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Review

New directions in testicular cancer; molecular determinants of oncogenesis and treatment success

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Abstract

Metastatic testicular cancer is highly curable with conventional cytotoxic drugs. This is in contrast to most other metastatic solid tumours which can only be palliated with chemotherapy achieving only a modest impact on overall survival. If we could understand at the molecular level why chemotherapy is so effective in the treatment of testicular cancer, we may be better able to move other forms of metastatic cancer into the curable bracket. Most cytotoxic drugs appear to induce cell death by activating intracellular apoptotic mechanisms. Thus, the ability of a cancer to activate and execute such mechanisms in response to treatment is paramount in determining the effectiveness of chemotherapy. The basic study of cancer molecular biology is providing some insight into the proteins involved in this process and the ability to apply this information to actual human tumours is essential to rationalise clinical treatment failures at a molecular level. Testicular cancer provides an excellent model system in this analysis. Whereas there are large numbers of patients that are cured by chemotherapy, there are some whose cancers become resistant to treatment. An understanding of testicular cancer molecular biology may allow the identification of the genes regulating such a crucial behavioural switch. It may then be possible to manipulate specific signalling pathways to overcome drug resistance. This review focuses on recent developments in our understanding of the molecular biology of testicular cancer. A number of key players have been implicated including p53, pRb, cyclin D2, p INK proteins, c-kit and the bcl-2 family of proteins. The exact manner by which cellular transformation occurs has still not been established, but it is clear that many of the above proteins also have important roles in normal spermatogenesis. This is a developmental phase when the generation of genetic diversity is at a premium, but when selective apoptotic mechanisms are paramount. We discuss why this may be relevant to the behaviour of germ cell tumours and address possible reasons why they can become resistant to conventional therapy.

Keywords: Seminoma; Teratoma; Apoptosis; Chemo-sensitivity; p53; bcl-2; Cyclin; Retinoblastoma

1. Introduction

Metastatic testicular cancer can be cured with chemotherapy in around 85% of cases and as such it is a gratifying disease to treat especially in such a young group of patients. Clearly oncologists would be thrilled at the prospect of being similarly upbeat in the treatment of other solid tumours. Unfortunately, this is not often possible and metastatic disease is mostly treated palliatively with only modest expectations of improving overall survival. There is therefore an understandable urge to rationalise the success of chemotherapy in testicular cancer and apply it to other tumour types.

The molecular biology of cancer has become increasingly well understood over recent years and this has been coupled with an insight into the mechanisms through which chemotherapeutic agents kill cells [1]. Most cytotoxic drugs work through the interuption of critical cellular events such as DNA synthesis and mitosis. Any growing cell must complete such activities in a particular sequence and failure to do so is detected at specific phases of the cell cycle known as checkpoints. Drug induced perturbation of cellular growth events detected at such checkpoints forces cells into apoptosis resulting in a reduced tumour bulk. The drugs associated with a successful outcome in testicular cancer

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include cisplatin and etoposide, both of which act as DNA damaging agents.

These drugs are not effective in all tumour types and indeed a minority of testicular cancers are resistant to chemotherapy in the first instance. In theory, a cell can be resistant to a drug due to a number of mechanisms (see Fig. 1). For example, it can reduce drug accumulation within the cell either by preventing access or promoting efflux via the cell membrane. However, if the drug accumulates to functionally significant levels then there are additional determinants of whether the cell will be forced into apoptosis. Firstly, the cell must be able to detect the damage that the drug has caused; secondly, if the damage can be successfully repaired then the checkpoint can be passed; and thirdly, additional signals can allow the cell to survive even if the damage has been detected, but cannot be fully repaired.

The identification of the proteins involved in determining these behaviours will be crucial to rationalise treatment success. Testicular cancer provides an attractive model in this analysis. The majority of cases respond well to chemotherapy, but there are a significant minority that are either resistant at first presentation or relapse in a chemo-resistant form after first-line chemotherapy. As such, there is heterogeneity in the chemo-sensitivity of this disease which will be reflected in the specific molecular characteristics of the different tumours. An understanding of the molecular biology of testicular cancer should therefore provide valuable information with regard to the determinants of chemotherapeutic efficacy.

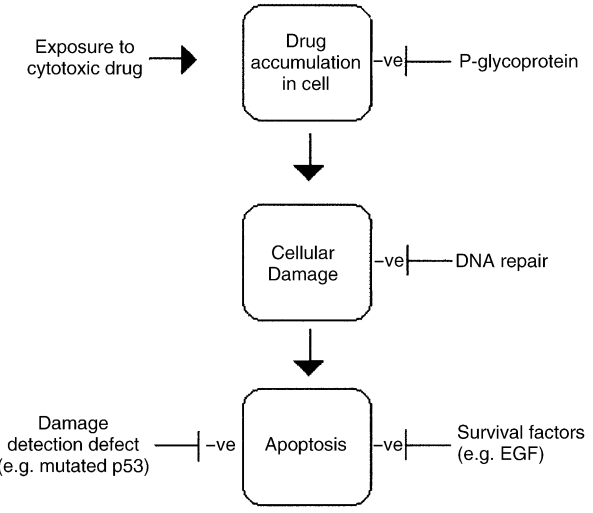

Fig. 1. Possible mechanisms of drug resistance in testicular cancer. Tumour cells (represented as a box) which are exposed to cytotoxic drugs can incur molecular damage that is detected at cell cycle checkpoints and can lead to apoptosis. However, there are a number of mechanisms (not all depicted here) that can help the tumour cell survive the insult. It can prevent drug accumulation within the cell (for example, by expressing high levels of a membrane protein that mediates drug efflux such as the multidrug resistance phenotype P-glycoprotein), or repair damage that has occurred (by expressing high levels of DNA repair proteins). In addition, mutation of proteins such as p53 may allow damage to go undetected. Activation of growth factor receptor pathways such as the epidermal growth factor receptor (EGFR) can provide signals that allow the cell to survive even if the damage is detected and remains unrepaired.

Germ cell tumours (GCTs) arise in both males and females, but for the purposes of this review, we will focus on testicular cancer. The main pathological subtypes of testicular cancer in adults are seminomatous and non-seminomatous germ cell tumours (NSGCT) (see Ref. [2] for a recent review). It is now generally accepted that both of these arise from a common progenitor lesion of carcinoma *in situ* (CIS). The cells within this lesion are derived from the primordial germ cell and as such the term CIS is somewhat misleading. The exact stage of development of the germ cell progenitor and the manner by which this transformation occurs is somewhat uncertain and is a topic of lively debate. This issue has been covered in a number of excellent recent review papers [3,4].

This review examines recent advances in the molecular biology of testicular cancer in relation to two important biological concepts. The first is the close relationship that appears to exist between normal tissue differentiation pathways and oncogenesis such that transformation of cells often appears to involve aberrant activity of proteins crucial to normal differentiation decisions. The second is the observation that some of the proteins involved in oncogenic transformation are themselves involved in determining the cellular response to cytotoxic drugs. By linking these two concepts, we will discuss the possibility that the intrinsic chemo-sensitivity of GCTs may be, in part, a consequence of the differentiation pathways followed by their normal tissue counterparts.

2. Issues in chemo-sensitivity

Most cytotoxic drugs kill cells by activating pathways which result in a commitment to apoptosis. The crucial decision as to whether to continue proliferating, growth arrest, or enter apoptosis is made at well defined points in the cell growth and division pathways called cell-cycle checkpoints. Only the decision to enter apoptosis will result in the eradication of the malignant clone. A commitment to apoptosis is determined by the relative balance of pro-apoptotic versus survival signals that the cell receives. Chemotherapy should provide a pro-apoptotic signal, but ultimately the ability to act on these signals depends on the integrity of the apoptotic pathway and the ability to direct the cell towards it. Two genes that have been consistently associated with this function in human cancers are *bcl-2* and *TP53* and as such it is no surprise that they have received particular attention in testicular cancer research.

2.1. Bcl-2

Bcl-2 itself has long been recognised as an inhibitor of apoptosis in a number of different tumour types. Its overexpression can protect cells from apoptotic stimuli and it is associated with the preservation of the malignant clone. More recently, it has become apparent that bcl-2 is one of a family of proteins that act in combination to either promote or inhibit apoptosis according to which family member dominates in a particular cell (see Fig. 2). Thus, for example two family members, bcl-X_L and bax, act antagonistically; bcl-X_L tends to inhibit apoptosis whereas elevated levels of bax tends to promote it (see Ref. [6] for review). In the clinical setting, high bcl-2 levels and low bax levels have been associated with a poor response to chemotherapy in a number of human cancers. Testicular tumours appear to contain very low levels of bcl-2 and relatively high levels of bax providing a possible explanation for the intrinsic sensitivity to chemotherapy [7]. In such a model one might expect that artificial overexpression of bcl-2 would confer relative resistance to chemotherapy-induced apoptosis. To test this, bcl-2 was expressed at high levels in a human GCT cell line but, surprisingly, this led to sensitisation of these cells to chemotherapy-induced apoptosis. A possible explanation became apparent when it was shown that overexpression of bcl-2 led to the downregulation of endogenous cellular levels of another bcl-2 family member, bcl-X_L [5]. As bcl-X_L itself normally acts to protect cells from apoptosis it is possible that downregulation of its expression is responsible for the paradoxical pro-apoptotic effect of bcl-2 (see Fig. 2). There is therefore a complex interplay between proteins within the *bcl-2* gene family and their relative ability to inhibit apoptosis may be, at least partly, tissue-specific. The above data would support a more important role for bcl-X_L as opposed to bcl-2 in protecting GCTs against programmed cell death, but there is no data relating to bcl-X_L as a determinant of response to chemotherapy.

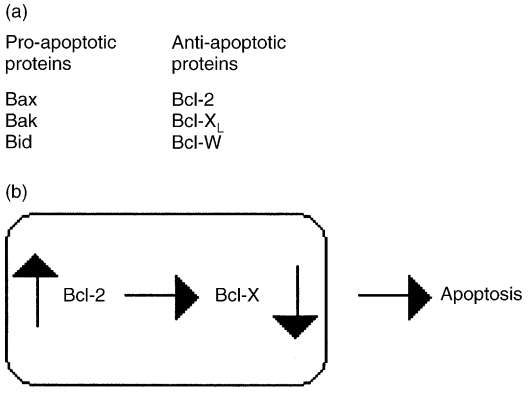

Fig. 2. The Bcl-2 gene family and apoptosis. (a) The bcl-2 gene family encode proteins that have a tendency to either induce cells into apoptosis (bax, bak, bid) or protect cells from apoptosis (bcl-2, bcl-X_L, bcl-W). (b) is a schematic representation of the data of Arriola and colleagues. Here the *bcl-2* gene was overexpressed in germ cell tumour (GCT) cells (represented as a rectangle) to see if this protected them from apoptosis. Surprisingly, this resulted in increasing sensitivity to apoptosis. Further analysis showed endogenous levels of another anti-apoptotic protein, bcl-X_L, were reduced in the presence of bcl-2 overexpression. Thus, bcl-X_L levels may be more important than bcl-2 in protecting GCTs from apoptosis.

2.2. p53

The role of p53 in testicular cancers has also been extensively investigated. *TP53* is a tumour suppressor gene and its activity is crucial for cells to be appropriately monitored during the cell cycle. The protein acts as a transcriptional activator that can be upregulated by a number of signals, particularly related to DNA damage. Once activated, it stimulates the expression of a number of genes that co-ordinately force cells into either cell cycle arrest or apoptosis. In order to avoid apoptosis, there is therefore a strong selection for malignant cells to neutralise *TP53* function and it is mutated to an inactive form in at least 50% of all known human cancers [8]. However, p53 is present in its wild-type form in most testicular cancers raising the question as to how these cells overcome p53-mediated apoptosis. One possible mechanism is derived from data suggesting that the wild-type p53 protein, seen in some testicular cancers, may be relatively non-functional. Lutzker and colleagues [9] showed p53 baseline activity, as measured by its ability to act as a transcriptional activator, was very low in teratoma cells, despite relatively high wild-type protein levels. Hence, the presence of p53 does not necessarily translate directly into activity and it is theoretically possible that a relatively non-functional p53 protein in the growing testicular cancer could mimic a mutated gene and therefore facilitate passage through the G1 checkpoint.

An important point from these experiments was that endogenous cellular p53 became activated by the addition of etoposide, a cytotoxic drug which indirectly damages DNA [9]. The mechanism of this activation is not clear, but other *in vitro* data has clearly demonstrated the importance of posttranslational modifications, such as phosphorylation, in the functional integrity of p53 [10]. Thus, a relatively inactive protein in the growing tumour cell may become activated by drug-induced DNA damage to facilitate cytotoxic-induced apoptosis and exquisite chemo-sensitivity. In such a scenario, one may predict that the development of chemotherapy resistance in GCTs may be associated with the generation of mutations in *TP53*. This is supported by the data of Houldsworth and colleagues [11] who investigated the *TP53* status of a panel of GCT specimens from 23 patients with tumours that were refractory to chemotherapy. Specimens from 4 of these patients had developed mutations in p53 and a cell line

derived from one of these was shown to be resistant to cisplatin-mediated apoptosis *in vitro*.

It would be of interest to reintroduce a wild-type *TP53* gene into these cells to observe if sensitivity to chemotherapy is restored. Thus far, this question has only been addressed in mouse teratocarcinoma cell lines. Here a cell line lacking p53 was not sensitive to DNA damaging drugs, but the introduction of p53 to the same cell line restored sensitivity in a dose-dependent manner, so that the higher the level of p53 the more sensitive the cells became [12].

Collectively, this data creates a strong argument for the role of p53 in mediating response to treatment. However, one must note that Houldsworth and colleagues [11] described a number chemo-refractory tumours that were not mutated for p53. In addition, the absolute role of p53 has been questioned recently by Kersemakers and colleagues [13] who found that the development of *TP53* mutations did not appear to be an important event in cisplatin resistance. The authors concluded that the presence of a high level of wild-type p53 was unlikely to explain the intrinsic chemosensitivity of GCTs. It is therefore clear that alternative mechanisms of resistance must exist. Interestingly, it appears that even in the presence of wild-type protein, p53 related regulatory pathways may still be involved in determining chemotherapy resistance. This has been suggested with *in vitro* experiments using two related human embryonal cell lines that differ in their expression of a retinoic acid receptor [14]. Here both cell types expressed wild-type p53 protein equally well, but the addition of cisplatin led to more effective activation of p53 in the cell line expressing the retinoic acid receptor. This in turn conferred a significantly increased apoptotic sensitivity to cisplatin in this cell line. The precise interrelationship of p53 activity with the retinoic acid receptor is not entirely clear. However, this experiment illustrates how mutations in regulatory pathways impinging on p53 may mediate changes in treatment sensitivity in the presence of a wild-type p53 protein.

2.3. MDM-2

A major regulatory interplay, characterised in other tumour systems, occurs between p53 and the ubiquitin ligase mouse double minute-2 (mdm-2) protein. p53 has been shown to stimulate the expression of mdm-2 which itself acts to promote the destruction of the p53 protein, thus mediating a negative regulatory feedback loop for p53 [15]. Activation of mdm-2 expression would therefore provide an additional mechanism to nullify p53 activity. There is circumstantial evidence of a role for mdm-2 in testicular carcinogenesis. Datta and colleagues [16] have examined a number of tumour specimens and found mdm-2 was expressed in the majority of invasive specimens, but only 7% of premalignant CIS lesions indicating mdm-2 may be important in the progression to the invasive phenotype. Additional work from Eid and colleagues found some correlation between the level of mdm-2 expression and how aggressively the tumour behaved [17].

2.4. DNA repair machinery

An additional issue in the pathogenesis and treatment of germ cell cancers is the status of the DNA repair machinery. If the cell is unable to repair damage inflicted upon it by cytotoxic drugs then it should be more inclined to enter apoptosis at cell cycle checkpoints where this is monitored. Some GCT cell lines have a reduced level of XPA, one of the proteins required for nucleotide excision repair (NER) of damaged DNA. NER is the major mechanism through which cisplatin-induced DNA damage is repaired and extracts from such testicular tumour cells are deficient in their ability to repair cisplatin-induced DNA damage [18]. This deficiency may therefore be important in the success of cisplatin-based treatments in these tumours. Indeed, other less chemo-sensitive tumour cell lines, from bladder for example, have higher levels of XPA and DNA repair activity. The addition of XPA to GCT extracts establishes their ability to mediate NER [18]. However, to fully assess the role of XPA in the chemo-sensitivity of GCTs it would be of interest to assay XPA levels in GCTs that have become resistant to platinum treatments and also test whether artificial over-expression of XPA in germ cell tumour cell lines can confer chemo-resistance.

The role of the AP endonuclease (Ape/ref-1) which is involved in DNA base excision repair has also been examined. Robertson and colleagues [19] found this protein in GCT specimens and when it was over-expressed in experimental sytems it appeared to confer resistance to cytotoxic agents. In a follow-up study, published in abstract form, the same group presented data on a further series of 80 tumour specimens indicating the cellular location of Ape1 was important. Although the data did not reach statistical significance, it appears a high level of protein located in the cytoplasm may be associated with a poor chemotherapeutic outcome [20].

More recently, chemotherapy resistance has been correlated with the degree of microsatellite instability seen in GCTs. Microsatellite instability represents alterations in the lengths of short repetitive sequences in the genome and has been studied as a prognostic and predictive factor in ovarian and colonic cancer. The phenotype is the result of inactivation of one or more genes involved in DNA repair such as *MLH1*, *MSH2* and *MSH6*. Mayer and colleagues [21] studied this phenotype in relation to chemotherapy resistance in 100 unselected GCT cases and 11 which were resistant to

cisplatin-based chemotherapy. When they examined the expression of the above proteins in tumour specimens, they found resistant specimens were much more likely to have inactivation of at least one (45% versus 6%, $P=0.001$) or more than one (36% versus 0%, $P=0.001$) of the loci. Although only a retrospective study, this phenomenon certainly merits further investigation. The reason why MSI should be related to chemotherapy resistance is not entirely clear, but it is possible that a defect in this repair mechanism may allow the accumulation of mutations that would produce the resistance phenotype.

2.5. Survival signals

Chemo-sensitivity also appears to relate to the presence of survival signals as some cancers are better able to avoid apoptosis after cytotoxic drug insult, even though the drug has successfully induced damage. The determinants of cell survival are not well defined, but it appears that growth factors often associated with a mitogenic function such as the epidermal growth factor (EGF) can also act as a survival factor. The expression of the EGF receptor in many cancers is associated with a poorer prognosis and this may partly be due to a reduced cellular tendency to undergo apoptosis [22]. Recent work has also indicated that this may be important in testicular cancer. Moroni and colleagues [23] examined EGF receptor expression in a number of testicular cancer specimens and demonstrated positive expression only in specific tumour sub-types, choriocarcinomas and choriocarcinoma components of mixed GCT specimens. None of the pure seminomas or embryonal carcinomas expressed the EGF receptor. Interestingly, the presence of choriocarconoma components in mixed GCTs is a poor prognostic indicator with a poor response to conventional chemotherapy.

Recently, small molecules and antibodies that inhibit EGF receptor activity have been developed [22]. When such agents are given in combination with chemotherapeutic agents or radiotherapy, they enhance apoptotic-mediated cell kill in a number of tumours that express the EGF receptor. This may reflect a loss of survival potential due to the neutralisation of the activated EGF receptor which would otherwise help endure the treatment insult. At present, there is limited and conflicting data on the association of EGF receptor expression and chemotherapy resistance. Kollmansberger and colleagues [24] examined 32 GCT specimens, of which 22 were classified as cisplatin-resistant. EGF receptor was expressed in less than 20% of samples and there was no association between receptor expression and sensitivity to chemotherapy. However, Madani and colleagues [25] retrospectively looked at a series of 23 NSGCT specimens from patients refractory to chemotherapy and found EGF receptor expression in 61%. Thus, it remains to be seen whether the EGF receptor inhibitors may have a role in the treatment of germ cell tumours, but there has been at least one anecdotal report of a chemoresistant cancer responding to trastuzumab (Herceptin), an antibody directed to another member of the EGF receptor family [26].

3. Disruption of developmental pathways in GCT oncogenesis

The process of oncogenic transformation involves the disruption of normal cell-cycle regulatory events and the differentiation processes that lead to normal tissue formation. Some growth regulation pathways are shared between different cell types but many differentiation steps will inherently vary between different tissues. It is theoretically easier for a cell to become cancerous by mutating a gene that is pivotal to its own differentiation fate as it is predetermined that such a mutation will be functionally relevant. Thus while certain oncogenic mutations will be shared between many types of cancers others will be more specific and should reflect the differentiation pathways followed by the tissue from which a particular cancer is derived.

It is now apparent that this principle holds true in GCTs and this is particularly well illustrated in genes controling the G1-S checkpoint. The full details of this control are complex and not fully resolved, but a subset of the molecular interactions are represented as a simplified schematic in Fig. 3. As discussed below a number of these proteins such as cyclin D2 and the cyclin-dependent kinase inhibitors appear to have roles in normal differentiation pathways as well as oncogenic transformation. Another protein that possesses such Jekyll and Hyde properties in testicular biology is c-kit. C-kit has recently been the target of biological therapy and inhibition of its activity is effective treatment in at least one other tumour type [27].

3.1. Cyclin D2 and the cyclin-dependent kinases

The cyclin D2 gene has received much attention in normal testicular development as well as in the pathogenesis of GCTs. It has an important role in cellular proliferation and its expression is tightly regulated throughout the cell cycle. When present it binds with other proteins known as cyclin-dependent kinases (cdk 4 or cdk 6) to form an active enzyme complex which facilitates passage of cells through the G1 cell-cycle checkpoint [32]. Cyclin D2 appears to be important in normal spermatogenesis as its expression is carefully regulated during normal germ cell development. For example, in newborn mice the protein can be detected only from days 7 to 13 in the postnatal spermatocytes after which expression is downregulated (Table 1).

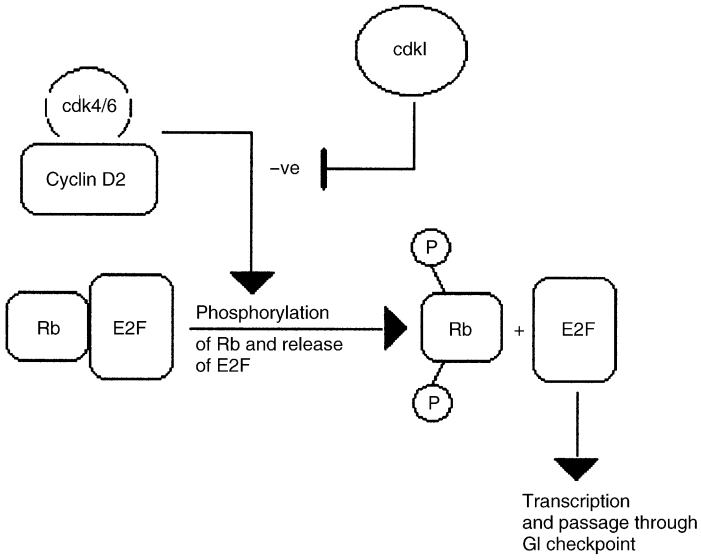

Fig. 3. The Retinoblastoma protein and the G1-S checkpoint. The key event here is the phosphorylation of the retinoblastoma tumour suppressor protein (Rb) that normally binds to and inactivates a transcription factor E2F. Phosphorylation causes a conformational change in pRb facilitating release of E2F that then stimulates transcription of key genes necessary for progression through the G1-S checkpoint. Phosphorylation is largely controlled by the interaction of three families of proteins, the cyclins, the cyclin-dependent kinases (cdks), and the cyclin-dependent kinases inhibitors (cdkIs) [37]. The cyclin family consists of a number of proteins with well established roles in cell cycle control. Within germ cell cancers cyclin D2 appears of particular importance. The expression of cyclin D2 protein expression is normally tightly regulated through the cell cycle so that it only achieves functionally relevant levels at the G1-S transition. At this point, it can bind with protein kinases dependent upon it for their activity, the cyclin-dependent kinases (cdk4 and cdk6). The active cyclin/kinase complex can then phosphorylate the tumour suppressor Rb causing it to release E2F. This process in turn can be negatively regulated by the cdkIs [36].

Deletion of the *cyclin D2* gene in transgenic mice results in no major abnormalities except a lowered testicular mass and decreased sperm counts [28,29]. This would indicate a particularly important role for this gene in testicular developmental pathways and thus it is a good candidate for disruption in testicular cancer.

Analysis of human cancer tissue has shown the *cyclin D2* gene is frequently amplified in germ cell tumours leading to a possible deregulation of the strict cell cycle control of its expression. The gene is located on the short arm of chromosome 12 and amplification is often achieved through the presence of an isochromosome comprising two fused p arms from chromosome 12 within which the gene lies. It is present regardless of histology or metastatic site and is also apparent in pre-malignant CIS implying it may be an important early event in oncogenesis (see Ref. [3] for review). This amplification likely to be relevant as it results in elevated mRNA levels. For example, Schmidt and colleagues [30] showed 69% of a series of 45 testicular GCTs overexpressed cyclin D2 mRNA by a mean factor of 8-fold. An excess of cyclin D2 protein would then be

Table 1
Summary of data implicating the involvement of candidate proteins in normal spermatogenesis and GCT development

Protein	Role in development	Role in oncogenesis
Cyclin D2	Expressed only on days 7–13 in mouse post-natal spermatocytes [28]	Gene amplified in GCTs [3]
	Gene knockout produces lowered testicular mass and sperm counts in mice [25]	mRNA over-expressed in GCTs [30]
p19 INK	Elevated expression in adult spermatocytes but not foetal germ cells [34]	Expression suppressed in CIS and invasive GCTs [34]
	Gene knockout leads to testicular atrophy in mice [33]	
Rb	Rb protein family differentially expressed during rat testis development [38]	Lowered expression in human GCT and CIS specimens versus normal testicular tissue [45]
		Variable Rb expression dependent on tumour differentiation [39]
c-kit	Expressed only until week 12 of development [41]	Activating mutation found in seminoma [42]

CIS, carcinoma *in situ*; GCT germ cell tumour.

available to form an active kinase complex with its partner proteins such as cdk4 thus facilitating passage through G1. Of interest, therefore Schmidt and colleagues also demonstrated that 41% of 51 GCT specimens overexpressed cdk4 by a factor of 6 and there was a statistically significant correlation for both proteins to be overexpressed in the same tumour specimen ($r^2 = 0.68$, $P = 0.000052$). Moreover, immunoprecipitation of tumour cell extracts containing high levels of cyclin D2 protein using a cyclin D2 antibody recovers both cyclin D2 with both cdk4 and cdk6 in a protein complex that exhibits protein kinase activity [31]. Together this provides persuasive data that overexpression of these proteins is functionally relevant in both spermatogenesis and GCT development.

3.2. Cyclin dependent kinase inhibitors

Cyclin-dependent kinase inhibitors (cdkIs) negatively regulate the activity of the cyclin/cdk complexes (see Fig. 3). They are therefore important in preventing inappropriate passage through checkpoints and loss of their activity has been associated with a number of different human cancers [32]. Like cyclin D2, the cdkIs have become increasingly implicated both in GCT pathogenesis and normal testicular development. Although the cdkIs form a large group of proteins, there is a family of four proteins, the INK 4 family of cdk inhibitors (p15 INK, p16 INK, p18 INK, and p19 INK), that appear particularly important in the testicular system. In transgenic mice experiments, the elimination of p19 INK4D produces testicular atrophy as the only obvious phenotype. In human tissue, p19 INK4D product is highly expressed in the spermatocytes of the adult testis, but is absent in foetal germ cells [33]. This provides reasonable grounds to suggest a particularly important role for this protein in testicular development. Data relating to a direct role in testicular oncogenesis are more circumspect. It is not expressed in either CIS or invasive germ cell tumour cells, hence providing circumstantial evidence that lack of expression may have a role in maintaining an undifferentiated oncogenic phenotype [34].

There is also limited evidence that the expression of another of the cdk inhibitors, p18 INK4C, appears to be important in GCTs. Immunohistochemical analysis of the protein shows it is expressed in CIS, but often not in seminomas and embryonal carcinomas [35]. This once again provides circumstantial evidence indicating that loss of function may be correlated with progression to the invasive phenotype.

Overall therefore, the data relating to the role cdkIs in testicular oncogenesis is less compelling than for cyclin D2 and cdk4. However, this is due to paucity of convincing causative evidence as opposed to the presence of negative data.

3.3. Retinoblastoma

The *pRb* tumour suppressor gene itself is clearly a target of mutation and it is often deleted or mutated to an inactive form in a variety of human cancers. It is a member of a family of proteins including p107 and p130 (see Refs. [36,37] for review). Its role in normal testicular development has been highly studied in the rat system. A recent analysis has shown a differential spatial and temporal pattern of expression of the various protein family members in rat testis development and established important roles for all three proteins in normal spermatogenesis including cell cycle control and apoptosis [38]. RNA analysis in human GCT and CIS specimens has revealed lowered levels of mRNA compared with normal testis tissue and this is confirmed by a virtual lack of protein. Interestingly, this did not reflect a grossly altered structure of the DNA coding regions, but instead likely relates to a potentially reversible transcriptional regulation through methylation of the promoter. Indeed the retinoblastoma protein appears to be differentially expressed according to the differentiation status of the tumour such that a number of teratocarcinoma specimens stain positively for Rb protein in comparison with the less differentiated negatively staining embryonal carcinoma specimens [39]. As such, the absolute functional importance of retinoblastoma expression and the relative roles of each family member in GCT development remains to be established.

3.4. C-kit

The *c-kit* proto-oncogene encodes a transmembrane receptor tyrosine kinase. Receptor tyrosine kinases form a group of proteins that include the EGF receptor family and have well established activity in human malignancies [40]. As for all the other proteins discussed in this section, c-kit appears to have a role in normal spermatogenesis as it is expressed in early foetal germ cells up to 12 weeks of gestation, but not beyond [41]. In addition, the protein has also been detected in CIS and seminoma cells reflecting a possible role in oncogenesis. Recently, an activating mutation of c-kit has been isolated from a germ cell tumour providing additional evidence that it may be important in the pathogenesis of seminoma neoplasms [42]. In addition, Madani and colleagues [25] showed c-kit expression (in an unmutated form) in 48% of a series of 23 tumour specimens from patients with chemotherapy-resistant NSGCTs. An inhibitor of c-kit, Imatinib mesylate (STI571), has been shown to mediate good clinical responses in the c-kit-expressing gastrointestinal stromal tumours (GISTs). These tumours are resistant to chemotherapy and were thus previously untreatable in the metastatic form [27]. Clearly this has important implications for

testicular cancer, particularly in those rare patients with chemo-resistant disease where STI571 may provide an alternative therapeutic strategy. However, it is important to note that in the case of GISTs, STI571 works most effectively in tumours that express a mutated form of the tyrosine kinase receptor and thus far mutations have only been detected in a very small minority of GCTs that express c-kit.

4. Conclusions

The work described support two important principles. Firstly, that proteins involved in normal developmental pathways can also play a role in oncogenic transformation and, secondly, that those proteins involved in oncogenesis can also be important in determining response to chemotherapy. By linking these two concepts, one can hypothesise that the unusual stage of development from which GCTs are derived is likely to contribute to their treatment success. If one looks at the biology of spermatogenesis and GCTs then there is a good grounding for such a theory. The developing gametocytes represent a meiotic phase where genetic diversification is occurring and indeed is essential for the continued evolutionary development that is inherent in sexual reproduction. It is therefore important for the cells to allow proliferative and diversification events to occur, but to subsequently monitor and eliminate any clones containing potentially unfavourable mutations that would otherwise form part of the reproductive germ line. Extensive cell death is a striking feature of spermatogenesis and the ability to rapidly switch from a proliferative to apoptotic state is likely to be an important property for this. Such a rapid switch could be mediated by a posttranslational modification of p53. We have already seen that p53 can be changed from an inactive to active form in GCT lines exposed to cytotoxic drugs and this likely reflects a property held by the germ cell progenitors from which they were derived [9]. It is therefore possible that rapid activation of an endogenous pool of p53 may be crucial for activating apoptotic pathways in normal spermatogenesis and also mediate the apoptotic response of GCTs to cytotoxic drugs. As such, the developmental requirements of spermatogenesis may determine treatment success in testicular cancer.

It may be of interest therefore that a comparable phase of development is also true for the progenitor cells of another chemo-sensitive tumour, diffuse large B cell lymphoma (DLBCL). Here it is thought the progenitor cell is derived from the germinal centre compartment of lymphocyte development. This is a phase where the immunoglobulin gene rearrangements have occurred and the cells are fine tuning their antibody binding specificity by somatic mutation of the hypervariable antigen binding region. The mutation process itself is random and thus the clones carrying antibody with the best fit for the antigen are subsequently positively selected and the other clones are eliminated. Thus, somewhat like the meiotic diversification of the germ cell, this is a period when generation of mutational diversity is at a premium, but in the context that strong apoptotic selection pressure can be subsequently applied to ensure only the appropriate cells survive. The detailed molecular biology mediating this remains to be fully resolved, but it is possible that this specific developmental stage might represent an important biological state for the apparent success of chemotherapy in this disease.

Recently, lymphocytes and lymphomas have been the subject of analysis of DNA microarray technology. This has produced molecular signatures characteristic for different phases of normal lymphocyte development and allowed comparison with the molecular profile of various lymphomas. As such, it appears that diffuse large B cell lymphoma is a heterogeneous entity and only a proportion carry a profile characteristic of the germinal centre developmental compartment discussed above [43]. When 5-year survival of DLBCL was analysed with respect to the molecular signature, those placed in the germinal centre-like subgroup had a significantly better prognosis than other molecularly defined subgroups (76% versus 16%, $P=0.01$). The 5-year survival for all DLBCL patients in this study was 52%. Thus, the treatment success in DLBCL may also be determined by the intrinsic developmental requirements of the progenitor cells from which they are derived.

We are at an exciting time in oncology as there is increasing evidence that the long haul of basic molecular cancer research is starting to translate into real therapeutic benefits. In testicular cancer, we have a head start as metastatic disease can often be cured with conventional cytotoxic agents. However, resistant disease does occur and this provides two major challenges. Firstly, to target specific molecular pathways not yet exploited by conventional cytotoxic drugs; the potential use Imatinib mesylate (STI571) in tumours that overexpress the c-kit tyrosine kinase is one example, but as the details of GCT oncogenesis become clearer, further approaches should become available. Secondly, to better understand the mechanism through which resistance and sensitivity to cytotoxics is mediated. The fact that so much metastatic disease is curable, but resistance does develop makes GCTs a particularly attractive model. Ultimately, DNA microarray analysis will facilitate a comprehensive molecular investigation of testicular cancer. It has already been used in a targeted fashion to identify overexpression of candidate genes in GCT development [44]. However, by comparing the molecular signatures of sensitive and resistant tumours with this technology it should also be possible to iden-

tify genes responsible for the development of chemoresistance. Once identified, the key therapeutic gain will only be achieved through manipulation of pathways through which these proteins operate. This is clearly some way off at present, but it is possible that lessons learnt in testicular cancer will be applicable to other tumour types.

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