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Germ cell pluripotency, premature differentiation and susceptibility to testicular teratomas in mice

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SUMMARY

Testicular teratomas result from anomalies in germ cell development during embryogenesis. In the 129 family of inbred strains of mice, teratomas initiate around embryonic day (E) 13.5 during the same developmental period in which female germ cells initiate meiosis and male germ cells enter mitotic arrest. Here, we report that three germ cell developmental abnormalities, namely continued proliferation, retention of pluripotency, and premature induction of differentiation, associate with teratoma susceptibility. Using mouse strains with low versus high teratoma incidence (129 versus 129-Chr19^{MOLF/EI}), and resistant to teratoma formation (FVB), we found that germ cell proliferation and expression of the pluripotency factor *Nanog* at a specific time point, E15.5, were directly related with increased tumor risk. Additionally, we discovered that genes expressed in pre-meiotic embryonic female and adult male germ cells, including cyclin D1 (*Ccnd1*) and stimulated by retinoic acid 8 (*Stra8*), were prematurely expressed in teratoma-susceptible germ cells and, in rare instances, induced entry into meiosis. As with *Nanog*, expression of differentiation-associated factors at a specific time point, E15.5, increased with tumor risk. Furthermore, *Nanog* and *Ccnd1*, genes with known roles in testicular cancer risk and tumorigenesis, respectively, were co-expressed in teratoma-susceptible germ cells and tumor stem cells, suggesting that retention of pluripotency and premature germ cell differentiation both contribute to tumorigenesis. Importantly, *Stra8*-deficient mice had an 88% decrease in teratoma incidence, providing direct evidence that premature initiation of the meiotic program contributes to tumorigenesis. These results show that deregulation of the mitotic-meiotic switch in XY germ cells contributes to teratoma initiation.

KEY WORDS: Teratoma, Pluripotency, Differentiation, Germ cell, Mouse

INTRODUCTION

Male germ cell development in the 129 family of inbred mice is an important *in vivo* experimental model system for studying fundamental questions about maintenance of pluripotency and induction of differentiation. Germ cells arise during embryogenesis as pluripotent primordial germ cells (PGCs) that differentiate into mature gametes and ultimately the cells and tissues of an adult organism (Aponte et al., 2005; Kunwar et al., 2006). Defects during male germ cell development can lead to the formation of testicular germ cell tumors (TGCTs), which are classified as teratomas, non-seminomas or seminomas (Almstrup et al., 2004; Looijenga et al., 2003; Oosterhuis and Looijenga, 2005; Stevens, 1966). In 129 mice, TGCTs are first evident microscopically at embryonic day (E) 15.5 as foci of pluripotent stem cells (embryonal carcinoma cells or EC cells) (Stevens, 1962). Mouse EC cells differentiate to form teratomas, disorganized cell masses consisting of embryonic and extra-embryonic tissue types at various stages of differentiation (Stevens, 1967a; Stevens, 1967b; Stevens and Hummel, 1957). The teratomas of 129 mice share many developmental characteristics with human pediatric teratomas and adult non-seminomas (Oosterhuis and Looijenga, 2005; Stevens and Hummel, 1957).

The capacity of germ cells to maintain pluripotency and to differentiate when perturbed is similar to the *in vitro* properties of embryonic stem (ES) cells, pluripotent stem cells derived from the inner cell mass (ICM) of the blastocyst (Smith, 2001). These developmental similarities and the shared expression of several markers have led to the hypothesis that ICM cells transition through an intermediate germ cell-like state during ES cell derivation (Chu et al., 2011; Zwaka and Thomson, 2005). Interestingly, 129, the only strain with an appreciable frequency of spontaneous TGCTs, is also the most permissive mouse strain for ES cell derivation (Smith, 2001; Stevens and Hummel, 1957; Stevens and Little, 1954). Thus, the genetic elements of strain 129 that induce the transformation of germ cells into pluripotent EC cells are likely to facilitate the efficient derivation of totipotent ES cells. In fact, we previously demonstrated that genetic factors on 129-derived chromosome 18 are essential for both increased teratoma risk and ES cell derivation efficiency (Anderson et al., 2009). Therefore, studies of testicular teratoma initiation in 129 mice not only provide insight into the etiology and pathogenesis of a common cancer in humans, but also into the genetics and cellular pathways involved in stem cell maintenance and differentiation.

It has long been hypothesized that testicular teratomas arise from germ cells that fail to become mitotically inactive in the G₀ phase of the cell cycle (Matin et al., 1998; Noguchi and Stevens, 1982; Stevens, 1966; Stevens, 1967b). The decision to enter mitotic arrest versus initiate meiosis, the so-called mitotic-meiotic switch, is a crucial event in the development of the germ cell lineage (McLaren, 2000; Park and Jameson, 2005). In mice, this switch coincides with germ cell sex specification at ~E13.5 (McLaren, 1984). In the embryonic ovary, retinoic acid induces germ cells to express *Stra8*, which initiates an anterior-to-posterior wave of

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meiotic differentiation that lasts for four days (E12.5 to E16.5) (Menke et al., 2003). During the same developmental period, *Cyp26b1* inactivates retinoic acid, thereby blocking *Stra8* expression in male germ cells (Bowles et al., 2006; Koubova et al., 2006; MacLean et al., 2007; Vernet et al., 2006). *Nanos2* expression also inhibits *Stra8* expression in male germ cells (Suzuki and Saga, 2008). Rather than initiating meiotic prophase, male germ cells remain quiescent until after birth when they re-initiate proliferation and differentiate to form the spermatogonial lineage (McLaren, 1984).

The influence of genetic modifiers on teratoma incidence in 129 mice supports the hypothesis that aberrant cell proliferation leads to tumor initiation. The *Ter* mutation of *Dnd1* (*Dnd1^{Ter}*), an engineered deletion of *Dmrt1*, and a *Pten* loss-of-function mutation increase both teratoma incidence and germ cell proliferation after E13.5 (Kimura et al., 2003; Krentz et al., 2009; Noguchi and Stevens, 1982). By contrast, deletion of *Eif2s2* decreases the number of aberrantly dividing male germ cells and tumor incidence (Heaney et al., 2009; Kimura et al., 2003). Importantly, continued proliferation cannot by itself explain the cellular events that must occur to transform germ cells into EC cells and ultimately the various tissue types found in TGCTs. Retention of pluripotency is likely to play an important role. Repression of *Oct4* (*Pou5f1* – Mouse Genome Informatics) significantly reduces the teratoma-forming capacity of ES cells in mouse xenograph assays (Gidekel et al., 2003), and it is likely that EC cells are similarly dependent on pluripotency factors for their teratoma-forming capacity. Following initiation of G₀ arrest at E13.5, male germ cells normally downregulate expression of pluripotency factors (e.g. *Nanog*, *Sox2* and *Pou5f1*) (Avilion et al., 2003; Pesce et al., 1998; Yamaguchi et al., 2005). In testicular teratoma-susceptible mice, germ cells that fail to enter mitotic arrest continue to express pluripotency factors through the transition to EC cells (Cook et al., 2011; Kimura et al., 2003; Krentz et al., 2009). Importantly, signaling pathways involving *POU5F1* and *NANOG* have been implicated in germ cell tumor initiation in humans (Clark et al., 2004; Looijenga et al., 2003; Oosterhuis and Looijenga, 2005).

Tantalizing evidence suggests that meiotic differentiation might also be involved in human and mouse testicular germ cell tumors. In humans, markers associated with both mitotic and meiotic cells, such as cyclin D2 (*CCND2*) and *SYCP3*, respectively, are expressed in EC cells and germ cell tumors (Adamah et al., 2006; Bartkova et al., 1999; Sicinski et al., 1996). In mice, *Dnd1^{Ter}* mutant male germ cells express *STRA8* and *SYCP3*, factors involved in meiotic commitment and meiotic prophase I, respectively (Cook et al., 2009). However, these factors were not expressed in EC cells. By contrast, male germ cells of *Dmrt1*-deficient embryos were reported to not express *Stra8* (Krentz et al., 2009). Thus, whether premature expression of genes involved in meiotic differentiation contributes to tumor initiation or is an unrelated phenotype associated with certain mutations remains unresolved.

In the present study, we demonstrate that teratoma-susceptible germ cells in mice delay entry into G₀ arrest, delay the repression of pluripotency, and prematurely express genes associated with pre-meiotic embryonic female and adult male germ cell differentiation. Importantly, we show that germ cell proliferation, and the expression of germ cell pluripotency and differentiation-associated factors at a specific developmental time point, E15.5, are directly correlated with increased teratoma risk. Furthermore, we demonstrate that genes involved in germ cell pluripotency (*Nanog*) and differentiation (*Cnd1*) are co-expressed in EC cells, and that

Stra8 deficiency reduces teratoma incidence. We propose that retention of pluripotency is required for the teratoma-forming capacity of EC cells and that premature expression of factors associated with germ cell differentiation contribute to the transformation of germ cells into tumorigenic EC cells. Together, our results suggest that TGCT initiation is a complex process involving several developmental abnormalities.

MATERIALS AND METHODS

Mice

129S1/SvImJ (JR#002448) and FVB/NJ (JR#001800) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). 129/SvImJ mice homozygous for the Chr19^{MOLF/Ei} chromosome substitution (M19) were obtained from our research colony (Matin et al., 1999). The germ cell-specific *Oct4ΔPE:GFP* (*Oct4::GFP*) transgene (Yoshimizu et al., 1999; Youngren et al., 2005) was backcrossed onto the 129/SvImJ, FVB/NJ and M19 backgrounds to establish congenic lines (Heaney et al., 2009). An engineered deletion of *Stra8* (*Stra8^{KO}*) was backcrossed onto a 129/Sv inbred background for at least ten generations to establish a congenic strain. *Stra8^{KO}* mice were PCR genotyped as previously described (Baltus et al., 2006). All protocols were approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

Timed matings and embryonic gonad dissections

For immunohistochemistry and fluorescence-assisted cell sorting (FACS), wild-type females were bred to males homozygous for the *Oct4::GFP* transgene to produce FVB, 129 or M19 transgenic embryos. For meiotic chromosome spreads, wild-type mice were bred to produce FVB, 129 or M19 embryos. E0.5 was assumed to be noon of the day the vaginal plug was observed. Pregnant females were euthanized by cervical dislocation and gonads were removed from embryos in ice-cold PBS. Embryos older than E14.5 were decapitated prior to dissection. PCR genotyping for *Sry* identified the sex of E12.5 embryos (Heaney et al., 2009). Gonad morphology identified the sex of E13.5 to E16.5 embryos.

Fluorescence-assisted cell sorting

FACS with the *Oct4::GFP* transgene has been previously described (Heaney et al., 2009; Molyneaux et al., 2003). Briefly, gonads were digested in 0.25% trypsin (Invitrogen, Carlsbad, CA, USA) for 15 minutes at 37°C. Tissues were triturated into single-cell suspensions and filtered through a 40 μm nylon mesh cell strainer (BD Biosciences, Franklin Lakes, NJ, USA). The mesh was washed with 2% bovine serum albumen (BSA) in PBS and the cells were kept on ice until FACS with the BD Biosciences FACSARIA system. The *Oct4::GFP* transgene was used to sort GFP-positive PGCs from GFP-negative somatic cells, which typically yielded 8000 GFP-positive germ cells (98% purity) from both gonads of a single embryo (Heaney et al., 2009; Molyneaux et al., 2003).

Quantitative real-time PCR expression analysis

Germ cell RNA was prepared using the RNeasy Micro Kit (Qiagen, Valencia, CA, USA). RNA was reverse transcribed with the SuperScript First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR (qPCR) was performed with the Chromo4 real-time PCR system (MJ Research/BioRad, Hercules, CA, USA) and the DyNAmo HS Sybr Green qPCR kit (Fisher Scientific) using manufacturer's suggested protocols. Serial dilutions of wild-type adult testis cDNA were used to generate standard curves for each primer set. Expression was normalized to the ubiquitously expressed housekeeping gene *Rpl7* as previously described (Heaney et al., 2009; Jeong et al., 2005). Female germ cell expression data from all strains was pooled. Significant differences in expression between male FVB germ cells (control) and male M19, male 129 and female germ cells were tested with unpaired *t*-tests with *P* values corrected for multiple testing. See supplementary material Table S1 for qPCR primer sequences.

Immunohistochemistry

Gonads were removed from E13.5 to E16.5 embryos and processed for sectioning and immunohistochemistry as previously described (Heaney et al., 2009). Sections were incubated with a 1:500 dilution of rabbit

polyclonal anti-KI67 (ab15580, Abcam, Cambridge, MA, USA), a 1:100 dilution of rabbit polyclonal anti-NANOG (IHC-00205, Bethyl Laboratories, Montgomery, TX, USA) or a 1:100 dilution of rabbit monoclonal anti-CCND1 (ab16663, Abcam) antibody overnight at 4°C. For some experiments, sections were co-incubated with a 1:400 dilution of rat monoclonal anti-E-cadherin (13-900, Invitrogen) or a 1:500 dilution of mouse monoclonal anti-phosphoSer139-Histone H2A.X (γ H2A.X) (05-636, Fisher Scientific) antibody. For secondary detection, sections were incubated with a 1:400 dilution of goat anti-rabbit Alexa Fluor 555 (A-21429), a 1:400 dilution of goat anti-rat Alexa Fluor 633 (A-21094, Invitrogen) or a 1:400 dilution of goat anti-mouse Alexa Fluor 633 (A-21052, Invitrogen) antibody for 2 hours at room temperature. Nuclei were counterstained with DAPI. Images were taken with a Leica SP2 Confocal Microscope.

Cell counts

Oct4::GFP-positive germ cells positive or negative for KI67, NANOG or CCND1 immunostaining were counted as previously described (Heaney et al., 2009). At E15.5 and E16.5, co-labeling for E-cadherin was used to exclude *Oct4::GFP*-positive EC cells from germ cell assays. Significant differences in the percentage of KI67, CCND1 or NANOG-positive germ cells were tested with unpaired *t*-tests with *P* values corrected for multiple testing.

Cell spreads for meiotic chromosome analysis

Gonads were removed at E14.5 and E16.5 and chromosome spreads were processed for immunohistochemistry as previously described (Anderson et al., 2008). Spreads were incubated with a 1:100 dilution of mouse monoclonal anti-SYCP3 (ab97672, Abcam) or a 1:100 dilution of rabbit polyclonal anti-SYCP1 (ab15090, Abcam) antibody overnight at 4°C. For secondary detection, spreads were incubated in blocking solution containing a 1:400 dilution of goat anti-mouse Alexa Fluor 555 (A-21424, Invitrogen) or goat anti-rabbit Alexa Fluor 555 (A-21429, Invitrogen) antibody for 2 hours at room temperature. Nuclei were counterstained with DAPI.

Tumor surveys

Crosses between 129 mice heterozygous for *Stras8^{KO}* were used to produce wild-type (129), heterozygous knockout (129-*Stras8^{KO/+}*) and homozygous knockout (129-*Stras8^{KO/KO}*) male offspring to survey for teratomas. Males at least 1 month of age were necropsied prior to genotyping and testes were visually and histologically examined for tumors, which are readily detected at this age (Lam et al., 2004; Matin et al., 1999). A χ^2 contingency table was used to assess statistical differences between the number of teratoma-affected control (129 wild type and 129-*Stras8^{KO/+}*) and experimental (129-*Stras8^{KO/KO}*) progeny.

Histology

Tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned (5 μ m), and stained with Hematoxylin and Eosin. Images were taken with the Leica SCN400 Slide Scanner and processed with SlidePath Digital Image Hub and Adobe Photoshop CS5.

RESULTS

Delayed entry into G₀ arrest associates with increased testicular teratoma risk

To test whether germ cell proliferation at a specific developmental time point associates with increased teratoma risk, we examined germ cell proliferation in two teratoma susceptible strains, the 129-Chr19^{MOLF/Ei} chromosome substitution strain (M19) and the 129/SvImJ (129) inbred strain, and a teratoma-resistant strain, FVB/NJ (FVB). M19 mice, in which both copies of chromosome 19 are derived from the MOLF/Ei inbred strain, have a high risk of developing teratomas (80% of males affected) (Matin et al., 1999). By contrast, 129 inbred mice have a low risk of developing teratomas (8% of males affected) (Stevens, 1967b). Importantly, most M19 and 129 germ cells develop normally (Matin et al.,

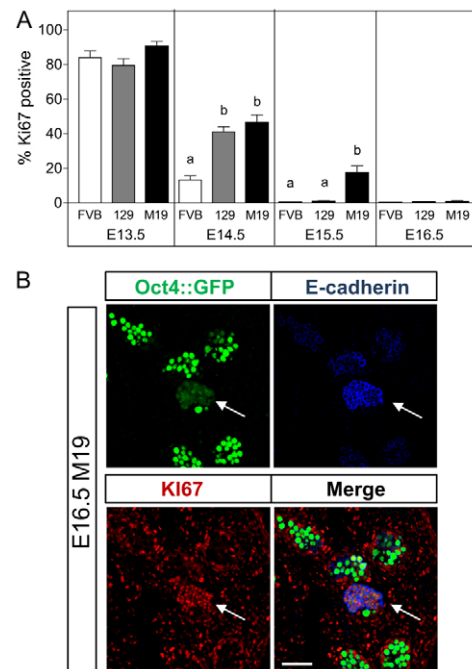


Fig. 1. Teratoma-susceptibility germ cells delayed entry into G₀ arrest. Male germ cell proliferation at E15.5 associates with increased teratoma risk. (A) *Oct4::GFP* transgenic FVB (teratoma-resistant), 129 (low teratoma risk) and M19 (high teratoma risk) embryonic testes were sectioned and immunostained for the nuclear mitotic marker KI67 (E13.5 and E14.5) or KI67 and the EC cell marker E-cadherin to exclude EC cells from the germ cell counts (E15.5 and E16.5). Data are plotted as the percentage of KI67-positive germ cells \pm s.e.m. ($n=6-8$). Means without a common letter differ, $P<0.05$. (B) Confocal microscopy images of an E16.5 M19 testis immunostained for KI67 and E-cadherin. A proliferating cluster of cells with an EC cell-like morphology, positive for E-cadherin and expressing low levels of the *Oct4::GFP* transgene is noted (arrows). E-cadherin-negative, *Oct4::GFP*-positive germ cells are not expressing KI67. Scale bar: 75 μ m.

1999; Stevens, 1967b). Thus, the developmental characteristics of teratoma-susceptible germ cells that do not transform to EC cells can also be studied in M19 and 129 mice.

We first assayed germ cell proliferation at E13.5 and E14.5 by immunostaining *Oct4::GFP* transgenic FVB, 129 and M19 embryonic testes with the proliferation marker KI67 (Fig. 1; supplementary material Fig. S1). At E13.5, the percentage of proliferating *Oct4::GFP*-positive germ cells was similar in the embryonic testes of all strains. At E14.5, the percentage of proliferating germ cells decreased in all strains, but the incidence of germ cell proliferation was statistically higher in teratoma-susceptible gonads (13% proliferating in FVB and 40-46% proliferating in 129 and M19). Importantly, the percentage of proliferating germ cells in 129 and M19 testes at E14.5 was statistically similar, suggesting that the occurrence of proliferation at this time point does not influence teratoma risk.

Having found no differences in germ cell proliferation in 129 and M19 testes through E14.5, we assayed germ cell proliferation at E15.5 and E16.5. For these assays, *Oct4::GFP* transgenic embryonic testes were co-labeled with KI67 and the EC cell marker E-cadherin. EC cells, which are transformed and no longer represent the developmental characteristics of germ cells, first appear at E15.5 and are often mistakenly included in germ

cell assays because they express markers common to embryonic germ cells (e.g. endogenous OCT4 and TRA98) (Kimura et al., 2003; Krentz et al., 2009). E-cadherin is expressed early in germ cell development, but is downregulated in male germ cells at E15.5, whereas EC cells continue to express E-cadherin (Cook et al., 2009; Di Carlo and De Felici, 2000). In addition, the *Oct4::GFP* transgene is expressed at low levels in EC cells (Yeom et al., 1996). Thus, E-cadherin and *Oct4::GFP* expression can be used to identify and exclude EC cell foci (Fig. 1B; supplementary material Fig. S1C,D). At E15.5, <1% of 129 and FVB germ cells were KI67-positive (Fig. 1A). By contrast, 25% of M19 germ cells were proliferative. Therefore, teratoma risk increases with the incidence of germ cell proliferation at E15.5, suggesting that the occurrence of aberrant germ cell proliferation at this specific time point increases the risk of germ cell transformation into EC cells. Interestingly, only 2% of M19 germ cells were KI67-positive at E16.5 (Fig. 1A), which was not significantly different from the percentage of germ cells proliferating in E16.5, 129 or FVB testes. Thus, at E16.5, most germ cells in teratoma-susceptible strains become quiescent regardless of tumor risk, suggesting that germ cells that have not transformed by this time point develop normally.

Delayed repression of pluripotency associates with increased teratoma risk

Downregulation of pluripotency in male germ cells coincides with the induction of G₀ arrest (Avilion et al., 2003; Yamaguchi et al., 2005). Because differences in germ cell proliferation in 129 (low teratoma risk) and M19 (high teratoma risk) embryonic testes were only observed at E15.5, we investigated whether pluripotency gene expression increased with teratoma risk only at E15.5. *Oct4* and *Nanog* expression was examined in teratoma-resistant and susceptible germ cells isolated by FACS with the *Oct4::GFP* transgene. Endogenous *Oct4* expression was similar in teratoma-resistant and teratoma-susceptible germ cells at all embryonic time points tested, with no observable decrease in expression from E13.5 to E15.5 (Fig. 2A). By contrast, *Nanog* expression increased with teratoma risk (Fig. 2B). At E13.5, *Nanog* was similarly expressed in teratoma-resistant and -susceptible germ cells. By E14.5, *Nanog* expression decreased in all male germ cells but was significantly higher (and similar) in teratoma-susceptible 129 and M19 germ cells. Importantly, at E15.5, *Nanog* expression decreased in 129 male germ cells to levels similar to those in FVB male germ cells, but remained significantly increased in M19 male germ cells. As expected, decreased *Oct4* and *Nanog* expression was observed in E13.5 to E15.5 female germ cells, coinciding with initiation of the meiotic program (Fig. 2A,B) (Massari and Murre, 2000; Pesce et al., 1998).

Next, we tested whether the number of NANOG-expressing germ cells increases with teratoma risk (Fig. 3). At E13.5 and E14.5, the percentage of NANOG-positive germ cells was similar in teratoma-resistant and -susceptible testes. Interestingly, at E15.5, the percentage of NANOG-positive germ cells decreased substantially in all testes; however, 30% of M19 germ cells remained NANOG-positive whereas only 1-5% of 129 and FVB germ cells were NANOG-positive. Curiously, at E16.5, only 1% of M19 germ cells were NANOG-positive, which was statistically similar to the percentage of NANOG-positive germ cells in 129 and FVB testes. Therefore, teratoma risk increases with the occurrence of NANOG-positive germ cells at E15.5 and germ cells that have not transformed by E16.5 repress *Nanog* expression.

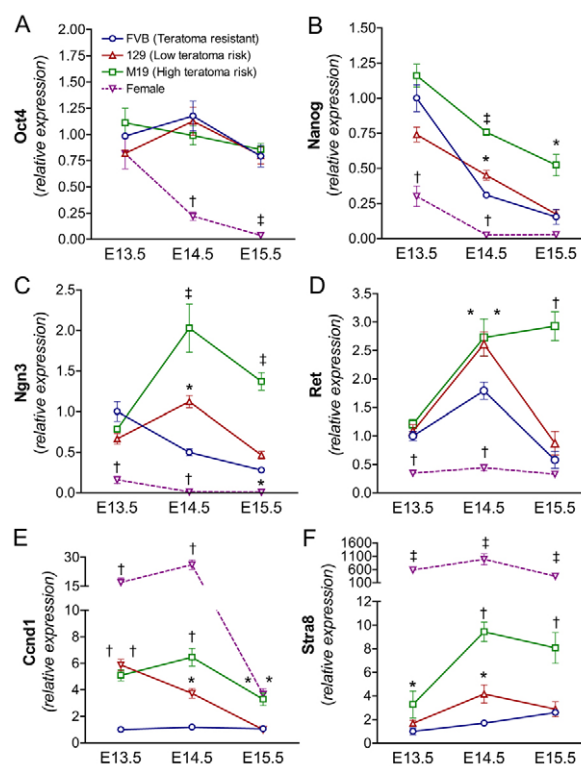


Fig. 2. Teratoma-susceptible germ cells express markers of germ cell pluripotency and male germ cell differentiation.

(A-F) Teratoma-susceptible germ cells express pluripotency (A,B) and differentiation (C-F) markers. *Oct4::GFP* transgenic FVB, 129 and M19 germ cells from E13.5-E15.5 gonads were FACS-isolated and gene expression was analyzed by qPCR ($n=4-15$). Female germ cell expression data from all strains was statistically similar and pooled. Gene expression at all embryonic time points is plotted relative to expression in E13.5 FVB male germ cells. *, $P<0.05$; †, $P<0.005$; §, $P<0.0005$.

Premature expression of male germ cell differentiation factors associates with increased teratoma risk

In addition to regulating pluripotency gene expression, entry into G₀ arrest might also serve to prevent inappropriate germ cell responses to signals produced by somatic cells within the developing testis. Because teratoma-susceptible germ cells continue to actively divide until E15.5, we tested whether a failure to enter mitotic arrest is followed by premature expression of genes associated with spermatogonial differentiation. E13.5 to E15.5 germ cells were examined for the expression of neurogenin 3 (*Ngn3*; *Neurog3* – Mouse Genome Informatics) and the ret receptor (*Ret*), which are expressed in undifferentiated adult spermatogonia (A_s, A_p and A_{al}) (Peters, 1970; Yoshida et al., 2004; Yoshida et al., 2006), and cyclin D1 (*Ccnd1*), which is expressed in neonatal and adult spermatogonia but not embryonic male germ cells (Beumer et al., 2000). Our expression analysis revealed that all three spermatogonial markers are induced in teratoma-susceptible germ cells (Fig. 2C-E). At E13.5, the expression of *Ngn3* and *Ret* was similar in teratoma-resistant and -susceptible male germ cells. However, the expression of *Ccnd1* in 129 and M19 male germ cells was significantly higher than in FVB male germ cells at E13.5. By E14.5, all three markers were significantly increased in 129 and M19 germ cells relative to FVB germ cells. By contrast, at E15.5

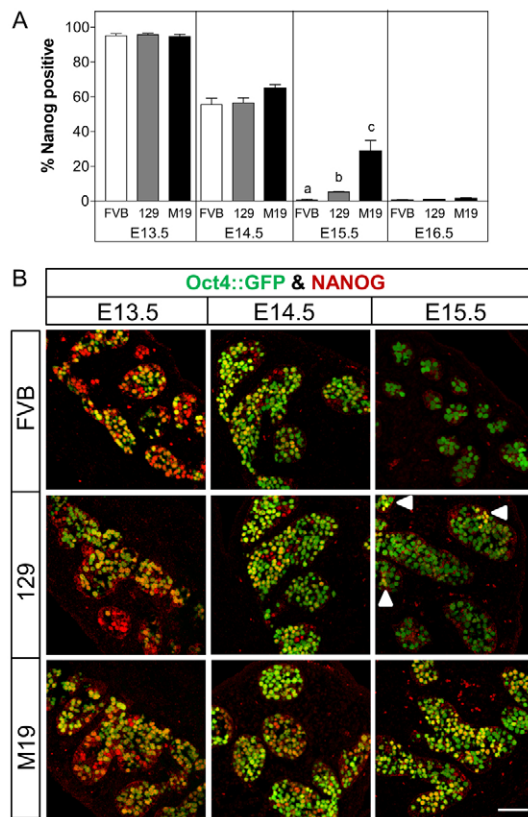


Fig. 3. Teratoma-susceptible germ cells retain pluripotency. NANOG expression at E15.5 associates with increased teratoma risk. **(A)** *Oct4::GFP* transgenic FVB, 129 and M19 embryonic testes were sectioned and immunostained for NANOG (E13.5 and E14.5) or NANOG and E-cadherin (E15.5 and E16.5). Data are plotted as the percentage of NANOG-positive germ cells \pm s.e.m. ($n=6-8$). Means without a common letter differ, $P<0.05$. **(B)** Confocal microscopy images of E13.5-E15.5 testis immunostained for NANOG. At E15.5 FVB germ cells are NANOG-negative, most 129 germ cells are NANOG-negative, and many M19 germ cells are NANOG-positive. Small groups of NANOG-positive germ cells are noted (arrowheads). Scale bar: 75 μ m.

expression of all three differentiation-associated factors in 129 male germ cells had decreased to levels similar to those observed in FVB male germ cells. However, expression of all three genes remained significantly increased in M19 male germ cells (Fig. 2C-E). Therefore, as observed with germ cell proliferation and *Nanog* expression, teratoma risk increases with the expression of male germ cell differentiation-associated genes at E15.5.

Ccnd1 was also strongly expressed at E13.5 and E14.5 in female germ cells and decreased substantially at E15.5 when most female germ cells entered meiosis (Fig. 2E). Similar to its association with pre-meiotic spermatogonia in the adult testis, cyclin D1 expression might be associated with pre-meiotic embryonic female germ cells. Furthermore, expression of *Ccnd1* in teratoma-susceptible germ cells and female germ cells at the same developmental time points implies that a shared signal induces *Ccnd1* expression and possibly pre-meiotic germ cell differentiation. However, *Ret* and *Ngn3* were not induced in female germ cells (Fig. 2C,D), suggesting that if a common signal induces germ cell differentiation, male germ cell identity is retained in teratoma-susceptible embryos.

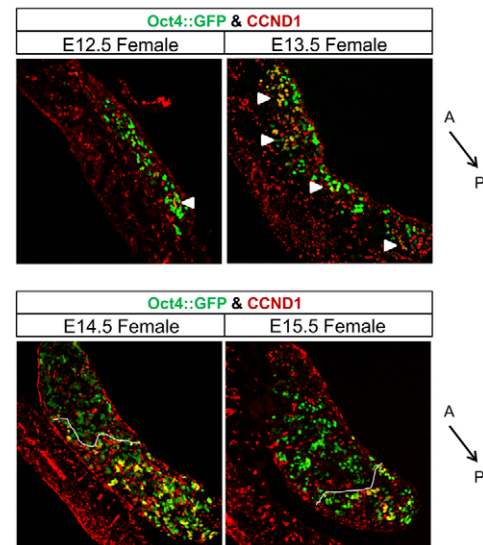


Fig. 4. Pre-meiotic female germ cells express CCND1. CCND1 is expressed prior to meiosis and is repressed in an anterior-to-posterior wave. Confocal microscopy images of E12.5 to E15.5, *Oct4::GFP* transgenic ovaries immunostained for CCND1. Dotted lines indicate the boundary at which germ cells transition from CCND1-negative to CCND1-positive. Small groups of CCND1-positive germ cells are noted (arrowheads). The anterior (A) to posterior (P) axis is indicated. Scale bars: 150 μ m.

Pre-meiotic female germ cells and teratoma-susceptible male germ cells express CCND1

Aberrant expression of cyclin D1 in teratoma-susceptible male germ cells has both developmental and tumorigenic implications (Deshpande et al., 2005). Therefore, we characterized the kinetics of CCND1 expression during the developmental stages associated with the mitotic-meiotic switch and teratoma initiation. We first examined the expression of CCND1 in female germ cells. At E12.5, germ cells of most female gonads did not express CCND1. However, some female gonads contained small groups of CCND1-positive germ cells at the anterior or posterior end (Fig. 4). By E13.5, we observed a strong induction of CCND1 expression in germ cells throughout the developing ovary (Fig. 4). Curiously, from E14.5 to E15.5 expression of CCND1 in female germ cells was lost in an anterior-to-posterior wave, so that by E15.5 only a few germ cells at the posterior end of the developing ovary expressed CCND1 (Fig. 4). Similar patterns of CCND1 expression were found in FVB, 129 and M19 female germ cells. Co-labeling of E14.5 and E15.5 female gonads for CCND1 and γ H2A.X, a marker of DNA double strand breaks produced during meiotic prophase (Paull et al., 2000), revealed that meiotic female germ cells did not express CCND1 (supplementary material Fig. S2). Therefore, CCND1 expression is induced at \sim E13.5 in pre-meiotic female germ cells and is downregulated during meiotic initiation.

We next examined the expression of CCND1 in teratoma-resistant and -susceptible male germ cells. CCND1 expression was not observed in male germ cells at E12.5 (Fig. 5B). However, as observed in the female gonad, CCND1 expression was induced at E13.5 in 28% of 129 and 51% of M19 male germ cells (Fig. 5). By E14.5, the majority (50-60%) of 129 and M19 male germ cells expressed CCND1 (Fig. 5). Interestingly, CCND1 expression was dramatically downregulated in 129 male germ cells at E15.5 and E16.5 (10% and 1% positive, respectively) (Fig. 5). By contrast,

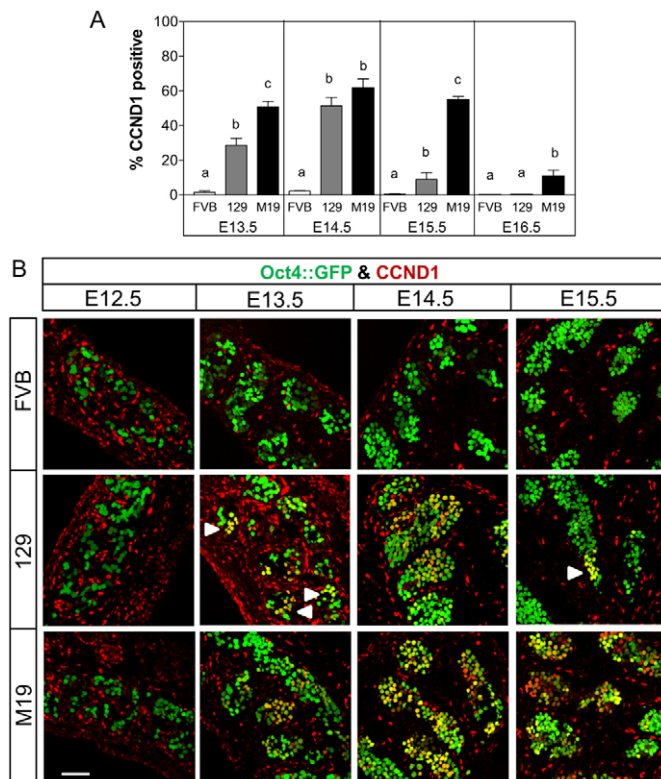


Fig. 5. Teratoma-susceptible germ cells ectopically express CCND1. CCND1 expression at E15.5 associates with increased teratoma risk. **(A)** *Oct4::GFP* transgenic FVB, 129 and M19 embryonic testes were sectioned and immunostained for CCND1 (E13.5 and E14.5) or CCND1 and E-cadherin (E15.5 and E16.5). Data are plotted as the percentage of CCND1-positive germ cells \pm s.e.m. ($n=6-8$). Means without a common letter differ, $P<0.05$. **(B)** Confocal microscopy images of E12.5 to E15.5 testis immunostained for CCND1. At E14.5, teratoma-resistant, FVB germ cells are CCND1-negative and teratoma-susceptible germ cells are mostly cyclin D1-positive. At E15.5, FVB are CCND1-negative, most 129 germ cells are CCND1-negative, and several M19 germ cells are CCND1-positive. Small groups of CCND1-positive germ cells are noted (arrowheads). Scale bar: 75 μ m.

50% of E15.5 and 10% of E16.5 M19 male germ cells expressed CCND1 (Fig. 5). In both 129 and M19 testes, there was no evidence of an anterior-to-posterior wave of CCND1 repression. In agreement with our RNA expression data, CCND1 protein expression was rarely observed in FVB male germ cells (0-3%) at any embryonic time points tested (Fig. 5). Therefore, as observed with the proliferation and pluripotency markers, the percentage of germ cells expressing CCND1 at E15.5 increases with teratoma risk and germ cells that have not transformed into EC cells by E16.5 repress *Ccnd1* expression and probably develop normally.

EC cells co-express NANOG and CCND1

Retention of pluripotency and induction of germ cell differentiation demonstrate that the mitotic-meiotic switch and normal male germ cell development are disrupted in teratoma-susceptible gonads. However, whether two independent populations of abnormal germ cells, one pluripotent and one prematurely differentiating, or a single population of abnormal germ cells that expresses both pluripotency and differentiation factors exists within teratoma-susceptible gonads was unclear. Thus, to test whether these

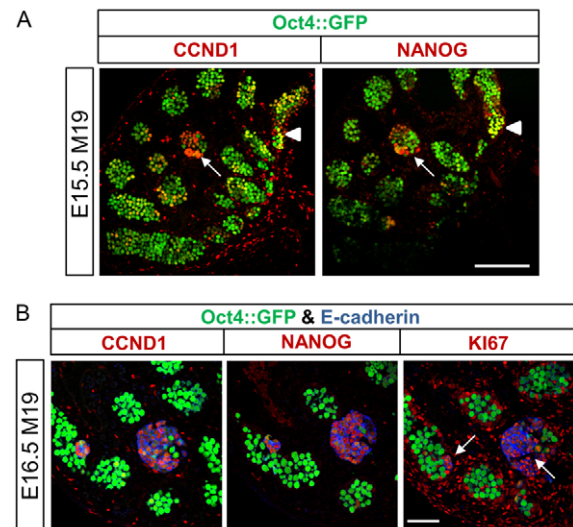


Fig. 6. CCND1 and NANOG are co-expressed in proliferating teratoma-susceptible germ cells and EC cells. **(A)** Confocal microscopy images of serial sections of an *Oct4::GFP* transgenic, E15.5 M19 testis immunostained for CCND1 or NANOG. Tubules with germ cells expressing both CCND1 and NANOG are noted (arrowhead). A cluster of cells with EC cell-like morphology and co-expressing CCND1 and NANOG is indicated (arrow). Scale bar: 150 μ m. **(B)** Confocal microscopy images of serial sections of an *Oct4::GFP* transgenic, E16.5 M19 testis immunostained for CCND1, NANOG, or KI67 and E-cadherin. E-cadherin-positive EC cell foci with low levels of *Oct4::GFP* and expressing CCND1, NANOG and KI67 are noted (arrows). *Oct4::GFP*-positive, E-cadherin-negative germ cells are not expressing CCND1, NANOG or KI67. Scale bar: 75 μ m.

disparate factors are co-expressed in germ cells, we examined NANOG and CCND1 expression in serial sections of *Oct4::GFP* transgenic, E15.5 M19 testes. Importantly, NANOG and CCND1 were expressed in germ cells within similar regions of the developing tubules and in small clusters of cells with weak *Oct4::GFP* expression and morphology consistent with nascent EC cells (Fig. 6A).

To corroborate that pluripotency and germ cell differentiation-associated factors are co-expressed in EC cells, serial sections of E16.5 M19 gonads were immunostained for E-cadherin and either CCND1 or NANOG (Fig. 6B). In agreement with our previous assays, few *Oct4::GFP*-positive, E-cadherin-negative germ cells expressed CCND1 or NANOG at E16.5 (Fig. 6B). However, CCND1 and NANOG colocalized to E-cadherin-positive EC cell foci with weak *Oct4::GFP* expression (Fig. 6B; supplementary material Fig. S3A). In addition, expression of the KI67 proliferation marker localized to the same EC cell foci as CCND1 and NANOG (Fig. 6B; supplementary material Fig. S3A). As with all other time points assayed, few teratoma-resistant FVB germ cells expressed CCND1 or NANOG at E16.5 (Fig. 3A, Fig. 5A; supplementary material Fig. S3B). Therefore, markers of germ cell differentiation and pluripotency colocalize to proliferative EC cells, suggesting that induction of genes associated with both premature differentiation and pluripotency contribute to tumor stem cell formation. Furthermore, restriction of CCND1 and NANOG to proliferating EC cell foci at E16.5 demonstrates that as most teratoma-susceptible germ cells enter G_0 arrest (Fig. 1A), expression of pluripotency and differentiation factors is repressed (Fig. 3A, Fig. 5A).

***Stra8* expression in teratoma-susceptible male germ cells influences teratoma susceptibility**

We tested whether teratoma-susceptible male germ cells are induced to initiate the meiotic program. In the adult testis, *Stra8* is required for the initiation of meiosis (Anderson et al., 2008; van Pelt et al., 1995; Wang and Kim, 1993). Thus, we assayed the expression of *Stra8* in E13.5 to E15.5 germ cells (Fig. 2E). At all embryonic time points, *Stra8* expression was significantly increased in M19 (high teratoma risk) germ cells compared with FVB (teratoma-resistant) germ cells. By contrast, *Stra8* expression was significantly increased in 129 (low teratoma risk) germ cells only at E14.5. As with the other markers of germ cell differentiation, *Stra8* expression in 129 male germ cells reduced to levels similar to those in FVB male germ cells at E15.5. Therefore, *Stra8* expression at E15.5 increases with teratoma risk.

To test whether premature *Stra8* expression during male germ cell development contributes to tumor initiation, we surveyed 129 male mice harboring an engineered deletion of *Stra8* (*Stra8*^{KO}) for teratomas. The *Stra8*^{KO} allele has been shown previously to be a null mutation that blocks the meiotic commitment of embryonic female and adult male germ cells in homozygotes (*Stra8*^{KO/KO}) (Fig. 7A) (Anderson et al., 2008; Baltus et al., 2006; Koubova et al., 2006). Heterozygotes (*Stra8*^{KO/+}) are phenotypically normal. 129-*Stra8*^{KO/+} mice were intercrossed to produce wild-type (129), 129-*Stra8*^{KO/+} and homozygous (129-*Stra8*^{KO/KO}) offspring. As expected, surveys of 129 and 129-*Stra8*^{KO/+} males revealed a teratoma incidence similar to the expected 129 incidence (not shown) and the two groups were pooled as controls. Importantly, the teratoma incidence of 129-*Stra8*^{KO/KO} males was decreased by 88% compared with 129 and 129-*Stra8*^{KO/+} siblings (Fig. 7C), with one teratoma observed in 90 males examined (Fig. 7B). Therefore, *Stra8* expression and possibly the initiation of meiosis influence teratoma susceptibility.

Teratoma-susceptible male germ cells initiate meiotic prophase

Next, we asked whether the aberrant expression of *Stra8* is sufficient to induce male germ entry into meiosis. If so, then the chromosomes of teratoma-susceptible male germ cells should be decorated with the synaptonemal complexes associated with meiotic prophase. At E14.5, synaptonemal complex protein 3

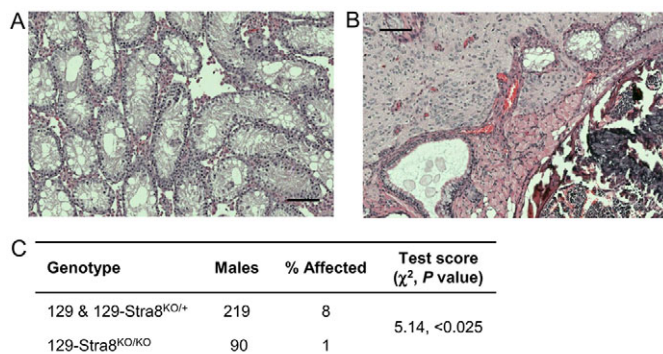


Fig. 7. *Stra8* is a modifier of teratoma susceptibility.

(A,B) Photomicrographs of a Hematoxylin and Eosin stained 6-week-old 129-*Stra8*^{KO/KO} testis lacking meiotic germ cells (A), and 6-week-old 129-*Stra8*^{KO/KO} testis with a teratoma consisting of neuronal, muscle, secretory epithelial, bone marrow, bone, and cartilage (B). (C) χ^2 analysis of the teratoma survey of 129 wild-type and 129-*Stra8*^{KO/+} (control) and 129-*Stra8*^{KO/KO} (experimental) siblings reveals that *Stra8* deficiency reduces teratoma incidence by 88%. Scale bars: 100 μ m.

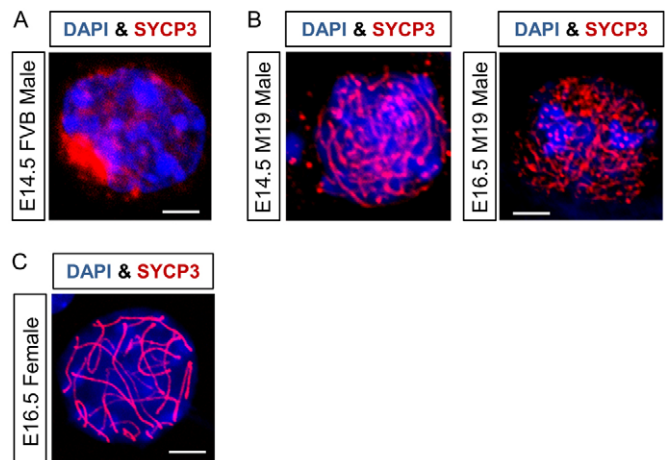


Fig. 8. Teratoma-susceptible germ cells initiate but do not complete prophase I of meiosis. Chromosome spreads from E14.5 and E16.5 gonads were immunostained for DAPI and SYCP3 and imaged by confocal microscopy. (A) An E14.5 male, FVB germ cell nucleus with pre-leptotene, nucleolar localization of Sycp3. (B) At E14.5 and E16.5, some male M19 germ cell nuclei have a leptotene to early zygotene SYCP3 distribution with no evidence of chromosome pairing or synapsis. (C) An SYCP3-immunostained, E16.5 M19 female germ cell in the pachytene stage of meiotic prophase. Scale bars: 5 μ m.

(SYCP3) localized to the nucleoli of teratoma-resistant, FVB and most teratoma-susceptible M19 germ cells (Fig. 8A; supplementary material Fig. S4A). However, in <1% of E14.5 and E16.5 teratoma-susceptible M19 germ cells, SYCP3 associated with chromosomes in a pattern consistent with leptotene to early zygotene stages of meiotic prophase (Fig. 8A,B).

Interestingly, unlike female germ cells, a SYCP3 distribution consistent with chromosome pairing and synapsis during later stages of meiotic prophase (late zygotene and pachytene) was not observed in teratoma-susceptible male germ cells (Fig. 8C). Thus, E16.5 chromosomes were immunostained for synaptonemal complex protein 1 (SYCP1) to test whether teratoma-susceptible male germ cells progress through the late stages of meiotic prophase. Importantly, SYCP1 associated with chromosomes of female germ cells but not teratoma-susceptible M19 male germ cells (supplementary material Fig. S4B). Therefore, teratoma-susceptible male germ cells do not complete meiotic prophase. Whether meiotic male germ cells become apoptotic or revert to mitotic cells and contribute to germ cell tumor initiation remains to be determined.

DISCUSSION

Male germ cell proliferation and retention of pluripotency after the mitotic-meiotic switch at E13.5 has been shown to be associated with increased teratoma susceptibility (Matin et al., 1998; Noguchi and Stevens, 1982; Stevens, 1966; Stevens, 1967b). Our analyses of germ cell development in low and high teratoma risk strains of mice not only agree with these observations, but also identify E15.5 as the specific time point at which germ cell proliferation and pluripotency increase with teratoma risk. As with ES cells, the teratoma forming-capacity of EC cells is dependent upon their initial pluripotency (Gidekel et al., 2003). Because EC cells first appear at E15.5 (Stevens, 1962; Stevens, 1967b), retention of proliferation and pluripotency through this time point is probably necessary to establish a tumor stem cell population.

Our results also demonstrate that teratoma-susceptible germ cells are induced to express genes associated with pre-meiotic embryonic female and adult male germ cells (e.g. *Ccnd1*, *Stra8*, *Ngn3* and *Ret*) following the failure to enter mitotic arrest. Importantly, despite the expression of some markers of adult male germ cells, it is unlikely that teratoma-susceptible embryonic male germ cells differentiate into bona fide spermatogonia. *Ccnd1* expression initiated at the same developmental time point in pre-meiotic female and teratoma-susceptible germ cells, implying that a signal shared by the female and teratoma-susceptible male gonad induces differentiation. Thus, teratoma-susceptible germ cells appear to adopt some characteristics of pre-meiotic female germ cells. In fact, the rapid commitment of some teratoma-susceptible male germ cells to a meiotic fate is more reminiscent of female germ cell differentiation than the prolonged proliferation/differentiation steps of spermatogenesis. Furthermore, we demonstrated that NANOG and CCND1 are co-expressed in teratoma-susceptible germ cells. Mouse spermatogonia and meiotically differentiating female germ cells do not express detectable levels of NANOG (Yamaguchi et al., 2005). Thus, teratoma-susceptible male germ cells acquire some but not all characteristics of germ cells differentiating towards a meiotic fate.

As observed with proliferation and pluripotency, the expression of germ cell differentiation markers decreases substantially in 129 male germ cells at E15.5 but remains elevated in M19 male germ cells. Given the tenfold increased risk of teratoma formation in M19 mice, expression of germ cell differentiation markers beyond E15.5 might be required for the transformation of germ cells into EC cells. Whether all or a subset of these genes influence teratoma risk remains to be determined. However, our expression analyses and teratoma surveys suggest that at least two differentiation-associated factors, *Ccnd1* and *Stra8*, contribute to tumor initiation.

Ectopic expression of *Ccnd1* in teratoma-susceptible male germ cells and EC cells might play a central role in inducing tumor initiation and maintaining pluripotency. D-type cyclins are normally expressed in response to mitogenic signals and activate cyclin-dependent kinases, which phosphorylate and inactivate retinoblastoma (pRB) to induce expression of genes required for G1 to S phase cell cycle transition (Deshpande et al., 2005). As with inactivating mutations of pRB, ectopic expression of cyclin D1 is sufficient to induce tumorigenesis by promoting growth factor independence, quiescent cells to re-enter the cell cycle, and rapid progression through the G1-S cell cycle transition (Deshpande et al., 2005; Musgrove et al., 1994). Importantly, rapid transition from the G1 to S phase might also facilitate the maintenance of pluripotency (Filipeczyk et al., 2007; Singh and Dalton, 2009). A short G1 and long S phase promotes the euchromatic state of chromatin in pluripotent cells and inhibits differentiation, which preferentially occurs during G1 in EC cells (Herrera et al., 1996; Jonk et al., 1992; Mummery et al., 1987). Therefore, ectopic *Ccnd1* expression might be required for, not only the neoplastic transformation of germ cells, but also the retention of pluripotency by germ cells and EC cells. Previous studies of human EC cells and TGCTs support a role for *Ccnd1* in stem cell maintenance and teratoma development. Inhibition of cyclin D1 reduces the proliferative capacity of EC cells, *CCND1* expression decreases with germ cell tumor differentiation, and *CCND1* expression increases with chemoresistance to cisplatin (Freemantle et al., 2007; Noel et al., 2010). Thus, understanding the contributions of CCND1 to testicular teratoma pathogenesis has both developmental and clinical relevance.

Stra8 is required for the initiation of the meiotic program and its expression is tightly regulated to ensure that entry into meiosis is induced at the appropriate developmental time points (Bowles et al., 2006; Koubova et al., 2006). Thus, the premature induction of *Stra8* expression in teratoma-susceptible embryonic male germ cells might be indicative of a breakdown in the regulation of germ cell fate. Furthermore, the reduced teratoma incidence of 129-*Stra8*^{KO/KO} mice implies that premature initiation of the meiotic program is directly involved in tumor susceptibility. Whether entry into meiosis and reversion to mitosis contributes to teratoma susceptibility, as observed in *Drosophila* and *Caenorhabditis elegans* (Biedermann et al., 2009; Parisi et al., 2001; Sugimura and Lilly, 2006), remains to be determined in the mouse model. The molecular function of *Stra8* also remains to be resolved, although it appears that it functions as a transcription factor (Tedesco et al., 2009). Thus, it is possible that if *Stra8* is expressed at an inappropriate developmental time point and in a cell type that has retained pluripotency, gene expression changes might be induced that do not result in meiotic initiation but instead lead to tumorigenesis.

Our results demonstrate that when the decision to enter mitotic arrest is not executed properly, pluripotency is retained and differentiation-associated genes are induced. These observations suggest that male germ cell commitment to mitotic arrest has two important developmental roles, namely to facilitate the transition of pluripotent PGCs into unipotent germ-line stem cells, and to prevent lineage-restricted germ-line stem cells from receiving signals that induce premature differentiation. Previous studies in ES and germ cells support our conclusion that mitotic arrest has a dual role in regulating male germ cell development. In both germ and ES cells, cell cycle arrest is accompanied by the reprogramming of pluripotency genes (Avilion et al., 2003; Pesce et al., 1998; Singh and Dalton, 2009; Wang and Bllulloch, 2009; Yamaguchi et al., 2005). In addition, G₀ arrest at E13.5 prevents the induction of the meiotic differentiation program in male germ cells (McLaren and Southee, 1997; Trautmann et al., 2008).

Importantly, our results also suggest that co-expression of pluripotency and differentiation-associated factors through E15.5 can alter germ cell fate from spermatogenic to tumorigenic. However, not all germ cells that express pluripotency and differentiation-associated factors lose germ cell identity and transform into EC cells. Germ cells that have not transformed by E16.5 become quiescent and downregulate pluripotency- and differentiation-associated genes. We propose that these germ cells retain or regain germ-line stem cell identity and develop normally to establish spermatogenesis within teratoma-susceptible testes. Understanding the developmental processes that control these germ cell fate decisions in teratoma-susceptible mice will help guide future studies in humans, and may provide new targets for the diagnosis and treatment of human TGCTs.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at

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