A MicroRNA Targeting Dicer for Metastasis Control

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SUMMARY

Although specific microRNAs (miRNAs) can be upregulated in cancer, global miRNA downregulation is a common trait of human malignancies. The mechanisms of this phenomenon and the advantages it affords remain poorly understood. Here we identify a microRNA family, miR-103/107, that attenuates miRNA biosynthesis by targeting Dicer, a key component of the miRNA processing machinery. In human breast cancer, high levels of miR-103/107 are associated with metastasis and poor outcome. Functionally, miR-103/107 confer migratory capacities in vitro and empower metastatic dissemination of otherwise nonaggressive cells in vivo. Inhibition of miR-103/107 opposes migration and metastasis of malignant cells. At the cellular level, a key event fostered by miR-103/107 is induction of epithelial-to-mesenchymal transition (EMT), attained by downregulating miR-200 levels. These findings suggest a new pathway by which Dicer inhibition drifts epithelial cancer toward a less-differentiated, mesenchymal fate to foster metastasis.

INTRODUCTION

microRNAs (miRNAs) are an evolutionarily conserved group of small RNAs (18–24 nucleotides) that inhibit gene expression. miRNAs are transcribed by RNA polymerase II as longer precursors and then processed into mature miRNAs by the sequential action of Drosha and Dicer endonucleases (Bartel, 2009). Mature miRNAs operate via sequence-specific interactions with the 3’ untranslated region (UTR) of cognate mRNA targets, causing suppression of translation and mRNA decay (Ambros, 2004; Bartel, 2009).

miRNAs coordinate the expression of entire sets of genes, shaping the mammalian transcriptome (Bartel, 2009). Loss of miRNA biosynthesis, as in Dicer knockouts, is lethal, owing to mitotic catastrophe and severely defective stem cell proliferation and differentiation (Bernstein et al., 2003; Fukagawa et al., 2004; Tang et al., 2007).

A large body of evidences suggests that the multigene regulatory capacity of miRNAs is dysregulated and exploited in cancer: miRNA loci are targeted by genetic and epigenetic defects, and miRNA “signatures” have been found informative for tumor classification and clinical outcome (Calin and Croce, 2006; Ventura and Jacks, 2009).

Although several miRNAs are upregulated in specific tumors (Volinia et al., 2006), a global reduction of miRNA abundance appears a general trait of human cancers, playing a causal role in the transformed phenotype (Kumar et al., 2007; Lu et al., 2005; Ozen et al., 2008). However, little is known on the underlying mechanisms or the phenotypic advantages afforded to cells by reduced miRNA expression and, if any, on the clinical relevance of this phenomenon. The present work sheds light on these questions as here we identify a microRNA family, miR-103/107, whose expression is associated with metastasis and poor outcome in breast cancer patients. miR-103/107 inhibit the expression of Dicer, causing global microRNA downregulation. miR-103/107 foster the acquisition of mesenchymal characteristics and are relevant for breast cancer cell migration and metastatic dissemination.

RESULTS

miR-103/107 Target Dicer

miRNAs constrain gene expression by binding to the 3’UTR of messenger RNA (mRNAs); thus, genes that must remain active
miR-15a levels were compared in parental and MDA-MB-231 cells stably expressing a (G) Expression of a miR-103/107 family
See also Figure S1 and Table S1.

During a survey of the 3'UTRs of several housekeeping genes, we were struck by the unusual conservation across different cell types, such as housekeeping genes, typically evolved 3'UTRs that are short, allowing them to escape this regulation (Bartel, 2009). During a survey of the 3'UTRs of several housekeeping genes, we were struck by the unusual length of the 3'UTR of Dicer (>4000 bp). As Dicer is essential for processing miRNA precursors (Filipowicz et al., 2008), this raised the possibility that some mature miRNAs could feed back to control Dicer expression. To explore this further, we used Pictar and TargetScan computational tools (Friedman et al., 2009; Krek et al., 2005) to search for miRNA binding sites in the 3'UTR of Dicer. From this analysis, the miR-103/107 family (composed of miR-103.1, miR-103.2, and miR-107) stood out for the presence of eight evolutionarily conserved binding sites, suggesting cooperative binding and biologically effective interaction (Figure 1A).

To test if miR-103/107 target Dicer, we first generated reporter constructs in which the full-length 3'UTR of Dicer, either wild-type or mutant in the miR-103/107-binding sites, was cloned downstream of the luciferase open reading frame (Lux-Dicer-3'WT or -MUT, respectively, Figure 1B). The activity of these two reporters was compared in human U2OS cells: the wild-type miR-103/107-WT causes a global reduction of mature miRNAs to antagomiR-MUT-treated cells. Global miRNA expression was measured with TaqMan Human miRNA Array and normalized to snRNA-U6b loading control. Absolute values are shown as mean and standard deviation (SD).

Figure 1. miR-103/107 Target Dicer
(A) Schematic representation of the 3'UTR of Dicer. Red bars show predicted miR-103/107' target sequences. Below, sequences of mature miR-103 and miR-107 aligned to one of these target sites, revealing evolutionary conservation in the seed-pairing sequence between amphibians (Xenopus, Xtr), birds (Gallus, Gga), and mammals (human, Hsa and mouse, Mmu).
(B) Schematic representation of the reporters for miR-103/107 activity against the Dicer 3'UTR. The CMV promoter drives constitutive transcription of a chimeric miRNA containing the firefly luciferase coding sequence fused to the full-length Dicer 3'UTR (Lux-Dicer-3'-WT) or to the same UTR mutated in all the miR-103/107 seed-pairing sequences (Lux-Dicer-3'-MUT). Below, the predicted miR-103/107-binding sites in the 3'UTR of Dicer miRNA are responsive to endogenous (compare lane 1 with lane 5) and overexpressed miR-103 or miR-107 in human U2OS cells. Luciferase reporters were transfected in parental (–) or in stable cell lines expressing pri-miR-103, pri-miR-107, or, as a control, the unrelated pri-miR-154. Absolute values are shown as mean and standard deviation (SD).
(C) Downregulation of endogenous Dicer protein expression by miR-103/107 as assayed by immunoblotting. LaminB serves as loading control. Controls are shGFP (U2OS) or scrambled siRNA (MDA-MB-231). Mature miR-107-MUT bears mutations in the seed sequence and is inactive compared to wild-type.
(D and E) Endogenous requirement of miR-103/107 on expression was measured with TaqMan Human miRNA Array and normalized to snRNA-U6b loading control. See Table S1 for expression values.
3'UTR reporter (Figure 1B, compare lane 5 with lanes 6 and 7) but not of the corresponding seed mutant reporter. As control, forced expression of the unrelated pri-miR-154 or shGFP had no effect on luciferase expression (Figure 1B, lane 8). Collectively, these data indicate that miR-103/107 target the Dicer 3'UTR.

We next monitored to what extent miR-103/107 affect the endogenous levels of Dicer protein. In multiple cell lines, Dicer protein was specifically downregulated (about 50%–60% reduction) by expressing pri-miR-103 or pri-miR-107 (Figure 1C, left panel and Figure S1A available online). To exclude any potentially confounding effect from the viral expression system—or from flanking sequences of the pri-miR constructs—we also transiently transfected cells with the mature form of miR-107 or, as control, a mutant miR-107 that contained three mismatches in the seed-binding sequence (miR-107-MUT). Dicer protein levels were downregulated by mature miR-107 but not miR-107-MUT (Figure 1C, right panel and Figure S1B). miR103/107 affect Dicer levels acting on its 3'UTR, as lentiviral expression of Dicer lacking the 3'UTR was insensitive to miR-107 (Figure S1C).

Next, we tested if miR-103/107 are causal for Dicer downregulation in a loss-of-function experimental setting. For this, we used antagomiR reagents (Krutzenfeld et al., 2005) to silence endogenous miR-103/107 (antagomiR-103/107); as a control, we used a mutant version of this reagent carrying six mismatches (antagomiR-MUT) (Figure S1D). As shown in Figure 1D, treatment of MDA-MB-231 cells with antagomiR-103/107 specifically promoted expression of the Dicer 3'UTR-wild-type reporter and, crucially, upregulated endogenous Dicer protein, as assayed by immunoblotting (Figure 1E). Thus, Dicer levels are limited by endogenous miR-103/107.

We then investigated the effects of the miR-103/107-Dicer interaction on miRNA biosynthesis, by comparing miRNAs levels in MDA-MB-231 cells expressing miR-107 or miR-107-MUT. As assayed quantitatively by qPCR, mature miRNAs were globally downregulated in the presence of miR-107, and this is phenocopied by Dicer knockdown (Figure 1F; Figures S1E and S1F; and Table S1). Sustaining Dicer expression by means of a miR-107-insensitive transgene rescues this effect (Figure 1G, Figure S1G, and data not shown).

If miR-103/107 restrict miRNA processing at the level of Dicer, then the levels of miR-103/107 should directly correlate with the abundance of Dicer substrate, that is, the 70 nt precursor miRNAs (pre-miRNAs). Indeed, pre-miRNAs, but not pri-miRNAs, accumulate in miR-107-expressing MDA-MB-231 cells (Figure S1H).

In sum, miR-103/107 lead to inhibition of miRNA biogenesis through Dicer downregulation.

Inverse Correlation between miR-103/107 and Dicer Levels in Cancer Cell Lines

At this point, we were intrigued by the analogy between the effects of miR-103/107 on Dicer function and the hampered maturation of multiple miRNAs observed in human tumors (Lu et al., 2005; Ozen et al., 2008; Ventura and Jacks, 2009). Following this lead, we first compared the endogenous levels of miR-103/107 and Dicer protein in a well-established cellular model of mammary tumor progression, consisting of four distinct cell lines, 67NR, 168FARN, 4T07, and 4T1, all derived from a single primary tumor and whose activity as xenografts reflects the sequence of multistep progression toward metastasis (Aalakson and Miller, 1992). We found that miR-103/107 expression levels increased from the nonaggressive cells (67NR, 168FARN) to metastatic lines (4T07 and 4T1). Conversely, endogenous Dicer protein levels decreased in metastatic lines (Figure 2A). To determine whether the expression of miR-103/107 increases with enhanced metastatic propensity in another cellular context, we analyzed SW480 and SW620 human colon cancer cell lines, derived, respectively, from the primary tumor and a metastasis of the same patient (Leibovitz et al., 1976). As shown in Figure 2B, an inverse correlation between miR-103/107 and Dicer level could also be observed in this case.

Clinical Association of miR-103/107 Expression to Breast Cancer Metastasis and Poor Prognosis

The data on cell lines suggested a possible link between miR-103/107 expression and tumor progression. We surmised that, if biologically meaningful, this mechanism might also be found in human tumors. For this, we measured mature miR-103/107 levels in a collection of breast cancer patients treated in our Institution with annotated clinical history. Patients were divided in two groups, with respectively high or low levels of miR-103/107 (Figure 2C, see Extended Experimental Procedures). Remarkably, when tested using the Kaplan-Meier survival analysis, the miR-103/107 “high” group displayed a significantly higher probability to develop metastasis when compared to the “low” group (Figure 2D). In line with our biochemical characterization of Dicer as target of miR-103/107, the “high” group tumors showed a reduced level of Dicer protein when compared to the “low” group, as assayed by immunohistochemistry (Figures 2E–2H).

We next wished to extend these analyses to independent cohorts of patients. For this, we took advantage of the fact that miR-103 and miR-107 are intronic miRNAs contained in three PANK (Pantothenate kinase) loci of the human genome (i.e., PANK1, 2, and 3 corresponding to pri-miR-107, pri-miR-103-2, and pri-miR-103-1, respectively). Expressions of PANK genes paralleled that of miR-103/107 in the series of cell lines described above (Figure S2); this coexpression is in line with a previous analysis in normal human tissues (Baskerville and Bartel, 2005).

We thus used PANK/pri-miR-103/107 expression as an approximation of miR-103/107 levels to interrogate several public gene expression datasets for which a wealth of molecular and associated clinical data is available (summing up to more than 1000 breast cancer patients, see Extended Experimental Procedures). For each dataset, tumors were divided in two groups, with respectively high or low levels of pri-miR-103/107 (see Extended Experimental Procedures for details). In agreement with our previous analyses on mature miR-103/107, the group expressing higher levels of pri-miR-103/107 displayed a significantly higher probability to develop metastasis and poor outcome when compared to the “low” group (Figure 2I and Figure S3). Taken together, our results suggest that high miR-103/107 expression is unlikely to represent a general feature of all tumors but rather identifies those associated to adverse and metastatic disease.
Given the significant association between miR-103/107 expression, Dicer protein levels, and clinical relapse, we wished to establish if a similar association existed for Dicer transcripts. However, when breast cancer patients’ stratification was repeated based on high or low Dicer mRNA levels, our analyses failed to detect a significant association with metastasis or outcome (Figure 2J and Figure S3).

miR-103/107 Downregulate Dicer to Promote Cell Migration and Invasion In Vitro

In light of the preceding data, we sought to determine more directly if the miR-103/107-Dicer connection plays a causal role in conferring aggressive traits to breast cancer cells. For this, we assayed how gain or loss of function of either miR-103/107 or Dicer impacted on cell migration, a hallmark of metastatic capacity. We first assayed 168FARN and SUM149: these cells are tumorigenic but...
display poor migratory capacities and contain relatively low levels of miR-103/107 (see Figure 2A and data not shown). As assayed in transwell migration assays, raising miR-103/107 in these cell lines increased migration by 8- to 10-fold, whereas overexpression of control-shGFP had no effect (Figure 3A). Complete loss of Dicer is detrimental for cell survival (Fukagawa et al., 2004), whereas here we show that miR-103/107 enhance motility with no effect on cell proliferation (Figures 3B and 3D). Different degrees of Dicer downregulation may reconcile these findings, as Dicer protein is only partially downregulated by miR-103/107. To tackle this quantitative issue, we transfected MDA-MB-231 cells with increasing doses of Dicer.

Induction of migratory capacity by miR-103/107 relies on attenuation of Dicer as expression of miR-103/107 is phenocopied by shDicer SUM149 cells (reducing Dicer to about 40% its normal levels) and is rescued by coexpression of a miR-insensitive Dicer transgene that restores Dicer protein to a level near-to-endogenous (Figure 3A, lanes 4 and 5, see immunoblots in Figure S4C). Similar results were obtained in wound-healing assays with another, more aggressive cell line, MDA-MB-231 cells (Figures 3C and 3D). Thus, miR-103/107 empower cell motility through Dicer inhibition.
siRNA, inducing a range of depletions, from negligible to more quantitative knockdown (Figure 3F and data not shown). As expected, full Dicer knockdown impaired cell viability and, consequently, secondarily reduced cell migration if compared to control cells (Figure 3E, compare lane 1 with lanes 2–4). Remarkably, however, partial attenuation of Dicer to levels similar to those achieved by miR-103/107 (i.e., 50%–60% reduction) potently fostered cell migration (Figure 3E, lanes 6–8 and 10 and Figure 3F). Similar results were obtained with immortalized mouse mammary epithelial cells NMuMG (Figures S4F–S4H). These findings suggest that cell migration is exquisitely sensitive to the levels of Dicer, and that the degree of Dicer downregulation imposed by miR-103/107 is sufficient to unleash aggressive cell behaviors.

We next tested if endogenous levels of miR-103/107 are required for cell migration in the highly metastatic tumor cell line 4T1. For this, we first silenced miR-103/107 by treatment with antagomiR-103/107. This leads to a 5-fold reduction in migratory properties similar to that one obtained by increasing Dicer expression (Figure 3G, lanes 1–3). Strikingly, loss-of-Dicer renders 4T1 cells insensitive to loss of miR-103/107, indicating that Dicer is epistatic to miR-103/107 (Figure 3G, compare lanes 1 and 2 with lanes 4 and 5). Taken together, the data suggest that the balance between miR-103/107 and Dicer is critical to controlling cancer cell motility.

Expression of miR-107 Endows Metastatic Potential

The data presented so far raised the possibility that the link between miR-103/107 and Dicer could configure in vivo a metastasis-promoter/suppressor pair. To test this idea, we assayed if miR-103/107 could foster metastasis in vivo. For this, we used SUM149 cells, which form nonmetastatic primary tumors in vivo after injection in the mouse mammary gland (Ma et al., 2007) but retain residual lung colonization capacity when delivered through the tail vein. Notably, expression of pri-miR-107, but not shGFP (control), strongly promoted metastatic colonization (Figure 4A, compare lanes 1 and 2, and Figure 4B). In agreement with our previous in vitro characterization, this is phenocopied by partial depletion of Dicer (Figure 4A, lane 3 and Figure 4B). Conversely, rescuing Dicer expression (Figure S4C) abolished the prometastatic effects of miR-107 (Figure 4A, lane 6).

Figure 4. miR-107 Induces Metastatic Dissemination

(A and B) Lung colonization assays of SUM149 derivatives after injection in the tail vein of SCID mice (8 mice per cell line). Four weeks after injection, lungs were analyzed for the presence of metastatic nodules. (A) Quantification of metastatic nodules formed by the indicated SUM149 derivatives. Analyses were carried out on histological sections of the lungs (4 sections per lung) stained with the anti-cytokeratin antibodies AE1/AE3. Data are represented as mean and SD. (B) Representative pictures of metastases embedded in the lung parenchyma. Macrometastases were observed only in mice injected with cells expressing pri-miR-107 or shDicer. (C) SCID mice were orthotopically injected in the fat pad with SUM149-shGFP or SUM149-miR-107 cells. The rates of primary tumor growth were not significantly different, showing, if anything, a reduced proliferation of SUM149-miR-107 in vivo. Data are represented as mean and SD. (D and E) Pri-miR-107 promotes distant metastatic dissemination of breast cancer cells from the orthotopic site. Lungs of mice injected in (C) were explanted after 12 weeks and scored for the presence of metastases as in (A). (E) Right panel: representative metastatic focus of SUM149-miR-107 cells embedded in the lung parenchyma. Graphs in (D) provide a quantification of metastatic dissemination measured as the percentage of sections displaying at least one metastasis out of n = 8 SUM149-shGFP injected mice and n = 8 SUM149-miR-107 injected mice. Four to six serial sections were sampled and analyzed for each mouse.
4 and Figure 4B). Thus, Dicer serves as metastasis suppressor downstream of miR-103/107.

Having established this relationship, we next asked whether miR-107 would also empower distant metastatic dissemination from primary tumor masses. For this, we implanted control (shGFP) and miR-107-expressing SUM149 cells in the mammary fat pad of immunocompromised mice. As previously shown in vitro, gain of miR-107 does not foster proliferation in vivo, not even within the competitive tumor microenvironment (Figure 4C). After 12 weeks, host mice were sacrificed and examined for the presence of metastatic lesions in the lung. Although no macroscopic metastases were detected, the staining of histological sections with anti-cytokeratin antibodies revealed the presence of micrometastatic foci in the lungs explanted from mice bearing the SUM149-miR-107 xenografts, whereas almost none were found in mice injected with control cells (Figures 4D and 4E). Thus, once overexpressed, miR-107 is a prometastatic factor that unleashes the ability to initiate distant dissemination in otherwise nonmetastatic cells. Further, these data provide functional support to the association between miR-103/107 and clinical relapse previously revealed in human tumors.

Silencing of miR-103/107 Inhibits Metastasis

We next asked if continuous repression of Dicer by endogenous miR-103/107 in aggressive cells is required for metastatic spread in vivo. For this, 4T1 cells were injected into the mammary fat pad of recipient mice and tumors treated either with antagomiR-MUT or antagomiR-MUT (see Experimental Procedures). As shown in Figure 5A, we found that the onset and size of primary tumors were comparable in the two groups of mice (p value > 0.05), despite the quantitative loss of endogenous miR-103/107 in antagomiR-103/107-treated primary tumors (Figure 5B). Strikingly, however, although the antagomiR-MUT receiving cells invaded the lung parenchyma, silencing of miR-103/107 efficiently reduced metastatic colonization (Figures 5C, 5D, and 5E). This occurred without detectable detrimental effects on normal mammary glands (Figure S5A). Thus, endogenous miR-103/107 is critical for efficient metastatic dissemination of breast cancer cells.

We also compared control and miR-103/107-depleted primary tumors for expression of a number of miRNAs. As expected from the rescue of endogenous Dicer activity, silencing of miR-103/107 enhanced global miRNA processing, as revealed by the increased levels of mature miRNAs (Figure 5F) and concomitant reduction of the 70 nt miRNA precursors (Figure S5B). This suggests that Dicer is limiting in metastatic tumors.

If the reduction of Dicer activity by high miR-103/107 is critical for the execution of the metastatic program, then sustaining Dicer expression should phenocopy the loss of miR-103/107 and oppose metastasis. To test this, we selected two Dicer-overexpressing 4T1 cell clones from a lentivirally infected cell population (Figure S5C). Dicer-4T1-derived tumors were strikingly deprived of metastatic capacity when compared to lesions from mock-infected cells (Figures 5G–5I). In sum, the data reveal a functional pathway in aggressive tumors, whereby endogenous miR-103/107 are instrumental to attenuating Dicer levels below a threshold for metastasis protection.

Because levels of Dicer are around 50% when miR-103/107 are elevated, one would expect that heterozygous loss of Dicer would represent one mechanism selected during tumor progression to favor metastasis. To explore this issue, we queried array CGH profiling of breast cancers and found that some tumors display a reduced copy number of the Dicer1 locus, a finding compatible with Dicer heterozygosity; intriguingly, this subset of tumors also display an increased propensity to develop metastasis (Figure S5D). This provides a genetic proof-of-principle that selective pressure for Dicer downregulation exists in aggressive breast cancer. Interestingly, pri-miR-103/107 expression levels are able to stratify patients according to outcome only in Dicer+/- tumors but not upon Dicer heterozygosity (Figure S5E). In other words, Dicer heterozygous tumors lost selective pressure for miR-103/107 upregulation, supporting the notion that these molecules are indeed in the same pathway. It is, however, remarkable that the number of tumors displaying elevated miR-103/107 and wild-type Dicer is higher (37%, n = 313) than those carrying copy-number variations of the Dicer gene (18%), suggesting that the use of an miRNA is preferred to genetic deletions, at least in this context (see Discussion).

miR-103/107 Promote Epithelial-to-Mesenchymal Transition

We next wished to understand the nature of aggressive cell behaviors leading to metastatic dissemination empowered by the miR-103/107-Dicer axis. We found that miR-107 expression did not significantly affect cell proliferation, growth after serum starvation, resistance to apoptotic stimuli, and anoikis in immortalized mammary cell lines (MCF10A, NMuMG) or tumor cell lines (SUM149, MDA-MB231) (data not shown). This is in line with our previous measurements of the growth rates of cancer cells in vitro and in vivo upon gain or loss of miR-103/107 (Figure 4C, Figure 5A, and Figures S4B, S4D, and S4E).

In contrast, our attention was attracted by a striking change in cellular shape promoted by overexpression of miR-107, whereby the cobblestone-like appearance of epithelial cells switched to a spindle-, fibroblast-like morphology with extensive cellular scattering and formation of lamellipodia (Figures 6A and 6B). These are hallmarks of epithelial-to-mesenchymal transitions (EMT). EMT is a pivotal cellular program to induce rapid changes in the shape and motility of epithelial cells, normally used during morphogenesis and tissue repair. EMT is also aberrantly activated in cancer cells to promote their malignant and stem cell characteristics (Polyak and Weinberg, 2009). The aggressive traits conferred by the EMT program to carcinoma cells presented clear similarities with those empowered by miR-103/107: both are clinically associated in breast cancer with poor clinical outcome and are functionally required for migration, invasion, and metastasis (Polyak and Weinberg, 2009).

To determine if the molecular alterations typical of an EMT occurred in miR-107-expressing cells, we examined the localization of adherent and tight junction markers, such as E-Cadherin andZO-1 in NMuMG cells, a well-established model system for the study of EMT (Miettinen et al., 1994). Immunofluorescence showed that these proteins were strongly downregulated in cells
expressing miR-107 (Figure 6B). EMT was also validated by gene expression analysis: in the presence of miR-107, expression of E-Cadherin mRNA was downregulated whereas the mesenchymal markers vimentin, ICAM-1, and fibronectin mRNAs were significantly increased (Figures 6C–6F). In agreement with the role of Dicer downregulation as mediator of miR-107 effects, lowering Dicer levels by shRNA similarly caused reduction of E-Cadherin and induction of vimentin. Thus, the ability to induce EMT parallels with the previously shown induction of cell motility by miR-107 or Dicer downregulation (see Figure S4F).

We next asked if modulating the levels of miR-103/107 altered the mesenchymal traits of metastatic cells. For this, we monitored the effects of gain and loss of miR-103/107 in MDA-MB-231. Gain of miR-107 massively induced expression of fibronectin, vimentin, and ICAM (Figure 6G), whereas antagonomiR-mediated depletion of endogenous miR-103/107 reduced expression of the same genes (Figure 6H).

In line with such an endogenous role of miR-103/107 in EMT, we found that high versus low levels of pri-miR-103 are associated with mesenchymal versus epithelial phenotypes in a panel of breast cancer cell lines previously stratified according to expression profiles and metastatic capacity (Charafe-Jauffret et al., 2009) (Figure 6I).

Taken together, the data indicate that expression of miR-103/107 is sufficient for inducing epithelial plasticity and required for maintenance of mesenchymal gene expression.
miR-103/107 Control Mesenchymal Traits by Regulating the Expression of the miR-200 Family of miRNAs

We next wished to define the identity of key miRNAs acting as downstream mediators of the miR-103/107-Dicer axis. We focused on the miR-200 family (Figure 7A) because previous studies showed that these miRNAs display properties opposite to those of miR-103/107: they are required to suppress EMT and migration while their attenuation unleashes mesenchymal gene expression (Inui et al., 2010; Polyak and Weinberg, 2009).

Figure 6. miR-103/107 Induce Epithelial Plasticity

(A) Morphology of MCF10A cells transiently transfected with mature miR-107 or the control miRNA (miR-107-MUT). Note the loss of cell-cell adhesion and acquisition of spindle morphology in cells expressing miR-107.

(B) NMuMG cells were transiently transfected with control miRNA (miR-107-MUT) or miR-107 and, after 3 days, analyzed for epithelial characters. Panels show the bright-field morphology of transfected cells (upper panels) and the immunofluorescence for the adherent junction marker E-Cadherin (green, middle panels) or for the tight junction marker ZO-1 (green, lower panels). Nuclei are stained in blue with DAPI.

(C-F) Expression of the epithelial marker E-cadherin (D) and of the mesenchymal markers vimentin (C), fibronectin (E), and ICAM-1 (F) was examined by qRT-PCR in NMuMG cells. Graphs show relative expression levels, normalized to GAPDH. Stable expression of pri-miR-107 by retroviral transduction, transient transfection of mature miR-107, or shRNA knockdown of Dicer (shDicer) upregulates mesenchymal while inhibiting epithelial markers. miR-107-MUT and shGFP are negative controls. TGF-beta treatment (TGFβ1 200 pM for 3 days) serves as positive control for EMT induction. Data are shown as mean and SD.

(G) Transient transfection of mature miR-107 increases the expression of the mesenchymal markers vimentin, fibronectin, and ICAM-1 in MDA-MB-231 cells, as quantified by qRT-PCR. Expression values were given as relative to those of miR-107-MUT-treated cells. Data are shown as mean and SD.

(H) Pri-miR-103/107 are required to support mesenchymal gene expression in MDA-MB-231 cells. Cells were treated for 5 days with antagomiR-MUT or antagomiR-103/107 and analyzed for mesenchymal markers by qRT-PCR. For each marker, expression values were given as relative to those of antagomiR-MUT-treated cells. Data are shown as mean and SD.

(i) Pri-miR-103 expression correlates with mesenchymal traits in a panel of breast cancer cell lines. Heatmap depicts the relative changes of standardized expression values of E-cadherin, vimentin, and pri-miR-103 for each cell line. Blue indicates low expression whereas yellow indicates high expression.

If members of the miR-200 family are functionally relevant downstream of miR-103/107, then miR-200 should oppose miR-107. Confirming this hypothesis, transfection of miR-200b in NMuMG cells reverts the EMT induced by miR-107, as assayed by morphology and gene expression (Figures 7B and 7C). Migration of MDA-MB-231 cells is inhibited by antagomiR-103/107 but, remarkably, this has no effect in miR-200-depleted cells (by means of antagomiR-200, targeting the whole miR-200 family) (Figure 7D).

Biochemically, mature miR-200 levels were increased by antagomiR-103/107 (Figure 7E). To confirm that this extent of miR-200 upregulation was biologically effective, we monitored the miR-200 targets ZEB1 and ZEB2. We found that these genes were downregulated (Figure 7F) in antagomiR-103/107-treated cells to about 50%, mimicking the effect of mature miR-200 overexpression (see Figure 7I). In agreement, we found that...
Figure 7. The miR-200 Family Members Are Inhibited by miR-103/107 to Promote Mesenchymal Traits

(A) A model for the miR-103/107-Dicer-miR-200 pathway in EMT control.

(B and C) Transfection of miR-200b reverts the EMT induced by pri-miR-107 in NMuMG, as assayed by cell morphology (B) and by qRT-PCR for the expression of epithelial (E-cadherin) or mesenchymal markers (vimentin, ZEB1, and ZEB2) (C). Graphs show relative expression levels.

(D) Transwell migration assays of MDA-MB-231 cells treated with antagoniR-103/107, alone or in combination with a mix of antagoniRs targeting the entire miR-200 family (antagoniR-200). AntagoniR-MUT serves as negative control. Depletion of miR-103/107 inhibits MDA-MB-231 cell migration but has no effect in cells depleted of the miR-200 family. See Figure S6A for controls of antagoniR-200 effects.

(E–H) miR-103/107 regulate the expression and activity of the miR-200 family.

(E) Expression of mature miR-200 family members (miR-429, miR-200b, and miR-200c) in MDA-MB-231 cells treated as in (D). Graphs show quantification of gene expression by qRT-PCR. For each marker, expression values were given as relative to those of antagoniR-MUT-treated cells.

(F) qRT-PCR analysis for the expression of the miR-200 direct targets ZEB1 and ZEB2 in MDA-MB-231 cells treated with antagoniR-MUT or antagoniR-103/107.

(G and H) Panels show qRT-PCR for mature miR-200 family members (G) and for their targets ZEB1 and ZEB2 (H) in MDA-MB-231 cells transiently transfected with miR-107-MUT or miR-107. Coexpression of a miR-insensitive form of Dicer transgene opposes the effects of miR-107. See Figure S6B for pre-miRNA levels upon miR-107 transfection.

(I and J) Forced expression of miR-200b inhibits the effects of gain of mir-107 on gene expression and cell migration. MDA-MB-231 cells were transiently transfected with the indicated combinations of miRNAs and assayed for ZEB2 expression by qRT-PCR (I) or for cell migration by transwell assay (J).

Data are shown as mean and SD. See also Figure S6.
overexpression of miR-107 downregulates miR-200 and upregulates ZEB1 and ZEB2 mRNA levels (Figures 7G and 7H). Importantly, these effects are potently rescued by adding-back Dicer (Figures 7G and 7H). Crucially, expression of miR-200 blocks the phenotypic effects of miR-107, as assayed by expression of ZEB2 and cell migration (Figures 7I and 7J), indicating that inhibition of miR-200 is critical for maintenance of mesenchymal and motile properties by the miR-103/107-Dicer axis.

DISCUSSION

The miR-103/107-Dicer Connection in Metastasis

The findings here presented provide evidence that cancer cells use global downregulation of the miRNA network to induce epithelial plasticity and foster invasive and metastatic behaviors. In breast cancer, a microRNA targeting Dicer, miR-103/107, plays a causal role in these events.

These data contribute to an unsettled issue in cancer biology. Genetically, as revealed by knockout of Dicer or Drosha in mice, miRNA biosynthesis is essential for basic cellular functions, such as stemness, cell-cycle progression, and mitosis (Bernstein et al., 2003; Fukagawa et al., 2004; Tang et al., 2007); these processes are ostensibly essential at all stages of tumorigenesis. Despite this, global downregulation of miRNA expression and processing appears a widespread phenomenon in cancer (Lu et al., 2005; Ozen et al., 2008; Ventura and Jacks, 2009). These observations beg the question of how cancer cells can seamlessly reconcile to lose miRNA activity without tackling cellular basal functions.

This study unveils a means by which breast cancer cells solve this dilemma. We find that in metastatic cells, high levels of miR-103/107 attenuate Dicer expression: this empowers invasive and metastatic properties without major impact on primary tumor growth. Thus, it appears that distinct cellular functions are differentially sensitive to Dicer fluctuations. miR-103/107 keep Dicer below a threshold required for metastasis protection. Conversely, the miRNA network sustaining tumor growth operates safely at lower Dicer levels (Kumar et al., 2009). An appeal of this system is its embedded robustness: miR-103/107 are both generated by, and regulators of, Dicer; this mutual feedback relationship allows scale-down of Dicer levels but is also intrinsically incompatible with complete depletion, maintaining sufficient Dicer for growth control and, likely, other cellular functions.

miR-103/107 and Epithelial Plasticity

Our clinical validation studies reveal that high levels of miR-103/107 earmark primary breast tumors with metastatic capacity. By facilitating the acquisition of a more plastic epithelial state, the miR-103/107-Dicer axis may assist early tumor dissemination, preceding or conspiiring with other genetic lesions that complete neoplastic conversion or endow distant colonization. Moreover, miRNAs have been envisioned as key players in “robustness loops” that prevent aberrant/ectopic gene expression, in so doing stabilizing cell identity and masking expression of genetic variation (Hornstein and Shomron, 2006; Inui et al., 2010). Thus, escaping miRNA control in cancer cells, as attained upon Dicer downregulation, may allow the phenotypic emergence of more aggressive genetic variants, accelerating cancer progression.

A significant finding was indeed the association of the miR-103/107-Dicer pair with EMT. In normal tissues, the EMT program is used to assist epithelial plasticity whereas it is exploited opportunistically in cancer to habilitate metastasis (Polyak and Weinberg, 2009). Thus, the ability of miR-103/107 to turn on this program may well represent a leading mechanism by which miR-103/107 foster breast cancer metastasis.

It is worth discussing why only EMT becomes manifest after the general downregulation of miRNAs induced by the miR-103/107-Dicer axis. For several biological processes, downregulation of miRNAs might not reveal immediate phenotypic consequences in virtue of the “balancing effect” between miRNAs favoring and opposing the same process, keeping the system in equilibrium; for example, increase of miR-103/107 downregulates miRNAs playing both positive (i.e., miR-17–92 cluster or miR-21) and negative (i.e., let-7 family) effects on proliferation (Ventura and Jacks, 2009). In contrast, the gene network controlling the maintenance of the epithelial phenotype appears mainly under positive control by miRNAs, as attested by multiple miRNAs inhibiting EMT (miR-200a, miR-200b, miR-200c, miR-141, miR-429, miR-205, and miR-125a) (Inui et al., 2010; Polyak and Weinberg, 2009). Our data suggest that this imbalance is exploited in breast cancer to favor acquisition of mesenchymal traits. Notably, leading targets of the miR-200 family are ZEB1 and ZEB2, pivotal genes for mesenchymal traits (Burk et al., 2008; Liu et al., 2008).

Additionally, the EMT bias can be also explained by the fact that different miRNAs may display differential sensitivity to the changes in levels of cognate miRNAs imposed by miR-103/107. From this perspective, it’s worth noticing that miR-200 genes are transcriptionally repressed by ZEB1. This configures an unusual double-negative circuit that magnifies fluctuations in its components, favoring a “switch-like” transition between two alternative states (i.e., epithelial versus fully mesenchymal) (Inui et al., 2010). Indeed, we found that levels of ZEB1/ZEB2 are controlled by miR-107 in a miR-200-dependent manner (Figures 7F and 7I). As comparison, mature miR-15/16 and let-7 family members and miR-17–92 cluster are downregulated similarly to miR-200 by miR-107, but the steady-state levels of some of their key targets (BCL2, KRas, and cMyc, respectively) are not affected (Figure S6C). Clearly, our focus on EMT does not exclude that miR-103/107 may regulate other biological processes regulated by Dicer and miRNAs in metastatic cancer cells or other cellular contexts.

Clinical Implications

In human primary breast tumors, we validated expression of miR-103/107, but not Dicer mRNA, as prognostic marker. This finding is consistent with miR-103/107 targeting Dicer translation; this endows miR-103/107 with a better patient stratification capacity than Dicer transcripts. That said, tumors may regulate Dicer by other means, including genetic inactivation; indeed low levels of Dicer are associated with poor survival in a fraction of lung and ovarian cancer patients and animal models (Kumar et al., 2009; Karube et al., 2005; Merritt et al., 2008). Here we found that, similarly to elevated miR-103/107, heterozygous
loss of Dicer also instills metastatic propensity in breast cancer patients. Likely, this result is not apparent from the analysis of Dicer miRNA levels because this genetic lesion occurs in a relatively minor fraction of patients, complicating in large datasets the assignments of reliable cutoff values for patients' correlations. It is tempting to speculate that the use of a miR-103/107 may be preferred over genetic deletion because it may represent a reversible and dynamic means to attenuate Dicer protein levels, perhaps corresponding to a physiological process requiring transient empowering of cell motility (i.e., during wound-healing or neural crest biology).

Finally, our findings have some implications for treatment of breast cancer. The positive effects of antagomiR-103/107 in our experimental models of metastasis at least suggest that modulation of miR-103/107 by RNA-based therapeutics may prove clinically useful.

**EXPERIMENTAL PROCEDURES**

**Biological Assays in Mammalian Cells**

For Transfection procedures and luciferase assays, see the Extended Experimental Procedures and Martello et al., 2007. For wound-healing experiments, cells were plated in 6-well plates, transfected as indicated, and cultured to confluency. Cells were serum-starved and scraped with a P200 tip (time 0), and the number of migrating cells were counted from pictures (five fields) taken at the indicated time points.

Transwell migration/invasion assays were performed in 24-well PET inserts (Costar 8.0 mm pore size). Cells were plated and transfected with miRNA as indicated. The day after, 100,000 cells were plated in serum-free media in transwell inserts (at least three replicates for each sample). Medium containing 1% FBS served as chemotacticant in the lower chamber. Cells in the upper part of the transwell were removed with a cotton swab; migrated cells were fixed in 4% PFA and stained with 0.5% Crystal Violet. Filters were photographed and the total number of cells counted. Each experiment was repeated at least three times independently.

For Dicer knockdown the sequences of the siRNA were: 5'-UCC AGA GCU GCU UCA AGC ATT-3' and 5'-UGC UUG AAG CAG CUC UGG ATT-3'. As for mature miRNAs: miR-107; 5'-AGC AGC AUU GUA CAG GGC UAU CA-3' and 5'-AUA GCC CUG UAC AUA AUU GUA CAG GGC UAU CA-3'; miR-107: 5'-AUA GCC CUG UAC AUA AUU GUA CAG GGC UAU CA-3'; miR-200b: 5'-AUC ACC AGG CAG GAU AAG A-3' and 5'-UAU UAC UC GCG UGA AUG AUG A-3'.

**Experimental Models of Metastasis and AntagomiR-Treatment**

Mice were housed in Specific Pathogen Free (SPF) animal facilities and treated in conformity with institutional guidelines. For xenograft studies of breast cancer metastasis, cells (500,000 cells/mouse for 4T1 cells, 1,000,000 cells/mouse for SUM149 cells) were unilaterally injected into the mammary fat pad, or in the tail vein, of SCID female mice, age-matched between 5 and 7 weeks. After the indicated periods, mice were sacrificed and their lungs explanted for histological analyses.

AntagomiRs were designed as described (Krutaizfeld et al., 2005) and purchased from Fidelity System. Sequences were “AntagomiR-103/107”: 5'-UUC AUU AGCCCUGUAAUUGCUCC’U’-Chol-3’; “AntagomiR-MUT”: 5’-UU’CAUAACCCUGUAAGAcAaUaCeTaU’-Chol-3’. “AntagomiR-200a”: 5’-G-A’ACACCGTGATT’A-G’-Chol-3’; “AntagomiR-200b”: 5’-U-A’CCCTAATCCGCCGACATT’A-G-Chol-3’; all the bases are 2’OMe modified, ‘ representing a phosphorothioate linkage, and ‘Chol’ represents linked cholesterol tail. For AntagomiR-200 we used a 1:1 mixture of the two oligos.

4T1 cells, after 4 days of AntagomiR treatment, were orthotopically injected in SCID mice (500,000 cells/mouse). After 3 days, 100 μl of AntagomiR-103/107 or control AntagomiR-MUT solutions (diluted in PBS at 2 mg/ml) were injected intratumorally three times per week for 2 weeks.