



REVIEW

The rise of testicular germ cell tumours: the search for causes, risk factors and novel therapeutic targets [v1; ref status: indexed, <http://f1000r.es/md>]

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Abstract

Since the beginning of the 20th century there has been a decline in the reproductive vitality of men within the Western world. The declining sperm quantity and quality has been associated with increased overt disorders of sexual development including hypospadias, undescended testes and type II testicular germ cell tumours (TGCTs). The increase in TGCTs cannot be accounted for by genetic changes in the population. Therefore exposure to environmental toxicants appears to be a major contributor to the aetiology of TGCTs and men with a genetic predisposition are particularly vulnerable. In particular, Type II TGCTs have been identified to arise from a precursor lesion Carcinoma *in situ* (CIS), identified as a dysfunctional gonocyte; however, the exact triggers for CIS development are currently unknown. Therefore the transition from gonocytes into spermatogonia is key to those studying TGCTs. Recently we have identified seven miRNA molecules (including members of the miR-290 family and miR-136, 463* and 743a) to be significantly changed over this transition period. These miRNA molecules are predicted to have targets within the CXCR4, PTEN, DHH, RAC and PDGF pathways, all of which have important roles in germ cell migration, proliferation and homing to the spermatogonial stem cell niche. Given the plethora of potential targets affected by each miRNA molecule, subtle changes in miRNA expression could have significant consequences e.g. tumourigenesis. The role of non-traditional oncogenes and tumour suppressors such as miRNA in TGCT is highlighted by the fact that the majority of these tumours express wild type p53, a pivotal tumour suppressor usually inactivated in cancer. While treatment of TGCTs is highly successful, the impact of these treatments on fertility means that identification of exact triggers, earlier diagnosis and alternate treatments are essential. This review examines the genetic factors and possible triggers of type II TGCT to highlight target areas for potential new treatments.

Article Status Summary

Referee Responses

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- 1 **Anis Feki**, Geneva University Hospitals Switzerland
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Introduction

Testicular cancers are generally grouped into three broad categories with type I testicular germ cell tumours (TGCTs) being observed primarily in neonatal boys and young children and consisting of benign teratomas and malignant yolk sac tumours¹. Type III TGCT, also called spermatocytic seminomas, affect older men above 50 years of age and are derived from a slow growing expansion of type B spermatogonia². Type II TGCTs mostly affect men aged between 20 and 40; however, the origins of this tumour type are much earlier during foetal development³. All type II TGCTs develop from a pre-invasive lesion termed Carcinoma *in situ* (CIS), which has been identified as a dysfunctional foetal germ cell^{2,4}.

In the developed world the incidence of type II TGCT (seminoma and non-seminoma), but not type I or II, has increased significantly over the last century to become the most common malignancy found in men aged between 20 and 40 years of age⁵⁻⁷. Such findings have led to speculation that environmental factors impact on the tumorigenesis of this cancer⁸. In addition, several genetic mutations have been discovered that positively impact TGCT rates in both sporadic and familial cases of TGCTs^{8,9}. While the characterisation of risk factors is far from being completed, one of the most promising indicators of TGCT is mutations in *c-KIT* and *KIT ligand*¹⁰⁻¹³.

The perceived association of TGCTs with infertility combined with evidence that pre-existing subfertility increases in patients with type II tumours, raises the possibility that these lesions may act as indicators of a general reduction in male reproductive health and rising fertility problems within the male population^{14,15}. Type II TGCT is known to develop from dysfunctional gonocytes located within the seminiferous tubules¹⁶, indicating that the risk factors predisposing an individual to TGCTs are active early in male foetal development, necessitating the investigation of early germ cell development in an effort to identify causative factors for type II TGCTs. In this review we examine normal germ cell development and potential areas of this differentiation that are modified during the development of type II TGCTs. We also discuss the genetic and environmental contributions to disease specification as well as risk factors and indicators of tumour progression.

Germ cell development

Germ cell specification

Primordial germ cells (PGCs) are signalled to develop by Bone Morphogenic proteins (BMPs) secreted from the extraembryonic ectoderm and the visceral endoderm¹⁷. BMPs (BMP4, BMP2, and BMP8b), in turn activate the expression of *Fragilis* (IFITMS) genes^{17,18}. In the mouse around six of the *Fragilis* positive cells begin to express *BLIMP1/PRDM1* and *PRDM14* at embryonic day (E) 6.5 and thereafter are committed to the germ line (Figure 1)¹⁹⁻²³. *BLIMP1* is a key component of germ cell specification as studies of the *BLIMP1* knockout mouse have revealed that PGCs cluster, but fail to migrate²⁴. The expression of *BLIMP* represses the expression of somatic genes such as *HOX*, *FDF8* and *SNAIL*, in addition to DNA methyl transferases²⁵. The combination of the highly proliferative nature of the germ cells i.e. expansion from six cells at E6.5, to 250 cells at E9.5, 1000 cells at E10.5, and 26,000 cells at E13.5, and the lack of maintenance DNA methyltransferases causes general demethylation of the DNA in primordial germ cells^{25,26}. The methylation status, and the repression of somatic cell

phenotype, permits the expression of pluripotency factors such as *NANOG* and *OCT 3/4* (but not *c-MYC* and *KLF* - required for total pluripotency)²⁶. *SOX2*, an essential pluripotency factor in mouse, is not expressed in human primordial germ cells. However, it is expressed in nonseminoma germ cell tumours²⁶.

OCT 3/4 is expressed in pluripotent cells of the inner cell mass of a blastocyst and, in contrast to the differentiating somatic cells, its expression is maintained in primordial germ cells^{17,27}. *OCT 3/4* is believed to control primordial germ cell survival, given that germ cells are lost through an apoptotic pathway in *OCT 3/4* null mice^{17,28}. In mice, *SOX2* acts in conjunction with *OCT 3/4* and its expression is maintained in primordial germ cells until they migrate, colonise the gonad, and become specified as pre-spermatogonia or oogonia, whereupon *SOX2* is down regulated^{17,29}. The pattern of *NANOG* expression tracks that of *SOX2*, and is required for the maturation of germ cells once they reach the genital ridges^{17,30}. The exact roles of *OCT 3/4*, *SOX2* and *NANOG* in primordial germ cells is currently unknown, but as they are important for maintaining pluripotency and proliferation in embryonic stem (ES) cells, it is considered likely that they also maintain pluripotency in germ cells and prevent their differentiation¹⁷. More recently, several other pluripotency factors have been identified in primordial germ cells, gonocytes and pre-spermatogonia²⁰. One such protein is *LIN28*, which is involved in maintaining pluripotency and survival. *LIN28* is located upstream of, but linked to, both *OCT 3/4* and *NANOG* expression. Additionally *LIN28* is not found in postnatal spermatogonia but is expressed in CIS, seminoma, and embryonal carcinoma, further demonstrating the importance of the differentiation of germ cells to maintain normal testis development^{20,31}. Another pluripotency protein identified upstream of the classical pluripotency regulator *OCT 3/4*, and capable of interacting with *NANOG*, is *SALL4*³². *SALL4* is expressed late primordial germ cells, pre-spermatogonia, and spermatogonial stem cells and is hypothesised to be involved in both the maintenance of primordial germ cells as well as the differentiation of spermatogonial stem cells^{32,33}.

Primordial germ cells and pluripotency

Interestingly, primordial germ cells are not natively pluripotent. Indeed, despite the fact that they express many pluripotency markers, they only differentiate into one cell type i.e. a germ cell²⁶. However, pluripotency can be induced in *in vitro* cultures of germ cells, if they are isolated before the colonisation of the gonad and incubated in the presence of the growth factors *SCF*, *FGF2*, and *LIF*¹⁷. In addition, germ cells are more efficiently transformed into pluripotent cells in the presence of *FFG2* in conjunction with *MAK2k* and *GSK3B*, as well as *TGFB* type 1 receptor inhibitors³⁴.

Germ cell maintenance

Once primordial germ cells are specified, germ cell specific genes that promote cell survival, such as *STELLA* and *NANOS3*, are up-regulated (Figure 1)¹⁷. Other markers of primordial germ cells include *SSEA1*, *PRDM14*, *DND1*, *Fragilis*, *LIN28*, *c-KIT* and *MVH*²⁶. *DND* (dead end/Ter) prevents miRNA mediated translational repression and serves as a survival factor for PGCs. Mutations in *DND* cause testicular teratomas and *DND* null mice lose their PGCs via apoptosis between E8.5 and E12.5⁹. At E7.5 in the mouse (3 weeks in humans) *PLAP* (Placental Like Alkaline

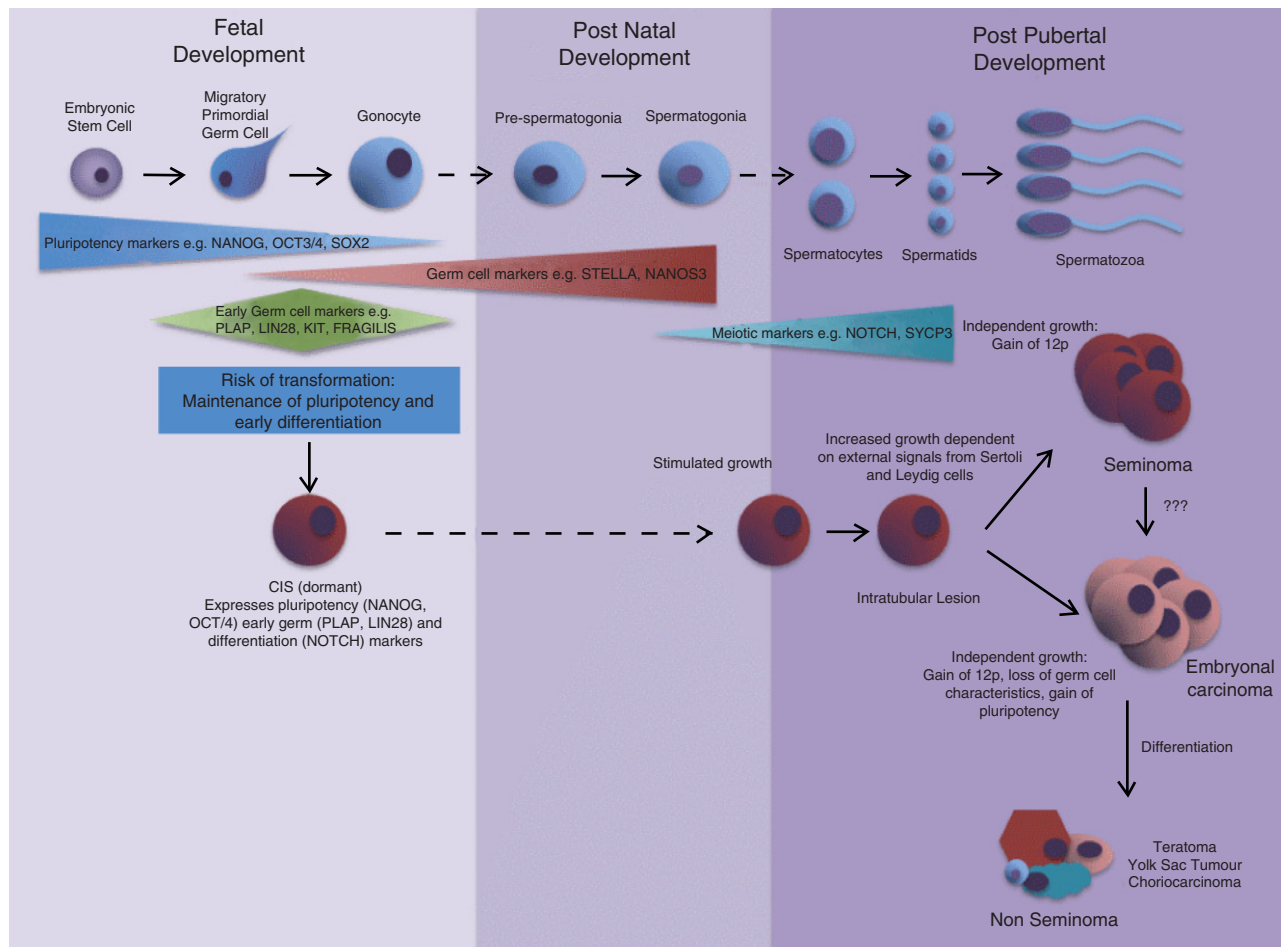


Figure 1. Proposed mechanism of type II testicular germ cell tumour (TGCT) differentiation. This is an update and adaption of the classic model of type II TGCT development first proposed by Rajpert-De Meyts⁹³. Current understanding indicates maintained pluripotency combined with incomplete premature differentiation of gonocytes causes the specification of Carcinoma *in situ* (CIS) cells. Signals caused by puberty cause these cells to proliferate and once additional mutations accumulate CIS cells differentiate into overt type II TGCTs.

Phosphatase)-positive PGCs reside in the posterior of the primitive streak and become motile shortly after this time^{35,36}.

Germ cell migration

Primordial germ cells initially migrate into the hindgut during its anterior extension (E8-9.5); they then move into the mesoderm (E9.5) and bilaterally travel to the genital ridges to contribute to the formation of the gonads (E10.5-11.5)^{19,37}. This process is complete by E33-37 in humans^{37,38}.

Steel factor (KIT-ligand) has been identified as a key survival and proliferative signal for developing germ cells as well as acting to guide PGCs along the hindgut and towards the genital ridges^{39,40}. The movement of PGCs out of the hindgut and into the gonads (E9.5) is dependent on E-cadherin (CDH1) and β 1-integrin (ITGB1)³⁷, and is directed by CXCL12⁴¹. On reaching the genital ridges at around E11 to E11.5, the PGCs proliferate and form gonocytes³⁵. At this time, active demethylation continues by UTX (histone demethylase), another pluripotency factor, before the cells then undergo sex specific epigenetic changes required to produce

viable germ cells^{25,42}. By E13.5 the gonocytes enter either mitotic arrest in the case of testis, or meiotic arrest in the ovary. Therefore the period of time between the arrival in the gonad and arrest is key to the primordial germ cell proliferation and differentiation⁴³.

Sex determination

Male sex determination is triggered by the expression of SRY (Sex-determining region on the Y chromosome), a high mobility group (HMG) transcription factor which activates SOX9 (SRY related HMG box 9), another transcription factor which in itself is sufficient for sex determination^{44,45}. SOX9-positive pre-Sertoli cells recruit cells from the mesonephros and the coelomic epithelium to form the testicular cords^{46,47} which occurs in concert with the commitment of male germ cells to the pre-spermatogonia cell fate⁴⁸. Sertoli cells also secrete paracrine factors (DHH and platelet-derived growth factors) initiating the differentiation of the testosterone producing Leydig cells⁴⁹.

Male germ cells are maintained in mitotic arrest within the seminiferous tubules by the enzyme CYP26B1 which facilitates degradation

of retinoic acid, preventing the expression of STRA8 (stimulated by retinoic acid 8) and hence entry into meiosis⁵⁰. When the expression of CYP26B1 decreases at E13.5, the RNA binding protein NANOS2 maintains mitotic arrest in male germ cells⁵¹. Shortly after birth in mice, and in late gestation in humans, gonocytes (prospermatogonia) migrate from the centre to the basement membrane of the seminiferous tubules and by postnatal day 6 they have begun to divide and are designated single spermatogonia (A_s) or spermatogonial stem cells (SSCs) and spermatogenesis is initiated⁵².

Risk factors and genetic predisposition of testicular cancer

The risk factors for type II TGCTs include family predisposition, cryptorchidism, disorders of sexual development, high maternal oestrogen during foetal development, environmental exposures, sub- or infertility, and previous TGCTs^{3,36,53}. Additionally, geographical regions with a high rate of TGCTs have lower sperm quality as well as increased rates of cryptorchidism and hypospadias when compared to regions with low rates of TGCTs⁵⁴. While not all sexual development disorders lead to an increased risk of TGCTs, they usually occur at higher frequency in less differentiated gonads⁵⁵. Identified genetic factors, including deletions on the Y chromosome, androgen insensitivity, and KIT mutations; negatively impact germ cell development supporting the notion that the risk of TGCTs is established early^{3,36,56}. It is proposed that the combination of both genetic and environmental factors prevents or delays the maturation of PGCs/gonocytes into pre-spermatogonia by retarding the development of the supporting Sertoli and Leydig cells, thus perturbing the microenvironment required for germ cell development^{3,57}.

Patients lacking the SRY gene on the Y chromosome required for Sertoli cell development, or WT1 required for ovary development, have a high risk of type II TGCTs, because of the lack of correctly differentiated support cells, which in turn inhibit germ cell development³. A partial deletion of the AZoospermia Factor C (AZFC) region (gr/gr mutation) on the short arm of the Y chromosome is also associated with an increased risk of TGCTs⁵⁶.

Mutations in KIT signalling and continued KIT expression are seen in CIS cells, but not more advanced stages of testicular cancer, indicating that this may be an initial feature of CIS cell specification. Furthermore, genetic association studies have identified mutations in genes encoding KIT, KIT ligand and its downstream signalling molecules such as KRAS, SPRY4, and BAK1, as likely predisposition genes for TGCTs^{3,58-60}. It is believed that a region of the Y chromosome encoding TSPY (testis specific protein Y-encoded), with a potential role in germ cell mitotic division, is required for the development of TGCTs. Interestingly, as with KIT, TSPY protein expression is lost when CIS becomes invasive^{36,55}, suggesting that it may have a role in the initiation, but not maintenance, of tumours.

The switch to meiosis from mitotic amplification is tightly controlled in normal spermatogenesis and there is some indication that this pathway may be prematurely initiated then aborted in CIS and TGCTs⁶¹. Several members of the NOTCH signalling pathway control the mitotic to meiotic switch, and mutations in the NOTCH

signalling pathway in *C. elegans* have been shown to elicit tumour-like expansion of germ cells⁶¹. This observation stimulated exploration of NOTCH signalling in human TGCTs and revealed that NOTCH1 and NOTCH4 are both over-expressed in CIS and seminoma. Additional genetic screening of affected individuals has identified mutations in members of the NOTCH family as primary risk factors for developing these pathologies^{61,62}. The meiotic protein SYCP3, a component of the synaptonemal complex which aligns sister chromatids in prophase I of meiosis allowing their proper segregation, is also expressed in CIS, seminoma and embryonal carcinoma, thus providing further indication of a premature activation of meiosis in TGCTs development⁶¹.

More recent genetic screens have identified transcription factors DMRT1 and ATF7IP, as well as the telomere regulator TERT, as susceptibility genes for TGCTs⁵⁸. DMTR1 is required for normal testicular differentiation and DMTR1 knockout mice have been shown to develop teratomas⁵⁸. ATF7IP is a transcription factor that regulates the expression of subunits of the enzyme complex responsible for maintaining telomere length such as the active component, TERT⁶³. Telomere regulation and length is tightly controlled within cells and usually only immortal cells, i.e. stem cells, express active telomerase. Telomerase is also extensively overexpressed in cancer cells and its activity plays a key role in the transformation process^{63,64}.

However, in most cases no known genetic factor contributing to the development of TGCTs is identified and therefore environmental factors may play an essential role in the development of tumours in these individuals. The rates of TGCTs within Western countries have risen sharply since the early 1900s; however, this rise is not consistent across all countries^{8,65}. The most notable differences in TGCT incidences and rates of increase are demonstrated in Europe. For example, increased TGCT rates were documented in England, Wales, and Germany before Denmark and Norway. By contrast, Finland, Eastern Germany, and Poland, still have lower TGCT rates than surrounding countries^{65,66}. The rapid rise in TGCT rates, as well as the variation between geographical locations, implicates exposure to one or more environmental toxicants as a key risk factor⁶⁵. For instance, it is believed that xeno-oestrogens and anti-testosterones negatively affect the development of Sertoli cells and Leydig cells causing a suboptimal environment for germ cell differentiation leading to reduced fertility and development of CIS cells³. Phthalates represent another possible environmental toxicant that could contribute to the incidence of TGCTs. Indeed, exposure to these toxins is known to be highly variable between different countries and this could, in part, contribute to geographical differences in cancer rates⁶⁵. Additionally, high levels of phthalates have been documented in the blood of mothers whose sons developed TGCT⁶. Similarly, rats exposed to phthalates exhibit symptoms similar to testicular dysgenesis, which is also associated with a high risk of TGCT⁶⁵. The possibility of toxicant exposure leading to TGCT has led to the investigation of detoxification mechanisms in cancer sufferers and their mothers⁶⁶. While the power of this study is compromised by the small sample size, the analysis did demonstrate a link between specific cytochrome p450 polymorphisms and an increased rate of TGCT development⁶⁶.

The development of type II testicular germ cell tumours

TGCTs originate from CIS cells, which are found on the basement membrane of abnormal seminiferous tubules that usually lack normal spermatogenesis. CIS cells were first identified in 1972 in biopsies of infertile men⁶⁷, and a causal link was subsequently established when, during follow-up, the men with this lesion developed tumours while control subjects exhibited no cases of TGCT⁵³. Furthermore, the incidence of CIS is about equal to that of type II TGCTs, suggesting that CIS cells will eventually develop into overt TGCT³⁶.

Morphologically, CIS cells resemble primordial germ cells/gonocytes and express a number of similar markers, in that they lack imprinting and express OCT 3/4, PLAP and c-KIT^{36,68}. In fact, the transcriptome of CIS cells is extremely similar to that of isolated normal human gonocytes, demonstrating a close relationship between these cell types¹⁶. CIS cells appear to be blocked from differentiating and entering spermatogenesis³. Instead they accumulate within the seminiferous tubule to become an intratubular lesion termed either seminoma or non-seminoma (embryonal carcinoma)^{3,36}. Interestingly, seminomas can later reprogram to become non-seminomas³⁶. These intratubular lesions proliferate to fill the lumen of the seminiferous tubule and rely on Sertoli cell-derived growth/survival signals and Leydig cell production of testosterone³⁶. Once these cells break their dependence on external signals they become overt testicular cancer³⁶.

Invasive TGCTs are characterised by a gain in copy number of the short arm of chromosome 12^{3,53}. Within this chromosomal region there are several genes encoding candidate virulence factors, including NANOG, KRAS2 and BCAT1; however, their role has yet to be confirmed³. BCAT1 for example, is only expressed in embryonal carcinoma and not in other germ cell lesions, indicating that the role of the genes on chromosome 12 may vary depending on the tumour type³. There are several markers which identify seminoma and non-seminoma tumours; for example, OCT 3/4 and NANOG are expressed in both seminoma and embryonal carcinoma, while SOX17 is specific to seminoma tumours and SOX2 is only expressed in non-seminoma⁶⁹. In extended culture, ES cells gain excess copies of chromosome 12, but they do not seem to exhibit malignant characteristics, indicating that the gain of chromosome 12 is not the sole initiation factor for virulence in TGCTs³.

Possible mechanisms of germ cell cancer specification

Several risk factors have been identified in a mouse model of teratoma formation (strain 129/Ter)⁷⁰. These include the continued expression of pluripotency markers and proliferation as well as premature differentiation, e.g. precocious entry into meiosis. Usually pluripotency factors such as NANOG, SOX2, and OCT 3/4, are down-regulated following cell cycle arrest at E13.5, but the germ cells of Ter mice continue to proliferate and express NANOG at E15.5⁷⁰. In this mouse model, PGCs also appear to prematurely differentiate with the detection of cyclin D1 as early as E13.5⁷⁰, compared to its normal expression in spermatogonia at postnatal day 4⁷¹. It is possible that a similar mechanism occurs for the development of CIS cells considering that CIS cells express markers for primordial germ cells including PLAP, OCT 3/4, and c-KIT, long past embryonic development³. Concurrent with this delayed

maturation is the expression of meiotic genes including NOTCH and SYCP3 in both CIS cells as well as seminoma cells⁶¹. Therefore maintenance of pluripotency in primordial germ cells while undergoing a defective maturation process, which may include premature activation of meiosis, could underpin the specification of these cells as CIS and tumorigenic (Figure 1).

Metastasis

Patients with stage I TGCTs and a concomitant high risk of metastasis are destined to undergo aggressive surgery and chemotherapy³. So far, vascular invasiveness, percentage of embryonal carcinoma and the proliferation index, have been the best predictors of metastasis risk. However, more recently the chemokine-mediated CXCR4 pathway has demonstrated some promise in metastasis prediction - with tumours containing localised high CXCL12 expression being less likely to metastasise^{3,72}. Gilbert and colleagues⁷² examined TGCTs and found that seminomas expressed higher levels of CXCR4 transcript and protein, than normal testis, but this trend was not maintained in non-seminomas. In addition Gilbert *et al.*⁷² demonstrated that a seminoma cell line (TCam2) migrates in response to CXCL12 α , via activation of the MAP kinase pathway. However, the non-seminoma cell line 2102EP does not, which is not surprising, as this cell line does not express CXCR4. We have independently looked at the expression of CXCL12 and CXCR4 in TGCTs (McIver SC, Loveland KL, Roman SD, Nixon B, Kitazawa R, McLaughlin EA, unpublished observations) and confirmed that CXCR4 mRNA was overexpressed in seminomas. However, at the protein level we did not find elevated levels of CXCR4 when its expression was examined via immunohistochemistry. Additionally, the tight correlation between CXCL12/CXCR4 with MAP kinase activation that is found in normal testis was abolished in the TGCT samples. We were able to confirm that both CXCL12 α and β caused cell migration in the seminoma cell line (TCam2) while no migration response was observed in the CXCR4 positive non-seminoma cell lines 833ke and NTera2/D1. Therefore the expression of CXCL12 and CXCR4 is more likely to be a better indicator of the possibility of seminoma metastasis rather than non-seminoma.

Treatment resistance

Generally seminoma cells (a category that includes CIS cells) are extremely sensitive to irradiation as well as cisplatin-based chemotherapy drugs, while non-seminomas only respond to chemotherapy³. However, the mature teratoma components of non-seminoma tumours are resistant to DNA damage therapies, which is consistent with the loss of embryonal cell characteristics these tumour exhibit³. Although the common chemotherapy and radiotherapy treatments are very effective and maintain quality of life, they can lead to infertility, hypogonadism and retrograde ejaculation, which new treatment options should seek to avoid^{2,15}. Both seminoma and non-seminoma tumours can become resistant to traditional oncology treatments³. Interestingly, evidence suggests that the methylation status of the cells DNA controls the expression of specific genes, such as c-FLAR, which inhibits caspase-dependant apoptosis, thus imparting resistance to cisplatin treatment³. Several other genes have been implicated in cisplatin resistance including Cyclin D1³. Cyclin D1 is expressed in murine germ cells from post natal day 3, which coincides with the appearance of spermatogonia in testis and

therefore may control the proliferation and differentiation of these cells⁷¹. The differentiation and abnormal proliferation of gonocytes is essential to the development of CIS, and therefore Cyclin D1 may play a key role in CIS evolution.

Role of miRNA in testicular germ cell tumours

A particularly exciting recent finding is that different types of TGCTs can be distinguished on the basis of their distinct miRNA expression profiles. For example, miR-122a is only expressed in yolk sac tumours^{69,73}. Also the regulator of miRNA maturation, LIN28, has been shown to be expressed in PGCS, gonocytes and pre-spermatogonia CIS, seminoma and non-seminoma, where it regulates totipotency, and functions upstream of the tumour/pluripotency transcription factors OCT1/3 and NANOG³¹.

The tumour suppressor p53 generally controls the exit from the cell cycle upon DNA damage, to allow for either repair or apoptosis. It follows that p53 mutation is usually a key step in tumorigenesis; however, in TGCTs p53 is only rarely inactivated⁷⁴. Voorhoeve *et al.*⁷⁵ provided an explanation for this phenomenon when they determined that the presence of microRNAs; miR-372 and miR-373; conveyed a growth advantage and protected against the cellular senescence response to DNA damage, in the presence of wild type p53. Analysis of the molecular basis for this effect revealed that these miRNA molecules act in conjunction with RAS downstream of p53 in order to mimic the phenotype of inactivated p53⁷⁵. Furthermore, the authors identified LATS2 (Large tumour suppressor homology 2) as a potential target of p53 signalling given that LATS2 deletion accelerates cellular proliferation and tumour development. This can be phenocopied in some seminomas, which do not overexpress miR-372 or miR-373, by mutations in LATS2. The LATS2 protein inhibits cyclin E/CDK2 activity and thereby arrests the cell cycle⁷⁵. Later studies found that this miRNA cluster is exclusively over expressed in almost all seminomas and most embryonal carcinoma tumours⁷⁴.

In addition to their role in the tumorigenesis of TGCTs, miRNA have an essential role in the development of primordial germ cells and in spermatogenesis⁷⁶. Therefore we decided to examine the change in the miRNA expression profile between postnatal gonocytes and spermatogonia in mice in the hope of gaining a better understanding of this essential step in both normal spermatogenesis and CIS development. This approach led to the identification of seven differentially expressed miRNA molecules (three of which were up regulated; miR-136, -743a and -463*, and four down regulated miR290-5p, -291a-5p, -294* and -293) during this developmental phase. Analysis of their potential targets indicated that these miRNA molecules were likely to impact the PTEN and Wnt/ β catenin signalling pathways⁷⁷ as well as the CXCR4 signalling pathway previously implicated in TGCT metastasis (McIver SC, Loveland KL, Roman SD, Nixon B, Kitazawa R, McLaughlin EA, unpublished observations). The PTEN tumour suppressor is known to inhibit PI3K signalling to negatively regulate cell growth and therefore its suppression causes increased proliferation and potentially tumorigenesis. The loss of PTEN is also associated with the transformation of CIS cells into overt cancerous tumours⁷⁸. Furthermore, increased

relapse rate in TGCTs has also been found to be associated with the loss of PIK3IP1, an additional negative regulator of PIK kinase⁷⁹. Both the PTEN and Wnt/ β catenin pathways converge to control the function of Cyclin D1, which is expressed in highly proliferative TGCTs where down-regulation instigates cell cycle arrest⁸⁰, and is associated with chemotherapy resistance in these tumours³. Indeed, on this basis, the control of Cyclin D1 activity has been suggested as a potential treatment target⁸⁰. Subsequently we selected the three most highly differentially expressed miRNA (Mir-743a, -291a-5p, -293) and instigated a strategy to directly identify their targets. The use of a pull down approach pioneered by Orum and Lund⁸¹ in which the miRNAs were used as bait, and bound mRNA analysed by microarray, revealed a significant number of predicted and non-predicted targets bound to each miRNA (McIver SC, Roman SD, Nixon B, McLaughlin EA., unpublished observations).

Interestingly the targets identified were postulated to regulate key germ cell migration and proliferation pathways controlled through PDGF⁸² and RAC⁸³ signalling (McIver SC, Roman SD, Nixon B, McLaughlin EA, unpublished observations). PDGF signalling is known to play a role in gonocyte migration and proliferation^{82,84}. In addition, PDGF signalling has been shown to work in conjunction with oestrogen signalling⁸⁴, a mechanism that is easily hijacked by the presence of xeno-oestrogens. In fact gonocytes have been shown to be more sensitive to xeno-oestrogens than endogenous ligands⁸⁴. This has implications for the development of CIS because high concentrations of xeno-oestrogens are associated with increased TGCT risk³. RAC on the other hand has been shown to be essential for the transmigration of spermatogonial stem cells through the blood-testis barrier during testis colonisation assays⁸³. Using a knockdown assay as an alternate technique to determine the targets of miR-291a-5p, -293 and -743a we identified one promising gene, IGFBP7 (Insulin-like growth factor binding protein 7) (McIver SC, Roman SD, Nixon B, McLaughlin EA, unpublished observations), which is known to act as either a positive (e.g. in glioma⁸⁵) or negative regulator (e.g. in breast and liver cancer^{86,87}) of cell proliferation and migration depending on the environment in which it is found. Therefore the disrupted expression of IGFBP7 by aberrantly expressed miRNAs during the gonocyte to spermatogonia transition could have implications for their growth and differentiation.

The miRNA molecules found to be down regulated during gonocyte differentiation into spermatogonia all belong to the miR-290 family. This cluster of miRNA is highly expressed in embryonic stem cells (ES) and is known to be a key regulator of ES cell pluripotency, controlling the expression of OCT4, SOX2 and NANOG⁸⁸⁻⁹⁰. Additionally, upon knockout of the miR-290 cluster, aberrant migration of germ cells has been observed with significantly fewer primordial germ cells reaching the bipotential gonad⁸⁸. Comparatively less is known about the miRNA molecules that were found to be upregulated during gonocytes differentiation. miR-136 is proposed to be a tumour suppressor in glioma and is capable of targeting the anti-apoptosis genes AEG-1 and BCL-2⁹¹. miR-743a on the other hand is involved in oxidative stress responses⁹². On the basis of these data, as well as our own observations of miRNA involvement in key germ cell regulatory pathways⁷⁷, it appears likely that the changing miRNA

profile between postnatal gonocytes and spermatogonia could have a fundamental role in the development of CIS within the testis.

Concluding remarks

Research into both the genetic and environmental factors that predispose an individual to type II TGCTs has been hampered by the lack of a suitable animal model for the study of this tumour type. Mouse models are suitable for the study of type I neonatal TGCTs and canines exhibit type III TGCTs caused by an expansion of type B spermatogonia. These models offer a number of advantages in terms of allowing genetic screens and treatments to be conducted to enhance our understanding of these cell types³⁶. In contrast, the study of seminoma and non-seminoma tumours is restricted to cell lines and, in the case of seminoma, these are scarce³⁶, severely limiting explorative experiments to study these tumour types. Type II TGCTs have been extensively characterised at the molecular level resulting in the identification of several predisposition genes, tumour markers, as well as the mode of tumour progression³. However, despite our extensive knowledge of mature tumours, the exact triggers for the development of CIS cells remain unknown. Unfortunately, until these mechanisms are identified, therapeutic interventions are limited. Current treatment techniques are very effective but their side effects often include a loss of fertility, a concerning fact given that these tumours primarily arise in men of reproductive age¹⁵. Therefore, a better understanding of the cellular mechanisms underlying germ cell development, are vital to establish novel treatments that are capable of preserving fertility in patients.

Author contributions

SM performed the initial research. SM, EM and BN drafted the manuscript. SR and KL performed critical review and editing of the manuscript. All authors provided input to the revision of the article and have agreed to the final content.

Competing interests

No competing interests were disclosed.

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Approved: 28 May 2013

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This article is a review discussing testicular germ cell tumours and how they may be triggered with an emphasis on miRNA expression. There are a few minor grammatical and spelling errors throughout this review but these do not impact on the quality of the manuscript. I would like to point out however that my area of expertise is primarily miRNA biology and I do not have a great background in TGCT.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.



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Approved: 21 February 2013

Referee Report: 21 February 2013

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.
