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Differential methylation of the *OCT3/4* upstream region in primary human testicular germ cell tumors

JEROEN DE JONG, SANNAH WEEDA, AD J.M. GILLIS,
J. WOLTER OOSTERHUIS and LEENDERT H.J. LOOIJENGA

Department of Pathology, Erasmus MC-University Medical Center Rotterdam, Josephine Nefkens Institute, Daniel den Hoed Cancer Center, Rotterdam, The Netherlands

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Abstract. Germ cell tumors show many similarities to normal embryogenesis. This is, for example, illustrated by the expression of the marker of pluripotency, *OCT3/4*, known to play a pivotal role in the early stages of normal development. Interestingly, it is found to be the most informative diagnostic marker for the early developmental stages of malignant germ cell tumors. Expression regulation of *OCT3/4* has been extensively studied in murine and human cell lines, including embryonic stem cell lines and tumor derived cell lines. We investigated for the first time the methylation status of the upstream region of the *OCT3/4* gene in normal and neoplastic testicular primary tissues using bisulfite genomic sequencing. The cell line JKT-1, supposedly seminoma-derived, was included in the survey. Normal testis parenchyma, peripheral blood lymphocytes, spermatocytic seminoma, yolk sac tumor and teratoma, and JKT-1 showed a consistent hypermethylation. In contrast, seminoma and embryonal carcinoma were hypomethylated, confirmed by analyses after tumor micro-dissection. Testicular lymphomas showed the most heterogeneous pattern, although specific regions were consistently hypermethylated. In conclusion, the results obtained from this set of adult normal and neoplastic *in vivo* derived samples is in accordance to the *in vitro* data that expression of *OCT3/4* is associated with specific changes in methylation. Moreover, the findings argue against OCT3/4 being a driving oncogenic factor in the pathogenesis of human germ cell tumors.

Introduction

Gene expression during normal development is tightly regulated by epigenetic modifications, including cytosine (CpG) methylation and histone acetylation (1). During the process of tumorigenesis, the physiological (normal) epigenetic patterns may become disturbed and as a result, genes under control of these epigenetic modifications, may become silenced by promoter hypermethylation (2). Testicular germ cell tumors of adolescents and young adults (TGCTs), i.e., the seminomas and non-seminomas, have a heterogeneous epigenetic phenotype (3). Overall, gene promoters in seminomas are highly demethylated, whereas the non-seminomas show methylation of the majority of promoters, as reported for other solid tumors (4,5). The methylation status of seminomas is consistent with the cell of origin of TGCTs, the primordial germ cell (PGC) (reviewed in ref. 3). During early embryogenesis, PGCs migrate from the yolk sac to the genital ridge, from which the gonad develops. During this migration the epigenetic marks of the PGCs are largely erased and only become re-established as the PGCs mature during gametogenesis eventually leading to either oocytes or spermatozoa (6). Another hallmark that PGCs share with some histological elements of TGCTs is the expression of *OCT3/4* (*POU5F1*), a member of the POU family of transcription factors. *OCT3/4* is regarded as one of the key regulators of pluripotency and is only expressed in pluripotent stem and germ cells (7-10). In the human embryo, *OCT3/4* expression starts at the morula stage and becomes restricted at first to the inner cell mass during blastocyst formation, subsequently to the epiblast during gastrulation and finally to the PGCs. In TGCTs mRNA and protein encoded by *OCT3/4* is present in carcinoma *in situ* (CIS), the precursor of all TGCTs (11), as well as in all seminomas and the undifferentiated component of non-seminoma, i.e., embryonal carcinoma (EC) (12,13). Yet, when EC cells differentiate, either into teratoma, yolk sac tumor or choriocarcinoma, expression of *OCT3/4* is consistently turned off (12). In murine and human EC-derived cell lines the same pattern of down-regulation is found upon induction of differentiation by retinoic acid (14,15). However, it must be emphasized that murine and human EC cells are not interspecies equivalents and have a different pathogenesis (3).

Methylation of the *OCT3/4* upstream region has been studied in several human and murine tumor-derived and embryonic stem cell (ESC) derived cell lines. In mouse ESCs, Hattori *et al* showed the hypomethylation of this region and compared it to trophoblast stem cells (TSCs) and mouse liver cells which were hypermethylated (16). In

Correspondence to: Dr Leendert H.J. Looijenga, Department of Pathology, Erasmus MC-University Medical Center Rotterdam, Daniel den Hoed Cancer Center, Josephine Nefkens Institute, Room 430b, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands
E-mail: l.looijenga@erasmusmc.nl

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addition, they showed that *in vitro* methylation suppressed *OCT3/4* enhancer/promoter activity in a reporter assay, and the demethylating agent 5-aza-2'-deoxycytidine induced aberrant *OCT3/4* expression in TSCs. In cloned mouse embryos the *OCT3/4* promoter underwent gradual demethylation during pre-implantation development and inefficient demethylation of the *OCT3/4* promoter was associated with developmental retardation at early cleavage stages (17). In the malignant equivalent of ESCs, the EC cells, it was demonstrated in 1993 that inhibition of *OCT3/4* expression in retinoic acid (RA)-induced differentiated murine EC cells is achieved through changes in methylation status, chromatin structure and transcriptional activity of the *OCT3/4* upstream regulatory region (18). These results were confirmed by independent studies (19,20). Loss of *OCT3/4* expression during differentiation of a human EC-derived cell line (NTera2) was found to be correlated with increased methylation in the *OCT3/4* upstream region (15). Recently, this correlation was also found in human ESCs (21).

To augment the current knowledge, we analyzed the methylation status of the 2.3-kb upstream region of the human *OCT3/4* gene in different types of primary TGCTs and non-germ cell tumors of the testis. Analysis was performed by PCR and sequencing of bisulfite-treated DNA from seminoma, histological variants of non-seminoma, spermatocytic seminoma, testicular lymphoma, normal testicular parenchyma and peripheral blood lymphocytes. In addition, micro-dissected seminoma and EC as well as the cell line JKT-1, supposedly derived from a seminoma (22), were included. We demonstrate a tissue-specific differential methylation of the upstream region of the *OCT3/4* gene correlating with gene expression, with a profound general hypomethylation in microdissected seminoma and EC and a heterogeneous hypermethylation in samples of differentiated histology. Some regions were identified that could be related to activity of the promoter.

Materials and methods

Tissue samples. Use of tissues for scientific reasons has been approved by an institutional review board (MEC 02.981). The samples are used according to the 'Code for Proper Secundary Use of Human Tissue in the Netherlands', as developed by the Dutch Federation of Medical Scientific Societies (FMWV) (version 2002).

Immunohistochemistry. Frozen sections were stained using immunohistochemistry with a monoclonal antibody for OCT3/4 (sc-5279; Santa Cruz Biotechnology) as described previously (23). The secondary antibody was a biotinylated rabbit anti-mouse antibody (E0413; Dako). Final visualization was made with diaminobenzidine (DAB, FLUKA), after which the slides were counterstained with haematoxylin.

DNA-isolation and PCR. Genomic DNA was isolated from peripheral blood lymphocytes, normal testis parenchyma, spermatocytic seminoma, testicular lymphoma, embryonal carcinoma, yolk sac tumor, teratoma and seminoma by standard phenol extraction. Of each type of material three independent DNA samples were used, except for yolk sac tumor of which only two samples were included. In addition, genomic DNA from the JKT-1 cell line was isolated. DNA was treated with bisulfite (EZ DNA methylation kit, Zymo Research) to convert unmethylated cytosines into uracil. On this converted DNA PCR was performed with seven primer pairs used in previous experiments (15). For the PCR reactions two different Taq polymerases were used. For primer pair 3 and 4 Taq polymerase (Qiagen) was used with the following program on a MJ Research Cycler (Waltham): 95˚C for 3 min, 35 cycles of 94˚C for 30 sec (denaturation), 58˚C for 45 sec (annealing), 72˚C for 1 min (extension), and finally an extension at 72˚C for 8 min. For primer sets 5-9 a High Fidelity Taq polymerase (Invitrogen) was used with this program: 95˚C for 3 min, 35 cycles of 94˚C for 30 sec, 58˚C for 45 sec, 68˚C for 45 sec, and an extension at 68˚C for 8 min. The obtained PCR products were purified (Qiagen PCR-28106 purification kit).

Sequencing. Forward primers were labelled with [γ^{32}P]ATP. PCR products were sequenced using a cycle reader DNA sequencing kit (K1711 Fermentas). The sequencing program was 95˚C for 3 min, 25 cycles of 94˚C for 30 sec, 58˚C for 45 sec, 72˚C for 1 min and 4˚C for 30 min. Gels (6 and 8%) ran from 1-4 h, depending on the size of the sequenced fragment.

Micro-dissected samples. Micro-dissection was performed with a PALM-microlaser system (Zeiss) on frozen slides guided by Alkaline Phosphatase staining found to be informative to distinguish seminoma and EC cells from other cell types (24,25). DNA from three micro-dissected ECs and seminomas was processed as described above by bisulfite treatment, PCR and sequencing.

Results

We examined the methylation status of 32 CpG sites between the transcription start site and position -2351 upstream of the human *OCT3/4* gene of *in vivo* derived human samples. In addition, matched micro-dissected samples were included to exclude misinterpretation of the data due to the presence of host tissue (Fig. 1). Based on sequence analysis each position was scored as complete methylation, partially methylated and unmethylated (Fig. 2). Because direct sequencing was applied to the tumor samples, we checked if this method was comparable to sequencing of cloned PCR products. Therefore, part of the upstream sequence (primer pair 9) was analyzed by both methods in undifferentiated and retinoic acid differentiated NTera2 cells. Results were similar [data not shown, in accordance to previous findings (26)] and confirmed the results obtained by Deb-Rinker *et al* (15). Therefore, we only investigated the direct sequencing derived data in this study. Results from the human tissue samples are schematically represented in Fig. 3.

Some of the positions could not be reliably scored due to sequencing limitations and these are omitted from further analysis as indicated (Fig. 3). The CpG sites at position -1891 and -2351 could not be conclusively analyzed in any sample. Deviating from the sequence of the upstream region of the human *OCT3/4* gene in the article by Nordhoff *et al* (27) we found two extra CpG sites at positions -1692 and -1755 generated by single nucleotide polymorphisms. For position -1692 the polymorphism was CG/CA and for position -1755

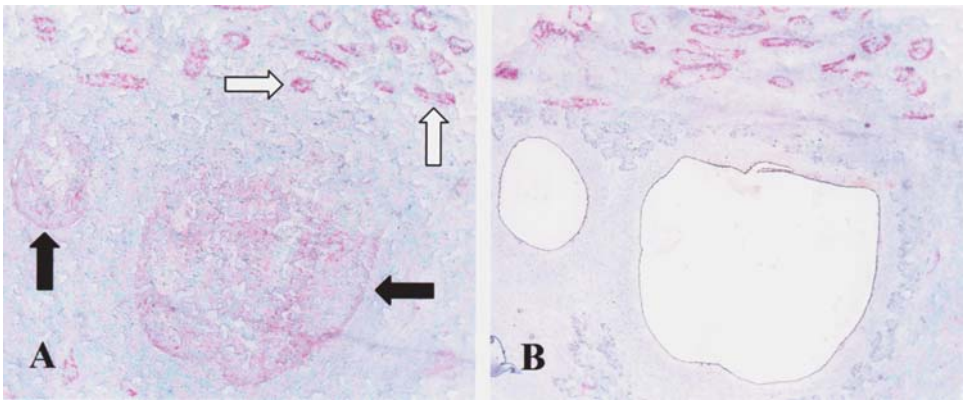

Figure 1. Microdissection of embryonal carcinoma. Embryonal carcinoma (black arrows) before (A) and after (B) micro-dissection. Enzymatic alkaline phosphatase stains embryonal carcinoma as well as the carcinoma *in situ* cells (white arrows).

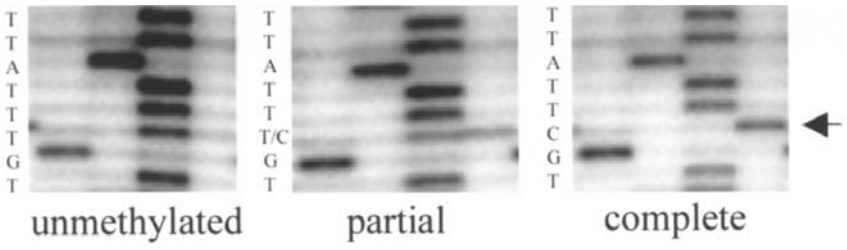

Figure 2. Sequencing analysis of bisulfite treated DNA. Position-1755 (arrow) in representative samples. Unmethylated CpG site shows only a band at the T position (left panel), partially methylated site has a band at both the T and C position (middle panel), and complete methylated site has only a band at the C position (right panel). Sequences are indicated on the left of each autoradiograph.

it was CG/CT. In addition, position -1772 was also a polymorph and consisted of a CG/CC polymorphism. Frequency and functionality of these specific polymorphisms in normal or patient populations were not investigated in this study, and remain to be elucidated.

To compare the methylation status of the different samples, the number of complete methylated CpG sites was divided by the total number of CpG sites analyzed. This resulted in the percentage of completely methylated CpG sites per sample (Fig. 3). Peripheral blood lymphocytes, spermatocytic seminoma and differentiated components of non-seminoma, i.e., yolk sac tumor and teratoma had a hypermethylated upstream region of the *OCT3/4* gene, ranging from 56 to 89%. The testicular lymphomas were heterogeneous with methylation percentages of 31 to 94%, while they were all immunohistochemically negative for OCT3/4. The JKT-1 cell line was hypermethylated, and also lacked expression of OCT3/4.

In contrast to the differentiated TGCT components, the seminomas and embryonal carcinomas had a relatively low level of CpG methylation, ranging from 9 to 42%. Matched micro-dissected samples of these two histologies, lacking (hypermethylated) host cells, such as lymphocytes and normal testis parenchyma, were all unmethylated (Fig. 3).

Discussion

The methylation status of the upstream region of the *OCT3/4* gene has been studied in various murine and human cell lines, both benign and malignant. Here we showed the differential methylation of this region in a range of primary human testicular tumors and normal tissue, an unexplored field so far. This differential methylation is in accordance with previous data obtained from *in vitro* grown cells. In fact, undifferentiated, *OCT3/4* expressing cells, have overall a hypomethylated upstream region, whereas differentiated cells, lacking OCT3/4 expression, are hypermethylated.

In our study, seminoma and the undifferentiated non-seminomatous EC, both characterized by OCT3/4 expression, are hypomethylated. However, tumor tissue can be quite heterogeneous and especially seminoma cells are intermingled with lymphocytic infiltrations that can drastically decrease the percentage of tumor cells to below 50%. Also, EC often constitutes a low percentage of cells in the tissue, because of growing in nests and their capacity to differentiate into extra-embryonic and embryonic tissues. Of course, this contamination distorts the results of the analyses, especially if total samples are investigated. Therefore, in this study, also micro-dissected seminoma and EC samples were investigated, and these samples showed almost no methylation of the region analyzed. In contrast to seminoma and EC, differentiated non-seminomatous components, teratoma and yolk sac tumor, which do not express OCT3/4, were indeed found to be hypermethylated. Spermatocytic seminoma, normal testis parenchyma and peripheral blood lymphocytes, all consisting of differentiated cells, are hypermethylated. These findings support the different cell of origin and pathogenesis of seminoma and spermatocytic seminoma (28). The cell line JKT-1, supposed to be derived from a seminoma (22), was hypermethylated (78%), adding

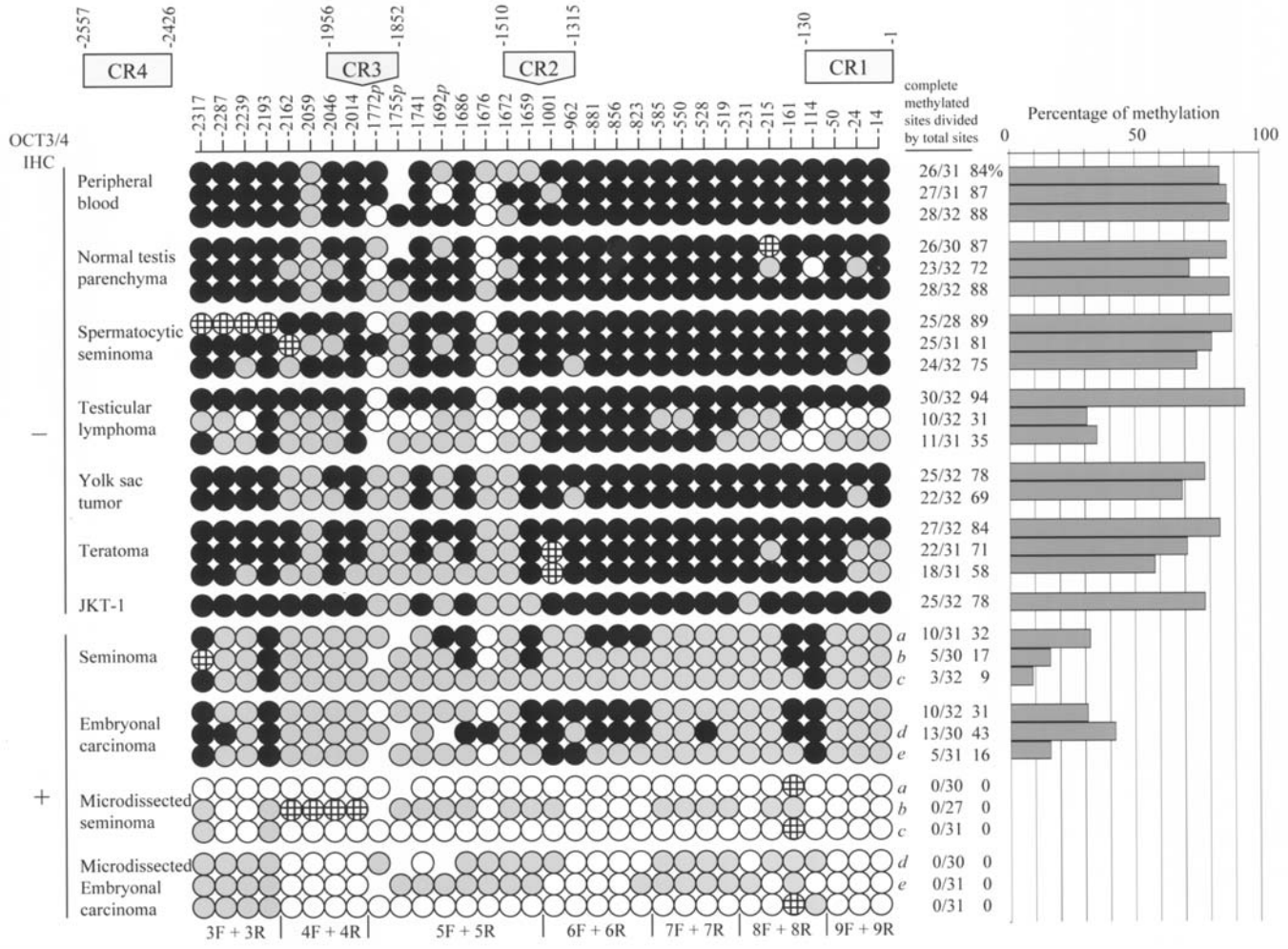

Figure 3. Methylation status of the upstream region of the *OCT3/4* gene. At the top conserved regions (CR) between human, murine and bovine sequences are represented as grey boxes and accompanied by their relative position to the transcription start site (TSS). Directly below the examined CpG sites and their position relative to TSS are indicated. On the left side are the histological groups, each group consisting of three independent DNA samples, except for the group of yolk sac tumors that contains two samples. In addition, OCT3/4 immunohistochemistry (IHC) results are indicated as positive (+) or negative (-). Each horizontal row of circles represents one sample. A black circle signifies a complete methylated CpG site, a grey circle partial methylation and a white circle no methylation. In addition, gritted circles signify an inability to analyse and polymorphic sites (indicated by *p*) not constituting a CpG are omitted. Letters *a* to *e* at the right end of (micro-dissected) seminoma and embryonal carcinoma samples indicate which samples were derived from the same tumor. The primer pairs that cover the CpG sites are represented at the bottom. At the right of each row is the fraction of complete methylated CpG sites and total CpG sites analysed. This fraction is represented as a percentage in the vertical histogram. Differentiated samples of peripheral blood lymphocytes, normal testis parenchyma, spermatocytic seminoma, yolk sac tumor and teratoma have a high level of methylation. Seminoma and embryonal carcinoma were hypomethylated, which was confirmed in the micro-dissected samples where no contaminating normal cells were present. In contrast, methylation in the group of testicular lymphomas was heterogeneous.

to the accumulating evidence that it is not of seminomatous origin (unpublished data). The testicular lymphomas are the only tumors that show a heterogeneous pattern in this limited set of samples. While all were negative for OCT3/4, two (both of T-cell origin) showed a low level of methylation, while one B-cell lymphoma showed a high level of methylation. This might be of interest, although it needs further investigation (29).

In the human EC-derived cell line NTera2 Deb-Rinker *et al* showed that a relatively small increase in methylation of specific CpG sites in the upstream *OCT3/4* region is sufficient to shut-down expression of the gene (15). These specific CpG sites correspond to positions -1001, -231, -50, +5 and +35 (Fig. 3). The sites upstream of the transcription start site were also differentially methylated in our analysis; the latter two were not represented in our search. In addition, the previous report identified CpG sites that were already methylated in the undifferentiated EC cells. This block of constitutive methylation (CpG sites -519 to -881) was not present in the micro-dissected EC samples and is possibly related to the clonal origin of the NTera2 cells and/or their *in vitro* propagation. It is indeed known that upon *in vitro* growth hypermethylation might occur compared to the original *in vivo* obtained cells (30).

Expression of the *OCT3/4* gene is, besides methylation, regulated by other factors, such as binding of transcription factors to specific regions in the upstream sequence. These regions are conserved between different species and designated CR1 to CR4 by Nordhoff *et al* (27). OCT3/4 expression is regulated by a minimal TATA-less promoter (31) present in CR1. In addition, there are two enhancer sites more upstream: a proximal enhancer (PE) mainly coinciding with CR2 that

activates *OCT3/4* in the epiblast (32), and a distal enhancer (DE) in CR4 driving gene expression in the morula, inner cell mass and PGCs (32). Next to the DE the CR4 also contains the OCT3/4-SOX2 binding site (33). These promoter and enhancers are the key regulatory sequences for *OCT3/4* gene expression and therefore methylation status of their associated CpG sites can be expected to be of more importance than the sites of non-regulatory upstream sequences. However, thus far this was not experimentally tested. The minimal *OCT3/4* promoter in CR1 has no CpG island, but harbors 4 of the investigated CpG sites (position -14, -24, -50 and -114 relative to the transcription start site). Unfortunately, the DE in CR4 lies outside the examined upstream region and CR2 containing the PE has no CpG sites (Fig. 3). Because of the limited number of CpG sites in the regulatory regions, no clear relation between methylation of these regions and gene expression could be made. However, some general patterns can be observed. Overall, CpG sites -14 to -1659 and -2193 to -2317 show differential methylation between differentiated and undifferentiated tissue, whereas this relation is not present in CpG -1672 to -2162 lying in between (Fig. 3). The same pattern was observed in NTera2 cells (15). Most of these differentially methylated CpG sites had no association with known transcription factor binding sequences, except for position -114 where Sp1 and Sp3 can bind.

Genomic methylation patterns in solid tumors might be disturbed compared to their normal counterpart, causing for example gene silencing by aberrant promoter methylation (34). If this is the case, these epigenetic alterations, together with mutations, can contribute to the process of tumorigenesis (reviewed in ref. 34). Thus far, no mutations or aberrant epigenetic patterns for *OCT3/4* have been described and *OCT3/4* is only expressed in the malignant counterpart of cells normally expressing the gene, i.e., CIS/seminoma and EC, respectively representing neoplastic PGCs and ESCs (35). This observation, together with our results presented here on the methylation status in human TGCTs supports the view that *OCT3/4* is not one of the driving oncogenic forces in these tumors. However, the data support the model that TGCTs can be used to study mechanisms involved in normal embryogenesis, as we previously reported for X-inactivation (36).

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