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Coordinated epigenetic repression of the miR-200 family and miR-205 in invasive bladder cancer

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MicroRNAs (miRNA) are small noncoding RNAs commonly deregulated in cancer. The miR-200 family (miR-200a, -200b, -200c, -141 and -429) and miR-205 are frequently silenced in advanced cancer and have been implicated in epithelial to mesenchymal transition (EMT) and tumor invasion by targeting the transcriptional repressors of E-cadherin, *ZEB1* and *ZEB2*. *ZEB1* is also known to repress miR-200c-141 transcription in a negative feedback loop, but otherwise little is known about the transcriptional regulation of the miR-200 family and miR-205. Recently, miR-200 silencing was also reported in cancer stem cells, implying that miR-200 deregulation is a key event in multiple levels of tumor biology. However, what prevents miR-200 expression remains largely unanswered. Here we report concerted transcriptional regulation of the miR-200 and miR-205 loci in bladder tumors and bladder cell lines. Using a combination of miRNA expression arrays, qPCR assays and mass spectrometry DNA methylation analyses, we show that the miR-200 and miR-205 loci are specifically silenced and gain promoter hypermethylation and repressive chromatin marks in muscle invasive bladder tumors and undifferentiated bladder cell lines. Moreover, we report that miR-200c expression is significantly correlated with early stage T1 bladder tumor progression, and propose miR-200 and miR-205 silencing and DNA hypermethylation as possible prognostic markers in bladder cancer. In addition, we observe that the mesoderm transcription factor *TWIST1* and miR-200 expression are inversely correlated in bladder tumor samples and cell lines. *TWIST1* associates directly with the miR-200 and miR-205 promoters, and may act as a repressor of miR-200 and miR-205 expression.

MicroRNAs (miRNA) are a class of ~22nt noncoding RNAs that regulate gene expression post-transcriptionally.[1] It is widely accepted that miRNA expression is broadly altered in most forms of cancer,[2] and loss of miR-200 family (miR-200a, -200b, -200c, -141 and- 429) expression has been reported in several types of advanced carcinoma, including bladder cancer.[3,4] Recently, several independent reports have implicated miR-200 and miR-205 in epithelial to mesenchymal transition (EMT), an important event in tumorigenesis associated with a decrease in E-cadherin levels, loss of cell adhesion and subsequent tumor invasion and metastasis.[5–9] The miR-200s and miR-205 are key determinators of the epithelial phenotype by directly targeting *ZEB1* (also known as *TCF8*) and *ZEB2* (also known as *SIP-1*), which are transcriptional repressors of E-cadherin (*CDH1*). Loss of miR-200 expression thus leads to accumulation of *ZEB1* and *ZEB2*, which is sufficient to silence *CDH1* and promote EMT and tumor invasion.[7]

Interestingly, *ZEB1* has been shown to act as a transcriptional repressor of miR-200c and miR-141 by directly interacting with E- and/or Z-box motifs in the miR-200c-141 promoter through a double-negative feedback mechanism.[8,9] However, the mechanisms regulating miR-200 and miR-205 expression remain otherwise largely uncharacterized. A recent study also concluded that *ZEB1* is necessary for development and maintenance of stemness in cancer cells, thereby

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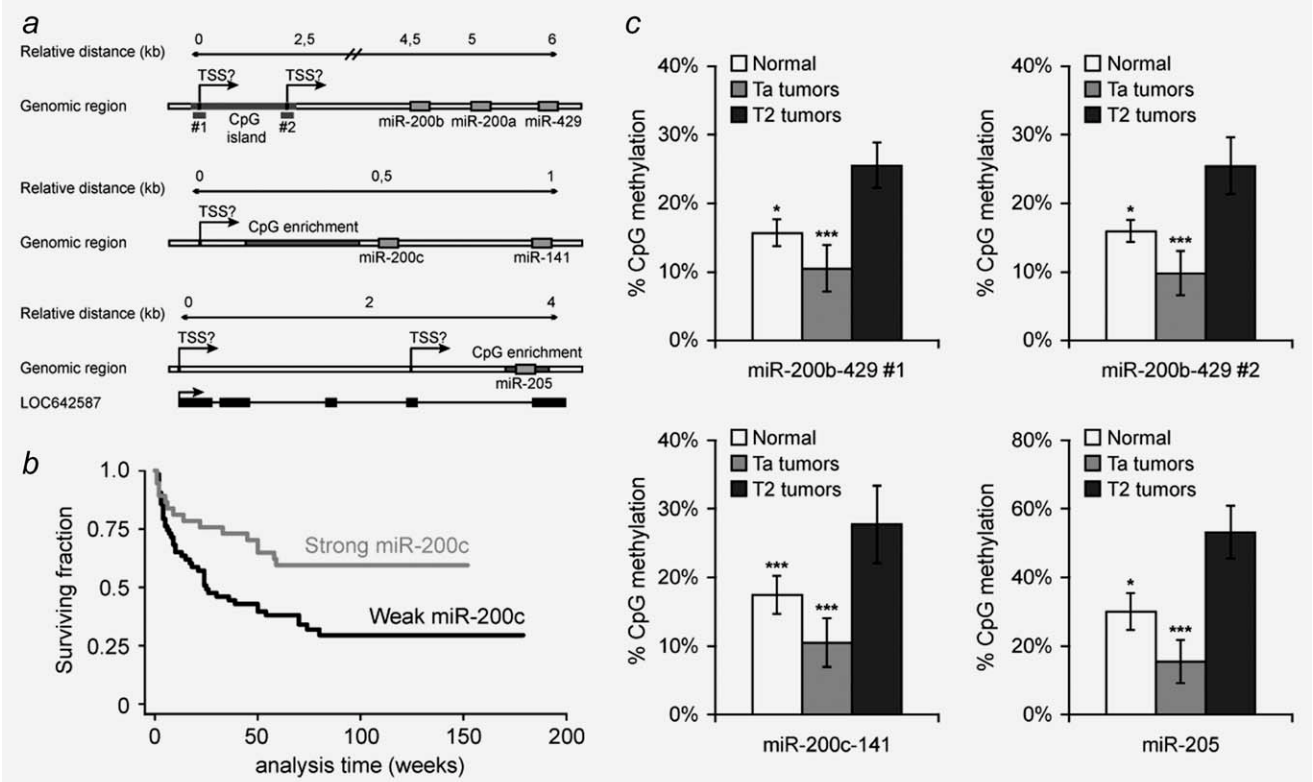

Figure 1. miR-200 and miR-205 DNA methylation status and miR-200c expression in clinical bladder specimens. (*a*) Graphical depiction of the miR-200b-429, miR-200c-141 and miR-205 genomic loci with putative transcription start sites (TSS) indicated by arrows. For miR-200b-429, regions marked #1 and #2 inside the CpG-island were analyzed for DNA methylation. For miR-200c-141 and miR-205 the regions tested for DNA methylation are indicated as "CpG enrichment." (*b*) Kaplan-Meier survival estimates for 100 patients diagnosed with T1 tumors displaying strong or weak miR-200c staining as determined by tissue microarray *in situ* hybridization ($p < 0.01$; an example shown in Supporting Information Figs. 1*c* and 1*d*). (*c*) Average percent CpG methylation levels (±SE) across the region shown in panel *a* for normal bladder tissue ($n = 5$), superficial Ta tumors ($n = 5$) and muscle invasive T2-4 tumors ($n = 5$) determined by Sequenom EpiTyper® MALDI-TOF mass spectrometry. Complete DNA methylation profiles for each individual sample are shown in Supporting Information Fig. 2. (*$p < 0.05$, ***$p < 0.001$; *p*-values determined by 2-tailed Student's *t*-test).

implying perturbation of the miR-200-*ZEB1* feedback loop in promoting migrating cancer stem cells.[10] Thus, aberrant miR-200 regulation is an important event at multiple levels of cancer development and progression, and discerning the underlying mechanisms that prevent miR-200 expression in aggressive tumors and cancer stem cells is key to improving our understanding of tumor biology.[11]

Epigenetic remodeling is a wide-spread hallmark of cancer affecting transcriptional regulation of a large number of genes.[12] Aberrant DNA methylation has been implicated in the deregulation of several miRNAs in different types of cancer.[13] A comprehensive bioinformatic analysis found that about 50% of miRNA genes are associated with CpG-islands,[14] suggesting that many more miRNAs are candidate targets of the DNA methylation machinery. As both of the miR-200 clusters (miR-200b-200a-429 and miR-200c-141) and miR-205 are associated with local CpG enrichments (Fig. 1*a*), we speculated that these miRNA loci might be aberrantly silenced by DNA hypermethylation in cancer.

In this study, we investigated miR-200 and miR-205 expression and DNA methylation status in a panel of 11 normal urothelium biopsies, 30 superficial Ta bladder tumors and 27 muscle invasive T2-4 bladder tumors (Bergkvist classification). We show that the 2 miR-200 clusters and miR-205 are concurrently silenced and DNA hypermethylated in muscle invasive T2-4 bladder tumors compared with superficial Ta bladder tumors and normal bladder tissue. Validating these results in 12 bladder cell lines, we confirm that miR-200 and miR-205 are repressed and gain DNA hypermethylation and repressive chromatin marks in undifferentiated compared with differentiated bladder cell lines. Moreover, we show that miR-200c deficiency is tightly correlated with disease outcome in patients with early T1 bladder tumors, for which it is intrinsically difficult to predict progression. We also observe an inverse correlation between miR-200/miR-

205 and the mesoderm specific transcription factor *TWIST1*, which is known to promote EMT. The ChIP data showed that *TWIST1* associates directly with the E-box rich miR-200 promoters, indicating that it may be a transcriptional repressor of the miR-200 family and miR-205.

Material and methods

Patient material

Bladder tumor samples were obtained directly from surgery and processed as previously described at Skejby University Hospital (Aarhus Denmark).[4,15] All tumors were graded by the Bergkvist classification system. Normal urothelium samples were laser microdissected from biopsies of the bladder wall of patients hospitalized for other indications than bladder cancer as described in Refs. 4 and 15. Informed written consent was obtained from all patients, and research protocols were approved by the Aarhus county science ethics committee.

Isolation of RNA and DNA

Total RNA from tissue samples, biopsies and cell lines was purified using the standard procedure for TRIzol® RNA extraction (Invitrogen, Carlsbad CA) and stored at −80°C. RNA quality was examined on a 2100 Bioanalyzer® chip (Agilent, Santa Clara CA), and samples with RIN < 5 were excluded from microarray analysis. DNA from tissue samples and cell lines was column-purified using the DNeasy® blood and tissue kit (Qiagen, Valencia CA) according to the supplied protocol and stored in aqueous solution at −20°C.

miRNA expression analysis

For the clinical samples, miRNA expression was analyzed by microarray of purified total RNA using the miRcury LNA array ready to spot v.7.1 oligonucleotide probe library (Exiqon, Vedbæk Denmark). Arrays were processed and analyzed using TIGR spotfinder 2.23, TIGR MIDAS 2.19 and TIGR MEV 3.1 software (Dana-Farber Cancer Institute, Boston, MA) as previously described.[16] MiRNA expression in cell lines was determined by miRNA TaqMan® qRT-PCR assays (Applied Biosystems, Foster City, CA) on a LightCycler® 480 (Roche, Basel, Switzerland) according to suppliers protocol. Expression was normalized to RNU48 ($2^{-\Delta Ct}$) (TaqMan® assay, Applied Biosystems).

In situ hybridization

The *in situ* detection was performed on 5-μm paraffin sections essentially as described in Ref. 16. The miR-200c probe had the sequence: TCCATCATTACCCGGCAGTATTA. Probe specificity was verified using a probe with 2 sequence mismatches (italics): TCCATGATTACCCCGCAGTATTA.

Tissue microarray construction

One biopsy (0.6-mm cores) from every tumor was taken from the area marked by the uropathologist and placed in the recipient paraffin block using a custom-made precision instrument (manual tissue microarrayer 1, Beecher Instruments, Sun Prairie, WI). This was carried out according to the method developed by Kononen *et al.*[17]

CpG cluster prediction

Regions significantly enriched for CpG dinucleotides were predicted using the online sliding window algorithm CpGPlot in the EMBOSS package from EMBL-EBI[18] (window = 100nt; step = 1; Obs/Exp = 0.6; MinPC = 50; Length = 50/100).

DNA methylation analysis

Bisulphite conversion of DNA was performed as described by Clark *et al.*,[19] using 0.4–2 μg DNA purified from patient bladder tissue and bladder cell lines. Bisulphite specific primers were designed according to standard principles for PCR amplicons of ca. 200–300 bp, adding forward and T7 promoter reverse primer tags required for Sequenom analysis (Fwd primer tag: AGGAAGAGAG; Re primer tag: CAGTAATACGACTCACTATAGGGAGAAGGCT) according to supplier and as described in Ref. 19. Primer sequences: miR-200b-429#1 Fwd: TTATGGGAGTTTAGGGGATATATT Re:ATTCAAACCTACACAAATAAA; miR-200b-429#2 Fwd:GGGGAGGGTTGGATTTTATAT Re:CCCCAACAAAAATTCTCTA; miR-200c-141 Fwd:AAGGTTATTAGGGGAGAGGTTT Re:CTTCAAACCCAAAATCCCTA; miR-205 Fwd:GTTTTTTTGGAGGATGTGATT Re:CACTCCAAATATCTCCTTCATTA. PCR was carried out at an annealing temperature of 55°C, 40 cycles, using Platinum® Taq DNA polymerase (Invitrogen).

DNA methylation analysis of bisulfite PCR amplicons for all tumor samples and cell lines was performed using the MassArray® MALDI-TOF mass spectrometry platform (Sequenom, San Diego, CA) according to the supplier's protocol and improvements described by Coolen *et al.*[20] The DNA methylation level was scored as percentage methylation of individual CpG units (comprising 1 or more CpG-sites) averaged for all samples of each class (normal tissue, Ta and T2 tumors). Overall, DNA methylation level was defined as the average percent methylation of each individual CpG-unit averaged across the regions. Methylation levels were also validated by standard clonal bisulfite sequencing of 10–12 clones for 2 primary bladder tumor samples[19,21] (Supporting Information Fig. 2).

Cell culture

All bladder cell lines were available at Aarhus University Hospital (Skejby) or supplied by Professor Pamela J. Russell (Prince of Wales Hospital, Sydney, Australia). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen), 50 U/ml penicillin and 50 mg/ml streptomycin (both Sigma-Aldrich, St. Louis, MO) under standard conditions (37°C, 5% CO_2).

Table 1. miR-200 deregulation in bladder cancer

microRNA loci	Fold change		Chromosome	CpG-island
	Cancer *vs.* normal	Invasive *vs.* superficial		
miR-200b-429 cluster			1 (p36.33)	#183
miR-200b	1.78***	−1.96***		
miR-200a	1.43*	−1.59*		
miR-429	NC	−1.25		
miR-200c-141 cluster			12 (p13.31)	CpG enriched
miR-200c	1.99***	−2.38***		
miR-141	1.92***	−2.33***		
miR-205/LOC642587			1 (q32.2)	CpG enriched
miR-205	2.49***	−1.51**		

Relative miRNA expression was determined by Exiqon LNA microarray for normal bladder urothelium ($n = 11$), superficial Ta tumors ($n = 30$) and muscle invasive T2-4 tumors ($n = 27$)
NC: No change
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

For the DNA demethylation and histone deacetylatse inhibition experiments, cells in culture were treated with 1–3 μM 5-Aza-2′deoxycytidine (5-Aza-dC) for 48 hr or 3 μM 5-Aza-dC for 24 hr followed by 50 nM trichostatin A (TSA) for 48 hr (both Sigma-Aldrich), as described in Hinshelwood *et al.*[22]

Gene expression analyses

First strand cDNA was synthesized using SuperScriptIII® (Invitrogen) according to the manufacturer's protocol from 1 μg of total RNA starting material. The reactions were diluted 1:10 and 4 μl cDNA product was used per 10 μl PCR reaction. Gene expression was analyzed by qRT-PCR using Power SYBR® Green master mix (Applied Biosystems) on a LightCycler® 480 (Roche) (40 cycles, 60°C annealing). Intron spanning primers were designed for all genes: *TWIST1* Fwd:CGGACAAGCTGAGCAAGATT Re:TGGAGGACCTG GTAGAGGAA; *ZEB1* Fwd:AGTGATCCAGCCAAATGGAA Re:TTTTTGGGCGGTGTAGAATC; *ZEB2* Fwd:AACAAGC CAATCCCAGGAG Re:GTTGGCAATACCGTCATCCT; pri-miR-200b-429 Fwd:CGCAGCAGTGGAACCTGT Re:GTGA GGAGGTGCTGGGATG; pri-miR-200c-141 Fwd:CTTAAA GCCCCTTCGTCTCC Re:AGGGGTGAAGGTCAGAGGTT; GAPDH Fwd:GTCAGCCGCATCTTCTTTTG Re:GCGCCC AATACGACCAAATC. Expression was normalized ($2^{-\Delta Ct}$) to GAPDH (SYBR green) and 18S rRNA (TaqMan® assay, Applied Biosystems).

For clinical sample gene expression, we used an independent dataset previously generated using HG-U133A GeneChips (Affymetrix, Santa Clara. CA)[23] on 87 bladder carcinomas (55 Ta tumors, 3 T1 tumors and 29 T2-4 tumors). Raw data were analyzed and normalized using the GCRMA method implemented in the GeneSpringGX 10.0 software (Agilent, Santa Clara, CA).[24]

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the Magna ChIP™ system (Millipore, Billerica, MA) and ChIP-grade antibodies against acetylated H3K9 (06-599; Millipore), tri-methylated H3K27 (07-449; Millipore) and *TWIST1* (sc-15393; Santa Cruz Biotechnology, Santa Cruz, CA). Cells were fixed in 1% formaldehyde, and 2×10^6 – 5×10^6 cells were used per IP. The chromatin was sheared into 200–500 bp fragments using a Bioruptor® sonicator (Diagenode, Liége, Belgium), and ChIPs were performed on a rotation wheel at 4°C for 12–16 hr with 5–8 μg of antibody. ChIP enrichment was quantified by SYBR green qRT-PCR and normalized to input material ($2^{-\Delta Ct}$) using the following primers: miR-200b-429#1 Fwd:CTGGCTGAGGCAGAGCAC Re:ACCGGCTTCGGAAGGAAT; miR-200b-429#2 Fwd:ACG CAGAGGGAAGAACCTG Re:GACGGGCTTAATCAAT GGTG; miR-200c-141 Fwd:AGGGGTGAGACTAGGCAGGT Re:CCACTGCCTTAACCCCTTC; miR-205 Fwd:CCTCCC TAAATCCTCCATCC Re:TCTAGGAAGGACAGCCTCCA; *CDH1* Fwd:CTCCAGCTTGGGTGAAAGAG Re:GGGCTTT TACACTTGGCTGA; GAPDH Fwd:TCGACAGTCAGCCGC ATCT Re:CTAGCCTCCCGGGTTTCTCT.

Statistical analyses

Statistical significance was determined by 2-tailed Student's *t*-test using Microsoft Excel® 2004 for Macintosh. We used Stata 10.0 statistical analysis software (Stata Corporation, College Station, TX) for calculation of log-rank tests for equality of survival function and for generation of Kaplan-Meier survival estimates.

Results and Discussion

Exiqon LNA microarrays were used to investigate miRNA expression patterns in superficial stage Ta tumors ($n = 30$), muscle invasive stage T2-4 tumors ($n = 27$) and normal urothelium ($n = 11$). The miR-200 family and miR-205 were found to be upregulated by an average of 1.5–2.5-fold in the bladder tumors compared with normal urothelium (Table 1). Conversely, miR-200 and miR-205 expression was 1.5–2.5-

fold lower in invasive compared with superficial tumors, suggesting that the miR-200/miR-205 may have oncogenic potential, but at the same time inhibit tumor invasion and metastasis of established tumors (Table 1).

The expression data also revealed a tight correlation in relative miR-200 expression (Table 1). This was apparent across all 3 miR-200/miR-205 loci, suggesting that expression of the miR-200 family and miR-205 are coordinately regulated in normal and cancer derived urothelial cells. Only miR-429 displayed a lesser relative change, which is probably a result of the low abundance of its mature form (barely above background), with miR-200a and -200b being the predominant species of the miR-200b-429 cluster (data not shown). Similarly, miR-200c is the prominent form of the miR-200c-141 cluster,[11,25] with at least 10-fold higher expression in all tested cell lines (Supporting Information Fig. 1*b*); thus, miR-200a, -200b and -200c are considered the major mature forms of the miR-200 family.

As miR-200c appears to be the dominant miR-200 form,[7,25] we performed tissue microarray *in situ* hybridization with 5′-DIG–labeled LNA probes against miR-200c on 100 stage T1 bladder tumors, for which it is intrinsically difficult to predict disease outcome (Fig. 1*b*).[4] Loss of miR-200c expression was significantly associated with subsequent disease progression to muscle invasive bladder cancer and poor outcome in patients followed up to 180 weeks ($p < 0.01$; Fig. 1*b*). Consequently, miR-200c expression may be a useful marker for predicting bladder tumor progression and could potentially direct treatment decisions.

The apparent coregulation of the miR-200s and the presence of nearby CpG-rich elements led us to speculate that all 3 miR-200/miR-205 loci might be repressed by DNA hypermethylation in invasive bladder tumors. Using the Sequenom MassArray® MALDI-TOF mass spectrometry platform, we estimated DNA methylation for 2 regions (#1 and #2) in a 2.5-kb large CpG-island 2-kb upstream of miR-200b-429, and for smaller CpG-enriched regions associated with miR-200c-141 and miR-205 (Fig. 1*a*). A significantly higher DNA methylation level in muscle invasive T2-4 tumors ($n = 5$) compared with superficial Ta tumors ($n = 5$; $p < 0.001$) and normal bladder tissue ($n = 5$; $p < 0.05$) was observed at all the 3 miR-200 and miR-205 loci (Fig. 1*c*). This increase in methylation was evident for all individual CpG units in the analyzed regions. We thus postulate that aberrant DNA hypermethylation is associated with miR-200/miR-205 silencing in invasive bladder tumors, and that the CpG methylation status of these loci is a possible prognostic marker in bladder cancer.

To validate the clinical data, we tested miR-200/miR-205 expression and DNA methylation in a panel of twelve bladder cell lines. These represent a broad group of cell types ranging from normal immortalized urothelial cells (HU609), carcinoma *in situ* (MGH-U4) and highly differentiated slowly dividing cells (SW780, SD, SW1710, RT112 and 5637) to poorly differentiated, rapidly dividing cell lines (VmCub1, VmCub3, J82, T24 and HT1376) (Fig. 2 and Supporting Information Table 1). Consistent with a hypothesis for coordinated regulation of miR-200 and miR-205, qRT-PCR analysis showed a tight correlation between the expression of the miR-200 family and miR-205 in all bladder cell lines, except for SW1710 and J82 (Fig. 2*a*). In accordance with the pattern observed for the clinical data, miR-200 expression was also up to 10^5-fold higher in the cancerous compared with normal cell lines, and decreased by 10^2- to 10^4-fold in poorly relative to highly differentiated cells (Fig. 2*a*). miR-200c and miR-205 were generally the most abundant mature forms in the bladder cell lines (Fig. 2*a*), agreeing with previous reports of miR-200c being the predominant miR-200 species.[7,25] Furthermore, CpG methylation analysis revealed near complete methylation of the miR-200 loci, correlating with the observed loss of miR-200 expression in all poorly differentiated cell lines, except HT1376 and J82 (Fig. 2*b* and Supporting Information Fig. 3). In contrast, the same regions were completely unmethylated in the differentiated cells demonstrating robust miR-200 expression.

The upstream region (#1) of the miR-200b-429 CpG island was generally more methylated than the downstream region (#2) in cells with repressed miR-200 expression, indicating that the upstream putative transcription start site (TSS) might be the more active (Figs. 1*a* and 2*b*). Indeed, this region has previously been verified as a miR-200b-429 TSS.[9] However, in SW1710 cells the deficiency in DNA methylation of region #2 appears sufficient to drive miR-200b-429 expression despite region #1 being hypermethylated, suggesting that the methylation status of both regions may play a role in the transcriptional regulation of this miRNA cluster. The unmethylated state of miR-200b-429 downstream region #2 in SW1710 cells also explain the apparent inconsistency in miR-200 co-regulation observed for this cell line (Figs. 2*a* and 2*b*).

To confirm that miR-200 expression is influenced by DNA methylation, we treated normal immortalized urothelial cells (HU609), differentiated (SW780) and undifferentiated (T24) bladder cells with the demethylating cytosine analog 5-Aza-2′-deoxycytidine (5-Aza-dC; 1-3 μM, 48 hr) and the histone deacetylase (*HDAC*) inhibitor trichostatin A (TSA; 50 nM, 48 hr) (Fig. 2*c*). Robust reactivation of all the mature miR-200 family members and miR-205 was only observed in T24 cells, where the 3 respective loci are all hypermethylated. In HU609, miR-200c, and to a lesser extent miR-200b and miR-205, reacted to 5-Aza-dC, but mature miRNA levels remained low (Figs. 2*b* and 2*c*). Concordantly, 5-Aza-dC treatment failed to increase miR-200/miR-205 expression in the DNA hypomethylated SW780 cells.

Overall, the increase in miRNA expression was attributable to 5-Aza-dC, and TSA did little to activate miR-200 and miR-205 (Figs. 2*b* and 2*c*), suggesting that DNA methylation is the primary epigenetic mark controlling transcription of these miRNAs. However, the persistent low level of miR-200 and miR-205 expression in HU609 despite 5-Aza-dC treatment, and complete or partial silencing of these loci in the absence of DNA hypermethylation in HU609 and HT1376,

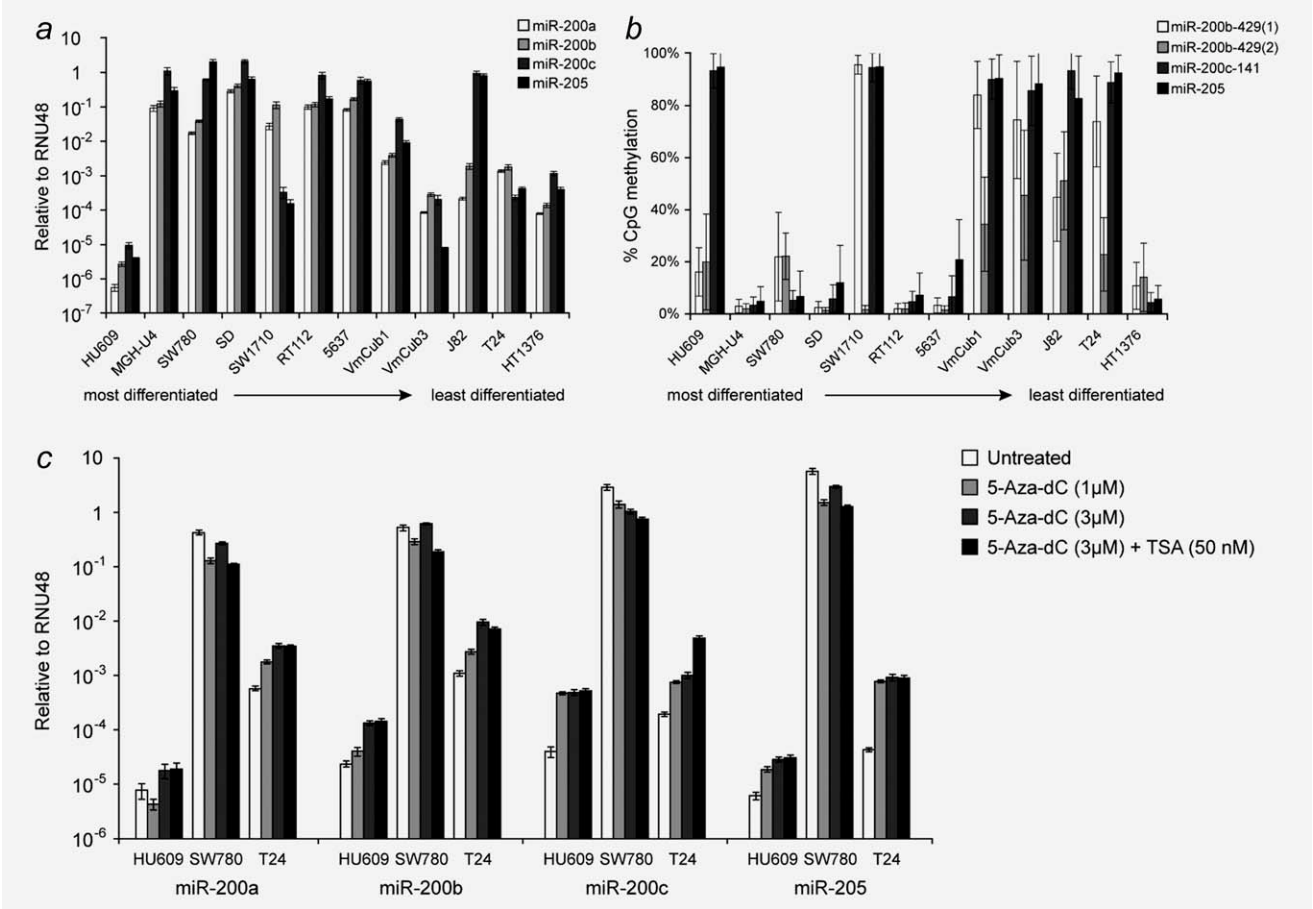

Figure 2. miR-200 and miR-205 DNA methylation levels in bladder cell lines. (*a*) Expression (±SD) of miR-200a, -200b, -200c and -205 in a panel of 12 bladder cell lines determined by TaqMan miRNA qRT-PCR assays. Expression was normalized to RNU48 ($2^{-\Delta Ct}$). All cell lines originate from primary bladder tumors and are sorted from most to least differentiated phenotype. HU609 was derived from normal bladder urothelium (see also Supporting Information Table 1). (*b*) Average percent CpG methylation level (±SD) of the miR-200 and miR-205 loci (Fig. 1*a*) in the 12 bladder cell lines determined by Sequenom EpiTyper® MALDI-TOF mass spectrometry. Complete DNA methylation profiles for each individual cell line are shown in Supporting Information Figure 3*a*. (*c*) Effect of 5-Aza-dC (1–3 μM, 48 hr) and TSA (50 nM, 48 hr) treatment on miR-200 and miR-205 expression (±SD) in HU609, SW780 and T24 cells.

indicates that DNA methylation is not the sole regulator of miR-200/miR-205 and that other factors may also be involved in transcriptional repression in these cells.

Epigenetic silencing of miR-200/miR-205 was validated by chromatin immunoprecipitation (ChIP) for the active histone mark H3 lysine 9 acetylation (H3K9Ac) and the repressive polycomb mark H3 lysine 27 trimethylation (H3K27me3) (Figs. 3*a* and 3*b*). Consistent with the expression and DNA methylation data, SW780 cells displayed a strong enrichment for H3K9Ac and low H3K27me3 enrichment for the miR-200/miR-205 loci, and *vice versa* for HU609 and T24, supportive of active and repressed transcription, respectively (Figs. 3*a* and 3*b*). A stronger H3K27me3 enrichment was observed for HU609 than for T24, consistent with the comparatively low miR-200 levels in HU609 cells (Figs. 2*a* and 3*b*). As expected based on previous reports,[5,7] epigenetic repression of the miR-200s and miR-205 was accompanied by high expression of *ZEB1* and *ZEB2* and transcriptional silencing of *CDH1* in the bladder cell lines (Fig. 3*a* and Supporting Information Fig. 4*a*).

Despite the clear inverse correlation between miR-200/miR-205 expression and CpG methylation, miR-200b-429 is silenced in the absence of DNA methylation in HU609 and HT1376 cells and expressed and hypermethylated in J82 cells (Figs. 2*a* and 2*b* and Supporting Information Fig. 3*b*). Therefore, we hypothesize that other factors may also be involved in miR-200 and miR-205 repression in invasive tumors. Indeed, *ZEB1* is known to bind the miR-200c promoter and silence transcription.[8] Using the online transcription factor binding site prediction tool MatInspector,[26] we found 6–8 E-box consensus *TWIST1* binding sites proximal to the putative miR-200 and miR-205 TSSs (Fig. 3*a*). Similar to *ZEB1*, *TWIST1* is known to transcriptionally repress *CDH1* during EMT[27,28]; thus, a role for *TWIST1* in silencing miR-200 transcription is highly plausible.

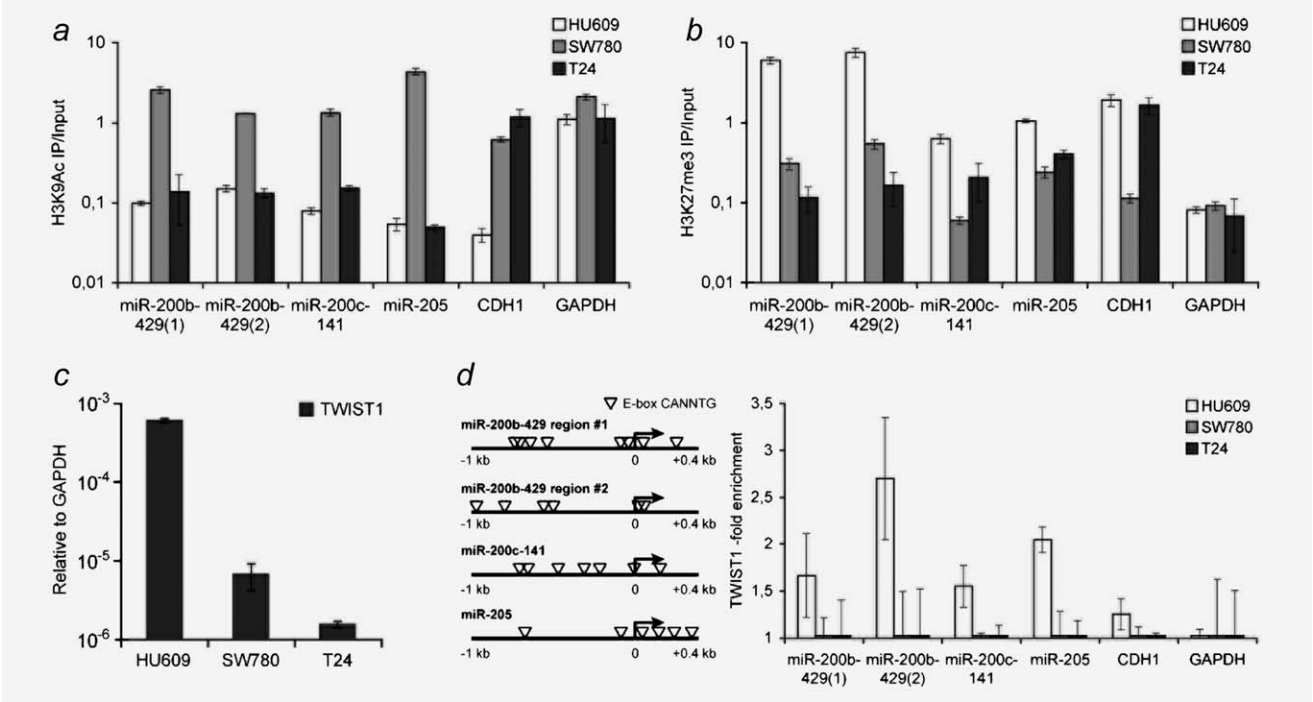

Figure 3. miR-200 and miR-205 chromatin status and *TWIST1* expression and promoter association in bladder cell lines. (*a*) Acetylated H3K9 and (*b*) tri-methylated H3K27 enrichment determined by ChIP for the miR-200, miR-205 and *CDH1* loci quantified by qPCR (±SD) (normalized to input material, $2^{-\Delta Ct}$). (*c*) Endogenous *TWIST1* expression levels (±SD) in HU609, SW780 and T24 cells determined by qRT-PCR (normalized to *GAPDH*, $2^{-\Delta Ct}$). (*d*) E-box consensus sequences (CANNTG) in the putative miR-200 and miR-205 promoter regions (triangles) and *TWIST1* ChIP showing binding to the promoters in HU609, SW780 and T24 cells. *TWIST1* binding was defined as fold enrichment relative to negative control *GAPDH* signal. *CDH1* is a positive control for *TWIST1* binding.

Consistent with a role in miR-200/miR-205 repression *TWIST1* mRNA levels were highest in HU609 cells, which displayed 10^5-fold lower miR-200/miR-205 and 100-fold higher *TWIST1* expression compared to SW780 (Figs. 2*a* and 3*c*; complete *TWIST1* expression profile for all 12 cell lines shown in Supporting Information Fig. 4). Moreover, *TWIST1* was 1.8-fold upregulated ($p < 0.00001$) in muscle invasive T2-4 compared with superficial Ta tumors, which displayed high miR-200/miR-205 expression (data not shown and Table 1). An analysis of the correlation between miRNA and mRNA expression in publicly available data for the NCI60 panel of cell lines revealed that *TWIST1* is in the top 25 genes negatively correlated with miR-200/miR-205 expression (data not shown). As the *TWIST1* mRNA does not contain any predicted miR-200 or miR-205 target sites, it seems unlikely that this negative correlation is due to miR-200s directly targeting *TWIST1*, hence supporting *TWIST1* as a general transcriptional repressor of the miR-200s.

To assess *TWIST1*-binding to the miR-200 and miR-205 promoters, we performed *TWIST1* ChIP in HU609, SW780 and T24 cells. Consistent with a role for *TWIST1* in transcriptional repression of miR-200 and miR-205, above background *TWIST1* binding to the miR-200/miR-205 promoters was only detected in HU609 cells (Fig. 3*d*), with enrichment stronger than for the bona fide *TWIST1* repression target *CDH1*.[27] Hence, *TWIST1* appears to directly associate with the miR-200 and miR-205 promoters and may act as a transcriptional repressor of miR-200/miR-205, thus further underpinning its role in silencing *CDH1* and promoting a mesenchymal phenotype. A possible causative link between *TWIST1* repression and epigenetic silencing of the miR-200 and miR-205 loci remains to be investigated.

In summary, we show that the miR-200 family and miR-205 are often concurrently silenced and gain DNA hypermethylation in muscle invasive bladder tumors and poorly differentiated bladder cell lines. This is the first evidence of coordinated regulation of the miR-200 family and miR-205. Repression of miR-200/miR-205 is accompanied by a loss of H3K9Ac and a gain of H3K27me3 in HU609 and T24 cells, demonstrating regional epigenetic remodeling encompassing the miR-200 clusters and miR-205.[29] This suggests that EMT and tumor invasion is linked to aberrant epigenetic architecture at these loci, and we propose that miR-200 and miR-205 DNA hypermethylation could serve as a prognostic marker in bladder cancer and probably also other types of neoplasms. In addition, we report that miR-200c expression is significantly correlated to disease progression of early stage T1 bladder tumors, therefore miR-200c levels could potentially be used to direct treatment decisions in patients diagnosed with such tumors.

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