MiR-1 Downregulation Cooperates with MACC1 in Promoting *MET* Overexpression in Human Colon Cancer

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Abstract

Purpose: MET, the tyrosine kinase receptor for hepatocyte growth factor, is frequently overexpressed in colon cancers with high metastatic tendency. We aimed to evaluate the role of its negative regulators, miR-1 and miR-199a*, and its transcriptional activator, the metastasis-associated in colon cancer 1 (MACC1), in controlling MET expression in human colon cancer samples.

Experimental Design: The expression of MET, miR-1, miR-199a*, and MACC1 was evaluated by real-time PCR in 52 matched pairs of colorectal cancers and nontumoral surrounding tissues. The biological role of miR-1 in controlling MET expression and biological activity was assessed in colon cancer cells either by its forced expression or by AntagomiR-mediated inhibition.

Results: MiR-1 was downregulated in 84.6% of the tumors and its decrease significantly correlated with MET overexpression, particularly in metastatic tumors. We found that concurrent MACC1 upregulation and miR-1 downregulation are required to elicit the highest increase of MET expression. Consistent with a suppressive role of miR-1, its forced *in vitro* expression in colon cancer cells reduced MET levels and impaired MET-induced invasive growth. Finally, we identified a feedback loop between miR-1 and MET, resulting in their mutual regulation.

Conclusions: This study identifies an oncosuppressive role of miR-1 in colorectal cancer in which it acts by controlling MET expression through a feedback loop. Concomitant downregulation of miR-1 and increase of MACC1 can thus contribute to MET overexpression and to the metastatic behavior of colon cancer cells. *Clin Cancer Res; 18(3); 737–47. ©2011 AACR.*

Introduction

Colorectal cancer represents the third most common cancer type worldwide, and it is responsible for a high percentage of cancer deaths (1). Metastatic disease is the most frequent reason of treatment failure (2, 3).

Many studies have implicated the product of the *MET* oncogene, encoding for the tyrosine kinase receptor for hepatocyte growth factor (HGF), in the progression of colon cancer toward metastasis (4, 5). Besides stimulating proliferation, *MET* also promotes cell scattering, invasion, and protection from apoptosis, thereby acting as a prometastatic gene for many tumor types (6). Even if the presence of gene amplification, activating point mutations, or ligand autocrine stimulation has been described, the most frequent alteration of the MET receptor in human tumors is, by far, overexpression (7, 8). Many works have shown that, in colorectal cancer, MET overexpression associates with development of distant metastases and with shorter metastasis-free survival (5, 9–12); thus, MET activation is considered important for the acquisition of metastatic potential. This idea is further supported by the observation that while the *MET* gene is very rarely amplified in primary colorectal cancer, an increased copy number can be found in liver metastases derived from the same tumors (4, 12). In a previous work, we found that overexpression was associated with amplification of the *MET* gene in only 10% of primary carcinomas, but in 8 out of 9 metastases examined (12). These data suggest that overexpression of *MET* oncogene provides a selective growth advantage to neoplastic colorectal cells; moreover, amplification appears to give a further selective advantage for the acquisition of metastatic potential. It was also shown that the seeding of metastatic

Translational Relevance

The *MET* oncogene and its transcriptional activator, metastasis-associated in colon cancer 1 (MACC1), were implicated in the progression of colon cancer toward metastasis. On these bases, phase I/II clinical trials are currently addressing the therapeutic efficacy of MET inhibitors in metastatic colorectal cancer. The most frequent alteration of MET in colorectal cancer is overexpression, the molecular bases of which are not yet defined. We aimed to evaluate if increased MET expression could be associated with deregulation of micro-RNAs. We found indeed that miR-1, which negatively controls MET, is downregulated in the majority of colon cancers and that MET and miR-1 reciprocally control their expression through a feedback circuit, modulating MET-induced invasive growth. Moreover, differently from what has been previously shown, miR-1 decrease and MACC1 increase are both required to elicit a significant increase of MET expression. This study thus identifies an oncosuppressive role of miR-1 in colon cancer, in which it modulates MET levels and shows that MACC1 alone is not sufficient as a predictor of the metastatic tumor behavior. Moreover, because we showed the presence of a feedback circuit between MET and miR-1, it is likely that MET targeting in colon cancer could result not only in abrogation of MET-dependent signaling but also in the partial recovery of miR-1 levels and, consequently, of the suppressive ability of this miRNA.

cells in the liver leads to a reactive hepatic response, resulting in an increase of circulating HGF, which further activates MET by systemic/paracrine stimulation (13). In light of these findings, clinical trials with MET and HGF inhibitors in advanced colorectal cancers are now ongoing (http://clinicaltrials.gov; http://www.amgentrials.com).

It has recently been shown that a key regulator of HGF–MET signaling in colon cancer is metastasis-associated in colon cancer 1 (MACC1), a MET transcriptional activator that was identified as an independent prognostic indicator of metastasis formation and metastasis-free survival in colon cancer (14).

Among the several mechanisms regulating gene expression, an important role is played by microRNAs (miRNAs), which are negative regulators acting mainly at posttranscriptional level (15). The involvement of miRNAs in cancer pathogenesis is now well established, and increasing evidence shows that they can behave either as oncogenes (16–18) or tumor suppressor genes (19–21), depending on the cellular function of their targets. Genetic analyses estimate that approximately 50% of miRNAs are located in regions known to be amplified or deleted in human cancers (22, 23). In addition, epigenetic modifications in miRNA loci, altering miRNA transcription and affecting the metastatic

ability of tumor cells, have been described (24). Finally, recent data suggest that miRNAs are deregulated in many tumor types, including colorectal cancer, during tumor development and progression (25–29).

In vitro studies carried out by us and other groups have recently identified miR-1 and miR199a* as negative regulators of MET expression (30–32). In this work, we aimed at evaluating if deregulation of these miRNAs can be responsible for MET overexpression in human colon cancer. We found that miR-1 is downregulated in the majority of colon cancers, and that the concomitant decrease of miR-1 and increase of MET shows a highly significant correlation, especially in metastatic tumors. We also provide evidence of a reciprocal interplay between miR-1 and MET because this receptor negatively controls the expression of miR-1. Moreover, the exogenous expression of miR-1 in colon cancer cells decreased MET levels and MET-induced invasive growth. Finally, we show that concomitant miR-1 downregulation and MACC1 increase functionally synergize in promoting MET overexpression and can contribute to the progression of colon cancer toward metastases.

Materials and Methods

Study population

Pairs of primary colorectal tumors and adjacent nontumorous specimens came from patients diagnosed with colorectal cancer and operated over the period of 2004 to 2009 at the Department of General Surgery, University of Cagliari. Clinical data and follow-up records of colorectal cancer patients were collected in a dedicated electronic database. For this study, cases with familial adenomatous polyposis or human nonpolyposis colorectal cancer were excluded. All patients provided informed consent. The study has been approved by the ethical committee of the University of Cagliari.

Immediately after surgical specimen extraction, the colon was opened and both tumoral tissue and normal mucosa were collected. To preserve only the mucosal layer, a mucosectomy was carried out after injecting saline solution to separate it from the submucosal layer.

Cell culture and transfection

SW48 (colon adenocarcinoma), DLD-1 (colorectal adenocarcinoma), A549 (lung adenocarcinoma), and Hep G2 (hepatocellular carcinoma) cell lines were obtained from American Type Culture Collection (ATCC), where they are regularly authenticated based on viability, recovery, growth, morphology, and isoenzymology. HT-29 (colorectal adenocarcinoma) cell line was obtained from National Cancer Institute. EBC-1 (lung adenocarcinoma) cell line was obtained from JCRB. All cell lines were used within 6 months from their arrival.

Transient transfections were carried out with 0.2 μmol/mL of synthetic pre-miRNAs and mirVana miRNA mimic Negative Control # 1 (Applied Biosystem), 0.2 μmol/mL of AntagomiR (Exiqon), or 0.1 μmol/mL of synthetic MET/Ctrl siRNAs (33, 34), according to standard protocols. For

GFP and MET transduction, viruses were produced as in ref. 35.

Quantitative analysis of miRNAs and mRNAs

Total RNA extraction, retrotranscription, and real-time PCR were carried out as in ref. 30. For primer sequences, see Supplementary Methods.

Copy number analysis

Genomic DNA was extracted with Blood and Cell Culture DNA Midi Kit (QIAGEN) according to standard protocols. *MET*, *miR-1-1*, and *miR-1-2* copy number was evaluated with TaqMan predesigned assays. *RNAse P* was used as internal reference (Applied Biosystem).

Protein extraction and Western blot

Western blot analysis was conducted according to standard methods. Used antibodies are listed in Supplementary Methods.

In vitro biological assays

Cell viability was evaluated with the Cell Titer GLO (Promega) according to the manufacturer protocol.

Wound healing ability was evaluated with the Oris Cell Migration Assay (Platypus Technologies) according to manufacturer protocol. Two days after the seeding, cells were fixed with 11% glutaraldehyde and stained with 0.1% crystal violet. Pictures were taken at $5\times$ magnification, with a Leica DMIL microscope connected to a Leica DFC320 camera. The area of the hole was calculated with ImageJ software.

Soft agar, scatter and migration assays were conducted as in refs. 30, 34. Every biological assay was done at least 3 times.

Immunohistochemistry

Immunohistochemical staining was carried out with the anti-MET antibody C-28, (Santa Cruz Biotechnology) according to standard methods. Positivity was evaluated as low ($+$, <10% of positive cells), intermediate ($++$, >10% to <50% of positive cells), or high ($+++$, >50% of positive cells), considering both the number of positive cells and the intensity of the staining. Pictures were taken at $40\times$ magnification, with a Leica DMIL microscope connected to a Leica DFC320 camera.

Statistical analyses

All the statistical analyses for the real-time PCR expression profiles were conducted in R-Bioconductor (36); after quality control, the C_t values of MET, MACC1, and miRNAs were normalized to β-actin and RNU48 CT, respectively.

The expression analyses of MET, miR-1, miR-199a*, and MACC1 were conducted on $\log_2$ signal. The correlation analyses between miR-1/MET, MACC1/MET, and miR-1/C/EBPα were done on paired $\log_2$ ratio values. For statistical analyses, we carried out Student t test.

In Fig. 3C, the levels of miR-1 and MACC1 were evaluated as the ratio between the expression in the tumor versus the corresponding normal tissue. Specimens were subdivided on the basis of the median gene expression (always normalized vs. the corresponding normal tissue). Specimens with "miR-1 low" display miR-1 expression below the median of all samples; conversely "MACC1 high" specimens display MACC1 expression above the median. "MiR-1 normal" samples show miR-1 levels not below the median range. "MACC1 normal" samples show MACC1 levels not above the median range.

Results

Patients

Matched pairs of colorectal cancers and nontumoral surrounding tissues were obtained from 52 patients who underwent surgical resection of the tumor. The characteristic of the patients included in this study are described in Table 1. Briefly, the analyzed group was composed of 53.8% males and 46.2% females; 44.2% of the patients were aged more than 74. According to the analysis of the pathologist, 5.8% of the cases were classified as T1, 9.6% as T2, 67.3% as T3, and 7.7% as T4; nodes were not compromised in 51.9% of the patients and 65.4% did not show metastasis.

MET is overexpressed in colon cancer samples

Several studies have shown that MET is frequently overexpressed in colon adenocarcinomas (8). Accordingly, real-time PCR on retrotranscribed total RNA, obtained from tumors as well as from their normal surrounding tissues, showed MET overexpression in 82.7% of tumors compared with their normal counterparts (Fig. 1A); the median level of MET expression was also significantly increased in the neoplastic tissue ($P < e^{-12}$; Fig. 1B).

To confirm that the enhanced level of MET mRNA corresponded indeed to an increase of the MET protein, we carried out immunohistochemistry (IHC) on paraffin-embedded slices derived from the same tumors. We compared the level of MET expression in the tumor versus the surrounding normal tissue. All the analyzed samples showing an increased level of MET mRNA, also displayed a proportional increased expression of the MET protein (a representative case—out of 15—is shown in Fig. 1C).

MET overexpression is very rarely due to gene amplification in primary colon cancers (4, 12). In agreement with the literature, real-time PCR carried out on tumor-derived genomic DNA did not detect *MET* amplification in any examined samples (Supplementary Fig. S1A), clearly showing that the observed MET overexpression is not due to increased gene copy number.

The expression of MET-targeting miR-1 and miR-199a* is decreased in colon cancers

Works done by our and other groups showed that miR-1, miR-199a*, and miR-34c are involved in the control of MET expression in several tissues (30–32). Because in the examined colorectal cancer samples MET overexpression is not due to gene amplification, we wondered if it could be due to

Table 1. Baseline characteristics of the patients

	N	%
Gender		
Male	28	53.8
Female	24	46.2
Total	52	
Age		
<65	16	30.8
65–74	9	17.3
>74	23	44.2
NA	4	7.7
Total	52	
Tumor stage		
T1	3	5.8
T2	5	9.6
T3	35	67.3
T4	4	7.7
NA	5	9.6
Total	52	
Nodes		
N0	27	51.9
N1	7	13.5
N2	13	25.0
NA	5	9.6
Total	52	
Metastases		
M0	34	65.4
M1	13	25.0
NA	5	9.6
Total	52	
Grading		
G0	1	1.9
G1	6	11.5
G2	26	50.0
G3	12	23.1
NA	7	13.5
Total	52	
Dukes		
A	4	7.7
B	21	40.4
C	10	19.2
D	13	25.0
NA	4	7.7
Total	52	
Tumor type		
Adenocarcinoma	47	90.4
Mucinous adenocarcinoma	5	9.6
Total	52	

Figure 1. MET is overexpressed in colorectal cancer. A, MET expression was evaluated by real-time PCR in 52 matched pairs of colorectal cancers and nontumoral surrounding tissues. Tumors (82.7%) showed MET overexpression (at least 2-fold) vs. the normal peritumoral tissue. B, box plot showing the median expression level of MET mRNA (measured by real-time PCR) in tumors versus the normal counterpart. The difference is statistically significant, $P < e^{-12}$. C, MET evaluation in a representative case. Top, immunohistochemical staining of a representative sample (out of 15) with anti-MET antibodies. As shown, the tumor displays a strong positivity for the MET protein. Bar, 0.025 mm. Positivity was evaluated as low ($+$, <10% of positive cells), intermediate ($++$, >10% to <50% of positive cells), or high ($+++$, >50% of positive cells), considering both the number of positive cells and the intensity of the staining. All the tumor area was evaluated, and the percentage of cells fitting in each category is shown in the circle graph. Bottom, real-time PCR evaluation of MET mRNA of the same tumor sample. The increased level of MET mRNA is paralleled by an increased expression of the MET protein.

decreased expression of miRNAs that negatively control MET. Expression of miR-1, miR-199a*, and miR-34c was thus analyzed by real-time PCR in colon adenocarci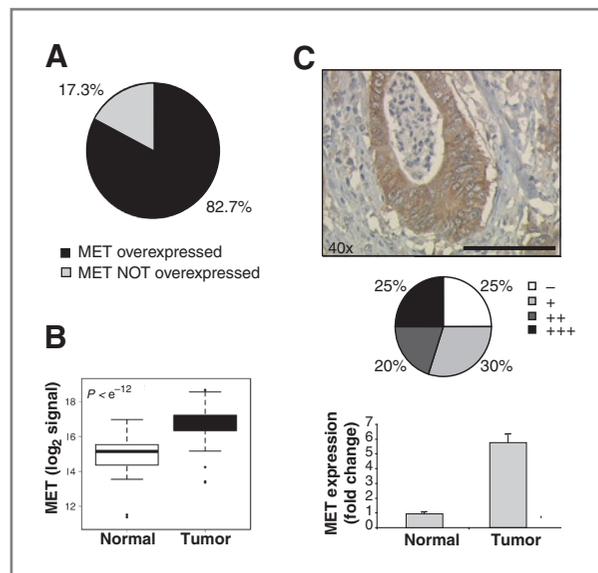nomas and in their normal counterparts. MiR-1 was downregu-lated in 84.6% of tumors compared with the normal peri-tumoral tissue (Fig. 2A, left); notably, the median expres-sion level of miR-1 was significantly decreased in neoplastic tissues ($P < 1.4e^{-9}$; Fig. 2A, right); a decrease of miR-199a* was observed in 30.8% of the cases, (Fig. 2B, left), but changes in expression level of miR-199a* were not signif-icant ($P < 0.17$; Fig. 2B, right). MiR-34c was barely expressed both in tumors and in the peritumoral mucosa and thus could not be evaluated (data not shown).

Because the decrease of miR-1 in tumor samples was strong and statistically significant, we focused on this miRNA. To evaluate whether the decrease of miR-1 could be due to the loss of gene copies, we carried out real-time PCR on genomic DNA (gDNA). As shown in Supplemen-tary Fig. S1B and S1C, no deletion of either gene encoding for this miRNA (37) was detected.

We also tested the possibility that the loss of C/EBPα, a transcription factor promoting miR-1 expression (31), could be involved in miR-1 downregulation. As shown in Supplementary Fig. S2, we did not observe any statistically significant downregulation of C/EBPα in samples with low miR-1 levels.
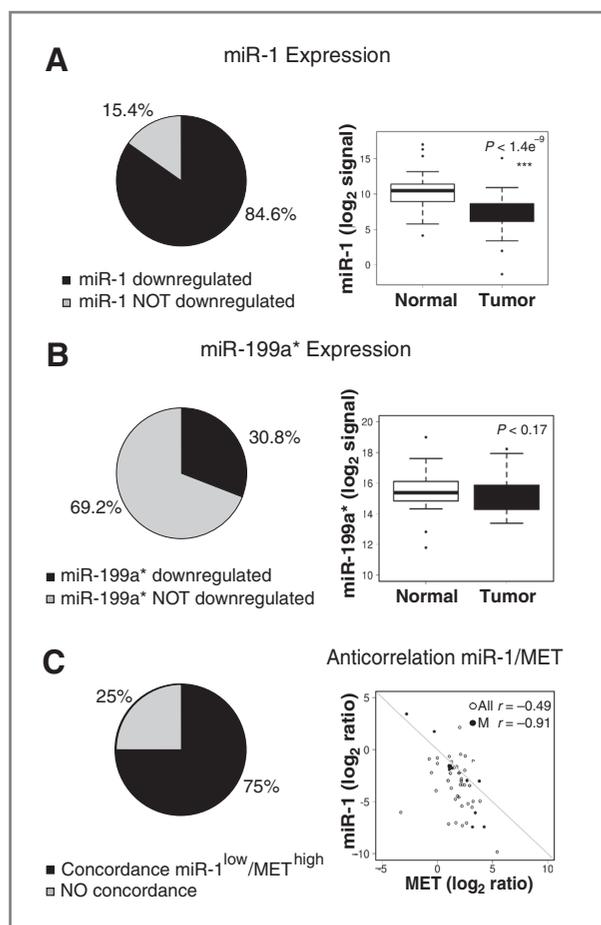
Figure 2. miR-1 expression is decreased in colorectal tumors and is anticorrelated with MET expression. A and B, expression of miR-1 (A) and miR-199a* (B) was evaluated by real-time PCR. As shown, miR-1 was downregulated (less than half in tumors vs. the corresponding normal mucosa) in 84.6% of the tumors whereas miR-199a* in 30.8%. The right part of each panel contains box plots showing the median expression level of miR-1 and miR-199a* in tumors versus the normal counterpart. The difference in miR-1 expression is statistically significant, $P < 1.4e^{-9}$. C, miR-1 and MET are often concomitantly deregulated in colorectal cancer samples. Left, the circle graph represents the percentage of tumors (75%) displaying both MET overexpression and miR-1 downregulation (concordance miR-1^{low}/MET^{high}). Right, the scatter plot shows the anticorrelation between miR-1 and MET expression (all samples: All, $r = -0.49$; tumor samples with synchronous or metachronous metastases: M, $r = -0.91$).

Because it had been reported that miR-1 expression can be modulated by epigenetic modifications (31, 38), we tested their possible involvement in controlling miR-1 expression in colon cancer cells. SW48 and HT-29 cells, treated with the histone deacetylase inhibitor Thricostatin A, did not show increased expression of miR-1 (Supplementary Fig. S3A). When cells were treated with 5-aza-cytidine, a DNA methyltransferase inhibitor, a decrease of miR-1-1 promoter methylation (Supplementary Fig. S3C) and an increase of miR-1 expression was observed (Supplementary Fig. S3B), suggesting that altered methylation could be involved in the regulation of miR-1 in colon

cancer cells. This result prompted us to investigate the methylation status of miR-1 promoter in tumors. MiR-1 is encoded by 2 different loci, miR-1-1 and miR-1-2 (37), but only the miR-1-1 promoter has evaluable CpG islands nearby (our unpublished data and ref. 31). The analysis of miR-1-1 promoter revealed a significant difference in methylation in tumor samples (Supplementary Fig. S4), associated with a decreased pri-miR-1-1 expression (Supplementary Fig. S5). To investigate the transcriptional activity of the miR-1-2 locus, we evaluated the expression of pri-miR-1-2 in normal and cancer samples. As shown in Supplementary Fig. S5, no significant modification of this pri-miR was found in samples showing a decrease of miR-1, suggesting that the amount of mature miR-1 is likely to be controlled both at transcriptional and posttranscriptional levels.

MiR-1 decrease correlates with MET increase in human CRCs

Because it is known that miR-1 negatively regulates MET, we evaluated if its downregulation in tumor samples was correlated to MET increase. Indeed, a concomitant decrease of miR-1 and increase of MET was observed in 75% of the samples (Fig. 2C, left). When the analysis was conducted in tumors of all stages, we found a significant anticorrelation between miR-1 and MET expression (All, $r = -0.49$), as shown in the scatter plot of Fig. 2C (right). This anticorrelation became even more significant when the analysis was restricted to tumors which developed synchronous or metachronous metastases (M, $r = -0.91$). These data strongly suggest a role for miR-1 in controlling MET expression during progression of colon cancer.

It was previously described that, in colon cancer, the increase of the transcription factor MACC1 is a good biomarker for the identification of poor prognosis patients, and that *MET* is a transcriptional target of MACC1 (14). Therefore, the expression of MACC1 and its correlation with MET levels were analyzed. As shown in Fig. 3A (left), MACC1 expression was increased in 71.2% of the tumors compared with normal peritumoral tissues; moreover, its median level was significantly higher in neoplastic tissues as well ($P < 5.55e^{-9}$; Fig. 3A, right). The scatter plot reported in Fig. 3B shows that MACC1 increase significantly correlated with enhanced MET expression. However, only a small difference in the correlation of these two genes was observed between nonmetastatic and metastatic tumors ($r = 0.73$ in all tumors vs. $r = 0.86$ in metastatic ones).

To evaluate the respective contribution of miR-1 and MACC1 in controlling MET levels, we examined MET expression in 4 groups of tumors, divided according to their respective levels of miR-1 and MACC1 (Fig. 3C). As shown, a significant difference of MET expression ($P < 0.0001$) was found only in tumors displaying both decreased miR-1 and increased MACC1 versus those with normal miR-1/MACC1. Indeed, the single decrease of miR-1 or increase of MACC1 did not result in a significant difference of median MET levels ($P > 0.05$). Due to the small number of samples available, we could not restrict this analysis on
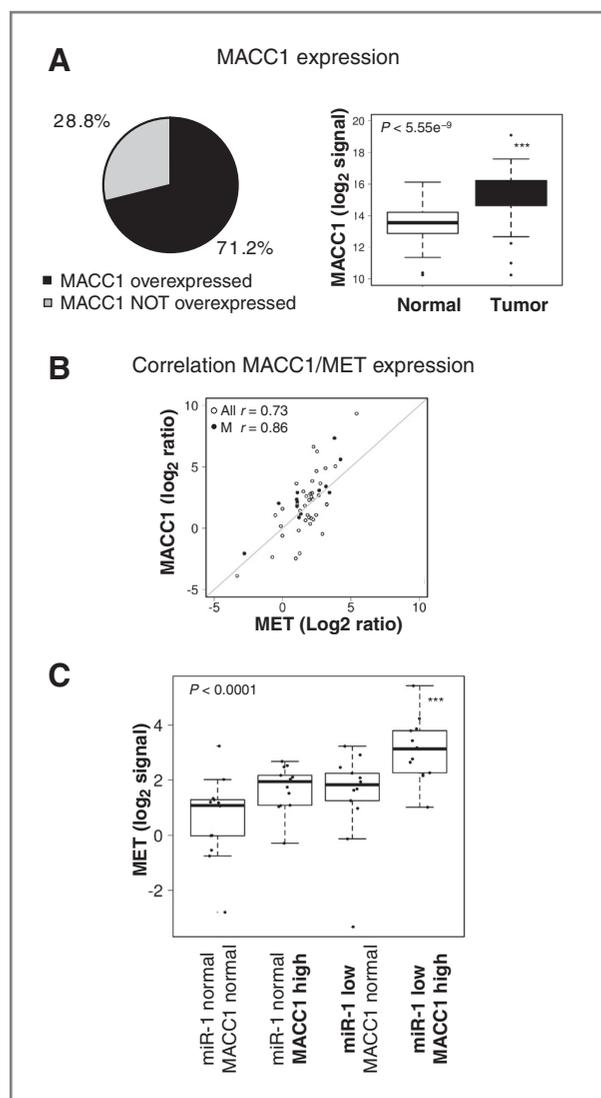
Figure 3. MACC1 expression is increased in colorectal tumors and correlates with MET expression. A, left, MACC1 expression was evaluated by real-time PCR. Tumors (71.2%) overexpressed MACC1 (at least 2-fold more in the tumor vs. the normal counterpart). Right, Box plot showing the median expression level of MACC1 in tumors versus the normal counterpart. The difference is statistically significant, $P < 5.55e^{-9}$. B, the scatter plot shows the correlation between MACC1 and MET expression (all samples: All, $r = 0.73$; tumor samples with synchronous or metachronous metastases: M, $r = 0.86$). C, miR-1 and MACC1 control MET levels. MET expression was evaluated in groups divided according to miR-1 and MACC1 expression (for details, see Materials and Methods). A significant difference of MET expression ($P < 0.0001$) was found only in tumors showing both miR-1 low and MACC1 high versus those with normal miR-1/MACC1 levels.

tumors which developed synchronous or metachronous metastases.

To assess if MACC1 is a posttranscriptional target of miR-1, as predicted by the TargetScan (http://www.targetscan.org/) algorithm, MACC1 expression was evaluated in HT-29 cells transfected with this miRNA. MiR-1 transfection did not reduce MACC1 mRNA or protein levels, while in the

same experimental setting it strongly decreased MET expression (Supplementary Fig. S6 and next paragraph).

Altogether, these data show that concomitant decrease of miR-1 and increase of MACC1 is required to significantly upregulate MET expression in colon cancers; moreover, miR-1 decrease shows a stronger correlation with the metastatic status compared with MACC1 increase.

MiR-1 reexpression in colon cancer cell lines impairs MET-induced invasive growth

To study the functional role of miR-1 in colon cancer cells, we first evaluated its level of expression in several colon cancer cell lines. As shown in Fig. 4A, miR-1 was barely expressed in almost every cell line tested. On the contrary, the level of MET was significantly increased in most of them compared with normal colonic mucosa (Fig. 4B).

To evaluate the physiologic role of miR-1 in colon cancer cells, different human carcinoma cell lines were transfected with chemically synthesized miRNA precursors. As shown in Fig. 4C, 72 hours after miR-1 transfection, MET protein was significantly reduced. On the contrary, AntagomiR-mediated miR-1 inhibition resulted in MET protein increase in SW48 cells, the only ones endogenously expressing miR-1. These data show that miR-1 is physiologically involved in the control of MET expression.

MET activation contributes to several important steps in tumor progression and metastatic dissemination (6). To assess if miR-1 expression could impair the execution of the invasive growth program, we selected HT-29 and DLD-1, two colon cancer cell lines able to activate a motogenic and invasive response to HGF (39, 40). HT-29 and DLD-1 cells were transfected with miR-1 and their motile/invasive properties were evaluated. As shown in Fig. 5, upon miRNA transfection, cells were impaired in their scattering ability in response to HGF; moreover, their ability to migrate or to reconstitute the wound in response to HGF was strongly decreased. In cells expressing exogenous miR-1, we also observed a decreased cell viability, which was more evident in HT-29 cells, displaying a moderate level of constitutive MET phosphorylation (41).

MET and miR-1 exert a reciprocal control through a regulatory feedback loop

As shown in Fig. 2C, miR-1 and MET expression showed a very significant anticorrelation. This led us to hypothesize the existence of a reciprocal regulation. To investigate this point, SW48, DiFi (colon cancer cells), and EBC1 (lung cancer cells) were transduced with a lentiviral vector expressing MET cDNA. As shown in Fig. 6A and Supplementary Fig. S7, in transduced cells, increased MET expression was accompanied by reduced miR-1 expression. To verify if this decrease was due to reduced transcription, the expression of the pri-miRs was evaluated. As shown in Supplementary Fig. S7, the decline of miR-1 was not paralleled by the decrease of both the two pri-miRs, suggesting that the control of miR-1 levels is not exerted uniquely at transcriptional level.
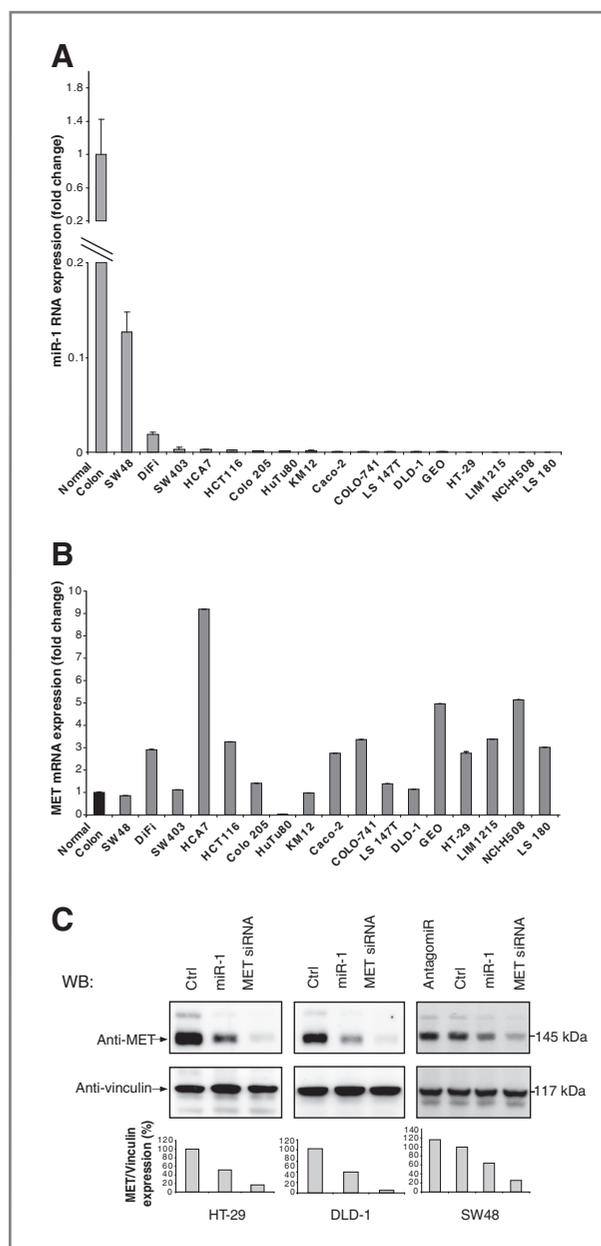
Figure 4. miR-1 is expressed at low levels in colon cancer cell lines and its forced expression downregulates MET. A and B, expression profiles of miR-1 and MET in a panel of colorectal cancer cell lines. As shown, miR-1 was expressed at very low levels in almost all the examined colorectal cancer cell lines, with the exception of SW48 cells. On the contrary, MET was more expressed, compared with the normal colonic mucosa, in most of the tested cell lines. C, miR-1 regulates MET level in colorectal cancer cell lines. HT-29, DLD-1, and SW48 colon carcinoma cells were transfected with miR-1, with a specific MET siRNA (33), or with a control mismatched siRNA (Ctrl). SW48 cells expressing detectable levels of miR-1 were also transfected with miR-1 AntagomiR. Expression of the MET protein was evaluated 72 hours later by Western blot. Vinculin was used as an internal control for Western blot loading. Bands were scanned and quantified. Columns, ratio between MET and Vinculin expression (%; bottom). As shown, the levels of MET protein were decreased upon exogenous expression of miR-1 and increased upon AntagomiR transfection. WB, Western blot.

Finally, the decrease of MET expression, obtained either by RNA interference with specific MET siRNAs or by exposure to Fab DN30, a monovalent form of a monoclonal antibody known to downregulate MET levels (42, 43), resulted in increase of miR-1 (Fig. 6B and C).

Discussion

The most significant findings of this study are the following: (i) MET overexpression significantly correlates with miR-1 downregulation in the vast majority of human CRCs, especially in advanced stages of progression; (ii) MET and miR-1 reciprocally control their expression through a feedback circuit; (iii) forced reexpression of miR-1 in colon cancer cells leads to a decrease of MET expression and of MET-induced invasive growth; (iv) miR-1 decrease and MACC1 increase are both required for maximal MET overexpression.

Concerning the first point, the results of this work show that the expression of miR-1 is downregulated in 84.6% of the examined human CRCs, with a significant decrease of average expression in tumors versus normal tissues; notably, in the same samples, MET was overexpressed (at least 2 folds) in 82.7% of cases, with a strongly significant increase of average expression in tumors versus the corresponding normal mucosa. A significant ($r = -0.49$) anticorrelation between the decrease of miR-1 and the increase of MET was observed in the examined tumors; interestingly, the significance of this anticorrelation was much stronger ($r = -0.91$) in metastatic tumors. This suggests that the paired miR-1 decrease and MET increase is likely to be associated to cancer progression and to the acquisition of an invasive phenotype.

MiR-1 was originally identified for its role in controlling muscular development and differentiation (44). Moreover, it was shown that it plays an important role in some cancers such as rhabdomyosarcoma, the most common soft tissue sarcoma in children (45, 46). These works showed that miR-1 was barely detectable in primary rhabdomyosarcomas, and that its reexpression in tumor cells promoted myogenic differentiation and blocked tumor growth in xenografted mice. MiR-1 was shown to have a tumor-suppressive effect also in bladder cancers, by regulating LASP1 (LIM and SH3 protein 1; ref. 47) and in lung cancer (31). All these observations suggest that misregulation of miR-1 can play an oncogenic role via abolition of the suppressive effect on specific target genes such as *MET*, *LASP1*, *IGF-1*, *IGFR-1*, or *BCL2* (45, 47–52).

The results of our study also show that decreased expression of miR-1 in colon cancer is not due either to gene loss, as shown by the normal number of gene copies in the examined samples, or to loss of the transcription factor C/EBPα. Our data also indicate a significant increase of DNA methylation of miR-1-1 promoter in tumor samples (as observed also by Suzuki and colleagues, ref. 53), accompanied by a reduction of pri-miR-1-1. In this context, it is interesting to note that we did not find a significant
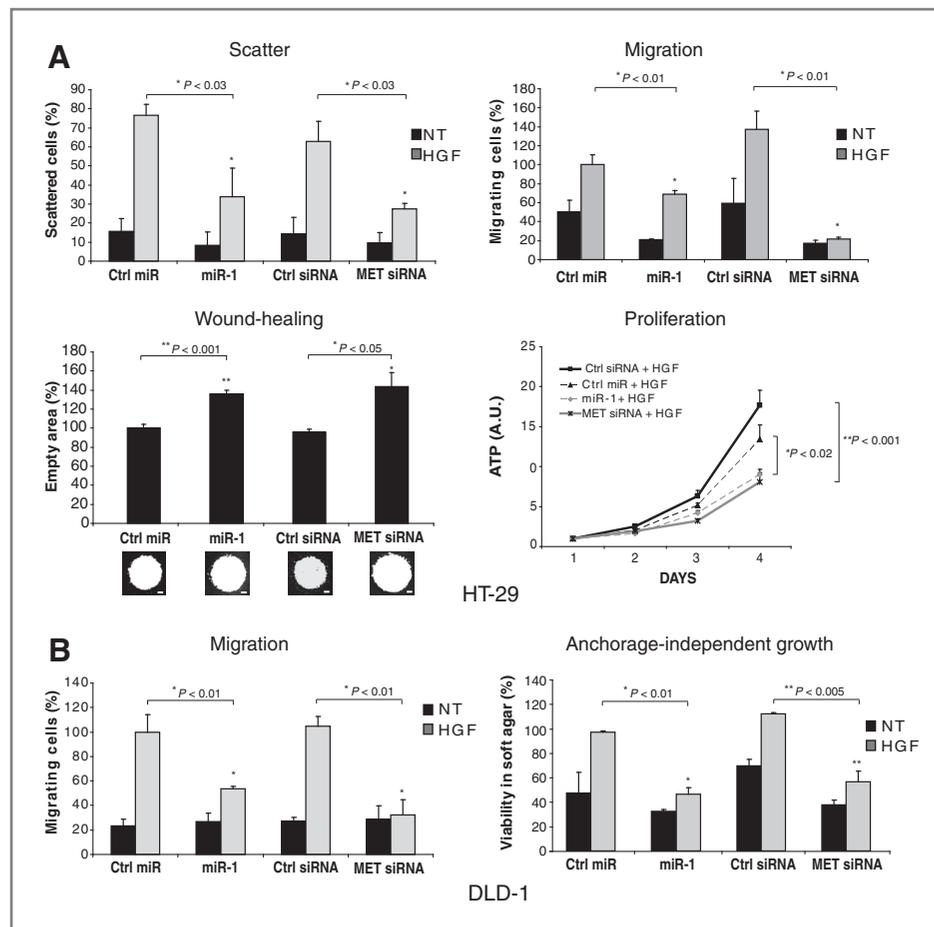
Figure 5. miR-1 impairs the ability of colorectal cancer cells to activate the invasive growth program. A, biological assays carried out on HT-29 cells transfected with miRNA mimic negative control (Ctrl miR), miR-1, control mismatched siRNA (Ctrl siRNA), and MET siRNA. Scatter, cells were seeded at low density in the presence of Dulbecco's Modified Eagle's Medium supplemented with 10% FBS. After the formation of the islets, cells were left untreated (NT) or stimulated for 16 hours with 20 ng/mL HGF. The chart shows the quantification of the scatter assay: The percentage of scattered colonies was counted and related to the total number of colonies (considered as 100%). As shown, cells transfected with miR-1 were severely impaired to scatter in response to HGF. The difference is statistically significant ($P < .03$). Migration, HT-29 cells expressing miR-1/Ctrl miR or MET/Ctrl siRNA were seeded on the upper face of Transwell filters, exposed to a gradient of HGF (20 ng/mL, gray bars), or left untreated (NT, black bars). As shown, cells transfected with miR-1 were significantly impaired in their ability to migrate in response to HGF ($P < 0.01$). Wound-healing assay, HT-29 cells were seeded at confluence in the presence of Oris Cell Seeding Stoppers. After Stoppers removal, cells were treated with HGF (20 ng/mL), fixed 2 days later with 11% glutaraldehyde, and stained with 0.1% crystal violet. The chart shows the quantification of the area not covered by migrated cells. Representative pictures are shown for each experimental point (bars, 0.25 mm). Cells transfected with miR-1 were severely impaired in their ability to heal the wound ($P < 0.001$). Viability assay, HT-29 cells transfected with miR-1/Ctrl miR and MET/Ctrl siRNA were treated with HGF (20 ng/mL). Viability was assessed at the indicated times by Cell titer Glo assay. As shown, miR-1 expression significantly impaired HT-29 viability ($P < 0.02$). B, biological assays conducted on DLD-1 cells transfected with miR-1/Ctrl miR, MET/Ctrl siRNA. Migration assays were done as in A. Anchorage-independent growth was evaluated by seeding cells in agar and assessing growth of colonies 14 days later. As shown, miR-1 expression significantly reduced cell migration ($P < 0.01$) and cell ability to grow in an anchorage-independent manner ($P < 0.01$) in response to HGF.

reduction of pri-miR-1-2. This suggests that the amount of mature miR-1 is controlled at several levels, both transcriptional and posttranscriptional. A mechanism of posttranscriptional regulation of miR-1 has recently been described in the heart of patients affected by myotonic dystrophy (54). In this study, it was shown that sequestering of MBNL1, a regulator of pre-miR-1 biogenesis, prevents DICER activity, leading to a decrease of mature miR-1. It can be speculated that analogous mechanisms, involving miRNA regulatory proteins, could contribute to miR-1 alterations found in colon cancer.

Another relevant finding of this work is the identification of miR-1 as a natural antagonist of MET in colon cancer cells because its reexpression impaired MET-dependent biological properties such as growth, motility, and invasion. We conclude that miR-1 loss can contribute to the acquisition of a more aggressive phenotype, and so this miRNA can be considered an oncosuppressor in colon cancer.

Our results also suggest that the invasive growth properties of colon cancer cells are controlled by a delicate balance between MET and miR-1 that results in a feedback loop in

Figure 6. Existence of a regulatory feedback loop between MET and miR-1. A, SW48 cells were transduced with either a lentiviral vector encoding for GFP or for MET. Left, Western blot showing that MET protein was constitutively phosphorylated (top) and overexpressed (intermediate) upon lentiviral transduction. Vinculin was used as an internal loading control. Right, real-time PCR showing a statistically significant decrease ($P < 0.01$) of miR-1 levels upon MET overexpression. B, left, SW48 cells were transfected with either Ctrl siRNAs or MET-specific siRNAs. The Western blot shows MET silencing. Vinculin was used as an internal loading control. Right, real-time PCR showing a significant miR-1 increase ($P < 0.0005$) upon MET silencing. C, SW48 cells were treated for 72 hours with the antagonist Fab DN30 monovalent antibody (40 μg/mL). Left, Western blot showing Fab DN30-induced MET downregulation. Right, real-time PCR showed a statistically significant ($P < 0.02$) miR-1 increase upon antibody-induced MET downregulation. WB, Western blot.
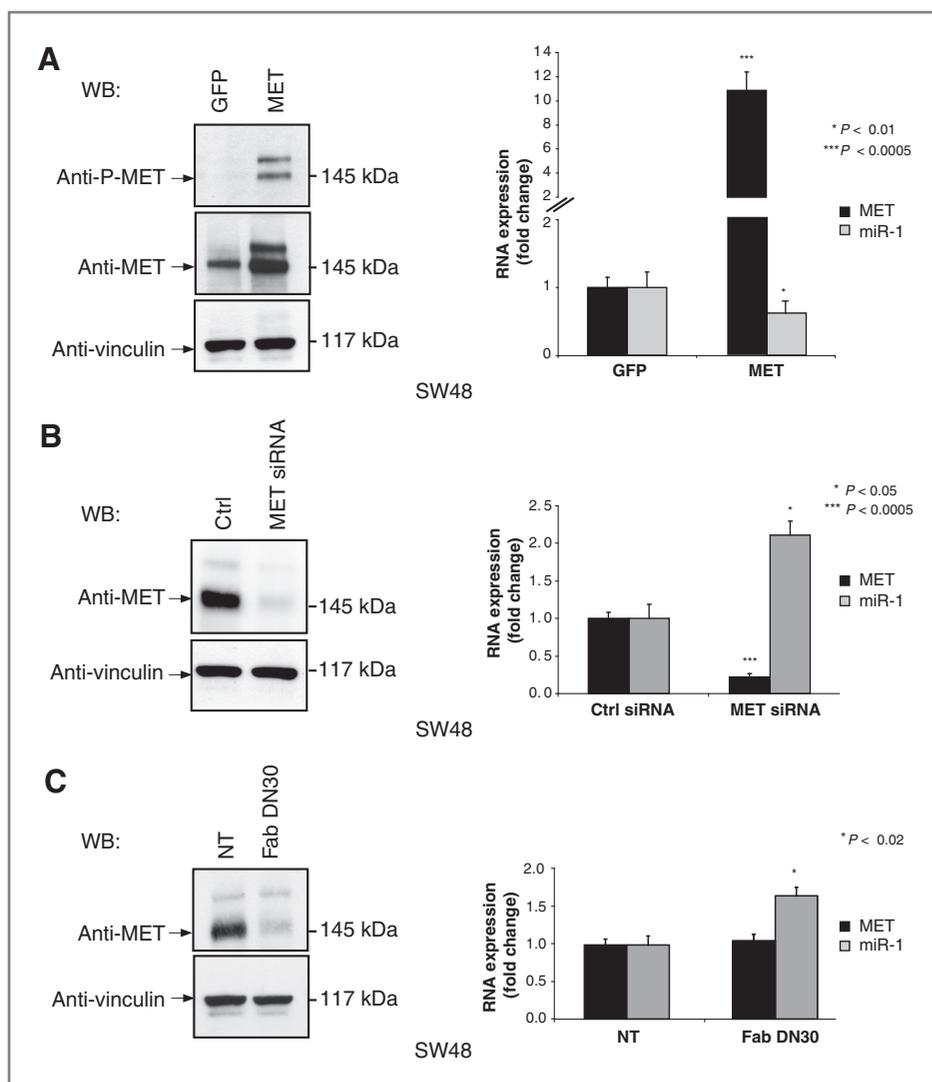
which each partner finely tunes the levels of the other. Notably, the existence of similar loops has been described in other tumors; among them is Ewing sarcoma, in which feedback regulation between EWS-FLI1 and miR-145 is critical for EWS-FLI1–mediated sarcomagenesis (51). Although further studies are required to carefully evaluate the biological significance of the interplay between MET and miR-1, it is tempting to speculate that it might play a critical role in advanced phases of progression—namely the metastatic phase—in which all tumors virtually display a highly significant anticorrelation between these two molecules.

Finally, in our work, we also considered the role of MACC1 expression in the interplay between MET and miR-1. MACC1 was originally identified as a key regulator of HGF–MET signaling and as an independent prognostic indicator of metastasis formation and metastasis-free survival in colon cancer (14). In our study, we could not confirm that MACC1 increase correlates with metastasis formation as previously reported (14). In fact, we found

increased levels of MACC1 in tumors of all stages of progression, irrespectively of formation of synchronous and metachronous metastases, suggesting that MACC1 deregulation takes place in the early stages of the tumor formation (as hypothesized also by Arlt and Stein; ref. 52). MACC1 is a transcriptional activator of *MET*; accordingly, its increased expression should lead to MET overexpression. However, we found that MACC1 increase *per se* is not sufficient to cause a significant MET upregulation. Indeed, MET was found significantly upregulated only when MACC1 increase was coupled with miR-1 downregulation. Notably, also miR-1 downregulation alone was not effective in determining a statistically significant MET increase.

In conclusion, this study identifies an oncosuppressive role of miR-1 in colon cancer in which it modulates MET levels. Because we showed the presence of reciprocal interaction between MET and miR-1, it is likely that MET targeting in colon cancer could result not only in abrogation of MET-dependent signaling but also in some recovery of

miR-1 levels and, consequently, of the suppressive ability of this miRNA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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MiR-1 Downregulation Cooperates with MACC1 in Promoting *MET* Overexpression in Human Colon Cancer

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