Oct-3/4 is a dose-dependent oncogenic fate determinant

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Summary

The Oct-3/4 transcription factor sustains embryonic stem (ES) cell self-renewal and is a dose-dependent cell fate determinant. In the adult male, its expression is restricted to type A spermatogonia. We show that Oct-3/4 is expressed in all human testicular germ cell tumors (GCTs) tested, even in the early premalignant component. We demonstrate that Oct-3/4 dictates ES cells' oncogenic potential in a dose-dependent manner; high levels increase the malignant potential of ES cell-derived tumors while *Oct-3/4* inactivation induces regression of the malignant component. Oct-3/4 expression in a heterologous cell system transforms nontumorigenic cells and endows tumorigenicity in nude mice. Our findings suggest that Oct-3/4 is not only a distinctive immunohistochemical marker for GCTs, but also plays a critical role in the genesis of these tumors.

Introduction

Testicular germ cell tumors (TGCTs) are the most common malignancy in young adult Caucasian males and are responsible for 1 in 7 deaths in this group (Hellerstedt and Pienta, 2002; Oliver, 1999). It is generally accepted that most types of TGCTs originate from the premalignant lesion intratubular germ cell neoplasia (ITGCN) (Ulbright, 1993). However, little is known about the genetic and molecular events that contribute to the initiation and progression of this neoplastic process (Looijenga and Oosterhuis, 1999). Understanding these mechanisms may bring about substantial advancements in understanding the genetic and environmental factors that predispose men to these tumors, as well as enable the development of targeted treatment modalities aimed at curing or preventing this disease in predisposed individuals.

The Oct-3/4 transcription factor plays a pivotal role as a key regulator of pluripotency in the earliest stages of mammalian development (Brehm et al., 1998). It has been shown that a critical amount of Oct-3/4 is required to sustain embryonal stem (ES) cell self-renewal, and any up- or downregulation induces divergent cell fates. Lack of expression drives the formation of trophoectoderm, and elevated levels result in differentiation into extra embryonic endoderm and mesoderm (Niwa et al., 2000). Oct-3/4 expression is downregulated in ES cells that are treated with retinoic acid (RA) (Okamoto et al., 1990; Palmieri et al., 1994; Rosner et al., 1990; Scholer et al., 1989, 1990), and this reduction is associated with increased DNA methylation and changes in the chromatin structure in the immediate upstream regulatory region (Ben-Shushan et al., 1993). In the embryo, Oct-3/4 expression is downregulated in trophoectoderm cells and becomes restricted to cells of the inner cell mass of the blastocyst (Palmieri et al., 1994). From 8.5 days postcoitum (dpc), expression cannot be detected in any somatic tissue of the mouse embryo (Palmieri et al., 1994; Yeom et al., 1996). We have previously shown that in embryonal cells as well, the *Oct-3/4* gene, which is unmethylated in the blastula stage, undergoes de novo methylation at 6.5 dpc and remains modified in all adult tissues (Gidekel and Bergman, 2002). The expression pattern of Oct-3/4 in embryonic and postnatal life suggests that it plays a role as a "stem cell survival" factor. This is supported by recent findings showing that loss of Oct-3/4 expression results in complete loss of pluripotent stem cells in early embryonic life (Nichols et al., 1998). Moreover, variations in Oct-3/4 levels alone (regardless of other genes) account for the majority of failures currently observed for somatic cell cloning (Boiani et al., 2002; Donovan, 2001). In the adult male mouse, expression of Oct-3/4 is restricted to type A spermatogonia—the earliest stage in the spermatocytic ontogeny. It is believed that Oct-3/4 maintains the pluripotency of spermatogonial stem cells and keeps them in an undifferentiated, self-renewing state (Pesce et al., 1998).

It has long been argued that tumors are maintained by a population of tumor stem cells (Reya et al., 2001). Because the ability of Oct-3/4 to maintain the stem cell state of germ cells may be advantageous to cancerous cells, we hypothesized that

SIGNIFICANCE

Germ cell tumors (GCTs) are the most common cause of cancer death in young adult Caucasian males. The molecular events leading to the development of these tumors are largely unknown. Here we provide evidence to suggest that the transcription factor Oct-3/4, a known gatekeeper of germ cell fate in normal spermatogenesis, also plays a key role in GCTs. Moreover, we demonstrate that it confers oncogenicity in a heterologous cell system and that downregulation of *Oct-3/4* in established tumors induces regression of the malignant component. Identification of the molecular mechanisms that underlie the generation of GCTs may open new avenues for better understanding of the pathogenesis of this disease and for developing targeted therapeutic modalities.

aberrant Oct-3/4 expression may contribute to the neoplastic process in germ cells. A possible role for Oct-3/4 in germ cell tumor evolvement is supported by several additional lines of evidence. Oct-3/4 was initially identified in embryonal carcinoma (EC) cells and is expressed in all EC cell lines assayed thus far (Rosner et al., 1990). Moreover, our search of the CGAP database identified *Oct-3/4* transcripts in several cDNA libraries prepared from primary GCTs (http://cgap.nci.nih.gov), and a recent study using RT-PCR has shown that *Oct-3/4* transcripts are present in human GCTs (Palumbo et al., 2002). Thus, we studied the possibility that aberrant expression of Oct-3/4 may contribute to the neoplastic process in germ cells.

Results

Overexpression of Oct-3/4 in human germ cell tumors

To study whether the Oct-3/4 protein is expressed in human cancer, we raised antibodies specific for the Oct-3/4 protein. These antibodies gave a specific nuclear staining pattern in type A spermatogonia cells in human testis (Figure 1A, arrowheads) and not in either more mature sperm ontogeny or the surrounding normal tissues (Figure 1A). Control antibody labeling with preimmune rabbit serum was negative (data not shown). A variety of human normal tissues and tumor samples (archival preserved primary patient material) were tested for the presence of Oct-3/4 protein using immunohistochemical staining. Oct-3/4 expression was seen in all 45 primary human GCTs tested exhibiting a range of expression levels (Figure 1). The variability observed in Oct-3/4 expression could be due to inherent differences in the tumors or to differences in the preservation of excised material. The GCTs that express Oct-3/4 include embryonal carcinomas (Figures 1B–1F), seminomas (Figures 1G and 1H), and mixed germ cell tumors (in the malignant component, Figures 1I and 1J). Interestingly, the strongest Oct-3/4 nuclear staining was observed in carcinoma in situ (CIS) cells of ITGCN, Figure 1A, arrows), which represent the premalignant component. Expression of Oct-3/4 in these CIS cells is higher than in the adjacent normal type A spermatogonia. Non-GCT testicular tumors, including lymphomas (Figure 1K) and Sertoli cell tumors, were negative (Figure 1L). These results suggest that aberrant overexpression of Oct-3/4 is an early and possibly a contributory event in germ cell neoplasia.

In contrast to abundant Oct-3/4 expression in GCTs, Oct-3/4 protein was not detected in an analysis of 182 solid tumors that were sampled in a tissue array. This array contained samples from 16 lung tumors, 19 colon tumors, 7 stomach tumors, 7 liver tumors (primary hepatocellular carcinoma and metastases to the liver), 15 breast tumors, 14 ovarian tumors, 8 endometrial carcinomas, 16 prostate carcinomas, 7 bladder carcinomas, 7 kidney carcinomas, 7 pancreatic cancers, 11 thyroid tumors, 14 skin tumors, 14 lymphomas, and 20 brain tumors. The array slide contained normal testicular tissue as well, which showed positive Oct-3/4 staining and served as a positive control for the experimental procedure (data not shown). Therefore, our findings clearly show that Oct-3/4 is specifically expressed in GCTs.

Aberrant expression of Oct-3/4 influences the malignant potential of the tumors generated from inoculation of ES cells

It was shown previously that mice injected with either human or murine ES clones develop mixed teratomas (Damjanov, 1987).

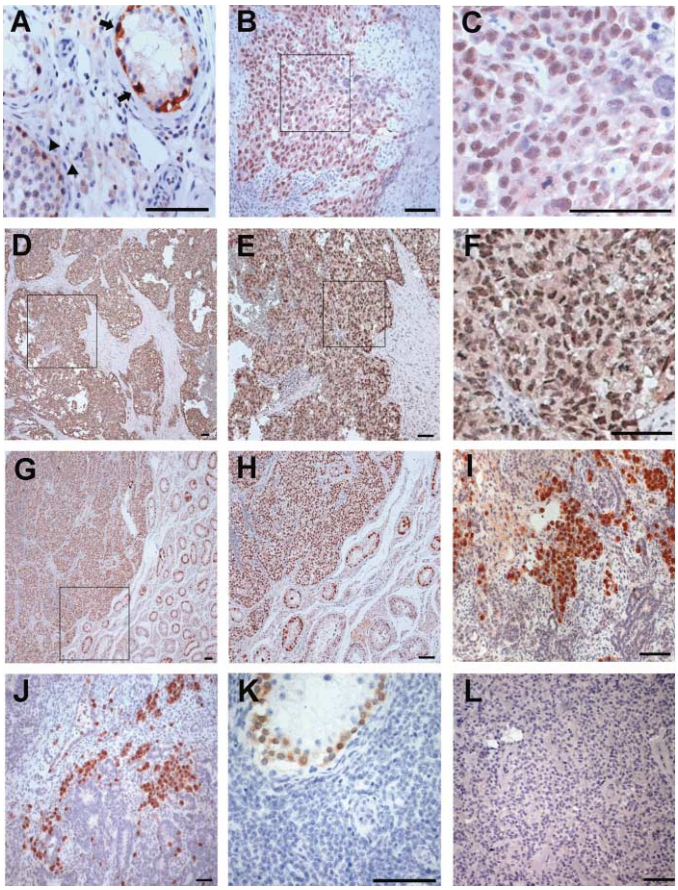

Figure 1. Oct-3/4 is overexpressed in human germ cell tumors

Immunohistochemical staining of Oct-3/4 protein expression in paraffin-embedded human germ cell tumors sections.

A: Intratubular germ cell neoplasia. The left seminiferous tubule contains normal germ cells while the right seminiferous tubule is colonized by in situ neoplastic cells. Note that in the lower left tubule, normal spermatogenesis is seen and only the basal most spermatogonia are stained. In contrast, the upper right tubule only contains Sertoli cells and neoplastic CIS/ITGCN cells that are intensely stained with anti-Oct-3/4.

B–C: Embryonal carcinoma. **D–F:** Embryonal carcinoma (different patient).

G–H: Seminoma. **I–J:** Teratoma with focal malignant component.

K: Testicular lymphoma. The tumor cells occupying most of the section's area are negative while residual germ cells in a nearby seminiferous tubule (upper left) show positive staining.

L: Testicular Sertoli cell tumor. The bar equals 100 μM. The boxed areas are enlarged in the photomicrograph to their right.

These tumors are morphologically similar to human immature teratomas, a subtype of GCTs, and are characterized by the presence of a mixture of mature and immature embryonal tissues (Ulbright, 1993). Human immature teratomas are graded into a four tiered system according to the relative abundance of primitive neural tissue present. Grade 0 tumors harbor no immature tissue whereas grade 3 denotes an immature teratoma with abundant primitive tissue (Norris et al., 1976). While grade 0 teratomas are benign lesions with a 5 year survival rate of 100%, grade 3 teratomas are highly malignant tumors with a 5 year survival rate of 33% for tumors that were considered to be localized on operation (Zalel et al., 1996). Thus, the prognosis is directly related to the percentage of immature tissue present in these tumors. Interestingly, immunohistochemical staining of

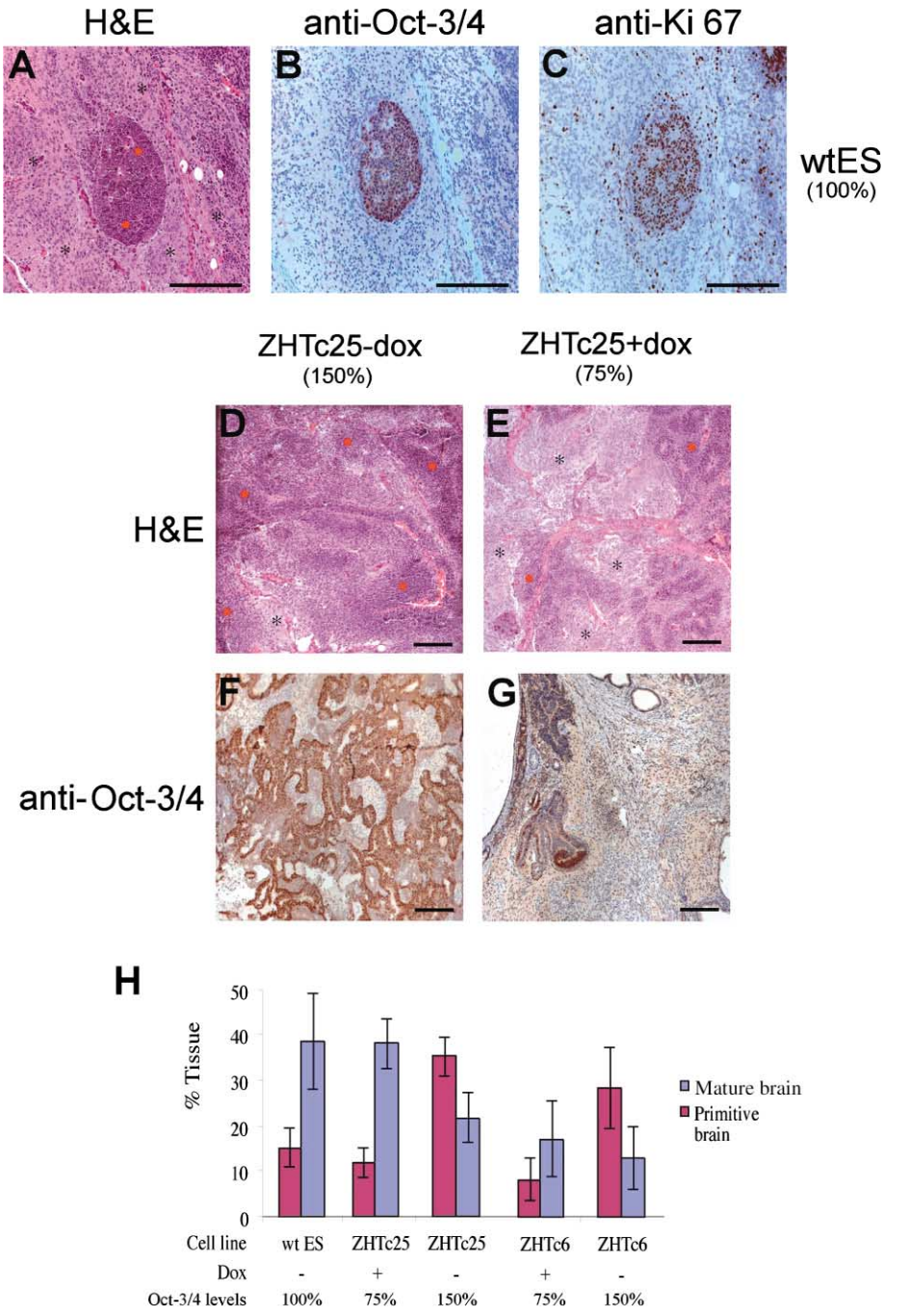

Figure 2. Alterations in Oct-3/4 expression change the tissue composition and malignant potential of ES cell xenografts in Ola129/Sv mice

A–G: Histology of subcutaneous tumors derived from: wt CGR8 ES cells (**A**–**C**); ZHTc25 ES cells (**D** and **F**), and ZHTc25 ES cells treated with doxycycline (dox, **E** and **G**). Panels **A**, **D**, and **E** were stained with hematoxylin and eosin (H&E). Panels **B**, **F**, and **G** show immunohistochemical staining with anti-Oct-3/4, and panel **C** is stained with anti-Ki-67. Black asterisks represent mature brain tissue and red asterisks represent primitive brain tissue. The bar equals 200 μM. The percentages reflect Oct-3/4 levels in the relevant cell line in vitro under the appropriate dox treatment.

H: Tissue composition of the different xenografts was determined by a systematic sampling analysis of H&E-stained sections from all tumors (described in Results). The relative frequencies of mature brain and primitive brain tissue are shown. Bars indicate standard error of the mean. *y* axis—percent tissue; *x* axis—cell line and dox treatment.

immature teratomas formed from wild-type (wt) ES cells showed that while most of the tissue was negative for Oct-3/4 expression, all malignant foci, and some of the areas composed of primitive brain tissue, were positive for Oct-3/4 expression (Figures 2A and 2B). Staining of serial sections with an antibody directed against the proliferation marker Ki-67 showed that Oct-3/4-expressing foci are highly proliferative (Figure 2C).

Oct-3/4 expression level was previously shown to be a key factor in determining the cell fate in the early embryo (Niwa et al., 2000). To address the possibility that various levels of Oct-3/4 expression may affect the tumorigenic capacity of ES cells, we inoculated mice with ES cells that harbor an inducible *Oct-3/4* transgene. We used two kinds of Ola129/Sv ES cell lines engineered by Niwa and collaborators (Niwa et al., 2000). Both types of cell lines harbor a tetracycline-inducible *Oct-3/4* transgene in which expression is induced after withdrawal of doxycycline (dox). The ZHTc6 and ZHTc25 clones are heterozygous for the endogenous *Oct-3/4* gene (*Oct-3/4*$^{-/+}$), whereas in the ZHBTc4 clone, both *Oct-3/4* alleles are disrupted (*Oct-3/4*$^{-/-}$). Upon induction of the inducible transgene in ZHTc6 and ZHTc25 cells, Oct-3/4 levels increase to 150% relative to wt ES cells. ZHBTc4 cells, harboring no functional endogenous *Oct-3/4* gene, express the inducible transgene at a level of 50% of wt levels, whereas in the presence of dox, these cells do not ex-

Table 1. Incidence of tumor formation with the different ES cell clones and relative abundance of primitive and mature tissue

Clone	dox	Oct-3/4 expression levels (%)	Incidence of tumor formation (%)	Primitive neuronal/ total tissue (%)	Primitive neuronal/total neuronal tissue (%)
wt CGR8 ES	−	100	83 (15/18)	15.1 ± 4.3 (n = 4)	28 ± 4 (n = 4)
ZHTc25	+	75	70[a] (7/10)	11.7 ± 3.3 (n = 5)	24 ± 7 (n = 5)
ZHTc25	−	150	64[a] (9/14)	35.1 ± 4.3[d] (n = 5)	64 ± 5[e] (n = 5)
ZHBTc4	+	0	4.3[b] (1/23)	NA	NA
ZHBTc4	−	50	40[c] (8/20)	NA	NA

Mice were inoculated with the various clones and followed for tumor development with or without dox, as indicated. The relative Oct-3/4 expression levels are inferred from the in vitro findings (Niwa et al., 2000). NA encodes not applicable.

[a]The difference in the incidence of tumor formation is not statistically significant from the incidence observed with wt ES cells; [b]$p < 0.0001$ compared with wt ES cells; [c]$p < 0.01$ compared with wt ES cells; [d]$p = 0.01$ compared with wt ES cells and with ZHTc25 cells with dox; [e]$p < 0.01$ compared with ZHTc25 cells with dox and $p < 0.02$ compared with wt ES cells. [a–c]Fisher's exact probability test; [d,e]Mann-Whitney test.

press the Oct-3/4 protein at all (Niwa et al., 2000). Thus, these cleverly engineered ES cells that express *Oct-3/4* at 0%–150% allowed us to study the effect of different levels of Oct-3/4 on the malignant potential of ES cells.

Cells from wt ES, ZHTc6, ZHTc25, and ZHBTc4 clones were injected subcutaneously into syngeneic Ola129/SV mice. Mice inoculated with the ZHTc25 or ZHTc6 cells were either treated or not with 0.2 mg/ml dox in their drinking water. Tumors became apparent at the site of injection within 2 to 4 weeks in 83% of the mice injected with wt ES. Mice injected with ZHTc25 and treated or not with dox-containing water developed tumors in 70% and 64% of the cases, respectively. In marked contrast, only 40% ($p < 0.01$ compared with wt ES) of mice inoculated with ZHBTc4 and given plain drinking water developed tumors, and this percentage dropped to 4.3% when dox was added to the water ($p < 0.0001$ compared with wt ES, Table 1). This is not a result of decreased proliferative potential since in vitro the proliferation rate of the various clones was found to be similar (data not shown). These results suggest that lower levels of Oct-3/4 expression (below 75%) hinder tumor formation.

To determine the effect of Oct-3/4 overexpression, mice injected with ZHTc25 cells were divided into groups maintained either on plain water or on water supplemented with dox. These animals were sacrificed when the tumors reached the size of 1 cm^2 for histological examination. The tissues were examined for the presence of primitive, mature, and malignant cells. Malignant phenotype is characterized by the appearance of pleomorphic and hyperchromatic nuclei and abundant mitoses as evidenced by high power view of the tumors. Primitive brain tissue is characterized by neuropil packed with small neuroblast-like cells with dark nuclei and high mitotic activity, while mature brain is characterized by less crowding and the appearance of differentiated neurons. Quantitation of these histological analyses were obtained from a systematic sampling analysis of hematoxylin- and eosin-stained sections from all tumors (Hammel et al., 1989). Briefly, we randomly sampled ten microscopic fields (1.1 mm × 1.4 mm each) from each tumor for analysis. A matrix of 20 × 20 dots was applied to each field, and tissue type for each dot was assigned and recorded. The reader was blinded to the ES cell clone as well as to the mouse treatment group. Thus, approximately 4,000 different points were analyzed per tumor sample. The relative frequency of each tissue type assigned to all dots corresponds to the proportional volume of this tissue type in the entire tumor. This labor intensive analysis has previously been shown to be the optimal method for evaluating the relative proportion of different components in a sampled tissue (Hammel et al., 1989).

To this end, we found that mice that were inoculated with ZHTc25 cells and given plain drinking water developed tumors that contained a higher proportion of primitive neural tissue compared with tumors from dox-provided mice or with tumors from mice injected with wt ES cells (35.1% ± 4.3%, n = 5 versus 11.7% ± 3.3%, n = 5 and 15.1% ± 4.3%, n = 4, respectively, $p = 0.01$, Figure 2H and Table 1). In addition, we calculated the percentage of primitive neural cells with regard to the total amount of neural tissue present in the tumor mass. Again, upregulation of Oct-3/4 (Figure 2, compare F and G) resulted in a higher percentage of primitive neural tissue in ZHTc25 tumors in the absence of dox (64% ± 5%) versus the presence of dox (24% ± 7%, $p < 0.01$, Figure 2, compare D and E), or in wt ES tumors (28% ± 4%, $p < 0.02$). Similar results were obtained with an additional independent clone, ZHTc6 (Figure 2H), indicating that this phenotype is a direct result of upregulation of Oct-3/4 expression. Taken together, we found that upregulating Oct-3/4 levels resulted in a higher proportion of tumors containing primitive neural and malignant-appearing tissues, compared with tumors having developed either from wt ES or from engineered ES cells in which Oct-3/4 levels are kept low (Figure 2H and Table 1). These data bear relevance to the corresponding human tumors since, as noted above, a higher proportion of immature tissue indicates a more malignant behavior in these human tumors.

Mice injected with ZHBTc4 cells, expressing Oct-3/4 from the transgene only, developed tumors that were enriched for trophoblastic tissue as compared with a very low level of this tissue in tumors evolving from injection of ZHTc25 cells (11.2% ± 8.9%, n = 5 versus 0.02% ± 0.03%, n = 5, $p < 0.005$, Figures 3A–3C). It is generally accepted that the different subtypes of GCTs correspond to different lineages or stages in the developing embryo. For instance, seminoma is regarded as a recapitulation of spermatogonia; embryonal carcinoma corresponds to inner cell mass cells and choriocarcinoma to trophoblastic tissues (Chaganti and Houldsworth, 2000). In accordance, the malignant component in ZHBTc4-derived tumors, expressing lower levels of Oct-3/4, was mainly composed of choriocarcinoma rather than embryonal carcinoma, typically found in tumors derived from ZHTc25 and ZHTc6 cells. As previously noted, only 1 mouse out of 23 (Table 1) injected with ZHBTc4 cells and fed with dox developed a tumor. This tumor was extremely small and composed of mature trophoblast cells

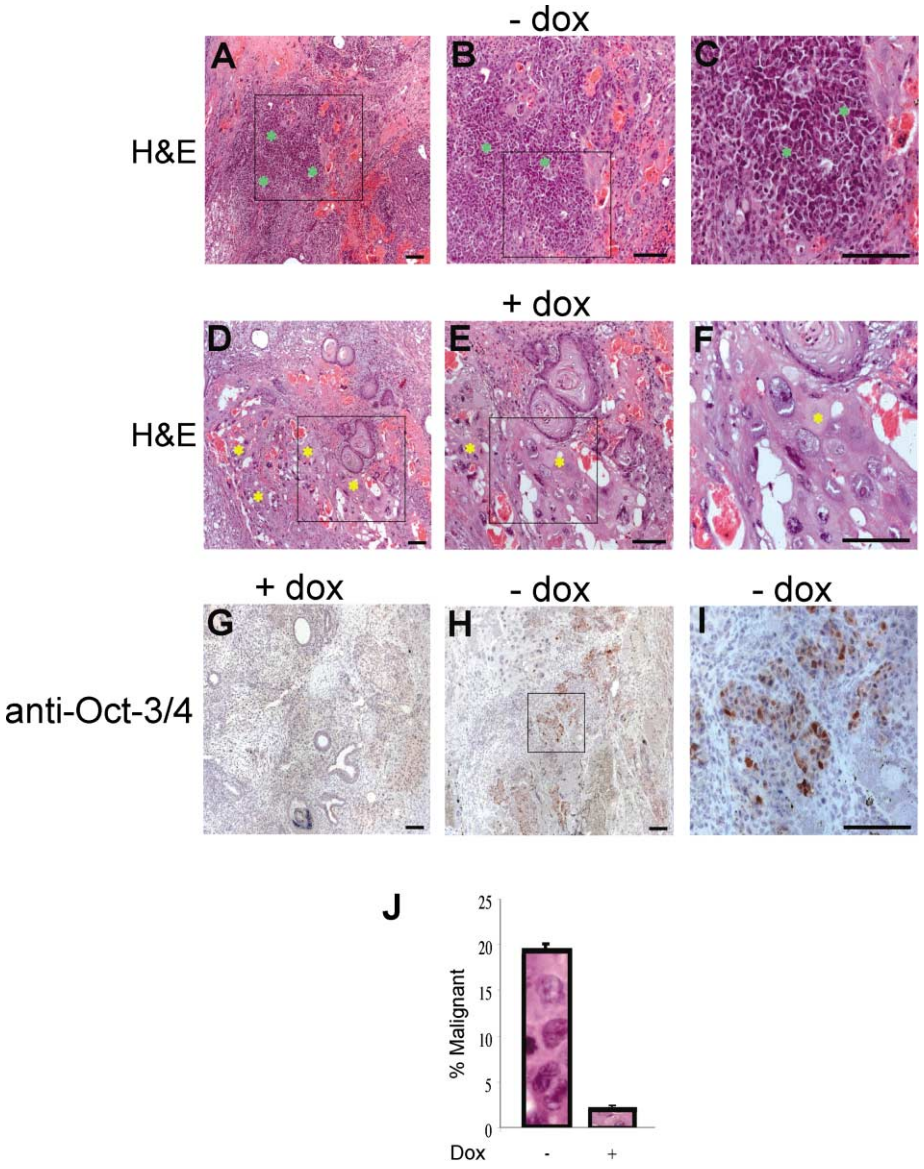

Figure 3. Oct-3/4 downregulation induces regression of the malignant component

A–I: ZHBTc4 ES cells were injected subcutaneously into the left flank of Ola129/Sv mice. After reaching a size of 1 cm^2, ZHBTc4 tumors were maintained in the absence (**A**–**C**, **H**, and **I**) or presence (**D**–**F** and **G**) of dox for another 2 weeks. Xenografts were subjected to histological analysis and stained with H&E (**A**–**F**) or with anti-Oct-3/4 (**G**–**I**). Green asterisks represent choriocarcinoma foci and yellow asterisks represent the mature trophoblastic component. The bar equals 100 μM. The boxed areas are enlarged in the photomicrograph to their right.

J: The proportion of immature and malignant tissue was recorded for each tumor (as described in the text) and the mean and standard deviation were calculated for each group. *y* axis—percent malignant tissue; *x* axis—dox treatment.

with no immature areas (data not shown). Interestingly, our in vivo results clearly corroborate previously published in vitro experiments showing that downregulating Oct-3/4 expression in ZHBTc4 cells results in differentiation toward the trophoblastic lineage (Niwa et al., 2000). Thus, our data suggest that also in ZHBTc4 cells, upregulating Oct-3/4 expression results in more aggressive tumors, as judged by the abundance of malignant tissue, whereas low levels of Oct-3/4 expression are associated with mature/differentiated tissue. Moreover, these results also emphasize that Oct-3/4 expression is cardinal for tumor formation since ES cells devoid of Oct-3/4 hardly generated any tumors.

Expression of Oct-3/4 is required for maintenance of the malignant phenotype

ZHBTc4 cells provided us with the opportunity to investigate whether Oct-3/4 expression is required for maintenance of the immature tumor phenotype. These cells express Oct-3/4 only from the stably integrated dox-regulated transgene since both endogenous *Oct-3/4* alleles were disrupted. To address this question, mice were injected with ZHBTc4 cells, kept in the absence of dox and followed for tumor appearance. Once tumors reached the size of 1 cm^2, mice were assigned to either one of two groups: one treated with dox (12 mice) and the other without (11 mice). After three weeks, the tumors were subjected to histological analysis. Several sections were examined from each tumor and representative images are shown in Figure 3. In all mice, administration of dox (resulting in downregulation of Oct-3/4 expression, Figure 3, compare G to H) was associated with tumor regression, as evidenced by histological analysis revealing mature tissue (Figures 3D–3F), large areas of fibrosis, granulation tissue, and signs of hemorrhage. Remarkably, in a subset of these tumors (4/12), complete disappearance of the malignant component was observed following dox addition. These findings are in complete contrast with abundant malignant tissue found in all (11/11) mice not treated with dox (Figures

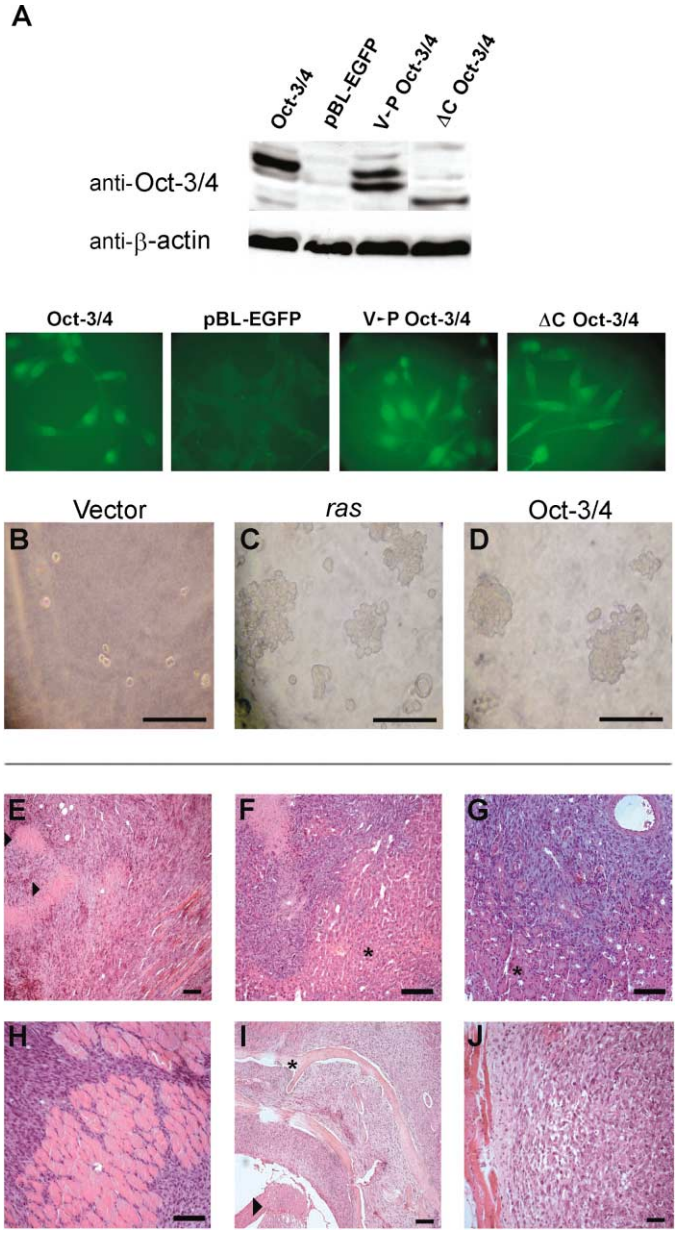

Figure 4. Oct-3/4-expressing Swiss 3T3 cells form colonies in soft agar and tumors in nude mice

A: Western blot analysis of nuclear extracts from all the stably transfected clones generated from Swiss 3T3 cells was performed using anti-mouse Oct-3/4 serum. Oct-3/4 protein was detected in the clones transfected with pBL-Oct-3/4-EGFP (shown is clone #16), V→P Oct-3/4 (shown is clone #7), and ΔC Oct-3/4 (shown is clone #1), but was absent in Swiss 3T3 cells stably transfected with the pBL-EGFP vector only (shown is clone #8). β-actin was used as a loading control. Immunofluorescence staining with antiserum against Oct-3/4 was used to further verify the nuclear localization of the protein generated form the transgene.

B–D: Colony formation in soft agar of Swiss 3T3 cells stably transfected with pBL-EGFP vector (**B**, negative control, clone #8); Ras expression vector (**C**, positive control), and pBL-Oct-3/4 expression vector (**D**, clone #16). Photomicrographs were taken with a phase contrast microscope.

E: Photomicrograph of a tumor derived from an Oct-3/4-expressing clone showing tumor necrosis (arrowheads).

F–I: Representative photomicrographs of hematoxylin- and eosin-stained tumors derived from an Oct-3/4-expressing clone showing invasion into liver (**F**, asterisk denotes liver tissue), kidney (**G**, asterisk denotes kidney tissue), muscle (**H**), and spinal cord and bone (**I**, arrowhead marks spinal cord and asterisk designates bone).

3A–3C). Quantitative assessment of the abundance of malignant tissue revealed a 10-fold reduction in the mean percentage of malignant tissue in tumors that grew in the presence of dox compared with the untreated group (1.7% $\pm$ 0.1% versus 19% $\pm$ 0.9%, respectively, $p < 0.02$, Figure 3J). These results indicate that Oct-3/4 expression is essential for maintenance of these tumors and suggest that Oct-3/4 functions as an oncogene.

Oct-3/4 is a candidate oncogene in Swiss 3T3 cells

Several homeobox-containing genes were previously shown to transform nontumorigenic fibroblasts in vitro (Maulbecker and Gruss, 1993; Qin et al., 1994; Stuart et al., 1995; Soubeyran et al., 2001). We set out to directly test the oncogenic potential of Oct-3/4 in this system as well. Nontumorigenic Swiss 3T3 fibroblast cells were transfected with the Oct-3/4 expression vector (pBL-Oct-3/4-EGFP), the empty vector (pBL-EGFP), and as a positive control the oncogenic *ras* expression vector (H-rasV12, pBpRas kindly provided by W.C. Hahn). Western blot analysis of nuclear extracts and immunofluoresence staining revealed that all the independent cell lines transfected with pBL-Oct-3/4-EGFP expressed the Oct-3/4 protein in the nucleus, whereas control cell lines did not (Figure 4A, showing representative clones). Four independent subclones from each group were assayed for their ability to grow in soft agar. This experiment was performed because loss of anchorage dependence is a hallmark of tumor cells, and the ability to promote anchorage-independent growth is a common property of oncogenes. Swiss 3T3 wt cells, as well as those harboring the pBL-EGFP vector only, did not form colonies whereas *ras*-expressing cells did. Remarkably, expression of Oct-3/4 stimulates formation of robust colonies in soft agar, with a cloning efficiency similar to oncogenic *ras* (Figures 4B–4D and Table 2). Moreover, either a point mutation in the DNA binding domain (Vigano and Staudt, 1996) or a deletion in the C-terminal region that harbors the specific transcriptional activation domain (Brehm et al., 1997) completely abrogated the ability of Oct-3/4 to form colonies in soft agar (Table 2). These results indicate that Oct-3/4 shares the ability to promote anchorage-independent growth with oncogene products, and both the DNA and the C-terminal transactivation domains play a crucial role in this ability.

To find out whether Swiss 3T3 cells expressing Oct-3/4 are tumorigenic in vivo, cells expressing Oct-3/4, vector only, or *ras* were injected subcutaneously into CD1 nude mice. None of the mice injected with the vector-only cells developed tumors within an eight-week observation period. In contrast, cells expressing either *ras* or Oct-3/4 formed bulky tumors in 10–18 days. Histological analysis of the Oct-3/4-expressing tumors showed that they are high-grade fibrosarcomas (Figure 4E) that aggressively invade adjacent tissues such as liver (F), kidney (G), muscle (H), and spinal cord and bone (I). This invasive phenotype was much more marked in the Oct-3/4-expressing tumors as compared with *ras*-expressing tumors, which tended to be localized and separated from the surrounding tissues by a thin pseudo-capsule (Figure 4J).

J: Photomicrograph of a tumor derived from a *ras*-expressing clone composed of a high grade fibrosarcoma, circumscribed by a thin pseudocapsule (left). The bar equals 100 μM.

Table 2. Incidence of anchorage-independent colonies in wt and transfected Swiss 3T3 cells

Clone	Colonies per dish (mean ± SEM)	Cloning efficiency (%)
wt	0	0
pBL-EGFP (#2, 8, 11)	0	0
Oct-3/4-#2	122.7 ± 0.8	24.5
Oct-3/4-#3	107.0 ± 1.6	21.4
Oct-3/4-#9	135.0 ± 2.9	27.0
Oct-3/4-#16	126.0 ± 1.2	25.2
V→P Oct-3/4 (#1, 6, 7)	0	0
ΔC Oct-3/4 (#1, 11, 19)	0	0
ras	149.3 ± 2.0	29.8

A total of 500 cells of each clone were seeded into 0.33% agar as described in Experimental Procedures. Four different Oct-3/4-expressing clones, designated Oct-3/4-#3, #16 (Figure 4A), #9, and #2 were used. As negative controls, three clones harboring the empty vector (designated pBL-EGFP #2, #8, and #11); three clones harboring an *Oct-3/4* gene carrying a nonfunctional DNA binding domain (a Val235 to Pro mutation, designated V→P Oct-3/4 #1, #6, and #7); or three clones harboring an *Oct-3/4* gene lacking the C-terminal region that encodes the specific transcriptional activation domain (designated ΔC Oct-3/4 #1, #11, and #19) were used. A clone harboring oncogenic *ras* was used as a positive control. Colonies (with >40 cells) were counted after 14 days of growth. Values represent averages of triplicate dishes with standard error of the mean (SEM). Cloning efficiency was calculated as the average number of colonies/number of cells seeded.

Discussion

In this study, we used ES cell lines with dox-dependent Oct-3/4 expression to investigate whether Oct-3/4 can influence the tumorigenic phenotype of ES cells in a dose-dependent manner. Mice injected with either ZHTc6 or ZHTc25 cells, overexpressing Oct-3/4, developed tumors with a more primitive and malignant phenotype as compared with tumors that arose from injection of the same cells in which Oct-3/4 expression is downregulated. Oct-3/4 was previously shown to serve as a dose-dependent cell fate/differentiation determinant. The level of Oct-3/4 expression dictates the cellular phenotype, with low levels driving differentiation toward trophoectoderm and high levels resulting in differentiation into mesoderm and primitive endoderm lineages (Niwa et al., 2000). In this study, we show that in mouse xenografts in vivo, Oct-3/4 influences the oncogenic fate in a dose-dependent manner. ES cells expressing low levels of Oct-3/4, ZHBTc4, gave rise to tumors that were enriched for trophoblast tissue, whereas mice injected with ES cells containing high levels of Oct-3/4, ZHTc6 and ZHTc25, developed embryonal carcinomas/primitive neural tumors. Moreover, we provide data to show that expression of Oct-3/4 in a heterologous cell system confers a malignant phenotype upon these cells, evidenced by colony formation in soft agar, tumorigenicity in nude mice, and the appearance of histological hallmarks of malignancy such as marked pleomorphism, invasiveness, and necrosis. This activity requires both the C-terminal and the DNA binding domains. Therefore, our findings that Oct-3/4 confers a malignant phenotype upon Swiss 3T3 cells, increases the malignant potential of ES cells, and is ubiquitously expressed in all human GCTs from the earliest detectable stage highly suggest that aberrant Oct-3/4 expression may play a cardinal role in genesis of GCTs.

We have found that the tumors obtained from injection of ES cells that express Oct-3/4 only from a dox-inducible *Oct-3/4* transgene, ZHBTc4, regress after dox addition. In a subset of these tumors, the malignant component completely disappeared, whereas in others, there was a dramatic reduction in this component as compared to abundant malignant tissue found in tumors expressing Oct-3/4 throughout their growth. Downregulation was accompanied by infiltration of inflammatory cells, granulation tissue formation, and hemorrhage, all signs of tumor regression.

Recent studies with switchable oncogenes in mouse tumor models suggest that some tumors depend on a single oncogene for survival. This was shown for H-ras-dependent melanomas, K-ras-induced adenocarcinomas of the lung, BCR-ABL-induced leukemias, and Myc-induced leukemias, lymphomas, and islet cell tumors (Chin et al., 1999; Felsher and Bishop, 1999a; Fisher et al., 2001; Huettner et al., 2000; Karlsson et al., 2003; van de Wetering et al., 2002). These findings suggest that human tumors may be similarly reversible with targeted therapy aimed at a single particular oncogene. Indeed, anti-Her-2/neu antibodies are part of the standard treatment regimen for metastatic Her-2/neu-overexpressing breast cancer (Yarden, 2001).

However, there are data to show that in other cases, tumors do not remain dependent on the initiator oncogene for maintenance of the transformed state (D'Cruz et al., 2001; Ewald et al., 1996; Felsher and Bishop, 1999b; Jain et al., 2002). Thus, it seems likely that the response of tumors to remediation of a single genetic lesion will vary with the nature of the initiating oncogene and the cellular lineage of the tumors. Our findings showing residual malignant component in the tumor mass in a subset of the tumors where Oct-3/4 expression was downregulated could result from secondary genetic changes that are responsible for the transformed phenotype. Alternatively, it is possible that we did not wait long enough for the disappearance of the entire malignant component.

Our results, along with a very recent extensive study (Looijenga et al., 2003), show that Oct-3/4 is overexpressed in human GCTs, as early as the carcinoma in situ stage all the way through metastatic lesions. Oct-3/4 is normally expressed in the earliest stages of spermatogenesis (Pesce et al., 1998). Thus, these results fit well with the growing body of evidence that links cancer with genes and pathways that are required for normal embryonic patterning. In many cases, cancer seems to be caused by the deregulation of transcription factors that affect cell fate and proliferation. Two well-studied examples are (1) the *wnt* pathway, which in normal intestine regulates cell fate and is involved in colon cancer through aberrant regulation of β-catenin (van de Wetering et al., 2002) and (2) the Sonic hedgehog-Gli signaling pathway, which normally regulates epidermal differentiation and whose inappropriate activation occurs in several types of tumors, including those of brain and skin (Dahmane et al., 2001; Grachtchouk et al., 2000; Nilsson et al., 2000). Interestingly, both Oct-3/4 and Gli proteins seem to be involved in tumors that are derived from tissues in which these proteins are active. These findings support the view that many molecular pathways that underlie carcinogenesis represent aberrations of the normal processes that control embryogenesis.

An additional example of deregulation of transcription factors that affect cell fate and proliferation are the homeobox genes, which encode transcription regulatory proteins that are widely used during development and are often aberrantly expressed in cancer (Abate-Shen, 2002; Stuart et al., 1995). A number of these genes were shown to induce transformation

of cells in culture and to form tumors in vivo (Maulbecker and Gruss, 1993). Oct-3/4 belongs to the POU transcription factor family, a subset of the homeobox superfamily (Ryan and Rosenfeld, 1997; Verrijzer and Van der Vliet, 1993). Thus, our results suggesting that Oct-3/4 plays an important role in malignant transformation, in addition to its recognized role in developmental decisions, are in agreement with previously reported data pertaining to the bona fide homeobox genes.

What is the molecular mechanism by which Oct-3/4 directs tumors to a more primitive and malignant phenotype and what causes the deregulation of *Oct-3/4* expression in cancer? *Oct-3/4* encodes a transcription factor that acts as a gatekeeper in early mammalian development (Pesce and Scholer, 2001). It has been implicated in a variety of cellular functions including maintaining the pluripotent phenotype of ES cells and directing differentiation to particular cellular lineages (Niwa et al., 2000). It is clear that Oct-3/4 cumulative activities provide a balance between proliferation and differentiation. Thus, it is reasonable to suggest that deregulated Oct-3/4 expression distorts the balance toward a transformed phenotype. In this context, it is interesting to note that expression of the stem cell-specific growth factor fgf-4 (Wilder et al., 1997), which is one of Oct-3/4's putative target genes, is associated with human testicular tumors (Strohmeyer et al., 1991; Suzuki et al., 2001; Yoshida et al., 1988). Furthermore, in the mouse testis, fgf-4-specific gain of function resulted in markedly enhanced spermatogenesis (Yamamoto et al., 2002). Therefore, the aberrant expression of the *Oct-3/4* gene in cancer cells may lead to inappropriate activation of growth factors, which, in turn, promote cell proliferation.

The widespread occurrence of Oct-3/4 expression in GCTs could be due to dysregulation of either upstream activators of *Oct-3/4* or mutations and deletions in the proteins that negatively regulate *Oct-3/4*. Such mutations were detected in the adenomatous polyposis coli (APC) gene and were found to lead to β-catenin overactivity in human cancers (Polakis, 2000; Taipale and Beachy, 2001). It was recently shown that β-catenin, together with TCF-4, constitutes the master transcriptional regulator that controls proliferation versus differentiation in intestinal stem cells (van de Wetering et al., 2002). These results bring the analogy between Oct-3/4 and β-catenin even closer. It seems that both genes are master regulators of differentiation, which when aberrantly overexpressed pave the way to the development of cancer.

The genetic mechanisms by which wt ES cells generate tumors in vivo are poorly understood. It is possible that since stem cells have the machinery for self-renewal already activated, maintaining this activation may be a simpler task than turning it on de novo. Our data clearly show that high levels of Oct-3/4 expression increase the malignant potential of ES cells and are detected in GCTs. If Oct-3/4 is essential for tumor survival in established human GCTs, then eliminating Oct-3/4 may be a valid gene-directed therapy. Indeed, our oct-3/4 downregulation studies support this notion. The fact that in the adult, Oct-3/4 is expressed in germ cells only and is probably not necessary for any other tissue makes this approach even more appealing as a possible safe target for gene-directed therapy.

Experimental procedures

Clinical samples

Forty-five GCTs including embryonal carcinoma, seminoma, and mixed germ cell tumor samples, in addition to 4 non-GCT testicular tumors, were collected from the archives of the Pathology Department of the Hadassah-Hebrew University Medical Center, Jerusalem. In addition, a tumor tissue array containing more than 180 different tumors was analyzed (Clinomics Biosciences Inc., Pittsfield, Massachusetts).

Antibodies

Polyclonal rabbit anti-sera directed against the human and the mouse Oct-3/4 proteins were raised in rabbits following injection of the full-length recombinant proteins (Ben-Shushan et al., 1998). Both antibodies react specifically with the human Oct-3/4 protein as illustrated by the specific immunohistochemical staining of Oct-3/4 in type A spermatogonia as well as by specific electrophoretic mobility shift assay experiments (data not shown). For most of the immunohistochemical experiments, the anti-mouse Oct-3/4 serum was used as it gave a better signal to noise ratio. An anti-mouse Ki-67 antibody (clone Tec-3, DakoCytomation, Denmark) was used for proliferation assays and an anti-mouse β-actin antibody (clone AC-15, Sigma, St. Louis, Missouri) was used for loading control in Western blot analyses.

Cell culture

Wild-type CGR8 ES cells, ZHTc25, and ZHBTc4 ES cells were cultured without feeders in LIF-supplemented medium in the presence or absence of dox as described previously (Niwa et al., 2000). Swiss 3T3 cells (Clontech Laboratories, Palo Alto, California) were maintained in DMEM supplemented with 10% fetal calf serum in the presence of G418 (250 μg per ml, Invitrogen Life Technologies, Carlsbad, California).

Plasmids

pBL-Oct-3/4-EGFP was constructed by cloning the full-length *Oct-3/4* cDNA into the PvuII site of pBL-EGFP (Clontech Laboratories). pBL-Oct-3/4-ΔC75-EGFP was constructed by deleting 400 bp harboring 75 codons from the 3′ end of the *Oct-3/4* cDNA and subcloning the construct in frame into the PvuII site of pBL-EGFP. pBp-Ras was received from William C. Hahn (Stanford University, California) and the V-P construct was received from Maria Alessandra Vigano (degli Studi di Pavia Universita, Italy).

DNA transfections

Transfection of Swiss 3T3 cells was carried out using FuGENE6 transfection reagent following the manufacturer's protocol (Roche, Basel, Switzerland). Cells (5×10^5) were plated 24 hr before transfection and cotransfected with 10 μg of either pBL-EGFP vector, pBL-Oct-3/4-EGFP, or pBp-Ras expression vectors together with 1 μg of a plasmid carrying the Hygromycin B selection gene. Stably transfected cells were selected by Hygromycin B (125 μg per ml, Roche) and clonally propagated. The *Oct-3/4* deletion mutants expression vectors (10 μg) were cotransfected together with 1 μg of a plasmid carrying the Puromycin selection gene. Stably transfected cells were selected by Puromycin (5 μg per ml, Roche) and clonally propagated.

Soft agar assay

The soft agar assay was carried out in 60 mm culture dishes. Each dish was coated with 4 ml of medium containing 0.55% soft agar. Triplicates of 0.5×10^3 cells were suspended in 3.6 ml of growth medium, mixed with 0.4 ml of 3.3% soft agar, and added to the dishes. Cells were fed weekly with 0.5 ml of medium. After 14 days, colonies were counted.

Protein analysis

For Western immunoblot analysis, nuclear extracts (Ben-Shushan et al., 1993) from the various Swiss 3T3-transfected cells were loaded on 6% SDS-PAGE (7.5 μg total protein/lane) along with extract from P19 EC cells (positive control) and transferred to nitrocellulose membrane (Protran, Schleicher and Schuell, Dassel, Germany). The blots were probed with antibodies against Oct-3/4 or β-actin, developed with HRP-conjugated secondary antibodies (ImmunoPure, Pierce, Rockford, Illinois), detected by chemiluminescence following the manufacturer's protocol (ChemiLucent detection system, Chemicon, Temecula, California), and visualized by exposure to X-ray film.

Mouse xenograft assay

Cells were trypsinized and resuspended in PBS. 6×10^6 viable wt Swiss 3T3 cells or transfected clones with either pBL-EGFP, pBL-Oct-3/4-EGFP, or pBp-Ras were injected subcutaneously into the left flank of Cd1 nude mice. In the case of wt CGR8 ES cells, ZHTc6 and ZHTc25, 2×10^6 viable

cells were injected subcutaneously into the left flank of Ola129/Sv mice. In the case of ZHBTc4 ES cells, 3×10^6 viable cells were resuspended in 0.2 ml PBS-Basement membrane matrix 1/1 (Matrigel™, Biological Industries, Kibbutz Beit Haemek, Israel) before subcutaneous injection into Ola129/Sv mice. When appropriate, dox was delivered by the addition of 0.2 mg/ml doxycycline (D-9891, Sigma-Aldrich Israel, Rehovot, Israel) to their drinking water. Bi-dimensional measurements of the tumors were obtained every 2 days with calipers, and animals were sacrificed either when the tumors reached the size of 1 cm^2 or 12 weeks after injection. Tumors were weighed, fixed in formalin, and embedded in paraffin for immunohistochemical analysis. For tumor regression experiments, mice were inoculated with 3×10^6 ZHBTc4 ES cells, as above, and followed for tumor development. When tumors reached a size of 1 cm^2, mice were randomly assigned to either one of two groups: one fed with plain water and the other with water supplemented with dox. Mice were kept for an additional 2 weeks.

Histology, immunohistochemistry, and immunofluorescence staining

Formalin-fixed, paraffin-embedded xenograft samples were cut into 5 μm sections, deparaffinized in xylene, and rehydrated through a series of decreasing concentrations of ethanol. Sections were stained with hematoxylin and eosin or with the indicated antibodies. For immunofluorescence staining, cells (5×10^5) were allowed to adhere to glass coverslips pretreated with 0.2% gelatine. Twenty-four hours later, cells were washed with 1× PBS and fixed in 3.7% paraformaldehyde, permeabilized with 0.2% triton x-100, and blocked with 1% BSA. The slides were incubated with antibody against the mouse Oct-3/4 protein for 40 min at 25°c and than washed with PBS. Secondary antibody conjugated to FITC (Jackson Immunoresearch Laboratory Inc., West Grove, Pennsylvania) was then used to detect Oct-3/4 immunoreactivity. The slides were examined and photographed.

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