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POU5F1 (OCT3/4) Identifies Cells with Pluripotent Potential in Human Germ Cell Tumors[1]

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ABSTRACT

Human germ cell tumors (GCTs) may have variable histology and clinical behavior, depending on factors such as sex of the patient, age at clinical diagnosis, and anatomical site of the tumor. Some types of GCT, *i.e.*, the seminomas/germinomas/dysgerminomas and embryonal carcinomas (the stem cell component of nonseminomas), have pluripotent potential, which is demonstrated by their capacity to differentiate into somatic and/or extraembryonic elements. Although embryonal carcinoma cells are intrinsically pluripotent, seminoma/germinoma/dysgerminoma cells, as well as their precursor carcinoma *in situ*/gonadoblastoma cells, have the phenotype of early germ cells that can be activated to pluripotency. The other types of GCT (teratomas and yolk sac tumors of infants and newborn, dermoid cyst of the ovary, and spermatocytic seminoma of elderly) are composed of (fully) differentiated tissues and lack the appearance of undifferentiated and pluripotent stem cells. OCT3/4, a transcription factor also known as OTF3 and POU5F1, is involved in regulation of pluripotency during normal development and is detectable in embryonic stem and germ cells. We analyzed the presence of POU5F1 in GCT and other tumor types using immunohistochemistry. The protein was consistently detected in carcinoma *in situ*/gonadoblastoma, seminomas/germinoma/dysgerminoma, and embryonal carcinoma but not in the various types of differentiated nonseminomas. Multitumor tissue microarray analysis covering >100 different tumor categories and 3600 individual cancers verified that POU5F1 expression is specific for particular subtypes of GCT of adults. No protein was observed in GCT of newborn and infants, spermatocytic seminomas, and the various tumors of nongerm cell origin. In addition, no difference in staining pattern was found in chemosensitive and chemoresistant GCT of adults. These results indicate preservation of the link between POU5F1 and pluripotency, as reported during normal development, after malignant transformation. Therefore, POU5F1 immunohistochemistry is an informative diagnostic tool for pluripotent GCT and offers new insights into the histological heterogeneity of this cancer.

INTRODUCTION

oct3/4, also known as *otf3* or *pou5f1*, is a member of the POU family of transcription factors, which is expressed in pluripotent mouse and human embryonic stem and germ cells, including PGCs[4] (1–6). Expression of this gene is down-regulated during differentiation (7). Furthermore, knocking out the *pou5f1* gene in mice causes early lethality because of lack of inner cell mass formation (8) because pou5f1 is critical for self-renewal of embryonic stem cells (9). Interestingly, pou5fl has been linked to the capacity of proper outgrowth of somatic cell clones (10). During human development, expression of *POU5F1* is found at least until the blastocyst stage (11) in which it is involved in gene expression regulation. The protein activates transcription via octamer motifs located distally or proximally from transcriptional start sites (12). POU5F1 binding sites have been identified in various genes, including *fibroblast growth factor 4* and the 1.5-kb alternative promoter of the *platelet-derived growth factor* α receptor (13). The data indicate that pou5f1/POU5F1 functions as a master switch in differentiation by regulating cells that have, or can develop, pluripotent potential.

We have previously demonstrated that *POU5F1* transcripts are found in a specific set of human testicular GCT of adolescents and young adults (TGCT): the seminomas and embryonal carcinomas (14). In addition, the precursor lesions of TGCT, known as CIS (15), also express *POU5F1* (14). These lesions are composed of cells that are considered to be the malignant counterpart of an embryonic germ cell, most likely a PGC (15–17). Interestingly, these cell types are in principle pluripotent or even multipotent (Ref. 18, for review). In contrast, no expression was found in the differentiated components of nonseminomas, *i.e.*, teratomas, yolk sac tumors, and choriocarcinomas (14). Indeed, expression of *POU5F1* has been reported in embryonal carcinoma cells lines, and down-regulation of expression is found upon differentiation (4, 13).

In contrast to our finding of a specific expression pattern of *POU5F1* in TGCT, expression of this gene has recently been reported in nonmalignant adult human tissues (19), as well as in a number of carcinoma cell lines (20). This latter finding was interpreted as the result of aberrant reactivation of embryonic genes during the process of malignant transformation. However, the conclusion that *POU5F1* is expressed in these cells was based solely on reverse transcription-PCR results, which can be misleading because of the presence of multiple *POU5F1* pseudogenes (Refs. 14, 19, 21, own unpublished observations). We are not aware of any previous studies reporting analysis of POU5F1 protein expression in normal and malignant human tissues to clarify whether the mRNA is translated to functional POU5F1 protein. Therefore, an extensive immunohistochemical screening for POU5F1 protein expression was done in various types of GCT at different sites and in a set of >3600 tumors of >100 different types using multitumor tissue microarrays. POU5F1 immu-

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[4] The abbreviations used are: PGC, primordial germ cell; GCT, germ cell tumor; CIS, carcinoma *in situ*; TGCT, testicular germ cell tumor of adolescents and young adults.

noreactivity was detected only in cells of CIS/gonadoblastoma, seminoma/germinoma/dysgerminoma, and embryonal carcinoma. These results convincingly demonstrate that the presence of POU5F1 protein is related to pluripotent capacity of human GCT and that reactivation of its expression is not a common mechanism in cancer. Conclusively, POU5F1 is a distinctive immunohistochemical marker to identify tumor cells resembling embryonic/primordial germ and embryonic stem cells.

MATERIALS AND METHODS

Sample Handling and Characterization. The (T)GCT not included in the tissue arrays were collected in the southwestern part of the Netherlands in collaboration with urologists and pathologists. Representative parts of the tumor (and adjacent tissue, if available) were snap frozen in liquid nitrogen and were fixed in 10% formalin for paraffin embedding. Tumors were diagnosed according to the WHO classification (22), as previously described (23), supported by immunohistochemistry using antibodies directed against germ cell/placental alkaline phosphatase, α-feto protein, human chorionic gonadotropin, the stem cell factor receptor c-KIT, and cytokeratin (CAM5.2). The testicular tumors included 35 seminomas, 50 nonseminomas [with 14 embryonal carcinoma components, 21 teratoma components (6 mature, 5 immature, and 10 mixed), 18 yolk sac tumor components, and 5 choriocarcinoma components], and 10 spermatocytic seminomas. In addition, CIS containing testicular parenchyma ($n = 16$, including both adjacent to seminoma and nonseminoma) and embryonic testes of different developmental stages, *i.e.*, from 17 to 40 weeks and 28 weeks postpartum, were included. These latter samples have been reported before (24). Moreover, 3 gonadoblastomas, found in dysgenetic gonads, of which 2 also contained dysgerminoma, as well as 4 GCT of the midline of the brain of adults (including 1 germinoma, 1 embryonal carcinoma, and 2 mixed differentiated nonseminomas) were included. To investigate possible difference in presence of POU5F1 between chemotherapy-sensitive and -resistant GCT, a series of 34 patients with known clinical course, including 12 high-risk patients that were relapse-free after high-dose chemotherapy and 22 refractory cases, were investigated. Part of this series has been reported before (25, 26). The clinical parameters are indicated in Table 1. Patients were considered refractory when progression or relapse occurred despite adequate initial and salvage treatment, including high-dose chemotherapy with autologous stem-cell transplantation.

Table 1 *Clinical parameters of the patients with chemosensitive and chemoresistant GCT*

	Sensitive	Resistant
Nos. of cases	12	22
Median age in years (range)	28 (20–47)	29 (17–56)
Histology[a]		
Seminoma	1	1
Nonseminoma	11	21
Stage at diagnosis (UICC[b])		
I	0	3
II	0	7
III	12	12
Initial treatment after surgery		
Surveillance	0	2
Chemotherapy	12	20
Follow-up (months)		
Median (range)	51 (14–69)	39 (11–180)
Relapse free survival (months)		
Median (range)	NR	7.1 (0–150)
Response to initial treatment		
Complete remission	8	5
Partial remission, marker −	4	8
Partial remission, marker +	0	3
Progressive disease	0	3
Unknown	0	3
No. of salvage regiments		
Median (range)	0	3 (1–9)

[a] For the POU5F1 immunostaining all seminoma, embryonal carcinoma, and CIS components of both the sensitive and resistant tumors (3 and 2; 6 and 8; and 4 and 6, respectively) are positive, whereas all differentiated nonseminoma components are negative.

[b] UICC, International Union Against Cancer; NR, not reached.

To expand the series of both GCT and non-GCT, three different multitumor tissue microarrays were investigated. One was generated at the Department of Pathology of the University of Basel (Basel, Switzerland), and included 3273 interpretable individual tumors of >100 different types, represented on seven different slides (Table 2 and Fig. 2). The second was generated at the Division of Pediatric Pathology of the Johns Hopkins Medical Institution (Baltimore, MD) and included 84 (T)GCT of newborn, infants, and adolescents (Table 3), represented on four different slides. Both arrays contained various positive and negative controls. The third array, generated at the Department of Pathology, Erasmus Medical Center (GE Rotterdam, the Netherlands), included 48 esophagus tumors and 100 prostate cancers (50 progressing and 50 nonprogressing), as well as benign prostatic hypertrophy ($n = 18$). The result of this latter array is included in Table 1.

Immunohistochemistry. Immunohistochemistry with anti-POU5F1 antibodies was performed on paraffin-embedded tissue sections of 3-μm thickness. The sections were incubated overnight at 4°C with a polyclonal goat anti-POU5F1 antibody (C20, sc 8629; Santa Cruz Biotechnology, Santa Cruz, CA), directed toward the COOH terminus of the protein, diluted 1:8000 (final concentration, 0.025 μg/ml). Subsequently, a biotinylated horse-antigoat secondary antibody was applied to the sections, and the bound antibody complex was visualized using the horseradish peroxidase avidin-biotin complex method. Double fluorescence staining was performed using the polyclonal goat-anti-POU5Fl antibody and a monoclonal mouse-anti-c-KIT antibody (final concentration 2 μg/ml; Neomarkers, Fremont, CA) on frozen tissue sections. c-KIT is a known marker for CIS and seminomas (27). Secondary antibodies were labeled with FITC and CY3 (Dako Diagnostics, Glostrup, Denmark, and Jackson Immunoresearch, West Grove, PA, with a final concentration of 5 μg/ml), respectively. The stainings were also performed separately. Every experiment was accompanied by appropriate positive and negative (without primary antibody) controls.

Cell Culture. The human cell lines Tera2 (28), 2102Ep (29), and NCCIT (30), all nonseminoma derived, were cultured and split under conventional conditions (37°C, 5% CO_2). Cell lines were cultured until 80% confluency, and differentiation was induced with retinoic acid as described previously (30). Immunohistochemistry was performed on cytospin preparations as described above.

RESULTS

POU5F1 Immunohistochemistry on TGCT. On the basis of mRNA analysis (14), we expected that POU5F1 protein is present in specific different histological subtypes of TGCT. We first analyzed the presence of POU5F1 by immunohistochemistry on a series of TGCT of various histological types. Representative images of representative stainings are shown in Fig. 1. All tumor cells of seminomas and embryonal carcinomas showed a nuclear staining (Fig. 1, *A* and *B*), whereas all nontumor cells were negative. A similar pattern of staining was found in the gonadoblastomas, dysgerminomas and germinomas, and embryonal carcinomas of the brain (see also below). In contrast, all teratomas, both mature and immature, choriocarcinomas, and yolk sac tumors were negative (Fig. 1, *C–E*), as were the differentiated nonseminomas of the brain (data not shown). The staining intensity in the positive cases varied between moderate and high. None of the spermatocytic seminomas showed a positive staining (Fig. 1*F*). We also tested a series of CIS containing testicular parenchyma samples, both adjacent to seminoma and nonseminoma. All CIS cells, identified by a double staining for c-KIT, irrespective of the histology of the invasive tumor, were positive for POU5F1 (Fig. 1*G* and *inset*). In contrast, no protein could be detected in any stage of spermatogenesis (see below). This makes POU5F1 one of the most informative immunohistochemical markers to identify CIS cells that our laboratory has tested.

To investigate whether a difference in presence of POU5F1 exists between chemosensitive and chemoresistant GCT, immunohistochemistry was done on a well-characterized series of GCT with known clinical outcome (Refs. 25, 26 and Table 2). The results

Table 2 *Results of tissue array immunohistochemistry of 3439 individual tumors of >100 different types for POU5F1*

The organ (in alphabetic order), tumor type, number of cases studied, and positive findings are indicated

Organ	Tumor	Subgroup	*n*	Positive
Adrenal gland	Carcinoma	Adeno-	19	0
Anus	Carcinoma	Squamous	3	0
Brain		Gliobl. multif./astrocytoma/	46/35	0
		oligodendroglioma/meningioma/	24/46	0
		PNET/ependymoma/	17/9	0
		gangioneuroma/	7	0
		medulloblastoma/estioneuroblastoma/	4/2	0
		opticus glioma/cranioph.[a]	1/8	0
Carotid body	Glomus tumor		11	0
Cervix	Carcinoma	Squamous/CIS/adeno	37/7/4	0
Colon	Adenoma	Tubular and villous	134	0
	Carcinoma	Adeno-	47	0
Endometrium	Carcinoma	Adeno-	61	0
	Sarcoma	Endometrial stromal sarcoma	3	0
Esophagus	Carcinoma	Squamous/adeno	21/116	0
Gall bladder	Carcinoma	Adeno-	23	0
Glomus tumor			11	0
Kidney	Carcinoma	Clear cell/papillary	50/45	1/0
		Chromophobic	13	0
	Oncocytoma		7	0
Larynx	Carcinioma	Squamous	46	0
Liver	Carcinoma	Hepatocellular	41	0
Lung	Carcinoma	Adeno-/squamous/	48/50	0/1
		large cell/small cell	47/41	1/0
Lymph nodes	Lymphoma	Hodgkin's	50	0
		Non-Hodgkin's high/low grade	69	0
		MALT	42	0
Breast	Carcinoma	Lobular/ductal	42/46	0
		Med./muc./tub.	27/23/26	0
		Cribriform/apocrine	5/3	0
	Phylloidees tumor		13	0
Mouth	Carcinoma	Squamous	57	0
Ovary	Carcinoma	Endometroid/serous mucinous	49/48/20	0
		Brenner/mixed Müllerian tumor	9/6	0
	Granulosa cell		7	0
	Dysgerminoma		2	2
	Gonadoblastoma		1	1
Pancreas	Carcinoma	Adeno-	47	0
Paraganglia	Paraganglioma		10	0
Parathyroid	Carcinoma	Adeno-	20	0
Parotid gland	Carcinoma	Mixed	15	0
		Acinic cell/mucoepithelial	5/6	0
Phaeochrom.			26	0
Penis	Carcinoma	Squamous	43	0
Prostate	Carcinoma	Adeno-, horm. (non-)refract.	200	0
	PIN/BPH		24/18	0
(Small) salivary gland	Pleom. adenoma		49	0
	Lymphoep. lesion		5	0
	Carcinoma	Adenoid cystic	43	0
		Acinic cell	36	0
	Adenolymph.		30	0
Skin	Carcinoma	Squamous/basal	81/37	0
	Melanoma		44	0
	Merkel cell tumor		6	0
	Benign	Hemangioblastoma	8	0
		Nevus	38	0
		Appendix tumors	25	0
(Small) intestine	Carcinoma	Adeno-	17	0
	GIST		14	0
Stomach	Carcinoma	Intestinal/diffuse	44/23	0
Soft tissue	Benign	Lipoma/fibrous histiocytoma/Schwann/	15/58/35	0
		neurofibroma/leiomyoblastoma/	31/7	0
		giant cell tumors of tendon sheet/	29/	0
		hemangioma	17	0
	Intermediate	Haemangiopericytoma	8	0
		Dermatofibrosarcoma protuberans	2	0
	Malignant	Kaposi/liposarcoma	25/26	0
		Angiomyolipo./malign. Schwann.	1/8	0
		Rhabdomyosarcoma/synovial sarcoma	13/3	0
		Angios./fibros./alv. soft part sarc.	4/6/1	0
		Epithelioid sarcoma	2	0
Testis		Seminoma/nonseminoma	49/47	49/33[b]
		Adenomatoid tumor	10	0
Thyroid	Carcinoma	Foll./papill./med.	42/48/36	0
Thymus	Thymoma		23	0
Urinary bladder	Carcinoma	TCC	101	0
		Small cell/squamous/adeno-	3/5/4	0
	Sarcoma		7	0
Uterus	Myoma		107	0
Vagina	Carcinoma	Squamous	5	0
Vulva	Carcinoma	Squamous	37	0
Various	Carcinoid		48	0
	Adenomatoid tumor		10	0
	Mesothelioma		23	0
Controls[c]			30	0

[a] Gliob. multif., glioblastoma multiforme; PNET, primitive neuro-ectodermal tumor; cranioph, craniopharyngioma; CIS, carcinoma in situ; MALT, mucosa associated lymphoid tissue; med, medullar; muc, mucinous; tub, tubular; horm (non-)refract, hormonal (non)refractory; angiomyolipo., angiomyoliposarcoma; malign. Schwann., malignant Schwannoma; anglo., angiosarcoma; fibros., fibrosarcoma; alv. soft part sarc., alveolar soft part sarcoma; foll., follical.

[b] If containing seminoma in case of combined tumors or embryonal carcinoma elements.

[c] Included in all seven slides: skin ($n = 4$); lymph node ($n = 2$); heart ($n = 2$); skeletal muscle ($n = 2$); smooth muscle ($n = 2$); myometrium ($n = 2$); thyroid gland ($n = 2$); liver ($n = 2$); kidney cortex ($n = 2$); prostate ($n = 2$); testis ($n = 2$); submand. gland ($n = 2$); proliferative endometrium ($n = 2$); gray matter cerebrum ($n = 2$).

Table 3 *Results of the multitissue microarray immunohistochemistry on 84 germ cell tumors of predominantly newborn, infants, and adolescents for POU5F1*

The histology and anatomical localization of the tumor, sex of the patient, number of studied cases (*n*), age at clinical presentation and number of positive findings are indicated.

Histology	Sex[a]	Anat. Local.	*n*	Age (yr)	Positive
Teratoma		*Total*:	25		0
Mature	M	Sacral	2	0/1	0
	F	"	2	0/0	0
	M	Nervous system	1	6	0
	F	Gonad	2	15/34	0
	M	"	1	0.3	0
Immature	F	Sacral	3	0/0/0	0
	M	"	1	0.3	0
	F	Gonad	11	5/6/8/10/10/11/12/12/13/15/?[b]	0
	M	"	1	0.8	0
	?	?	1	?	0
Yolk sac tumor		*Total*:	33		
	F	Sacral	6	0.2/1/1/2/2.6/5	0
	M	"	1	1.5	0
	?	"	6	0/0.5/?/?/?/?	0
	M	Gonad	10	0.7/0.7/1.3/1.3/1.9/2/2/2.8/3/4	0
	F	"	9	7/8/9/10/10/13/18/20/24	0
Gonadoblastoma/Dysgerminoma	D	Gonad	1	?	1
Dysgerminoma	F	Gonad	11	12/12/12/17/18/14/14/?/?/?/?	1
Dysgerminoma/Nonseminoma	F	Gonad	1	12	1
Seminoma	M	Gonad	4	27/30/41/?	4
Mixed GCT[c]	M	Gonad	4	27/28/29/?	4[d]
Nonseminoma	M	Gonad	5	23/?/?/?	5[e]
Controls[f]	M/F		30		0

[a] M, male; F, female; ?, unknown; D, dysgenetic gonad.
[b] ?, unknown.
[c] Germ cell tumor.
[d] When containing either a seminoma of embryonal carcinoma component.
[e] When containing an embryonal carcinoma component.
[f] POC ($n = 9$); fetal kidney ($n = 4$), fetal ovary ($n = 1$), fetal uterus ($n = 2$), Wilms' tumor ($n = 2$), hepatoblastoma ($n = 3$), embryonal neuroblastoma ($n = 5$), testis ($n = 3$), MMMT ($n = 1$).

indicate that the staining pattern is determined by histology, *i.e.*, positive in CIS, seminoma and nonseminoma, irrespective of sensitivity to chemotherapy.

Immunohistochemistry for POU5F1 in Normal Spermatogenesis and Embryonic Germ Cells. Although POU5F1 could be detected by immunohistochemistry in CIS cells and cells of seminoma and embryonal carcinoma (see above), no staining was observed in seminiferous tubules containing normal spermatogenesis (Fig. 1*G*), neither on frozen nor formalin-fixed, paraffin-embedded tissue sections. In contrast, embryonic germ cells in embryos and fetuses were occasionally positive. The highest number and most intensely stained germ cells were found in gonads between week 17 and 24 of gestational age (Fig. 1*H* and see below). Earlier stages of development were not available for investigation. Only sporadic positive cells were identified in the samples obtained from developmental stages weeks 28, 35 and 37, whereas no staining was found in samples of 38 and 40 weeks gestational age and after birth. Germ cells are present in these samples, as identified by staining for various markers, including c-KIT and VASA (24, 31). These data indicate that POU5F1 is present in germ cells at least from the 17th to 37th week of gestational age, after which the protein is no longer detectable by immunohistochemistry.

POU5F1 in Undifferentiated and Differentiated Cells of TGCT-derived Cell Lines. In the nonseminomatous TGCT-derived cell line Tera2, expression of the *POU5F1* is only observed in undifferentiated cells, whereas induction of differentiation of embryonal carcinoma cells with retinoic acid results in down-regulation of *POU5F1* expression. The potential of Tera2 cells to differentiate has been described before (4, 14). Here, we confirm this finding on the protein level by immunohistochemistry (Fig. 1*I, left and right panel*). In contrast, both the cell lines NCCIT and 2102Ep showed *POU5F1* expression and protein before and induction with retinoic acid differentiation (data not shown). This is in accordance to the finding that these cell lines, in contrast to Tera2, do not show complete differentiation after exposure to retinoic acid (28, 30).

POU5F1 Immunohistochemistry on Multitumor Tissue Microarrays. To investigate whether POU5F1 protein can be detected in other tumors than GCT, we performed immunohistochemistry using the same antibody on three different multitumor tissue microarrays. One contained a large series of tumors of different origin (Table 1), one of the other contained specifically prostate and esophageal carcinomas (of which the results are also included in Table 1), and one contained mainly GCT of newborn and infants (Table 3). None of the control tissues, both adult and embryonal (with one exception, see below), included in the arrays showed positive staining for POU5F1. Of the 3439 tumors (from the first arrays), all testicular seminomas and nonseminomas (containing either a seminoma or an embryonal carcinoma component), as well as two dysgerminomas and one gonadoblastoma were positive, confirming the results presented above. Representative examples of the first arrays are shown in Fig. 2. In addition, one clear cell carcinoma of the kidney and two lung carcinomas (one squamous cell carcinoma and one undifferentiated large cell carcinoma) were positive, suggesting that POU5F1 protein can be found in a small percentage (1–2%) of other tumors.

The multitumor tissue microarray of the (T)GCT confirmed the specificity of the POU5F1 immunohistochemistry for the histological types CIS/gonadoblastoma, seminoma/dysgerminoma, and embryonal carcinoma, as described above (Table 3). None of the immature and mature teratomas and yolk sac tumors showed a positive staining. Again, embryonic germ cells, included as arrays in the array, were also positive (data not shown). These data establish POU5F1 as highly specific marker for normal and malignant embryonic germ cells and embryonic stem cells, both in precursor (CIS) lesions and invasive tumors.

DISCUSSION

Human GCTs comprise a heterogeneous group of neoplasms, which are predominantly found along the midline of the body. This localization can be explained by the migration route of the PGC during embryogenesis from the yolk sac to the genital ridge (32). The histology and clinical behavior of the various types of GCT depends on the sex of the patient, the age at clinical diagnosis, the anatomical location, and histology of the tumor (Ref. 33 for review). Overall, four different entities can be distinguished: (*a*) the teratomas and yolk sac tumors of newborn and infants; (*b*) the seminomas/dysgerminomas/germinomas and nonseminomas of the gonads (both ovary and testis, and dysgenetic gonad), anterior mediastinum, and midline of the brain; (*c*) the dermoid cyst (mature cystic teratoma) of the ovary; and (*d*) the spermatocytic seminoma of the elderly testis. From a biological point of view, the pluripotent tumors (entity *b*) are the most intriguing. Histologically, they are subdivided into seminomas (known as dysgerminoma when identified in the ovary and dysgenetic gonad, and germinoma when arising in extragonadal sites) and nonseminomas (Ref. 34 for review). The seminoma-like tumors are composed of cells with a similar morphology as CIS cells. The nonseminomas can contain different histological elements, including embryonal carcinoma as the stem cell component, teratoma (representing somatic differentiation), yolk sac tumor, and choriocarcinoma (representing extraembryonic differentiation). The occurrence of embryoid bodies in nonseminomas (22), as well as certain patterns of

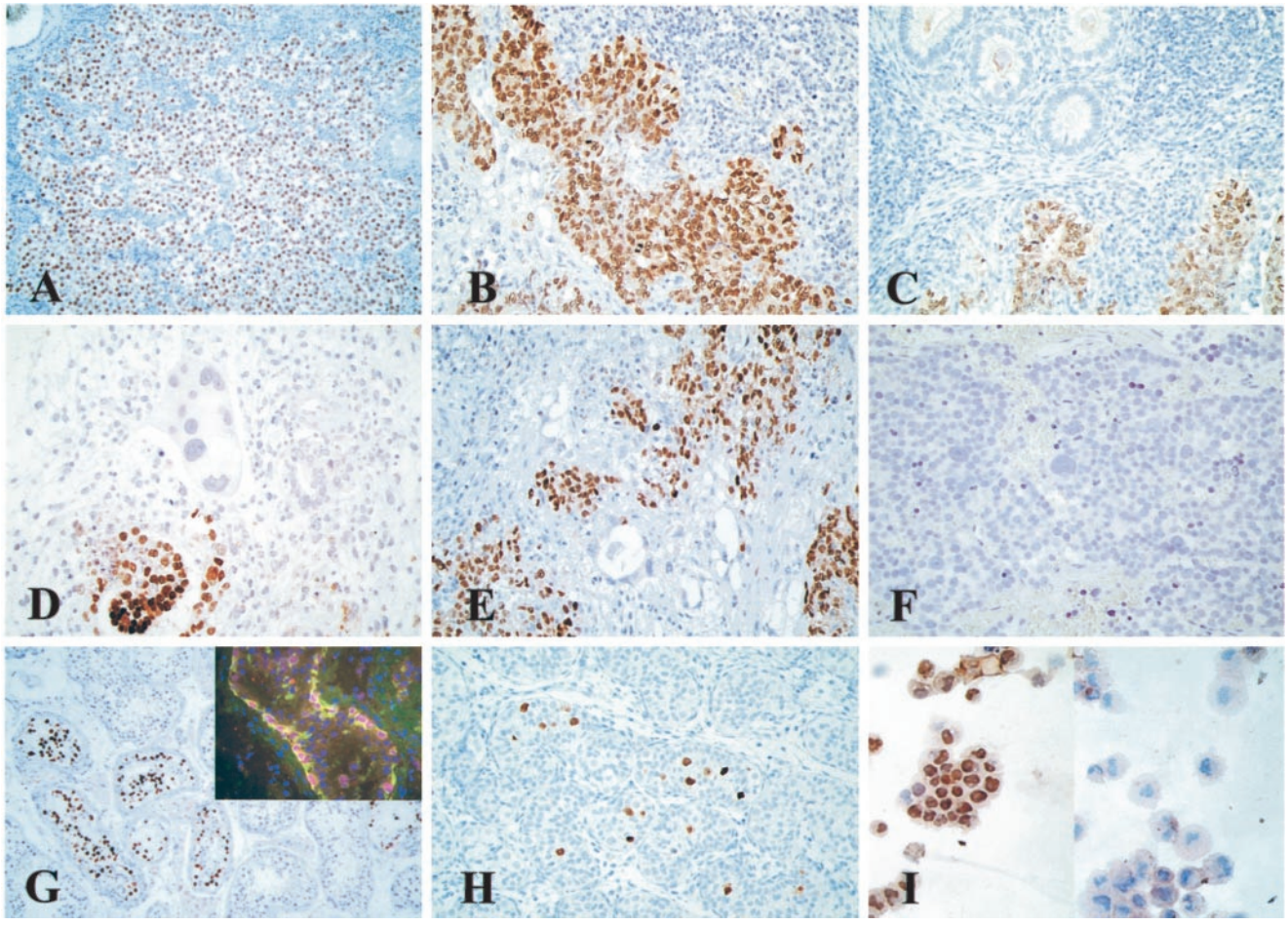

Fig. 1. Visualization of POU5F1 protein by immunohistochemistry on formalin-fixed, paraffin-embedded tissue sections of a seminoma (*A*); pure embryonal carcinoma (*B*); mixed embryonal carcinoma and teratoma (*C*); mixed embryonal carcinoma and choriocarcinoma (*D*); mixed embryonal carcinoma and yolk sac tumor (*E*); spermatocytic seminoma (*F*); testicular parenchyma containing seminiferous tubules with normal spermatogenesis and CIS cells. *Inset* shows a seminiferous tubules with CIS cells double stained for c-KIT (*green*) and POU5F1 (*red*) (*G*); an embryonic testis of 21 weeks of gestation (*H*); cytospins of Tera2 cells before (*left panel*) and after (*right panel*) exposure to retinoic acid, inducing differentiation, and resulting in loss of POU5F1 (*I*).

gene expression, including POU5F1 (4, 13, 14, 35–38), illustrates the similarities between developmental patterns in the embryo and nonseminomas. This study aimed at a further understanding of the nature of the pluripotent (T)GCT compared with the other types of GCT and tumors of nongerm cell origin.

Here, we convincingly show that testicular CIS, seminoma, and embryonal carcinoma cells are consistently positive for POU5F1, irrespective of chemosensitivity or chemoresistance. Gonadoblastomas and associated dysgerminomas, as well as germinomas and embryonal carcinomas of the midline of the brain, showed a similar pattern. In contrast, none of the differentiated nonseminomatous derivatives (teratoma, yolk sac tumor, and choriocarcinoma) stained positive, irrespective of anatomical localization. This suggests that loss of protein expression is because of down-regulation of gene expression upon differentiation/maturation. These data are in accordance with earlier findings in mice, indicating that *pou5f1* is indeed a marker for embryonic stem cells and germ cells (1–3) and that expression is lost upon additional differentiation and maturation (7). The fact that we observed a homogeneously positive staining for POU5F1 in the embryonal carcinoma components of both pure and mixed nonseminomas (and representative cell lines) supports the model that the encoded protein is crucial but not sufficient for pluripotency. This has also been implied by the recent finding that *POU5F1* overexpression had no effect on HeLa cells (39). Apparently, particular auxiliary proteins are needed. In accordance with the histology-dependent pattern of POU5F1 staining, both mature and immature teratomas, as well as the yolk sac tumors of newborn and infants, are negative. This is also true for the spermatocytic seminomas. Both tumor types have a different pathogenesis from TGCT (22, 24, 40–47). The absence of POU5F1 in these tumors is in agreement with their inability to generate pluripotent stem cells.

Our findings within *in vivo* tumor samples and representative cell lines are concordant with previous findings in mice (8, 9), showing that *pou5f1* is expressed in pluripotent cells and is down-regulated upon differentiation. Therefore, we conclude that POU5F1 is involved in regulation of differentiation of human cells in line with a report on human embryos (6). No POU5F1 was detectable in adult testicular samples with normal spermatogenesis by immunohistochemistry, which is consistent with findings in mice using fluorescent protein-tagged *pou5f1* expression analysis (48). However, a positive staining by immunohistochemistry has been reported in mouse spermatogonia A (7). This difference might be because of sensitivity differences of the methods applied and/or differences in protein level. The finding of POU5F1 in human embryonic germ cells and the down-regulation of expression before spermatogonia A formation supports an embryonic initiation of the pathogenesis of TGCT (15, 16, 27, 49–52).

We did not find POU5F1 protein in any of the differentiated components of nonseminomas or in the majority (>99.9%) of 3340 nongerm cell tumors. This indicates that reactivation of *POU5F1* expression and translation, as previously suggested based on cell line

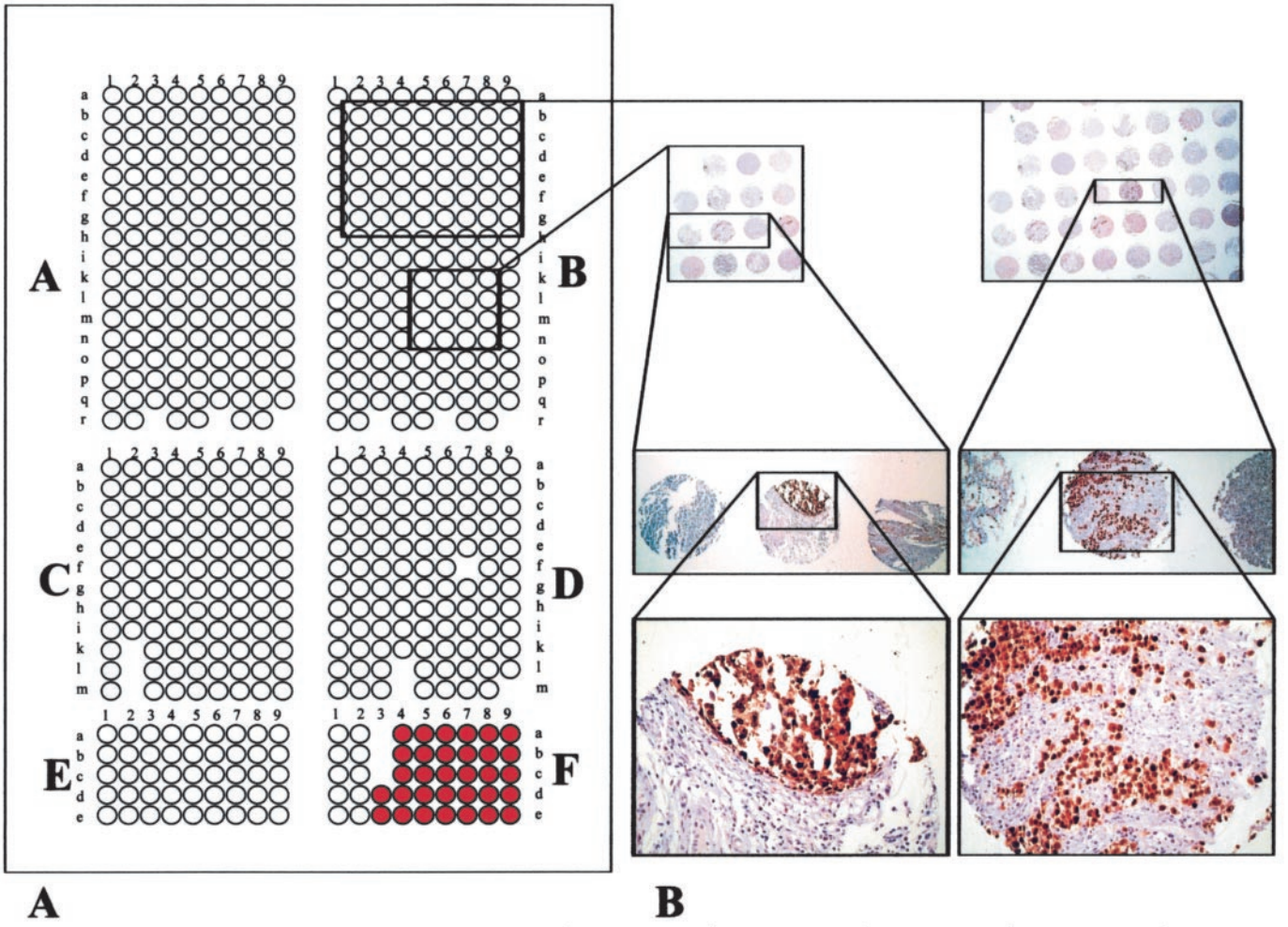

Fig. 2. *A*, schematic representation of one of the multitumor tissue microarrays used for the immunohistochemical detection of POU5F1, of which higher magnification examples are indicated in *B*, including a testicular seminoma (*left panel*) and an ovarian dysgerminoma (*right panel*).

data (20), is not a frequent finding in cancer. The positive tumors were one kidney carcinoma (of 50 clear cell carcinomas) and two lung carcinomas (1 of 50 squamous and 1 of 47 large cell carcinomas analyzed). We do not currently have any plausible biological explanation for POU5F1 expression in these tumor types. Nevertheless, rare occurrence of POU5F1 immunoreactivity in kidney and lung must be kept in mind when performing immunohistochemistry for POU5F1 to demonstrate a pluripotent GCT in these organs. We think that the discrepancy between our study and the previous report suggesting reactivation of *POU5F1* occurring as part of the malignant transformation process (20) can be attributable to the different techniques used. The mRNA expression analyses by Monk and Holding (20) were performed using reverse transcription-PCR with primers that may amplify both *POU5F1* and different intron-less *POU5F1*-related sequences (unpublished observations).

In conclusion, this study demonstrates the usefulness of POU5F1 immunohistochemistry to support that TGCT originate from PGC and have pluripotent potential. However, POU5F1 is also present in nullipotent embryonal carcinoma and thus does not predict whether an embryonal carcinoma is able to generate various lineages of differentiation. Overall, POU5F1 is a highly specific immunohistochemical marker to confirm the diagnosis of CIS/gonadoblastoma, seminoma/dysgerminoma, and embryonal carcinoma. In addition, these data support the hypothesis that (T)GCT are excellent models to study the molecular mechanisms of pluripotency and differentiation, and therefore, a deeper understanding of these mechanism might also reflect into the rapidly evolving field of stem cell biology. Conversely, advances in the rapid evolving field of stem cell biology regarding the control of differentiation might also provide innovative therapeutic alternatives in (T)GCT in the future.

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