The p63 Protein Isoform ΔNp63α Inhibits Epithelial-Mesenchymal Transition in Human Bladder Cancer Cells

**ROLE OF MIR-205**

Received for publication, August 5, 2012, and in revised form, December 13, 2012 Published, JBC Papers in Press, December 13, 2012, DOI 10.1074/jbc.M112.408104

Mai N. Tran,‡‡‡ Woonyoung Choi,§ Matthew F. Wszolek, Neema Navai, I-Ling C. Lee, Giovanni Nitti,§§ Bogdan Czerniak,‡‡ Colin Dinney, Michelle Barton,¶ and David J. McConkey

From the Departments of ‡‡‡ Urology, **Genitourinary Medical Oncology, ‡‡ Cancer Biology, ‡ Biochemistry and Molecular Biology, §§ Pathology, and † Biostatistics, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77054 and the ‡‡‡ University of Texas Graduate School of Biomedical Sciences (GSBS) at Houston, Houston, Texas 77030

**Background:** ΔNp63 expression correlates with an epithelial phenotype and adverse clinical outcome.

**Results:** ΔNp63α suppressed ZEB1/2 and invasion in part by promoting miR-205 transcription, and tumor miR-205 expression is a marker of poor survival.

**Conclusion:** ΔNp63α inhibits EMT in part via miR-205.

**Significance:** We show that ΔNp63α directly regulates miR-205 and that these effects contribute to EMT suppression. The results provide important insight into the biology of lethal bladder cancer.

Epithelial-mesenchymal transition (EMT) is a physiological process that plays important roles in tumor metastasis, “stemness,” and drug resistance. EMT is typically characterized by the loss of the epithelial marker E-cadherin and increased expression of EMT-associated transcriptional repressors, including ZEB1 and ZEB2. The miR-200 family and miR-205 prevent EMT through suppression of ZEB1/2. p53 has been implicated in the regulation of miR-200c, but the mechanisms controlling miR-205 expression remain elusive. Here we report that the p53 family member and p63 isoform, ΔNp63α, promotes miR-205 transcription and controls EMT in human bladder cancer cells. ΔNp63α, E-cadherin, and miR-205 were coexpressed in a panel of bladder cancer cell lines (n = 28) and a cohort of primary bladder tumors (n = 98). Stable knockdown of ΔNp63α in the “epithelial” bladder cancer cell line UM-UC6 decreased the expression of miR-205 and induced the expression of ZEB1/2, effects that were reversed by expression of exogenous miR-205. Conversely, overexpression of ΔNp63α in the “mesenchymal” bladder cancer cell line UM-UC3 induced miR-205 and suppressed ZEB1/2. ΔNp63α knockdown reduced the expression of the primary and mature forms of miR-205 and the miR-205 “host” gene (miR-205HG) and decreased binding of RNA Pol II to the miR-205HG promoter, inhibiting miR-205HG transcription. Finally, high miR-205 expression was associated with adverse clinical outcomes in bladder cancer patients. Together, our data demonstrate that ΔNp63α-mediated expression of miR-205 contributes to the regulation of EMT in bladder cancer cells and identify miR-205 as a molecular marker of the lethal subset of human bladder cancers.

Epithelial-mesenchymal transition (EMT) is a reversible process that plays important roles in development and wound healing by which cells exhibit decreased cell polarity, cell-cell and cell-basement membrane adhesion, and increased migratory and invasive capacity. EMT plays important roles in tumor metastasis, “stemness,” and drug resistance (1, 2). Therefore, identifying the molecular mechanisms that control EMT is a top priority in ongoing cancer research. The hallmark change that characterizes EMT is loss of the epithelial marker E-cadherin, which is mediated by up-regulation of transcriptional repressors (ZEB1, ZEB2, Twist, Snail, and Slug) that directly bind to bipartite E-box elements located within the proximal E-cadherin promoter (1). Conversely, recent studies demonstrated that members of the miR-200 family and miR-205 promote E-cadherin expression and the “epithelial” phenotype by repressing the expression of ZEB1 and ZEB2 (3–6). However, the upstream molecular mechanisms that control the expression of these epithelial miRNAs remain unclear.

The p63 proteins are homologues of p53 that play central roles in epithelial development (7, 8). They exhibit substantial sequence and structural homology to p53, especially in their DNA binding domains, and they bind to p53 response elements (p53REs) in vitro and in vivo (9). The TP63 gene contains two promoters that produce two groups of protein isoforms: the full-length TAp63 group that contains functional N-terminal transcriptional transactivation (TA) domains and the ΔNp63 group, which lacks TA domains and is deficient in transcriptional transactivation. Alternative splicing at the C termini of both groups generates three different isoforms: α, β, and γ (7, 9). Only the α isoforms contain sterile α motif domains, which are involved in protein-protein interactions. Various p63 iso-
forms are highly expressed in the basal layers of epithelial tissues (including the urothelium), where they appear to play essential roles in stem cell homeostasis (10, 11). Interestingly, TAp63 can inhibit tumorigenesis and metastasis in vivo, suggesting its role as an important tumor suppressor (12, 13). ΔNp63, especially the ΔNp63α isoform, promotes survival, proliferation, and tumorigenesis, which are characteristics of an oncogene (14–16).

Bladder cancer (BC) is the fourth most common malignancy among men in the United States and is the costliest to clinically manage (17). The vast majority of BCs (90%) are carcinomas of the urothelium, the stratified transitional epithelium lining the urinary tract (National Cancer Institute, Ref. 55). BC progresses along two distinct tracts that pose distinct challenges for clinical management (18). Most tumors (75–80%) present as low-grade papillary non-invasive tumors that rarely progress to become lethal but almost always recur, and they require very expensive long-term management. This type of cancer is called “superficial” BC. The rest are high-grade tumors that invade the surrounding muscularis and can progress very rapidly to become metastatic. Despite multimodal therapy involving radical surgery and systemic chemotherapy, approximately half of patients with muscle-invasive bladder cancer (MIBC) succumb to the disease. Recent work has demonstrated that this lethal subset of MIBCs expresses high levels of ΔNp63 (10, 19). Therefore, obtaining a deeper understanding of the effects of ΔNp63 should provide important insights into the biology of the most lethal subset of MIBCs.

We noted recently that a close correlation between expression of ΔNp63 and E-cadherin exists in BC cell lines and primary tumors (19, 20). To determine whether a cause-effect relationship exists between the two, we used RNAi and whole genome expression profiling to identify EMT-related targets of ΔNp63 in human BC cells. Our data reveal a novel role for ΔNp63α in the regulation of miR-205 transcription. Specifically, ΔNp63α directly binds to a highly conserved region just upstream of the miR-205 start site and promotes increased binding of RNA polymerase II to the proximal promoter of the host gene of miR-205, resulting in increased transcription of both RNAs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Cell lines were obtained from the MD Anderson Bladder SPORE Tissue Bank and cultured as in Ref. 19. Their identities were verified by DNA fingerprinting using AmpFISTR® Identifiler® amplification (Applied Biosystems/Life Technologies, Grand Island, NY) or AmpFISTR® Profiler® PCR amplification (Applied Biosystems/Life Technologies) in the MD Anderson Characterized Cell Line core facility.

**Protein Overexpression and Gene Knockdown**—TAp63α (Open Biosystems/Thermo Scientific, Lafayette, CO, catalog no. EHS1001-7380111) and ΔNp63α (GeneCopoeia, Rockville, MD, catalog no. EX-Z5740-M02) were transfected into cells using Lipofectamine 2000 (Invitrogen/Life Technologies, catalog no. 11668-019) following the instructions provided by the manufacturer. The ΔNp63-specific siRNA (5’-AACAUGCCAGACUCAUUU-3’) was designed on the basis of a previous publication (21) and was synthesized by Dharmacon/Thermo Scientific. The non-targeting siRNA was from Dharmacon (catalog no. D-001810-10-20). siRNAs were transfected into cells using Lipofectamine RNAiMAX (Invitrogen/Life Technologies, catalog no. 13778-075). The pSilen1 lentiviral shRNA construct (V3LHS_397885) that targets all p63 isoforms and the pGIPZ empty vector (RHS4339) were purchased from Open Biosystems. The ΔNp63α stable expression construct was cloned from the ΔNp63α-pReceiver-M02 expression vector (Genecopoeia, catalog no. EX-Z5740-M02) and packaged into a lentivirus. The pre-miR-205 vector was from System Bioscience (Mountain View, CA, catalog no. CD511B-1). Virus production, virus infection, and infected cell selection were performed in the MD Anderson Vector Core as described in Ref. 20.

**RNA Isolation and Real-time Reverse Transcription PCR (qRT-PCR) Analysis**—RNA was isolated from cells using the mirVana™ miRNA isolation kit (Ambion/Life Technologies). The AgPath-ID One-Step RT-PCR kit (Applied Biosystems/Life Technology) was used for real-time reverse transcription PCR. To qualify mature miRNAs, 10 ng of total RNA was reverse-transcribed to cDNA using the Taqman microRNA reverse transcription kit (Applied Biosystems/Life Technologies) and miRNA-specific primers. After that, real-time PCR was performed to measure mature miRNA expression. Gene expression was calculated by the comparative ΔΔct method and displayed as relative quantity (RQ) ± RQ min and RQ max. Cyclophilin A was used as an endogenous control for mRNA expression, and U6snRNA was the endogenous control for mature miRNA expression. Taqman primers and probes were obtained from Applied Biosystems. Assay ID numbers and sequences are listed in supplemental Table 1. All PCR reactions were performed using either the ABI PRISM 7500 or the StepOne Plus PCR systems (ABI).

**Flow Cytometry**—Cells were detached by 10 mM EDTA. One million cells were used for each immunoreaction. Blocking was performed in incubation buffer (0.5% BSA in PBS) for 15 min at room temperature. A direct staining method was employed for detection of N-cadherin using an allophycocyanin-conjugated anti-human N-cadherin antibody (R&D Systems, Minneapolis, MN, catalog no. FAB6426A) following the protocol of the company. Allophycocyanin-conjugated sheep IgG was used as a negative control. Indirect staining was performed for P-cadherin using a polyclonal rabbit anti-P-cadherin antibody (Cell Signaling Technology, Boston, MA, catalog no. 2130) and Alexa Fluor 594-conjugated goat anti-rabbit IgG (H+L) (Invitrogen/Life Technology, catalog no. A11037) following a protocol from Cell Signaling Technology. Negative control samples were stained with the secondary antibody alone.

**Nuclear Run-on**—Experiments were performed as described in the short technical report (22) with minor modifications. Briefly, cells were lysed on ice, and nuclei were collected by centrifugation. Nuclei were then incubated with riboATP, riboCTP, rGTP (Epicenter Biotechnologies, Madison, WI, catalog no. RN02825), and Biotin-16-UTP (catalog no. BU6105H) in transcription buffer for 30 min at 29 °C. Reactions were then halted by adding a “stop” buffer containing 250 mM CaCl2 and 10 units/μl DNase I. RNA was purified, and biotin-labeled RNA was precipitated using magnetic beads coated with streptavidin.
(Dynabeads® M-280 Streptavidin, Invitrogen/Life Technologies, catalog no. 112.05D). High-capacity cDNA reverse transcription kits (Applied Biosystems/Life Technologies) were used to generate cDNA from the precipitated RNA, and qPCR was performed using the Fast SYBR Green Master Mix (Applied Biosystems/Life Technologies).

**Invasion Assays**—Cells were seeded into invasion inserts (UC6, 25 × 10^3 cells/insert; UC3, 15 × 10^3 cells/insert) of BD BioCoat™ Matrigel™ invasion chambers (BD Biosciences, catalog no. 354480) in triplicate. 3T3 conditioned medium was used as a chemotactic agent. The chambers were incubated at 37 °C in a 5% CO₂ incubator for 48 h. After incubation, Matrigel membranes were fixed in 1% glutaraldehyde and stained with gentian violet. Micrographs of the membranes were captured using an inverted microscope, and the numbers of invaded cells were counted using ImageJ software (Bethesda, MD).

**Immunoblotting**—Immunoblotting experiments were performed as described previously (20). Primary antibodies used in this study were anti-panp63 (clone 4A4, Santa Cruz Biotechnology, Santa Cruz, CA, catalog no. sc-8431), anti-ZEB1 (Cell Signaling Technology, catalog no. 3396), anti-N-cadherin (Invitrogen, catalog no. 33-3900), and anti-Slug (Santa Cruz Biotechnology, catalog no. sc-15391).

**ChIP Assay**—Experiments were performed using the ChIP-IT-Express kit from Active Motif (Carlsbad, CA, catalog no. 53009) according to the instructions from the manufacturer. For each ChIP reaction, we used 1–8 µg of antibody, either anti-panp63 (clone 4A4, Santa Cruz Biotechnology), anti-p53 (Millipore, Billerica, MA, catalog no. 17–13), anti-Pol II (Millipore, catalog no. 17–620), or normal mouse IgG (Millipore, catalog no. 12–371B). Precipitated DNA and the DNA input were amplified and analyzed by quantitative real-time PCR with SYBR Green qPCR Master Mix (Applied Biosystems/Life Technologies) using the primers listed in supplemental Table 1. Input DNA was used to normalize the values in each real-time PCR reaction. The relative enrichment of protein binding to target sequences is represented as RQ values (RQ = 2^(-ΔΔCt) × 100; ΔCt = Ct(ChIP) - Ct(Input)). Real-time-PCR reactions were performed in triplicate, and the results are presented as mean ± S.D. for the triplicate samples. Data are representatives of two to three independent experiments.

**Human Specimens**—Fresh frozen tumors from 98 patients obtained from the MD Anderson Genitourinary Cancer Tissue Bank were macrodissected to enrich for tumor content. Sample information and processing methods were described previously (19).

**Statistical Methods**—The primary objectives were to examine correlations between p63 and miR-205 expression and to evaluate the association between marker expression and overall survival (OS) and disease-specific survival (DSS). Tumors at stage Ta or T1 were classified as superficial, and stage ≥ T2 tumors were considered as muscle-invasive. Correlations among expression of markers were quantified using Spearman’s ρ coefficients. The Kaplan–Meier estimate of survival distribution was displayed by the investigated biomarker expression characterized as high and low (e.g. p63, miR-205), where the cutoff point to define high and low was obtained from regression tree analyses. The log-rank test was used to compare survival distributions between groups. All p values presented are two-sided. p < 0.05 were considered to be statistically significant. Statistical analyses were carried out using Splus 7 (Insightful Corp., Seattle, WA).

**Results**

**Δp63α Is the Most Abundant Isoform in Human BC Cell Lines**—Because p63 proteins exist as two groups of isoforms, TAp63 and ΔNp63, that potentially have different functions in cells, we compared their mRNA expression levels in a panel of human BC cell lines (n = 28) using primers that detect all p63 isoforms (panp63) as well as TA and ΔN isoform-specific primers. The levels of ΔNp63 were substantially higher than the levels of TAp63 in the majority of the cell lines (Fig. 1A, right panel). Moreover, the patterns of panp63 and ΔNp63 expression were very similar (Fig. 1A, compare the left and right panels), indicating that ΔNp63 is the most abundant mRNA isoform group in the BC cell lines. Immunoblot analyses of p63 protein expression using the monoclonal mouse anti-human panp63 antibody 4A4 in a representative subset of 14 BC cell lines revealed a strong band migrating at ~75 kDa in all cell lines that expressed high ΔNp63 mRNA levels (Fig. 1B). Among the six p63 isoforms, TAp63α, TAp63β, and ΔNp63α are each ~75 kDa in size (23). Because ΔNp63 was the most abundant isoform subgroup (Fig. 1A), the 75-kDa immunoreactive band most likely corresponded to ΔNp63α. To more directly test this idea, we overexpressed TAp63α and ΔNp63α in a cell line with very low endogenous panp63 expression (UC3) and analyzed the expressed proteins by immunoblotting with 4A4. The results confirmed that the endogenous 75-kD immunoreactive band corresponded to ΔNp63α (Fig. 1B).

**ΔNp63α Suppresses EMT**—Previous studies showed that p63 isoforms play crucial roles in maintaining the stem cell compartments of epithelial tissues (24, 25) and that p63 directly regulates the expression of several epithelial markers, including cytokeratins (CKs) 5 and 14 and P-cadherin (26, 27). Furthermore, we recently reported that p63 and E-cadherin expression correlated closely with one another in human BC lines and primary tumors (19, 20). However, other recent work suggests that normal epithelial stem cells and cancer stem cells from epithelial tissues possess features of EMT (28). Therefore, we first examined the expression of epithelial and mesenchymal markers in our whole panel of BC cell lines (n = 28) by qRT-PCR. As we had observed previously, expression of ΔNp63 correlated closely with E-cadherin expression and correlated inversely with the expression of ZEB1 and ZEB2 (Fig. 2A).

We then used a panp63 lentiviral shRNA construct to stably knock down the expression of all p63 isoforms in UC6, a representative "epithelial" BC cell line that expresses high levels of ΔNp63 mRNA and protein. Because ΔNp63α is the most abundant p63 isoform in BC cells (Fig. 1), we concluded that ΔNp63α is the primary isoform targeted by the panp63 shRNA construct. We also stably overexpressed ΔNp63α in UC3, a "mesenchymal" BC cell line that expresses low levels of all p63 isoforms at the RNA and protein levels (Fig. 1). Strikingly, the UC6 ΔNp63αKD cells exhibited morphological changes consistent with EMT, from displaying a characteristic epithelial polygonal appearance with discrete colonies to an elongated...
**ΔNp63α Regulates miR-205 Expression**

**FIGURE 1. ΔNp63α expression in urothelial carcinoma (BC) cells.** A, qRT-PCR quantification of panp63, TAp63, and ΔNp63 mRNA expression in a panel of BC cell lines (n = 28). The bars display the RQs of gene expression ± RQ max and RQ min. B, immunoblotting (IB) using the panp63 antibody (4A4, Santa Cruz Biotechnology) to detect all p63 isoforms in wild-type cells (n = 14) and in TAp63α- and ΔNp63α-overexpressing cells.

Spindle-like shape, whereas the UC3 ΔNp63α overexpressing cells acquired morphological characteristics that resembled epithelial cells (Fig. 2B). Functionally, cells that have undergone EMT display increased invasion. Consistent with the effects of ΔNp63α modulation on cell morphology, the UC6 ΔNp63αKD cells exhibited increased invasion compared with the UC6 cells infected with a non-targeting construct, whereas the UC3 ΔNp63α-overexpressing cells became less invasive than the corresponding empty vector-infected controls (Fig. 2C).

At the molecular level, EMT is characterized as the loss of epithelial markers and gain of mesenchymal markers. Therefore, we performed qRT-PCR and/or immunoblotting to examine the effects of modulating ΔNp63α expression in the UC6 and UC3 cells on the expression of epithelial and mesenchymal markers. Interestingly, the levels of several mesenchymal markers (ZEB1, ZEB2, and N-cadherin) were increased significantly in the UC6 ΔNp63αKD cells and decreased in the UC3 ΔNp63α-overexpressing cells, whereas expression of the epithelial markers CK-5 and CK-14 was decreased in the UC6 ΔNp63αKD cells and increased in the UC3 ΔNp63α-overexpressing cells (Fig. 3, A and B).

Cadherins, a family of calcium-dependent transmembrane glycoproteins, are major cell-cell adhesion molecules that play important roles in development and carcinogenesis (29). P-cadherin is a basal cell-specific epithelial marker in the prostate and the bladder (30, 31). On the other hand, N-cadherin, the widely accepted mesenchymal marker (32), is absent in normal bladder mucosa but aberrantly expressed in bladder tumors. To more precisely define the effects of ΔNp63α modulation on EMT, we measured surface P- and N-cadherin expression by two-color surface staining and flow cytometry (Fig. 3C). The results demonstrated that the UC6NT cells were double-positive for P- and N-cadherin, consistent with partial EMT (33) at baseline (Fig. 3C and data not shown). UC6 ΔNp63αKD exhibited reduced expression of P-cadherin and increased expression of N-cadherin, and a new population of cells emerged (~50% of the total) that were N-cadherin-positive but P-cadherin-negative (data not shown). These analyses demonstrate that ΔNp63αKD modulated the functionally relevant (surface) pools of P- and N-cadherin in the UC6 cells and that they were modulated across the entire cell population.

 Slug (SNAI2) was the only EMT-related marker that did not conform to this pattern. Expression of Slug was decreased by ΔNp63αKD in all of the cell lines we examined and was increased in the UC3 cells transduced with ΔNp63α (Fig. 3, A and B, and supplemental Fig. S1). This observation indicates that ΔNp63α promotes some mesenchymal characteristics and may help to explain the “partial EMT” (33) phenotype that is observed in the parental UC6 cells at baseline.

ΔNp63α Expression Correlates with miR-205 Expression in BC Cell Lines and BC Primary Tumors—ZEB1 and ZEB2 are canonical EMT markers that function to directly suppress E-cadherin expression (34, 35). The close correlation between ΔNp63α and E-cadherin expression as well as the inverse correlation between ΔNp63α and ZEB1/2 drew our interest to the possible relationship between ΔNp63α and ZEB1/2. Because p63 interacts with p53REs (9), we first searched for p53REs in the ZEB1 and ZEB2 promoters but failed to find any, suggesting that ΔNp63α does not control expression of ZEB1 and ZEB2 directly. We then used gene expression profiling (Illumina HT12V4 chips) to identify all of the EMT-related changes induced by ΔNp63αKD in triplicate RNA isolates obtained from UC6, and another p63-positive BC line (UC14), cells transduced with the non-targeting lentiviral vector, and cells...
transduced with the panp63 shRNA construct. One of the most striking and consistent alterations was down-regulation of the primary form of miR-205 (data not shown), a known direct inhibitor of ZEB1 and ZEB2 (3, 5).

A recent study concluded that p53 also inhibits EMT by regulating the expression of miR-200c (36). Therefore, we measured expression of the five members of the miR-200 family in the isolates, but we did not observe down-regulation of miR-
200c or any of the other family members in either cell line (data not shown).

To confirm our gene expression profiling data, we performed qRT-PCR using primers for panp63, \( \Delta \text{Np63} \), the primary form of miR-205 (pri-miR-205) and the mature form of miR-205 (miR-205) in RNA isolated from the 28 BC cell lines in our panel. Statistical analyses revealed a strong correlation among the expression levels of these markers (Spearman \( \rho > 0.79, p < 0.0001 \)) (Fig. 4, A and B, and supplemental Table 2). The close correlation between the expression of the primary and mature forms of miR-205 in the majority of the cell lines suggests that transcription rather than miRNA processing plays a central role in maintaining mature miR-205.

We also compared the expression of panp63 and mature miR-205 in a cohort of 32 superficial and 66 muscle-invasive primary BCs from patients. Again, the results indicated that a close correlation existed between the two (Spearman \( \rho = 0.44, p < 0.00001 \)) (Fig. 4C). Because \( \Delta \text{Np63} \alpha \) is the major isoform present in BC cell lines (Fig. 1) and BC primary tumors (19, 37), the results support the data obtained from our gene expression profiling studies implicating \( \Delta \text{Np63} \alpha \) in the regulation of miR-205 expression.

\( \Delta \text{Np63} \alpha \) Regulates ZEB1/2 by Modulating miR-205—To further examine the relationship between \( \Delta \text{Np63} \alpha \) and miR-205, we used quantitative RT-PCR to measure the primary and mature forms of miR-205 in the UC6 \( \Delta \text{Np63} \alpha \)-KD and UC3 \( \Delta \text{Np63} \alpha \)-overexpressing cells. Consistent with the gene expression profiling data, \( \Delta \text{Np63} \alpha \)-KD in UC6 decreased the expression of both primary and mature forms of miR-205, whereas overexpression of \( \Delta \text{Np63} \alpha \) in UC3 resulted in the opposite effects, indicating that \( \Delta \text{Np63} \alpha \) directly or indirectly modulated miR-205 expression (Fig. 5A). We confirmed these results in four additional “epithelial” BC lines (UC14, UC17, UC5, and SW780) (supplemental Fig. S2A). We also confirmed the results using an independent \( \Delta \text{Np63} \)-specific siRNA, which also decreased miR-205 expression in the UC6 cells (supplemental Fig. S2B). To determine whether decreased miR-205 expression mediates the effect of \( \Delta \text{Np63} \alpha \)-KD on ZEB1 and ZEB2 expression, we overexpressed miR-205 in the UC6 \( \Delta \text{Np63} \alpha \)-KD cells. Overexpression of exogenous miR-205 largely reversed the increased ZEB1 and ZEB2 expression induced by \( \Delta \text{Np63} \alpha \)-KD (Fig. 5B), confirming that decreased miR-205 expression plays an important role in the response. The relationship between \( \Delta \text{Np63} \alpha \) and EMT is summarized in Fig. 5C.

\( \text{MiR-205 Is Regulated via Its “Host” Gene} \) —Genomic localization analyses of miRNAs indicate that they can be grouped into two classes, intergenic miRNAs and intragenic miRNAs. Intergenic miRNAs are located between genes and are controlled as independent transcriptional units. Intragenic miRNAs are located within annotated genes which are considered the “host” genes for the miRNAs (38). The transcription patterns of intragenic miRNAs and their “host” genes suggest that this class of miRNAs is transcribed in parallel with their “host” genes (39, 40). The genomic location of miR-205 overlaps the junction between the last intron and the last exon of a “host” gene that has been termed miR-205HG (miR-205 “host” gene), formerly known as LOC642587. MiR-205HG is a protein coding gene that contains four exons and three introns (Fig. 6A). We performed quantitative RT-PCR using primers hybridizing to the exon 2 and 3 junctions of miR-205HG to determine the effects of \( \Delta \text{Np63} \alpha \) knockdown or overexpression on miR-205HG expression. The results showed that the expression of miR-205HG was changed in parallel with miR-205 when \( \Delta \text{Np63} \alpha \) expression was modified (Fig. 7A and Fig. S2A). The data confirm that there is a link between expression of miR-205 and its “host” gene and that expression of both is coordinated by \( \Delta \text{Np63} \alpha \).

Steady-state mRNA levels are controlled by a balance between transcription and RNA degradation. To determine the role of \( \Delta \text{Np63} \alpha \) in the transcriptional control of miR-205 and miR-205HG, we performed nuclear run-on experiments using biotin-labeled dUTP. This technique allowed us to directly measure the rates of transcription for miR-205HG and pri-miR-205 by real-time PCR. The rates of transcription for both pri-miR-205 and miR-205HG were decreased by over 50% in the UC6 \( \Delta \text{Np63} \alpha \)-KD cells compared with those observed in the UC6 NT cells (Fig. 7B).

Even though intragenic miRNAs may be transcribed together with their host genes, some reports have concluded that intragenic miRNAs can also have their own promoters and be transcribed independently (41, 42). Analysis of the 1-kb region upstream of the miR-205 start site using the University of California, Santa Cruz Genome Browser revealed a region that was highly conserved across 46 different vertebrate species (region 2), similar to the promoter region of miR-205HG, which is the 1-kb region upstream of the first exon (region 1) (Fig. 6B). Moreover, region 2 is also hypersensitive to DNaseI (Fig. 6B), indicating its likely role as a regulatory region or functional promoter. Intriguingly, we identified a p53RE within region 2 that was also detected by Genomatix (Fig. 6, A and B). A p53 response element generally contains two tandem copies of a 10-bp sequence homologous to the consensus binding motif 5′-PuPuPuC(A/T)(A/T)GPyPyPy, separated by a 0- to 13-bp spacer (43). Each binding motif, which is comprised of a core sequence (C(A/T)(A/T)G) and its flanking sequences (PuPuPu and PyPyPy), is considered to be a half-site of the p53RE. The p53RE identified in region 2 is a canonical whole-site p53RE with only one mismatch in the flanking sequence (Fig. 6A). However, there were no canonical p53REs within the proximal promoter of miR-205HG. ChiP using primers specific for region 1, region 2, or an intronic region 2.5 kb away from the last exon of miR-205HG (region 5) confirmed that \( \Delta \text{Np63} \alpha \) only binds to region 2 (Fig. 7C). The binding of \( \Delta \text{Np63} \alpha \) to...
region 2 was reduced in the UC6 ΔNp63αKD cells, indicating that the binding was specific (supplemental Fig. S3). To determine whether region 1 or region 2 is the promoter for miR-205, we performed ChIP using an anti-RNA Pol II antibody. We observed strong enrichment of Pol II binding at region 1 and less binding at region 2, strongly suggesting that region 1 serves as the promoter for both miR-205HG and miR-205 (supplemental Fig. S4). More importantly, ΔNp63α knockdown significantly reduced the binding of Pol II at regions 1 and 2, demonstrating the importance of ΔNp63α in Pol II recruitment to miR-205 (Fig. 7D).

High miR-205 Expression Correlates with Adverse Clinical Outcomes—Recent studies concluded that high ΔNp63 expression correlates with unfavorable clinical outcomes in patients with MIBC (10, 19). Given that it is a downstream transcriptional target of ΔNp63, we wondered whether miR-205 might also serve as a biomarker for the lethal BC subset. Regression tree analyses were used to determine the cutoff point of miR-
205 expression as 1.76 within our dataset. In the whole cohort of tumors (superficial plus muscle-invasive), elevated expression of miR-205 was associated with a median DSS of 13.4 months and a median OS of 12 months, whereas low miR-205 expression was associated with a significantly better median DSS of 140 months and median OS of 69.1 months (p = 0.0001 for DSS and p = 0.0004 for OS) (Fig. 8A). When we confined the analyses to the MIBC subgroup, the association between high miR-205 expression and adverse clinical outcome was even more significant. Patients whose MIBCs expressed high miR-205 levels had a median DSS and OS of only 8.11 months, whereas those with tumors that expressed low miR-205 levels also had a median DSS of 140+ months and OS of 69.1 months (p < 0.0001 for DSS and p = 0.0004 for OS) (Fig. 8B). Therefore, like Np63, high miR-205 expression identifies the lethal BC subset.

DISCUSSION

Our data implicate ΔNp63α, the most abundant isoform of p63 expressed in BC, in the control of EMT. We also show, for the first time, that ΔNp63α binds to a highly conserved regulatory region upstream of the miR-205 start site, participates in the recruitment of RNA Pol II to the promoter of the miR-205 host gene (miR-205HG), and coordinates the transcription of both miR-205HG and miR-205. miR-205 transcriptional regulation is one mechanism by which ΔNp63α controls EMT because up- or down-regulation of ΔNp63α results in parallel changes in miR-205 levels and reciprocal effects on the canonical EMT inducers ZEB1 and ZEB2. However, our results also show that ΔNp63α controls the expression of several other EMT-related targets, and we do not think that they are all directly or indirectly regulated via miR-205. Therefore, future studies should be designed to identify the molecular mechanisms involved in these other EMT-related effects of ΔNp63α.

Interestingly, ΔNp63α has been shown to promote TGFβ-induced EMT in normal human keratinocytes (44). Our data demonstrate that ΔNp63α promotes the expression of at least one important mesenchymal marker in BC cells, Slug (SNAI2), consistent with the conclusion that ΔNp63α has some EMT-promoting effects. These data also show that the parental UC6
cells are not purely epithelial but exhibit a partial EMT phenotype at baseline (33). However, the overall EMT-promoting impact of ΔNp63α-dependent Slug expression on cellular morphology and invasion appears to be outweighed by the suppressive effects of ΔNp63α on EMT in our cell lines.

Because the domain (region 2) that physically interacts with ΔNp63α contains a whole-site p53RE, it is possible that p53 and p73 also interact with this region. Indeed, a previous paper (45) concluded that p53 binds to region 2 and controls the expression of miR-205 in breast cancer. We performed ChIP experiments to directly test this possibility but did not observe any enrichment of p53 binding at region 2 in the UC6 cells, which express wild-type p53 (supplemental Fig. S5). Furthermore, there was no correlation between the mutational status of p53 and the expression of miR-205 (or for that matter members of the miR-200 family) in our BC cell lines (46), suggesting that p53 is not centrally involved in maintaining expression of these epithelial miRNAs in BC cells. Importantly, our conclusions regarding the importance of ΔNp63α in regulating miR-205 expression are consistent with recent work in prostate cancer cells (47).

TAp63 plays a crucial role in suppressing metastasis via regulation of Dicer expression, which leads to downstream global effects on micro RNA expression (12). In our BC cell lines, the panp63 shRNA produced no changes in Dicer mRNA expression (supplemental Fig. S6), and in fact it actually led to increased miR-200c expression in the UC14 cells (data not shown). Given that our BC cells generally expressed very low levels of TAp63 and that the effects of ΔNp63α were associated with increased miR-205HG and miR-205 transcription, these observations are not surprising and do not contradict previous findings (12).

Similar to the majority of intragenic miRNAs, miR-205 is transcriptionally coregulated with its "host" gene, and ΔNp63α is somehow critical for this regulation. However, exactly how ΔNp63α promotes Pol II recruitment to miR-205HG promoter remains unresolved. In contrast to clear binding of ΔNp63α to region 2, our ChIP results indicate that ΔNp63α does not inter-

**FIGURE 6.** miR-205HG sequence analysis. A, map showing the positions of the p53REs and the positions of the primers for the examined regions (regions 1, 2, and 5). The positions were numbered on the basis of the potential transcription start site (TSS) directly 5′ of miR-205 (red, below) or on the basis of the TSS of the miR-205 host gene (miR-205HG, black, above). The sequence of the p53RE in region 2 was compared with the consensus p53 binding site in detail. The base that does not correspond to the p53RE consensus sequence is shown in lowercase.

B, conservation of region 2 (sequence from Blast search) among 46 vertebrate species plotted using the UCSC Genome Browser. Evolutionary conservation is measured by the PhyloP method. Predicted conserved sites are assigned positive scores (blue), whereas predicted fast-evolving sites have negative scores (red). The Digital DNaseI hypersensitive tract shows DNase hypersensitive regions tested in a large number of cell lines. The gray boxes represent the extent of the DNase hypersensitivity of the regions. The darkness is proportional to the signal strength in the cell lines. The number of the cell lines hypersensitive in the region is shown on the left. The location of p53RE is also denoted.
ΔNp63α binds to a regulatory region upstream of miR-205 and regulates the transcription of miR-205 and miR-205HG. A, qRT-PCR results for miR-205HG mRNA expression in ΔNp63αKD UC6 and ΔNp63α-expressing UC3. The Taqman probe for miR-205HG spans the junction of exons 2 and 3. The bars show the RQ of mRNA expression ± RQ max and RQ min. NT, non-targeting; Vec, vector. B, real time PCR results for miR-205HG and pri-miR-205 expression. Nuclear run-on experiments were used to measure the nascent transcripts generated from miR-205HG and miR-205. HG1 primers were located within exon 1 of miR-205HG. Amplicons generated from Pri1 overlap with the amplicons generated from the Taqman pri-miR-205 primers (ABI). Expression of GAPDH was used as an endogenous control. C, ChIP results showing that ΔNp63α binds upstream of the miR-205 start site in UC6. The bars represent the mean ± S.D. of RQ values for target proteins (IgG, ΔNp63α, and H3) in triplicate samples. Data are representative of two to three independent experiments. D, ChIP results comparing Pol II binding to target regions in UC6 NT and UC6 ΔNp63αKD cells. RQ values of Pol II binding to regions 1, 2, and 5 were normalized to RQ values of Pol II binding to GAPDH promoter. The bars represent the mean ± S.D. of normalized RQ values in triplicate samples. Two-tailed, unpaired Student’s t test was used to analyze the significance of the difference. *, p < 0.050; **, p < 0.01; ***, p < 0.001.

act directly with the miR-205HG proximal promoter. These negative results do not rule out the possibility that region 2 serves as a downstream enhancer or that ΔNp63α binds to an unidentified distal miR-205HG enhancer element. However, the fact that ΔNp63α lacks a full-length N-terminal transcriptional transactivation domain, generally associated with direct regulation of transcription, suggests that a different mechanism is probably involved.

ΔNp63α and its downstream target, miR-205, are markers of the epithelial phenotype. p63 is uniformly expressed in the basal layer of the normal urothelium, which contains urothelial stem cells (48), and in superficial BC, which is usually low grade and non-lethal (10). We have reported a correlation between elevated expression of ΔNp63α and adverse outcomes in patients with MIBCs (19). In this study, we observed that high miR-205 expression also correlates with poor outcomes in MIBC patients. Our conclusion that ΔNp63α coordinates the expression of multiple genes in BC cells to reinforce the “epithelial” phenotype and at the same time is associated with poor clinical outcomes in patients may seem somewhat paradoxical given that EMT is considered essential for tumor metastasis (2) and that metastasis is invariably associated with BC mortality. Our observation that ΔNp63α promotes the expression of Slug and therefore a partial EMT phenotype (33), coupled with the fact that ΔNp63α controls the expression of BC stem cell markers (including CK-5 and CK-14) (49), helps to resolve this paradox. Furthermore, emerging evidence indicates that EMT “plasticity” is crucial for productive metastasis (33, 50). Even though preclinical studies have clearly established the importance of EMT in metastasis, tumor metastases in patients express epithelial markers (51, 52), which has raised doubts about the relevance of the preclinical observations to the process of tumor metastasis in patients. However, a recent study provides an elegant resolution to this apparent contradiction. Using an inducible Twist expression construct in a popular mouse model of carcinogen-induced head and neck squamous cell carcinoma, the authors demonstrated that primary tumors use EMT to escape from the primary tumor, form circulating tumor cells, and extravasate into lymph nodes and distant organs but remain dormant unless they subsequently undergo mesenchymal-to-epithelial transition, which facilitates proliferation. Importantly, the circulating tumor cells in this model and in patients still express epithelial cytokeratins, indicating that the process involves a “partial EMT” (33). Therefore, it is possible that ΔNp63α expression is dynamically regulated during this process and that the cells in transit (i.e. circulating tumor cells) actually express lower levels of ΔNp63α than do cells within the pri-
mary tumor or metastases. In our future studies we plan to directly address this possibility in preclinical mouse models and circulating tumor cells from BC patients.

Although miR205 expression is associated with a lethal BC phenotype, this does not necessarily mean that miR205 drives lethal biology. Instead, it appears that miR205 is associated with poor outcomes because it is a marker of $\Delta Np63$ activity. Support for this conclusion comes from an ongoing study where we are using unsupervised hierarchical clustering of gene expression profiling data from MIBCs to determine whether discrete biological subsets exist within them (as has been demonstrated in breast cancers) (53). We have identified three discrete subsets within our MIBCs and in three other independent gene expression profiling datasets. Ingenuity pathway analyses revealed that BCs within the subset that is associated with the worst clinical outcomes is enriched for expression of $\Delta Np63$ downstream targets, including P-cadherin, CK-5, and CK-14. These cancers may possess a “basal” phenotype because they arose via neoplastic transformation of normal urothelial basal stem cells, whereas the other two subsets appear to have evolved from independent, more well differentiated “luminal” progenitors. Our results are also consistent with other recent work that identified CK-5 and CK-14 as markers of BC stem cells and poor outcomes in other cohorts of MIBCs (49, 54).

**Acknowledgments**—We thank the Vietnam Education Foundation for fellowship support to Mai Tran. We also thank Dr. Santosh Chauhan (Cancer Biology Department, University of Texas M.D. Anderson Cancer Center) for technical help with the ChIP experiments and Dr. Russell Braeuer (Cancer Biology Department, University of Texas M.D. Anderson Cancer Center) for help with the nuclear run-on experiments.

**REFERENCES**

1. Kalluri, R., and Weinberg, R. A. (2009) The basics of epithelial-mesenchymal transition. *J. Clin. Invest.* **119**, 1420–1428

2. McConkey, D. J., Choi, W., Marquis, L., Martin, F., Williams, M. B., Shah, J., Svatik, R., Das, A., Adam, L., Kamat, A., Siefker-Radtke, A., and Dinney, C. (2009) Role of epithelial-to-mesenchymal transition (EMT) in drug sensitivity and metastasis in bladder cancer. *Cancer Metastasis Rev.* **28**, 335–344

3. Gregory, P. A., Bert, A. G., Paterson, E. L., Barry, S. C., Tsykin, A., Farshid, G., Vadas, M. A., Khew-Goodall, Y., and Goodall, G. J. (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat. Cell Biol.* **10**, 593–601

4. Korpel, M., and Kang, Y. (2008) The emerging role of miR-200 family of
microRNAs in epithelial-mesenchymal transition and cancer metastasis. RNA Biol. 5, 115–119.

5. Gregory, P. A., Bracken, C. P., Bert, A. G., and Goodall, G. J. (2008) MicroRNAs as regulators of epithelial-mesenchymal transition. Cell Cycle 7, 3112–3118.

6. Adam, L., Zhong, M., Choi, W., Qi, W., Nicoloso, M., Arora, A., Calin, G., Wang, H., Siefker-Radtke, A., McConkey, D., Bar-Eli, M., and Dinney, C. (2009) miR-200 expression regulates epithelial-to-mesenchymal transition in bladder cancer cells and reverses resistance to epidermal growth factor receptor therapy. Clin. Cancer Res. 15, 5060–5072.

7. Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dötsch, V., Andrews, N. C., Caput, D., and McKeon, F. (1998) p35 homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol. Cell 2, 305–316.

8. Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R. T., Tabin, C., Sharpe, A., Caput, D., Crum, C., and McKeon, F. (1999) p35 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 398, 714–718.

9. Westfall, M. D., and Pietrenpol, J. A. (2004) p63. Molecular complexity in development and cancer. Carcinogenesis 25, 857–864.

10. Karni-Schmidt, O., Castillo-Martin, M., Shen, T. H., Gladoun, N., Domingo-Domenech, J., Sanchez-Carbaya, M., Li, Y., Lowe, S., Prives, C., and Cardon-Cardo, C. (2011) Distinct expression profiles of p63 variants during urothelial development and bladder cancer progression. Am. J. Pathol. 178, 1350–1360.

11. Blanpain, C., and Fuchs, E. (2007) p63. Revving up epithelial stem-cell potential. Nat. Cell Biol. 9, 731–733.

12. Su, X., Chakravarti, D., Cho, M. S., Liu, L., Gi, Y. J., Lin, Y. L., Leung, M. L., El-Naggar, A., Creighton, C. J., Suraokar, M. B., Wistuba, I., and Flores, E. R. (2010) TAp63 suppresses metastasis through coordinate regulation of Dicer and miRNAs. Nature 467, 986–990.

13. Guo, X., Keyes, W. M., Papazoglou, C., Zuber, J., Li, W., Lowe, S. W., Vogel, H., and Mills, A. A. (2009) TAp63 induces senescence and suppresses tumorigenesis in vivo. Nat. Cell Biol. 11, 1451–1457.

14. Yugawa, T., Narisawa-Saito, M., Yoshimatsu, Y., Haga, K., Ohno, S., Egawa, N., Fujita, M., and Kiyono, T. (2010) ΔNp63α repression of the Notch1 gene supports the proliferative capacity of normal human keratinocytes and cervical cancer cells. Cancer Res. 70, 4034–4044.

15. Keyes, W. M., Pecoraro, M., Aranda, V., Vermersson-Lindahl, E., Li, W., Vogel, H., Guo, X., Garcia, E. L., Michurina, T. V., Enikolopov, G., Muthuswamy, S. K., and Mills, A. A. (2011) ΔNp63α is an oncogene that targets chromatin remodeler Lsh to drive skin stem cell proliferation and tumorigenesis. Cell Stem Cell 8, 164–176.

16. Rieger-Christ, K. M., Cain, J. W., Braasch, J. O., Dugan, J. M., Silverman, M. L., Bouyounes, B., Libertino, J. A., and Summerhayes, I. C. (2001) Expression of classic cadherin type I in urothelial neoplastic progression. Hum. Pathol. 32, 18–23.

17. Jarrard, D. F., Paul, R., van Bokhoven, A., Nguyen, S. H., Bova, G. S., Wheelock, M. J., Johnson, K. R., Schalken, J., Bussmakers, M., and Isaacs, W. B. (1997) P-Cadherin is a basal cell-specific epithelial marker that is not expressed in prostate cancer. Clin. Cancer Res. 3, 2121–2128.

18. Lee, J. M., Dedhar, S., Kalluri, R., and Thompson, E. W. (2006) The epithelial-mesenchymal transition. New insights in signaling, development, and disease. J. Cell Biol. 172, 973–981.

19. Tsai, J. H., Donaher, J. L., Murphy, D. A., Chau, S., and Yang, J. Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. Cancer Cell 22, 725–736.

20. Comijn, J., Berg, G., Vernassen, P., Verschueren, K., van Grunsven, L., Bruyneel, E., Mareel, M., Huybrechts, K., and Van Roy, F. (2001) The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol. Cell 7, 1267–1278.

21. Eger, A., Aigner, K., Sonderegger, S., Dampier, B., Oehler, S., Schreiber, M., Berx, G., Cano, A., Beug, H., and Foisner, R. (2005) ΔE1F is transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. Oncogene. 24, 2375–2385.

22. Chang, C. J., Chao, C. H., Xia, W., Yang, J. Y., Xiong, Y., Li, C. W., Yu, W. H., Rehman, S. K., Hsu, J. L., Lee, H. H., Liu, M., Chen, C. T., Yu, D., and Hung, M. C. (2011) p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. Nat. Cell Biol. 13, 317–323.

23. Koga, F., Kawakami, S., Kumagai, I., Takizawa, T., Ando, N., Arai, G., Kageyama, Y., and Kihara, K. (2003) Impaired ΔNp63 expression associates with reduced β-catenin and aggressive phenotypes of urothelial neoplasms. Br. J. Cancer 88, 740–747.

24. Saini, H. K., Griffiths-Jones, S., and Enright, A. J. (2007) Genomic analysis of human microRNA transcripts. Proc. Natl. Acad. Sci. U.S.A. 104, 17719–17724.

25. Rodriguez, A., Griffiths-Jones, S., Ashurst, J. L., and Bradley, A. (2004) Identification of mammalian microRNA host genes and transcription units. Genome Res. 14, 1902–1910.

26. Baskerville, S., and Bartel, D. P. (2005) Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. RNA 11, 241–247.

27. Ozsolak, F., Poling, L. L., Wang, Z., Liu, H., Liu, X. S., Roeder, R. G., Zhang, X., Song, J. S., and Fisher, D. E. (2008) Chromatin structure analyses identify miRNA promoters. Genes Dev. 22, 3172–3183.

28. Corcoran, D. L., Pandit, K. V., Gordon, B., Bhattacharjee, A., Kaminiski, N., and Benos, P. V. (2009) Features of mammalian microRNA promoters.
emerge from polymerase II chromatin immunoprecipitation data. *PLoS ONE* 4, e5279
43. el-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992) Definition of a consensus binding site for p53. *Nat. Genet.* 1, 45–49
44. Oh, J. E., Kim, R. H., Shin, K. H., Park, N. H., and Kang, M. K. (2011) $\Delta$Np63$\alpha$ protein triggers epithelial-mesenchymal transition and confers stem cell properties in normal human keratinocytes. *J. Biol. Chem.* 286, 38757–38767
45. Piovan, C., Palmieri, D., Di Leva, G., Braccioli, L., Casalini, P., Nuovo, G., Tortoreto, M., Sasso, M., Plantamura, I., Triulzi, T., Taccioli, C., Tagliaabue, E., Iorio, M. V., and Croce, C. M. (2012) Oncosuppressive role of p53-induced miR-205 in triple negative breast cancer. *Mol. Oncol.* 6, 458–472
46. Sabichi, A., Keyhani, A., Tanaka, N., Delacerda, J., Lee, I. L., Zou, C., Zhou, J. H., Benedict, W. F., and Grossman, H. B. (2006) Characterization of a panel of cell lines derived from urothelial neoplasms. Genetic alterations, growth in vivo, and the relationship of adenoviral mediated gene transfer to Coxsackie adenovirus receptor expression. *J. Urol.* 175, 1133–1137
47. Gandellini, P., Profumo, V., Casamichele, A., Fenderico, N., Borelli, S., Petrovich, G., Santilli, G., Callari, M., Colecchia, M., Pozzi, S., De Cesare, M., Folini, M., Valdagni, R., Mantovani, R., and Zaffaroni, N. (2012) miR-205 regulates basement membrane deposition in human prostate. Implications for cancer development. *Cell Death Differ.* 19, 1750–1760
48. Kurzrock, E. A., Lieu, D. K., Degraffenried, L. A., Chan, C. W., and Isseroff, R. R. (2008) Label-retaining cells of the bladder. Candidate urothelial stem cells. *Am. J. Physiol. Renal Physiol.* 294, F1415–1421
49. Volkmer, J. P., Sahoo, D., Chin, R. K., Ho, P. L., Tang, C., Kurtova, A. V., Willingham, S. B., Pazhanisamy, S. K., Contreras-Truillo, H., Storm, T. A., Lotan, Y., Beck, A. H., Chung, B. I., Alizadeh, A. A., Godoy, G., Lerner, S. P., van de Rijn, M., Shortliffe, L. D., Weissman, I. L., and Chan, K. S. (2012) Three differentiation states risk-stratify bladder cancer into distinct subtypes. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2078–2083
50. Polyak, K., and Weinberg, R. A. (2009) Transitions between epithelial and mesenchymal states. Acquisition of malignant and stem cell traits. *Nat. Rev. Cancer* 9, 265–273
51. Chao, Y. L., Shepard, C. R., and Wells, A. (2010) Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition. *Mol. Cancer* 9, 179
52. Hugo, H., Ackland, M. L., Blick, T., Lawrence, M. G., Clements, J. A., Williams, E. D., and Thompson, E. W. (2007) Epithelial-mesenchymal and mesenchymal-epithelial transitions in carcinoma progression. *J. Cell Physiol.* 213, 374–383
53. Perou, C. M., Serlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S. X., Lamming, P. E., Berresen-Dale, A. L., Brown, P. O., and Botstein, D. (2000) Molecular portraits of human breast tumours. *Nature* 406, 747–752
54. Chan, K. S., Espinosa, I., Chao, M., Wong, D., Ailles, L., Diehn, M., Gill, H., Presti, J., Chang, H. Y., van de Rijn, M., Shortliffe, L., and Weissman, I. L. (2009) Identification, molecular characterization, clinical prognosis, and therapeutic targeting of human bladder tumor-initiating cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 14016–14021
55. Johansson, S. L., and Cohen, S. M. (1997) Epidemiology and etiology of bladder cancer. *Semin. Surg. Oncol.* 13, 291–298