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Aberrant expression and distribution of the OCT-4 transcription factor in seminomas

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Summary

Testicular germ cell tumors (TGCTs), comprised of seminomas and non-seminomas, are derived from premalignant and noninvasive intracellular germ cell neoplasias. Among TGCTs, seminomas are believed to resemble a transformed state of primordial germ cells (PGCs) and are known to exhibit a gene expression profile similar to that of embryonic stem (ES) cells, such as transcription factor OCT-4. OCT-4 has recently been recognized as a diagnostic marker for clinical aspects of seminomas. However, the role of the OCT-4 protein in seminomas has not been clarified. To determine a possible role of the OCT-4 protein in seminomas, in this paper, we studied a series of 41 testicular tumor tissues and four cell lines by immunohistochemistry, Western blotting, and reverse-transcriptase polymerase chain reaction (RT-PCR) to examine the expression and distribution of the OCT-4 transcription factor in seminomas. By utilizing immunohistochemical staining and Western blotting, we demonstrated that the OCT-4 transcription factor was aberrantly localized in the cytoplasm and nuclei of cells in the collected seminoma tissues. This observation was further confirmed using immunocytochemical staining of NCCIT (seminoma-embryonal carcinoma) and NT2 (embryonal carcinoma) cells. In addition, the RT-PCR results indicated that *Oct-4* mRNA was relatively highly expressed in NCCIT, NT2 cells, and seminoma tissues when compared with human embryonic stem cells. The aberrant expression and distribution of the OCT-4 transcription factor in seminomas may provide some important clues concerning the cell transformation between germ line stem cells (like PGC) and testicular germ cell tumors.

Introduction

Carcinomas in the testes (especially germ cell tumors) are the most common malignancy in men aged 15~35 years [1]. The most common type of human germ cell tumor, which is derived from premalignant and noninvasive intracellular germ cell neoplasias (also called carcinoma in situ, CIS), is testicular germ cell tumors (TGCTs). TGCTs are histologically classified into seminomas, non-seminomas, and a mixed combination. Seminomas are generally histologically uniform and seem to

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resemble a transformed state of primordial germ cells (PGCs). Non-seminomas are a more-heterogeneous group of tumors which typically includes teratocarcinomas with undifferentiated/pluripotent embryonal carcinomas (ECs), yolk sac tumors (YSCs), and choriocarcinomas [2]. Among TGCTs, seminomas and ECs are thought to be pluripotent tumors [3].

The gene profiles of testicular tumors have been investigated by gene microarray analysis [4]. When compared with differentiated tissues, the most significantly upregulated genes in seminomas and ECs are early embryogenic genes, such as *Oct-4* [4]. The OCT-4 protein is a nuclear transcription factor belonging to class V of the Pit-Oct-Unc (POU) family (*Pou5f1*) [5]. It was first identified in mice as an ES cell- and germ line-specific transcription factor [6]. In ES cells, the OCT-4 protein is known to be a master regulator controlling self-renewal and differentiation processes. In embryo development, OCT-4 is highly expressed during the period of blastomeres to implanted-epiblasts, and then is downregulated at gastrulation [7]. In germ cell development, OCT-4 is first found in PGCs, and is then expressed at gonocytes (prospermatogonia) in the gonadal ridge (where PGCs migrate to proliferate). Following sexual development, OCT-4 continues to be expressed until undifferentiated A spermatogonia occurs in the testes (spermatogonial stem cells, SSCs) [7]. Apparently, regulation of OCT-4 protein expression plays a critical role in the specific fate of a cell.

In germ cell tumors, OCT-4 has been well-documented as being expressed in CIS which is considered to be the malignant counterpart of embryonic germ cells, most likely PGCs [8]. In addition, OCT-4 was also shown to be expressed in seminomas, ECs [9], dysgerminomas, and gonadoblastomas [10]. These observations suggest a tight link between the OCT-4 transcription factor and germ cell tumors. However, the role of the OCT-4 protein in germ cell tumors (like seminomas) remains unclear. To uncover possible roles of the OCT-4 protein in seminomas in this paper, immunohistochemical staining and Western blotting were utilized to examine seminoma tissues and four cell lines, and we demonstrated the aberrant distribution of the OCT-4 transcription factor (in both the cytoplasm and nuclei) in seminomas. In addition, using RT-PCR analysis with NCCIT (seminoma-EC) and NT2 (EC) cells, we further demonstrated that *Oct-4* mRNA is relatively highly expressed in seminoma tissues/NCCIT/NT2 cells compared with human embryonic stem (hES) cells. The cellular redistribution and overexpression of the primitive embryonic OCT-4 transcription factor in seminomas may provide important clues for understanding testicular germ cell transformation and developing clinical tumor treatments.

Materials and methods

Tissue samples and cell lines

Forty-one formalin-fixed, paraffin-embedded testicular tumor tissue samples were obtained from the surgical pathology files of Taipei Medical University Hospital (TMUH), Taipei, Taiwan. This study was approved by the institutional ethical and review board (IRB) of Taipei Medical University (TMU) for the protection of human subjects. All testicular tumors, including 16 cases of seminomas, 4 cases of ECs, 15 YSCs, and 6 unclassified germ cell tumors, were reviewed and reclassified according to the 2004 WHO classification system [2]. NCCIT (human seminoma–EC, CRL-2073^{TM}), NT2 (human embryonal carcinoma, CRL-1973 ^{TM}), and MRC5 (normal human lung epithelium, CCL-171 ^{TM}) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). hES cells were kindly provided by Dr. Hung-Chih Kuo, Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan.

Immunohistochemistry

For paraffin-embedded tissues, 4-μm-thick sections were dewaxed with xylene and rehydrated in graded alcohol. After being deparaffinized, tissue sections for immuno-recognition of OCT-4, cytokeratin, CD30, and CD117 were heated in 0.01 M citric buffer (pH 6.0) for 10 min with autoclaving. This pretreatment of sections was not required for alpha-1-fetoprotein (AFP) recognition. After being allowed to cool down to room temperature, sections were then treated with 3% H_2O_2 in phosphate-buffered saline (PBS) at room temperature for 30 min. Slides were washed, and then blocked with 5% normal horse serum in PBS and incubated with each primary antibody (Ab) at

Table 1. Antibody conditions of the immunohistochemical staining.

Antibody	Clone	Dilution	Company	Paraffin/Frozen section
OCT-4	sc-9081	1: 200	Santa Cruz Biotech	Frozen
OCT-4	sc-9081	1: 500	Santa Cruz Biotech	Paraffin
Cytokeratin	AE1/AE3	1: 100	DakoCytomation	Paraffin
AFP		1: 400	DakoCytomation	Paraffin
CD30	BER-H2	1: 50	DakoCytomation	Paraffin
CD117	c-KIT	1: 50	DakoCytomation	Paraffin

4 °C overnight. The experimental conditions of the primary antibodies (Abs) are listed in Table 1. For the OCT-4 Ab neutralizing assay, the OCT-4 Ab was preincubated with human OCT-4 recombinant protein (sc-4420, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a molar ratio of 1: 10 at 4 °C overnight. The enzyme activity was detected using an LSAB system with 3,3′-diaminobenzidine tetrachloride as the chromogen according to the manufacturer's instructions (DakoCytomation, Carpinteria, CA, USA). Non-specific binding sites were blocked using Protein Block (DakoCytomation). The immunostained sections were then counterstained with hematoxylin, dehydrated, and mounted.

For confocal analysis, frozen seminoma sections were fixed in 100% ethanol and rehydrated with PBS. NCCIT, NT2, and MRC-5 cells on cover glasses were washed with PBS, followed by fixing with 4% paraformaldehyde. All the seminoma sections, NCCIT, NT2, and MRC-5 cells were then blocked with 5% normal horse serum in PBS, and treated with a rabbit polyclonal anti-OCT-4 Ab (1 μg/ml, sc-9081, Santa Cruz Biotechnology). Cy3-conjugated anti-rabbit immunoglobulin (IgG) (Jackson ImmunoResearch, West Grove, PA, USA) was used as the secondary Ab.

Subcellular fractionation and Western blot analysis

Fresh seminoma tissues were frozen and homogenized in liquid N_2 to prepare the whole cells of the tissue. Total proteins of seminomas, hES, NCCIT, NT2, and MRC5 cells were extracted with lysis buffer containing 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, and 10 mM Tris–HCl (pH 7.5) with a protease inhibitor cocktail (Roche Diagnostics, NA, USA). The cytoplasm and nuclear extracts of the whole seminoma cells were prepared as described previously [11]. Briefly, cell pellets were suspended in a hypotonic buffer (20 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 10 mM KCl, 0.5% Nonidet P-40, 0.5 mM dithiothreitol, and the protease inhibitor cocktail) at 4 °C for 30 min. After centrifugation at $4000 \times g$ at 4 °C for 10 min, the supernatant was collected as the cytoplasmic fraction. To collect the nuclear fraction, the cell pellets of nuclei were washed twice with ice-cold hypotonic buffer, and then resuspended in a high-salt buffer (20 mM HEPES (pH 7.4), 0.4 M NaCl, 1 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and the protease inhibitor cocktail) and incubated on ice for 30 min. The supernatant fraction recovered from centrifugation was treated as the nuclear extract. The protein concentration was measured by a BCA protein quantification kit (Pierce, Rockford, IL, USA). Twenty micrograms of total protein and 10 μg of each nuclear or cytoplasmic fraction protein were boiled in Laemmli buffer, loaded on a mini 10% SDS-PAGE gel, transferred to a PVDF membrane, and then subjected to Western blot analysis. We used a rabbit polyclonal anti-OCT-4 Ab (0.2 μg/ml, sc-9081, Santa Cruz Biotechnology) and an anti-PARP Ab (0.1 μg/ml, sc-8007, Santa Cruz Biotechnology) as the primary Abs, followed by an HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) as the secondary Ab. In brief, the PVDF membranes were blocked in 5% skim milk in Tris-buffered saline (TBS) for 1 h at room temperature, and then incubated with the primary Abs at 4°C overnight. After washing with TBST (TBS containing 0.05% Tween-20, v/v), the blots were incubated with the secondary Ab at a dilution of 1:5000 at room temperature for 1 h. After washing in TBST four times, the ECL system (Amersham Pharmacia Biotech, Buckinghamshire, UK) was used to develop the immunoreactive bands.

Reverse-transcription polymerase chain reaction (RT-PCR)

Frozen human seminoma tissues were crushed and homogenized in liquid nitrogen. The total RNA of the human seminoma tissues, and hES, NCCIT, NT2, and MRC5 cells was extracted with an RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For the RT-PCR assay, random primers (Invitrogen, Carlsbad, CA, USA) and 1 μg total RNA were used with Superscript III reverse transcriptase (Invitrogen) for cDNA synthesis. The PCR was conducted with PlatinumTaq™ polymerase (Invitrogen) and the following primer pairs: *Oct-4* (forward primer, 5′-CAACTCCGATGGGGCCT-3′ and reverse primer, 5′-CTTCAGGAGCTTG-GCAAATTG-3′, 148-bp amplified product) and beta 2-microglobulin (*β2M*) (forward primer, 5′-GTCTCGCTCCGTGGCCTTA-3′ and reverse primer, 5′-TGAATCTTTGGAGTACGCTGG-ATA-3′, 81-bp amplified product). The mixture was first heated to 95 °C for 5 min in a DNA thermal cycle (GenAmp PCR System 2400; Perkin-Elmer, Quebec, Canada). Amplification was performed for 25 and 30 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, followed by 72 °C for 5 min.

Statistical analysis

All results were obtained from at least three independent experiments. The data are expressed as the mean ± SD. Differences in the means were assessed by one-way ANOVA, followed by the Tukey–Kramer multiple comparisons test.

Results

Aberrant localization of the OCT-4 transcription factor in human germ cell tumors

We collected 41 cases of TGCT tissues from TMUH to identify the cellular distribution of the OCT-4 transcription factor in human germ cell tumors. These tumor tissues were identified and classified into different tumor types using specific Abs (see Table 1 and "Materials and Methods"). OCT-4$^+$ CD117$^+$ CD30$^-$ cytokeratin$^-$ AFP$^-$ was for seminomas; OCT-4$^+$ CD117$^-$ CD30$^+$ cytokeratin$^+$ AFP$^-$ was for ECs; OCT-4$^-$ CD117$^-$ CD30$^-$ cytokeratin$^+$ AFP$^+$ was for YSCs; while others were classified as unspecified cases. OCT-4 protein expression was immunodetected in all classified tissue sections using a rabbit polyclonal anti-OCT-4 Ab. As shown in Table 2, the OCT-4 transcription factor was highly expressed in human seminoma (16 of 16) and EC tissues (4 of 4). From the aspect of cellular distribution, the OCT-4 protein was basically located in the nuclei of cells; however, we interestingly observed strong cytoplasmic expression of the OCT-4 transcription factor in seminomas (6 of 16) and faint cytoplasmic expression in EC tissues (2 of 4) (N+C indicated). Figure 1 demonstrates the nuclear and cytoplasmic localization of OCT-4 protein in seminomas by immunohistochemical staining with the anti-OCT-4 and anti-c-KIT Abs. We observed strong expression of the OCT-4 protein in seminoma cells, but not in the surrounding lymphocytes (Figure 1A, C). Higher-magnification images emphasized the OCT-4 transcription factor distribution in both the nuclei

Table 2. TGCTs collected from Taipei Medical University Hospital.

		Seminoma	Embryonal carcinoma	Yolk sac tumor	Unspecified cases
Immunogen	OCT-4 positive	(16/16)	(4/4)	(1/15)	(5/6)
OCT-4	N only	10/16	2/4	0/15	1/6
	C only	0/16	0/4	1$^{\#}$/15	3/6
	N$^{\#\#}$ + C	6/16	1/4	0/15	1/6
	N + C$^{\#\#}$	0/16	1/4	0/15	0/6
CD 117		15/16	0/4	2/15	6/6
CD 30		0/16	4/4	2/15	0/6
Cytokeratin		1$^{\#}$/16	4/4	15/15	6/6
AFP		0/16	0/4	15/15	2/6

N$^{\#\#}$ + C, OCT-4 protein dominantly localized to the nucleus; N + C$^{\#\#}$, OCT-4 protein dominantly localized to the cytosol; $^{\#}$ weak positive staining.

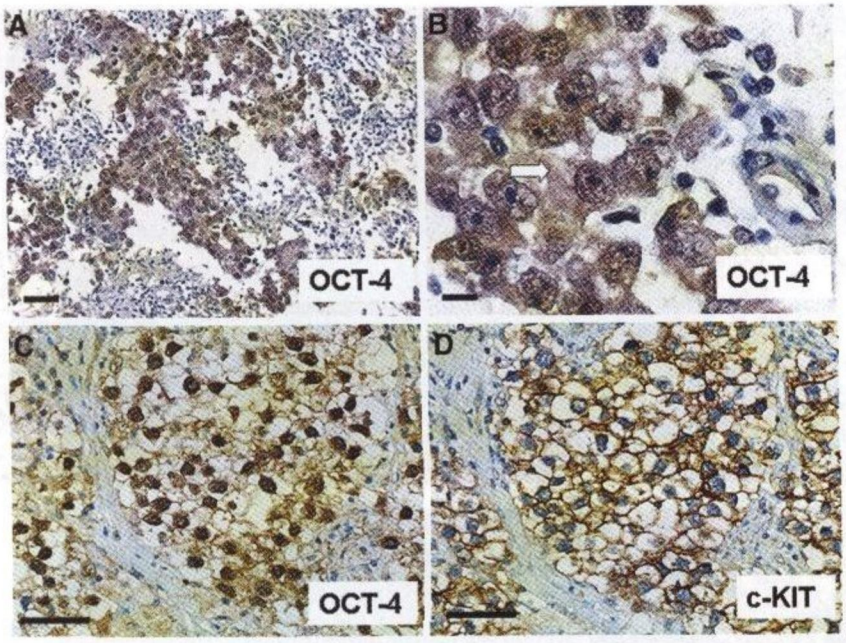


Figure 1. Immunolocalization of the OCT-4 transcription factor in paraffin-embedded seminoma tissues. The photograph shows OCT-4 transcription factor localization in paraffin-embedded seminoma tissue sections by immunostaining with the anti-OCT-4 Ab. Note the intense labeling of the nuclei and cytoplasm of the seminoma cells, but not the surrounding lymphocytes (A, C). Higher magnification shows strong staining of the cytoplasm in seminomas (B, white arrow). OCT-4-stained cells were positively recognized with an anti-c-KIT Ab (D). No staining was observed when using control rabbit immunoglobulin (IgG) and a second Ab alone (data not shown). Magnification: A, 200×; B, 1000×; C and D, 400×. Bar = 5 μm (A, C, and D); bar = 1 μm (B).

and cytoplasm of seminoma cells (Figure 1B, as indicated by a white arrow). C-KIT (CD117) staining of cytoplasmic OCT-4-positive cells further confirmed the seminoma characters of these cells (Figure 1C, D). Six cases of seminomas with cytosolic OCT-4 protein expression are shown in Figure 2A. In addition, the specificity of OCT-4 immunostaining (Figure 2B-b) was further confirmed by utilizing an antibody-neutralizing assay with the human OCT-4 recombinant protein (sc-4420, Santa Cruz Biotechnology) (Figure 2B-c), or a control with a secondary Ab alone (Figure 2B-d). In addition, in line with previous reports, the OCT-4 protein was not expressed in differentiated non-seminoma tissues, such as YSCs (Table 2) [12–14].

To rule out a nonspecific interaction of the OCT-4 Ab with paraffin-embedded sections, we collected freshly frozen seminoma tissues to detect the location of OCT-4 protein using confocal microscopy. As shown in Figure 3A, the OCT-4 protein was consistently observed to be localized in both the nuclei and cytoplasm of seminoma cells (as indicated by the arrows, upper panels). The surrounding lymphocytes were not positively stained (as indicated by arrowheads, upper panels). Immunostaining of hES cells with the same OCT-4 Ab showed exclusive specific positive nuclear staining, with no cytoplasmic signal (data not shown). In addition, the Cy3-conjugated second Ab alone (Figure 3A, lower panels) and control IgG (data not shown) also revealed negative staining. The fact of cytosolic expression of the OCT-4 transcription factor in seminomas was further confirmed by examining NCCIT, NT2, and MRC-5 cells (Figure 3B). In this experiment, NCCIT and NT2 cells were used as positive controls to show the cytosolic OCT-4 expression, and MRC-5 served as a negative control which produced no OCT-4 expression in cells. CD30 was used to distinguish with NCCIT ($CD30^-$) and NT2 ($CD30^+$) cells. Taken together, these observations further support the specific interaction of the OCT-4 Ab and both the cytoplasmic and nuclear distributions of the OCT-4 transcription factor in seminomas.

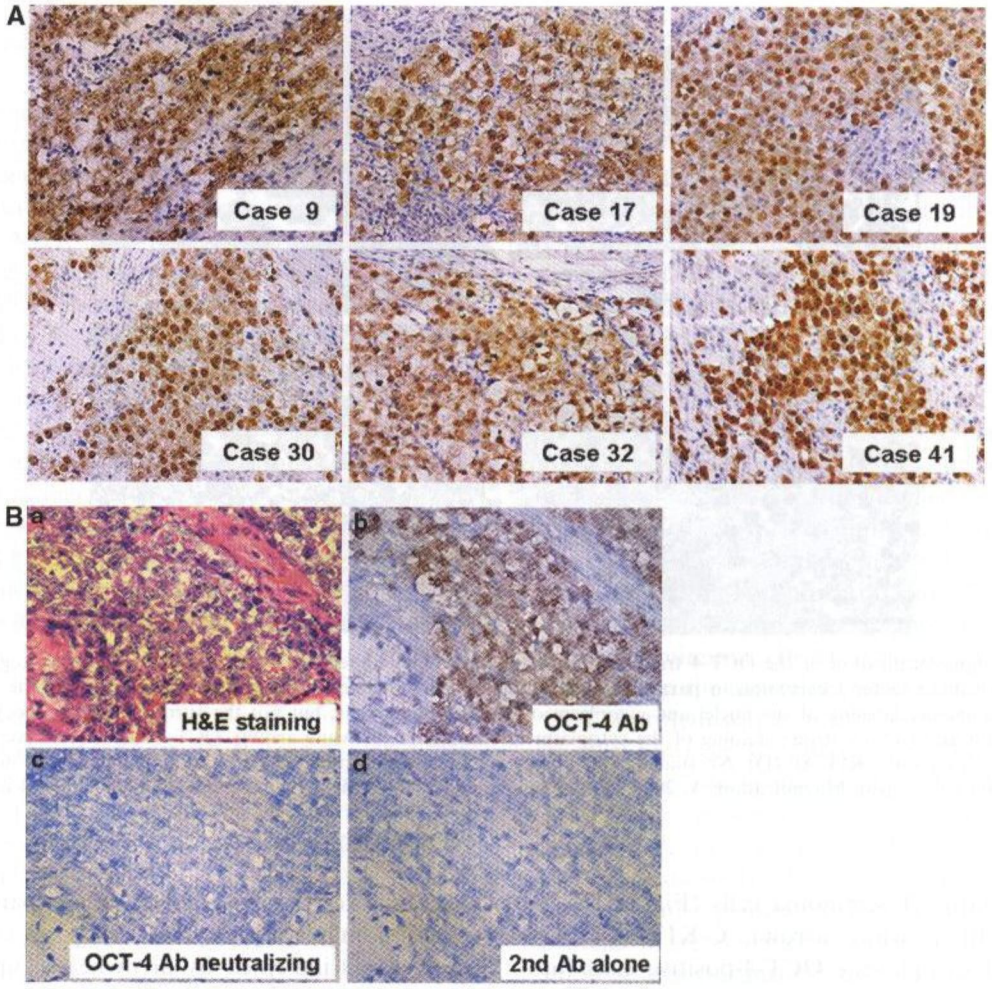


Figure 2. Specific localization of the OCT-4 transcription factor in paraffin-embedded seminoma tissues. Six seminoma cases with cytosolic OCT-4 protein expression are shown in (A). Specific localization of the OCT-4 transcription factor in paraffin-embedded seminoma tissues was verified by immunostaining with an anti-OCT-4 Ab (B-b); OCT-4 Ab neutralizing assay (B-c), and a control with secondary Ab alone (B-d). No staining was observed in the OCT-4 Ab neutralizing assay (B-c) or the secondary Ab control (B-d).

Western blot analysis of the cellular localization of the OCT-4 transcription factor in seminomas

The cytoplasmic expression of the OCT-4 transcription factor in seminoma tissues was further confirmed by Western blotting. For this purpose, fresh seminoma tissues were collected, and the nuclear and cytoplasmic fractions of total cells were separated as described previously [11]. The total extracted cell lysate, nuclear, and cytoplasmic proteins were loaded into a 10% SDS-PAGE gel to perform a Western blot analysis with an anti-OCT-4 Ab (sc-9081, Santa Cruz Biotechology). Cell lysates of hES, NCCIT, NT2, and MRC5 cells were used as controls to verify the specificity of the OCT-4 Ab. As shown in Figure 4A, in congruence with a previous report [15], there were two protein bands ranging 40–45 kDa in hES and NCCIT cells recognized by the OCT-4 Ab. MRC-5, a differentiated somatic cell line, showed no OCT-4 immunoreactivity. We used the same OCT-4 Ab to detect the total protein extracts of seminoma tissues. As shown in Figure 4B, two to three major bands with molecular weights of 40–45 kDa were detected in the whole-protein extract of seminoma tissues (lane 1). The OCT-4 protein was detected in both the nuclear fraction and the cytoplasmic part with different protein patterns of molecular sizes

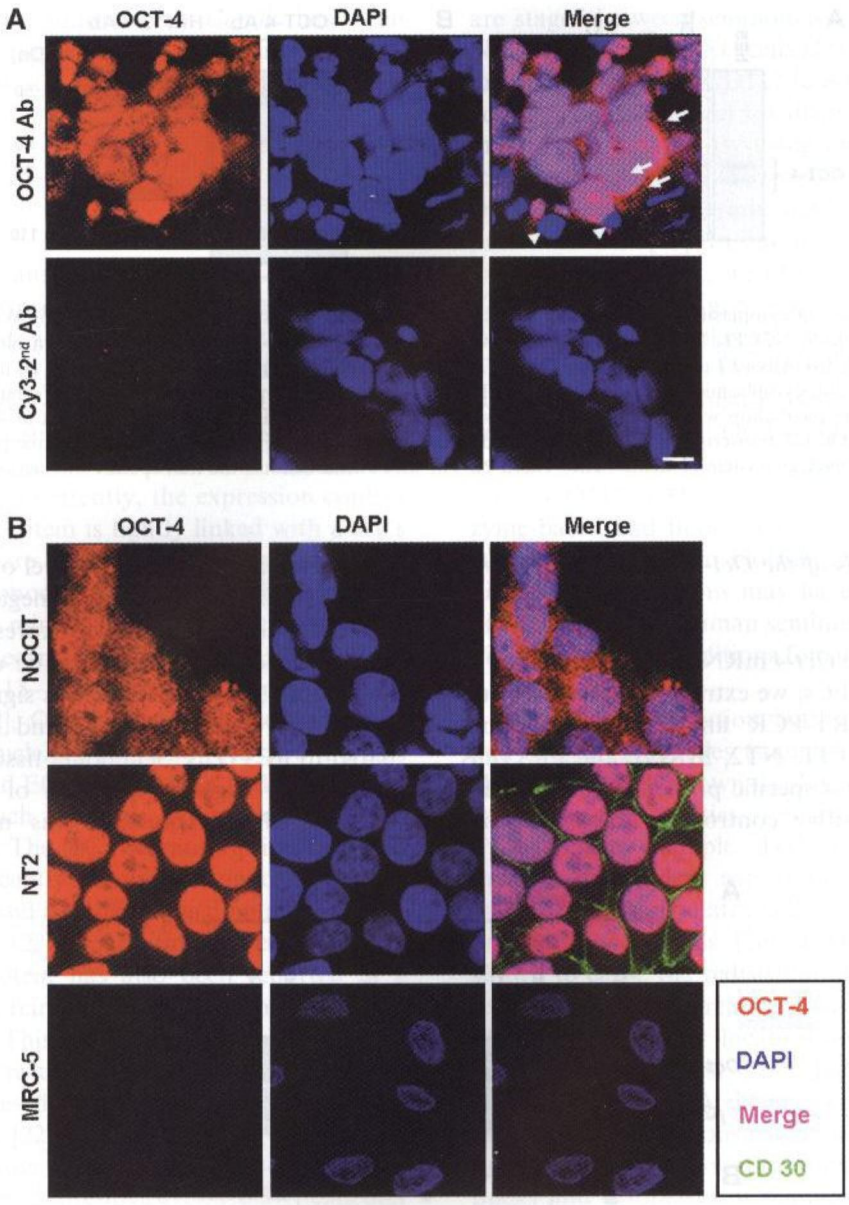


Figure 3. Immunolocalization of the OCT-4 transcription factor in frozen seminoma tissues and NCCIT/NT2/MRC-5 cells by confocal microscopic analysis. Frozen seminoma tissue sections (A) and NCCIT, NT2, and MRC-5 cells (B) stained with the anti-OCT-4 Ab were analyzed by confocal microscopy. Confocal fluorescence (OCT-4, in red) merged with the DAPI image (in blue) is shown in the "Merged" panel. Intense red immunofluorescence is highly associated with the nucleus as well as the cytoplasm in seminoma cells (A, upper panel, as indicated by arrows), and NCCIT/NT2 cells (B). Neither lymphocytes (A, upper panel, as indicated by arrowheads) nor MRC-5 (B) were stained with the anti-OCT-4 Abs. CD30 (in green) was used to distinguish NCCIT and NT2 cells (B). The secondary Ab alone showed negative staining (A, lower panels).

(lanes 2 vs. 3). This result suggests the existence of diverse characteristics of the nuclear and cytoplasmic OCT-4 proteins. PARP, a nucleus-specific poly-(ADP-ribose) polymerase, was also detected specifically in nuclear fraction proteins. PARP was shown to exclusively be expressed in the nuclear fraction (lanes 1 and 2), not in the cytoplasmic part (lane 3). This observation indicates that no contamination of the cytoplasmic fraction by nuclear OCT-4 protein occurred.

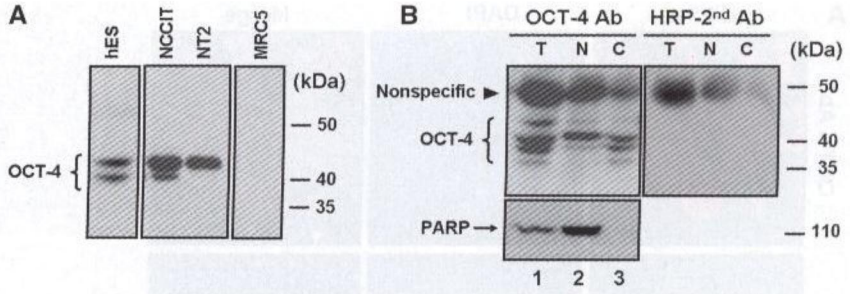


Figure 4. Identification of cytoplasmic localization of the OCT-4 transcription factor in seminoma tissues by Western blot analysis. (A) Cell extracts of hES, NCCIT, NT2, and MRC5 cells were separated by SDS-PAGE, and the Western blot analysis was performed with anti-OCT-4 Abs (0.2 μg/ml, sc-9081, Santa Cruz Biotechnology). (B) Total cell extracts (T, 20 μg), nuclear fraction extracts (N, 10 μg), and cytoplasmic fraction extracts (C, 10 μg) were separated by SDS-PAGE, and a Western blot analysis was performed. Following incubation with the anti-OCT-4 Abs (0.2 μg/ml), three specific major protein bands of 40–45 kDa were detected (lane 1). The OCT-4 transcription factor was located in the nuclear fraction as well as the cytoplasmic part (lanes 2 and 3). PARP indicates the nuclear protein contents (lanes 1 and 2). The arrowhead denotes the non-specific interaction of the second Ab staining.

RT-PCR analysis of the Oct-4 mRNA expression levels

To determine the *Oct-4* mRNA expression levels in seminomas and ECs, we extracted total RNA and performed an RT-PCR analysis of seminoma tissues from NCCIT, NT2, MRC5, and hES cells with human *Oct-4*-specific primers. We used hES cells as the positive control, as these cells are known to express a normal level of *Oct-4* mRNA; MRC5 cells were used as a negative control to demonstrate the negative expression of *Oct-4* mRNA in differentiated somatic cells. As shown in Figure 5, *Oct-4* mRNA was significantly more-highly expressed in NCCIT and NT2 cells compared to hES cells. Seminoma tissues also showed a relatively higher expression of *Oct-4* mRNA. *Oct-4* mRNA expression was not detected in

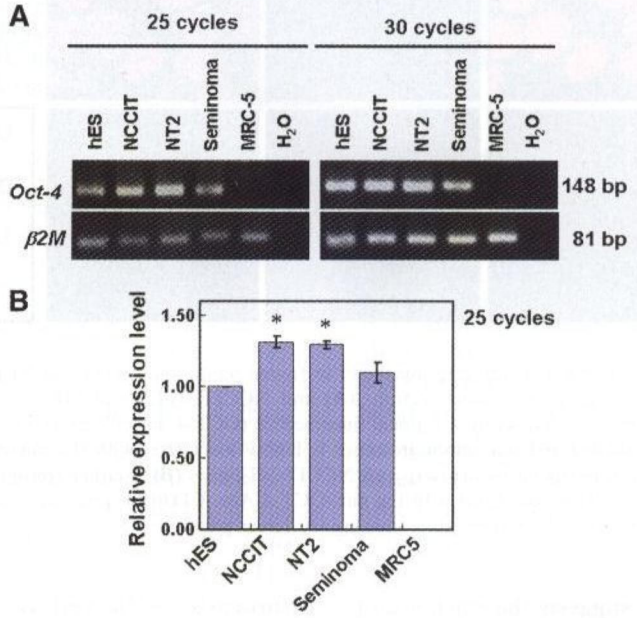


Figure 5. Identification of the *Oct-4* mRNA expression level by RT-PCR. Fresh seminoma tissues were collected from Taipei Medical University Hospital. The *Oct-4* mRNA expression levels in seminoma tissues, and hES, NCCIT, NT2, and MRC5 cells were determined by RT-PCR with 25 and 30 PCR cycles (A). *β2M* served as the internal control. The relative expression levels of *Oct-4* mRNA detected by 25 PCR cycles were normalized to *β2M* expression, and the intensity of hES cells is shown as 100% (B). * Denotes that the difference from the hES group was significant at $p < 0.05$.

MRC5 cells. In addition, as *Gapdh* has been reported to be upregulated in seminomas [16], *β2M* was used as the internal control in this experiment.

Discussion

The OCT-4 transcription factor is well-documented to be expressed in pluripotent mouse and human ES and germ line stem cells, including PGCs [6, 17]. In mouse and human ES cells, inhibition of OCT-4 expression results in trophoectoderm differentiation [18]. In PGCs, loss of the OCT-4 protein has been shown to induce cell apoptosis [19]. Apparently, the expression control of the OCT-4 protein is tightly linked with a cell's fate. TGCTs are known to originate from an erased PGC/gonocyte [20], and OCT-4's expression in TGCTs mimics the development pattern of germ cells. Recently, by immunohistochemical staining [10, 13–15] and a multitumor tissue microarray [12], OCT-4 was demonstrated to be expressed in nuclei of testicular CIS and TGCTs (seminomas and EC cells), but not in differentiated components such as YSCs, teratomas, and choriocarcinomas. The OCT-4 transcription factor in TGCTs has recently become a diagnostic marker of seminomas and ECs due to its dominant nuclear location [9, 10, 12, 15]. Additionally, expression of the OCT-4 protein has also been reported in a number of carcinoma cell lines by RT-PCR analysis [21]. This observation has been interpreted as the result of aberrant reactivation of embryonic genes during the process of malignant transformation [22].

The diagnostic value of the OCT-4 transcription factor for both pre-invasive/invasive testicular germ cell tumors and somatic carcinoma cell lines is based on its dominant nuclear expression. Interestingly, in our results, by collecting patients' testicular tumor tissues and using immunohistochemical staining and Western blot analysis, we found that the OCT-4 transcription factor was redistributed between the cytoplasm and nuclei of cells of seminoma tissues (Table 2, Figures 1–4). The characteristics of seminoma cells in this experiment were further confirmed by c-KIT Ab recognition (Table 2, Figure 1C vs. D) [23]. Further study using NCCIT and NT2 cells also supported this result (Figure 3B). NCCIT cells are staged between seminomas and ECs [24]; and NT2 cells belong to EC cells [25]. Two cell surface markers, CD30 and CD117 (c-KIT), when used in combination are useful for distinguishing seminomas from ECs [23]. By using immunohistochemical staining, previous work of de-Jong's lab showed faint cytoplasmic staining of the OCT-4 protein in ECs [15]. In our study, partially in line with de-Jong's work, we observed that NT2 cells ($CD117^{-}CD30^{+}$, Figure 3B) showed strong nuclear and faint cytoplasmic staining of the OCT-4 transcription factor. Furthermore, in contrast to de-Jong's work, we further observed strong nuclear and cytoplasmic OCT-4 protein staining in human seminoma tissues (Figure 3A) and NCCIT cells ($CD117^{+}CD30^{-}$, Figure 3B) by both enzyme-based and fluorescent immunocytochemical staining and Western blotting (Figures 1–4). The divergent observations may have been due to the different collected human seminoma tissues and/or different culture conditions for cells used for OCT-4 immunostaining.

Protein redistribution between the cytoplasm and specific organelles (such as nuclei and mitochondria) is well known to play important roles in regulating cell activities of normal and cancer cells [26, 27]. For example, TGF-β was reported to mediate the nuclear export of prohibitin to the cytosol, which regulates cell apoptosis in human prostate cancer cells [26]; ERK has also been shown to cause the redistribution of ATR (ataxia telangiectasia-Rad3-related kinase, with both cytoplasmic and nuclear localization) from the cytoplasm to the nucleus, which facilitates activation of the S-phase DNA damage checkpoint [27]. In this paper, we demonstrated the aberrant distribution of the OCT-4 transcription factor in the nuclei and cytoplasm of seminoma cells. First, in terms of OCT-4's expression and aberrant distribution, we observed two or three major bands of the OCT-4 protein ranging 40–45 kDa expressed in seminoma tissues by Western blot analysis; notably, the size pattern of the OCT-4 protein in the nuclear fraction differed from that in the cytoplasm part (Figure 4B). This observation suggests post-modification differences in nuclear and cytoplasmic OCT-4 proteins in seminomas. In addition, the *Oct-4* mRNA levels in NCCIT/NT2 cells were relatively higher than those of hES cells ($p < 0.05$, Figure 5). Human seminoma tissues also showed relatively higher expression levels of

Table 3. Clinicopathological correlations of OCT-4 distribution in seminomas.

OCT-4 expression	N + C n = 6	N only n = 10	p value
Age (medium ± SD)	32.6 ± 2.42	35.5 ± 10.52	0.59
Tumor size (medium ± SD)	11.6 ± 3.83	7.0 ± 4.66	0.14
Local tenderness			
Yes	0	4	0.1
No	6	6	
Associated with NSGCT			
Yes	1	2	1.0
No	5	8	
Local invasion #			
Yes	2	2	1.0
No	4	8	
Tumor necrosis*			
Presence	4	1	0.015
Absence	2	9	
Lymph node metastasis			
Presence	1	1	1.0
Absence	5	9	
Distant metastasis			
Presence	1	1	1.0
Absence	5	9	

NSGCT, non-seminomatous germ cell tumor; #, tumor had invaded the regional epididymis and spermatic cord; *, necrosis noted in the area of the seminoma; N, nuclear; C, cytoplasmic.

Oct-4 mRNA than hES cells, although there was no statistical significance between the two groups. This result may have been due to the presence of other cell types (like lymphocytes) in the collected tumor tissues (Figures 3A and 5). Second, in terms of clinical pathology, we found the cytosolic OCT-4 expression may correlate with tumor necrosis ($p < 0.05$, Table 3). How the mislocalization of the OCT-4 transcription factor leads to seminoma necrosis, or, vice versa, remains unclear.

The most significant finding of this study is the first demonstration of the aberrant distribution and overexpression of the OCT-4 transcription factor and its possible clinicopathological association in seminomas. Given that the control of OCT-4's expression is tightly correlated with a cell's fate, the aberrant localization and expression of the OCT-4 protein in seminomas may provide some hints as to the cell transformation process between germ line stem cells (like PGCs) and germ cell tumors. Whether epigenetic modification or other molecular regulatory processes mediate the expression level and cellular localization of the OCT-4 transcription factor in seminomas remains to be determined in further studies. Understanding the regulatory mechanisms associated with the OCT-4 transcription factor and seminomas would be helpful in the clinical treatment of this disease.

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