A microRNA DNA methylation signature for human cancer metastasis

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MicroRNAs (miRNAs) are small, noncoding RNAs that can contribute to cancer development and progression by acting as oncogenes or tumor suppressor genes. Recent studies have also linked different sets of miRNAs to metastasis through either the promotion or suppression of this malignant process. Interestingly, epigenetic silencing of miRNAs with tumor suppressor features by CpG island hypermethylation is also emerging as a common hallmark of human tumors. Thus, we wondered whether there was a miRNA hypermethylation profile characteristic of human metastasis. We used a pharmacological and genomic approach to reveal this aberrant epigenetic silencing program by treating lymph node metastatic cancer cells with a DNA demethylating agent followed by hybridization to an expression microarray. Among the miRNAs that were reactivated upon drug treatment, miR-148a, miR-34b/c, and miR-9 were found to undergo specific hypermethylation-associated silencing in cancer cells compared with normal tissues. The reintroduction of miR-148a and miR-34b/c in cancer cells with epigenetic inactivation inhibited their motility, reduced tumor growth, and inhibited metastasis formation in xenograft models, with an associated down-regulation of the miRNA oncogenic target genes, such as C-MYC, E2F3, CDK6, and TGIF2. Most important, the involvement of miR-148a, miR-34b/c, and miR-9 hypermethylation in metastasis formation was also suggested in human primary malignancies (n = 207) because it was significantly associated with the appearance of lymph node metastasis. Our findings indicate that DNA methylation-associated silencing of tumor suppressor miRNAs contributes to the development of human cancer metastasis.

MicroRNAs (miRNAs) are small, noncoding RNAs, ≈22 nucleotides long, that repress gene expression in a variety of eukaryotic organisms (1, 2). In animals, these single-stranded RNAs interact with specific target mRNAs through an almost perfect complementarity with sequences located in the 3′ UTR, where they induce mRNA degradation or translational inhibition (1, 2). miRNAs play important roles in several cellular processes, such as proliferation, differentiation, apoptosis, and development, by simultaneously controlling the expression levels of hundreds of genes (1, 2). In human cancer, recent studies have shown that miRNA expression profiles differ between normal tissues and derived tumors and between tumor types (3, 4). miRNAs can act as oncogenes or tumor suppressors, exerting a key function in tumorigenesis (5, 6). Recently, a new function mediating tumor metastasis in breast cancer has been assigned to miRNAs, by which this malignant step is promoted (7, 8) or suppressed (9). However, the precise contribution of miRNAs to human metastasis and the mechanism underlying their dysregulation remain largely unexplored.

The metastatic process consists of sequential, interrelated steps by which primary tumor cells acquire the capacity to invade the adjacent tissue, enter the systemic circulation, translocate through the vasculature, arrest in distant capillaries, extravasate into the surrounding tissue parenchyma, and, finally, proliferate from micrometastases into macroscopic secondary tumors (10, 11). The lymphatic route of metastasis is particularly relevant for carcinomas, where regional lymph nodes are often the first organs to develop metastasis and might serve as a potential "bridgehead" in further dissemination (12, 13). Metastasis is responsible for 90% of deaths in patients with solid tumors (10, 11), so understanding its pathogenesis at the systemic, cellular, and molecular levels has become one of the most ambitious goals in cancer research (10, 11). The discovery of the contribution of miRNAs to metastasis has given us great insight into the process. On one hand, three metastasis-promoting miRNAs have been described (7, 8): miR-10b promotes cell migration and invasion through homeobox D10 inhibition (7), whereas miR-373 and miR-520c cooperate in metastasis by suppressing CD44 (8). On the other hand, miRNAs like miR-126 and miR-335 (9) can act as metastasis suppressors, the latter by targeting oncogene SOX4 and tenascin C (9).

However, one question remains largely unanswered: what are the defects leading to the imbalance of miRNAs in cancer cells? Genetic alterations (14, 15) and failure of posttranscriptional regulation might cause the dysregulation of subsets of miRNAs, but epigenetic alterations also appear to be likely culprits (16, 17). In classical tumor suppressor genes, promoter CpG island hypermethylation occurs in genes involved in cell adherence, invasion, and angiogenesis, such as cadherins, tissue inhibitors of metalloproteinases, and thrombospondins (18). For miRNAs, we and others have recently identified the presence of CpG island methylation-associated silencing of miRNAs with tumor suppressor features in human cancer (19–22), such as miR-127 and miR-124a. Thus, aberrant hypermethylation events in the regulatory regions of miRNAs

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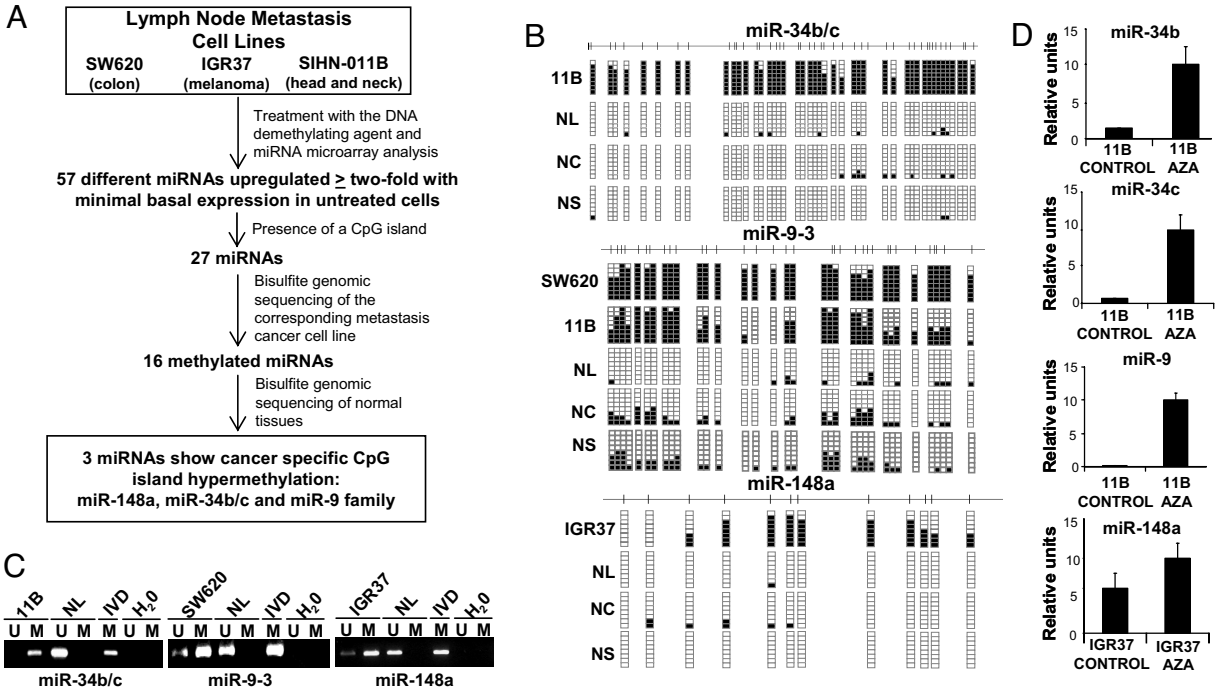

Fig. 1. Pharmacological epigenomic unmasking of cancer-specific hypermethylated miRNAs in metastasis cancer cells. (*A*) Schematic strategy used to identify DNA methylation-associated repression of miRNAs in metastatic cancer cell lines. (*B*) Bisulfite genomic sequencing analyses of the miR-34b/c cluster, miR-9-3, and miR-148a CpG islands in normal tissues and metastatic cell lines. Eight single clones are represented for each sample. The CpG island is depicted, and each vertical bar illustrates a single CpG. Black and white squares represent methylated and unmethylated CpG, respectively. NL, normal lymphocytes; NC, normal colon; NS, normal skin. These miRNAs show cancer-specific CpG island hypermethylation. (*C*) Methylation-specific PCR analyses of miR-148a, miR-34b/c, and miR-9-3. Normal lymphocytes (NL) and *in vitro*-methylated DNA (IVD) are shown as positive controls for the unmethylated and methylated sequences, respectively. (*D*) Expression analyses of mature miRNAs by qRT-PCR in methylated metastatic cell lines in untreated cells and upon treatment with the DNA demethylating agent 5-aza-2′-deoxycytidine (AZA). The use of the DNA methylation inhibitor restores miRNA expression.

might also play a role in the establishment of human metastasis. Herein we present a pharmacological and genomic unmasking of hypermethylated miRNAs, such as miR-148a, miR-34b/c, and miR-9, that contribute *in vitro* and *in vivo* to the formation of lymph node metastasis, one of the first steps in the dissemination of human tumors.

Results and Discussion

Identification of miRNAs Undergoing Transcriptional Silencing in Metastatic Cancer Cells by miRNA Expression Microarray Analysis upon DNA-Demethylating Drug Treatment. To identify miRNAs with putative DNA methylation-related inactivation involved in metastasis, we followed the experimental algorithm shown in Fig. 1*A*. First, we treated three representative human cancer cell lines established from lymph node metastasis from colon (SW620), melanoma (IGR37), and head and neck (SIHN-011B) with the DNA methylation inhibitor 5-aza-2′-deoxycytidine. To measure miRNA levels, total RNA was extracted before and after treatment with the DNA demethylating drug in each case and profiled by using our previously validated miRNA platform (23). Unsupervised principal component analysis of normalized and filtered datasets showed clear separation between various classes (SW620, SIHN-011B, and IGR37 untreated and treated cell lines), showing high similarity among samples that belong to the same group. Our pharmacological screening revealed that, considering all three metastatic cancer cell lines, 57 of 389 (15%) different human miRNAs printed on the microarray had minimal basal expression in the untreated cells and ≥2-fold up-regulation upon use of 5-aza-2′-deoxycytidine [supporting information (SI) Table S1 and Table S2]. Of these 57 significantly up-regulated miRNAs, 27 (47%) were embedded in a canonical CpG island (Table S2). miR-126 and miR-126*, which originated from the same genomic locus, were the only two miRNAs commonly up-regulated upon administering the DNA methylation inhibitor in SW620, IGR37, and SIHN-011B cells. Interestingly, miR-126 is one of the first miRNAs for which a role as a metastasis suppressor has been proposed (9).

CpG Island DNA Methylation Analysis and Expression Studies of Candidate Metastasis Suppressor miRNAs. To demonstrate the presence of CpG island methylation in these 27 up-regulated miRNAs, we undertook bisulfite genomic sequencing analyses of multiple clones in the metastatic cancer cell from which the miRNA expression microarray data were obtained. We found dense CpG island hypermethylation in 16 (59%) of the described miRNAs (Fig. 1*A* and Table S2). We wanted to focus on the cancer-specific DNA methylation changes, so we used bisulfite genomic sequencing to analyze the DNA methylation status of these 16 miRNAs in normal tissues ($n = 32$), including colorectal mucosa, lymphocytes, and skin, to exclude tissue-specific DNA methylation patterns. The expression of many miRNAs is tightly regulated according to cell type (1, 2), so it was not surprising to observe that 11 (65%) of the miRNAs were also densely methylated in normal tissues (Fig. 1*A*, Table S2, and Fig. S1). The previously mentioned miR-126 was one of these. However, and most importantly, miR148a, miR-34b/c, miR-9-1, miR-9-2, and miR-9-3 were always unmethylated in all normal tissues studied (Fig. 1 *A* and *B*, Table S2, and Fig. S2). The DNA methylation results were also confirmed by using methylation-specific PCR (Fig. 1*C*). Thus, the CpG island hypermethylation of these miRNAs was cancer-specific.

To demonstrate the transcriptional silencing of these miRNAs in metastatic cancer cells in association with the presence of CpG

island hypermethylation, we measured mature miRNA levels by quantitative RT-PCR (qRT-PCR). The expression of miR148a, miR-34b, miR-34c, and miR-9 transcripts was not detectable in those cells showing CpG island methylation of the corresponding miRNA (Fig. 1*D*). Most important, a restoration of miRNA expression was observed upon treatment with the DNA demethylating agent (Fig. 1*D*). Thus, our data obtained from the miRNA microarray and the DNA methylation assays suggested that miR148a, miR-34b, miR-34c, and miR-9 transcripts were induced from their own CpG island-associated promoter after treatment with the DNA methylation inhibitor. These results were confirmed by the determination of the potential transcription start sites for the primary transcripts of these miRNAs after 5-aza-2′-deoxycytidine treatment using 5′ RACE (24) in the corresponding metastatic cell lines. We were able to identify a start site for each miRNA transcript (miR-9-1, miR-9-2, miR-9-3, miR-148, and miR-134b/c) within the described corresponding CpG island (Fig. S3). Interestingly, miR-9-3 and miR34b/c might also be part of the larger transcripts CR612213 and BC021736, respectively (http://genome.ucsc.edu/cgi-bin/hgGateway) (25), but those start sites are located in CpG poor sequences that were mostly unmethylated in both normal and transformed cells (Fig. S4). In the case of miR-34b/c, a recent report shows that a promoter region for this miRNA overlaps with the described CpG island and that treatment with a DNA demethylating agent induces the expression of miR-34b/c but not of BC021736 (26).

Most important, we analyzed alterations in chromatin structure in the CpG islands where we mapped the putative transcription start sites of miR-9-1, miR-9-2, miR-9-3, miR-148, and miR-34b/c by chromatin immunoprecipitation. We observed that in the corresponding metastatic cancer cell lines the five described hypermethylated miRNA CpG islands were depleted in histone marks associated with open chromatin structure and active transcription, such as acetylated histone H4 and trimethylated lysine-4 of histone H3 (Fig. S5). Remarkably, these CpG islands showed a pronounced increase in both active marks upon treatment with the DNA demethylating agent (Fig. S5). Identical chromatin setting has been described for the hypermethylated CpG islands of miR-127 and miR-34b/c (19, 26). Treatment with an inhibitor of histone deacetylases (trichostatin A) as a single agent did not achieve restoration of miRNA expression or miRNA CpG island DNA hypomethylation (Fig. S6).

Hypermethylated miRNAs Show Metastasis Tumor Suppressor Features *in Vitro* and *in Vivo*. Once we had observed the specific CpG island hypermethylation-associated silencing of miR-148a, miR-34b/c, miR-9-1, miR-9-2, and miR-9-3 in metastatic cancer cells, we sought to demonstrate that the epigenetic inactivation of these miRNAs contributed to metastasis formation. We took two approaches: *in vitro* and *in vivo*. For the former, we stably transfected the metastatic carcinoma cell line SIHN-011B, hypermethylated and silenced for miR-148a and miR-34b/c, with expression vectors containing the flanking regions of the mature miR-148a or miR-34b/c. The efficiency of the transfection was tested by measuring the mature miRNA levels by qRT-PCR (data not shown). We then measured the migration capability of the transfected control short hairpin RNA (c-shRNA) vector (Ambion) compared with the miRNA transfected metastatic SIHN-011B cells using the wound-healing assay (27). miR-148a and miR-34b/c transfected SIHN-011B cells had a significantly lower capability of migration than did c-shRNA vector cells ($P = 0.0001$ in both cases) (Fig. 2*A*). For the *in vivo* approach, we used tumor and metastasis formation assays in nude mice. Three million SIHN-011B cells transfected with the c-shRNA vector, miR-148a, or miR-34b/c were s.c. injected into nude mice, and the tumor progression was studied over time. Both miRNA transfected cell lines showed less tumor growth than seen with

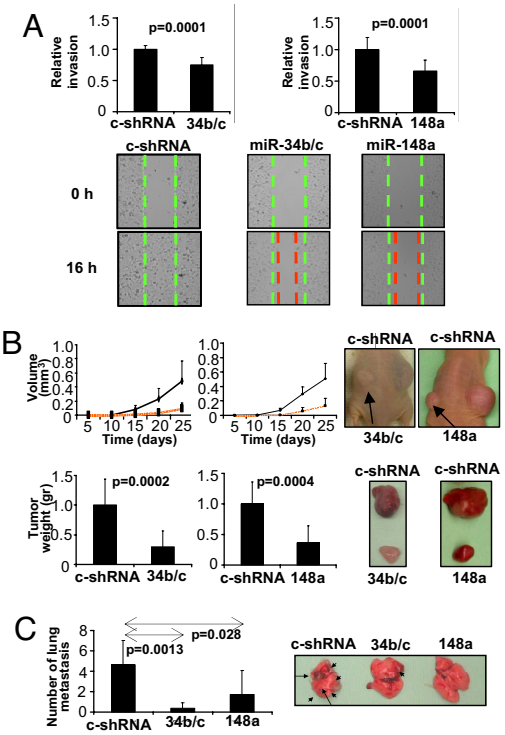

Fig. 2. miR-148a and miR-34b/c suppress tumor invasion and dissemination *in vitro* and *in vivo*. (*A*) Wound-healing assay. (*Upper*) The relative invasion of c-shRNA cells compared with miRNA transfected cells after 16 h. (*Lower*) Illustrative examples of the wound-healing assay in the c-shRNA and miRNA transfected metastatic cell lines at 0 h and 16 h. Green and red lines represent the initial and final leading edges of the invasion front, respectively. miR-148a and miR-34b/c transfected cells show minor invasion capability. (*B*) Effects of miRNA transfection on *in vivo* growth of metastatic SIHN-011B cells xenografted into nude mice. (*Left*) Tumor volume was monitored over time, and tumor weight was measured at the end of the experiment. (*Right*) Representative large tumors on the right flank (c-shRNA cells) and the small tumor on the opposite flank, corresponding to miR-34b/c or miR-148a transfected cells. Examples of the resected tumors are also shown. (*C*) Metastatic SIHN-011B cancer cells with stable transfection of miR-148a, miR-34b/c, or a control vector were transplanted into athymic nude mice by tail-vein injection. A graphical representation of the number of lung metastases observed and illustrative examples are shown.

c-shRNA vector transfected cells (Fig. 2*B*). Thirty days after injection, all mice were killed and tumor weights were measured. Again, these were significantly lower when arising from miR-transfected cells than in the tumors originating from c-shRNA vector cells (Fig. 2*B*). Most importantly, the inhibitory migration and invasion activities of miR-148a and miR-34b/c were also measured in athymic mice via tail-vein injection. Whereas metastatic nodules developed in the lung over a period of 6–8 weeks after injection in the c-shRNA vector transfected SIHN-011B cells (Fig. 2*C*), the metastasis events were not observed with miR-148a or miR-34b/c transfected SIHN-011B cells in the same period (Fig. 2*C*). These findings are evidence of the role of miR-148a and miR-34b/c as suppressors of tumor dissemination.

Our data also corroborate the findings of recent studies showing that miR-34 activation recapitulates tumor suppressor events associated with p53 activity (28, 29) and that the down-regulation of miR-148a, miR-9-2, and miR-34 is part of a unique 20-miRNA metastasis signature in liver tumors (30). The mechanisms underlying the inactivation of these miRNAs in human tumors were mostly unknown, and our results now indicate that CpG island hypermethylation is a likely molecular lesion responsible for these alterations.

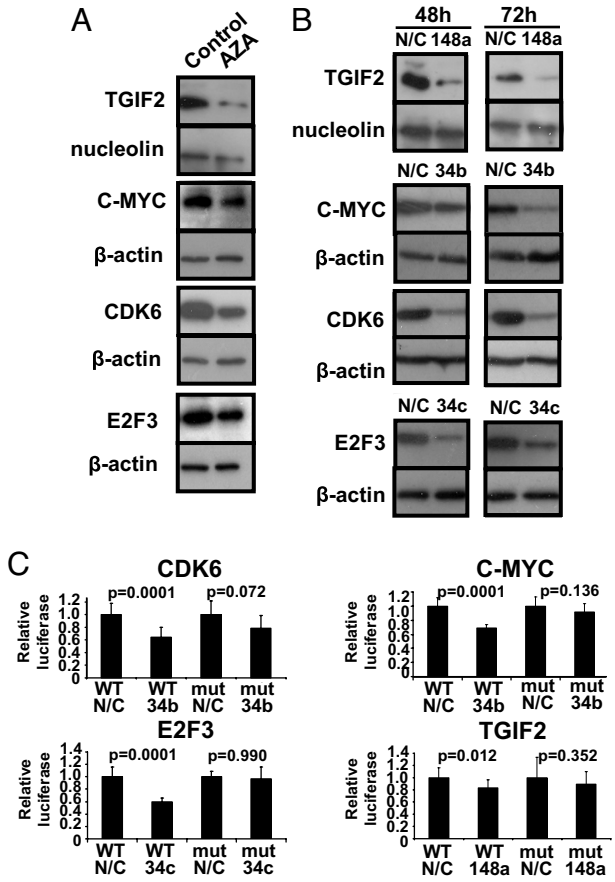

Fig. 3. miR-148a and miR-34b/c target and down-regulate cancer progression and metastasis-related target genes. (*A*) Protein expression analysis by Western blot for TGIF2, C-MYC, CDK6, and E2F3 in untreated and 5-aza-2′-deoxycytidine-treated SIHN-011B cells. The DNA demethylating agent induces down-regulation of the oncoproteins. (*B*) Transfection of metastatic SIHN-011B cells with the miR-148a, miR-34b, and miR-34c precursor molecules also causes a reduction in the protein levels measured. (*C*) Interaction between the miRNAs and the 3′ UTR of the target genes. Luciferase assays of SIHN-011B cancer cells, transfected with firefly luciferase constructs containing WT or mutant (MUT) target sites, and cotransfected with negative control (N/C) or the corresponding precursor of miRNA (miR-148a, miR-34b, or miR-34c) are shown.

miR-148a and miR-34b/c Epigenetic Silencing Mediates the Activation of Oncogenic and Metastasis Target Genes. To determine whether the epigenetic silencing of miR-148a and miR-34b/c had functional cancer relevance to overcome their putative metastasis tumor suppressor function, we examined its effect on the regulation of presumed target genes with oncogenic and metastatic capacity. Using computational prediction for miR-34b/c target genes, we observed that C-MYC, CDK6, and E2F3 were excellent potential targets, whereas for miR-148a the TGIF2 gene was one of the best candidates. Several lines of evidence relate the described genes to metastasis potential, such as the finding that metastasis-associated protein 1 (MTA1) is an essential downstream effector of the C-MYC oncoprotein (31), CDK6 is involved in cell-cycle progression and differentiation (32), and TGIF2 is overexpressed and amplified in aggressive ovarian tumors (33). Using Western blot analyses for C-MYC, CDK6, E2F3, and TGIF2, we observed that, whereas the untreated metastatic cancer cell line SIHN-011B with miR-148a and miR-34b/c methylation-associated silencing strongly expressed the four proteins, the cells treated with the DNA demethylating agent were down-regulated (Fig. 3*A*). To further confirm these putative miRNA targets, SIHN-011B cells were transfected with miR-148a and miR-34b/c precursor molecules, which are designed to mimic endogenous miRs. We observed that overexpression of miR-148a induced a reduction of TGIF2 protein levels (Fig. 3*B*). For miR-34b/c, miR-34b transfection decreased CDK6 and C-MYC protein levels, whereas miR-34c transfection induced the down-regulation of E2F3 protein levels (Fig. 3*B*). These latter results relate to the different predicted targeting scores of each miR-34b and miR-34c for the corresponding proteins, as has also been recently described in ovarian cancer cells (34).

Most importantly, a functional link was established by performing a luciferase reporter assay with vectors containing the C-MYC, CDK6, E2F3, and TGIF2 WT putative 3′ UTR target sites and corresponding mutant forms (MUT) (Fig. S7). The luciferase activities of miR-148a and miR-34b/c nonexpressing SIHN-011B cells transfected with the WT and mutant vectors for the four putative target proteins showed no measurable differences (Fig. 3*C*). However, when we cotransfected miR-148a, luciferase activity of SIHN-011B TGIF2-WT-transfected cells was significantly lower than with TGIF2-MUT ($P = 0.012$; Fig. 3*C*). A similar pattern was encountered for miR-34b/c: upon cotransfection of miR-34b, the luciferase activity of C-MYC- and CDK6-WT-transfected cells was significantly lower than that of the mutant forms ($P = 0.0001$; Fig. 3*C*), whereas cotransfection of miR-34c caused a significant reduction of luciferase activity in E2F3-WT-transfected cells in comparison to E2F3-MUT ($P = 0.0001$; Fig. 3*C*). These findings suggest that DNA methylation-associated silencing of miR-148a and miR-34b/c in metastatic cancer cells prevents the down-regulation of the corresponding miR targets, such as the oncoproteins C-MYC, CDK6, E2F3, and TGIF2, and stimulates the progression and dissemination of the tumor.

Hypermethylated miRNAs in Human Primary Malignancies Are Associated with Metastasis Development. The presence of CpG island hypermethylation of miR-148, miR-34b/c, and the miR-9 family was not a specific feature of the cancer cell lines or an *in vitro* phenomenon. We analyzed a comprehensive collection of human primary tumor samples ($n = 278$) from the most common tumor types, such as colon, lung, breast, and head and neck carcinomas and melanomas, and frequently observed hypermethylation in the described miRNA metastasis tumor suppressors (Fig. 4*A* and Table S3). Hypermethylation of the miRNAs was absent from the corresponding normal tissue counterparts ($n = 32$). We subsequently tested whether the observed *in vitro* loss of targeting oncoproteins mediated by miRNA methylation-associated silencing was also observed in human primary tumors. For this purpose, we studied the DNA methylation status of miR34b/c in human lung carcinomas and melanomas ($n = 66$) in relation to the expression of C-MYC and CDK6, two of the miRNA targets we had characterized in cell culture. Remarkably, the immunostaining analyses of C-MYC and CDK6 expression in the primary tumors patients showed that miR-34b/c hypermethylation was associated with strong expression of C-MYC and CDK6 (Pearson's χ^2 test; $P = 0.0001$ and $P = 0.040$, respectively; Table S3). Illustrative examples are shown in Fig. 4*B*. These data indicate that these two oncogenes are also targeted by miR-34b/c *in vivo* and that epigenetic silencing of miR-34b/c leads to their up-regulation in cancer patients.

However, the most compelling data concerning the role of miR-148, miR-34b/c, and miR-9 epigenetic silencing in tumor dissemination from the original location of the malignancy was obtained when the miRNA hypermethylation status was determined with respect to the existence or not of lymph node metastasis in the 207 tumors for which data were available (Table S4). The presence of miR-34b/c, miR-148, and miR-9-3 CpG island hypermethylation in the primary tumor was significantly associated with those tumors that were positive for metastatic cancer cells in the corresponding lymph nodes (Fisher's exact

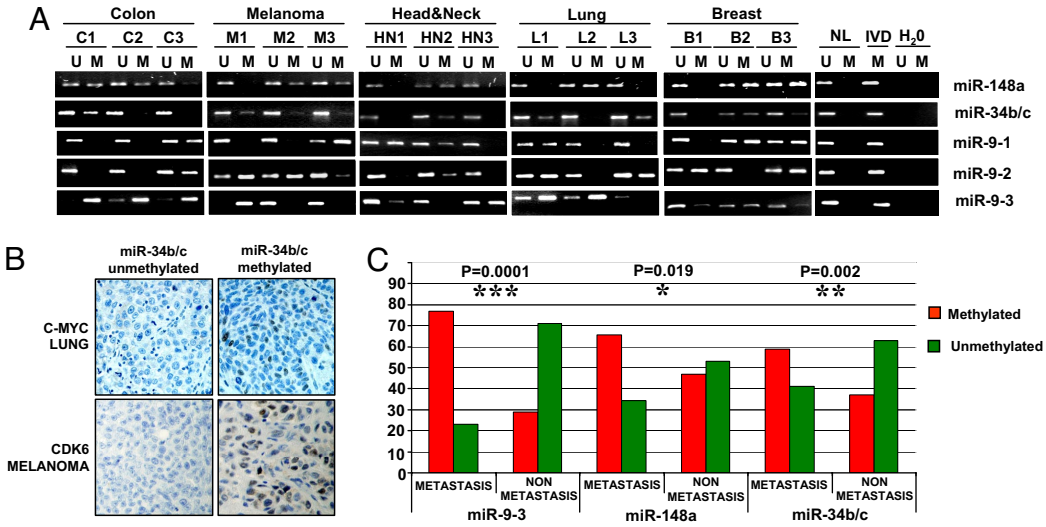

Fig. 4. The role of miRNA hypermethylation in the metastatic behavior of human primary tumors. (*A*) Methylation-specific PCR analyses for miR-148a, miR-34b/c, miR-9-1, miR-9-2, and miR-9-3 in primary human tumors derived from different tissues. The presence of a band under the U or M lanes indicates unmethylated or methylated sequences, respectively. Normal lymphocytes (NL) and *in vitro* methylated DNA (IVD) are shown as positive controls for the unmethylated and methylated sequences, respectively. (*B*) Association between miRNA methylation and up-regulation of their corresponding oncoprotein targets in primary tumors. Illustrative immunohistochemical examples demonstrate that C-MYC and CDK6 overexpression is associated with miR-34b/c hypermethylation in lung tumors and melanomas, respectively. (*C*) Graphical representation of the distribution of miR-9, miR-34b/c, and miR-148a CpG island hypermethylation in primary tumors according to the presence or absence of lymph node metastasis. The presence of hypermethylation of the miRNAs is significantly associated with the existence of metastasis in human malignancies.

test; $P = 0.002$, $P = 0.019$, and $P = 0.0001$, respectively) (Fig. 4*C* and Table S4), which highlights the importance of the *in vivo* role of miRNA epigenetic silencing in metastasis formation.

Our findings not only reinforce the concept that CpG island hypermethylation-mediated silencing of miRNAs with tumor suppressor features contribute to human cancer, but also show that particular DNA methylation signatures of miRNAs are associated with the metastatic behavior of tumors. The demonstration that the epigenetic silencing of miRNAs, and their associated loss of regulation of oncogenic target genes, is one factor that contributes to metastasis formation in cell cultures; xenograft tumor models and primary malignancies might also have important clinical and therapeutic consequences. One application could be the use of miRNA methylation markers to predict tumor prognosis and metastatic behavior. Most importantly of all, it might provide a mechanistic and molecular basis for a new therapeutic use for pharmacological compounds with DNA demethylating activity in the treatment of cancer patients with metastatic disease.

Materials and Methods

Human Metastatic Cancer Cell Lines. Several human cancer cell lines derived from lymph node metastasis were acquired for our study: SW620 (colorectal cancer) (American Type Culture Collection), IGR37 (melanoma) (German Collection of Microorganisms and Cell Culture), and SIHN-011B (head and neck cancer). The cell lines were treated with 1 μM 5-aza-2′-deoxycytidine (Sigma) for 48 h (35). The primary tumor samples ($n = 278$) and corresponding normal tissues ($n = 32$) from various tissue types were obtained under the approval of the respective ethics committees.

RNA Isolation and miRNA Expression Analysis. Total RNA was isolated from SW620, IGR37, and SIHN-011B cells, before and after 5-aza-2′-deoxycytidine treatment, by TRIzol extraction (Invitrogen). miRNA microarray profiling was conducted as described (23). Details are provided in *SI Materials and Methods*.

DNA Methylation Analyses. The miRNA sequences were analyzed by using miRBase (http://microrna.sanger.ac.uk/) and the University of California at Santa Cruz Human Genome Browser (http://genome.cse.ucsc.edu). The CpG Island Searcher Program (36) was used to determine which miRNAs were embedded in a CpG island, because it has been predicted that >90% of the human miRNA promoters are located 1,000 bp upstream of the mature miRNA (37). DNA methylation status was established by PCR analysis of bisulfite-modified genomic DNA, which induces chemical conversion of unmethylated, but not methylated, cytosine to uracil. Details are provided in *SI Materials and Methods*.

Quantification of miRNAs with Real-Time PCR. TaqMan miRNA assays were used to quantify the level of mature miRNAs as described previously (38). Details are provided in *SI Materials and Methods*.

RACE. RACE was developed as previously described (24). Details are provided in *SI Materials and Methods*.

Chromatin Immunoprecipitation Assay. Standard chromatin immunoprecipitation assays were developed as previously described (20). Details are provided in *SI Materials and Methods*.

Stable Transfection of miRNAs. Cells were transfected with pSILENCER/TM 4.1-CMV Expression Vector (Ambion) containing the flanking regions of the mature miRNAs (miR-148a and miR-34b/c). The primers used are described in Table S5. Cells were selected with puromycin 48 h after transfection and then diluted to perform clonal selection. The mature miRNA expression in the selected clones was assessed by qRT-PCR as described above.

Wound-Healing Assay. Transfected cells (miR-148a and miR-34b/c) were grown to confluence and wounded by dragging a 1-ml pipette tip through the monolayer. Cells were washed to remove cellular debris and allowed to migrate for 16 h. Images were taken at 0 and 16 h after wounding under a DMI6000 inverted microscope. The relative surface traveled by the leading edge was assessed by using LAS AF 6000 1.8.0 software. Five replicates each of two independent experiments were performed.

Mouse Xenograft and Metastasis Models. Four- to 5-week-old male athymic *nu/nu* mice (Charles River) were used in this study. Details are provided in *SI Materials and Methods*.

Expression Analyses of miRNA Target Genes by Western Blot and Immunohistochemistry. Information of base pairing comparison among miR-34b, miR-34c, and miR-148a and their targets sites in the 3′ UTR of c-MYC, E2F3, CDK6, and TGIF2 mRNA is available at Targetscan (www.targetscan.org/). Western blot was done as previously described (20). Details are provided in *SI Materials and Methods*.

Transfection with miR-34b, miR-34c, and miR-148a Precursor Molecules. miRNA precursor molecules and negative control miRNA were purchased from Ambion. Experiments involving transient transfections of miRNAs were done using oligofectamine (Invitrogen) and 100 nmol/liter RNA duplexes. The cells were collected 48 h and 72 h after transfection, and the expression of c-MYC, E2F3, CDK6, and TGIF2 was analyzed by Western blot.

Luciferase Reporter Assay. Luciferase constructs were made by ligating oligonucleotides containing the WT or mutant putative target site of the C-MYC, E2F3, CDK6, and TGIF2 3′ UTR into the multicloning site of the p-MIR Reporter Luciferase vector (Ambion). Details are provided in *SI Materials and Methods*.

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