miR-153 Supports Colorectal Cancer Progression via Pleiotropic Effects That Enhance Invasion and Chemotherapeutic Resistance

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Introduction

Colorectal cancer is the second commonest cause of cancer-related death in western societies (1). Disease progression and metastases are the principal causes of death, and advanced disease occurs in up to 30% at presentation (2). In addition, in patients with localized disease who proceed to apparently curative surgery, 50% subsequently develop recurrence (2, 3). Despite increasingly sophisticated techniques for therapy to recurrent colorectal cancer, the majority of patients remain incurable, highlighting the need for a continued effort to better understand the complexities of disease progression, and identify new directions for treatment strategies.

MicroRNAs (miRNA) are short noncoding RNA molecules and play a critical role in malignant transformation, underscored by the observation that over 50% of miRNA genes are located within or close to cancer-associated genomic regions (4, 5). miRNAs regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of protein-coding mRNAs through sequences in the 5'-end of the miRNA that are only partially complementary (6). One consequence of this imperfect complementarity is that each miRNA can regulate multiple target mRNAs and thereby impact functionally diverse programs of gene expression. miRNA expression is deregulated in colorectal cancer with a growing number of oncogenes and tumor suppressor genes under miRNA regulation (7, 8). Importantly, miRNA expression patterns correlate with distinct colorectal cancer subtypes and clinical phenotype (8–11). Emerging data have also implicated miRNAs in disease progression and acquisition of metastatic capabilities in colorectal malignancies, through promotion of epithelial–mesenchymal transition and regulation of metastasis-associated genes (12, 13). To date however, little is understood about the role of miRNAs in disease progression in colorectal cancer and few candidate miRNAs and target genes have been implicated.

Although microRNAs (miRNA) have been broadly studied in cancer, comparatively less is understood about their role in progression. Here we report that miR-153 has a dual role during progression of colorectal cancer by enhancing cellular invasiveness and platinum-based chemotheraphy resistance. miRNA profiling revealed that miR-153 was highly expressed in a cellular model of advanced stage colorectal cancer. Its upregulation was also noted in primary human colorectal cancer compared with normal colonic epithelium and in more advanced colorectal cancer stages compared with early stage disease. In colorectal cancer patients followed for 50 months, 21 of 30 patients with high levels of miR-153 had disease progression compared with others in this group with low levels of miR-153. Functional studies revealed that miR-153 upregulation increased colorectal cancer invasiveness and resistance to oxaliplatin and cisplatin both in vitro and in vivo. Mechanistic investigations indicated that miR-153 promoted invasiveness indirectly by inducing matrix metalloprotease enzyme 9 production, whereas drug resistance was mediated directly by inhibiting the Forkhead transcription factor Forkhead box 03a (FOX03a). In support of the latter finding, we found that levels of miR-153 and FOX03a were inversely correlated in matched human colorectal cancer specimens. Our findings establish key roles for miR-153 overexpression in colorectal cancer progression, rationalizing therapeutic strategies to target expression of this miRNA for colorectal cancer treatment. Cancer Res; 73(21); 1–13. ©2013 AACR.

Abstract

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In this study, we hypothesized that deregulated miRNAs contribute to colorectal cancer progression. miR-153 was identified as overexpressed in more advanced colorectal cancer using cell models and human tumor samples. Functional and mechanistic studies show that overexpression of miR-153 can increase colorectal cancer invasiveness through upregulation of matrix metalloprotease enzyme 9 (MMP-9), and additionally lead to enhanced platinum-based chemotherapy resistance. This latter effect may be through an inhibitory effect on the Forkhead box O3 (FOXO3) transcription factor, which we show is targeted by miR-153, and inversely correlates with miR-153 in human colorectal cancer samples. Together, these results point to a significant and novel contribution of upregulated miR-153 in colorectal cancer, and suggest that modulation of miR-153 may be a useful strategy in limiting colorectal cancer progression.

Materials and Methods
Cell and organotypic cultures, transfections, and cloning
Details of the cell lines, cloning, and transfections are provided in supplementary methods (14–16, 44). Organotypic cultures were prepared as previously described (17). Gels comprised a 50:50 mixture of Matrigel and type I collagen with $5 \times 10^5$ human fetal fibroblast cells, to which were added $5 \times 10^5$ SW480 cells stably transfected with 2 µg plasmid expression constructs for miR-153 or control scrambled miRNA (CmiR00001-MR04; GeneCopoeia). Media were changed every 2 days, and gels harvested and formalin fixed after 14 days.

Patients and samples
Samples from patients with biopsy-proven colorectal cancer were obtained fresh at the time of surgery and snap frozen before being deposited in a UK Human Tissue Act approved tumor bank. In each case, matched tumor and uninvolved proximal mucosa were obtained. All patients provided informed consent, and the study was approved by the regional research ethics committee. Pathologic verification of diagnosis and staging was in accordance with the Association of Coloproctology of Great Britain and Ireland guidelines (18). All specimens were reviewed by clinicians with a specialist interest in gastrointestinal pathology using paraffin-embedded tissue that was adjacent to or in close proximity to tumor bank tissue. Tumors used in this study were adenocarcinomas only, and selected at random from the tumor bank before laser capture microdissection (LCM) and RNA extraction. Exclusion criteria included evidence of a hereditary tumor, presence of multiple tumors, and tumors with histologically identified extensive necrosis. A total of 100 human tumors were examined. RNA extraction from tumors is described in supplementary methods.

miRNA expression profiling, in silico analyses, and bioinformatics
miRNA expression profiling and in silico analyses are detailed in supplementary methods. Array data are MIAME compliant and available in the EBI database (http://www.ebi.ac.uk/arrayexpress/experiments/; accession number E-MEXP-3270).

Array-based comparative genomic hybridization
Array-based comparative genomic hybridization was conducted as previously described and is detailed in supplementary methods (19).

Cell lysis, real-time PCR, Western blotting, and functional assays
Cell lysis, downstream analyses, and functional assays to determine biological effects of overexpression of miR-153 are described in supplementary methods.

Tissue microarray development and immunohistochemistry
Tissue microarray development and immunostaining is detailed in supplementary methods.

In vivo xenograft invasion assay
Development of a severe combined immunodeficient (SCID) mouse miR-153 knockdown xenograft model is detailed in supplementary methods.

Results
MicroRNA expression profiling in SW480 and SW620 cells
Expression of 328 human miRNAs was determined in the paired colorectal cancer lines SW480 and SW620. Figure 1A and B show that the top 20 miRNAs up- or downregulated >4-fold in the metastatic SW620 cells compared with SW480. Some candidates identified have been previously found to be deregulated in colorectal cancer by other groups (9–11). To further verify microarray data, 9 miRNAs of variable fold change were selected for real-time PCR validation. Consistent with the microarray data, similar fold changes in the selected miRNAs were found with high correlation between microarray and PCR results (Supplementary Fig. S1A and S1B).

Expression of miR-153 is increased in colorectal cancer and advanced disease
To prioritize differentially expressed miRNA candidates for further study, putative mRNA targets of the top 15 miRNAs were predicted computationally using TargetScan (www.targetscan.org). This gene list was annotated and then subjected to gene-set enrichment analysis using the DAVID bioinformatics resource (see supplementary methods) to identify overrepresented biological processes for each candidate miRNA. This data were collated and compared for all 15 overexpressed miRNA candidates, to inspect for enrichment of biological processes associated with disease progression and cancer spread (summarized in Supplementary Fig. S1C). One miRNA identified in our cell line profiling as upregulated over 12-fold in higher stage colorectal cancer was miR-153. Clustered and nonclustered analyses for miR-153 show that based on a $P$ value of 0.001, 55% of the 36 biological processes identified were cancer associated. In addition, the highest enrichment score obtained for all candidate miRNAs was associated with miR-153 and the biological processes of motility and adhesion with an enrichment score of 12.5, suggesting that deregulation...
Figure 1. miR-153 is upregulated in advanced stage colorectal cancer cell lines and human tumors. A and B, miRNA expression profiles were determined in SW480 and SW620 cells. Upregulated (A) and downregulated (B) miRNAs in the metastatic SW620 cell line compared with SW480. miRNAs previously linked to colorectal cancer are indicated with an asterisk. C, endogenous miR-153 expression by quantitative RT-PCR in a panel of colorectal cancer cell lines (**, P < 0.05; ††, P < 0.01). D and E, expression levels of miR-153 were examined by qPCR in 83 human samples comprising 23 normal mucosa; 20 stage 1 (T1-2/N0/M0 tumors); 20 stage 3 or 4 (any T/N1-2 or M1); and 20 metastases (liver and lung). Significantly increased miR-153 expression was noted in tumor compared with normal tissue and with increasing disease stage (**, P < 0.005). F, disease-free survival for patients according to low or high expression of miR-153 (Kaplan-Meier).
of miR-153 may have important consequences in carcinogenesis and disease progression.

Further assessment with Panther, BioCarta, and KEGG annotation categories for miR-153 in DAVID showed enrichment of genes associated specifically with colorectal cancer (P < 0.005), and the Wnt, Cadherin, and TGF-β (P < 0.001) signaling pathways, lending additional support for a link between miR-153 deregulation and colorectal tumorigenesis in particular (Supplementary File S1).

Prompted by this, we investigated expression of miR-153 in a panel of human colorectal cancer cell lines. Five of 10 cell lines examined showed significant upregulation of miR-153 levels compared with SW480 cells (Fig. 1C). The pattern of miR-153 expression closely correlated with the expression of matching cell lines in the NCI-60 panel as determined through the web application CellMiner (43). To determine miR-153 expression in vivo, we conducted a small scale pilot to examine 10 random tumors of varying stage by LCM and miRNA expression analysis. Significantly increased expression of miR-153 was noted in tumor compared with normal tissue (Supplementary Fig. S2A; P < 0.0001). As transition between SW480 and SW620 cells principally reflects a change toward a metastatic phenotype, we examined expression of miR-153 in metastatic compared with nonmetastatic tumors. Expression of miR-153 in 5 nonmetastatic stage 1 tumors was compared with 5 stage 3 and 5 stage 4 tumors with lymphatic or distant organ metastases (Supplementary Fig. S2B). Higher expression of miR-153 was noted in more advanced colorectal cancer (P < 0.005).

We extended our analysis to an additional unselected group of 83 human samples with known clinical outcome (clinicopathologic data presented in Supplementary Table S1) comprising 23 normal mucosa; 20 stage 1 (T1-2/N0/M0 tumors); 20 stage 3 or 4 (any T/N1-2 or M1); and 20 metastases (liver and lung). Significantly increased miR-153 expression was noted in tumor compared with normal tissue (Fig. 1D; P < 0.005) and with increasing disease stage. Specifically, higher levels were noted in tumors with nodal or distant organ spread (Fig. 1E; P < 0.005). When median expression of miR-153 was used to stratify patients, after a follow up time of 50 months, 21 of 30 patients in the high miR-153 group developed disease progression compared with 9 of 30 in the low miR-153 group (Fig. 1F). Factors associated with improved disease-free survival by univariate analysis included the absence of extramural vascular invasion (EMVI) and low miR-153 expression (log rank P = 0.007; χ² = 7.3; Supplementary Table S2). Demographic characteristics, histological grading, and the type of surgery did not have a significant impact on survival. Multivariate analysis indicated that both EMVI and miR-153 status were independent prognostic factors [for EMVI, P < 0.001; HR = 9.6; 95% confidence interval (CI), 3.63–49.12; for miR-153 status, P = 0.024; HR = 4.7; 95% CI, 1.17–8.74].

**miR-153 overexpression is not due to genetic copy number change**

Deregulation of miRNAs has been attributed to genomic copy number changes (5) and miRNAs are overrepresented in regions of genomic gain in colorectal cancer (20). We therefore sought to determine if miR-153 upregulation in colorectal cancer was due to copy number change. SW620 cells were analyzed using a Genome-Wide Human SNP Array 6.0 with the hapmap270.422 dataset as reference. miR-153 is located on chromosome 2q35, however no copy number changes at this locus were identified (Supplementary Fig. S3). To clarify if the miR-153 locus is subject to copy number change in primary colorectal cancer, we examined the Mitelman Database of Chromosome Aberrations (21). A total of 346 cases of colorectal cancer were identified and examined for recurrent and nonrecurrent cytogenetic band 2q35 abnormalities. No abnormalities affecting this locus were identified.

**Functional consequences of miR-153 overexpression**

To determine any potential functional roles of overexpressed miR-153 in promoting colorectal cancer progression, we investigated the effects of transiently overexpressed miR-153 on a series of cancer-relevant *in vitro* cell-based assays testing growth and proliferation, invasion and motility, apoptosis, and chemosensitivity in SW480 cells. miR-153 overexpression (Supplementary Fig. S4A) had no effect on proliferation (Fig. 2A), anchorage-independent growth (Fig. 2B), or cell migration using scratch and transwell invasion assays without matrigel (data not shown). Overexpression of miR-153 promoted a more invasive phenotype in SW480 cells however, when matrigel-coated transwell invasion assays were examined (Fig. 2C and D; P < 0.05). Apoptosis was assessed in the presence or absence of cisplatin. Overexpression of miR-153 had little effect on viability in the absence of cisplatin, however protection against cell death was observed in cells overexpressing miR-153 when treated with cisplatin (Fig. 2E and F). These findings suggested that miR-153 may have a dual role in colorectal cancer progression, promoting enhanced invasiveness and chemosensitivity. Consequently both these processes were assessed further and are described later.

**Overexpression of miR-153 leads to increased invasiveness in colorectal cancer via MMP-9**

The effect of miR-153 on colorectal cancer invasion was tested in additional cell lines with low baseline expression of miR-153. Transient overexpression of miR-153 in DLD-1, HT29, and AAC1/82 cells (Supplementary Fig. S4E) led to enhanced invasiveness in Matrigel-coated transwell assays (Fig. 3A). Using antimiR-153, inhibition of miR-153 in colorectal cancer cell lines with high baseline levels of miR-153 (Supplementary Fig. S4F) significantly reduced invasion compared with control (Fig. 3B) without any alteration to proliferation (Supplementary Fig. S4B and S4C). To more closely recreate and model *in vivo* circumstances, we examined the effect of stably transfected GFP-tagged miR-153 in SW480 cells using a 3-dimensional organotypic coculture of SW480 cells and human fetal fibroblast cells (Fig. 3C). Ectopic expression of miR-153 but not control resulted in increased invasion of SW480 cells into the underlying stroma of organotypic cultures (Fig. 3C, i and ii). Although stable overexpression was achieved in only 50% of cells, immunostaining against GFP illustrated enrichment of miR-153–expressing cells at the invasive front (Fig. 3C, iii to vi).
To determine if miR-153 would stimulate invasion in vivo, we conducted subcutaneous implantation of SW620 cells in SCID mice. Cells were transfected with antimiR-153 or control, and injected into opposite flanks of each animal. Inhibition of miR-153 resulted in more spheroid tumors with clean edges compared with control tumors with more locally invasive phenotypes (Fig. 3D). No difference in size of tumors was noted between control and miR-153 inhibited xenografts (Supplementary Fig. S4D). Analysis of depth of invasion and number of invasive tumor spikes into surrounding stroma and adjacent tissue showed a significant reduction in invasive ability of xenografts transfected with antimiR-153 (Fig. 3E).

Our initial findings showed that miR-153 overexpression promoted a more invasive phenotype only in Matrigel-coated transwell invasion assays but not in non-Matrigel-coated assays. A principal constituent of Matrigel is type IV collagen, which is also one of the main substrates for MMP-9 (24). MMP-9 is a key effector in extracellular matrix degradation and strongly implicated in colorectal cancer associated invasion, with levels progressively increasing from early node-negative to metastatic colorectal cancer, making it an optimal candidate to examine (22, 23). To test if MMP-9 may play a role in miR-153-induced invasion, SW480 cells were transfected with miR-153 or control miRNA and medium supernatants sampled for MMP-9 activity. Figure 4A and B show that raised levels of MMP-9 activity were detected after transfection with miR-153, but not control miRNA. We also assessed levels of the other member of the Gelatinase subfamily, MMP2, however no effect
was identified (data not shown). We next tested invasiveness of SW480 cells transduced with miR-153 in the presence of a selective MMP-9 inhibitor. Inhibition of MMP-9 using an MMP-9 inhibitor or siRNA to MMP-9 abrogated the enhanced invasiveness mediated by miR-153 (Fig. 4C and D). Finally, to determine if increased activity of MMP-9 was because of raised
levels of MMP-9 transcript, we examined for MMP-9 mRNA after forced expression of miR-153 (Fig. 4E). No change in MMP-9 mRNA was identified, indicating that increased gelatinolytic activity is not consequent to a change in MMP-9 transcript production or stability. Collectively, these studies suggest that miR-153 may enhance invasiveness through an increase in MMP-9 activity.

miR-153 overexpression enhances platinum-based chemoresistance in colorectal cancer via a direct effect on FOXO3a

Our initial functional screen identified a possible role for miR-153 in mediating chemoresistance to cisplatin (Fig. 2E and F). miRNAs are also known to have powerful regulatory effects on viability and target pathways associated with chemosensitivity and chemoresistance (25, 26). Although cisplatin is an effective platinum-based antineoplastic agent with activity against a variety of gastrointestinal and other solid tumors (27, 28), it is infrequently used in the current management of colorectal cancer. Conversely, oxaliplatin, a newer generation platinum-based compound, now represents a standard of care for patients with stages III and IV colorectal cancer when combined with fluoropyrimidine-based agents (18, 28). We therefore evaluated effect of miR-153 overexpression on both cisplatin- and oxaliplatin-mediated apoptosis. Figure 5A shows that overexpressed miR-153 had a similar effect in protecting against oxaliplatin-mediated apoptosis. We also examined sensitivity of SW480 and SW620 cells to oxaliplatin. As might be predicted from the higher levels of miR-153 in SW620 cells, SW620 cells were more resistant to oxaliplatin-mediated apoptosis, even with high doses of oxaliplatin (Fig. 5B; ref. 16). Upregulation of miR-153 but not scrambled miRNA reduced abundance of activated caspase 3 when SW480 cells were treated with cisplatin, indicating that miR-153 is functioning as an antiapoptotic factor in this context (Fig. 5C and D). To explore gene targets of miR-153 that may mediate this effect, we examined for putative targets using 4
established miRNA target prediction programs, miRanda (www.microrna.org), PicTar (www.pictar.org), TargetScan (www.targetscan.org), and Diana-microTv3.0 (http://diana.cslab.ece.ntua.gr/microT/). To reduce false positives, candidates were only considered if they were predicted by at least 3 methods. One candidate identified by this approach was the Forkhead/winged helix box class O3a (FOXO3a) tumor suppressor protein. FOXO proteins are a conserved subfamily of transcription factors and orchestrate programs of gene expression involved in differentiation, apoptosis, and DNA damage responses. Deletion of FOXO alleles confers a tumorigenic phenotype in mice models (29), whereas inactivation or silencing leads to a well-described chemoresistance to cisplatin in colon, ovarian, bladder, and oral squamous cell carcinomas, making it an optimal target to investigate (30–36). FOXO3a protein levels were examined in the SW480/620 cell model, and found to inversely correlate with miR-153 expression levels (Fig. 6A). To experimentally verify FOXO3a as a target of miR-153, we examined the effect of miR-153 overexpression on endogenous FOXO3a protein levels. Figure 6B and C show a significant reduction in FOXO3a protein levels in different cell lines after miR-153 overexpression. We also evaluated if miR-153 could impact the transcript levels of FOXO3a, and noted a significant reduction in FOXO3a mRNA in the presence of miR-153 (Fig. 6D).

We next determined if the effect of miR-153 in promoting colorectal cancer chemoresistance to oxaliplatin could be altered by introduction of exogenous FOXO3a. As shown by other groups, FOXO3a was found to mediate chemosensitivity to oxaliplatin (Supplementary Fig. S5B; refs. 30–36). In addition, Fig. 6E shows that overexpression of FOXO3a can reverse the observed effect of miR-153 on oxaliplatin chemosensitivity. To determine if the miR-153–FOXO3a interaction may also be contributing to the enhanced invasiveness observed with miR-153 overexpression, we first examined the effect of FOXO3a overexpression in mediating colorectal cancer invasiveness. Figure 6F shows that although miR-153 can successfully mediate increased invasiveness in transwell invasion assays, overexpression or siRNA-mediated inhibition of FOXO3a is unable to replicate this effect. In addition, when FOXO3a was overexpressed in SW480 cells, medium supernatants sampled for MMP-9 showed no increase in MMP-9 activity compared with controls, unlike miR-153 overexpression (Fig. 6G). Similarly, other groups have noted no changes in invasiveness or MMP-9 activity from overexpressed wild-type FOXO3a, further supporting the hypothesis that the observed effects of miR-153 on invasion and MMP-9 are unlikely to be mediated through FOXO3a (30, 37).

The 3’-UTR of FOXO3a contains one phylogenetically conserved miR-153 binding site (Fig. 6H) with a 7mer-A1
Figure 6. FOXO3a levels are reduced in SW620 cells and FOXO3a is a target of miR-153. A, Western blotting for FOXO3a in SW480 and SW620 cells with corresponding analysis of signal intensities using polyclonal anti-FOXO3a. Results are presented relative to SW480 cells, which were set at 1.0. B, Western blot analysis for endogenous FOXO3a in SW480, DLD1, and Ht29 cells after transfection with control or miR-153 precursor miRNA. Representative figure for miR-153 relative levels is presented in Supplementary Fig. S4I. C, relative signal intensities for FOXO3a in Western blots were determined by densitometry. D, effect of miR-153 or control miRNAs on FOXO3a transcript levels. Total RNA was extracted 48 hours after transfection of SW480 cells and qRT-PCR conducted. Results illustrate data from 3 independent experiments and are expressed as means ± SD. E, overexpression of miR-153 in SW480 cells provides resistance to apoptosis from oxaliplatin (30 μg/mL), which can be reversed by exogenous FOXO3a. Relative expression of miR-153 is shown in Supplementary Fig. S4J. F, knock-in or knockdown of FOXO3a does not influence invasion compared with miR-153. G, zymographic activity in medium supernatants of SW480 cells after transfection with pre-miR-153, FOXO3a expression construct, or control. Comparative expression of miR-153 is shown in Supplementary Fig. S4K. H, the 3’-UTR of mammalian FOXO3a mRNA contains a phylogenetically preserved miR-153 binding site. F, dual luciferase reporter assays were conducted with FOXO3A-3’-UTRwt (containing wild-type 3’-UTR of FOXO3a) and FOXO3A-3’-UTRmut (containing a mutation of 4 of the amino acids in the predicted miR-153 binding site) vectors. * , P < 0.05.
seed-matched site in the 5′ region of miR-153, ranking as one of the top scoring probabilities of conserved targeting (\(P_C\)) as described by Friedman and colleagues (38). To determine if the effect on FOXO3a is mediated via this predicted miR-153 binding site, we cloned a 542bp region of the 3′-UTR of FOXO3a containing the single putative miR-153 binding site downstream of the Renilla luciferase open reading frame. Wild-type 3′-UTR and a mutant form in which the putative seed-binding site was mutated were evaluated. As shown in Fig. 6I, when miR-153 precursor was cotransfected with wild-type FOXO3a 3′-UTR reporter construct, significant repression in activity was noted. Mutation of the miR-153 binding site eliminated the observed repression, supporting a direct association between miR-153 and FOXO3a. Similar results were obtained in HCT116 colorectal cancer cells, indicating this is not unique to SW480 cells.

We next sought to determine if expression of FOXO3a was altered in human colorectal cancer. Using a custom-designed tissue microarray, we examined and scored the intensity and proportion of cells staining for FOXO3a in 10 normal colorectal mucosa samples, 10 adenomatous polyps, 20 stage 1 colorectal cancer, and 20 stage 3/4 tumors. Representative sections are presented in Fig. 7A and analysis of the scoring presented in Fig. 7B. FOXO3a expression was significantly lower in advanced cancer stages (\(P < 0.0005\)). Analysis of matched tumors for expression of miR-153 and FOXO3a showed a significant and inverse correlation between miR-153 and FOXO3a (Fig. 7C; \(R = -0.75; P < 0.0001; 95\% \text{ CI} = -0.86 \text{ to } -0.52\)). Taken together, these results suggest that miR-153-mediated resistance to platinum-based chemotherapy could be mediated through reduced levels of FOXO3a.

**Discussion**

Disease progression in colorectal cancer is a complex multistep process. During progression, cancer cells acquire the ability to invade beyond normal cellular boundaries, intravasate into blood and lymphatics, journey to distant organs, extravasate and proliferate in a different microenvironment, concomitantly eluding antitumor host immunity and defying chemotherapeutic agents. Although to date miRNAs have been principally identified as mediators of tumorigenesis, emerging data is also uncovering their role as important modulators of different steps in metastasis and disease progression, serving as promoters (7, 12), or inhibitors (13), of these processes.

Here we report on the pleiotropic actions of a poorly understood miRNA, miR-153 in promoting colorectal cancer progression. We used the SW480/SW620 cell model for colorectal cancer progression and conducted miRNA profiling to identify differentially expressed miRNAs between the 2 cell lines. One candidate identified by our screen was miR-153, which was upregulated by over 12-fold in the more advanced SW620 cells. miR-153 is a conserved miRNA first detected at

![Figure 7. FOXO3a expression in colorectal cancer. A, representative images from immunostaining on a tissue microarray, showing FOXO3a expression in normal, stage 1, and stage 3/4 colorectal cancer. B, FOXO3a expression is lower in more advanced disease stages (\(P < 0.0005\)). C, correlation of FOXO3a expression and miR-153 levels in human tumor samples.](image-url)
high levels in brain tissue, and implicated in development of neurodegenerative conditions (39, 40). Intriguingly, miR-153 levels are reduced in human Glioblastoma Multiforme (41), and increased in endometrial adenocarcinomas (42). To our knowledge, miR-153 has not been implicated in colorectal cancer before. To determine the relevance of this miRNA, we examined miR-153 expression in a panel of colorectal cancer cell lines, in human colorectal cancer tumor samples compared to normal mucosa, and subsequently across a panel of predefined colorectal cancers of different clinical stage. miR-153 expression in the colorectal cancer cell lines examined in our study showed similar expression patterns to matching colorectal cancer lines in the NCI-60 panel, further verifying our findings (43). miR-153 was overexpressed in tumors compared to normal tissue, and consistent with our cellular model, expressed at higher levels in metastatic compared with non-metastatic tumors. As the main mechanism for development of colorectal cancer is chromosomal instability (44), and miRNAs are overrepresented in regions of genomic copy number change in colorectal cancer, we examined the miR-153 locus for copy number change. Little is understood about the regulation of miR-153 and systematic evaluation of the literature did not identify any studies describing genetic or epigenetic regulation of miR-153 specifically. miR-153 resides in the gene locus for the PTPRN gene, which encodes a member of the protein tyrosine phosphatase family (Supplementary Fig. S3). Analysis of our cell line model and the Mitelman database suggest that copy number change in this region is a rare event however. Intriguingly, recent data from DNA methylation profiling in ovarian cancer has shown that promoter hypermethylation of the PTPRN gene is significantly associated with longer patient survival, supporting a model in which miR-153 silencing may be protective against disease progression (46). At present however, no data showing coregulation of PTPRN and miR-153 has been described.

To better understand how deregulation of miR-153 impacts colorectal cancer, we conducted functional studies to examine effects of ectopic miR-153 and inhibition of miR-153. We observed 2 broad effects of miR-153 upregulation, leading to increased cellular invasiveness, and enhanced platinum-based chemotherapy resistance. To dissect the mechanisms behind miR-153-mediated increase in invasiveness, we examined production of MMPs after miR-153 overexpression. The gelatinase MMP-9 is strongly implicated in colorectal cancer progression, and secretion is higher in metastatic murine colorectal cancer compared with nonmetastatic tumors (45), whereas in the SW480/620 human colorectal cancer model, MMP-9 expression is higher in metastatic SW620 cells (14). SW620 cells are more invasive than SW480 cells in a variety of in vitro assays, and overexpression of MMP-9 in SW480 cells leads to a more invasive phenotype (14, 47). In addition, multiple in vivo studies have showed increased MMP-9 transcript (23), protein (48), and bioactivity (49) in more advanced human colorectal cancer compared with earlier stages, and increased MMP-9 is an independent predictor of overall, cancer specific, and disease-free survival (23, 49). In this study, we noted increased MMP-9 activity after transfection of cells with miR-153. miR-153 transfected cells showed enhanced invasion through matrigel, a basement membrane-like matrix rich in collagen type IV (the main substrate of MMP-9), and inhibition of MMP-9 was sufficient to abrogate this effect. The mechanism by which miR-153 upregulates MMP-9 activity remains unclear at present, however, the results of our gene set enrichment analysis for putative miR-153 targets showed overrepresentation of a number of transcription factors, which may provide insights into this process (Supplementary Files S1 and Supplementary Fig. S1C).

To better understand how miR-153 increases resistance to platinum-based agents, in silico analysis was conducted with miRNA target predicting algorithms. One candidate identified was the FOXO3a transcription factor. Functionally, FOXO3a has striking similarities to p53, and the 2 transcription factors share many downstream targets (50). Although inactivating mutations in FOXO3a in human cancer have not been described, loss-of-function of FOXO3a frequently occurs by posttranslational modifications. FOXO3a is a critical molecule in initiating apoptotic programs, and upregulates proapoptotic genes such as Bim and PUMA, while downregulating antiapoptotic genes (50). FOXO3a is a well-described regulator of the response to cisplatin in several malignancies including colorectal cancer, and silencing endogenous FOXO3a impairs cytotoxicity of this chemotherapeutic agent (31–36). Accordingly, we tested if miR-153 could be mediating its effect through FOXO3a. Our results show a consistent and inverse relationship between miR-153 and FOXO3a levels in colorectal cancer cell lines and tumors, and support a model in which tumor overexpression of miR-153 reduces FOXO3a transcript and protein levels, impairing the apoptotic response to cisplatin, and mimicking the role of oncogenic kinases such as Akt in inactivating FOXO3a. Our results verify that the miR-153/FOXO3a interaction is a direct one, as miR-153 interacts with the 3′-UTR of FOXO3a through a specific seed-binding site to bring about repression of a FOXO3a-3′-UTR luciferase fusion gene. Expression of FOXO3a is clearly a tightly regulated process, and our findings show that miR-153 represents a further layer of regulation and providing further fine tuning of FOXO3a function.

Collectively, our findings suggest that overexpression of miR-153 has an important and dual role in promoting disease progression in colorectal cancer through enhanced cellular invasion and reduced chemosensitivity. The ability of one miRNA to have a dual function in disease promotion is not unprecedented (14) and indeed the predicted promiscuity of miRNAs by target prediction has long hinted at this. Such pleiotropy also exposes opportunities for exploiting the miRNA system for therapeutic manipulation with potential to correct more than one corrupted pathway by targeting one molecule only, giving added value in the design and development of any novel-targeted therapy. Our present findings also add to the weight of evidence incriminating miRNAs in the mechanisms behind cancer progression and metastasis, and identify miR-153 as a further molecule in the new class of "metastamirs" (51).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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