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Epithelial–mesenchymal transition promotes SOX2 and NANOG expression in bladder cancer

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Bladder cancer is the most common malignant tumor of the urothelium and is classified into non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). Stemness markers such as SOX2 and NANOG are frequently overexpressed in various aggressive cancers, including MIBC; epithelial–mesenchymal transition (EMT) has been proposed as a potential trigger of stemness in cancers. To determine whether cancer stemness is acquired via EMT in bladder cancer, we studied the effect of EMT on the expression of SOX2 and NANOG in bladder cancer cell lines. We also analyzed their expression in clinical tissue samples. Our results revealed that a potent EMT inducer (transforming growth factor β1) reduced the expression of the epithelial marker E-cadherin and increased expression of both SOX2 and NANOG in epithelial-type bladder cancer cells. As for clinical bladder cancer samples, in NMIBC, E-cadherin expression was slightly diminished, and the expression of both SOX2 and NANOG was negligible. In contrast, in MIBC, E-cadherin expression was highly and heterogeneously diminished, while the expression of both SOX2 and NANOG was increased. We also noticed that either E-cadherin or SOX2 (or NANOG) was expressed (ie, in a manner exclusive of each other). In addition, the concentration of E-cadherin showed a significant negative correlation with tumor grade and stage, while expression of SOX2 and NANOG positively correlated with those clinicopathological parameters. These findings suggest that EMT promotes stemness of bladder cancer cells, contributing to tumor aggressiveness. This EMT–cancer stemness axis may also play an important role in the pathogenesis of NMIBC and MIBC.

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Bladder cancer, originating from the urothelial epithelium, is the most common cancer of the human urinary tract and is the ninth most common cancer worldwide; it is relatively frequent in developed countries.[1] Bladder cancers are categorized into two subtypes based on their clinicopathology: non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC).[2,3] Approximately 80% of patients with bladder cancer present with NMIBC, which is associated with lower mortality despite a high risk of intravesical tumor recurrence.[2,3] The remaining 20% of patients present with MIBC, which frequently invades surrounding tissues and metastasizes to lymph nodes and distant organs and poses a higher mortality risk.[2,3]

Epithelial–mesenchymal transition (EMT) is involved in cancer invasion and metastasis and is associated with poor prognosis of bladder cancer.[4] Cancer cells that have undergone EMT share numerous molecular characteristics with cancer stem cells or cancer stem-like cells (CSCs).[5] Notably, EMT was reported to have a role in the formation of CSCs in breast cancer.[6] Thereafter, EMT has been known to be important for acquisition of the CSCs phenotype.[7–10] Bladder CSCs were first reported in 2009;[11] since then, considerable evidence supporting the existence of bladder CSCs has been presented.[12–14] Unlike other tumor cells, bladder CSCs likely emerge from pre-existing cancer cells at the late stages of bladder cancer,[14] suggesting that

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bladder cancer cells acquire the phenotype of CSCs through EMT.[15]

Three transcription factors—SOX2, OCT3/4, and NANOG—coordinately determine self-renewal and differentiation in embryonic stem cells.[16] These molecules also perform an important function in oncogenesis-related processes. The target genes activated by these molecules are more frequently overexpressed in poorly differentiated tumors than in well-differentiated ones.[17] SOX2 and NANOG regulate self-renewal and maintenance in CSC populations.[18,19] SOX2 and NANOG expression levels have been reported to correlate well with the malignancy of bladder cancer.[20,21]

Here, our hypothesis was that EMT increases the expression of stemness markers SOX2 and NANOG in bladder cancer cell lines. We assessed the associations between an epithelial marker, E-cadherin, and stemness markers SOX2 and NANOG in clinical tumor samples. Our findings further support the significance of EMT and CSCs in the pathogenesis of bladder cancer.

MATERIALS AND METHODS

Cell Lines and Reagents

Human bladder cancer cell lines RT4, T24, and UM-UC-3 were acquired from the American Type Culture Collection (Manassas, VA, USA). The establishment of JTC-30 cells has been previously reported.[22,23] Both RT4 and JTC-30 cells were derived from patients with NMIBC and showed an epithelial phenotype,[22,24] whereas T24 and UM-UC-3 cells were derived from patients with MIBC and had a mesenchymal phenotype.[22,24,25] The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 10% of fetal bovine serum (Life Technologies, CA, USA). All the cells were cultured with 1% penicillin/streptomycin (Life Technologies) at 5% CO_2 and 37 °C. For induction of EMT, RT4 and JTC-30 cells were grown in serum-free DMEM for 24 h, and then cultured in the presence or absence of recombinant human transforming growth factor β1 (TGF-β1; Peprotech, NJ, USA) for additional 48 h.

Patients and Clinical Samples

The study protocol was reviewed and approved by the institutional review boards of the Tokyo Metropolitan Institute of Medical Science as well as the Tokyo Metropolitan Cancer and Infectious diseases Center Komagome Hospital. We analyzed surgical specimens obtained by transurethral resection or a biopsy from 63 patients with bladder cancer between 2013 and 2014. Histological types were determined according to the 2004 WHO classification of bladder cancers.[26] All the patients received a diagnosis of urothelial carcinoma, except one, who had adenocarcinoma. The average age of the patients was 71 years (ranging from 39 to 94 years). Patients were subdivided into pairs of groups based on clinical parameters for statistical analysis (age, ≤ 70 and ≥ 71; sex, men and women; primary tumor and recurrence; TNM stage, ≤ Stage I and ≥ Stage II; tumor grade, low-grade and high-grade; Ki-67 labeling index (LI), < 20% and ≥ 20%).

Real-Time PCR

Total RNA from the cultured cells was extracted using the ISOGEN II kit (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from the total RNA using the SuperScript VILO cDNA Synthesis Kit (Life Technologies). FastStart Universal SYBR Green Master Mix (Roche, Basel, Switzerland) and gene-specific primers were added, and real-time PCR for transcript quantification was conducted using the Stratagene Mx3005P multiplex quantitative PCR system. Sequences of the primers are given below (5′ → 3′):

E-cadherin, forward: 5′-CCCGGGACAACGTTTATTA-3′, reverse: 5′-GCTGGCTCAAGTCAAAGTCC-3′;

N-cadherin, forward: 5′-ACAGTGGCCACCTACAAAGG-3′, reverse: 5′-CCGAGATGGGGTTGATAATG-3′;

vimentin, forward: 5′-AAAGTGTGGCTGCCAAGAAC-3′, reverse: 5′-AGCCTCAGAGAGGTCAGCAA-3′;

and β-actin, forward: 5′-GTGGGGCGCCCCAGGCACCA-3′, reverse: 5′-TGGGTCATCTTCTCGCGGTT-3′.

Relative quantities of mRNA were calculated using the comparative C_T method after normalization to β-actin. All the experiments were carried out in triplicate and repeated at least twice.

An Immunoblot Assay

For cell lysis, the cells were washed with phosphate-buffered saline and resuspended in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% DOC [deoxycholic acid], 0.1% SDS, 50 mM Tris-HCl [pH 7.5], and 5 mM EDTA) with protease inhibitor cocktail tablets (Roche) and Benzonase Nuclease (Santa Cruz Biotechnology, Dallas, TX, USA). The protein concentration in each lysate was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). After SDS-PAGE, the proteins were transferred onto Immobilon-P transfer membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 20 min in Blocking One buffer (Nacalai Tesque) and probed overnight with primary antibodies for E-cadherin, ZO-1, SOX2, NANOG, tubulin (all from Cell Signaling Technology, Danvers, MA), fibronectin (Novus Biologicals, Littleton, CO), and β-actin (Sigma-Aldrich, St. Louis, MO, USA). A horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG antibody (GE Healthcare, Buckinghamshire, UK) served as the secondary antibody. Immunoreactive bands were visualized by means of Chemi-Lumi One (Nacalai Tesque). All the assays were performed at least three times. The protein levels were quantified by densitometry in the ImageJ software (National Institutes of Health, Bethesda, MD, USA) and were normalized to β-actin or tubulin.

Immunohistochemistry (IHC)

IHC was performed on 5-μm-thick formalin-fixed paraffin-embedded tissue slices. Hematoxylin and eosin (HE) staining was performed on all the samples. Antigen retrieval was carried out by autoclaving for 20 min in 50 mM citrate buffer. The slides were then reacted with primary antibodies against E-cadherin, SOX2, NANOG, and the proliferation marker Ki-67 (MIB-1; Dako Corporation, Carpenteria, CA, USA).

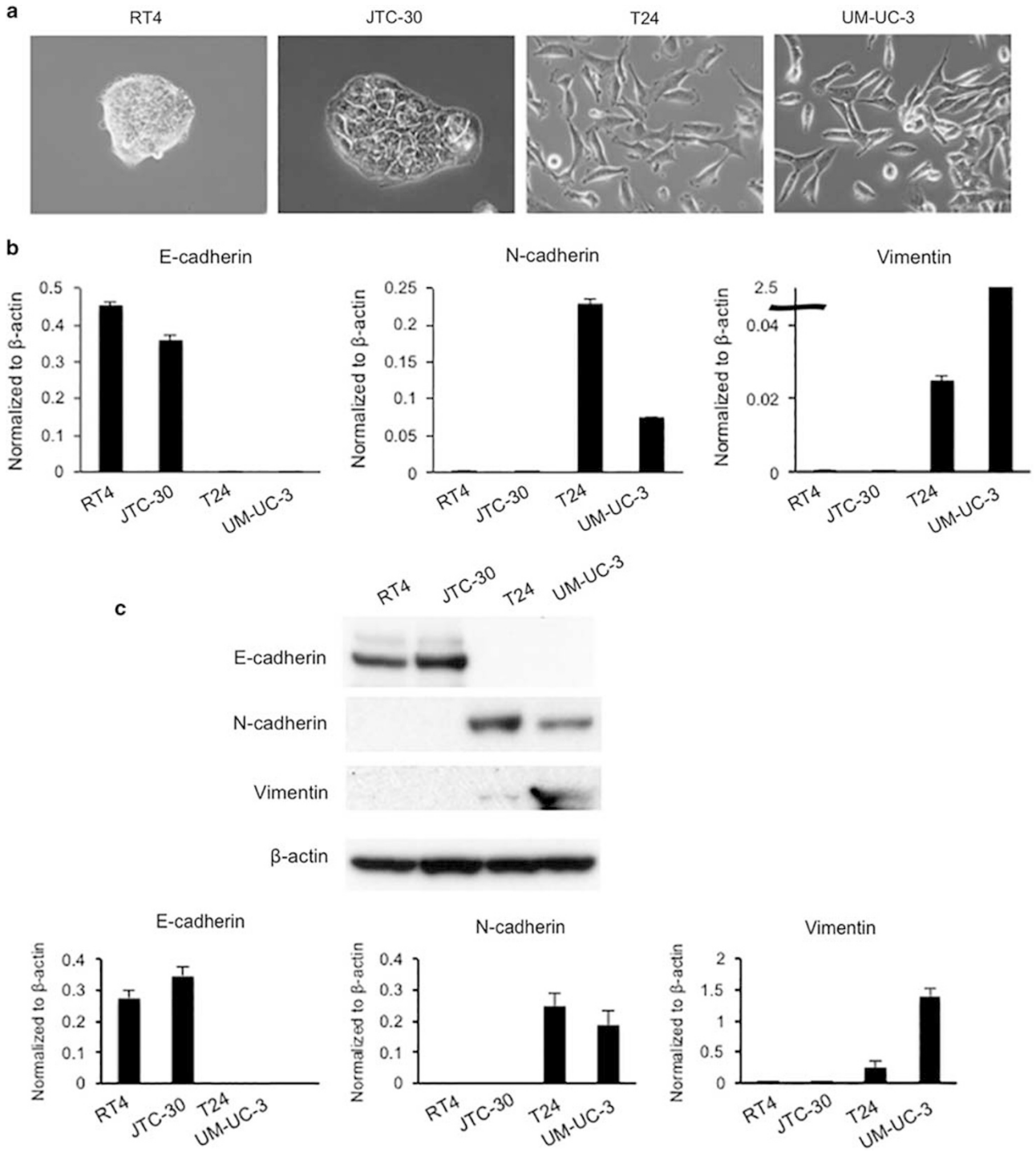


Figure 1 Phenotypic classification of bladder cancer cell lines by epithelial or mesenchymal markers. (**a**) Morphology of bladder cancer cell lines RT4, JTC30, T24, and UM-UC-3 in culture dishes. Cells were examined by phase contrast microscopy. Original magnification: ×200. (**b**) cDNA was synthesized from total RNA of each cell line, and real-time PCR was conducted for E-cadherin, N-cadherin, and vimentin. β-Actin served as an internal control. (**c**) An immunoblot assay for E-cadherin, N-cadherin, vimentin, and β-actin expression was performed on all cell lines. Bar graphs represent the average levels of each protein normalized to β-actin, as determined by densitometry.

The slides were developed by means of the VECTASTAIN Elite ABC kit and 3,3′-diaminobenzidine tetrahydro-chloride (DAB; Vector Laboratories, CA, USA). Hematoxylin was used as the counterstain. The expression and staining intensity on tissue slices were scored semiquantitatively by three authors (TM, AU, and SH) who did not have knowledge of the patients' clinical data. The scoring scale was as follows: 0 = no; +1 = weak; +2 = moderate; and +3 = strong. The Ki-67 labeling index (LI) was determined by enumerating more than 1000 tumor cells including Ki-67-positive and -negative cells, and was expressed as the percentage of cells positive for nuclear staining.

The Cancer Genomics Database

Gene expression datasets of E-cadherin, SOX2, and NANOG in bladder cancer were retrieved from the public database cBioPortal for cancer genomics (www.cbioportal.org). We obtained the profile of expression of these genes in bladder cancer from a study on Bladder Urothelial Carcinoma (TCGA, Provisional; 413 samples from 412 patients). The data from massively parallel high-throughput RNA sequencing (RNA-Seq) were processed by means of RNA Seq V2 RSEM (RNA-Seq by Expectation-Maximization) to estimate the abundance of the transcripts of these genes.

Statistical Analysis

Continuous variables were analyzed by two-tailed Student's *t* test with unequal variance. The statistically significant differences among the IHC scores on E-cadherin, NANOG, and SOX2 were examined by the Wilcoxon rank-sum test or Spearman's rank correlation coefficient test. The correlations between the clinicopathological parameters and the expression levels of E-cadherin, NANOG, and SOX2 were evaluated by Pearson's χ^2-test. The cBioPortal database provided Spearman's correlation coefficients for the correlations between E-cadherin and SOX2 and those between E-cadherin and NANOG. All statistical analyses were conducted in the JMP software, version 10 (SAS Institute, Cary, NC, USA). In all the analyses, data with $P<0.05$ were considered statistically significant.

RESULTS

EMT Induces Expression of Cancer Stemness Markers in Bladder Cancer Cells

Bladder cancer cell lines RT4, JTC-30, T24, and UM-UC-3 were cultured for 48 h, and cell morphology was examined by phase contrast microscopy. RT4 and JTC-30 cells proliferated while maintaining cell–cell adhesion, forming island-like colonies (Figure 1a, left two panels). By contrast, T24 and UM-UC-3 cells showed lesser adhesion and tended to proliferate individually (Figure 1a, right two panels). We next examined the expression levels of epithelial (E-cadherin) and mesenchymal markers (N-cadherin and vimentin) in these cells. RT4 and JTC-30 cells had high levels of the E-cadherin transcript; however, the N-cadherin and vimentin transcripts were undetectable (Figure 1b). This finding is consistent with other studies, which showed that both N-cadherin and vimentin are not expressed in these cell lines.[22,27–29] In contrast, T24 and UM-UC-3 cells had undetectable levels of the E-cadherin transcript but had plenty of N-cadherin and vimentin transcripts (Figure 1b). Furthermore, the E-cadherin, N-cadherin, and vimentin protein concentrations corresponded to the mRNA expression, as confirmed by the immunoblot assay (Figure 1c). These findings indicated that RT4 and JTC-30 cells had an epithelial phenotype, whereas T24 and UM-UC-3 cells had a mesenchymal phenotype.

TGF-β1 treatment induces EMT in urothelial cancer cells showing epithelial features.[30,31] In RT4 cells, which had an epithelial phenotype, we induced EMT by TGF-β1 treatment. The protein expression of the epithelial makers, E-cadherin and ZO-1, was reduced by TGF-β1 treatment in a dose-dependent manner (0–5 ng/ml), whereas protein concentration of the mesenchymal maker fibronectin increased (Figure 2). Furthermore, expression of SOX2 and NANOG increased in response to TGF-β1 treatment in a dose-dependent manner (Figure 2). We also confirmed TGF-β1-mediated SOX2 and NANOG expression in another epithelial cell line, JTC30 (Supplementary Figure 1). These results suggested that TGF-β1-induced EMT promoted the expression of stemness markers in bladder cancer cells.

Differential Expression Patterns of EMT and Stemness Markers in Clinical NMIBC and MIBC Samples

We next evaluated EMT and cancer stemness in clinical samples of bladder cancer tissues including NMIBC (pT1 or lesser) and MIBC (pT2 or greater) and performed IHC analysis for E-cadherin, SOX2, and NANOG.

In normal urothelial cells, E-cadherin was localized to the cell membrane, and its expression was abundant although both NANOG and SOX2 expression levels were miniscule in serial sections (Supplementary Figure 2). In cancer cells, E-cadherin expression was slightly decreased in NMIBC cells and remarkably decreased in MIBC cells (Figure 3a, upper panels). Cytoplasmic and nuclear NANOG expression was low or undetectable in NMIBC cells; however, it was strong in MIBC cells (Figure 3a, middle panels). In concordance with this expression pattern of NANOG, cytoplasmic and nuclear SOX2 expression was also low or undetectable in NMIBC cells but strong in MIBC cells (Figure 3a, lower panels).

Statistical analysis revealed that E-cadherin expression was significantly lower in MIBC cells than in NMIBC cells ($P<0.0001$, Figure 3b). In contrast, the expression levels of NANOG and SOX2 were significantly higher in MIBC cells as compared to NMIBC cells ($P<0.0001$ and $P<0.0001$, respectively; Figure 3b). To confirm that the decreased level of E-cadherin and increased levels of NANOG (or SOX2) are generally observed in MIBC, we analyzed the mRNA expression of E-cadherin, NANOG, and SOX2 in tumor samples from 412 patients with bladder cancer using a public

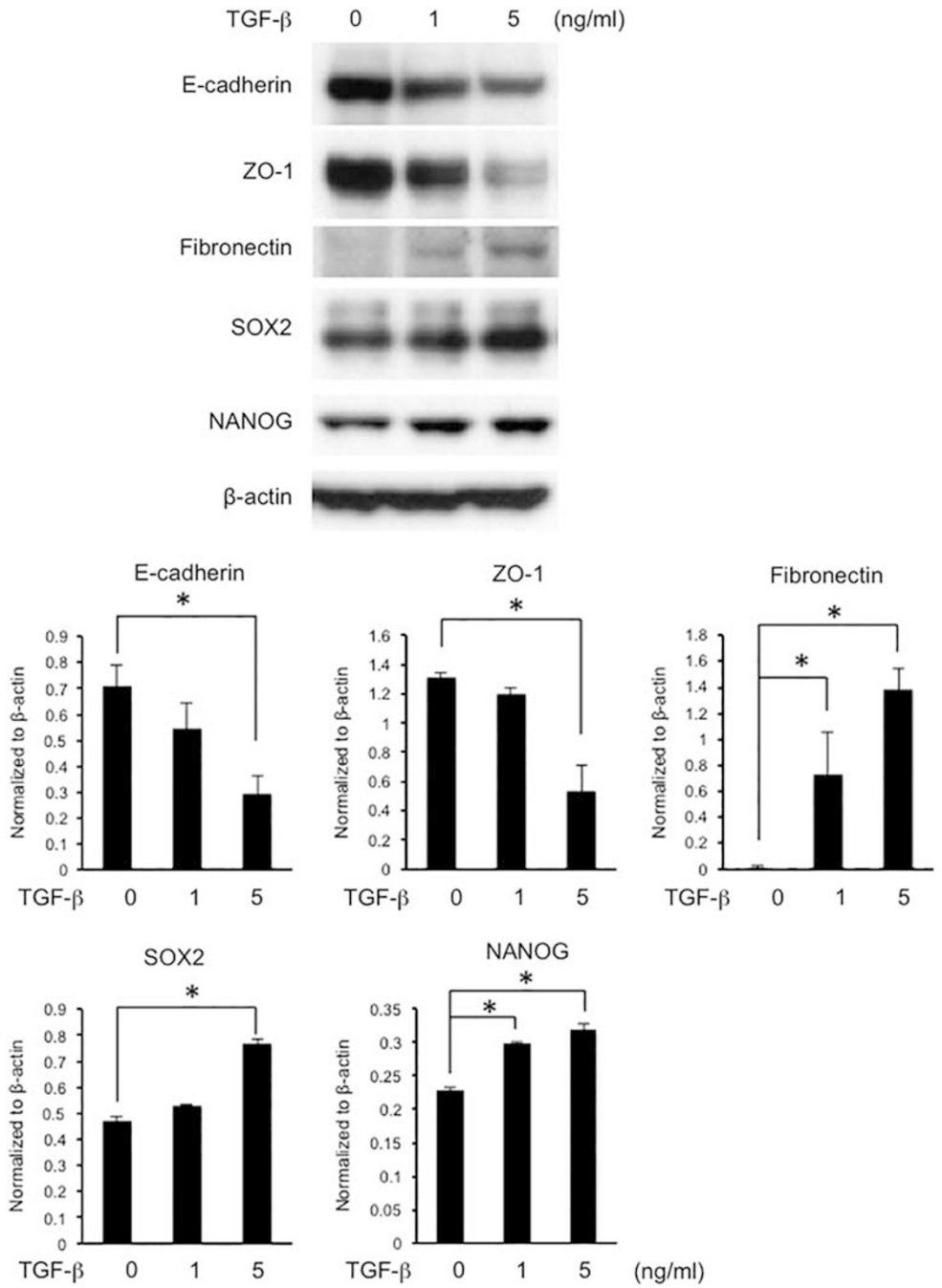


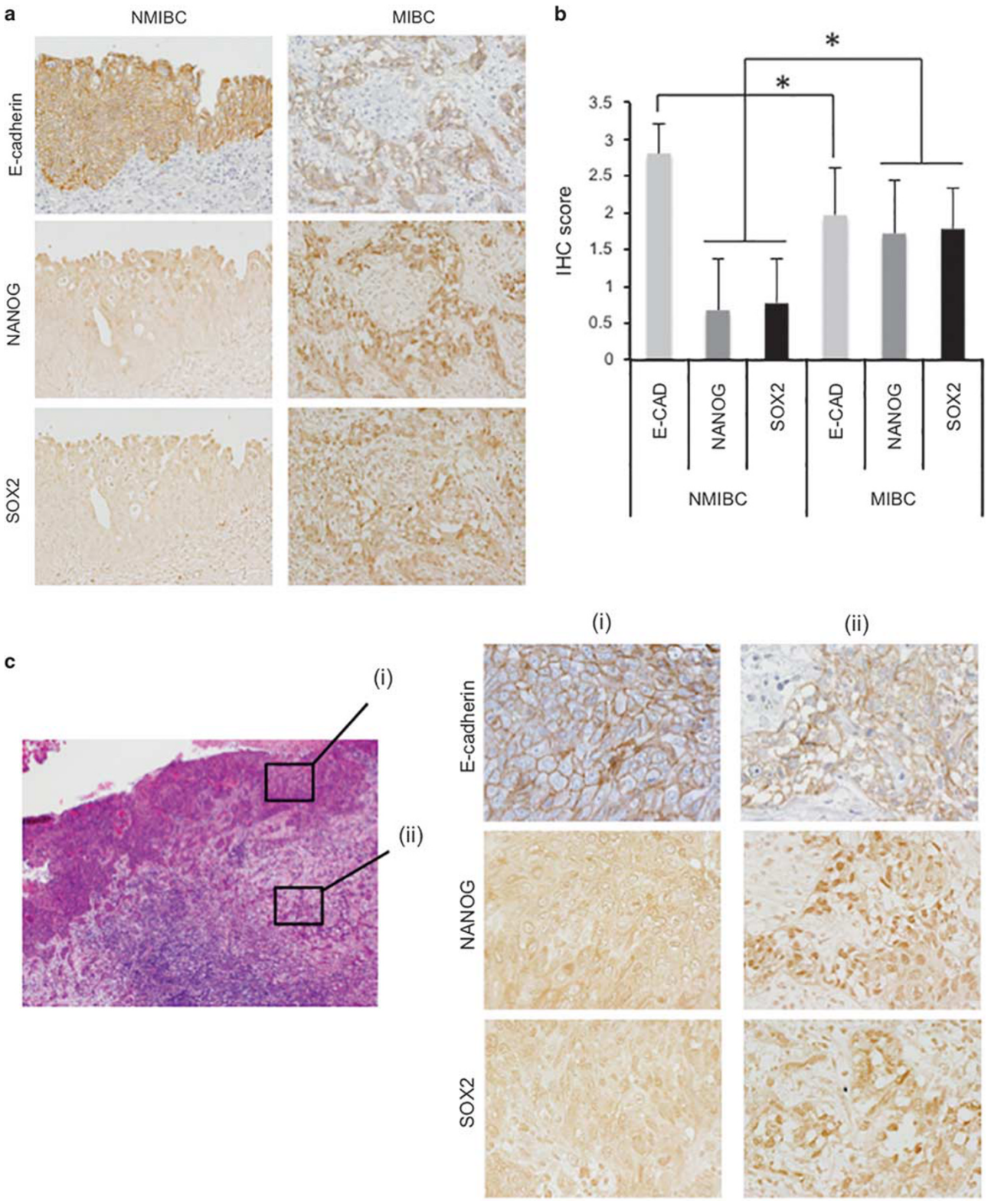
Figure 2 Transforming growth factor β1 (TGF-β1)-induced EMT increases the expression of SOX2 and NANOG. RT4 cells were cultured in the absence of serum for 24 h and then treated with the indicated concentrations of TGF-β1 for 48 h. Protein was extracted from the cells and an immunoblot assay for E-cadherin, ZO-1, Fibronectin, SOX2, NANOG, and β-actin was carried out. Bar graphs represent the average concentrations of each protein normalized to β-actin, as determined by densitometry. *Statistically significant. EMT, epithelial–mesenchymal transition.

database cBioPortal for cancer genomics. Although the differences were not significant, decreased expression of E-cadherin and increased expression of NANOG (or SOX2) were clearly observed in tumors at stages II–IV compared with those with stage I (Supplementary Figure 3a).

EMT frequently occurs at the invasive front of various cancers. As expected, E-cadherin expression was high in noninvasive areas, while that of both NANOG and SOX2 was low in the same areas of a serial section (Figure 3c, (i)). In contrast, at the invasive front, E-cadherin expression was found to be reduced, while both NANOG and SOX2 expression levels were increased (Figure 3c, (ii)). These findings suggested that EMT and cancer stemness were promoted in MIBC.

An Inverse Relation Between E-cadherin and SOX2 (or NANOG) Expression in Clinical Bladder Cancer

E-cadherin expression was likely to have an inverse correlation with NANOG or SOX2 expression (Supplementary Figure 4a). The expression of E-cadherin and NANOG was mutually exclusive in MIBC serial sections (Figure 4a). Similarly, the expression patterns of E-cadherin and SOX2 were also mutually exclusive in a patient with MIBC



(Figure 4a). To assess the relation between E-cadherin and SOX2 (or NANOG), we compared the expression levels of E-cadherin and NANOG (SOX2) in clinical samples including NMIBC and MIBC. We found a significant inverse correlation between E-cadherin and NANOG expression ($\rho = -0.3078$, $P = 0.0141$), and between E-cadherin and SOX2 expression ($\rho = -0.4763$, $P < 0.0001$; Figure 4b). The public database cBioPortal also showed that although not significant, there was an inverse correlation between mRNA levels of E-cadherin and NANOG ($\rho = -0.012$) and between mRNA levels of E-cadherin and SOX2 ($\rho = -0.057$; Supplementary Figure 3b). In addition, there was a significant positive correlation between SOX2 and NANOG expression ($\rho = 0.4173$, $P = 0.0007$; Supplementary Figure 4b). These findings suggested that there was an inverse correlation between E-cadherin and SOX2 (or NANOG) expression in clinical bladder cancer.

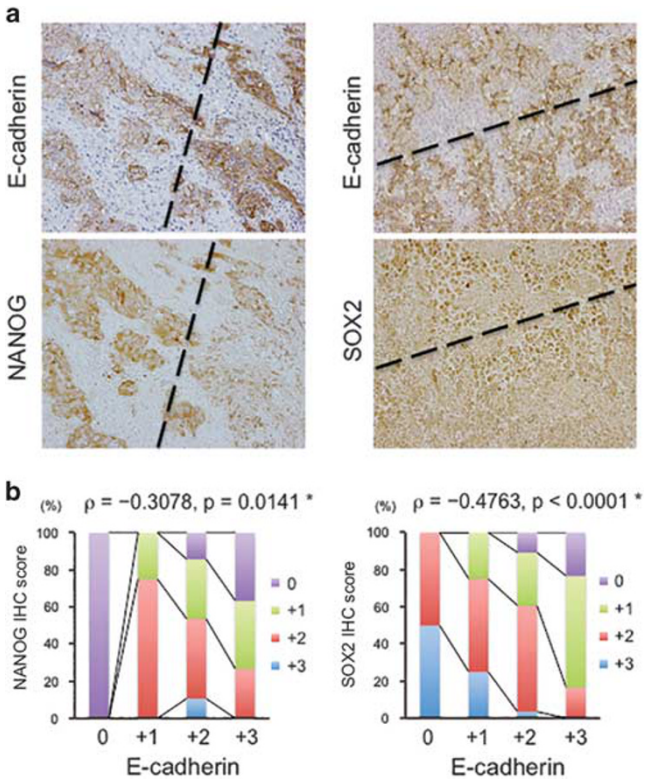


Figure 4 Inverse correlation between E-cadherin and NANOG (or SOX2) expression in bladder cancer. Immunohistochemical analysis for E-cadherin, NANOG, and SOX2 was performed on serial sections of bladder cancer. (**a**) Left panels: representative images of E-cadherin and NANOG expression in a serial section of a patient with MIBC. Reduced E-cadherin expression corresponded to increased NANOG expression on the left side separated by dotted line, and vice versa on the right-hand side. (right) a representative image of E-cadherin and SOX2 expression in a serial section of another patient with MIBC. Reduced E-cadherin expression corresponded to increased SOX2 expression in the upper region separated by the dotted line, and vice versa in the lower region. Original magnification: ×200. (**b**) The left bar graph shows the percentages of patients with NANOG scores for each E-cadherin score. The right-hand bar graph shows the percentages of patients with SOX2 scores for each E-cadherin score. Statistical analysis was performed by Spearman's rank correlation coefficient test; ρ: correlation coefficients. *Statistically significant. MIBC, muscle-invasive bladder cancer.

Expression of E-cadherin, NANOG and SOX2 Correlates with Tumor Aggressiveness in Bladder Cancer

The relations among E-cadherin, NANOG, SOX2, and clinicopathological features of all the studied cases are summarized in Table 1. E-cadherin expression significantly correlated with a lower TNM stage ($P = 0.0009$) or lower tumor grade ($p = 0.0017$). NANOG expression significantly correlated with a higher TNM stage ($P = 0.0109$), higher tumor grade ($P = 0.0005$), or higher Ki-67 LI ($P = 0.029$). SOX2 expression was significantly higher in recurrent tumors ($P = 0.0303$) and tumors with a higher TNM stage ($P = 0.0108$), higher grade ($P < 0.0001$), or higher Ki-67 LI ($P < 0.0001$). In addition, Ki-67 LI was significantly higher in MIBC than in NMIBC (Supplementary Figure 5a). Ki-67 LI was negatively associated with the E-cadherin expression level and positively associated with both NANOG and SOX2 expression levels (Supplementary Figure 5b).

These findings suggested that bladder cancer progression involved decreased protein levels of E-cadherin and increased protein levels of NANOG (or SOX2).

DISCUSSION

EMT and CSCs share a molecular network and both are functionally linked to bladder cancer biology including transdifferentiation, resistance to treatment, tumor heterogeneity, and tumor progression.[5,32] As stated in our review focused on the origin of bladder CSC, bladder cancer may acquire the CSC phenotype later in tumor development.[15] EMT may contribute to this process because of the following evidence: (1) stem-like characteristics of bladder cancer are not observed until late in tumor development;[14] (2) most bladder CSCs have been identified in MIBC, which is frequently associated with EMT but not identified in

Figure 3 Differential expression of E-cadherin, NANOG, and SOX2 in NMIBC and MIBC. (**a**) A representative image from IHC analysis for E-cadherin, NANOG, and SOX2 in serial sections. Original magnification ×200. (**b**) E-cadherin, NANOG, and SOX2 expression levels were semiquantitatively scored in NMIBC and MIBC cells. Data are the percentages of patients with E-cadherin scores in each group. Statistical analysis was performed by the Wilcoxon rank-sum test. *Statistically significant. (**c**) Hematoxylin and eosin staining shows a noninvasive area (i) and invasive area (ii) in a single tumor. A representative image from IHC analysis of the noninvasive and invasive areas in serial sections. Original magnification: ×400. IHC, immunohistochemical; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle-invasive bladder cancer.

Table 1 Correlation between the expression of E-cadherin, NANOG, and SOX2 and clinicopathological features

	E-cadherin					NANOG					SOX2				
	0	+1	+2	+3	*P*-value	0	+1	+2	+3	*P*-value	0	+1	+2	+3	*P*-value
Age															
≤ 70 ($n=25$)	1	3	11	10	0.2497	8	5	10	2	0.2644	5	9	11	0	0.4584
≥ 71 ($n=38$)	0	1	17	20		8	16	13	1		5	18	13	2	
Sex															
Women ($n=24$)	1	1	10	12	0.5683	4	7	12	1	0.3439	3	11	10	0	0.6328
Men ($n=39$)	0	3	18	18		16	21	23	3		7	16	14	2	
Recurrence															
Primary ($n=31$)	1	3	17	10	0.3165	6	8	16	1	0.1965	3	8	18	2	0.0303*
Recurrent ($n=32$)	0	1	11	20		10	13	7	2		7	19	6	0	
TNM stage															
≤ Stage I ($n=45$)	0	1	16	28	0.0009*	15	17	12	1	0.0109*	10	22	12	1	0.0108*
≥ Stage II ($n=18$)	1	3	12	2		1	4	11	2		0	5	12	1	
Tumor grade															
Low-grade ($n=25$)	0	0	6	19	0.0017*	12	11	2	0	0.0005*	9	15	1	0	<0.0001*
High-grade ($n=34$)	1	4	20	9		4	10	17	3		1	9	22	2	
Ki-67 LI															
Low Ki-67 LI ($n=31$)	0	0	12	19	0.0529	11	13	6	1	0.0290*	10	17	4	0	<0.0001*
High Ki-67 LI ($n=32$)	1	4	16	11		5	8	17	2		0	10	20	2	

*$P<0.05$, statistical significance.

NMIBC;[11,15] (3) some of NMIBCs (10–15%) eventually progress to MIBC but not the other way around; this situation corresponds to the process of EMT within solid tumors.[3]

We demonstrated that EMT is associated with the expression of stemness markers SOX2 and NANOG in bladder cancer. TGF-β1 treatment decreased the expression of epithelial markers (including E-cadherin) and increased SOX2 and NANOG expression in bladder cancer cell lines. NMIBC clinical samples showed a slight and homogeneous reduction in E-cadherin expression without any changes in SOX2 or NANOG expression. In contrast, a strong reduction in E-cadherin expression and increased SOX2 and NANOG expression were frequently observed in MIBC. Thus, there is likely a threshold of the E-cadherin level below which EMT and EMT-induced cancer stemness are initiated. It has been reported that a direct knockdown of E-cadherin increases the number of CSCs, drug resistance, and metastasis in breast epithelial cells[33,34] and enriches CSCs among colorectal cancer cells.[35] Moreover, the E-cadherin knockdown enhances expression of pluripotency genes including SOX2 in A549 lung cancer cells.[36] On the other hand, there is evidence that SOX2 and NANOG regulate EMT and inhibit E-cadherin expression in various types of cancers.[37–39] Therefore, there is likely some reciprocal regulation between E-cadherin and SOX2 (or NANOG). A loss of E-cadherin may trigger the development of cancer stemness and tumor aggressiveness.

On the basis of our results as well as those of others, we hypothesized a mechanism of bladder cancer formation and progression (Figure 5). Bladder cancers are believed to arise from distinct types of cells in normal urothelium.[40] Primary bladder cancer cells may have different amounts of E-cadherin depending on their site of origin. A small reduction in E-cadherin expression does not alter SOX2 and NANOG expression but allows cancer cells to form a relatively organized structure with loose cell–cell adhesion. These cancer cells take root and get implanted at other sites in the bladder mucosa and proliferate via clonal expansion,

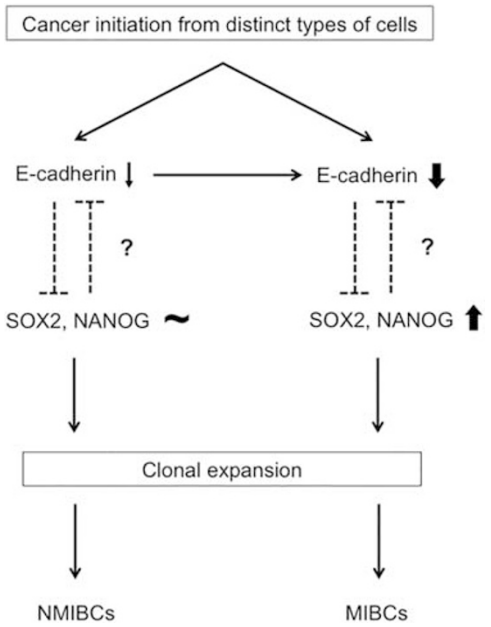


Figure 5 A hypothetical mechanism of the development of NMIBC and MIBC. Bladder cancer may originate from a different site of the bladder urothelium, which expresses E-cadherin at various magnitudes. There may be a mechanism of reciprocal inhibition between E-cadherin and SOX2 (or NANOG) expression. Strong downregulation of E-cadherin may lead to cancer stemness. Bladder cancer cells may develop into NMIBCs or MIBCs via clonal expansion. MIBC, muscle-invasive bladder cancer; non-muscle-invasive bladder cancer, NMIBC.

becoming a highly recurrent tumor of the NMIBC type. In contrast, a severe reduction in E-cadherin expression, some of which may develop from small downregulation of E-cadherin, may enable the upregulation of SOX2 and NANOG and subsequently enhance cancer stemness, contributing to tumor heterogeneity. These cancer cells proliferate via clonal expansion, becoming a highly invasive or metastatic tumor of the MIBC type. Further research is needed to determine whether this hypothesis is correct.

This study, to the best of our knowledge, is the first to show a relation between EMT and cancer stemness in bladder cancer; it also provides a rationale for a treatment strategy targeting EMT or cancer stemness. We believe that our findings will provide new insights into the pathogenesis of NMIBC and MIBC, and that EMT inhibitors may improve the clinical outcomes of bladder cancer treatment.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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