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Testicular teratomas: an intersection of pluripotency, differentiation and cancer biology

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ABSTRACT Teratomas represent a critical interface between stem cells, differentiation and tumorigenesis. These tumors are composed of cell types representing all three germ layers reflecting the pluripotent nature of their cell of origin. The study of these curious tumors became possible when Leroy Stevens identified the 129 mouse strain as a model of spontaneous testicular teratoma and later isolated a substrain carrying the *Ter* mutation, a potent modifier of tumor incidence. Early studies with 129 mice lead to the discovery of embryonal carcinoma (EC) cells which played a foundational role in the embryonic stem (ES) cell field and the study of pluripotency. The cells of origin of testicular teratomas are germ cells. During early development, primordial germ cells diverge from somatic differentiation and establish their pluripotent nature, maintaining or re-expressing core pluripotency genes; *Oct4, Sox2* and *Nanog*. It is believed that a misregulation of male germ cell pluripotency plays a critical role in teratoma development. Several mouse models of teratoma development have now been identified, including a chromosome substitution strain, 129-Chr19^{MOLF}, conditional *Dmrt1* and *Pten* alleles and the *Ter* mutation in the *Dnd1* gene. However, it is still unknown what role somatic cells and/or physiology play in the sensitivity to teratoma development. These unusual tumors may hold the key to understanding how pluripotency is regulated *in vivo*.

KEY WORDS: *testicular teratoma, germ cell, Dnd1, stem cell*

Introduction

Testicular teratomas, which originate from germ cells, are tumors in which cell types representing all three germ layers are found. From their earliest discovery, teratomas fascinated scientists because they implied the presence of pluripotent cells within a differentiated organism that could escape along a disordered differentiation pathway and undergo tumorigenesis. Teratomas represent a critical interface between fields devoted to the study of stem cells, differentiation, and tumorigenesis. Investigators hope that these unusual tumors hold the key to understanding how pluripotency is secured versus how unrestrained growth and/or differentiation are activated in stem cells. These issues are central to cancer, stem cell biology, and regenerative medicine. In this review, we will briefly place teratomas (benign) and teratocarcinomas (malignant) within the classification of testicular germ cell tumors (TGCTs). We will discuss the history of the teratoma/teratocarcinoma field (hereafter referred as teratoma) and review how the early work on teratomas in mice led to embryonic stem cell research. We will then recap recent progress toward a molecular understanding of teratoma susceptibility and origins in the context of our current understanding of germ cell development.

Teratoma is the most common TGCT in young men

TGCTs account for approximately 96% of all testicular tumors, and are the most common cancer in men between the ages of 20 and 39 (Richardson *et al.*, 2008). The worldwide incidence of TGCT has doubled in the past 30 years. In the United States approximately 8,590 new TGCT cases and 360 TGCT-related deaths are estimated for 2012 (NCI, 2012). All TGCTs are believed to arise from a common precursor called carcinoma *in situ* (CIS), which has variable differentiation potential (Looijenga *et al.*, 2011). The *International Classification of Diseases for Oncology* grouped TGCT

Abbreviations used in this paper: DND1, dead-end homolog 1; DMRT1, Doublesex and mab-3 related transcription factor 1; EC, embryonal carcinoma cell; EG, embryonic germ cell; ES, embryonic stem cell; iPS, Induced pluripotent stem cells; PGC, pri¬mordial germ cell; PTEN, phosphatase and tensin homolog; Sl or Sl-J, alleles of Steel, aka Kitl; TGCT, testicular germ cell tumor.

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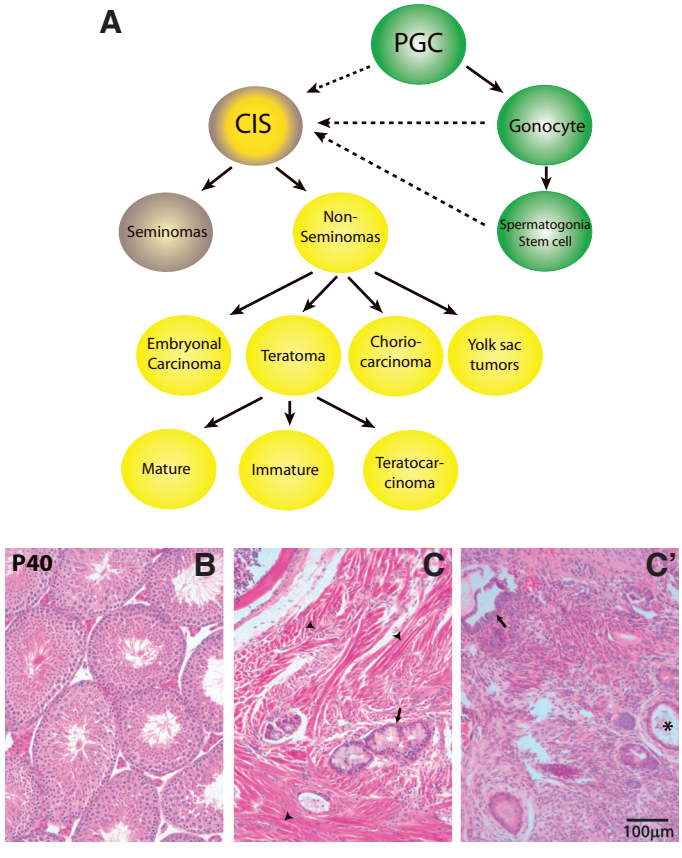
into two broad categories of tumors: seminoma and non-seminoma. Seminomas, which are relatively low in malignancy, arise from differentiated germ cells whereas non-seminomas arise from pluripotent germ cells and have a more clinically aggressive phenotype. The International Agency for Research on Cancer recognizes specific subtypes of non-seminomas initially proposed by Dixon and Moore in 1953 (Dixon and Moore, 1953; Egevad *et al.*, 2007): embryonal carcinomas, teratomas, yolk sac tumors, and choriocarcinomas. The histology of these tumors frequently shows a mixture of tumor subtypes (Fig. 1A).

The most common type of TGCT in young men and during early childhood is testicular teratoma (TCS, 2012). World Health Organization (WHO) classification recognizes three histological subtypes of teratoma: mature, immature, and teratocarcinomas. These tumors show high histological diversity and contain a variety of tissue elements derived from all three germ layers (Fig. 1C,C') (Stevens and Little, 1954), consistent with the pluripotent state of the cells that give rise to them. Teratomas are among the oldest known tumors, referenced first in a Babylonian document four thousand years ago (Pantoja and Rodriguez-Ibañez, 1976). These tumors were recognized as a curiosity from the earliest days of their discovery in humans. Perhaps the disorganized embryonic features of these tumors explains why in 1863 Rudolf Virchow, a German Pathologist, introduced the term "teratoma" from the Greek root *teras* meaning marvel or monster (Pantoja and Rodriguez-Ibañez, 1976).

Establishing an experimental model for teratoma

Apart from their clinical relevance, teratomas occupy a critical position along the timeline of stem cell research (Solter, 2006).



Teratomas found in humans and horses attracted the interest and curiosity of investigators, who recognized that the histological diversity of teratomas implied the presence of a cell or cells with pluripotent capability within a differentiated organism. However, it was not until the 1950's, when Leroy Stevens identified an inbred mouse strain, 129/Sv, with a 1% incidence of spontaneous testicular teratoma (Stevens and Little, 1954), that experimental work began and played a foundational role in the development of the stem cell field.

Stevens recognized that mice in which a high percentage of males developed testicular teratomas would be a valuable tool to study the origin and biology of these tumors. In subsequent years, Stevens isolated a few substrains with slightly higher incidence of spontaneous teratomas, one by introducing a Steel mutation onto the 129/Sv genetic background (129/Sv^{Sl}) (Stevens and Mackensen, 1961). In the course of other backcrosses, Stevens isolated a single female with a spontaneous mutation, whose offspring showed a high teratoma incidence. From this female, Stevens established the strain, 129/Sv^{Ter}. Intercrosses between individuals in this strain yielded a 30% teratoma incidence (Stevens, 1973), rendering the study of the etiology and biology of these rare tumors experimentally tractable for the first time.

Through an exhaustive histological analysis of testicular teratomas, Stevens observed continuity between the germinal epithelium of testicular tubules and the neoplastic epithelium that formed teratomas. The testicular tubules were enlarged by the presence of neoplastic cells and the basement membrane was disrupted, giving the appearance that pressure due to growth had ruptured the seminiferous epithelium, spilling out tumor cells (Stevens, 1959). These observations and other reports (Falin, 1940, Melicow, 1955), supported the hypothesis that testicular teratomas originate within the tubules, most likely from germ cells.

Stevens found that he could induce teratomas by grafting the genital ridges from developing 129/Sv embryos onto the testes of adult mice; however, the incidence of teratomas generated in this manner sharply declined when the graft came from an embryo older than E12.5 (Stevens, 1964), suggesting that the pluripotency of the testicular cell of origin is stage specific (Fig. 2A Table 1). These observations suggested to Stevens that teratomas originate from germ cells prior to their stable differentiation to spermatogonia. To address the question of the origin of testicular teratomas experimentally, Stevens grafted genital ridges of embryos from a 129/

Fig. 1. Germ cell origins of testicular tumors. (A) *In normal development PGCs differentiate into gonocytes, which become spermatogonial stem cells. Neoplastic development of PGCs, gonocytes, or spermatogonial stem cells is believed to give rise to carcinoma* in situ *(CIS) cells, the precursors of all types of TGCT. The* International Classification of Diseases for Oncology *divided TGCT in two broad categories: seminoma and non-seminoma. In humans approximately 61% of all germ cell tumors are pure seminomas. The remaining are non-seminomas which originate from pluripotent germ cells and include five histological groups: embryonal carcinomas, teratomas, choriocarcinomas, yolk sac tumors, and mixtures of two or more of these tumor types. Teratomas can be composed of differentiated cell types (mature), embryonic cell types (immature), or transformed undifferentiated EC cells (teratocarinoma).* **(B)** *Histological section of control 40 dpn 129/Sv testis stained with H&E.* **(C,C')** *Examples of testicular teratomas in a 40 dpn 129/Sv mouse testis. The normal morphology of the seminiferous epithelium has been replaced by the tumor with a diverse array of tissue types including muscle (arrow head) and glandular (arrow) tissue. A remnant of a degenerating tubule (*) can be observed.*

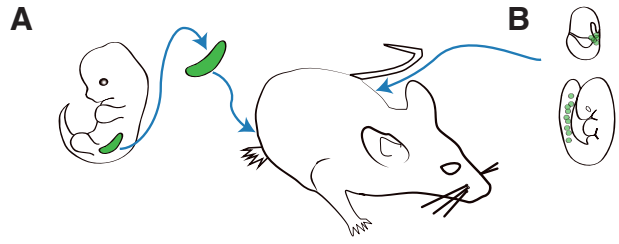


Table 1: Stevens's Experiments

Genital ridge age	E12.5	E13.5
Graft site	Teratoma (%)	Teratoma (%)
Testis	82	8
Spleen	9	—

Table 2: Solter's Experiments

Embryo stage	E6.5	E7.5	E8.5
Graft site	Teratoma (%)	Teratoma (%)	Teratoma (%)
Kidney capsule	100	100	100

Fig. 2. Experimental induction of teratoma development. *Two lines of experiments were conducted to investigate the origin of teratomas.* **(A)** *In Stevens's experiments, teratocarcinogenesis was induced by grafting the genital ridges of developing 129/Sv$^{Sl\text{-}J}$ heterozygous mice into the testes of adult mice. He observed that the incidence of teratomas was strongly influenced by the age and site of the graft.* **(B)** *In Solter's experiments, when post-implantation embryos at stage: E6.5, E7.5, and E8.5 were transplanted under the kidney capsule, 100% developed into teratomas. Solter deduced that germ cells and embryonal carcinomal cells have similar characteristics.*

Sv$^{Sl/+}$ x 129/Sv$^{Sl/+}$ cross to the testes of adults. Seventy-five percent of the heterozygous and wild type genital ridges developed into testes with teratomas as expected. However, few teratomas were found in testes that developed from homozygous 129/Sv$^{Sl/Sl}$ gonads, which were depleted of germ cells (Stevens, 1967). This experiment bolstered the evidence that teratomas arise from germ cells.

Solter also generated teratomas by grafting post-implantation rat and mouse embryos to the subcapsular space of the kidney (Damjanov *et al.*, 1971, Solter *et al.*, 1980) (Fig. 2B Table 2). The teratomas generated by this method were histologically identical to those that arose spontaneously in 129/Sv mice. From these results Solter deduced that the cell(s) of origin of teratomas, both those derived from the embryo and those derived from the genital ridge, had similar characteristics. These results implied that germ cells and embryonal cells from the egg cylinder had a similar susceptibility to develop teratomas, but the nature of that susceptibility was unknown.

From teratomas to stem cell biology

It was conceivable that the complex array of tissues detected in a teratoma arose from a mixture of originating cells. Previous experiments showed that teratomas could be serially propagated by transplantation of a tumor fragment (Stevens and Little, 1954), suggesting that a tumor stem cell might exist within the tumor. Histological analysis of teratomas revealed clusters of undifferentiated cells within the tumors (Stevens, 1959). Direct evidence for the presence of tumor stem cells was provided by Kleinsmith and Pierce in 1964 through an *in vivo* cloning technique. Embryonal carcinoma (EC) cells were purified from a subline of a transplanted teratoma isolated by Stevens. A single EC cell was injected intraperitoneally into 129/Sv mice and produced teratomas composed of different cell types (Kleinsmith and Pierce, 1964). The demonstration that a single cell derived from a teratoma could produce all cell types encountered in a teratoma was a crucial experiment. With this finding began the derivation and characterization of mouse and human cell lines that could retain their pluripotency (Holden *et al.*, 1977). The microinjection of EC cells into the blastocoel of developing embryos, showed that EC cells could contribute to all tissues of the host embryo (Brinster, 1974, Mintz and Illmensee, 1975). However, their contributions were often limited, and tumors frequently arose in the chimeric animals (Papaioannou *et al.*, 1975). Despite this, investigators began to realize that the availability of a population of stem cells that could be cultured indefinitely presented an opportunity to modify the genome (Mintz and Fleischman, 1981). It was shown that when EC cells were mutagenized, selected, and introduced into a blastocyst, they could contribute to germ line progeny that perpetuated the induced/introduced-genetic change in a new mouse strain. In the first experiment, EC cells deficient for hypoxantine phosphoribosyl transferase (*Hprt*-) were selected and injected into a blastocyst. In the chimeric mice that resulted, mature functional cells retained the HPRT deficiency (Mintz and Fleischman, 1981). The idea that this experimental system could be used to directly generate animal models of human genetic disease was emerging.

The derivation of teratomas from pre- and post-implantation embryo transplants suggested that pluripotent stem cells could be isolated directly from embryos. Evans and Kaufman developed a technique for deriving cell lines through outgrowth from 129/SvE mouse blastocysts. In 1981, these experiments successfully gave rise to pluripotent embryonic stem (ES) cells that could contribute extensively to the host embryo and did not lead to tumors in adult life (Evans and Kaufman, 1981). At the same time, Gail Martin independently optimized a method to isolate and culture ES cells from mouse early blastocytes (Martin, 1981). These methods were widely adopted, and ES cells rapidly became the most popular model used in stem cell research. In subsequent years, a third type of pluripotent stem cell was obtained by outgrowth from post-implantation embryos and gonads cultured on a supporting cell layer, similar to ES cell methods. These pluripotent cells were called embryonic germ (EG) cells (Matsui *et al.*, 1991, Resnick *et al.*, 1992), and showed similar properties to ES cells (e.g. they could give rise to teratomas when injected into an adult host, and they could contribute to the embryo when injected into a blastocyst). ES-like cells were later derived from rhesus monkeys and marmosets, and in 1998, from human blastocysts (Thomson *et al.*, 1998). Decades of work aimed at the identification of the transcriptional regulators that determine and maintain pluripotency in the embryo and in ES cells enabled the induction of pluripotent stem (iPS) cells from somatic cells by molecular reprogramming. iPS cells share the defining properties of ES cells, including contribution to the germline of chimeras following blastocyst injection (Takahashi and Yamanaka, 2006).

Germ cell teratomas provide an opportunity to investigate the intersection of pluripotency, differentiation and cancer biology

While the short-lived popularity of EC cells as the key model of pluripotency ended with the establishment of mouse ES cell and, more recently iPS cell technologies, teratomas are of continued

interest to both germ cell and cancer biologists because they provide a window onto the critical interface between stem cells, differentiation, and tumorigenesis. Stem cells afford enormous potential for regeneration and repair. However the risk that they will escape from growth or differentiation control (as occurs in teratomas) is a significant danger for the organism. Teratomas may hold the key to understanding how pluripotency is secured versus how unrestrained growth and/or differentiation are activated in stem cells—issues that lie at the heart of cancer, stem cell biology, and regenerative medicine, which seeks to direct the differentiation of pluripotent cells.

Although many types of stem cells are now known, germ cells are in a class of their own. They are the only known population of stem cells that retain the potential to develop into a complete organism while undergoing an elaborate differentiation process. Germ cells represent a significant regulatory challenge for the organism. They are set aside early in development and anointed as the cells that retain the blueprint for the next generation. They are responsible for carrying both the genome itself and the information about how to re-program it to the next generation. However, throughout most of an animal's lifetime, the underlying potential of germ cells is repressed, while they undergo sex-specific differentiation to form male and female gametes with highly specialized characteristics of sperm and oocytes. Despite their specialized differentiation, the underlying totipotency of germ cells is somehow preserved, and reactivated only when gametes unite to form a zygote. Testicular teratomas arise from germ cells as the result of escape from the mechanisms that repress their underlying pluripotency at a critical point during their differentiation. These tumors may result from a cell autonomous programming error in a pluripotent cell and/or an extracellular signaling interaction.

In mammals, germ cells are specified as a pluripotent cell population during early implantation stages. After specification, they migrate through the gut to the site of the developing gonads where they initiate sex-specific differentiation at E12.5 in mouse. Germ cells maintain a pluripotent state (similar to ES cells) throughout their early development, as evidenced by their ability to give rise to EG cells (Matsui *et al.*, 1991, Resnick *et al.*, 1992). However, once their fate is established in the testis or ovary, germ cell differentiation is unipotent, leading to sperm or oocyte development. These terminally differentiated cells do not retain pluripotent capabilities, but their fusion reinitiates a program of totipotency in the zygote. In the following section, we will review what is known about the specification of germ cells and both the establishment and repression of their underlying pluripotency.

Germ cell specification and migration to the gonads

In the mouse, germ cell precursors are specified around E6.25 from those cells of the pluripotent epiblast proximal to the extraembryonic ectoderm (Fig. 3A). BMP signals originating in the extraembryonic ectoderm and embryonic endoderm result in the expression of *Blimp1/Prdm1* and *Prdm14* in a handful of cells (Lawson *et al.*, 1999, Ohinata *et al.*, 2005, Yamaji *et al.*, 2008). This process gives rise to the initial PGC population of about 40 cells near the base of the allantois by E7.5 (Ginsburg *et al.*, 1990). BLIMP1 and PRDM14 activities lead to repression of somatic differentiation genes, expression of PGC-specific genes including a group of RNA-binding proteins, initiation of epigenetic changes, and re-expression or maintenance of core pluripotency genes (Magnusdottir *et al.*, 2012, Ohinata *et al.*, 2009, Yamaji *et al.*, 2008).

In the early embryo, the core transcription factors of pluripotency, *Nanog*, *Sox2* and *Oct4* are expressed in pluripotent cells of the inner cell mass and epiblast. These core transcription factors are necessary for the maintenance of pluripotency in the early embryo and in ES cells (Lawson *et al.*, 1999), and ectopic *Sox2* and *Oct4* expression are critical to iPS cell technology (Takahashi and Yamanaka, 2006). As gastrulation begins and the germ layers are formed, these pluripotency factors are downregulated in somatic cells (Chambers *et al.*, 2007, Yabuta *et al.*, 2006, Yeom *et al.*, 1996). However, *Oct4* is maintained throughout germ cell specification, while *Sox2* is transiently repressed then reactivated downstream of Prdm14 expression during germ cell specification (Fig. 3). It is unclear whether *Nanog* expression is simply maintained or reactivated upon germ cell specification (Yabuta *et al.*, 2006, Yamaji *et al.*, 2008). The role of these transcription factors in PGCs is incompletely understood, but they are likely to be involved in the suppression of somatic differentiation as the germ cells undergo epigenetic reprogramming. Expression of all three genes persists until after PGCs initiate sex-specific differentiation in the gonads (Western *et al.*, 2011, Yamaguchi *et al.*, 2005).

After specification, PGCs use both active and passive mechanisms to travel through the developing embryo and arrive at the genital ridge between E10.5-11.5 (Molyneaux and Wylie, 2004, Saitou *et al.*, 2012). PGCs proliferate during the early and late stages of migration surrounding a transient period of mitotic arrest in G2 at E8.5. A series of epigenetic changes to the germ cell genome begins upon specification and continues into sex-specific differentiation and after mitotic arrest in male germ cells (Fig. 3B).

Two primary forms of epigenetic reprogramming that occur in germ cells are changes in DNA methylation patterns and histone modifications (Fig. 3B). The earliest DNA demethylation begins around E8.0 when the maintenance methyltransferase, *Dnmt1*, and the *de novo* methyltransferases, *Dnmt3a* and *Dnmt3b*, are suppressed, suggesting a passive loss of methylation. During G2 mitotic arrest, demethylation continues despite the re-expression of *Dnmt1*, likely by an active mechanism (De Felici, 2011). Histone marks, are also modified during G2 arrest. Germ cells progressively lose H3K9me2 and increase H3K27me3, both generally repressive histone modifications that also occur in ES cells (Seki *et al.*, 2005). Coincident with an intermediate phase when many genes have neither of these repressive marks, PGCs are transcriptionally silent due to repression of RNA polymerase II (Fig. 3B) (Seki *et al.*, 2007).

Germ cells continue to proliferate and undergo epigenetic changes as they colonize the developing gonad. As germ cells enter the genital ridge H3K4me2, H3K4me3 and H3K9ac sharply increase (Hajkova *et al.*, 2008, Seki *et al.*, 2005). Further active DNA demethylation occurs at imprinted genes and at other loci associated with germ cell-specific expression at this stage (De Felici, 2011, Maatouk *et al.*, 2006, Seki *et al.*, 2005). While specific interdependencies of the changes that occur in germ cells are not completely understood, it is apparent that they are highly coordinated. Collectively, these changes lead to reprogrammed gametes, prepared for their differentiated fate, but retaining their underlying pluripotency.

Male-specific differentiation of germ cells

Germ cells maintain a pluripotent state (similar to ES cells)

throughout their early development, as evidenced by their ability to form EG cells. The underlying pluripotency of germ cells during their migration and initial period of residence in the gonad (eg. prior to E12.5 in mouse) represents a risk to the animal and is under tight regulation. Once their fate is established in the testis or ovary, germ cells are reprogramed to follow a unipotent differentiation pathway, giving rise to sperm or oocytes. These differentiated gametes do not retain the ability to give rise to EG cells, but their fusion reinitiates a program of totipotency in the zygote.

Soon after colonizing the genital ridge, sex-specific differentiation of germ cells begins according to the sex of the gonad (Adams and McLaren, 2002). Here we will focus on the differentiation of male gonadal germ cells, which are incorporated into the testis cords, the nascent seminiferous tubules. Shortly thereafter, male germ cells enter mitotic arrest in G0, which persists until after birth (Western *et al.*, 2011). The window of time in which Stevens found transplanted 129/Sv PGCs to be prone to transformation to teratoma is bracketed by PGC arrival at the gonad and their arrest in G0 of the cell cycle (Fig. 2A Table 1, Fig. 3 C,D).

Most germ cells in male gonads enter mitotic arrest between E12.5 and E14.5. At E12.5 greater than 60% of germ cells can be found in S or G2/M phases of the cell cycle. By E14.5 80% or more of germ cells are in G1/G0 in 129T2, CD-1, and C57BL/6 mice (Western *et al.*, 2011). Mitotic arrest in G0 likely involves regulation of the G1/S checkpoint (Fig. 3D). Proteins involved in progression of mammalian cells through the G1/S checkpoint have been investigated in male germ cells. A close look at the expression of these factors, both at the transcript and protein level, showed an initial increase in negative cell cycle regulators *p27* and *p15* along with a decrease in *Cyclin E* expression at E13.5. Activation of retinoblastoma protein (pRB), which also occurs at this stage, was proposed to be critical to arrest at the G1/S checkpoint (Sorrentino *et al.*, 2007, Western *et al.*, 2008, Western *et al.*, 2011). However, a null mutation in pRB had a modest effect, where most germ cells still entered mitotic arrest even in the absence of pRB (Spiller *et al.*, 2010). While many proteins involved with G1/S regulation are present in germ cells at this stage, the key pathway to mitotic arrest has still not been genetically determined. It is likely that there is significant redundancy in the regulation of this critical event.

The process of reprogramming in male germ cells spans the period of mitotic arrest and likely involves genome-wide changes. Active repression of the genes associated with pluripotency is required at the stage when fetal male germ cells undergo mitotic arrest and embark on a program of differentiation as spermatogonia (Western, 2009). Failure to repress pluipotency (specifically, expression of *Sox2*, *Nanog* and *Oct4*), and/or failure to maintain cell cycle arrest may render germ cells susceptible to tumor formation during this critical window of development and perhaps at later stages (Cook *et al.*, 2011, Heaney *et al.*, 2012). However, it is not clear whether either of these events is causative. Stevens's transplantation experiments suggested that germ cells are most susceptible to teratoma transformation prior to E12.5 (Stevens, 1964). However, the earliest stage when clusters of precursor cells have been identified by elevated levels of E-cadherin and up-regulation of pluripotent markers *in vivo* is E15.5 (Cook *et al.*, 2011, Heaney *et al.*, 2012). Because likely differences in growth rates make it difficult to determine when a transformation event initiated, the period of susceptibility has not been firmly established.

At birth the mitotically quiescent germ cells, called gonocytes, are localized at the center of the seminiferous tubules. Soon after

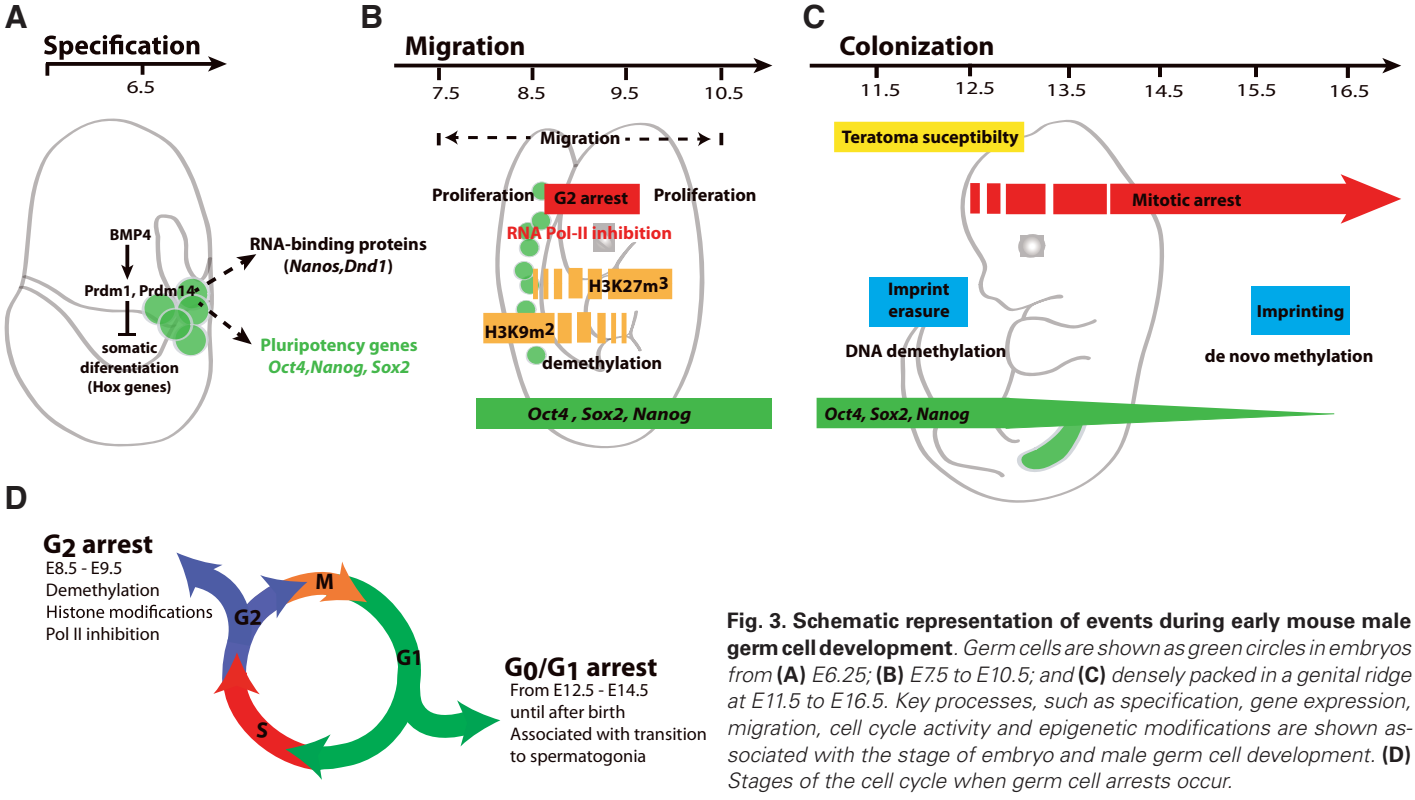


Fig. 3. Schematic representation of events during early mouse male germ cell development. *Germ cells are shown as green circles in embryos from* **(A)** *E6.25;* **(B)** *E7.5 to E10.5; and* **(C)** *densely packed in a genital ridge at E11.5 to E16.5. Key processes, such as specification, gene expression, migration, cell cycle activity and epigenetic modifications are shown associated with the stage of embryo and male germ cell development.* **(D)** *Stages of the cell cycle when germ cell arrests occur.*

birth, some germ cells are lost through apoptosis, while others relocalize to the basement membrane of the seminiferous tubules, resume mitosis and differentiate into spermatogonial stem cells (SSCs), the germ line stem cells that produce sperm throughout male reproductive life (Yoshida *et al.*, 2006) (Fig. 4). Although their differentiation trajectory is established at this stage, SSCs harbor an underlying totipotency and can still give rise to EG-like cells under appropriate explant conditions (Mochizuki and Matsui, 2010). Whether SSCs or some less differentiated progenitor is the origin of CIS leading to TGCTs in adult life is a subject of debate (Fig. 1) (Looijenga *et al.*, 2011).

Genetic influences on teratoma incidence

The goal of much of the work on teratomas is to achieve an understanding of the molecular pathways involved in the initiation of these tumors in the hope that this will reveal something fundamental about how the pluripotent genome is regulated in germ cells, and by analogy, in other natural stem cell populations. Stevens recognized that one way of identifying the pathways involved is to investigate the factors that influence teratoma incidence (Stevens and Mackensen, 1961).

In humans, Caucasian decent and familial occurrences are both risk factors for TGCTs. While recent genome wide association studies have identified loci associated with TGCT occurrence, these loci have modest effects, suggesting that TGCT occurrence is a complex multigenic phenotype. Variants of *KITLG, SPRY4, BAK1, TERT,* and *DMRT1* have been associated with TGCTs in genome-wide association studies. Of these associations, *KITLG* and *DMRT1* have been repeated (Kanetsky *et al.*, 2011). Importantly, these two genes have also been shown to influence teratoma incidence in mouse models, which are discussed below.

Consistent with evidence that the incidence of human teratomas varies with ethnic background (Garner et al., 2005), experiments in mice clearly indicate that genetic background influences teratocarcinogenesis. The highest incidence of spontaneous teratomas in an inbred strain occurs in the 129/Sv testis. In addition, Stevens observed that the susceptibility to develop teratomas in the graft induction experiments was also strain dependent, with grafts from C57BL/6 mice showing no incidence of tumors (Regenass *et al.*, 1982). Solter also classified the C57BL/6 strain as a nonpermisive strain for teratocarcinoma based on his embryo transplant experiments (Solter *et al.*, 1979). The basis for the high susceptibility of the 129/Sv strain is still unclear.

Stevens showed that by crossing on mutations known to show germ cell phenotypes, he could alter the spontaneous frequency of teratomas in the 129/Sv strain. For example, as mentioned earlier, 129/Sv mice heterozygous at the steel locus showed an increase in tumor incidence from 1% to ~10% (Stevens and Mackensen, 1961). More recently, Nadeau and colleagues derived a chromosome substitution strain, 129-Chr19^{MOLF}, with chromosome 19 from the MOLF/Ei strain introduced into the 129 background. This strain showed an increased tumor incidence from 5.6% in mice from his starting 129 substrain to 23.7% and 82% in mice heterozygous or homozygous for MOLF chromosome 19, respectively (Matin *et al.*, 1999). Strains that had only segments of MOLF chromosome 19 were generated from 129-Chr19^{MOLF}. Analysis of teratoma incidence in these strains revealed that several regions of chromosome 19 confer teratoma susceptibility, and that interactions between these segments have both additive and epistatic relationships (Youngren *et al.*, 2003). Heterozygous deletion of the eukaryotic initiation factor, Eif2s2, suppresses tumor incidence in the 129-Chr19^{MOLF} strain (Heaney *et al.*, 2009). In a related experiment, MOLF chromosome 18 was found to be protective, decreasing the incidence of teratomas in an otherwise 129 background. Interestingly, derivation of ES cells from the blastocysts of 129-Chr18^{MOLF} mice is significantly less efficient than derivation from 129-Chr19^{MOLF} mice (Anderson *et al.*, 2009).

The transcriptional regulator, DMRT1, is another 129 specific, dose sensitive modifier of teratoma incidence. 129 *DMRT1*$^{+/-}$ and DMRT1$^{-/-}$ mice have a 4% and 90% teratoma incidence, respectively, while homozygosity for the hypomorhic *DMRT1*^{fl} allele shows an intermediate incidence of 42%. DMRT1 binds the *Sox2* promoter in E13.5 germ cells, and *Sox2* is overexpressed in DMRT1 deficient germ cells (Krentz *et al.*, 2009). These findings suggest that DMRT1 acts as a potent repressor of pluripotency, although it remains unclear whether misregulation of *Sox2* is the primary defect. Teratomas appear to occur slightly later in fetal development in DMRT1 deficient mice, which may represent a later escape of germ cells from mitotic arrest.

Independent of the 129 strain background, homozygous disruption of *Pten* in mouse germ cells leads to a 100% incidence of teratomas, while teratomas are absent in *Pten* heterozygotes (Kimura *et al.*, 2003). Downregulation of *Pten* has been implicated in increased germ cell transformation into tumorigenic cells in culture experiments (Moe-Behrens *et al.*, 2003). *Pten,* one of the most common tumor suppressor genes mutated in human cancers, codes for a lipid phosphatase that negatively regulates proliferation through dephosphorylation of phosphatidylinositol(3,4,5)-triphosphate (PIP3). EG cells can be derived from the germ cell specific *Pten* null mice with highly increased efficiency (Kimura *et al.*, 2003), suggesting that releasing the brakes from the cell cycle promotes pluripotency. This is consistent with findings that ES cells transit rapidly through

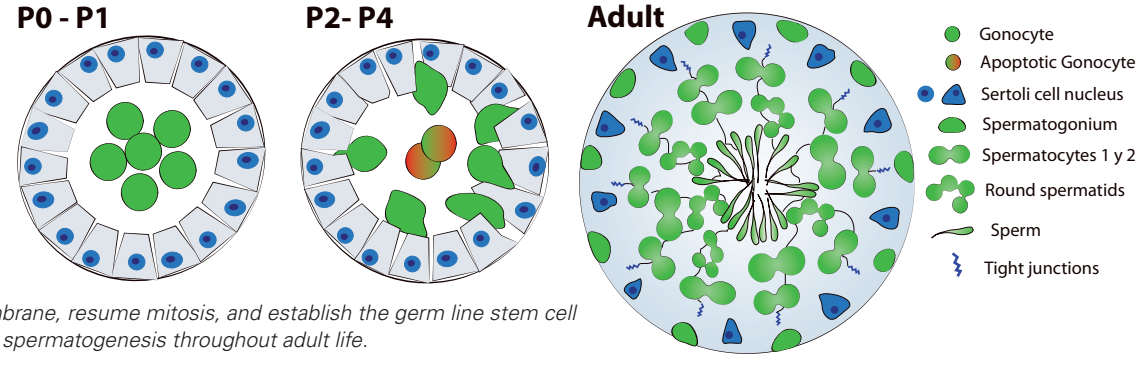


Fig. 4. Schematic representation of the establishment of the spermatogonial stem cell niche and initiation of spermatogenesis. *At birth (P0-P1) mitotically quiescent gonocytes are localized in the center of the seminiferous tubules. Between P2-P4, gonocytes undergo apoptosis or migrate to the basement membrane, resume mitosis, and establish the germ line stem cell population that gives rise to spermatogenesis throughout adult life.*

the cell cycle, but slow down upon differentiation (White *et al.*, 2005). Also in accord with this idea, the maintenance of mitotic activity beyond the normal stage of mitotic arrest is associated with teratoma susceptibility in *Dmrt1*$^{-/-}$, *Pten* null, and 129-Chr19^{MOLF} mice (Heaney *et al.*, 2012, Kimura *et al.*, 2003, Krentz *et al.*, 2009).

Interestingly, in *Pten* deleted mice, 129-Chr19^{MOLF} strain, and in *Dnd1Ter/Ter* mutants, some male germ cells express early markers of meiosis (Cook *et al.*, 2011, Heaney *et al.*, 2012, Kimura *et al.*, 2003). However, no overlap has been reported between cells expressing meiotic markers and cells expressing tumor markers; thus, it is unclear whether meiosis is one of the possible differentiation pathways when mitotic arrest is blocked or delayed, or whether an initial entry into meiosis is a first step toward teratoma formation. Heterozygous deletion of *Stra8*, a gene associated with meiotic entry, exerted a protective effect in the context of the 129-Chr19^{MOLF} strain, leading to the suggestion that premature initiation of the meiotic program may promote tumorigenesis (Heaney *et al.*, 2012).

Dnd1Ter: the best-studied modifier of teratogenesis

The sharp increase in tumor incidence in 129 mice carrying the *Ter* mutation suggested that *Ter* normally plays an important role in suppressing pluripotency, and might offer a means to identify and understand critical processes that control the balance between germ cell development and pluripotent neoplastic transformation.

The increase in the occurrence of teratomas in 129 mice caused by the *Ter* mutation was first reported in 1973 (Stevens, 1973), but the gene responsible was not identified until 2005. The *Ter* mutation acts as a potent modifier of the teratoma phenotype, but the background tumor incidence in 129/Sv mice, and the incomplete penetrance of the mutation indicated that *Ter* was neither necessary nor sufficient to cause teratomas (Matin *et al.*, 1998). This made it difficult to use the teratoma phenotype alone to map the allele. However, the *Ter* mutation also caused a dramatic loss of germ cells in both sexes and in all genetic backgrounds, which allowed investigators to map the mutation to a region of chromosome 18 in 1994, and later, to a specific point mutation that introduced a premature stop codon in the dead-end homolog 1 gene (*Dnd1*^{Ter}) (Asada *et al.*, 1994, Youngren *et al.*, 2005).

Of the various factors that influence teratoma incidence, *Dnd1* is the best studied from a molecular perspective. *Dnd1* is the mouse ortholog of the zebrafish dead end *(dnd*) gene (Youngren *et al.*, 2005). Zebrafish *dnd* was named for its germ cell phenotype. When *dnd* was depleted by antisense morpholinos in the early embryo, germ cells were normally specified based on early germ cell markers, but they failed to initiate migration and died by 24 hours post fertilization. Zebrafish *dnd* mRNA and protein localize to the germ plasm and germ granules, respectively; two related structures that are enriched for RNA binding proteins and mRNAs (Voronina *et al.*, 2011, Weidinger *et al.*, 2003). The major protein motif recognized in both *dnd* and mouse *Dnd1* is a putative RNA recognition motif (RRM), suggesting that this gene plays a conserved role in germ cell post-transcriptional regulation (Weidinger *et al.*, 2003, Youngren *et al.*, 2005). Mouse *Dnd1's* RRM showed the highest similarity to mouse apobec complementation factor (ACF), a member of the RNA editing complex that converts specific cytidines to uridines in target RNAs (Mehta *et al.*, 2000).

Throughout animal evolution, germ cell development is characterized by the presence of cytoplasmic granules containing RNAs and proteins. While these granules are referred to differently in different species, they all contain an abundance of RNA binding proteins (RBPs) (Voronina *et al.*, 2011). Many of these RNA binding proteins themselves are highly conserved and essential for germ cell development. For example, the NANOS proteins play critical roles in germ cell specification from *Drosophila* to mammals. In mouse, inactivation of *Nanos3* results in the disappearance of germ cells in both sexes soon after their specification, while *Nanos2,* which is specifically expressed in the XY gonad beginning just after germ cell colonization of the genital ridge, plays a role in suppression of meiotic genes and promotion of the male pathway. Interestingly, ectopic *Nanos2* expression can rescue the *Nanos3* phenotype, but *Nanos3* does not rescue *Nanos2*, indicating incomplete overlap in their functions (Suzuki *et al.*, 2007). NANOS2 has been shown to associate with members of the deadenylation complex and appears to negatively regulate mRNA target translation, in part by destabilization of target mRNAs (Suzuki *et al.*, 2012).

Experimental insight into the molecular function of *Dnd1* came in 2007, when it was shown in cell culture that human and mouse *Dnd1* can stabilize translation of target transcripts by binding to their 3'UTRs to antagonize miRNA mediated repression of translation. *Dnd1* was shown to interrupt miRNA-mRNA interactions, including the interaction between *p27* and miR-221, and *Lats2* and miR-372. Additionally, *in vivo* evidence for this mechanism in zebrafish was provided for DND target transcripts *nanos1* and *tdrd7's* interactions with miR-430 (Kedde *et al.*, 2007).

These findings demonstrated a mechanistic role for *Dnd1* and its homologs at the molecular level. The targets of DND1*, Lats2* and *p27*, both function as cell cycle inhibitors. Mouse *Dnd1* has since been shown by RNA immunoprecipitation to pull down transcripts of a group of negative regulators of the cell cycle (Cook *et al.*, 2011). Two of these, *p27* and *p21*, normally expressed during the transition to G0 in male germ cells (Western *et al.*, 2008), were not translated in *Dnd1*$^{Ter/Ter}$ mutants, and this was correlated with a failure of male germ cells to enter G0 in the mutants (Cook *et al.*, 2011).

Questions remain about whether there are additional roles for *Dnd1.* ACF, the protein to which DND1 shows the greatest homology, binds with APOBEC1 to form the RNA editing complex. In the mouse there are 4 other APOBEC family members, APOBEC 1, 2, 3 and AID, and all four have deaminase activity. DND1 was shown in pull-down and colocalization experiments to interact with APOBEC3 (Bhattacharya *et al.*, 2008). Interestingly, independent of its deaminase activity, APOBEC3 was previously shown to antagonize miRNA activity much like DND1 (Bhattacharya *et al.*, 2008). These findings may indicate that DND1 antagonism of miRNAs requires or is regulated by other cofactors. However, DND1 could also function in RNA editing through its interaction with APOBEC3. As an RNA binding protein, DND1 may be regulating multiple mRNAs through different mechanisms at different stages of germ cell development.

The explanation for the sensitivity of the 129/Sv strain to teratoma is not clear. However, this issue has been addressed in *Dnd1*$^{Ter/Ter}$ mice in which teratomas arise in 129/Sv mice, but not in other strains. In most genetic backgrounds, germ cells are almost completely lost in *Dnd1*$^{Ter/Ter}$ mice consistent with the early defect in germ cell specification (Noguchi *et al.*, 1996). This led to the idea that more efficient cell death pathways might protect certain strains by eliminating errant germ cells prior to tumor initiation. To explore the question of whether germ cell loss was a protective factor in non-129/Sv strains, a mutation in the pro-apoptotic gene *Bax* was

introduced into mice of several genetic backgrounds carrying the *Dnd1*^{Ter} mutation. Elimination (*Bax*$^{-/-}$) or reduction (*Bax*$^{+/-}$) of *Bax* function in mice partially rescued the germ cell loss phenotype in all strains. As predicted, a high incidence of teratomas was detected in double mutant *Dnd1*$^{Ter/Ter}$, *Bax*$^{-/-}$ as well as in *Dnd1*$^{Ter/Ter}$, *Bax*$^{-/+}$ mice on mixed genetic backgrounds, where teratomas were not seen in the absence of the *Bax* mutation. However, on a pure C57BL/6 background where ~50% of germ cells were rescued, no teratomas were seen, even in double mutants (Cook *et al.*, 2009).

In a gene expression comparison in E14.5 XY germ cells, cell cycle regulators, cell death pathways, and tumor suppressors were found to be among the genes enriched in C57BL/6 compared to 129/Sv (Cook *et al.*, 2011). These findings suggest that an elevation in the factors that promote cell cycle arrest or apoptotic pathways prior to the mitotic arrest stage of germ cell development may be sufficient to prevent teratoma formation, even in the presence of a homozygous mutation in *Dnd1*. A better understanding of the genetic basis for 129/Sv tumor susceptibility versus C57BL/6 tumor resistance could lead to the identification of additional genetic factors/modifiers that allow germ cells to escape from the regulators that control their underlying pluripotency.

Environmental influences on left-right asymmetry in teratoma incidence

Importantly, it is still unknown what role somatic cells and/or whole animal physiology plays in the sensitivity of the 129/Sv strain to teratoma development. Although expression of *Dnd1* is limited to germ cells (Cook *et al.*, 2009), decisions about cell death versus cell cycle progression are strongly influenced by the signaling environment in the testis and perhaps in the whole animal. Left-right asymmetry in tumor incidence is an *in vivo* example of how subtle micro-environmental differences may affect tumor incidence.

Stevens reported that 75% of teratomas were found in the left testis, suggesting a heritable genetic or physiological difference that affects the left gonad. An interesting possibility to explain the bias in teratoma development is the genetic control of laterality. Asymmetric left/right gene expression begins at gastrulation when several regulatory genes are expressed in the left lateral plate mesoderm (Lee and Anderson, 2008). The molecular networks that regulate left-right asymmetry are different in different species; however, some mechanisms seem to be highly conserved (Levin, 2005). The earliest gene expressed asymmetrically in the lateral plate mesoderm is *Nodal*, which belongs to the TGF-β superfamily (Lee and Anderson, 2008, Levin, 2005). *Nodal* and its receptor *Cripto* are expressed transiently in male germ cells between E12.5-E13.5, and compromised expression leads to premature differentiation of germ cells (Spiller *et al.*, 2012). *Nodal* promotes plasticity of both embryonic and tumor stem cells and re-expression of *Nodal* in tumorigenic cells (Postovit *et al.*, 2007) and in testicular tumors (Spiller *et al.*, 2012) has been associated with a poor prognosis. Although there is currently no evidence that *Nodal* is expressed asymmetrically in the developing gonad, these studies suggest a possible link between the molecular control of left-right asymmetry and teratoma bias to the left side.

Stevens proposed that the lateral asymmetry could be related to vascular differences between the left and right testis (Stevens and Little, 1954). To test this idea, Stevens crossed the *situs inversus viscerum* gene onto the 129/Sv strain and found that the sidedness of the teratoma incidence reversed to the right testis. Based on this work, Stevens proposed that vasculature influences teratocarcinogenesis (Stevens, 1982). It is accepted that the microenvironment plays a role in establishing the niche for stem cells. For example, nutrient availability influences germ line stem cell fate in *Drosophila* (Hsu *et al.*, 2008). In addition, low oxygen tension environment (hypoxia) maintains the undifferentiated states of embryonic, hematopoietic, mesenchymal, and neural stem cells, and can also influence proliferation, cell-fate commitment, and cancer (Mohyeldin *et al.*, 2010). It is possible that left-right variations in testis vascularization could cause differential delivery of nutrients or a hypoxic environment during early germ cell development that result in asymmetric tumor incidence in the 129/Sv *Dnd1*^{Ter} mice. The study of left-right asymmetry in tumor development could lead to important discoveries of additional environmental or physiological mechanisms that are involved in susceptibility of stem cells to tumorigenesis.

Conclusions

Teratomas, the most common form of TGCT affecting young men and boys, were among the earliest documented tumors. The capacity to produce all three germ layers places testicular teratomas at the crux of stem cell and cancer biology, and the study of these unusual tumors may hold clues about how natural populations of stem cells are regulated and how their differentiation is controlled. 129 mice have historically been and continue to be the most researched animal model of germ line teratomas. Early research with these teratomas paved the way for the development of ES cell technology. A variety of modifiers of teratoma incidence have been identified, the most widely studied, and one of the most potent of which is a mutation in the *Dnd1* gene. This RNA binding protein post-transcriptionally regulates multiple target RNAs by antagonizing miRNA mediated repression and possibly through RNA editing or other mechanisms. Perhaps the most critical question is how misregulation of DND1 targets leads specifically to teratoma development rather than to more uniform neoplasms resulting from unbridled proliferation. Although other forms of TGCTs arise at later stages, presumably also resulting from misregulation of the cell cycle, development of teratomas seems to be limited to stages of male germ cell development surrounding the initiation of mitotic arrest in fetal life. This implies that totipotent pathways of embryonic differentiation are still available to male germ cells prior to the reprogramming events that occur during mitotic arrest. Exactly what those reprogramming events entail, and how they are mechanistically achieved, may reveal the links between regulation of the cell cycle, differentiation and neoplastic transformation.

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