanisms through which these small RNAs can contribute to cell transformation.

Altogether, our data provide the evidence that miRNAs are a novel and additional system to regulate MET expression in tumor cells and, thus, to control MET ability to orchestrate the invasive growth program.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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