MIR-1 DOWNREGULATION COOPERATES WITH MACC1 IN PROMOTING MET OVEREXPRESSION IN HUMAN COLON CANCER

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TRANSLATIONAL RELEVANCE

The MET oncogene and its transcriptional activator 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 over-expression, whose molecular bases are not yet defined. We aimed at evaluating if increased MET expression could be associated to deregulation of microRNAs. 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. The present study thus identifies an oncosuppressive role of miR-1 in colon cancer, where it modulates MET levels and shows that MACC1 alone is not sufficient as a predictor of the metastatic tumor behavior. Moreover, since we demonstrated 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.
ABSTRACT

Purpose: MET, the tyrosine kinase receptor for Hepatocyte Growth Factor (HGF), is frequently over-expressed in colon cancers with high metastatic tendency. We aimed at evaluating 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 non tumoral 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 over-expression, particularly in metastatic tumors. We found that concurrent MACC1 up-regulation 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 feed-back loop between miR-1 and MET, resulting in their mutual regulation.

Conclusions. The present study identifies an oncosuppressive role of miR-1 in colorectal cancer where it acts by controlling MET expression through a feed-back loop. Concomitant downregulation of miR-1 and increase of MACC1 can thus contribute to MET over-expression and to the metastatic behavior of colon cancer cells.
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 pro-metastatic 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, over-expression(7,8). Many works have shown that, in colorectal cancer, MET over-expression 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 over-expression 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 over-expression 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 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.

It has recently been shown that a key regulator of HGF-MET signaling in colon cancer is 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 post-transcriptional level(15). The involvement of miRNAs in cancer pathogenesis is now well established and increasing evidence demonstrates that they can behave either 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 microRNAs are deregulated in many tumor types, including colorectal cancer, during tumor development and progression(25-29).

*In vitro* studies performed 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 over-expression 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 since 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 over-expression and can contribute to the progression of colon cancer toward metastases.
MATERIALS AND METHODS

Study population.

Pairs of primary colorectal tumors and adjacent non-tumorous specimens came from patients diagnosed with colorectal cancer and operated over the period 2004-2009 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 performed after injecting saline solution to separate it from the submucosal layer.

Cell culture and transfection.

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

Transient transfections were performed with 0.2μmol/mL of synthetic pre-microRNAs and mirVana miRNA mimic Negative Control #1 (Applied Biosystem, Foster City, CA), 0.2μmol/mL of AntagomiR (Exiqon, Woburn, MA) or 0.1μmol/mL
of synthetic MET/Ctrl siRNAs(33,34), according to standard protocols. For GFP and MET transduction, viruses were produced as in(35).

Quantitative analysis of microRNAs and mRNAs.
Total RNA extraction, retrotranscription and Real Time PCR were performed as in(30). For primer sequences see Supplementary Methods.

Copy number analysis.
Genomic DNA was extracted with Blood and Cell Culture DNA Midi Kit (Qiagen, Valencia, CA) according to standard protocols. MET, miR-1-1 and miR-1-2 copy number was evaluated with TaqMan pre-designed assays. RNAse P was used as internal reference (Applied Biosystem).

Protein extraction and Western blot.
Western blot analysis was performed according to standard methods. Used antibodies are listed in Supplementary Methods.

In vitro biological assays.
Cell viability was evaluated using the Cell Titer GLO (Promega, Madison, WI) according to the manufacturer protocol.
Wound-healing ability was evaluated with the Oris™ Cell Migration Assay (Platypus Technologies, Madison, WI) 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 5X 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 performed as in(30,34). Every biological assay was performed at least three times.

**IHC.**

Immunohistochemical staining was performed using the anti-MET antibody C-28, (Santa Cruz Biotechnology, INC.) according to standard methods. Positivity was evaluated as low (+, <10% of positive cells), intermediate (+++, >10%<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 40X 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 performed in R-Bioconductor(36); after quality control the CT 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 performed on Log2 Signal. The correlation analyses between miR-1/MET, MACC1/MET and miR-1/C/EBPα were performed on paired Log2 Ratio values. For statistical analyses we performed Student t test.

In figure 3C, the levels of miR-1 and MACC1 were evaluated as the ratio between the expression in the tumor vs. 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 non tumoral 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 Table1. Briefly, the analyzed group was composed of 53.8% males and 46.2% females; 44.2% of the patients were aged over 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 retro-transcribed total RNA, obtained from tumors as well as from their normal surrounding tissues, showed MET over-expression in 82.7% of tumors compared to their normal counterparts (Fig.1A); the median level of MET expression was also significantly increased in the neoplastic tissue ($P<e^{-12}$; Fig.1B).

In order to confirm that the enhanced level of MET mRNA corresponded indeed to an increase of the MET protein, we performed 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 over-expression is very rarely due to gene amplification in primary colon cancers (4,12). In agreement with the literature, Real Time PCR performed on tumor-derived genomic DNA did not detect MET amplification in any examined samples (Suppl. Fig.1A), clearly demonstrating that the observed MET over-expression is not due to increased gene copy number.

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

Works performed 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). Since in the examined colorectal cancer samples MET over-expression is not due to gene amplification, we wondered if it could be due to decreased expression of microRNAs that negatively control MET. Expression of miR-1, miR-199a* and miR-34c was thus analyzed by Real Time PCR in colon adenocarcinomas and in their normal counterparts. MiR-1 was downregulated in 84.6% of tumors, compared to the normal peritumoral tissue (Fig.2A, left); notably, the median expression 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 significant ($P<.17$; 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).

Since the decrease of miR-1 in tumor samples was strong and statistically significant, we focused on this microRNA. To evaluate whether the decrease of miR-1 could be due to the loss of gene copies, we performed Real Time PCR on genomic DNA
(gDNA). As shown in Suppl. Fig. 1B,C no deletion of either gene encoding for this microRNA(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 Suppl. Fig.2, we did not observe any statistically significant downregulation of C/EBPα in samples with low miR-1 levels.

Since 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 Tricostatin A (TSA), did not show increased expression of miR-1 (Suppl. Fig.3A). When cells were treated with 5-Azacytidine (5-AZA), a DNA methyltransferase inhibitor, a decrease of miR-1-1 promoter methylation (Suppl. Fig. 3C) and an increase of miR-1 expression was observed (Suppl. Fig.3B), 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 two 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 31). The analysis of miR-1-1 promoter revealed a significant difference in methylation in tumor samples (Suppl. Fig.4), associated with a decreased pri-miR-1-1 expression (Suppl. Fig.5). In order 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 Suppl. Fig. 5, 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 post-transcriptional levels.
MiR-1 decrease correlates with MET increase in human CRCs.

Since 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 performed in tumors of all stages we found a significant anti-correlation between miR-1 and MET expression (All, \( r = -0.49 \)), as shown in the scatter plot of Fig.2C (right). This anti-correlation 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 to 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 non metastatic and metastatic tumors (\( r = 0.73 \) in all tumors vs. \( r = 0.86 \) in metastatic ones).

In order to evaluate the respective contribution of miR-1 and MACC1 in controlling MET levels, we examined MET expression in four groups of tumors, divided according to their respective levels of miR-1 and MACC1 (Fig.3C). As shown, a significant difference of MET expression (\( P<.0001 \)) was found only in tumors
displaying both decreased miR-1 and increased MACC1 vs. 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 ($P > .05$). Due to the small number of samples available, we could not restrict this analysis on tumors which developed synchronous or metachronous metastases.

To assess if MACC1 is a post-transcriptional target of miR-1, as predicted by the TargetScan\textsuperscript{b} 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 (Suppl. Fig.6 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 to MACC1 increase.

\textit{MiR-1 re-expression 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 to normal colonic mucosa (Fig.4B).

To evaluate the physiological 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

\textsuperscript{b}http://www.targetscan.org/
significantly reduced. On the opposite, AntagomiR-mediated miR-1 inhibition resulted in MET protein increase in SW48 cells, the only ones endogenously expressing miR-1. These data demonstrate 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). In order 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 feed-back loop.**

As shown in Fig.2C, miR-1 and MET expression showed a very significant anti-correlation. This led us to hypothesize the existence of a reciprocal regulation. In order 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 Suppl. Fig. 7, 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 Suppl. Fig. 7, 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.

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,C).
DISCUSSION

The most significant findings of the present study are the following: i) MET over-expression 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 feed-back circuit; iii) forced re-expression 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 over-expression.

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 over-expressed (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) anti-correlation between the decrease of miR-1 and the increase of MET was observed in the examined tumors; interestingly, the significance of this anti-correlation 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 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 re-expression 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)(47) and in lung cancer(31). All these observations suggest that mis-regulation 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 et al., 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 reduction of pri-miR-1-2. This suggests that the amount of mature miR-1 is controlled at several levels, both transcriptional and post-transcriptional. A mechanism of post-transcriptional 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 since its re-expression 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 feed-back
loop in 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, where 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 anti-correlation 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(52)). MACC1 is a transcriptional activator of MET; accordingly, its increased expression should lead to MET over-expression. However, we found that MACC1 increase per se is not sufficient to cause a significant MET up-regulation. Indeed, MET was found significantly up-regulated 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, the present study identifies an oncosuppressive role of miR-1 in colon cancer, where it modulates MET levels. Since we demonstrated 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.
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FIGURE LEGENDS

Fig.1: MET is overexpressed in colorectal cancer. A: MET expression was evaluated by Real Time PCR in 52 matched-pairs of colorectal cancers and non-tumoral surrounding tissues. 82.7% of tumors showed MET over-expression (at least two-folds) vs. the normal peritumoral tissue. B: Box plot showing the median expression level of MET mRNA (measured by Real Time PCR) in tumors vs. the normal counterpart. The difference is statistically significant, P<e^{-12}. C: MET evaluation in a representative case. Upper panel. 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. Intermediate panel. Positivity was evaluated as low (+, <10% of positive cells), intermediate (++, >10% <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 cake. Lower panel. 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.

Fig.2: miR-1 expression is decreased in colorectal tumors and is anti-correlated with MET expression. A-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, while 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 vs. the normal counterpart. The
difference in miR-1 expression is statistically significant, $P<1.4\times10^{-9}$. C: miR-1 and MET are often concomitantly deregulated in colorectal cancer samples. Left side. The chart represents the percentage of tumors (75%) displaying both MET overexpression and miR-1 downregulation (concordance miR-1$_{low}$/MET$_{high}$). Right side. The scatter plot shows the anti-correlation between miR-1 and MET expression (all samples: All, $r = -0.49$; tumors samples with synchronous or metachronous metastases: M, $r = -0.91$).

**Fig. 3:** MACC1 expression is increased in colorectal tumors and correlates with MET expression. A: left side. MACC1 expression was evaluated by Real Time PCR. 71.2% of the tumors overexpressed MACC1 (at least two folds more in the tumor vs. the normal counterpart). Right side. Box plot showing the median expression level of MACC1 in tumors vs. the normal counterpart. The difference is statistically significant, $P<5.55\times10^{-9}$. 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 ($P<.0001$) was found only in tumors showing both miR-1 low and MACC1 high vs. those with normal miR-1/MACC1 levels.

**Fig. 4:** miR-1 is expressed at low levels in colon cancer cell lines and its forced expression downregulates MET. A-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 to 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 72h later by Western Blot. Vinculin was used as an internal control for Western Blot loading (upper panels). Bands were scanned and quantified. Columns, ratio between MET and Vinculin expression (%) (lower panel). As shown, the levels of MET protein were decreased upon exogenous expression of miR-1 and increased upon AntagomiR transfection.

**Fig.5: miR-1 impairs the ability of colorectal cancer cells to activate the invasive growth program. A:** Biological assays performed 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 DMEM supplemented with 10% FBS. After the formation of the islets, cells were left untreated (NT) or stimulated for 16h with 20ng/mL HGF (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 (20ng/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<.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 (20ng/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<.001). **Viability assay:** HT-29 cells transfected with miR-1/Ctrl miR and MET/Ctrl siRNA were treated with HGF (20ng/mL). Viability was assessed at the indicated times by Cell titer Glo assay. As shown, miR-1 expression significantly impaired HT-29 viability (P<.02). **B:** Biological assays performed on DLD-1 cells transfected with miR-1/Ctrl miR, MET/Ctrl siRNA. Migration assays were performed 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<.01) and cell ability to grow in an anchorage-independent manner (P<.01) in response to HGF.

**Fig.6:** Existence of a regulatory feed-back 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 (upper panel) and overexpressed (intermediate panel) upon lentiviral transduction. Vinculin was used as an internal loading control. **Right.** Real Time PCR showing a statistically significant decrease (P<.01) of miR-1 levels upon MET over-expression. **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 ($P<.0005$) upon MET silencing. *C:* SW48 cells were treated for 72h with the antagonist Fab DN30 monovalent antibody (40μg/mL). *Left.* Western Blot showing Fab DN30-induced MET downregulation. *Right.* Real Time PCR showed a statistically significant ($P<.02$) miR-1 increase upon antibody-induced MET downregulation.
Figure 1

A

- 17.3%
- 82.7%

- MET over-expressed
- MET NOT over-expressed

B

- MET expression
- MET (Log2 signal)
- P < e^{-12}
- Normal Tumor

C

- 25% 25%
- 20% 30%

- 40x
- 22% 78%

- MET over-expressed
- MET NOT over-expressed

- 17.3% 82.7%

- Normal Tumor

- P < e^{-12}

- MET expression

- MET (Log2 signal)
Figure 2

A. miR-1 expression

- miR-1 down-regulated: 15.4%
- miR-1 NOT down-regulated: 84.6%

B. miR-199a* expression

- miR-199a* down-regulated: 30.8%
- miR-199a* NOT down-regulated: 69.2%

C. Anti-correlation miR-1/MET

- Concordance miR-1 low / MET high: 25%
- NO concordance: 75%

All r = -0.49, M r = -0.91, P < 1.4e-9
Figure 3

A  
MACC1 expression

- 71.2%
- 28.8%
- MACC1 over-expressed
- MACC1 NOT over-expressed

B  
Correlation MACC1/MET expression

$\text{MACC1 (Log2 ratio)}$

$\text{MET (Log2 ratio)}$

*** $P < .0001$

C  
All $r = 0.73$

M $r = 0.86$

$P < .0001$

MACC1 normal

MACC1 high

miR-1 normal

miR-1 low
Figure 5

A

Scatter

Migrating cells (%)

<table>
<thead>
<tr>
<th></th>
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<th>miR-1</th>
<th>Ctrl siRNA</th>
<th>MET siRNA</th>
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<tr>
<td>HGF</td>
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Migration

Migrating cells (%)

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<th>MET siRNA</th>
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<td>HGF</td>
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Wound-healing

Empty area (%)

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<tr>
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<th>miR-1</th>
<th>Ctrl siRNA</th>
<th>MET siRNA</th>
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<tr>
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Proliferation

ATP (A.U.)

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<tr>
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<th>Ctrl miR+HGF</th>
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</tr>
<tr>
<td>4</td>
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B

Migration

Migrating cells (%)

<table>
<thead>
<tr>
<th></th>
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<th>Ctrl siRNA</th>
<th>MET siRNA</th>
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<tr>
<td>HGF</td>
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Anchorage-independent growth

Viability in soft agar (%)

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Figure 6

A

WB:

- GFP
- MET
- Anti-P-MET
- Anti-MET
- Anti-vinculin

RNA expression (Fold change)

* P < 0.01
*** P < 0.0005

B

WB:

- Ctrl
- MET siRNA
- Anti-MET
- Anti-vinculin

RNA expression (Fold change)

* P < 0.05
*** P < 0.0005

C

WB:

- NT
- Fab DN30
- Anti-MET
- Anti-vinculin

RNA expression (Fold change)

* P < 0.02
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MIR-1 DOWNREGULATION COOPERATES WITH MACC1 IN PROMOTING MET OVEREXPRESSION IN HUMAN COLON CANCER.

Cristina Migliore, Valentina Martin, Vera P. Leoni, et al.

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