miR-23a Promotes the Transition from Indolent to Invasive Colorectal Cancer

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Colorectal cancer is a classic example of a tumor that progresses through multiple distinct stages in its evolution. To understand the mechanisms regulating the transition from indolent to invasive disease, we profiled somatic copy number alterations in noninvasive adenomas and invasive adenocarcinomas from Apc and DNA mismatch repair (MMR) mutant mouse models. We identified a recurrent amplicon on mouse chromosome 8 that encodes microRNA (miRNA) 23a and -27a (miR). miR-23a and -27a levels are upregulated in mouse intestinal adenocarcinomas, primary tumors from patients with stage I/II colorectal cancers, as well as in human colorectal cancer cell lines and cancer stem cells. Functionally, miR-23a promotes the migration and invasion of colorectal cancer cells and stem cells, whereas miR-27a primarily promotes proliferation. We computationally and experimentally validated that metastasis suppressor 1 (MTSS1) is a direct miR-23a target and similarly validated that the ubiquitin ligase FBXW7 is a direct miR-27a target. Analyses of computationally predicted target genes in microarray data sets of patients with colorectal cancers are consistent with a role for miR-23a, but not miR-27a, specifically in invasive colorectal cancers.

**SIGNIFICANCE:** Understanding the mechanisms regulating the transition from indolent adenomas to invasive and metastatic colorectal cancers is critical to improving patient outcomes. Our study highlights roles of miR-23a and miR-27a in tumor progression and supports a potential mechanistic role for miR-23a in the transition from indolent to invasive colorectal cancers. *Cancer Discov; 2(6); 540–53. © 2012 AACR.*

**INTRODUCTION**

Colorectal cancer is the second leading cause of cancer death in the United States (1). Colorectal cancer is a classic example of a tumor that progresses through multiple distinct stages in its evolution. Mutations activating the WNT (most often APC) and KRAS pathways occur as an early event in cancer cells (2, 3). Subsequently, mutations in TGF-β, phosphoinositide 3-kinase, TP53 pathways, DNA mismatch repair genes (MMR), FBXW7, and others accumulate in these long-lived cells (4, 5). Morphologically, inappropriate proliferation causes formation of adenomas. Adenomas progress to carcinoma *in situ* and early-stage colorectal cancers. This process typically lasts several years (6, 7). Then, in a relatively short time, early-stage colorectal cancers acquire the ability to invade through the colon wall, metastasize, and survive outside the colon niche microenvironment (6, 7). As 5-year survival rate for indolent colorectal cancer is approximately 90% versus 10% to 15% for metastatic colorectal cancers, understanding the mechanisms that regulate the transition from indolent adenomas and carcinoma *in situ* to invasive and metastatic colorectal cancers is critical to improving patient outcomes (8).

MicroRNAs (miRNA) are small, endogenous, noncoding RNAs that simultaneously regulate levels of multiple proteins, primarily by binding to the 3′ untranslated region (UTR) of targets and inhibiting protein translation (9). Important roles for miRNAs have been shown in multiple types of cancer, including roles in tumor progression by modulating mechanisms of differentiation, proliferation, invasion, and metastasis (10). Expression of the miR-23a/27a/24-2 cluster is altered in many cancers and has diverse effects (11). These include orchestration of target genes important for increasing proliferation, cell differentiation, and growth (12, 13). The 3 miRNAs of this cluster are derived from a single primary transcript, but the levels of each can vary because of posttranscriptional processing (i.e., levels of 1 or 2 of these miRNAs can increase, while the third does not; ref. 12).

Previously, we described a mouse model system to study the mechanisms regulating the transition from preinvasive to invasive intestinal/colon tumors (14). Apc<sup>−/−</sup> and MMR-deficient (Mlh3<sup>−/−</sup>;Pms2<sup>−/−</sup>) mice develop almost exclusively adenomas and preinvasive adenocarcinomas, whereas dual Apc<sup>−/−</sup>;MMR-deficient mice develop almost all invasive adenocarcinomas (14). Here, we use this model system to identify and characterize a novel mouse chromosome 8 locus that is amplified in invasive versus preinvasive intestinal adenocarcinomas and contains the miR-23a/24-2/27a cluster. Expression levels of miR-23a and -27a are upregulated in Apc mutant/MMR-deficient invasive adenocarcinomas. miR-23a is upregulated specifically in invasive primary colorectal cancers from stage I/II patients, whereas miR-27a levels are upregulated in primary colorectal cancers from patients with disease that has spread beyond the colorectum (stage III/IV). Both miRNAs are also highly expressed in colorectal cancer cell lines and stem cells. Mechanistically, in colorectal cancer cell and cancer stem cell lines, the ubiquitin ligase F-box protein FBXW7 (the
fourth most commonly mutated gene in colorectal cancers) is a direct miR-27a target (15). In colorectal cancer stem cells, FBXW7 promotes proteasomal degradation of the transcription factors MYC and JUN and downregulates Notch signaling components. Consequently, FBXW7 inhibition by miR-27a increases MYC, JUN, and Notch signaling; promotes proliferation; and prevents secretory lineage differentiation (16). Similarly, we show that metastasis suppressor 1 (MTSS1) is a direct miR-23a target; MTSS1 interacts directly with cortactin to promote filopodia formation and upregulates Src signaling (17). Reduced MTSS1 levels promote colorectal cancer cell and cancer stem cell migration, invasion, and metastasis. In vivo, miR-27a is required for subcutaneous colorectal cancer xenograft (18, 19) tumor growth, and both miR-23a and -27a are required for formation of hematogenous metastases. Computational analyses of publically available colorectal cancer gene expression profiling data sets are consistent with a role for miR-23a, but not miR-27a, specifically in invasive colorectal cancers. Overall, these data support a potential mechanistic role for miR-23a and its target genes in the transition from indolent to invasive colorectal cancers.

RESULTS

High-Resolution Tiling Array Profiling of Mouse Intestinal Adenomas and Adenocarcinomas

To investigate the mechanisms that cause progression of intestinal adenomas to adenocarcinomas, we conducted high-resolution tiling array-based somatic copy number profiling of mouse chromosomes 6, 7, 8, and 9 in Apc<sup>−/−</sup>;MMR-deficient adenocarcinomas versus MMR-deficient adenomas, which we had previously shown using array comparative genomic hybridization (aCGH) to contain amplified loci (14). This identified a recurrent 5-Mb amplicon on mouse chromosome.
miR-23a Accelerates Colorectal Cancer Progression

8 with increased copy number in Apc−/−;MMR-deficient invasive adenocarcinomas versus normal mucosa (Fig. 1A; Supplementary Fig. S1). This amplicon contains both multiple coding genes and miRNAs. First, we evaluated the coding genes in the amplicon critical interval using quantitative real-time PCR (qRT-PCR). However, none of the protein coding genes in the amplicon had significantly or consistently increased mRNA expression levels in Apc−/−;MMR-deficient invasive adenocarcinomas (Supplementary Fig. S2). Therefore, we next evaluated noncoding genes in this amplicon.

miR-23a and -27a Expression Levels Are Increased in Mouse Intestinal Invasive Adenocarcinomas and Human Invasive/Metastatic Colorectal Cancers

miRNA-23a, -24-2, -27a, and -181c are also contained in the critical interval for this amplicon. Using a stem-loop miR-qRT-PCR assay, we confirmed that miR-23a and -27a levels were increased in Apc−/−;MMR-deficient adenocarcinomas as compared with MMR-deficient adenomas or normal mucosa (Fig. 1B and C), consistent with a potential role in tumor progression. In contrast, neither miR-24-2 nor miR-181c expression was significantly elevated (data not shown).

To understand whether miR-23a and -27a are upregulated in human colorectal cancers as well, we measured their expression levels in (i) preinvasive tumors (adenomas and carcinoma in situ), (ii) primary colorectal cancers from patients with locally invasive disease (stage I/II), or (iii) primary colorectal cancers from patients with tumors that had metastasized outside the colorectum (stage III/IV), each normalized to adjacent normal colon tissue from the same patient as control (Fig. 1D). Expression levels of miR-23a were upregulated in primary colorectal cancers from stage I/II patients versus preinvasive adenomas and carcinoma in situ (P = 0.0001). miR-23a upregulation was specific to colorectal cancers from stage I/II patients, as primary colorectal cancers from stage III/IV patients had lower miR-23a levels versus colorectal cancers from stage I/II patients (P = 0.0001; Fig. 1E). miR-27a expression levels were higher in both comparisons of either stage I/II or III/IV colorectal cancers versus preinvasive colon adenomas and carcinoma in situ (Fig. 1F). In summary, the human colorectal cancers and mouse intestinal tumor expression data are consistent with potential roles for miR-23a and -27a in colorectal cancer progression from preinvasive to locally invasive and metastatic disease. Importantly, miR-23a expression is specifically higher in colorectal cancers from patients with locally invasive (stage I/II) disease.

miRNA Profiling Reveals High Endogenous Levels of miR-23a and -27a in Colon Cancer Stem Cells and Commonly Used Colon Cancer Cell Lines

Recent studies have highlighted the important role of colon cancer stem cells (CCSC) in colorectal cancer progression and metastasis (20). CCSCs have high tumorigenicity and self-renewal capacity and are often found at the leading edge of invasive colorectal cancers. CCSCs are also proposed to play critical roles in seeding extracolonic metastases. To understand the roles of miR-23a and -27a in CCSCs, we conducted locked nucleotide analogue (LNA)-microarray-based miRNA profiling of CCSCs. miRNA profiling of CCSCs showed that both miR-23a and -27a are among the most highly expressed miRs in CCSCs, consistent with potential functional roles (Fig. 1G; Supplementary Table S1). We also tested several commonly used non-CCSC colorectal cancer cell lines for miR-23a and -27a expression and found that both miRNAs were highly expressed (Supplementary Fig. S3A). Overall, these data confirm mouse intestinal tumor and human colorectal cancer data and show that miR-23a and -27a are highly expressed in both CCSC and non-CCSC colorectal cancer cell lines. There was no difference between expression levels of miR-23a or -27a in patients with colorectal cancer MMR-proficient versus-deficient tumors (data not shown).

FBXW7 Is a Direct miR-27a Target

We used computational target prediction algorithms (21–24) to identify miR-27a target genes, with putative miR-27a–binding motifs in their 3′UTRs. One of the targets shared by all algorithms tested included F-box and WD repeat domain-containing 7 (FBXW7), which is mutated in 6% to 9% of colorectal cancers (25–27). FBXW7 encodes a ubiquitin ligase that regulates proteosomal degradation of binding substrates (15, 16, 25). FBXW7 is a key regulator of several signaling pathways, including Notch, MYC, and JUN (16), and FBXW7 mutation stimulates cell proliferation (16). The miR-27a–binding site in the FBXW7 3′UTR (nucleotides 502–508 and 1,378–1,384; Fig. 2A, top) is highly conserved in different species. Using a luciferase reporter construct expressing the FBXW7 3′UTR, we confirmed that FBXW7 is a direct target of miR-27a (Fig. 2A, bottom). We then used a lentiviral construct expressing anti-miR-27a and infected colorectal cancer cell lines and CCSCs. Western blot analysis confirmed that miR-27a knockdown significantly increases FBXW7 levels versus control short hairpin RNA (shRNA)-transduced cells (Fig. 2B). Together, these data are consistent with FBXW7 as a direct target of miR-27a.

miR-27a Knockdown Increases FBXW7 Protein Levels and Downregulates MYC, JUN, and Notch Signaling

c-MYC, c-JUN, cyclin E, and several Notch pathway components are FBXW7 substrates (Fig. 2C; refs. 28, 29). To test whether miR-27a regulates these downstream targets, we used lentiviral anti-miR-27a knockdown in CCSCs (Supplementary Fig. S3B), which have active MYC, JUN, and Notch signaling (20, 30). Compared with lentiviral expression of a control scrambled sequence shRNA, lentiviral miR-27a knockdown reduced protein levels of FBXW7, JUN, and MYC. Similarly, phosphorylated c-JUN and c-MYC were also reduced, consistent with downregulated JUN and MYC signaling (Fig. 2B).

Next, we evaluated whether miR-27a knockdown in CCSCs impairs Notch signaling. The downstream canonical Notch pathway target genes HES1 and HES5 are upregulated by active Notch signaling (31). Consistent with miR-27a upregulation of Notch signaling, CCSCs with miR-27a knockdown had lower HES1 and HES5 levels than control cells (Fig. 2D). In CCSCs, as well as normal intestinal stem
cells, Notch signaling suppresses secretory lineage differentiation (20). To confirm that \( \text{miR-27a} \) knockdown functionally downregulates Notch, we conducted immunostaining for the secretory lineage marker MUC2. Consistent with \( \text{miR-27a} \) knockdown downregulating Notch signaling, the number of MUC2\(^+\) CCSCs increased versus control shRNA–infected cells (Fig. 2E and F). In contrast, CCSC levels of cyclin E levels, another FBXW7 target, were not affected by \( \text{miR-27a} \) knockdown (data not shown), suggesting that additional signaling feedback pathways regulating cyclin E exists in CCSCs.

**miR-27a Knockdown Inhibits CCSC Proliferation In Vitro and Tumor Formation In Vivo**

Next, we tested the impact of \( \text{miR-27a} \) knockdown on FBXW7 levels; downstream MYC, JUN, and Notch signaling; and cell proliferation. For these experiments, we used 2 different methods to knock down \( \text{miR-27a} \): LNAs and shRNA knockdown (Supplementary Fig. S3B and S3C). Both experimental approaches showed that lowering \( \text{miR-27a} \) levels consistently and significantly inhibited CCSC proliferation (Fig. 3A; Supplementary Fig. S3D). By comparison, \( \text{miR-23a} \) knockdown had a more modest effect on cell proliferation (Supplementary Fig. S4A). \( \text{miR-27a} \) knockdown also significantly impaired CCSC clonogenicity, whereas \( \text{miR-23a} \) knockdown had less effect (Fig. 3B and C; Supplementary Fig. S4B).

Next, we tested the impact of \( \text{miR-27a} \) knockdown on xenograft tumor formation. For both CCSCs and LoVo subcutaneous xenograft tumors, compared with lentiviral expression of a control, scrambled sequence shRNA, \( \text{miR-27a} \) knockdown significantly inhibited tumor growth (\( P = 0.0004; \) Fig. 3D–F). In contrast, \( \text{miR-23a} \) knockdown in CCSCs did not cause a significant reduction in xenograft tumor volume (Supplementary Fig. S4C).

**miR-27a Overexpression In Vitro Promotes Cell Growth and Proliferation in DLD1 Cells**

To understand in more detail the mechanistic role of \( \text{miR-27a} \), we used lentiviral infection to constitutively express \( \text{miR-27a} \) in the DLD1 colorectal cancer cell line, which has low endogenous levels (Supplementary Fig. S5). Complementary to shRNA knockdown studies, upregulation of...
miR-27a levels significantly increased DLD1 cell proliferation ($P < 0.01$) and in vitro clonogenicity (Supplementary Fig. S6A–S6C). Overall, these data are consistent with a functional role of miR-27a to stimulate proliferation of both CCSCs and colorectal cancer cells.

**miR-23a Knockdown Reduces Colorectal Cancer and CCSC Cell Motility and Invasion by Upregulating MTSS1 and Downregulating Src Signaling and Filopodia Formation**

To understand the role of miR-23a in colorectal cancer progression, we conducted in vitro migration and invasion assays in colorectal cancer and CCSC cells. Consistently, in both colorectal cancer and CCSC cells, lentiviral miR-23a knockdown caused a dramatic decrease in the number of cells migrating to or invading the lower Boyden assay chamber versus control shRNA–transduced cells (Fig. 4A–C). We used a computational approach to identify putative miR-23a targets (21–24), with putative miR-23a–binding motifs in their 3′UTRs which could reveal the mechanism of miR-23a knockdown inhibition of cell motility. One high-ranking predicted target was MTSS1 [also known as missing in metastasis (MIM) or BEG4], a recently identified actin-binding protein (17, 19, 32). The MTSS1 3′UTR contains a putative miR-23a–binding site (nt 1,949–1,955) that is highly conserved across multiple species (Fig. 4D, top).

Using a luciferase reporter construct expressing the MTSS1 3′UTR, we confirmed that MTSS1 is a direct target of miR-23a (Fig. 4D, bottom). Next, using Western blotting, we showed that miR-23a knockdown in CCSCs significantly increases MTSS1 levels versus control shRNA–transduced cells (Fig. 4E).

MTSS1 is expressed in several embryonic tissues including the developing central nervous system (20). There, MTSS1 interacts with Src and cortactin to inhibit cell migration (33). Also consistent with a role in cell motility, MTSS1 is downregulated in several metastatic cancer cell lines (18, 34). Cell migration is accomplished by the formation of cellular protrusions including lamellipodia and filopodia (35). These protrusions result from actin filament (F-actin) rearrangement at the cell cortex by WASP/WAVE, RHO family proteins and other factors (35), which
is an important mechanism for enabling cell motility. Small RHO family GTPases, including CDC42, Rac, and RHO, control the formation and extension of filopodia, lamellipodia, and de novo actin polymerization and enable cell migration (36–38).

When plated onto laminin-coated chamber slides, CCSCs attach and extend filopodia along the surface. In contrast, CCSCs with miR-23a knockdown do not attach well to laminin-coated plates (Fig. 5A). CCSCs with lentiviral miR-23a knockdown also show morphologic changes as compared with cells infected with a control lentivirus. These include extension of significantly fewer filopodia and growth with spherical morphology (Fig. 5A). To confirm that filopodia are being reduced in cells with miR-23a knockdown, we immunostained for phalloidin, which tracks the distribution of F-actin in filopodia. Staining revealed that miR-23a knockdown significantly reduced the number of phalloidin+ filopodia (Fig. 5B and C), consistent with a role for MTSS1 to inhibit colorectal cancer cell motility.

RHOB is a mainly endosomal small GTPase that regulates actin organization, vesicle trafficking, and RHO/Rac signaling. In multiple types of cancer cells, RHOB inhibits cellular invasion and metastasis, and during malignant progression, RHOB levels decrease (39). Because MTSS1 has been shown to directly affect RHO/Rac signaling (40), we assayed CCSCs with lentiviral miR-23a knockdown for RHOB protein levels and Rac1 activity. CCSCs with miR-23a knockdown and increased MTSS1 levels versus control knockdown cells had increased levels of RHOB and reduced Rac1 activity (Fig. 5D and E), consistent with impaired cell motility.

MTSS1 has also been shown to regulate actin cytoskeleton dynamics and interact with cortactin (CTTN), a key substrate of the oncogenic Src kinase and a major activator of actin branching and polymerization (41, 42). Consistent with a role for miR-23a regulation of MTSS1 and downstream inhibition of activated Src, p-Src (Thr416) is decreased in the miR-23a knockdown CCSCs (Fig. 5F).

**Figure 4.** miR-23a knockdown increases MTSS1 protein levels and inhibits colorectal cancer cell and CCSC migration and invasion. **A,** crystal violet staining of a Transwell migration assay of CCSCs transduced with lentivirus containing anti-miR-23a shRNA or control sequences. **B** and **C,** anti-miR-23a or control sequence lentiviral–transduced LoVo, SW480, and CCSC migration and invasion, 95% confidence interval error bars are shown. For all comparisons, P < 0.002 by Student t test. **D,** miR-23a-binding site in MTSS1 mRNA 3′ UTR (nt 1,949–1,955; top); bottom, miR-23a–binding activity luciferase reporter assay in CCSCs. CCSCs were transfected with a plasmid containing the MTSS1 miR-23a–binding site fused to the 3′ UTR of firefly luciferase and cotransfected with plasmids driving expression of pre-miR-23a or a control insert sequence. Luciferase protein levels and activity are suppressed when a miRNA binds specifically to the 3′ UTR target sequence. **E,** MTSS1 protein levels in CCSCs infected with lentivirus expressing a control or anti-miR-23a shRNA. Ctrl, control; KD, knockdown.
miR-23a Accelerates Colorectal Cancer Progression

**RESEARCH ARTICLE**

JUNE 2012  CANCER DISCOVERY | 547

In sum, these data are consistent with a mechanism whereby miR-23a downregulates MTSS1, which decreases RHOB and increases Rac1 and Src activity. This in turn causes F-actin dysregulation and filopodia extension, which upregulates migration and invasion by CCSCs and non-CCSC colorectal cancer cell lines.

**miR-23a Overexpression In Vitro Promotes Cell Migration and Invasion in DLD1 Cells**

DLD1 cells have low endogenous miR-23a levels (Supplementary Fig. S5). Similar to miR-27a overexpression studies, we used a lentiviral vector to overexpress miR-23a in DLD1 cells. DLD1 cells expressing high miR-23a levels showed a significant increase in migration and invasion ability as compared with the control cells (Supplementary Fig. S7A–S7C). Overall, these data are complementary to miR-23a shRNA knockdown studies and consistent with a role for miR-23a to promote migration and invasion.

**miR-23a and 27a Inhibit CCSC Tumor Formation in a Mouse Model of Metastasis**

Hematogeneous injection of cells into the tail vein of immunodeficient mice is a commonly used in vivo assay of metastasis. We used this system to study the roles of miRs-23a and 27a in metastasis using CCSCs expressing anti-miR-23a, anti-miR-27a, or a control shRNA. Both miRs-23a and 27a knockdown resulted in significantly fewer lung tumors versus control CCSC (Fig. 6A–D). Also consistent with inhibition of metastatic tumor formation, knockdown of either miRs-23a or 27a significantly increased overall survival of mouse hosts versus cells expressing the control shRNA (Fig. 6E and F). In summary, these results are consistent with important in vivo roles for miR-23a and -27a in metastatic tumor formation. For miR-27a, the data are consistent with a mechanism of promoting cell proliferation, and for miR-23a, a mechanism primarily caused by increased cell motility.

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**Figure 5.** Filopodia formation is dramatically decreased following knockdown of miR-23a in CCSCs. A, light microscope image of CCSCs transduced with lentivirus containing control virus or anti-miR-23a shRNAs showing presence of filopodia (left, inset at ×40) or absence of filopodia (right, inset at ×40). B, F-actin staining (phalloidin) of CCSCs with control (left, inset at ×40 showing filopodia formation) or anti-miR-23a knockdown (right, inset at ×40 with no filopodia formation). C, mean number of filopodia per colony of CCSCs infected with anti-miR-23a or control knockdown lentiviral shRNA. (Student’s t test, P < 0.001). D, RHOB levels are increased after restoration of MTSS1 protein by knockdown of miR-23a. E, Rac1 activity is decreased by 25% in CCSCs expressing anti-miR-23a, tested using the G-LISA assay (Student’s t test, P < 0.05). F, phosphorylated Src416 levels are decreased in the miR-23a knockdown cells. Ctrl, control; KD, knockdown.
Analysis of Colorectal Cancer Gene Expression Microarray Data Sets for Predicted miR-23a and -27a Target Genes

To further investigate our findings for miRs-23a and 27a in colorectal cancer tumor progression, we examined relevant whole-genome colorectal cancer gene expression profiles annotated with clinical information from the Gene Expression Omnibus (GEO). While there were no gene expression (or miRNA) data sets specifically comparing preinvasive (adenomas and carcinoma in situ) with early-stage colorectal cancers available, we were able to analyze data sets generated by other investigators profiling the transitions from (i) normal colon to adenocarcinoma and (i) early- to late-stage colorectal cancers. These include human clinical samples from (i) GSE20916 (43), which compares normal colon with colorectal cancer adenocarcinoma and (ii) GSE14333 (44) and GSE17536 (45), which compare early- (locally invasive) versus late-stage (metastatic) colorectal cancers. The latter 2 data sets have been previously used by several investigators for colorectal cancer prognostication (44, 45) and the identification of an intestinal stem cell signature in poor-prognosis colorectal cancers (46). We queried the changes in expression profiles of putative miR-23a and -27a target genes computationally predicted by different algorithms, including PicTar, TargetScan, miRanda, and DIANA-microT (21–24). These methods assess the complementarity of miRNA seed sequences to binding motifs in the 3′UTRs of their putative target genes. We pooled the individual predictions from each method to form a master list of putative targets for miR-23a (1,171 genes) and miR-27a (1,179 genes). Globaltest analysis (47) of GSE20916 differential expression levels of predicted genes in the respective miR-23a and -27a master target lists showed consistent downregulation of both miR-23a and -27a predicted targets with statistically significant P values in comparisons of adenocarcinoma versus normal colon mucosa. Similarly, we confirmed upregulation of predicted miR-23a and -27a target expression levels with statistically significant P values in both GSE14333 and GSE17536 clinical data sets in comparisons of late- versus early-stage colorectal cancers. These scores are summarized in Supplementary Table S2.
and Supplementary Fig. 5A and 5B show the distribution of the permuted test statistic of miR-23a and -27a in data set GSE14333 from 10,000 times permutation of samples. Finally, because of its potential role as an miR-23a target, we confirmed using immunohistochemistry that MTSS1 protein is expressed at high levels in primary colorectal cancers from stage IV patients, as well as secondary liver metastases (Supplementary Fig. S9A–S9D). Overall, these data corroborate our mouse and human tumor expression data and support potential mechanistic roles for miR-23a in primary colorectal cancers from patients with early-stage but not late-stage disease. However, for miR-27a, these data are less clear because predicted miR-27a target genes are upregulated in primary colorectal cancers from patients with late- versus early-stage disease.

DISCUSSION

Understanding the molecular mechanisms that regulate the transition from preinvasive to invasive colorectal cancer is critical to the development of novel approaches to arrest tumor progression and improve outcomes of patients with colorectal cancer. Here, we describe roles for miR-23a and -27a in mechanisms that are associated with tumor progression. Importantly, results from both functional and patient colorectal cancer expression analyses are consistent with potentially important roles for miR-23a specifically to stimulate migration and invasion and promote the transition from indolent to invasive colorectal cancers.

miR-23a levels are upregulated during the evolution of mouse intestinal adenomas to adenocarcinomas and, importantly, are specifically upregulated during the transition from preinvasive (adenomas and carcinoma in situ) to locally invasive (stage I/II) primary colorectal cancer tumors. Subsequently, miR-23a levels decrease in primary colorectal cancers from patients with cancer cells that have metastasized outside the colorectum (stage III/IV).

MTSS1 is a potentially important mechanistic target of miR-23a because in tumors from patients with colorectal cancers, increased MTSS1 expression is strongly associated with metastatic disease (48). MTSS1 levels are lower in adenomas than in colorectal cancer tumors. High MTSS1 levels correlate with stage III/IV disease, as well as lymph node metastasis, poor histomorphologic differentiation, and local tumor invasiveness. Strikingly, high MTSS1 expression levels in colorectal cancer tumors are associated with reduced patient 5-year overall survival, and in multivariate analysis, high MTSS1 expression is an independent indicator of poor prognosis (48). Functionally, our studies show that MTSS1 is an important direct target for miR-23a and that reduced MTSS1 levels accelerate mechanisms that promote colorectal cancer migration. miR-23a knockdown decreases filopodia formation, Rac1 signaling, Src416 phosphorylation, cell motility, and hematogeneous metastasis, all of which are consistent with a causal role for miR-23a in invasive colorectal cancers. This finding complements previous studies proposing roles for MTSS1 as a metastasis suppressor in breast, prostate, and bladder cancers (18, 19, 49). To validate our findings, we reanalyzed predicted miR-23a target gene expression levels in publically available data sets. Corroborating our data, expression levels of miR-23a predicted target genes including MTSS1 in these data sets are lower in colorectal cancers from patients with early- versus late-stage disease, and we confirmed using immunohistochemistry that MTSS1 is highly expressed in both primary colorectal cancers and secondary metastases from patients with stage IV disease.

To further validate our findings, we searched for large-scale gene expression and miRNA data sets specifically comparing preinvasive (adenoma and carcinoma in situ) with invasive (stage I/II) colorectal cancers. However, to our knowledge, these data sets are currently not available. Therefore, as genome-wide data sets comparing preinvasive with invasive colorectal cancers become available, it will be important in the future to analyze whether they further validate our findings of miR-23a target gene upregulation during this transition.

miR-27a levels are upregulated in mouse intestinal adenocarcinomas as well as in invasive and metastatic colorectal cancers. Functionally, in colorectal cancer stem cells and commonly used colorectal cancer cell lines, miR-27a can directly downregulate the tumor suppressor FBXW7 and promote cell proliferation. Consistent with these data, miR-27a knockdown and the subsequent increase in FBXW7 protein levels inhibits Notch, JUN, and MYC signaling. Functionally, this causes colorectal cancer secretory lineage differentiation, as shown by expression of the colon goblet cell marker MUC2. Overall, these data are consistent with roles for miR-27a to promote general mechanisms associated with tumor progression. However, analyses of predicted miR-27a target gene expression levels in publically available large-scale gene expression data sets show upregulation of predicted targets in primary colorectal cancers from patients with late- versus early-stage disease even though miR-27a levels do not decrease. Although there are clearly many potential confounding factors that could cause this discrepancy (e.g., the lack of information in these data sets on which patients did or did not receive chemotherapy, cross-regulation by other miRNAs, stochastic noise), together they limit the specific conclusions that can be drawn at present about the role of miR-27a specifically in tumor progression of patients with colorectal cancers. Again, as genome-wide data sets comparing preinvasive with invasive colorectal cancers become available, it will be important to investigate in more detail the potential role of miR-27a to promote the transition from preinvasive to invasive colorectal cancers, as well as in other tumor types.

METHODS

Mice

All animal studies were conducted under an approved Weill Cornell (New York, NY) Institutional Animal Care and Use Committee protocol. Wild-type, Apc1638N+/−, Mlh3+/-, Pms2−/−, and Apc1638N−/−; Mlh3−/−; Pms2−/− mice have all been described previously and were maintained on the C57BL/6 genetic background (50). All lines of mice were necropsied when the mice became morbid or moribund. Sacrificed mice were surveyed for tumors and suspicious masses.
Mouse Tiling Array Studies

Genomic DNA was isolated from tumor and normal tissue from each mouse by using the PUREGENE DNA Isolation Kit (Gentra Systems). NimbleGen tiling array hybridization of mouse chromosomes 6 to 9 was conducted at NimbleGen Systems Inc. The 385K oligonucleotide tiling array produced by NimbleGen Systems Inc. was used. Probe design, array fabrication, aCGH experiments including DNA labeling, hybridization, array scanning, data normalization, and log2 copy number ratio calculation were conducted by NimbleGen Systems Inc.

miRNA Isolation and qRT-PCR

miRNA was extracted from cell lines and tissues using mirVana spin columns (Ambion). All primary colorectal cancer tissues in this study were taken from colorectal adenomas and stage 0 to IV colorectal cancers collected by the New York Presbyterian Center for Advanced Digestive Care Colon Cancer Biobank in accordance with the Weill Cornell Institutional Review Board. Briefly, RT-PCR studies were conducted using 10 ng of total RNA and gene-specific PCR primers for miRNAs purchased from Life Sciences (Applied Biosystems). PCR cycling conditions used 32 cycles (95°C for 15 seconds, 60°C for 30 seconds) after an initial denaturation step (95°C for 3 minutes). Expression levels are the average of 3 or more independent experiments. All expression levels are normalized to the average of small nuclear U6 and RNU48 snRNA levels.

RNA Isolation and qRT-PCR

Total RNA was extracted using the Qiagen RNeasy Kit and reverse-transcribed using the ABI RT Reversi Transcription Kit. Gene expression was quantified on a BioRad real-time PCR analyzer (CFX96). Expression levels are the average of 3 or more independent experiments. All expression levels are normalized to gliceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.

Culture of CCSC and Colon Cancer Cell Lines

CCSC lines used in this study are established cell lines from human colon cancer resections generated by our laboratory and have been described previously (19). CCSCs were cultured in vitro in ultra-low attachment flasks with Dulbecco’s Modified Eagle’s Media (DMEM)/F12 containing nonessential amino acids, antibiotic antimycotic, N2 supplement (Invitrogen), B27 supplement (Invitrogen), heparin (4 μg/mL; Sigma), epidermal growth factor (40 ng/mL; Sigma), basic fibroblast growth factor (bFGF; 20 ng/mL; Sigma), epidermal growth factor (40 ng/mL; Sigma), basic fibroblast growth factor (bFGF; 20 ng/mL) for 3.5 hours. The media were replaced with fresh media containing 2 μg/mL of puromycin, essentially as previously described (20).

Cell Viability Assays

Viability of cells was measured by using the MTT reduction method. Cells at a density of 3.0 × 10^4 per well were seeded into 96-well dishes in triplicate for each independent experiment. Cells were incubated with 2.5% MTT solution (5 mg/mL) for 3.5 hours at 37°C. Dimethyl sulfoxide was then used to dissolve the formazan product for spectrophotometric analysis at 540 nm.

Cell Migration, Invasion, and Clonogenicity Assays

To assay cell migration, the Boyden chamber assay was used. A total of 1 × 10^4 of CCSCs were seeded onto fibronectin-coated polycarbonate membrane inserts (6.5 mm in diameter with 8.0-μm pores) in a Transwell apparatus (Costar) and cultured in CCSC media. FBS (5%) was added to media in the lower chamber. After incubation for 12 hours at 37°C in a CO2 incubator, the inserts were washed with PBS, and cells on the top surface of the insert were removed by wiping with a cotton swab. Cells were then fixed with methanol, stained with 0.4% crystal violet solution, and inspected microscopically at ×200 magnification. For the invasion assay, the Transwell membrane was coated with a 300 ng/μL Matrigel solution (BD), and the cells were incubated for 24 hours at 37°C. Cells that migrated to the bottom surface of the insert were fixed with methanol and stained with 0.4% crystal violet solution. Cells in 5 separate randomly chosen fields were counted. Clonogenicity assays were conducted essentially as previously described (20). The value for each assay represents the mean ± SEM of triplicate measurements from at least 3 independent experiments.

Protein Isolation and Western Blotting

CCSC spheres and cell pellets were lysed in ice-cold NP-40 lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, and 1% NP-40). Protein quantification was carried out using Bio-Rad protein quantification assay. Proteins were separated by SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride (PVDF; Millipore). Membranes were blocked with 5% nonfat dry milk and incubated overnight at 4°C with the indicated primary antibody. Detection was carried out by peroxidase-based chemiluminescence (Amer sham). Antibodies used are listed in Supplementary Methods.

Immunohistochemistry

Immunohistochemistry was conducted on 5-μm paraffin-embedded sections from 3-dimensional Matrigel cultures as previously described (20). Paraffin-embedded sections were deparaffinized in xylene and rehydrated in a graded alcohol series and water. The slides were heated in antigen retrieval solution and incubated with…

Jahid et al.
miR-23a Accelerates Colorectal Cancer Progression

anti-Myc-tag (1:500) or anti-Muc2 (1:250) antibodies overnight at 4°C. The ABC kit (Vector) was used to visualize antigen. Counterstaining was done using hematoxylin. Immunofluorescence was conducted on CCSCs grown on laminin-coated chamber slides that had been fixed with 3% to 4% paraformaldehyde for 10 to 20 minutes and permeabilized with 0.5% Triton X-100 for 2 to 10 minutes. Chamber slides were then stained with Alexa Fluor 488 phalloidin at a dilution of 1:250 and analyzed with fluorescence microscopy.

Subcutaneous Xenograft Assay

Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were obtained from The Jackson Laboratory. All animals used were between 6 and 8 weeks of age and housed in microisolator cages in accordance with the Institutional Animal Welfare Guidelines of Weill Cornell Medical College. Mice were injected subcutaneously in the flank with either (1 × 10⁶) CCSCs or 0.5 × 10⁶ LoVo) mixed in a 1:1 ratio with Matrigel (BD). Tumor size was measured weekly using calipers.

Tail Vein Metastasis Assay

A total of 1 × 10⁶ of CCSC cells were injected into the tail vein of 6- to 8-week-old NOD.Cg-Prkdcsid1Ile2rgtm1Wjl(SzJ) mice obtained from The Jackson Laboratory. Moribund mice were sacrificed immediately, necropsy was conducted, and tumors were harvested using a dissecting microscope. All animal protocols in this study were in accordance with the Institutional Animal Welfare Guidelines of Weill Cornell Medical College.

Luciferase Assay

HEK293 cells were plated in 96-well plates at 5,000 cells per well. On the second day, the cells were transfected with 1.0 μg of (i) plasmid carrying the 3′UTR miRNA-binding site for miR-23a or -27a cloned in pEZX-MT01 (GeneCopoeia); (ii) empty vector or precursoir miRNA expression clone sequences in pEZX-MR03 (GeneCopoeia); or (iii) “scrambled” control sequences in pEZX-MR03, using Lipofectamine (Invitrogen) according to the manufacturer’s recommendations. Both firefly luciferase and Renilla luciferase activities were measured 2 days after transfection, and data were recorded on the GLOMAX system. Firefly luciferase activity was then normalized with Renilla luciferase activities in the same well. Luciferase activity was measured in triplicate with 3 different clones in at least 4 independent experiments and is presented as mean ± SEM.

miRCURY LNA miRNA Array Studies

Total RNA was extracted using the Qiagen RNeasy Kit. miRNA profiling was conducted at Exiqon. The hybridization was conducted according to the miRCURY LNA Array Manual using a Tecan HS4800 hybridization station. After hybridization, the microarray slides were scanned and stored in an oxygen-free environment (ozone level <2.0 parts per billion) to prevent potential bleaching of the fluorescent dyes. The miRCURY LNA array microarray slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc.), and the image analysis was carried out using the ImaGene 8.0 software (BioDiscovery, Inc.).

Clinical Colorectal Cancer Gene Expression Microarray Data Set Analyses

To compare the expression of miR-23a and -27a predicted target genes between (i) normal colon versus all colorectal cancers and (ii) primary tumors from patients with stage I versus stage IV colorectal cancers, and 3 human whole-genome microarray data sets (GSE20916, GSE14333, and GSE17536) were downloaded from the public functional genomics data repository at the National Center for Biotechnology Information GEO. All of these 3 data sets used the Affymetrix HGU-133Plus2 chip. The GSE20916 data set includes normal colon, adenocarcinoma, and carcinoma array data (43). The GSE14333 data set includes primary colorectal cancer tumor array data from 44 patients with stage I and 61 patients with stage IV colorectal cancer, respectively (44). The GSE17536 data set includes primary colorectal cancer tumor array data from 24 stage I and 39 stage IV patients, respectively (45).

Genes targeted by miR-23a and -27a were predicted by several miRNA target prediction methods, including PicTar, TargetScan, DIANA-microT, and miRanda (21–24). The latter 2 methods were used with tight filter thresholds—an miTG score of 0.45 for DIANA-microT and an miSRV score of −0.8 for miRanda. For each analysis of primary colorectal cancer tumor gene expression data, expression level changes in miR-23a and -27a predicted target genes were compared between (i) normal colon versus adenocarcinoma and carcinoma combined or (ii) stage I versus stage IV colorectal cancers using the Globaltest algorithm (47). Briefly, Globaltest uses a generalized linear model with a random effect for gene set analysis. A score test statistic is used for testing the null hypothesis, and the P value can be calculated either from asymptotic distributions of the test statistics or by permutation distributions of the test statistics.

Statistical Data Analysis

All statistical data analysis was conducted with GraphPad Prism 5 software (GraphPad Software, Inc.), with the exception of the clinical colorectal cancer gene expression microarray data set and NimbleGen tiling array analyses as described above.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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