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Testicular Germ Cell Tumors: A Paradigm for the Successful Treatment of Solid Tumor Stem Cells

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Abstract

Treatment of testicular germ cell tumors (TGCTs) has been a success primarily due to the exquisite responsiveness of this solid tumor to cisplatin-based therapy. Despite the promise of cure for the majority of TGCT patients, the effectiveness of therapy for some patients is limited by toxicity and the problem of resistance. There is compelling rationale to further understand the biology of TGCTs in order to better treat other solid tumors and to address the shortcomings of present TGCT therapies. TGCTs contain undifferentiated pluripotent stem cells, known as embryonal carcinoma, that share many properties with human embryonic stem cells. The importance of cancer stem cells in the initiation, progression and treatment of solid tumors is beginning to emerge. We discuss TGCTs in the context of solid tumor curability and targeted cancer stem cell therapy.

Keywords

Cisplatin; cancer stem cell; chemosensitivity; embryonal carcinoma; Nanog; Oct4; self-renewal

INTRODUCTION

There is a recent appreciation for the importance of cancer ‘stem cells’ although the identification and isolation of such cells in common solid tumors is challenging [1]. In contrast, homogenous lines of human embryonal carcinoma (EC) have been available since the 1970s [2, 3]. EC are the stem cell component of testicular germ cell tumors (TGCTs), are pluripotent, and have remarkable genetic and biologic similarity to human embryonic stem (ES) cells [4–6]. EC is the stem cell of a rare and curable disease. They are a valuable tool to understand the general principles governing mechanisms of self-renewal and pluripotency of cancer stem cells and importantly, how such cells can be effectively targeted. While the specific genes and regulators of self-renewal may differ between specific varieties of cancer stem cells, the general pathways and principles of cancer stem cell renewal may be similar. Hence, EC can be viewed as a well-characterized model of malignant stem cells [4, 7].

There are three classes of testicular germ cell tumors with distinct clinical and pathologic features: teratomas and yolk sac tumors of newborns and infants (type I); seminomas and nonseminomas of young adults (type II); and spermatocytic seminomas of the elderly (type III) [8]. This review will focus on type II TGCTs. Although rare in the general male population, TGCTs are the most common carcinoma in men between the ages of 15 and 35 [9]. Greater than 85 percent of TGCT patients are cured with standard surgical, radiological and chemotherapeutic regimens, even in those patients with advanced disease [10]. The

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successful treatment of TGCTs has benefited from the availability of diagnostic serum markers, such as alpha-fetoprotein and human chorionic gonadotropin, and most importantly, the development of cisplatin-based therapies, innovative chemotherapeutic combinations, and combined treatment modalities [10].

The precise mechanism(s) for TGCT sensitivity to therapy remains unclear. Type II TGCTs are a heterogeneous group of malignancies that can be broadly divided into two clinically and histologically distinct categories: seminomas and nonseminomas [6, 8]. Seminomas are composed uniformly of cells that resemble primordial germ cells (PGCs). In comparison, nonseminomas consist of a mixed population of cells that recapitulate the different stages of embryonic development, including cells from all three germ layers. Within these different cell types are undifferentiated, pluripotent stem cells, known as EC. EC share characteristics with the pluripotent cells of pre-implantation embryos, including the capacity for unrestrained proliferation, which resembles the self-renewal of stem cells [5]. EC cells are able to differentiate toward extra-embryonic tissues (yolk-sac and choriocarcinomas) and embryonic tissues (immature and mature teratocarcinoma) [6, 8]. Nonseminoma and seminoma share a common precursor condition, known as carcinoma *in situ* (CIS) [11]. It is generally accepted that approximately 50% of patients diagnosed with CIS will develop testicular cancer within five years [12]. Teratomas are observed in a number of nonseminoma patients after chemotherapy [13, 14]. Teratoma represent derivatives of the three primary germ layers and are benign. Mature teratomas are chemoresistant despite an apparently identical genetic composition compared to other histologically distinct nonseminomas. Interestingly, on rare occasions teratoma undergo secondary transformation to somatic-like tumors, for example, leukemia, lymphomas and sarcomas [15].

The biologic understanding of TGCTs will continue to benefit from the recent attention given to the characterization of adult and embryonic stem cells [5, 16, 17]. Comparisons between normal human ES cells and EC have supported the view that self-renewal and tumorigenicity may be linked [4, 5, 18, 19]. However, there is lack of understanding concerning the mechanisms regulating stem cell renewal and the events facilitating malignant transformation of TGCTs. The treatment success of TGCTs may be contingent on the fact that they likely derive from transformed PGCs that possess inherent sensitivity to genotoxic stress and distinct epigenetic characteristics compared to other solid tumors. Based on high-cure rates it appears that current drug regimens are effective at targeting the EC or stem cell component of TGCTs. Elucidation of the mechanisms of TGCT chemosensitivity may improve targeted therapies for other tumor types. In this review, we summarize recent findings and emerging perspectives on the transformation events of TGCTs and their sensitivity to conventional cytotoxic therapies.

ETIOLOGY OF TGCTS

Epidemiologic evidence suggests that TGCT incidence can be associated with a birth-cohort effect and that risk is likely determined by environmental and hormonal exposure *in utero* [20, 21]. Risk factors include cryptorchidism, infertility, and family history [22, 23]. The CIS lesion is considered a precursor for the diverse histology of TGCTs in the young adult [24]. Approximately 20% of TGCT patients present with seminoma and nonseminoma elements, supporting a common cellular origin. CIS is often adjacent to TGCTs and affords an opportunity for comparative marker, genetic, and gene expression analysis between precursor and invasive lesions [25]. CIS is proposed to arise from the transformation of PGCs or early gonocytes during fetal development [26]. The case that initiation occurs *in utero* is supported by similarities in phenotypic markers (Oct4, PLAP, Kit), morphology, genomic imprinting and gene expression between CIS and PGCs [11, 27–30]. In addition, certain human syndromes that are associated with arrest or delay of germ cell development

are associated with a higher incidence of TGCTs [21]. Rare, cytogenetically similar extragonadal TGCTs also tend to occur along the midline, which is the migratory route of PGCs in the fetus [31]. This has led to the proposal that TGCTs arise from PGCs or fetal gonocytes that have arrested in development *in utero*, for example due to aberrant Kit/SCF signaling, and further progress to invasiveness by hormonal stimulation during puberty [26]. Interestingly extra-gonadal germ cell tumors tend to be less sensitive to chemotherapy compared to disease arising in the gonad. This difference is so far unexplained.

An alternative hypothesis of origin is that transformation occurs later in the germ cell cycle, during a proposed 'recombination checkpoint' during the pachytene stage of spermatogenesis. This stage of meiosis shares several molecular and cytogenetic features with CIS including high levels of p53, hyperploidy, and cyclin D2 expression [32]. The two hypotheses, in theory, are not necessarily mutually exclusive if TGCT pathogenesis is a progressive process by which aberrant PGCs undergo an inappropriate and futile attempt to complete spermatogenesis. What is clear is that the likely initiating cell type of TGCTs during the germ cell cycle is the PGC.

Compared to most other tumors, the genetic alterations participating in the initiation and progression of TGCTs have been particularly elusive. Mouse strains prone to germ cell tumors do exist but it is unclear whether they are good models for the pathogenesis of the young adult form of TGCTs since they appear to have distinct features more similar to type I teratomas [33]. Identified alleles associated with mouse TGCTs include deletions of PTEN and the conditional overexpression of E2F [34, 35]. The 129/sv mouse strain has a high spontaneous rate of testicular tumors that also appears to model Type I teratomas found in infants and young boys [33]. These tumors have been established to derive from PGCs and susceptibility is under multigenetic control [36]. The mouse tumors are also pluripotent and sensitive to DNA-damaging agents, which mimic the human condition. Two modifier mutations Steel (Kit-ligand) and Ter (the RNA editing gene deadend1, Dnd1) increase the incidence of TGCTs in the 129/sv strain [37, 38]. Dnd1 is expressed in fetal gonads and has homology to the RNA binding protein, apobec complementation factor (ACF)[38]. ACF recruits the RNA editing protein Apobec1 to apolipoprotein B mRNA and other mRNAs [39]. Apobec1 is a cytidine deaminase that converts cytidine to uridine in apolipoprotein B and neurofibromatosis-1 mRNA [40]. Apobec1 and its homologs exhibit potent DNA mutator activity in an *E. coli* assay and appear to trigger DNA mutation through cytosine deamination with possible implications for cancer [41]. These results suggest that Ter may adversely affect essential aspects of RNA or DNA editing during PGC development [38]. Interestingly, ACF^{-/-} mice are embryonic lethal at the blastocyst stage and the Apobec1 gene is located on 12p13.31 directly adjacent to GDF3 in a hot-spot locus of TGCTs [42] (see last section).

In humans there appears to be a low penetrant inherited form of TGCTs and linkage analysis has only been able thus far to identify weak linkage with large chromosomal regions [43]. TGCTs are aneuploid (hypotetraploid) and associated with gross chromosomal gains and losses. There is often gain of chromosome 7, 8, 12p, 21 and X and loss of 1p, 11, 13 and 18 [6, 8, 44]. Gain of 12p is the only uniformly recurrent genetic aberration in TGCTs (see last section).

Mutation, amplification, or loss of specific candidate oncogenes or tumor suppressors has been noted to occur in TGCTs but at low frequency [45]. One impediment to functional characterization of candidate pathogenic events in TGCTs is the lack of cell lines which mimic CIS. Kit is important for mouse PGC survival and is expressed in CIS and seminomas [46, 47]. Somatic gain-of-function mutations in c-Kit have been reported primarily in seminomas, and significantly, in relatively rare, bilateral disease affecting both

testes [48, 49]. Looijenga et al. reported 93% of patients (57 of 61) with bilateral disease had codon 816 mutations in c-Kit in both testes while only 1.3% of patients (3 of 224) with unilateral disease carried the mutation [49]. This supports the notion that TGCTs, at least in cases of bilateral disease, initiate from PGCs *in utero* before colonization of the gonad. This activating mutation is dissimilar to mutations found in gastrointestinal stromal tumors and appears to be insensitive to the tyrosine kinase inhibitor STI571 [50]. However, the prospect of screening patients for development of bilateral disease is predicted to be of significant value as is the development of novel kinase inhibitors specific for the mutation [48].

The tumor suppressor PTEN was found to be expressed in normal testis germ cells but absent in 56% of seminomas and 86% of EC [51]. Interestingly, PTEN was expressed in adjacent CIS suggesting an associated loss with invasive phenotype. A LOH of the PTEN locus was found in 36% of TGCTs [51]. Other genes studied include the homeobox gene NKX3.1 that is expressed in normal testis and CIS but reduced in EC and seminomas [52]. Several Rho GTPases involved in cytoskeletal regulation were expressed to a higher extent in high stage TGCTs compared to low stage and normal tissue [53].

One unique feature of TGCTs is a nearly universal amplification of chromosome 12p, as indicated by isochromosome 12p, i(12p), or by tandem amplification of segments of chromosome 12p [8, 54]. Further the 12p amplification occurs in CIS, although this is still somewhat controversial [55, 56]. Whether 12p amplification is a cause or consequence of TGCT formation is unclear. The expectation is that one or more genes on chromosome 12p plays a role in the initiation or progression of TGCTs. Positional cloning efforts have thus far failed to identify a cancer susceptibility gene on 12p, although two large regions have been highlighted as additionally over-represented in a small minority of patients, 12p11.2–12p12.1 [57] and 12p13 [18, 58, 59]. Select genes on 12p11.2–12p12.1 such as KRAS, DAD-R, SOX5, JAW1 and EKI1 have been studied but none have been established as initiating factors [8, 60]. Cyclin D2, located on 12p13 has been shown to be highly expressed in CIS and seminomas and is an attractive candidate pathogenic factor [61].

Recent studies have begun to characterize the DNA-methylation status of TGCTs. PGCs undergo erasure of imprinting during the normal germ cell cycle [62]. The proposed origin of TGCTs from PGCs or early gonocytes predicts that the initiated cell of TGCTs may have undergone complete or partial loss of imprinting [26]. In fact, biallelic expression of IGF2 and H19 has been noted in TGCTs [27]. In general, it has been observed that TGCTs have significantly less gene specific and global DNA-methylation compared to other solid tumors. Seminomas appear to have severely hypomethylated DNA and nonseminomas may possess an intermediate and unique pattern of methylation between seminomas and somatic tissues [63–65]. This has been interpreted to support a progression from CIS to seminoma to nonseminoma. Interestingly, array expression analysis identified DNMT3B and DNMT3L, DNA methyltransferases that function in early embryogenesis, as highly expressed in EC but not in seminomas [18, 66]. Certain tumor suppressors including DNA repair genes RASSFL1A and MGMT and the tumor suppressor, HIC1 have been shown to be hypermethylated in TGCTs especially in nonseminoma [67, 68] and have even been associated with cisplatin resistance in one study [68]. It will be important to characterize other chromatin modifications in TGCTs since this may shed further light on TGCT pathogenesis and chemosensitivity. In summary, the etiology of human TGCTs appears to be complex with susceptibility genes and pathogenic factors still to be determined.

CISPLATIN-BASED THERAPY FOR TGCTS

TGCTs have served as a testing ground for the development of modern day therapy for solid tumors. TGCTs are highly aggressive and are often widely metastatic at the time of

diagnosis [69]. In contrast to other solid tumors, metastatic TGCTs can be cured at an overall rate of greater than 80% (Table 1). Seminomas are highly sensitive to radiation and chemotherapy and are highly curable (>90%) at all stages [10, 70]. Nonseminoma are typically treated with surgery and chemotherapy and the cure rates vary from 50% to 95% depending on tumor grade and stage [10, 70]. Approximately 10–20% of nonseminoma patients are refractory to treatment and most of these patients eventually die from progressive disease [71]. Better ways to identify refractory patients are needed [10, 72, 73]. Germ cell tumors have been an excellent testing ground for experimental anti-tumor drugs, a number of which were first approved by the Food and Drug Administration primarily on the basis of studies in testicular cancer [10]. Historically, TGCTs was one of the primary clinical setting in which the concept of combination therapy was validated [10]. A brief summary of the development of TGCT therapy will be presented.

Prior to 1970, 95% of men with metastatic TGCTs eventually died of progressive disease. Before the introduction of cisplatin, disseminated germ cell tumors were treated using single agents with a broad spectrum of activity [10]. Single agent therapy suffered from issues of severe side effects, resistance, remissions of short duration, and low response rates. An advance in therapy for TGCTs was the employment of combination therapy. A regimen of dactinomycin, methotrexate, and chlorambucil was shown to substantially improve response rates [74]. An advance in the effectiveness of chemotherapy for metastatic disease was achieved by using vinblastine in combination with bleomycin that resulted in a long-term disease-free survival rate of 25% [75].

The major breakthrough came with the introduction of cisplatin in the mid-1970s [10]. Rosenberg had earlier noted that bacterial cell division was inhibited in the presence of an electric field [76]. This inhibition was due to a toxic compound derived from the platinum electrode. The molecule was identified as cisdiamminedichloroplatinum or cisplatin. Cisplatin was shown in animal models to have antitumor activity [77]. The efficacy of cisplatin in testicular cancer was highlighted in a study where cisplatin treatment of 11 progressive patients resulted in 3 partial and 3 complete remissions [78]. It was later shown that cisplatin was the most active single agent in the treatment of germ cell tumors. Cisplatin was soon incorporated into combination regimens since its dose-limiting toxicity of renal dysfunction afforded an attractive theoretical coupling with myelosuppressive agents [10]. Multicenter trials were conducted to find the most efficacious drug combination and treatment regimen for metastatic germ cell tumors and led to the establishment of a standard regimen of cisplatin, vinblastine and bleomycin (PVB). This afforded disease-free rates of up to 74% [79–81]. Therapy, while effective, was associated with adverse neuromuscular events. With the intent to diminish toxicity, a randomized clinical trial was designed that replaced vinblastine with etoposide (BEP) [82]. A total of 83% of patients receiving this modified regimen achieved sustained tumor-free survival [82]. Today it appears that approximately 85% of metastatic TGCTs can be permanently cured with BEP therapy [10, 83].

WHY ARE TGCTS SENSITIVE TO CHEMOTHERAPY?

TGCTs are valuable to model curative therapy of solid malignant tumors [13, 70, 84, 85]. TGCTs and TGCT cell lines have been shown to be hypersensitive to drug-induced apoptosis [86–89]. The mechanism for this is currently unclear. An assumption commonly put forth is that TGCTs are inherently sensitive to DNA damage because they are the malignant counterparts of normal germ cells [6, 8, 26]. Such cells are assumed to possess unique mechanisms of hypersensitivity to genotoxic stress to guard against germline transmission of deleterious mutations [90, 91]. Mechanisms of inherent or acquired cisplatin resistance in other tumors, including cellular detoxification, platinum accumulations, DNA

repair, and expression of anti-apoptotic proteins have not been generally accepted as explanations for the inherent sensitivity of TGCTs, suggesting multi-factorial mechanisms of sensitivity [13, 70, 84, 85]. In this section we will review suggested mechanisms for the inherent sensitivity of TGCTs to DNA damage and highlight an emerging postulate that the pluripotent and unique epigenetic context of EC may be linked to the curative response of TGCTs. Since cisplatin is the chief agent responsible for cure of TGCTs we will focus our discussion on this agent.

Drug transport and detoxification

The fact that TGCTs are sensitive to a variety of DNA damaging agents of diverse pharmacology and mechanism of action argues against favorable drug retention and detoxification as mechanisms of sensitivity, since each agent has, at least in part, distinct pathways for these processes. For example, while etoposide is often associated with multi-drug resistance mediated by P-glycoprotein, cisplatin is not, but can be a substrate for multi-drug resistant-associated proteins (MRPs) when conjugated to glutathione [92]. It is generally accepted that cisplatin is transported into the cell by passive or facilitated diffusion, although recent evidence suggests that the copper transporter Ctrl plays a role in the cellular uptake of cisplatin [93]. Comparing cisplatin uptake between TGCT and other cancer lines did not reveal significant increased transport of cisplatin in TGCTs [94]. A survey of Pgp, MRP1, MRP2, breast cancer resistance protein, and lung resistant protein levels in TGCT samples revealed low levels of these transporters but no correlation with sensitive and resistant clinical samples [95].

Resistant to cisplatin is often associated with increased levels of thiol-containing proteins such as glutathione and metallothionein which detoxify cisplatin through conjugation. Although not universally reported, in general it appears that the level of these detoxifiers are low in TGCTs compared to some other tumor types [96]. Interestingly, one study noted high levels of a glutathione-S-transferase, the enzyme that conjugates glutathione, only in inherently resistant teratoma [95]. A number of studies have correlated sensitivity of TGCT lines and clinical samples with decreased levels of these cisplatin conjugation systems. However, other studies failed to find a correlation, as reviewed in [13]. These findings suggest that low levels of exporters and detoxifiers may play at least a contributory role in TGCT sensitivity to cisplatin. However, resistance appears not to be mediated by these mechanisms; an apparently common theme suggesting that inherent TGCT hypersensitivity and resistance are mechanistically distinct (see below).

Recognition and repair of DNA adducts

Several reports have shown that the level of DNA adducts upon initial exposure to cisplatin is similar in TGCT cell lines compared to other cancer lines [89, 96]. This supports the notion that increased access of cisplatin to DNA in TGCTs via greater drug accumulation or activation is not associated with hypersensitivity *per se*. However, several reports suggest that TGCTs may have an inherent lower capacity to repair cisplatin lesions compared to other tumor lines [97, 98]. The major pathway that repairs cisplatin adducts is nucleotide excision repair (NER) [84]. Low levels of the NER proteins XPA, ERCC1 and XPF have been noted in TGCT cell line extracts and in clinical TGCT samples [99, 100]. Importantly, adding back these limiting components restores NER activity to TGCT cell extracts and specific siRNA mediated repression of ERCC1 increases sensitivity to cisplatin in non-germ cell tumor lines [99, 101]. This is an excellent example of how an understanding of TGCT chemosensitivity can translate into a strategy to sensitize other solid tumors and suggests that a low inherent ability to undergo NER in response to cisplatin may increase TGCT sensitivity. However, a recent study did not find a correlation between acquired resistance to cisplatin and XPA levels in clinical TGCT samples [102]. This again suggests that

hypersensitivity and resistance in TGCTs may perhaps best be viewed as separate phenomena since there are many avenues by which TGCTs can become resistant that may not relate to the inherent sensitivity of TGCTs compared to other tumor types. A related point worth mentioning is the potential mechanistic difference between intrinsic and acquired TGCT resistance.

High Mobility Group (HMG) proteins are involved in various aspects of chromatin function, including DNA repair processes, as reviewed [103]. Certain HMG proteins have been shown to specifically recognize and bind cisplatin-induced DNA intrastrand cross-links, inhibiting NER [103]. In TGCTs, elevated levels of testis-specific HMG proteins like sex-determining factor (SRY) and testis-specific HMG-domain protein (ts-HMG) have been associated with increased cisplatin sensitivity through inhibition of NER [104, 105]. Overexpression of ts-HMG in human cervical carcinoma cells increased apoptosis under certain cisplatin treatment conditions but not all [106]. Inefficient cisplatin adduct repair due to HMG domain protein shielding may therefore be another factor in TGCT hypersensitivity.

Mayer *et al.* found that increased microsatellite instability correlated significantly with chemotherapy resistance in 100 TGCTs [107]. Some of the resistant tumors were shown to have a decrease in expression of mismatch repair proteins, MLH1, MLH2, or MSH6, suggesting that decreased mismatch repair may participate in TGCT resistance to therapy.

Thus, it appears that one mechanism that contributes to cisplatin sensitivity is low capacity to repair DNA-adducts, although more work needs to be done to assess its relative importance to the overall cisplatin sensitivity of TGCTs. DNA repair is likely not the whole story, since the same caveat mentioned above for differential retention and detoxification of cisplatin holds true for DNA repair; that is, TGCTs are sensitive to a range of genotoxic stresses associated with distinct DNA lesions recognized by distinct repair pathways. This suggests that the main mechanisms responsible for TGCT sensitivity are likely downstream of genotoxic stress and relate to how TGCTs respond to DNA damage. This response is likely driven by the unique cellular context of TGCTs compared to other solid tumor types. Reports related to downstream pathways of apoptosis in TGCTs will now be discussed.

FAS/FASL

Programmed cell death plays a vital role in normal germ cell development during spermatogenesis by eliminating damaged cells [91]. Cisplatin-based chemotherapy for testicular cancer results in temporary infertility in most men. Treatment with irradiation or certain alkylating agents like procarbazine can result in permanent infertility, highlighting the sensitivity of normal germ cells to DNA damage [90]. Thus, it appears in order to maintain the integrity of the germline, germ cells are sensitized to DNA insults.

The role of specific apoptotic pathways in TGCTs have until recently focused on the intrinsic (mitochondrial) pathway since it was believed that this pathway mediates the majority of chemotherapeutic drug effects. Although not entirely established, there has been a recent appreciation that the extrinsic death receptor pathway. In particular, the FAS pathway can contribute to cytotoxic drug-induced apoptosis in a number of tumor types [108]. In some models, FAS-FASL modulates the apoptotic threshold for chemotherapeutics while in other models the FAS death pathway can be triggered by FAS trimerization in the absence of FASL upon drug exposure [109, 110].

The FAS-FASL system between Sertoli (expressing FASL) and germ cells (expressing FAS) has been proposed to be a crucial paracrine-signaling mechanism regulating germ cell apoptosis as well as mediating the immune privileged nature of the testis [111]. Most reports suggest that TGCTs and cell lines express both FAS and FASL and this may result in bypass

of the FASL requirement from Sertoli cells resulting in cell autonomous activation of the death receptor pathway during therapy [112, 113]. Several FAS receptor pathways components are inducible upon genotoxic stress in both p53-dependent and p53-independent manner [114]. In our global expression analysis to assess the acute transcriptional response of the EC cell line, NT2/D1, to cisplatin many genes involved in death receptor signaling pathways were induced in a p53-dependent manner implying the importance of this pathways in response to cisplatin [115]. These genes include the death receptor gene FAS, the FAS adaptor LRDD, and a gene implicated in positive FAS regulation, PHLDA3 (see below).

Spierings *et al.* demonstrated that in two TGCT cell lines cisplatin induced FAS expression and death inducing signaling complex (DISC) formation prior to the onset of apoptosis and that FAS blocking and activating antibodies repressed and potentiated cisplatin-mediated apoptosis, respectively [112]. Further, cisplatin induced apoptosis was decreased in the presence of vFLIP and a caspase-8 inhibitor [112]. This data provides compelling evidence that TGCT lines engage the FAS receptor pathway and that this may be an important contributor to their sensitivity.

Bcl2/Bax and ROS

The Bcl2 family of proteins is made up of pro- and anti-apoptotic members and it is their balance that defines a cells apoptotic potential, as reviewed in [116]. High levels of pro-apoptotic Bax relative to anti-apoptotic Bcl-2 were reported in TGCT cell lines and linked to therapeutic hypersensitivity [117]. Several mouse knockout and transgenic models of Bcl2 family members possess germ cell apoptotic phenotypes, as reviewed in [86]. A Bcl2 family member, Bcl-G(S), a potent inducer of apoptosis, is found only in testis [118]. Further work is needed to establish whether the balance of Bcl-2 family members in TGCTs before and/or during cisplatin treatment is involved in drug sensitivity as other reports have not been able to correlate Bcl-2, Bax or Bcl-X_L levels with cisplatin sensitivity [119]. It has been reported that high levels of Bax are induced with cisplatin, suggesting that distantly treated or treatment naive specimens may not provide the necessary information to assess the role of Bcl2 proteins in mediating TGCT hypersensitivity. This underscores a drawback to the vitally important assessment of hypersensitivity mechanism in clinical samples of TGCTs. That is, many of the downstream pathways of potential importance including p53, Bax, and FAS are acutely and transiently regulated during the therapeutic response, an aspect of expression which is difficult to assess in tumor specimens. Comparing gene expression profiles of TGCT lines [115] with other tumors as they respond to cisplatin will be informative. Mechanisms associated with resistance in TGCT lines include downregulation of the serine/arginine-rich protein-specific kinase (SRPK1) and failure to activate caspase-9 [120, 121].

Reactive oxygen species (ROS) generation is suggested to play a role in chemotherapeutic responses in a number of tumor types. ROS is known to be an upstream mediator/activator of p53, mainly resulting in p53 stabilization [122]. In addition genes known to modulate ROS levels are known p53 target genes [123, 124]. Hence, p53 is suggested to mediate ROS generation, which results in the execution of apoptosis by mechanisms including induction of mitochondrial damage. In expression array studies we found several genes induced with cisplatin that are involved in redox regulation. These include ferredoxin reductase (FDR), Sestrin1, PLK3 and DNA-damage inducible transcript 4 (DDIT4 or REDD1) [115]. Few studies have assessed the importance of ROS in TGCT drug sensitivity. Schweyer *et al.* demonstrated that cisplatin induced ROS in the TGCT line NCCIT leading to apoptosis through MAP kinase and caspase 3 activation [125].

Deficient cell cycle checkpoints

Deregulation of cell cycle checkpoints is a frequent event in carcinogenesis. It promotes uncontrolled growth and genomic instability. It has been proposed that a deregulated G1/S checkpoint may sensitize TGCT cells to therapy [126]. If cells are to survive cisplatin treatment, DNA adducts must be repaired for cells to successfully replicate their DNA. Premature entry into S-phase results in cell death [126]. The G1/S checkpoint is frequently deregulated in cancer, as reviewed by Eastman [126]. In TGCT-CIS levels of the retinoblastoma tumor suppressor (pRB) and the CDK inhibitor p19 are reduced and levels of cyclin D2 are increased [127, 128]. Progression to invasive TGCTs is associated with loss of p16 and p18. These perturbations would be predicted to enhance cell cycle progression, suggesting that TGCTs may have a deregulated G1-S checkpoint [95, 129]. Recently, mouse ES cells have also been suggested to lack a normal G1-S checkpoint [130].

A classic p53 target gene is p21 which induces G1 cell cycle arrest through the inhibition of cyclin dependent kinases. Failure of p53 to robustly increase p21 levels in TGCTs has been noted, and proposed to contribute to a compromised G1/S phase arrest that favors p53-dependent apoptosis [95, 129].

P53

Evidence suggests an important role for p53 in mediating stress-induced apoptosis in the male germ line [131]. Yin *et al.* showed a higher rate of spontaneous apoptosis in p53^{+/+} compared to p53^{-/-} germ cells of mice [132]. In a *C. elegans* model of germ cell apoptosis a genome-wide RNAi screen revealed 21 genes whose knockdown caused a moderate to strong increase in germ cell death. A total of 16 of the knockdowns required p53 for germline apoptosis to proceed [133]. This suggests an important role for p53 is protection of the germline from genotoxic damage.

One relatively unique feature of TGCTs is the expression of higher than normal levels of wild-type p53 that is otherwise commonly mutated in over 50% of human solid tumors and in almost all tumor types [134, 135]. We and others have shown that p53 is latent in EC but can be activated by DNA-damage or differentiation agents like retinoic acid (RA) [136–138]. Thus, the lack of p53 mutations in TGCTs may be due to a lack of selective pressure during TGCT progression [136]. However, the evidence is conflicting as to the importance of p53 in the hypersensitivity of TGCTs to genotoxic stress. Several *in vivo* and *in vitro* studies suggest an important role for p53 in cisplatin responses [117, 129, 139–141]. In contrast, other studies have failed to support a role for p53 [95, 119, 142, 143]. This conflict may be due to inherent differences in murine versus human models of TGCTs and to differences in the dosages and assays used to assess relative cisplatin sensitivities. Conflicting reports also exist as to the importance of p53 mutations in mediating TGCT resistance [141, 143].

Further work is clearly needed to resolve the issue of p53 and cisplatin sensitivity of TGCTs. It is likely that both p53-dependent and p53-independent pathways contribute. It would be particularly interesting to address the role of the recent non-transcriptional, mitochondria-direct, functions of p53 in TGCTs [144]. Further the role of functionally relevant p53 polymorphisms have not been assessed in TGCTs [145, 146]. We would like to emphasize two points pertaining to p53 in TGCTs. First, it is again worth stressing that robust expression of wild-type p53 in resistant TGCTs does not necessarily rule out participation of p53 in mediating the curative response in sensitive TGCTs if resistance is mediated downstream of p53. Second, we would like to postulate that it is the acute transcriptional response mediated by p53 in the unique context of TGCTs that may be of critical importance, not necessary the basal levels of p53 or p53 targets in treatment naive or

relapsed tumors that predicts sensitivity of TGCT. It is clear that cytotoxic drugs are known to transiently activate and stabilize p53 in TGCT cell lines. We have shown that cisplatin treatment of an EC cell line results in a global p53-dominant transcriptional response [115]. Two classes of p53 targets were particularly represented as previously mentioned, those of the extrinsic death receptor pathway and those of ROS signaling. The relative contribution of these and other p53-dependent pathways in TGCT chemosensitivity needs to be further addressed.

We speculate that since EC are pluripotent and have not committed to a specific cell lineage they possess inherent plasticity in transcriptional potential not shared by somatic-derived tumors. This would likely be related to both genetic and especially epigenetic features that reflect their germ cell origins. The response is likely mediated by both p53-dependent and p53-independent mechanisms. Hence in this model, epigenetic reprogramming and lineage commitment associated with loss of pluripotency would be predicted to be potential mechanisms of chemotherapeutic resistance in teratomas and perhaps refractory nonseminoma (Fig. 1). A greater understanding of the epigenetic status of TGCTs and mechanisms of pluripotency in general, as well as global expression analysis comparing acute DNA damage responses in sensitive and resistant TGCTs and other tumor types not cured by chemotherapy, will help address this issue.

Interestingly, a recent study has proposed a molecular link between pluripotency and p53 in mouse ES cells. Lin *et al.* reported p53-dependent downregulation of the pluripotent factor Nanog in response to RA or doxorubicin [147]. Nanog is essential to maintain pluripotency in ES cells and is highly expressed in TGCTs (see next section). Nanog was found to contain two functional p53 response elements in its promoter [147]. It was suggested that p53-dependent repression of Nanog by DNA damage may be a mechanism to promote the elimination of damaged male germ cells through a process of loss of self-renewal [147]. This is an intriguing potential contributory mechanism for teratoma formation seen after TGCT therapy. We have previously shown that RA and cisplatin modulate the activity of p53 in the EC line, NT2/D1 but not in a subline co-resistant to RA and cisplatin [138]. Further study will be necessary to elucidate the role of Nanog and other pluripotent genes in the DNA damage response of TGCTs.

In summary, hypersensitivity of TGCTs to DNA damage is likely multi-factorial. Several mechanisms have been suggested to make important contributions (Fig. 1.). However, the majority of these pathways are shared with most solid tumors and fail to explain why TGCT are cured with chemotherapy while other tumors are not. It has recently been appreciated that the unique ES cell-like properties of TGCTs may provide clues to their pathogenesis and treatment success. The next section will summarize recent findings that pertain to the similarities between EC, the stem cells of TGCTs, and ES cells.

EMBRYONIC STEM CELL PROPERTIES OF EMBRYONAL CARCINOMA

There is a growing appreciation that TGCTs may be inherently sensitive to DNA-damaging agents due to their germ cell origins. EC cells appear to be the malignant counterpart of ES cells in which they share many genetic and phenotypic features. Recently, it has been proposed that ES cells, though derived from the ICM, may more closely resemble early germ cells selected for during their isolation [17], the very cell type proposed to be the origin of EC. Understanding the mechanisms of self-renewal, pluripotency, and epigenetic reprogramming of the ES cell may directly relate to the etiology and curability of TGCTs. What follows is a brief review on genes known to be important for the self-renewal of pluripotent cells and how these genes may impact TGCT biology.

Stem cell maintenance

Oct4 is a member of the POU family of transcription factors, and plays an essential role in maintaining pluripotency of the inner cell mass (ICM) and in the survival of primordial germ cells [148, 149]. Oct4 expression is restricted to pluripotent cells including ES, EC, PGCs, and cells of the ICM [150]. Oct4 deletion results in early embryonic lethality due to lack of ICM formation at day 3.5 of gestation [148]. Oct4 siRNA in ES and EC cells results in loss of cell proliferation and induction of differentiation [151]. These data indicate that Oct4 is critical in maintaining the self-renewal of pluripotent cells. Oct4 regulates pluripotent genes, including FGF4, Rex1 and UTF1 [152–154]. Oct4 activates genes cooperatively with Sox2, a high mobility group protein that functionally interacts with POU domain proteins [152, 153]. Oct4 and Sox2 expression overlap during development and Sox2 is important for the maintenance of the pluripotent state [155]. Evidence suggests that DNA-methylation may regulate the expression of Oct4 in ES and EC cells [156]. RA-mediated differentiation is associated with downregulation of Oct4 and hypermethylation of the Oct4 promoter [157]. These data suggest that repression of Oct4 may be mediated by the epigenetic modification of chromatin.

Oct4 is highly expressed in patient-derived CIS, seminoma and EC but not differentiated components of nonseminoma and is proposed to be a new and specific diagnostic marker of TGCTs [28, 158]. Interestingly, recent findings suggest that Oct4 may have tumorigenic properties [158, 159]. Hochedlinger *et al.* induced ectopic Oct-4 expression in somatic tissues of adult mice using a doxycycline-dependent expression system and found dysplastic growths in epithelial tissues that are dependent on continuous Oct-4 expression. Dysplastic lesions had evidence of expansion of progenitor cells and increased beta-catenin activity [159]. Oct4 has recently been found to be expressed in a few other tumor cell lines and certain adult stem cell compartments, including human breast and human epidermal stem cells [160]. This suggests that Oct4 may be a general marker of adult stem cells and perhaps even cancer stem cells. Whether Oct4 has a pathologic role in TGCTs has not been tested.

Nanog is a homeodomain-containing protein that has recently been identified as an important transcriptional regulator in the maintenance of the pluripotent phenotype and self-renewal [161, 162]. Like Oct4, Nanog expression is restricted to pluripotent cells and is required to maintain pluripotency of the ICM and ES cells [161, 162]. Nanog overexpression results in ES self-renewal independent of cytokines, such as leukemia inhibitory factor (LIF) [161]. *In vivo*, Nanog deletion results in failure of the ICM to form epiblast [162]. Nanog appears to be directly regulated by Oct4/Sox2 [163, 164]. Similar to Oct4, methylation of the Nanog promoter is associated with RA-induced differentiation [157]. Recently, Nanog has been shown to be expressed in patient-derived CIS, seminoma, and EC, but not differentiated somatic elements of TGCTs [165]. Further, Nanog expression was found in normal human fetal gonocytes and mouse migratory PGCs that undergo reprogramming, but not in adult testes, strengthening the notion that TGCTs originate from early PGCs or early gonocytes [165, 166].

Self-renewal of ES cells depends upon several signaling pathways. LIF and related cytokines bind to the gp130 receptor and activate the transcription factor STAT3 [167]. LIF promotes self-renewal of undifferentiated mouse, but not human ES cells in culture. However, LIF by itself is not able to maintain ES cell self-renewal in the absence of serum. This has led to the idea that another factor in addition to LIF is required for self renewal [5]. Bone morphogenic protein (BMP) has recently been identified as important for maintaining stem cell renewal [168]. In the presence of serum-free media, LIF in conjunction with BMP2, BMP4 or growth differentiation factor 6 (GDF6) is able to support ES cell survival [168]. Nanog has the ability to bypass the requirement for LIF [161]. However, Nanog and LIF act synergistically to enhance self-renewal [5].

Sox2 is a SRY family member, which is expressed in the ICM and ES cells and plays a role in cell fate decisions during development [155]. Sox2^{-/-} embryos die shortly after implantation [155]. The classic Sox/Oct target gene is fibroblast growth factor 4 (FGF4) [152]. FGF4 belongs to a family of growth factors. FGF4 expression in the early embryo is restricted to the ICM [169]. FGF4 is essential for embryonic development, as embryos deficient in FGF4 do not develop beyond implantation [170]. FGF4 expression decreases significantly after differentiation of EC cells due to repression of Oct/Sox activity at the distal enhancer of the FGF4 gene [152]. Other proposed pluripotency genes include Wnt, PLZF, FoxD3, Rex1 and Cripto [171–176]. The exact function of these genes in ES/EC biology needs to be clarified and it is likely that key genes and pathways that regulate self-renewal await discovery.

Epigenetics

A series of important epigenetic events take place in early development, laying the groundwork for sex specific imprints and histone modifications in the developing organism [177, 178]. One modification is CpG methylation resulting in gene silencing. As stated previously, PGCs undergo genome wide demethylation during migration to the genital ridge, that resets imprints and restores totipotency [62]. A reprogramming of genome-wide methylation is associated with dynamic changes in the levels of DNA methyltransferases (DNMTs), including DNMT3 β and DNMT3L, in the developing embryo [177, 178]. DNA hypo- and hyper-methylation is associated with tumorigenesis.

Another mechanism by which a methylation imprint can be modified is deamination. It has recently been shown that activation induced cytidine deaminase (AID) and Apobec1 can deaminate 5-methylcytosine in DNA, resulting in transition mutations [179]. If the mismatch is repaired, it is predicted that a methylated cytosine will be replaced with an unmethylated cytosine. If the mismatch is not repaired, the result is a C:T transition mutation which can lead to a mutator phenotype in bacteria and mammalian cells [41]. Ectopic expression of Apobec1 in mice enhances tumorigenesis [180–182] and as previously stated, a mutation in a gene related to an Apobec1 auxiliary factor is associated with mouse TGCTs [38]. This proposed mechanism of demethylation suggests a role for Apobec1 and AID in epigenetic reprogramming [179]. Interestingly, Apobec1 is located on 12p13.31 the ‘hot spot’ region within the common 12p amplicon [183] and Apobec1 and AID are expressed in oocytes, embryonic germ cells and ES cells [179]. This suggests that Apobec1 may have a role in the pathogenesis and epigenetic remodeling of TGCTs.

Histone modification is an important mechanism of epigenetic regulation [184]. Polycomb family members assemble into distinct multi-protein complexes that repress homeotic (Hox) gene expression and regulate stem cell fate via chromatin modification [185–187]. Polycomb repression complex 2 (PRC2) contains histone deacetylase and histone methyltransferase activities that initiate silencing via deacetylation of histone H3 and trimethylation of Lysine 9 and Lysine 27 on histone H3. The PRC complex 1 (PRC1) recognizes H3 Lysine 27 trimethylation and is implicated in the maintenance of gene repression [185–187]. Deregulation of polycomb genes, including Bmi1, Pc2, Cbx7 and EZH2 is linked to aberrant proliferation of cancer cells [185, 188, 189]. Polycomb genes Bmi1 and Rae28/PHC1/EDR1 are known to play an important role in the maintenance of hematopoietic, neural, and leukemia stem cells [190–192].

Expression profiling

Expression profiling of EC has reinforced the notion that EC cells can be viewed as a model of embryogenesis [4]. Our laboratory has studied RA-induced differentiation of the human EC cell line NT2/D1 [7, 193, 194]. This line is highly malignant, but with RA can be

induced to undergo terminal neuronal like differentiation associated with cell cycle exit and loss of tumorigenicity [195–197]. The cell cycle arrest is associated with RA-mediated ubiquitination and degradation of cyclin D1 [198]. Importantly cells commit to lineage selection and exit self-renewal within 48 hours of RA-treatment [4, 199]. We, along with Houldsworth *et al.*, performed large scale microarray analysis of RA-treated human EC and found that prior to, and during differentiation commitment a number of genes associated with early development are acutely regulated [193, 200]. Surprisingly, in this cell autonomous differentiation model, many genes associated with cell-cell communication, patterning, and fate determination are regulated by RA. Developmental genes involved in embryogenesis and regulated by RA include those of TGF β (Lefty2, NMA follistatin), homeodomain (HoxD1, Meis2, Meis1, Gbx2), IGF (IGFBP3, IGFBP6, CTGF), Notch, Wnt (Frat2, SFRP1) and telomerase (TERF1) pathways [193]. This suggests that multiple signals from diverse signaling pathways are involved in the maintenance of stem cell renewal in human EC. That global regulation of developmental and pluripotency genes is associated with human EC differentiation was noted in several additional studies [66, 201].

Sperger *et al.* directly compared human ES and EC cell lines and human TGCTs in order to identify genes important for the pluripotent phenotype [18]. A remarkably similar expression profile between human ES and EC was noted. The EC/ES expression profile was distinct from that of normal testis or somatic tumors. The genes included pluripotency regulators, Oct4, DNMT3 β , FoxD3, Sox2, Gal, and several genes involved in Wnt signaling [18]. Seminomas had a distinct profile and validated, on the global gene expression level, the concept put forth based on epidemiologic, morphologic, and marker studies that seminomas are more similar to PGCs and nonseminomas (EC) are more similar to ES cells [4, 6, 26]. The major difference in expression between EC and ES was over-representation of the genes on 12p, in particular 12p13[18]. Several detailed and highly comprehensive array analyses have now been performed and have also demonstrated the expression of pluripotency associated genes in TGCT samples including CIS [29, 66, 202, 203]. Other pluripotency associated genes highlighted as expressed in TGCTs include DPPA4, DNMT3L, TERF1 and Frat2 [29, 66, 202, 203]. Further, studies have suggested distinct expression signatures for seminoma, and each component of nonseminoma (EC, teratoma, choriocarcinoma, yolk sac tumors) that may be useful in diagnostics and undoubtedly for understanding the etiology of TGCT subtypes [18, 203–205].

Chromosome 12p13: a pluripotency ‘hot spot’ region?

Interestingly, RA treatment of EC/ES cells results in downregulation of the expression of a number of genes on chromosome 12p, including GDF3, Nanog, CD9 and Stella (DPPA3, PGC7) [161, 162, 183, 206–209]. Through further global transcriptional profiling of human EC we recently identified a cassette of 7 genes on 12p13.31 that are downregulated by RA in parallel with induced loss of tumorigenicity [199]. These genes include Nanog, GDF3, Stella, EDR1 (PHC1), Glut3, SCNN1A, and CD9, all of which, with the exception of SCNN1A, are associated with pluripotency (Fig. 2.). The gene cluster GDF3, Stella and Nanog had previously been noted to localize within a 100 kb TGCT ‘hot spot’ region on chromosome 12p13 [183, 208]. Early evidence suggests that these genes may be abundantly expressed in TGCTs [18, 165, 183, 199, 207, 210].

The location of Nanog on the previously established 12p amplicon characteristic of TGCTs exemplifies how ES and TGCT fields are beginning to merge and how important findings in one field can yield valuable insights in the other. Stella encodes a DNA-binding protein containing a SAP-like domain, which is suggestive of a role in chromatin organization and/or RNA processing [208, 211]. Stella expression is restricted to the preimplantation embryo and pluripotent germline stem cells [208, 212, 213]. Null mice reveal that Stella is a

maternal effect gene important for the early development of preimplantation embryos [208]. Growth differentiation factor 3 (GDF3) is a member of the TGF β -superfamily of the BMP class. Members of this class play a role in cell growth and differentiation in embryonic and adult tissues [207]. GDF3 is expressed in TGCTs with EC and yolk sac elements and is downregulated as EC cells differentiate with RA [207]. The fact that other members of the BMP-family, BMP2, BMP4, and GDF6, are known to drive stem cell renewal in mouse ES cells implicates GDF3 as a potentially important factor in mediating pluripotency of EC [5].

EDR1/PHC1 is a component of the above mentioned polycomb complex, PRC1, implicated in epigenetic reprogramming and repression of homeotic genes via chromatin modification [185–187]. Evidence suggests that EDR1 (termed Rae28 in the mouse) is a regulator of stem cell fate during hematopoiesis [191, 192]. Another gene within the cluster, Glut3, is a facilitative glucose transporter, expressed primarily in tissues with high glucose demand such as brain, nerves and vascular endothelium [214]. Glut3 expression is evident at the 8-cell stage blastocyst and is coincident with a switch from pyruvate/lactate to glucose utilization [214]. Its role in glucose utilization suggests that Glut3 may be an important factor in the survival of pre-implantation embryos [214]. Enhanced Glut3 expression has been suggested to contribute to tumor cell growth, as reviewed in [215]. Cell Differentiation Antigen 9 (CD9) is a member of the tetraspanin or transmembrane 4 family of integral membrane proteins. This family is known to mediate signal transduction events that have important functional roles in cell development, growth and motility [216]. CD9 was identified as the epitope of a monoclonal antibody raised against undifferentiated EC and is highly expressed in ES/EC cells [217]. Interestingly, CD9 may physically interact with c-Kit and modulate its activity in hematopoietic progenitors [218]. CD9 deletion results in infertility due to failure of sperm-egg fusion [219]. CD9 has been proposed to be involved in LIF-mediated maintenance of mouse ES cells [206].

These findings suggest that 12p genes may be coordinately regulated as cells exit stem cell renewal (Fig 2.). One candidate mechanism of coordinate regulation is Oct4. Nanog has recently been shown to possess a functional Oct4/Sox2 regulatory sequence within its promoter region [163, 164]. Oct/Sox functional sites have also been reported in the Sox and Oct promoters themselves and Glut3 was isolated in a subtraction screen for downstream targets of Oct4 [220–222]. Interestingly, we noted that siRNA to Oct4 in human EC cells downregulated basal expression of Nanog as well as GDF3, Stella and EDR1 [199]. These genes contain several near consensus Oct4 and Sox2 sites (Giuliano and Spinella unpublished observation). Another mechanism of coordinate regulation involves the orphan nuclear receptor GCNF that can directly regulate Oct4 and Nanog expression [223]. It is also likely that epigenetic-like chromatin remodeling of the region may play a role. Early indication is that Nanog, Oct4 and Sox2 regulation is tightly controlled and complex [5]. A recent study employing ChIP-Chip analysis indicated that Nanog, Oct4 and Sox2 have the potential to autoregulate one another and to collaborate in the regulation of a common set of genes in ES cells [224]. Additional study will be required to unravel the complex regulation of 12p associated pluripotency genes.

Thus, a deeper understanding of human embryology may also enhance our understanding of the unique biology of TGCTs. An untested but intriguing hypothesis is that “enforced” pluripotency through overexpression of the 12p13.3 genes mentioned above as well as Apobec1 and Cyclin D2 (also located on 12p13) may play a role in the pathogenesis of TGCTs (Fig. 1.). Interestingly, gain of chromosome 12 and 17q is observed in human ES cells upon extended passage, suggesting selective gain of these chromosomes improves survival and pluripotency of ES cells [225].

CONCLUSIONS

TGCTs are one of the few solid tumors cured at a high rate even when disseminated. One view to account for this curability is that the stem cells of TGCTs, EC, are effectively targeted by standard chemotherapy, whereas the stem cells of other solid malignancies are not. The precise mechanisms responsible for this critical difference are not entirely clear, but likely relate to the germ cell origins and pluripotent characteristics of EC. Adult tissue stem cells may not share these traits to the same degree. Knowledge gained from the rapid advances in developmental embryology, especially the genetic and epigenetic control of pluripotency and 'stemness', will likely continue to provide insights related to the pathogenesis and curability of TGCTs. This may lead to novel TGCT treatments for those patients refractory to current therapy. Understanding TGCT curability may uncover strategies that target the stem cells of other solid tumors.

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Fig. (1). Model depicting pluripotent features of embryonal carcinoma that may impact on the pathogenesis and chemosensitivity of testis cancer. Transcriptional plasticity due to stem cell-like chromatin structure, p53, deregulated cell cycle checkpoints, and low DNA repair are highlighted. These features may be lost during *in vivo* maturation of resistant teratoma. Mechanisms of non-teratoma resistance may or may not be related to mechanisms that confer inherent sensitivity. MSI, microsatellite instability; GST, glutathione-S-transferase.

Fig. (2).

Genes on chromosome 12p13.31 with roles or putative roles in regulating stem cell renewal. Genes repressed by retinoic acid in EC and ES during induced differentiation are highlighted with *. Distance between genes is approximate. Cyclin D2 is on 12p13.32 and not depicted here. Cyclin D2 is not downregulated with RA treatment of EC cells (Freemantle unpublished observation). See text for details.

Table 1

5-year survival rate of select solid-tumors stratified for metastases.

Site	Metastatic Stage			
	All	Local	Regional	Distant
Testis	95.9	99.4	96.3	71.8
Ovary	44.0	93.5	68.8	28.5
Breast	87.7	97.5	80.4	25.5
Cervix	72.7	92.2	53.3	16.8
Colon	63.4	89.9	67.3	9.6
Bladder	81.7	94.1	48.8	5.5
Esophagus	14.3	29.3	13.3	3.1
Lung	15.2	49.4	16.1	2.1
Pancreas	4.4	15.2	6.8	1.8

Source: American Cancer Society, Cancer Facts and Figures, 2005.