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Human Cancer Biology

Clinical Cancer Research

DNMT3L Is a Novel Marker and Is Essential for the Growth of Human Embryonal Carcinoma

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Abstract

Purpose: Testicular germ cell tumors (TGCT) have a unique epigenetic profile distinct from that of other types of cancer. Elucidation of these properties has a potential to identify novel markers for TGCTs.

Experimental Design: We conducted comprehensive analysis of *DNA methyltransferase* (*DNMT*) gene expression in TGCTs. Based on the expression profiles of *DNMT* genes in TGCTs, we generated a rabbit polyclonal anti-human DNMT3L antibody. We then studied the role of DNMT3L in TGCTs by the treatment of two embryonal carcinoma (EC) cell lines with a small interfering RNA system. Finally, we evaluated the immunohistochemical detection of DNMT3L in TGCT tissues. We also compared the patterns of DNMT3L immunohistochemistry with those of CD30 and SOX2.

Results: Among the *DNMT* genes, we found that mRNA for *DNMT3L* was specifically expressed in TGCTs, but neither in normal testicular tissues nor in cancer cells of somatic tissue origin. DNMT3L protein was strongly expressed in two EC cell lines, but not in the cell lines of somatic tissue origin. Transfection of small interfering RNA for *DNMT3L* significantly reduced DNMT3L expression and resulted in growth suppression and apoptosis in EC cells. Immunohistochemical analysis showed that DNMT3L protein was present only in EC cells, but not in the other types of TGCT components and cancer cells of somatic tissue origin. DNMT3L staining was more prominent and specific than CD30 or SOX2 staining for detecting EC cells.

Conclusion: DNMT3L is a novel marker and is essential for the growth of human embryonal carcinoma.

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Testicular germ cell tumors (TGCT) are the most common malignancy in young men, with a peak incidence between 20 and 40 years of age. The incidence of TGCT has increased dramatically over the last century (1). TGCTs are classified into two major histologic subgroups, seminoma and nonseminoma: nonseminomatous TGCTs show embryonal and extraembryonal differentiation patterns, which include primitive zygotic (embryonal carcinoma, EC), embryonal-like somatic differentiated (teratoma), and extraembryonally differentiated (choriocarcinoma, yolk sac tumor) phenotypes (2).

We and others have shown that TGCTs have a unique DNA methylation profile distinct from that of somatic tissue–derived neoplasms (3–6). TGCT tissues show less frequent methylation than somatic tissue cancers (3). This unmethylated DNA profile is significant particularly in seminomatous TGCTs (7). We have also shown that X-linked genes on super numerical X chromosomes in seminomatous and nonseminomatous TGCTs were predominantly hypomethylated, regardless of XIST expression (8). These studies suggest that seminoma and EC at least carry epigenetic profiles similar to those of fetal germ cells or embryonic stem (ES) cells.

By characterizing the epigenetic profiles of TGCTs, it may be possible to identify novel markers for TGCTs. In the present study, we have performed a comprehensive expression analysis of *DNA methyltransferase* (*DNMT*) genes in TGCTs. We found that mRNA for the fetal-specific methyltransferase-like protein *DNMT3L* is specifically expressed in TGCTs. By generating the anti-human DNMT3L antibody, we examined the biological role of DNMT3L on TGCTs. Here, we report that DNMT3L is a novel marker and essential for the growth of human ECs.

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

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Materials and Methods

Cell lines and culture conditions. We used two TGCT (EC) cell lines (NEC8 and NEC14), 15 renal cell carcinoma cell lines (CAKI1, CAKI2, NC65, ACHN, A704, SW839, VMRC-RCW, OS-RC-2, RCC10RGB, THUR4TKB, THUR10TKB, TUHR14TKB, KMRC-1, KMRC-2, and KMRC-3), 9 breast

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Translational Relevance

In this study, we have shown that DNMT3L, a fetal-specific DNA methyltransferase–like protein, is a novel marker and is essential for the growth of human embryonal carcinoma (EC).

Accurate diagnosis of EC is critical for the management of patients with stage I nonseminomatous testicular germ cell tumors because the percentage of EC in the primary tumor is predictive of occult metastasis. Our data convincingly show that the application of DNMT3L immunohistochemistry is a useful tool for identifying EC cells in pathology practice.

We have also shown that suppression of DNMT3L in EC cells results in growth inhibition through apoptosis. The data warrant further elucidation of the roles of DNMT3L in EC cells *in vivo* to evaluate the significance of DNMT3L as a therapeutic target of ECs.

cancer cell lines (MCF7, T47D, SK-BR-3, YMB-1E, MRK-NU1, CRL1500, MDA-MB-453, OCUB-F, and HMC-1-8), 7 cervical cancer cell lines (HeLa, D98-AH2, CaSki, ME-180, SKG-IIIa, BOKU, and SKG-II) and 7 ovarian cancer cell lines (RMG-1, RMG-II, RKN, RTSG, RMUG-L, TYK-nu, and OVK18). These cell lines were purchased from either the American Type Culture Collection, Riken Cell Bank, Cell Resource Center for Biomedical Research in Tohoku University, or the Japanese Collection of Research Bioresources. All the cell lines were maintained in DMEM or RPMI 1640 containing 10% fetal bovine serum supplemented with penicillin, streptomycin, and glutamine under humidified 5% CO_2 and passaged when they reached 80% confluence.

Tissue samples and pathologic data. Fifty-three primary TGCT specimens (30 seminomas and 23 nonseminomatous TGCT tissues), five primary non–Hodgkin's lymphomas of the testis (diffuse large-type non–Hodgkin's lymphomas), and three infantile testicular tumors (two teratomas and one yolk sac tumor) were surgically resected at Shiga University of Medical Science. Representative parts of the tumor were fixed in 10% formalin overnight for paraffin embedding. Tumors were classified according to the WHO's classification for testicular cancer (9). Representative H&E-stained tissue sections of each case were reviewed by two of the authors (R.K. and H.O.). For these samples, we used liquid nitrogen snap-frozen tissues for reverse transcription-PCR (RT-PCR) analysis (43 primary TGCTs, 23 seminomas, and 18 nonseminomatous TGCT tissues; 5 primary non–Hodgkin's lymphomas; and 18 surrounding testicular parenchyma tissues adjacent to the TGCTs). Three normal testis tissues of young adults were obtained through orchiectomy due to testicular injury and peripheral blood leukocytes (PBL) from five healthy volunteers were also included in the RT-PCR study. Written informed consent was obtained in advance for all human tissue samples. Sample collection and analysis were monitored and approved by the Institutional Review Board.

RNA extraction and cDNA synthesis. Total RNA was extracted from cell lines or tissue fragments using Trizol reagent (Invitrogen). All RNA preparations were treated with DNase I for 15 minutes at room temperature immediately before conversion to cDNA using the SuperScript II kit (Invitrogen) according to the manufacturer's instructions.

Reverse transcription-PCR. The expression of *DNMT* genes in cell lines and tissue samples was assessed by RT-PCR. Primer pairs used were DNMT3L-F, 5′-AAGTTCCTGGATGCCCTCTT-3′ (NM013369); DNMT3L-R, 5′-GCCGTACACAAGATCGAAGG-3′ (NM013369); DNMT1-F, 5′-ACCGCTTCTACTTCCTCGAGGCCTA-3′ (NM001379); DNMT1-R, 5′-GTTGCAGTCCTCTGTGAACACTGTGG-3′ (NM001379); DNMT2-F, 5′-GTCAACACTGTCGCTAATGA-3′ (NM004412); DNMT2-R, 5′-TATTCGTCCTTGAATCAGTC-3′ (NM004412); DNMT3A-F, 5′-CACACAGAAGCATATCCAGGAGTG-3′ (NM153759); DNMT3A-R, 5′-AGTGGACTGGGAAACCAAATACCC-3′ (NM153759); DNMT3B-F, 5′-AATGTGAATCCAGCCAGGAAAGGC-3′ (NM175850); and DNMT3B-R, 5′-ACTGGATTACACTCCAGGAACCGT-3′ (NM175850). The expression of β-actin was used as a control. Primers for β-actin were β-actin-F, 5′-ACCCCCACTGAAAAAGATGA and β-actin-R, 5′-ATCTTCAAACCTCCATGATG, and were used to monitor *β-actin* expression.

Generation of anti-DNMT3L antibody. We tested the specificity of three commercial DNMT3L antibodies: Dnmt3L (N-14; sc-10239), Dnmt3L (H-85; sc-20705; Santa Cruz Biotechnology), and Dnmt3L (ab17687; Abcam). Because none of the antibodies tested showed specificity for endogenous human DNMT3L, we generated specific polyclonal antibody against human DNMT3L: we designed recombinant glutathione *S*-transferase (GST)-fusion proteins containing the full-length DNMT3L peptides, which corresponds to the residues 2-387 of DNMT3L. The recombinant GST-fusion proteins were expressed and purified. Anti-human DNMT3L polyclonal antibodies were generated by immunizing rabbits with the GST-fusion peptides: rabbit IgGs were purified then the GST-specific fractions were excluded through GST-conjugated Sepharose 4B-column.

Plasmids and transfection. The cDNA-encoding full-length DNMT3L protein was amplified from NEC8 cells by RT-PCR, using the primers of 5′-ACTAGTATCCCCATGGCGGCCATC-3′ and 5′-CTCGAGTTATAAAGAGGAAGTGAGTTCTGTTGAAAAATACTT-3′. After double digestion with *SpeI* and *Xho*I, the PCR products were cloned into pLenti6 vector containing cytomegalovirus promoter and the blasticidin-resistant gene (Invitrogen). The resultant DNMT3L expression vector was transfected into human embryonic kidney 293 cells by Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Western blot analysis. Cells were lysed in Laemmli-SDS buffer and the lysate was boiled for 5 minutes, then sonicated. Samples containing equal amounts of protein were

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electrophoresed in 15% SDS-PAGE before transfer to a polyvinylidine fluoride membrane (Millipore). The membrane was immunoblotted with anti-DNMT3L polyclonal antibodies or anti–β-actin monoclonal antibody (Sigma) and developed using enhanced chemiluminescence systems (Amersham). Anti–caspase-3 antibody (Cell Signaling Technology) was used for the detection of the cleavage form of caspase-3. Anti-SOX2 (Cell Signal Technology) antibody was used to compare the data with those of DNMT3L. Cell extracts from human ES cells were used to study the expression of DNMT3L and SOX2.

Transfection with DNMT3L small interfering RNA. We selected two DNMT3L-specific small interfering RNA (siRNA) duplexes (3Lsi-1 and 3Lsi-2). We also used a pair of nonsilencing siRNA duplexes (NSsi) as control. All siRNA duplexes consisted of two complementary 21-nucleotide RNA strands with 3′ dTdT overhangs and were chemically synthesized (Qiagen). siRNA sequences were as follows: 3Lsi-1, (Sense) CGACGAUGACGGGUACCAAdTdT, (Antisense) UUGGUACCCGUCAUCGUCGdTdA; si3L-2, (Sense) CGGCCAAGUGGCCCACCAAdTdT, (Antisense) UUGGUGGGCCACUU GGCCGdTdA. NEC8 and NEC14 cells were transfected with siRNA for DNMT3L using Lipofectamine RNAi MAX reagents (Invitrogen) according to the manufacturer's protocol.

Trypan blue exclusion assay. The cell death induced by siRNA transfection was evaluated by trypan blue exclusion. In brief, NEC8 and NEC14 cells were harvested on days 2, 4, and 6 followed by siRNA for DNMT3L transfection or NSsi transfection. The cells were washed thrice with PBS and resuspended in 100 μL PBS. After mixing with 100 μL of 0.8% trypan blue, the cells were counted using a hemocytometer. Percentage of cell death was calculated as blue cells/total cells. The harvested cells at each period were also used for Western blot and Annexin V-FITC assay. The data were obtained through quadruple experiments. Data are presented as the means ± SEMs and statistical comparisons were done with paired *t* test. The significance level was set at $P < 0.05$.

Detection of apoptosis by flow cytometry. The Annexin V-FITC apoptosis kit (MBL) was used to evaluate apoptotic cells upon DNMT3L suppression. Briefly, 4 days after siRNA treatment for DNMT3L, cell pellets of NEC8 and NEC14 cells were resuspended in Annexin V buffer and incubated with FITC-conjugated Annexin V (1 μL/mL) for 15 minutes. Samples were analyzed on a FACSCalibur flow cytometer with the CELLQUEST software (Becton Dickinson).

Cell staining and fluorescence microscopy. NEC8 and NEC14 cells were cultured on MAS-coated TM glass slides (Matsunami Glass) and transfected with Alexa Fluor 647–labeled siRNA for DNMT3L. On day 4, after transfection, the cells were fixed with 1% buffered formaldehyde and 70% ethanol and stained with 4′,6-diamidino-2- phenylindole. The slides were then mounted with glycerol and photographed using a fluorescence microscope with a charge-coupled device camera (DMI4000 B; Leica Microsystems).

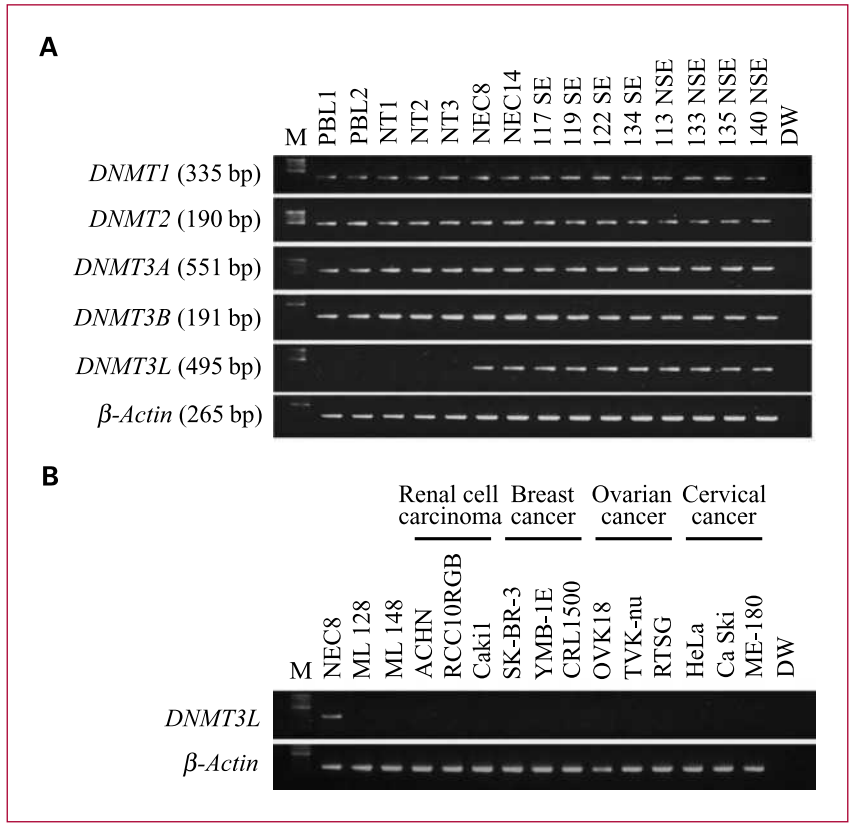

Fig. 1. *DNMT* gene mRNA expression in TGCTs and cancer cell lines derived from somatic tissues. A, RT-PCR analysis of *DNMT1, DNMT2, DNMT3A, DNMT3B,* and *DNMT3L* in TGCT (EC) cell lines and TGCT tissues. Bottom lane, RT-PCR results using β-actin primers as a control. Numbers in parenthesis, transcript size. NT, normal testis tissue; SE, pure seminomatous TGCT tissue; NSE, nonseminomatous TGCT tissue. Number of each tissue, the serial code number of our laboratory. The histologic components of each nonseminomatous TGCTs are 113NSE (YST/TE/EC), 133NSE (SE/EC/YST), 133NSE (EC/SE), and 140NSE (EC/YST/TE). YST, yolk sac tumor; TE, teratoma. Note that both TGCT-derived EC cell lines (NEC8 and NEC14) and TGCT tissues show expression of *DNMT3L,* whereas PBL and normal testis tissue show absence of *DNMT3L* expression. The other *DNMT* genes except for *DNMT3L* are ubiquitously expressed through out the sample tested. M, molecular weight marker. DW, distilled water as a negative control for PCR. B, expression of *DNMT3L* mRNA in cancer cell lines derived from somatic tissues. Note that cancer cell lines derived from somatic tissues (renal cell carcinoma, breast cancer, ovarian cancer, and cervical cancer) do not express *DNMT3L.*

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Immunohistochemistry. Immunohistochemistry with anti-DNMT3L antibodies was done on formalin-fixed and paraffin-embedded tissue sections. The sections were serially sliced into 5-μm sections. Deparaffinized sections were autoclaved at 120°C for 1 minute, immersed in 0.3% H_20_2, and rinsed with 1×PBS before incubation overnight at 4°C with each of the primary antibodies. The sections were rinsed with 1×PBS and incubated with the secondary antibody (Simple Stain MAX-PO, Nichirei) at room temperature for 1 hour. The sections were then stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and counter-stained with hematoxylin. Statistical differences between histologic subtypes and the results of DNMT3L staining were examined by use of Fisher's exact test. All *P* values are two sided. We also performed immunohistochemistry using anti-CD30 (Novocastra) and anti-SOX2 (Cell Signal Technology) antibodies in the serial sections and compared the data with those of DNMT3L.

Results

DNMT3L mRNA is specifically expressed in TGCT-derived cell lines and TGCT tissues. To see the roles of *DNMT* genes in TGCTs, we initially conducted RT-PCR analysis of the *DNMT* genes (*DNMT1*, *DNMT2*, *DNMT3A*, *DNMT3B*, and *DNMT3L*) in TGCT (EC) cell lines, TGCT tissues, normal testis tissues, and PBLs. Representative data are shown in Fig. 1A. *DNMT1*, *DNMT2*, *DNMT3A*, and *DNMT3B* were ubiquitously expressed in all the samples tested (Fig. 1A). However, *DNMT3L* was expressed specifically in TGCT (EC) cell lines and TGCT tissues, but in neither normal testis tissues nor PBLs (Fig. 1A). The transcripts of *DNMT3L* were positive both in seminomatous TGCTs (23 of 23) and nonseminomatous TGCTs (20 of 20). We also studied the expression of *DNMT3L* in 18 testicular tissues adjacent to the TGCTs: no expression of *DNMT3L* was found (Supplementary Fig. S1).

Next, we performed the RT-PCR analysis of DNMT3L in cancer cells of somatic tissue origin (5 primary non–Hodgkin 's lymphoma of the testis and 38 cancer cell lines of somatic tissue origin). *DNMT3L* expression was negative in all the cancer cells of various somatic tissue origin (0 of 5 lymphomas; 0 of 38 cancer cell lines; Fig. 1B).

Production and testing of novel human DNMT3L antibody. The specific expression of *DNMT3L* mRNA observed in TGCTs tempted us to search for antibodies against DNMT3L. A full-length encoding region of human *DNMT3L* cDNA was inserted into the pLenti6 vector and was expressed transiently in human emryonic kidney 293 cell lines. Using the human DNMT3L-expressing 293 cells, we tested the specificity of the four commercial DNMT3L antibodies. However, none of the antibodies tested reacted against DNMT3L (data not shown). Therefore, we generated specific polyclonal rabbit IgGs directed against the full-length DNMT3L protein. As shown in Fig. 2A, the generated DNMT3L antibody recognized the 42-kDa

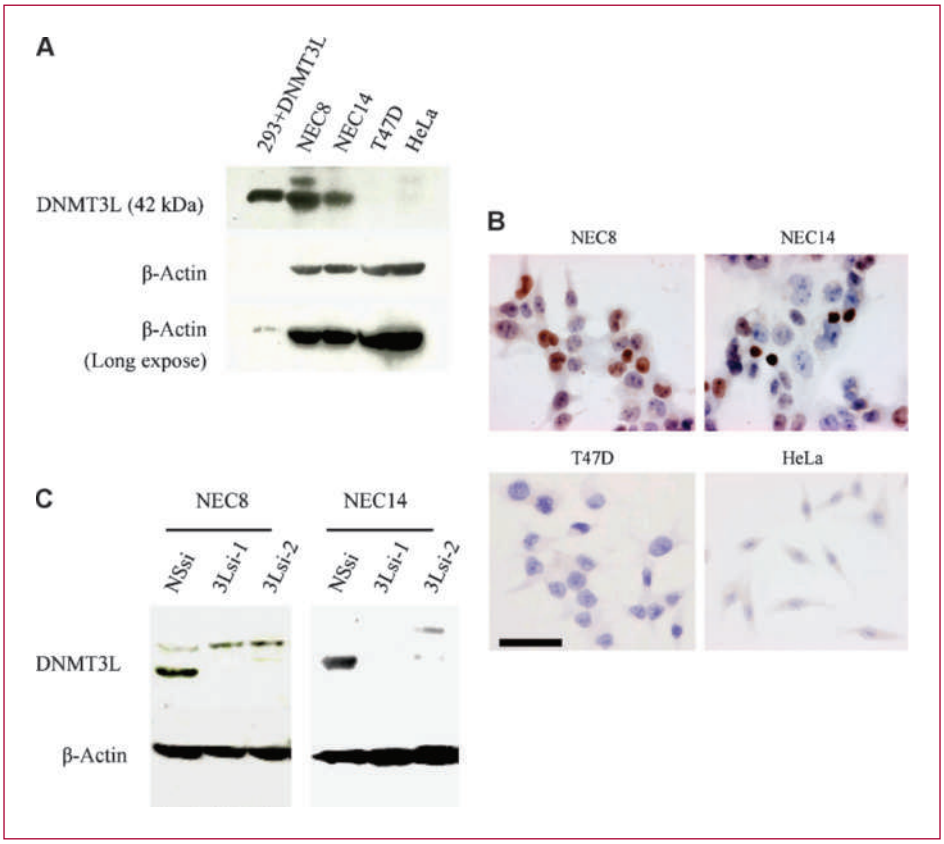

Fig. 2. Generation and validation of human DNMT3L antibody. A, DNMT3L protein expression in EC cells. The extracts from recombinant human *DNMT3L* gene–transfected 293 cells showed the optimal DNMT3L signal using the generated DNMT3L antibody. Note that both of the EC cell lines (NEC8 and NEC14) show intense DNMT3L signals but the two cancer cell lines derived from somatic tissues (T47D and HeLa) show absence of DNMT3L signals. B, subcellular localization of endogenous DNMT3L in EC cells. NEC8, NEC14, T47D, and HeLa cells were immunostained using the anti-DNMT3L antibody. NEC8 and NEC14 cells show DNMT3L-positive signals in the nucleus, whereas T47D and HeLa cells do not show any signal in neither cytoplasm nor nucleus. Scale bar, 60 μm. C, inhibition of DNMT3L protein in EC cells with siRNA treatment. Effect of siRNA for DNMT3L upon NEC8 and NEC14 cells was tested using two different siRNA constructs (3Lsi-1 and 3Lsi-2). Note that treatment with 3Lsi-1 and 3Lsi-2 shows significant reduction of DNMT3L protein in NEC8 and NEC14 cells, whereas DNMT3L expression was maintained by the treatment with NSsi. Membranes were probed with the antibody for β-actin as control to confirm equal amount of protein loading.

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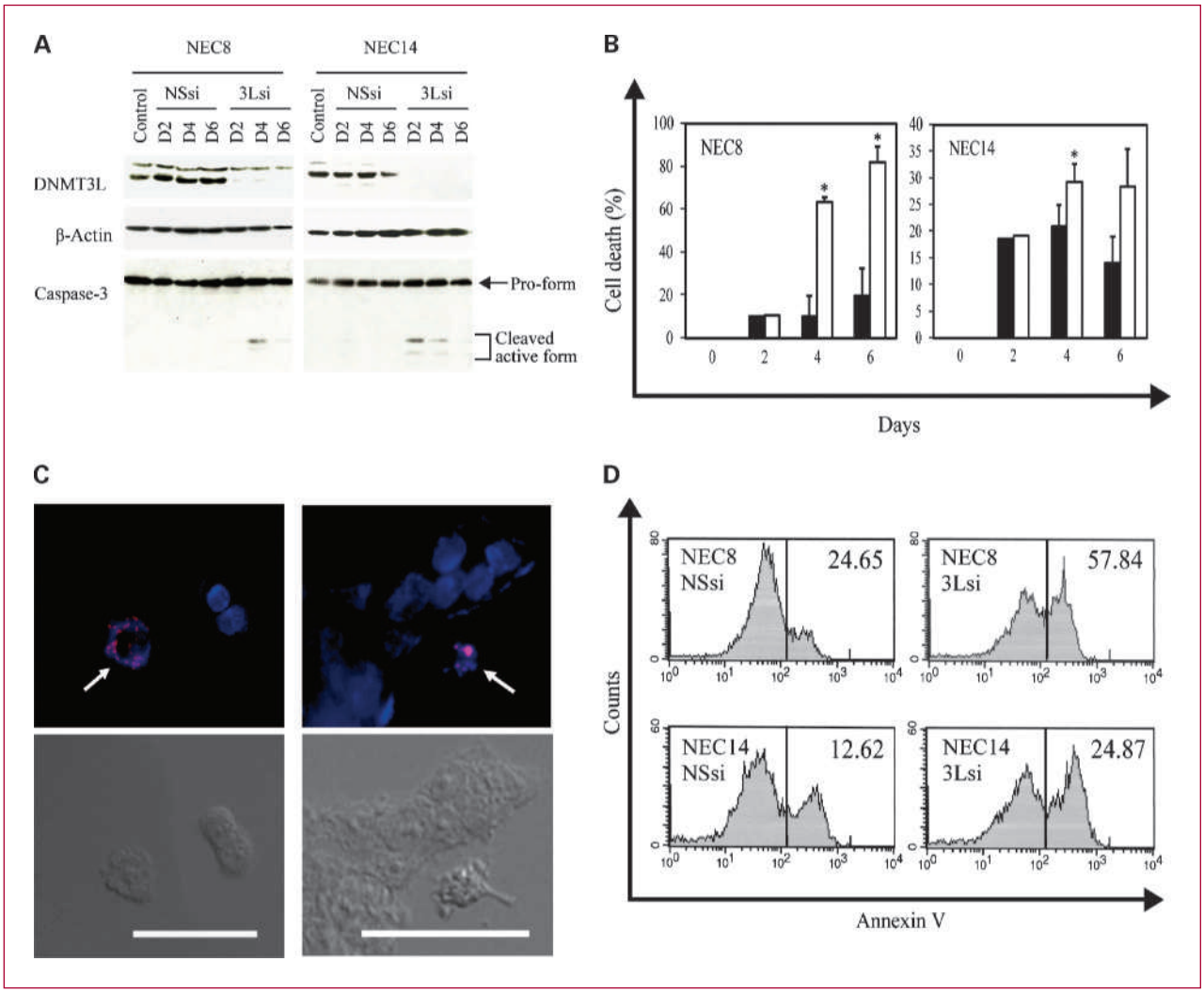

Fig. 3. Effect of DNMT3L inhibition in EC cells. A, DNMT3L-inhibiting effect in EC cells through siRNA treatment. After siRNA treatment, DNMT3L silencing was evaluated at days 2, 4, and 6. Silencing of DNMT3L was maintained over day 6 in NEC8 and NEC14 cells (3Lsi, top left lane). Treatment with NSsi (top right lane) did not affect the expression of DNMT3L in NEC8 and NEC14 cells. D number, the days after transfection. Control, nonsilenced control (Lipofectamine mixture only). Membranes were probed with the anti–β-actin monoclonal antibody to confirm equal amount of protein loading (middle lane). Membranes were also probed with anti–caspase-3 antibody. The treatment of NEC8 and NEC14 cells with DNMT3L siRNA induced a cleaved active form of caspase-3 (bottom lane). B, trypan blue exclusion assay. The results of cell death through DNMT3L siRNA transfection are shown. NEC8 and NEC14 cells harvested at day 2, 4, and 6 followed by the treatment with DNMT3L siRNA (□) or NSsi (■) are analyzed by the trypan blue exclusion. Columns, mean values from four independent experiments; bars, SE. *, $P < 0.05$, compared with NSsi-treated cells. C, morphologic effect of DNMT3L siRNA on EC cells. Immunofluorescence microscopy images of NEC8 and NEC14 cells treated with DNMT3L-siRNA labeled with Alexa Fluor 647 are shown. Arrows, NEC8 and NEC14 cells with DNMT3L-siRNA incorporation particularly show chromatin condensation. Scale bar, 60 μm. D, flow cytometry analysis of Annexin V staining. Cells were assayed by flow cytometry for Annexin V staining 4 d after siRNA treatment: top left, NEC8 cells treated with NSsi; top right, NEC8 cells treated with DNMT3L siRNA; bottom left, NEC14 cells treated with NSsi; bottom right, NEC14 cells treated with DNMT3L siRNA. Number of the each flow cytometry data indicates percentage of positive cells with Annexin V staining. Note that increased percentage of Annexin V–positive cells were observed in NEC8 and NEC14 cells treated with DNMT3L siRNA compared with those treated with NSsi.

DNMT3L protein. DNMT3L protein was strongly expressed in the two human TGCT (EC) cell lines (NEC8 and NEC14) but not in the cell lines of somatic tissue origin (T47D and HeLa; Fig. 2A). To study the expression of DNMT3L in normal embryonic development, we conducted Western blotting with DNMT3L antibody using human ES cells.

We also compared the data on DNMT3L expression with those on SOX2 expression: both DNMT3L and SOX2 proteins were expressed in human ES cells as well as human EC cell lines (Supplementary Fig. S2). Using the same antibody for DNMT3L, we examined the cellular localization of endogenous DNMT3L in the cell lines by immunocytochemistry. The results showed that distinctive DNMT3L-positive signals were present in the nucleus of NEC8 and NEC14 cells, whereas DNMT3L-positive signals were identified in neither cytoplasm nor nucleus of T47D or HeLa cells (Fig. 2B).

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Table 1. Summary of immunostaining results of DNMT3L for primary TGCTs and malignant lymphomas of the testis

Tumor Type	No. of DNMT3L-positive cases (%)	Total no. of cases
TGCTs of adolescents		
Pure seminomas	0 (0%)	30
Nonseminomatous TGCTs	21 (91.3%)	23
Foci of nonseminomatous TGCTs		
EC	21 (100%)*	21†
Seminoma	0 (0%)	7
Teratoma	0 (0%)	9‡
Yolk sac tumor	0 (0%)	12†
TGCTs of infants		
Teratoma	0 (0%)	2
Yolk sac tumor	0 (0%)	1
Malignant lymphomas of the testis	0 (0%)	5

*$P < 0.0001$ embryonal carcinoma versus nonembryonal carcinoma.
†Foci of embryonal carcinoma include six pure embryonal carinomas; foci of yolk sac tumor includes one pure yolk sac tumor.
‡Foci of teratoma include four immature teratomas.

Suppression of DNMT3L in EC cells results in growth inhibition and cell death through apoptosis. Next, we transfected siRNA for DNMT3L into NEC8 and NEC14 cells. Introduction of the siRNA significantly silenced the expression of DNMT3L in NEC8 and NEC14, thus validating the siRNA duplexes targeting the DNMT3L sequence (Fig. 2C). To see the biological significance of DNMT3L expression in EC cells, we studied the effect of DNMT3L silencing in EC cell lines (NEC8 and NEC14). As shown in Fig. 3A, DNMT3L silencing with the siRNA treatment was initiated at day 2 and maintained over day 6 in NEC8 and NEC14 cells.

Figure 3B shows the cell death rate at each period. Inhibition of DNMT3L resulted in significant cell death of both NEC8 and NEC14 cells (Fig. 3B). However, the effect of DNMT3L inhibition was stronger in NEC8 cells than in NEC14 cells (Fig. 3B). We speculate that growth of NEC14 cells accompany pile up formation (Fig. 3C) and this may cause less efficient siRNA incorporation in NEC14 cells than in NEC8 cells.

To examine whether the cell death induced by apoptosis, we evaluated morphologic and biochemical changes during the process. We analyzed the cell death by flow cytometry using Annexin V-FITC. The results showed that inhibition of DNMT3L in NEC8 and NEC14 led to increased Annexin V–positive cells indicating cell death by apoptosis (Fig. 3D). To see the morphologic effects of DNMT3L siRNA incorporation, we analyzed by immunofluorescence microscopy NEC8 and NEC14 cells treated with DNMT3L-siRNA labeled with Alexa Fluor 647. As illustrated in Fig. 3C, irregular chromatin condensation was typically seen in NEC8 and NEC14 cells with DNMT3L-siRNA incorporation. In agreement with apoptosis progression into the later stage, a cleaved form of active caspase-3 was observed on a Western blot (Fig. 3A, bottom lane).

Immunohistochemical detection of DNMT3L for the diagnosis of EC. Detection of DNMT3L protein in NEC8 and NEC14 raised the question of whether DNMT3L protein can be identified in seminomatous and nonseminomatous TGCT tissues with positive *DNMT3L* transcription. Therefore, we evaluated the immunohistochemical staining for DNMT3L in a series of testicular tumor tissues (TGCTs of adolescent, infantile TGCTs, and malignant lymphomas of the testis tissues). The immunohistochemical staining results are summarized in Table 1 and the representative data are shown in Fig. 4. DNMT3L protein was expressed specifically in the nuclei of tumor cells in all foci of EC (21 of 21; 100%; Table 1; Fig. 4), but was negative in all foci of seminoma (0 of 30 pure seminoma cases; 0% and 0 of 7 seminomas of mixed TGCTs; 0%), teratoma or immature teratoma (0 of 9; 0%), and yolk sac tumor (0 of 12; 0%; Table 1; Fig. 4). DNMT3L protein was also negative in all foci of infantile TGCTs (0 of 3; 0%) and malignant lymphomas of the testis (0 of 5; 0%; Table 1; Fig. 4). Interestingly, DNMT3L protein disappeared through differentiation from EC cells to teratoma cells or yolk sac tumor cells in the same mixed TGCT tissues (Table 1; Fig. 4).

We also compared the immunostaining of DNMT3L with that of CD30 and SOX2 for evaluating the specificity of detecting EC cells. The representative data are shown in Fig. 4. With regard to pure EC components, DNMT3L- positive cells corresponded to the CD30- or SOX2-positive cells. The data illustrate that DNMT3L-positive cells are indeed EC cells in TGCT tissues. CD30 was positive not only in EC cells, but also in malignant lymphoma of the testis (Fig. 4). SOX2 was present not only in EC cells but also in primitive gut in teratoma elements (Fig. 4). Thus, positivity in EC cells was more prominent and specific with DNMT3L staining than with CD30 or SOX2 staining. Overall, immunohistochemical detection of DNMT3L strongly indicates the diagnosis of EC.

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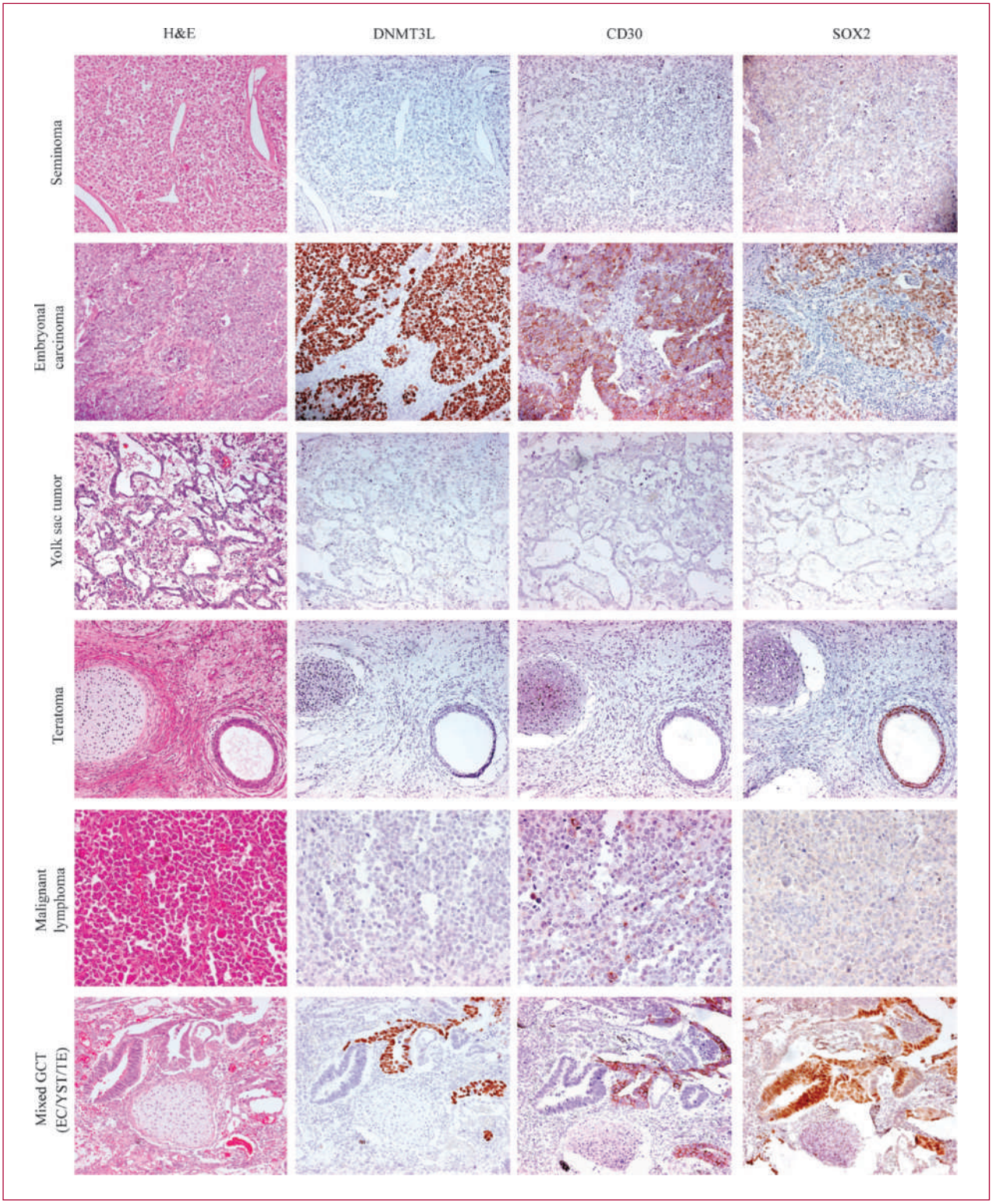

Fig. 4. Immunohistochemical detection of DNMT3L for the diagnosis of ECs. Primary TGCT tissues and malignant lymphomas of the testis tissues stained with H&E and immunostained for DNMT3L. To test the significance of immunohistochemical detection of DNMT3L for the identification of ECs, the staining results of CD30 and SOX2 in the same samples are also compared. Strong nuclear staining of DNMT3L is seen only in EC cells, which are also positive for CD30 and SOX2. In the same mixed TGCT tissue, DNMT3L protein vanishes through differentiation from EC to teratoma (TE) or yolk sac tumor (YST). CD30 shows positivity in the cell membranes of both EC and malignant lymphoma of the testis. SOX2 shows nuclear positivity in EC and endodermal cells of primitive gut (columnar epithelium cells of endodermal origin) in teratoma.

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Discussion

In the present study, we have shown that DNMT3L, a fetal specific DNMT-like protein, is a novel marker and essential for the growth of human EC: immunohistochemical detection of DNMT3L is highly sensitive and specific for the diagnosis of human EC. Suppression of DNMT3L in EC cells results in growth inhibition through apoptosis.

In mice, Dnmt3L is expressed in ES cells and is downregulated in differentiated embryonic body (10). In germ cells, Dnmt3L is expressed in testes during a brief perinatal period in the nondividing precursors of spermatogonial stem cells at a stage in which retrotransposons undergo *de novo* methylation (11). Expression of Dnmt3L declines rapidly after birth and is extinguished by 6 days postpartum, when most prospermatogonia have differentiated into dividing spermatogonial stem cells (11). Targeted disruption of Dnmt3L causes azoospermia in homozygous male mice (12). In detail, loss of Dnmt3L from early germ cells leads to meiotic failure in spermatocytes, which do not express Dnmt3L (11).

The present report shows for the first time that a critical fetal determinant for future spermatogenesis, DNMT3L, is a specific marker for human ECs. This finding may shed light on the mechanisms of frequent TGCT development in patients with male infertility (13, 14).

Recent data further show that Dnmt3L is expressed in embryonic germ cells but not in PGCs (15). Takashima et al. (16) have shown that Dnmt3L is positive in ES cells and multipotent GS cells. We have also shown that human ES cells indeed express DNMT3L protein. These lines of data imply that DNMT3L is specifically expressed in cells with pluripotency such as ES or multipotent GS cells. EC cells belong to a group of mammalian pluripotent cells that includes ES cells and embryonic germ cells (17). The mechanisms involved in the activation of pluripotency in EC cells remain largely unknown. The specific expression of DNMT3L protein observed in EC, ES, and mouse embryonic germ cells suggests a possible involvement of DNMT3L in the maintenance of pluripotency. Thus, further analysis of DNMT3L function in pluripotent cells is warranted.

OCT3/4 and NANOG are nuclear transcription factors that are expressed in early embryonic cells and germ cells (18–24). Both seminomas and EC express OCT3/4 and NANOG. These transcription factors are now used to identify germ cell tumors (25–27). However, OCT3/4 and NANOG do not help with the distinction between seminomas and ECs.

Seminomas are composed of malignant cells mimicking PGCs/gonocytes (28, 29). In contrast, EC cells resemble pluripotent ES cells and have the capacity to differentiate: EC is regarded as the malignant counterpart of the pluripotent ES (29). Based on gene expression profiles, others postulated that seminomas closely resemble primordial germ cells, whereas EC represents a primitive ectoderm-like cell (17). Our data show that the mRNA of *DNMT3L* was widely expressed in a variety of TGCT cell types including seminomas. However, DNMT3L protein was specifically expressed in ECs but not in seminomas. Similar discrepancy between mRNA and protein expression of SOX2 has been reported in seminoma (30). These findings indicate that the presence of posttranscriptional downregulation may also be a distinctive trait among seminoma and EC.

Currently, SOX2 and CD30 are used as markers to detect EC cells (31–34). However, both SOX2 and CD30 antibodies react not only with ECs but also with other histologic components. As shown in the present study, others have shown that SOX2 is positive in endodermal cells of primitive gut in teratoma (34, 35). CD30 is positive in malignant lymphoma of the testis or lymphatic cells (32). In terms of specificity, DNMT3L seems to be the most prominent marker for ECs.

Diagnosis of germ cell origin is critical for patient management, especially for young adult men with primary tumors of unknown origin (27). Furthermore, accurate diagnosis of EC is critical for the management of patients with stage I nonseminomatous TGCTs because the percentage of EC in the primary tumor is predictive of occult metastasis (36). In this regard, the application of DNMT3L immunohistochemistry is a useful tool to identify EC cells in clinical practice. The growth inhibition and cell death observed in EC cells through DNMT3L suppression warrant further elucidation of the roles of DNMT3L in EC cells *in vivo* to evaluate the significance of DNMT3L as a therapeutic target of ECs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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