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Research Article

miR-205 Exerts Tumor-Suppressive Functions in Human Prostate through Down-regulation of Protein Kinase Cε

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Abstract

Limited information is available concerning the expression and role of microRNAs in prostate cancer. In this study, we investigated the involvement of miR-205 in prostate carcinogenesis. Significantly lower miR-205 expression levels were found in cancer than in normal prostate cell lines as well as in tumor compared with matched normal prostate tissues, with a particularly pronounced reduction in carcinomas from patients with local-regionally disseminated disease. Restoring the expression of miR-205 in prostate cancer cells resulted in cell rearrangements consistent with a mesenchymal-to-epithelial transition, such as up-regulation of E-cadherin and reduction of cell locomotion and invasion, and in the down-regulation of several oncogenes known to be involved in disease progression (i.e., *interleukin 6, caveolin-1, EZH2*). Our evidence suggests that these events are driven by the concurrent repression of specific predicted miR-205 targets, namely N-chimaerin, ErbB3, E2F1, E2F5, ZEB2, and protein kinase Cε. Strikingly, the latter seemed to play a direct role in regulating epithelial-to-mesenchymal transition. In fact, its down-regulation led to a cell phenotype largely reminiscent of that of cells ectopically expressing miR-205. Overall, we showed for the first time that miR-205 exerts a tumor-suppressive effect in human prostate by counteracting epithelial-to-mesenchymal transition and reducing cell migration/invasion, at least in part through the down-regulation of protein kinase Cε. [Cancer Res 2009;69(6):2287–95]

Introduction

Prostate cancer (PCa) is the most frequent tumor in men and the second cause of cancer-related death in occidental male populations. Although surgery and radiotherapy are generally effective against clinically localized PCa, androgen ablation, the treatment of choice for advanced disease, only leads to temporary tumor regression (1). The lack of available treatment options for effectively eradicating advanced PCa makes the development of alternative approaches urgent. Understanding the molecular alterations that distinguish progressive from nonprogressive disease will allow the identification of novel prognostic markers or therapeutic targets.

Recent findings indicate that, compared with normal tissues, human cancers have distinct expression profiles of microRNAs (miRNA)—small noncoding regulatory RNAs—suggesting that their aberrant expression may be implicated in tumorigenesis (2, 3). At present, little is known about the PCa-specific miRNA signature. Studies conducted thus far, performed on limited series of clinical samples that generally did not comprise cancer and normal tissues from the same patient, have indicated that miRNA expression profiles may distinguish PCa from nonneoplastic specimens (4–6) and classify tumors according to their androgen dependence (5) or perineural invasion (7). However, a consensus on PCa-related miRNAs has not yet been achieved. Moreover, only a few miRNAs have been characterized as potential oncogenes or tumor suppressors in PCa (8–14).

Taking advantage of different available PCa cell models and a relatively large set of carcinoma and matched normal prostate tissues, we investigated the potential involvement of miR-205—which our preliminary microarray data[6] had shown to be markedly down-regulated in PCa compared with normal cells—in the development and progression of PCa. We show that miR-205 is markedly down-regulated in PCa and, based on a gain-of-function approach, provide, for the first time, significant clues regarding its role as a tumor-suppressor miRNA in human prostate.

Materials and Methods

Experimental models. All cell lines but VCaP (provided by K.J. Pienta, University of Michigan, Ann Arbor, MI) were obtained from American Type Culture Collection, maintained at 37°C/5% CO_2 in culture medium with 10% FCS (DU145, PC-3, LNCaP, VCaP) or in K-SFM (Invitrogen) with 5 ng/mL epidermal growth factor and 0.05 mg/mL bovine pituitary extract (RWPE-1). Carcinoma and matched normal prostate tissues were obtained, with appropriate informed consent and Institutional Review Board approval, from 31 untreated PCa patients subjected to radical prostatectomy. Freshly frozen surgical blocks, stored in the Institutional Frozen Tumor Bank, were carefully dissected by the pathologist using H&E-stained sections as a template to identify areas containing at least 70% of tumor or normal cells.

miRNA and gene expression analysis. miR-205 and mRNA expression was assessed by end-point and quantitative reverse transcription-PCR (qRT-PCR) as detailed in Supplementary Data.

Cell-based experiments. Cells seeded at the appropriate density were transfected for 4 h at 37°C with 20 nmol/L miRNA precursors or 50 nmol/L siRNAs (Supplementary Data) using Lipofectamine-2000 (Invitrogen), and processed at different time intervals.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

P. Gandellini and M. Folini contributed equally to this work.

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Cell morphology was evaluated at day 3 after transfection by ×10 objective magnification using an Eclipse TS100 microscope (Nikon). Images were acquired by a DC290 digital camera (Eastman Kodak Company).

In the migration assay, cells were transferred to the upper chamber (2×10^5 cells per well) of 24-well Transwell plates (Costar; Corning Incorporated) in serum-free medium. Conditioned medium (chemoattractant), obtained by incubating growing cells in a medium without serum for 24 h, was added to the lower chamber. After a 5-h incubation at 37°C, filters were fixed in 99% ethanol and stained with a 0.4% sulforhodamine B/1% acetic acid solution. Migrated cells were counted under a microscope. The same procedure was used for invasion assay, except that cells were seeded at 3×10^5 cells per well, Transwell chambers coated with 12.5 μg of Matrigel/well (BD Biosciences), and samples processed after a 24-h incubation.

Immunofluorescence and immunoblotting analyses. For immunofluorescence analyses, cells grown on glass coverslips (coated or not with polylysine) were fixed with 4% formaldehyde, stained with FITC-labeled phalloidin [Filamentous actin (F-actin); Sigma] or probed with anti–E-cadherin and anti–β-catenin antibodies (Abcam). Nuclei were counterstained with 0.1 μg/mL Hoechst 33258 dye. Images were acquired by Nikon Eclipse E600 microscope using ACT-1 software (Nikon) and processed with Adobe Photoshop Image Reader 7.0.

For immunoblotting, proteins (40 μg) were fractioned by SDS-PAGE and transferred onto Hybond nitrocellulose membranes (GE Healthcare Europe GmbH). Filters were blocked in PBS-Tween 20/5% skim milk and probed with anti–E-cadherin, anti–protein kinase Cε (PKCε; Santa Cruz Biotechnology), anti-ERK1/2, anti-phosphoERK1/2 (Cell Signaling Technology), anti–β-catenin (Abcam) antibodies, which were visualized by SuperSignal West PICO chemiluminescent detection system (Pierce Biotechnology). β-actin was used as equal protein loading control.

Gene expression profiling, target prediction, and pathway analyses. mRNA expression profiles were analyzed on an Illumina microarray platform as detailed in Supplementary Data. Prediction of miR-205 putative target genes and an *in silico* search for their connection with differentially expressed genes were performed by miRGen and Ingenuity Pathway Analysis softwares (Supplementary Data).

Statistical analyses. Two-sided Student's t test was used to analyze the differences in miR-205 expression, migration, invasion, matrix metalloproteinase (MMP)-2 activity and gene expression. P values of <0.05 were considered statistically significant. Data are reported as mean values ± SD of at least three independent experiments. Spearman's correlation coefficient with associated P value was calculated for E-cadherin mRNA and miR-205 expression in clinical samples.

Results

miR-205 is down-regulated in PCa cell lines and clinical specimens. End-point RT-PCR showed that miR-205 precursor was highly represented in normal prostate tissue and RWPE-1 cells, whereas it was almost undetectable in both androgen-dependent

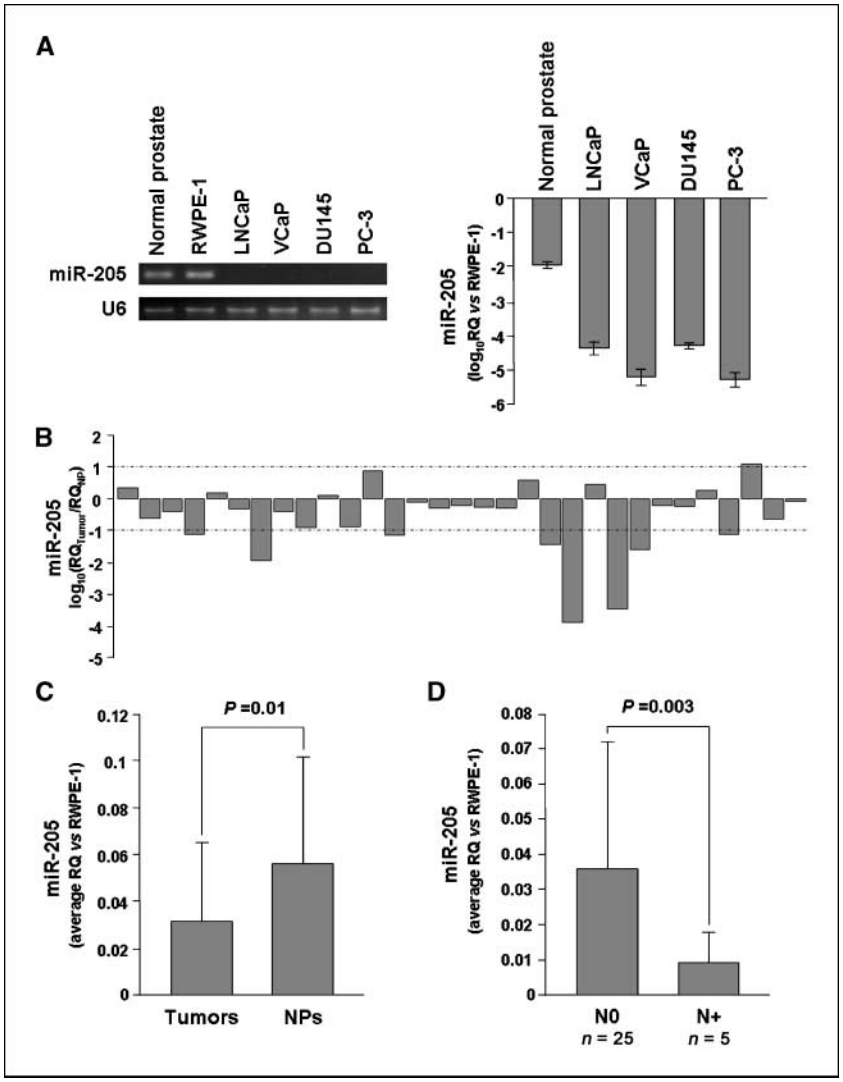

Figure 1. miR-205 is down-regulated in PCa cells and clinical specimens. *A,* representative RT-PCR (*left*) showing the expression of miR-205 precursor and quantification (*right*) of mature miR-205 expression by qRT-PCR in prostate cells. Expression values are reported as $\log_{10}$-transformed relative quantity (*RQ*) with respect to RWPE-1 cells. *Normal prostate,* normal prostate RNA from Ambion. *B,* expression of mature miR-205 in carcinoma versus matched normal prostate tissue (NP) samples from 31 PCa patients, reported as $\log_{10}(RQ_{tumor}/RQ_{NP})$, as determined by qRT-PCR. *C* and *D,* average miR-205 expression levels (RQ ± SD) in carcinomas and NP samples (*C*) and in carcinomas from N0 and N+ patients (*D*). n = number of patients within each subgroup.

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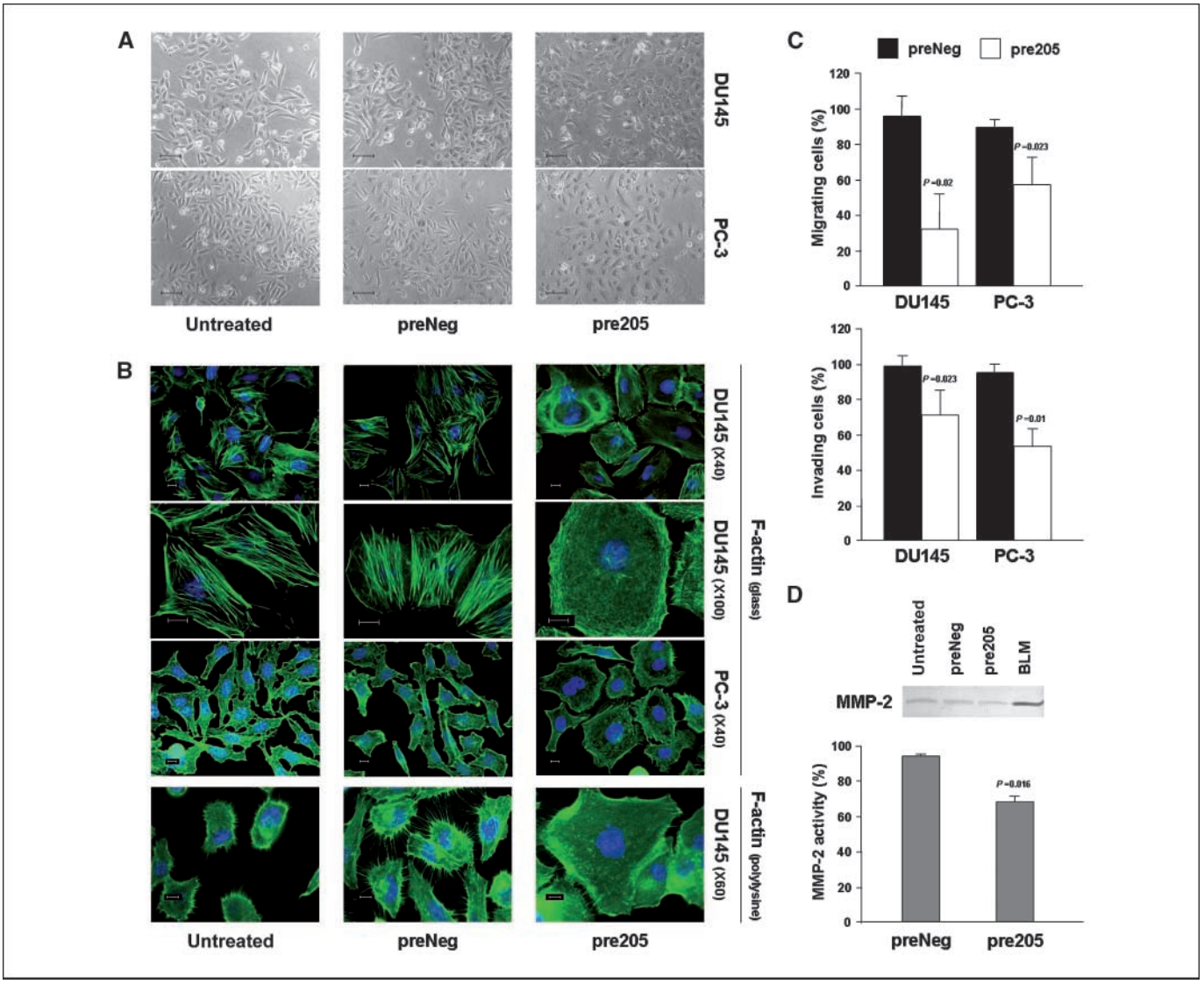

Figure 2. miR-205 induces morphologic changes and impairs the migratory and invasive properties of PCa cells. Representative microphotographs showing the morphology (*A*) and the organization of actin cytoskeleton (*B*) of untreated and negative control (*preNeg*)– or pre205-transfected PCa cells. *Scale bar,* 100 and 10 μm, respectively. *C,* percentage of migrating and invading transfected PCa cells with respect to untreated cells. Migrating and invading untreated cells were, respectively, 89 ± 9 and 1,242 ± 252/mm^2 (*DU145*) and 184 ± 18 and 1,490 ± 172/mm^2 (*PC-3*). *D,* MMP-2 gelatinolytic activity (*top*) in untreated and transfected DU145 cells. Quantification of MMP-2 activity (*bottom*), expressed as percentage of untreated cells.

(VCaP, LNCaP) and androgen-independent (DU145, PC-3) PCa cells (Fig. 1*A, left*). Similarly, the mature form showed ~10^4- to 10^5-fold reduced expression levels in all PCa cells with respect to RWPE-1 cells or normal prostate, as determined by qRT-PCR (Fig. 1*A, right*).

To examine the clinical relevance of miR-205, its expression was analyzed in carcinoma and matched normal prostate samples obtained from 31 untreated PCa patients. In most patients (23 of 31; 74.2%), carcinomas showed reduced miR-205 expression with respect to normal counterparts (0.88- to 0.0001-fold change; Fig. 1*B*), and an overall lower average relative expression was observed in carcinoma than in normal prostate tissues (0.031 and 0.056, respectively; Fig. 1*C*). In addition, tumors from patients with disease spread to regional lymph nodes (N+) showed significantly lower miR-205 levels than those from node-negative (N0) patients (0.009 and 0.036, respectively; Fig. 1*D*). No correlation between miR-205 expression and grade could be established because most tumors were graded Gleason 7.

miR-205 induces morphologic changes and impairs migratory and invasive properties in PCa cells. To investigate whether miR-205 down-regulation plays a causative role in PCa, we assessed the regulatory effect of miR-205 on cellular pathways relevant for the development and/or progression of the disease using a gain-of-function approach. Transfection of DU145 cells with miR-205 precursor (pre205) resulted in its persistent endogenous expression at levels comparable with those of RWPE-1 cells (Supplementary Fig. S1) and in marked morphologic changes. Specifically, a decrease in the fraction of cells with an elongated shape was paralleled by an increase in that of large, flattened, polygonal-shaped cells (Fig. 2*A*), which tended to aggregate in tightly packed colonies forming sheet-like structures, probably due to enhanced cell-cell adhesion. Similar evidence was also appreciable in PC-3 cells (Fig. 2*A*). Moreover, staining of the actin cytoskeleton with FITC-labeled phalloidin confirmed the increase in size and highlighted substantial disruption of the actin network, with a tendency toward

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a predominantly subcortical distribution and loss of stress fibers or filopodia (Fig. 2*B*).

Based on this evidence suggesting transition from a motile fibroblastic-like toward an epithelial-like phenotype, a transwell migration assay was carried out to evaluate whether miR-205 affects the migratory and invasive capabilities of PCa cells. Upon stimulation with conditioned medium, pre205-transfected cells showed a significantly lower migration ability than cells exposed to preNeg (32% ± 20% versus 96% ± 11% in DU145 cells, and 57% ± 15% versus 89% ± 5% in PC-3 cells, compared with untreated controls; Fig. 2*C*). When the assay was performed in the presence of Matrigel, the percentage of invading cells transfected with pre205 was significantly lower than that of preNeg-transfected cells (71% ± 14% versus 99% ± 6% in DU145 cells and 53% ± 9% versus 94% ± 5% in PC-3 cells, compared with untreated controls; Fig. 2*C*). In addition, zymography (performed as described in Supplementary Data) revealed that miR-205–expressing DU145 cells were characterized by significantly decreased MMP-2 activity compared with preNeg-transfected cells (67.7% ± 3.1% versus 93.3% ± 0.8%, with respect to untreated cells; Fig. 2*D*).

miR-205 affects the expression of epithelial and mesenchymal markers. Because the phenotype of pre205-transfected cells, including change in morphology, pronounced cell-cell adhesion and reduced migration/invasion, was reminiscent of a reverse transition from a mesenchymal to an epithelial state (MET), we set out to confirm the ability of miR-205 to revert the classic epithelial-to-mesenchymal transition (EMT) through evaluation of the expression of EMT-specific markers. Immunofluorescence analyses, carried out 3 days after transfection of DU145 cells with pre205, revealed a dramatic increase in staining for the epithelial marker E-cadherin—which mainly localized at cell-cell contacts—as well as of β-catenin (Fig. 3*A, top*), confirming enhanced cell-cell adhesion. Conversely, E-cadherin was almost undetectable in untreated or preNeg-transfected cells, except for small groups of cells characterized by weak staining and enlarged morphology (Fig. 3*A, top*). These observations were confirmed by immunoblotting in DU145 and PC-3

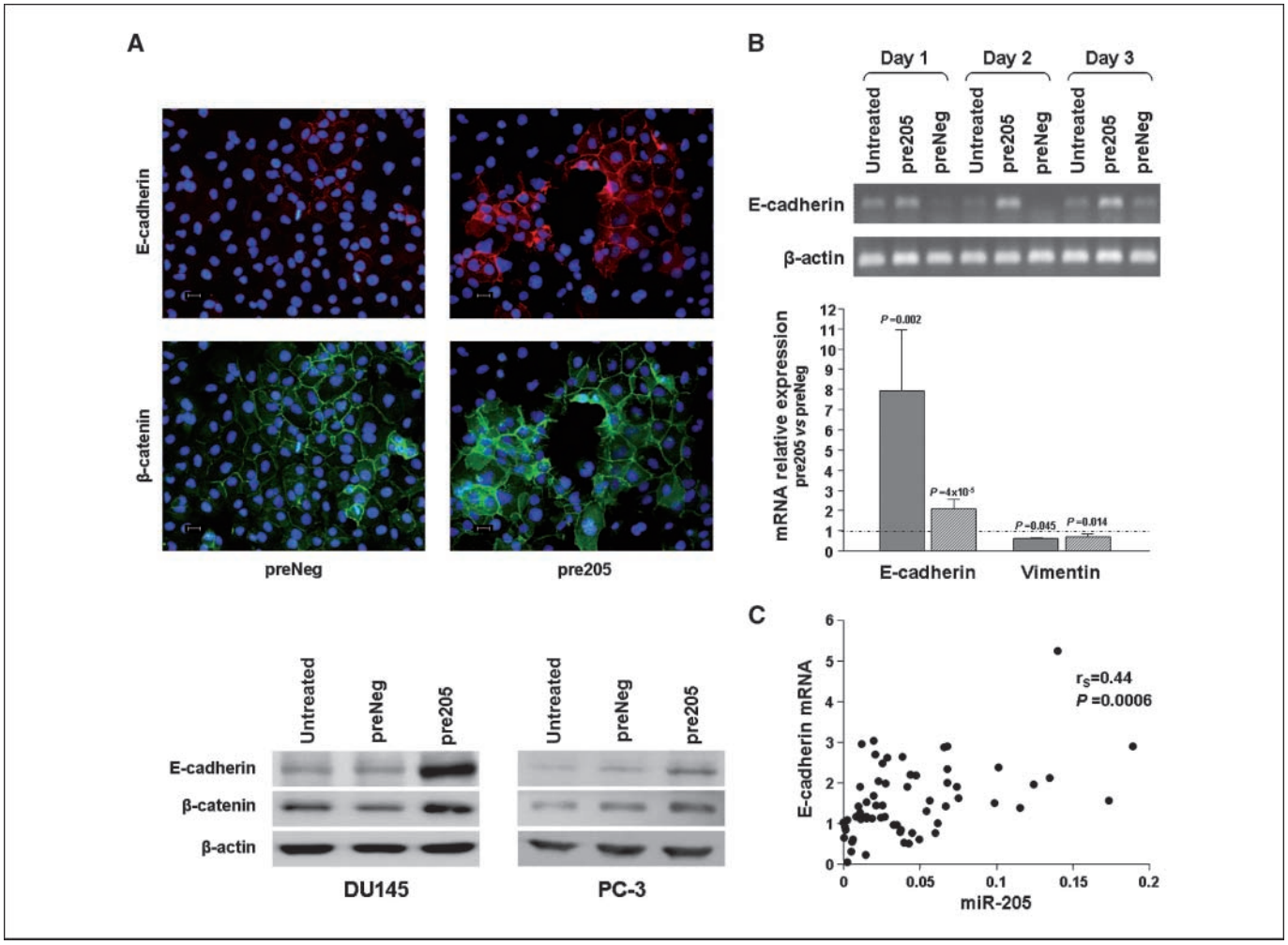

Figure 3. miR-205 affects the expression of epithelial and mesenchymal markers. *A,* representative microphotographs showing E-cadherin (*red*) and β-catenin (*green*) expression in transfected DU145 cells (*top*). *Scale bar,* 10 μm. Immunoblottings showing E-cadherin and β-catenin expression in untreated and transfected PCa cells (*bottom*). *B,* time course RT-PCR (*top*) showing the expression of E-cadherin in untreated and transfected DU145 cells. Quantification of E-cadherin and vimentin mRNA levels (*bottom*) performed by qRT-PCR in DU145 (*gray column*) and PC-3 (*hatched column*) cells. Results are reported as relative expression in pre205- versus preNeg-transfected cells. *C,* scatterplot showing the correlation between RQ values of E-cadherin mRNA and miR-205 in clinical specimens. r_S, Spearman's correlation coefficient.

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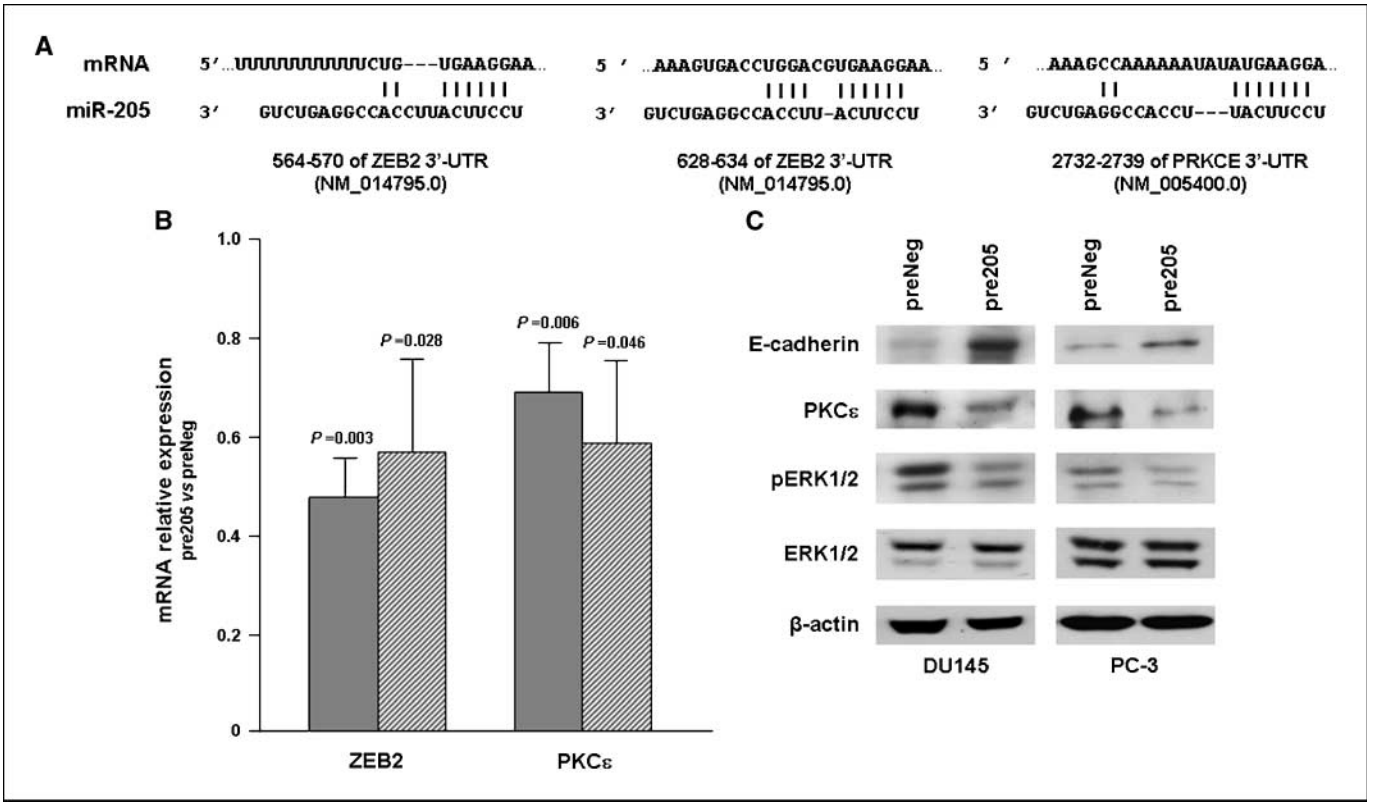

Figure 4. ZEB2 and PKCε are plausible miR-205 targets. *A,* miR-205 binding sites within *ZEB2* and *PRKCE* 3′-UTRs, as determined by TargetScan. Genbank accession numbers and positions are reported. *B,* quantification of ZEB2 and PKCε mRNA levels in DU145 (*gray column*) and PC-3 (*hatched column*) cells. Data are reported as relative expression in pre205- versus preNeg-transfected cells. *C,* representative immunoblottings showing E-cadherin, PKCε, phosphorylated ERK1/2, and total ERK1/2 levels in transfected PCa cells.

cells, where ectopic miR-205 expression resulted in a net increase in E-cadherin and β-catenin protein levels (Fig. 3*A, bottom*).

Because loss of E-cadherin function in tumor progression is caused by various mechanisms, we wondered whether miR-205 primarily affected E-cadherin mRNA expression. RT-PCR experiments showed a net increase in E-cadherin mRNA upon ectopic expression of miR-205. Such an increase reached its maximum at day 3 (7.95- ± 3.01-fold and 2.11- ± 0.4-fold change versus preNeg, in DU145 and PC-3 cells, respectively; Fig. 3*B*). In parallel, a significant reduction (−37% ± 3% and −28% ± 10% in DU145 and PC-3 cells, respectively) of the mesenchymal marker vimentin mRNA was observed (Fig. 3*B*). A direct correlation between the expression of mature miR-205 and E-cadherin mRNA was also observed in our set of clinical specimens (Fig. 3*C*).

miR-205 alters the expression of genes involved in epithelial organization, cell-cell adhesion, and PCa progression. To identify putative targets modulated at mRNA level by miR-205, we evaluated the gene expression profiles of DU145 cells exposed to preNeg or pre205. A set of 845 genes was found to be differentially expressed under the two experimental conditions, with overrepresentation of genes related to cellular components such as laminin complex, basal lamina, and tight junctions (Supplementary Table S4), as revealed by gene ontology enrichment analysis. Such evidence would suggest that miR-205 regulates genes involved in the organization of epithelia and cell-cell adhesion. Genes encoding for proteins involved in the assembly of adherens junctions, tight junctions, gap junctions, and desmosomes were actually overexpressed in pre205-transfected cells (Supplementary Table S5).

In addition, down-regulation of several factors known to contribute to PCa progression, such as interleukin (IL)-6, caveolin-1, and EZH2, was also observed (Supplementary Table S5), suggesting that miR-205 might also function as a tumor suppressor in prostate cells. Finally, several genes predicted to be putative miR-205 targets by at least two prediction programs, i.e., *CHN1* (N-chimaerin), *ERBB3, E2F5, E2F1*, and *PRKCE* (PKCε), were found to be down-regulated (Supplementary Table S5). Down-modulation of several potential targets was further confirmed by RT-PCR (Supplementary Fig. S2).

miR-205–mediated MET: role of putative targets ZEB2 and PKCε. To identify the mediators of miR-205–induced cell rearrangements, we focused mainly on two predicted miR-205 targets, ZEB2 and PKCε. ZEB2 is a well-known repressor of E-cadherin transcription. Different computational tools predicted two highly conserved sequences that are at least partially complementary to miR-205 within the 3′-untranslated region (UTR) of ZEB2 mRNA (Fig. 4*A*). Due to the lack of reliable antibodies, ZEB2 expression levels in pre205- or preNeg-transfected cells were assessed by qRT-PCR. The observation that ZEB2 mRNA levels were significantly reduced (−55.9% ± 7.8% and −44.2% ± 19.1% in DU145 and PC-3 cells, respectively) upon transfection with pre205 (Fig. 4*B*) suggested that miR-205 might mediate the repression of ZEB2, resulting in E-cadherin up-regulation and acquisition of an epithelial-like phenotype.

A highly conserved putative miR-205 binding site was also predicted within the 3′-UTR of PKCε mRNA (Fig. 4*A*). Microarray data showed down-regulation of PKCε mRNA in pre205-transfected

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compared with preNeg-transfected DU145 cells (Supplementary Table S5). Exposure to pre205 resulted in a significant reduction of PKCε mRNA levels accompanied by a pronounced decrease in protein abundance in DU145 and PC-3 cells (Fig. 4*B* and *C*). In addition, phosphorylation of ERK1/2 kinase—a downstream substrate of PKCε—was markedly reduced (Fig. 4*C*), suggesting that miR-205 can down-regulate PKCε and interfere with its function.

To corroborate the hypothesis that miR-205–mediated tumor-suppressive effect, including MET, occurs through the down-regulation of ZEB2 and/or PKCε, we performed phenocopy experiments using RNAi. When transfected into DU145 cells, siZEB2 and siPKCε were able to specifically and comparably down-regulate ZEB2 and PKCε mRNAs (Supplementary Fig. S3). Down-regulation of ZEB2 mRNA did not induce a marked change in cell morphology, except for the appearance of small clusters of polygonal cells (Fig. 5*A*), and failed to significantly up-modulate E-cadherin mRNA and protein (Fig. 5*B* and *C*) with respect to control siRNA (siCTR)-transfected cells. In addition, transfection of siZEB2 did not affect cell migration or invasion (Fig. 5*D*). However, knockdown of PKCε led to marked morphologic rearrangements (Fig. 5*A*) resembling those observed in cells transfected with pre205. Although to a lesser extent than observed in miR-205–expressing cells, exposure to siPKCε also resulted in E-cadherin up-modulation at the mRNA and protein level (Fig. 5*B* and *C*), as well as in a reduced migratory and invasive potential (Fig. 5*D*).

To tentatively summarize the molecular events responsible for the phenotypic rearrangements induced by miR-205 in PCa cells, we integrated microarray data with our novel findings and built a network connecting miR-205 with its target and downstream effector genes (Fig. 6).

Discussion

Expression of miR-205 in cancer is controversial because it was found to be either up-regulated (15, 16) or down-regulated (17, 18) in tumor compared with normal tissues. However, no functional evidence for a role of miR-205 as an oncogenic or tumor-suppressive miRNA has been documented. We found that miR-205 was markedly down-regulated in PCa cell lines, irrespective of their androgen responsiveness, compared with normal cells. Reduced expression of both the precursor and mature forms suggested that mechanisms other than defects in the miRNA processing machinery, such as genetic or epigenetic aberrations, could be responsible for miR-205 down-modulation. Reduction of miR-205 expression was also observed in carcinoma compared with matched normal tissues, and it was particularly pronounced

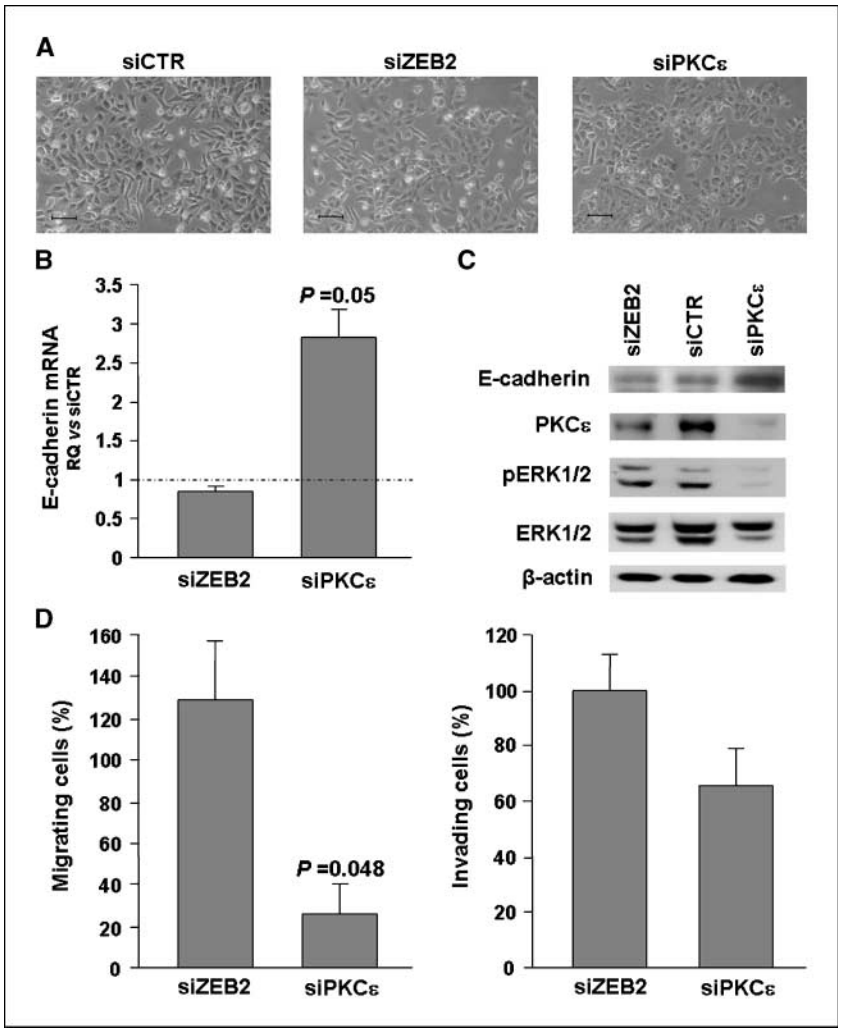

Figure 5. Knockdown of PKCε rather than ZEB2 phenocopies miR-205 ectopic expression in DU145 cells. *A,* representative microphotographs showing the morphology in siRNA-transfected cells. *Scale bar,* 100 μm. *B,* quantification of E- cadherin expression in siZEB2- or siPKCε-transfected cells. Data are reported as mean RQ ± SD with respect to siCTR-transfected cells. *C,* representative immunoblotting showing E-cadherin, PKCε, pERK1/2, and total ERK1/2 levels in siRNA-transfected cells. *D,* percentage of migrating and invading cells after transfection with siZEB2 and siPKCε compared with siCTR-transfected cells. Migrating and invading siCTR-transfected cells were 82 ± 16 and 1,163 ± 185/mm^2, respectively.

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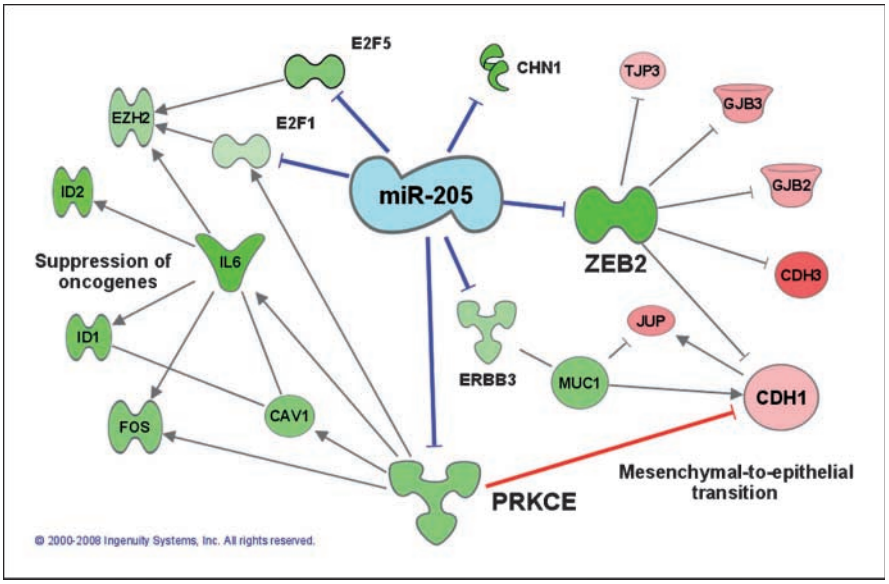

Figure 6. Hypothetical miR-205 network. The network was built on the basis of microarray data using Ingenuity Pathway Analysis and modified according to our findings. Down-regulated (*green*) and up-regulated (*red*) genes upon miR-205 ectopic expression are reported. →, induction; ⊣, repression; —, binding/interaction.

in tumor samples from patients with local-regionally disseminated disease.

These findings, together with those of Porkka and colleagues (5), showing reduced miR-205 expression in androgen-refractory tumors with respect to benign prostatic hyperplasias, suggest that miR-205 down-regulation may concur to PCa progression. To examine this possibility, excluding that miR-205 deregulation could merely represent a response to distinct oncogenic events, we restored miR-205 levels in DU145 and PC-3 cells. Its ectopic expression did not induce appreciable perturbations of cell proliferation or cell cycle progression (data not shown) but rather resulted in morphologic changes usually associated with the acquisition of a more epithelial phenotype. Consistent with loss of stress fibers and filopodia and accumulation of F-actin under the cell cortex (19), pre205-transfected cells showed impaired migratory and invasive capabilities. Altogether, our findings suggest a reversal of EMT, usually defined as MET, and the acquisition of a less invasive phenotype.

The essential features of EMT are the disruption of intercellular contacts and the enhancement of cell motility, thereby leading to the release of cells from the parent epithelial tissue, allowing cancer cell migration and, consequently, tumor invasion and dissemination (20, 21). One of the hallmarks of EMT is the loss of E-cadherin, the key player in inducing cell polarity and organizing epithelia, which is lost in most cancers of epithelial origin concomitantly with the progression toward malignancy (22). In PCa, E-cadherin was shown to correlate with dedifferentiation, local invasiveness, and dissemination of tumor cells into the blood. In addition, a contrasting increase in the expression of proteins associated with the mesenchymal phenotype (e.g., vimentin) has also been documented in high-grade PCa (23).

DU145 and PC-3 cells inherently show low levels of E-cadherin and high expression of vimentin. Moreover, DU145 subpopulations with increased invasiveness or metastatic potential were reported to have markedly decreased E-cadherin levels (24). We found that restoration of miR-205 in PCa cells resulted in a reduction of vimentin and in an increased expression of E-cadherin (although to a different extent in the two cell lines) and its intracellular partner β-catenin, which were mainly localized at cell-cell junctions. E-cadherin up-modulation occurred through overexpression of its mRNA, indicating that miR-205 directly or indirectly represses some of its transcriptional repressors, namely members of the ZEB, SNAIL, and bHLH families (25). In contrast, posttranscriptional mechanisms were presumably responsible for the increase in β-catenin protein level, given that β-catenin mRNA expression levels were not noticeably altered in either cell line upon miR-205 expression (data not shown). Suggestive of a functional link between miR-205 and E-cadherin, the expression levels of mature miR-205 and E-cadherin mRNA were directly correlated in our set of clinical prostate samples. Altogether, these findings strongly suggest that changes in morphology, enhancement of cell-cell adhesion, and reduction of motility/invasion observed in pre205-tranfected cells are part of a MET. In line with this, Gregory and colleagues (26) recently reported that miR-205 cooperates with miR-200 family members to initiate MET in mesenchymal Madin Darby canine kidney cells stably transfected with the protein phosphatase Pez (MDCK-Pez).

Gene expression profiling revealed that ectopic expression of miR-205 in DU145 cells mainly resulted in the up-modulation of factors involved in the organization of epithelia and in the assembly of cell-cell junctions. In addition, miR-205 induced the down-regulation of several genes known to contribute to PCa progression, such as *IL6, EZH2, FOS, MUC1*, and *CAV1* (1, 27), supporting the hypothesis that it might function as a tumor suppressor in prostate cells. In a preliminary *in vivo* experiment, pre205-transfected DU145 cells produced smaller tumors than preNeg-transfected cells after xenotransplantation into nude mice, although a comparable proliferation rate in terms of Ki67 staining was observed in the two groups of tumors (data not shown). It could be hypothesized that miR-205–expressing cells, which are characterized by decreased motility and invasive capability, have a reduced potential to grow *in vivo* as a result of limited access to the vasculature. In fact, recent results have shown that mice injected with SUM149 breast cancer cells overexpressing miR-10b bore larger tumors than mice injected with control cells, despite an overall comparable number of Ki67-positive cells, suggesting that increased invasiveness, rather than proliferation, might afford miR-10b overexpressing cells better access to the vasculature (28).

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Microarray profiles also showed down-regulation of several genes that are predicted to be putative miR-205 targets. We hypothesize that the tumor-suppressive functions of miR-205 are connected with the concurrent regulation of some of these, such as *CHN1, ERBB3, E2F5, E2F1, ZEB2*, and *PRKCE*. N-chimaerin is a GTPase-activating protein showing activity toward the small GTPase Rac (29). In our experimental model, its down-regulation could result in the loss of filopodia and reduction of cell migration, based on the evidence that microinjection of N-chimaerin into fibroblasts and neuroblastoma cells mediates the simultaneous formation of lamellipodia and filopodia (30). Similarly, the motility and invasion of PCa cells could be impaired by the suppression of ErbB3, a member of the epidermal growth factor receptor family, which was reported to enhance the invasiveness of PC-3 cells by stimulating the secretion of osteonectin by bone (31). miR-205 could further halt PCa progression through the down-regulation of E2F5 and E2F1. In fact, the pRB-E2F pathway was shown to induce expression of the polycomb group protein EZH2 (32), which is overexpressed in metastatic PCa and is a marker of aggressiveness in clinically localized solid tumors (33).

ZEB2 could represent another key miR-205 target because of its involvement in sustaining EMT and invasion of cancer cells through the repression of E-cadherin transcription (25). ZEB2 is predicted to have two highly conserved binding sites for miR-205 within its 3′-UTR. We showed that ectopic expression of miR-205 in PCa cells reduced ZEB2 mRNA levels and increased E-cadherin transcription. In addition, other genes coding for crucial proteins of tight junctions, desmosomes, and gap junctions, which have been reported to be repressed by ZEB2 (34), were up-modulated in DU145 cells ectopically expressing miR-205. Consistent with our findings, Gregory and colleagues (26) showed that miR-205 and miR-200 family members initiate MET in MDCK-Pez cells through the down-regulation of ZEB1 and ZEB2. Unexpectedly, when we silenced ZEB2 using a specific siRNA, neither a dramatic change in morphology nor significant up-regulation of E-cadherin was observed. Accordingly, the migratory and invasive properties of DU145 cells were not impaired. An explanation for this discrepancy could reside in the degree of suppression of the ZEB2 protein. Although pre205 and siZEB2 produced comparable down-regulation of ZEB2 mRNA, it is plausible that the former further affects ZEB2 expression at the translational level, resulting in a more marked modulation of its downstream targets. However, using the commercially available antibodies, we failed to properly detect the ZEB2 protein. Alternatively, despite being a validated target of miR-205 (26), ZEB2 may not play a major role in regulating EMT, or MET, in our model. The role of ZEB2 in PCa has not yet been investigated, and little is known about the determinants of EMT in prostate tissue. Very recently, it has been reported that the other member of the ZEB family, ZEB1, is expressed in highly aggressive PCa cells, and its inhibition results in E-cadherin up-regulation with concomitant reduction of mesenchymal markers (35). We can speculate that in prostate tissue miR-205 might regulate E-cadherin transcription mainly through repression of ZEB1 rather than ZEB2.

Another plausible miR-205 target is PKCε, a serine/threonine kinase member of the novel PKC subfamily (36). PKCε was reported to play a role in sustaining migration and invasion in different experimental models as well as to promote autocrine cell-signaling events in PCa (37), where its expression levels have been recently shown to correlate with aggressiveness of the disease (38). Its overexpression is sufficient to transform androgen-dependent LNCaP cells into an androgen-independent variant that rapidly initiates tumor growth *in vivo* in both intact and castrated male nude mice (37). Such transformation is associated with accelerated proliferation, resistance to apoptosis, hyperphosphorylation of ERK1/2 and Rb protein, and increased expression of E2F1, c-myc and caveolin-1 (37). PKCε-mediated induction of caveolin-1 was also shown to support the growth of CWR-R1 cells, selected for androgen-independent growth from recurrent CWR22 PCa tumors, which themselves inherently up-regulate PKCε (39). Moreover, PKCε overexpression in PCa was associated with increased levels of IL-6 (38)—an autocrine and paracrine growth factor (1)—and its RNAi-mediated knockdown impaired the invasive ability of DU145 cells (38).

We showed that ectopic expression of miR-205 in PCa cells resulted in the reduction of PKCε mRNA and protein, an event that was sufficient to affect some of its downstream targets. Specifically, it reduced the expression of IL-6, caveolin-1, and E2F1, as evidenced in microarray profiles, and the phosphorylation of ERK1/2 kinase. In addition, miR-205 impaired the motility and invasion of DU145 and PC-3 cells, which is consistent with the well-known functions of PKCε.

We wondered whether other tumor-suppressive functions of miR-205 could themselves rely on the suppression of PKCε, in particular the capability to initiate MET. Strikingly, RNAi-mediated silencing of PKCε in DU145 cells produced a phenotype that was largely reminiscent of that of miR-205–expressing cells, with marked alteration of cell morphology and reduction of migratory and invasive potential. In parallel, a net up-modulation of E-cadherin was observed, suggesting that PKCε suppression may itself mediate MET induced by miR-205. No evidence of the regulation of E-cadherin expression and EMT by PKCε had been previously reported.

Overall, our results highlight tumor-suppressive functions of miR-205 in PCa. In this context, miR-205 may contribute to sustain epithelial cell phenotype and tissue organization, repressing several factors known to be involved in the acquisition of a motile and invasive behavior and increasing cell-cell adhesion. Therefore, down-regulation of miR-205 could represent an oncogenic event that drives the progression toward a cell phenotype with reduced E-cadherin expression and enhanced invasive properties. Presumably, loss of miR-205 might also favor metastasis. We showed that miR-205–mediated regulation of EMT, recently reported to occur via the repression of ZEB1 and ZEB2 in a canine model (26), widely relies on the suppression of PKCε in prostate cells. These findings suggest miR-205 as a possible tool to reprogram the phenotype of PCa cells toward a less malignant state and unravel novel functions for PKCε in supporting the development and progression of the disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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