Elucidating human male germ cell development by studying germ cell cancer

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Abstract

Human germ cell development is regulated in a spatio-temporal manner by complex regulatory networks. Here, we summarize results obtained in germ cell tumors and respective cell lines and try to pinpoint similarities to normal germ cell development. This comparison allows speculating about the critical and error-prone mechanisms, which when disturbed, lead to the development of germ cell tumors. Short after specification, primordial germ cells express markers of pluripotency, which, in humans, persists up to the stage of fetal/infantile spermatogonia. Aside from the rare spermatocytic tumors, virtually all seminomas and embryonal carcinomas express markers of pluripotency and show signs of pluripotency or totipotency. Therefore, it appears that proper handling of the pluripotency program appears to be the most critical step in germ cell development in terms of tumor biology. Furthermore, data from mice reveal that germline cells display an epigenetic signature, which is highly similar to pluripotent cells. This signature (poised histone code, DNA hypomethylation) is required for the rapid induction of toti- and pluripotency upon fertilization. We propose that adult spermatogonial cells, when exposed to endocrine disruptors or epigenetic active substances, are prone to reinitiate the pluripotency program, giving rise to a germ cell tumor. The fact that pluripotent cells can be derived from adult murine and human testicular cells further corroborates this idea.

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Normal germ cell development

Germ cells (GCs) are the progenitor cells of sperm and oocytes, which are a prerequisite for the development of new life. GC development is initiated early during embryogenesis. In mice, few cells of the proximal epiblast acquire GC competence by BMP2, BMP4 and BMP8B stimuli (Hayashi *et al.* 2007). These primordial germ cell (PGC) precursors escape the somatic differentiation program and are specified by upregulation of PRDM1/BLIMP1, PRDM14 and Fragilis (Hayashi *et al.* 2007, Saitou *et al.* 2012). The PGCs migrate along the developing hindgut to the genital ridges, which eventually differentiate into testes or ovaries. During this migration, PGCs undergo crucial changes, like a G2-arrest, global DNA demethylation and erasure of imprinted genes as shown in mice and partially for humans (Saitou & Yamaji 2012, Meyenn & Reik 2015).

After arrival in the genital ridge, the GCs undergo a transition, which prepares them for meiosis. This phase is denominated ‘licensing’ and is controlled by the RNA-binding protein DAZL (deleted in azoospermia like) (Lin *et al.* 2008). In the C57/Bl6 strain of mice, it was shown that DAZL-deficient PGCs migrate to the gonad, but fail

to respond to feminizing or masculinizing cues leading to a failure of oogenesis or spermatogenesis respectively (Gill *et al.* 2011).

As observed in mice, male sexual differentiation is initiated by the *Sry* gene (sex determining region on the Y chromosome), which triggers the expression of *Sox9* and therewith Sertoli cell differentiation that is essential for proper testis development (Kashimada & Koopman 2010). In females, ovarian development is supported by inhibition of the SOX9 signaling cascade via beta-catenin and FOXL2, respectively (Kashimada & Koopman 2010). The expression of CYP26B1, a member of the cytochrome p450 family, prevents developing male embryonic germ cells from a premature initiation of meiosis (Koubova *et al.* 2006, Kumar *et al.* 2011).

Data dealing with the molecular mechanisms leading to PGC specification in humans are scarce. However, studies have demonstrated that human and murine germ cells display a highly similar expression profile like *OCT3/4*, *NANOG*, *BLIMP1* and *TFAP2C*. However, two main differences have been reported so far. Using ‘*in vitro*’ differentiation protocols, SOX17 was identified as a key player of human PGC fate inducing *PRDM1* expression and downstream GC genes (Irie *et al.* 2015).

Vice versa, human PGCs lack the expression of the pluripotency marker SOX2, which is expressed in murine PGCs aside with OCT3/4 and NANOG (Perrett *et al.* 2008). Further, PRDM14, which is essential for murine PGC maintenance, shows only low levels of expression in human PGC-like cells and seminomatous Tcam-2 cells, which also share a similar gene expression profile with PGCs (Irie *et al.* 2015, Nettersheim *et al.* 2015, Sugawa *et al.* 2015, Tang *et al.* 2015).

In human males, undifferentiated GCs (A_{dark} -spermatogonia), which are discussed to be the testicular stem cells, settle down at the basement membrane of the developing seminiferous tubules (Clermont 1966). They give rise to A_{pale} -spermatogonia, which divide mitotically and differentiate into B-spermatogonia (de Rooij 2001). These diploid B-spermatogonia eventually initiate meiotic divisions of spermatogenesis. In a step-wise process, four haploid spermatids are formed out of each diploid B-spermatogonium. Accordingly, self-renewing testicular stem cells in mice are described as A_{single} -spermatogonia, which progress into A_{paired} - and A_{aligned} -spermatogonia (de Rooij 2001). The differentiation into mitotically active A_1 – A_4 spermatogonia marks the entry into spermatogenesis, giving rise to B-spermatogonia (de Rooij 2001). With the onset of puberty and increased retinoic acid (RA) signaling, B-spermatogonia develop into spermatocytes that undergo meiotic divisions to generate haploid spermatids (Bowles & Koopman 2007). These spermatids enter the process of spermiogenesis/spermatohistogenesis, putatively involving genes like *AEP1*, *GOPC*, *PICK1*, *MNS1* and *TAF7L* (Yao *et al.* 2002, Luk *et al.* 2006, Xiao *et al.* 2009, Zhou *et al.* 2013). Finally, the majority of histones are replaced by protamines resulting in DNA hypercondensation (Balhorn 2007). Development of the flagellum and the acrosome gives sperm cells their characteristic morphology, necessary for full functionality, i.e. motility and penetration of the egg respectively.

Germ cell malignancies

Germ cell tumors (GCTs) are subdivided into different types (I–V), depending on cell of origin, sex of the patient, age of diagnosis, growth/differentiation behavior and morphology (Oosterhuis & Looijenga 2005).

Type I GCTs occur during early childhood and present mainly as non-seminomas (embryonal carcinomas (EC), teratomas and yolk-sac tumors). They are thought to originate directly from a PGC or gonocyte and, therefore, do not contain the precursor lesion germ cell neoplasia *in situ* (GCNIS) (Fig. 1A).

Type II GCTs arise via a GCNIS (also referred to as: carcinoma *in situ* (CIS); intratubular germ cell neoplasia unclassified (IGCNU)) (Fig. 1A) (Berney *et al.* 2016). Although not formally proven, the generally accepted hypothesis is that GCNIS cells represent arrested PGCs, which had acquired genetic or epigenetic aberrations

Figure 1 (A) Model of GCT development from PGCs. (B) Venn diagram summarizing commonly shared gene expressions and cellular/molecular features between PGCs, GCNIS, seminomas and ECs.

(Skakkebaek *et al.* 1987, Hoei-Hansen *et al.* 2005) (Fig. 1A). Morphologically, GCNIS cells resemble PGCs, i.e. they appear round with a big, round nucleus and a clear cytoplasm and grow uniformly within the confines of the seminiferous tubules. GCNIS and PGCs further share a similar gene expression profile, i.e. expression of pluripotency-related genes *LIN28*, *OCT3/4* and *NANOG* as well as PGC markers *PRDM1/BLIMP1*, *TFAP2C* and *cKIT* (Fig. 1B). Within 5 (50% of cases) to 7 (70% of cases) years, GCNIS lesions eventually develop into a seminoma or a non-seminoma (Fig. 1A) (Giwerzman & Skakkebaek 1993).

Seminomas are considered to be the default developmental pathway of GCNIS, since they show only limited differentiation abilities (Oosterhuis & Looijenga 2005, de Jong *et al.* 2008). Like GCNIS and PGCs, seminoma cells display a big round nucleus and a clear cytoplasm, but appear more polygonal in shape. They further share the gene expression profile of PGCs, expressing the pluripotency markers *LIN28*, *OCT3/4* and *NANOG* and the germ cell markers *PRDM1*, *cKIT*, *TFAP2C* as well as *PLAP*. In addition, they express *SOX17* and *D2-40* (Fig. 1B). The non-seminomas initially present as ECs and display features of totipotency: They are able to differentiate into all three germ layers

(meso-, endo-, ectoderm) as well as extraembryonic tissues, thereby giving rise to teratomas, yolk-sac tumors and choriocarcinomas (Oosterhuis & Looijenga 2005) (Fig. 1A). ECs share expression of TFAP2C (variable), GDF3 (high in EC), DPPA3 (high in EC), OCT3/4 and NANOG with seminomas and/or GCNIS, but additionally express DNMT3B, DNMT3L, NODAL, CRIPTO, CD30 and SOX2 (Hoei-Hansen *et al.* 2004, Almstrup *et al.* 2005, Ezech *et al.* 2005, de Jong *et al.* 2008) (Fig. 1B). Furthermore, ECs show only weak to absent expression of cKIT as well as cytoplasmic localization of PRDM1 (Rajpert-De Meyts & Skakkebaek 1994, Leroy *et al.* 2002, Almstrup *et al.* 2005, Eckert *et al.* 2008) (Fig. 1B). Outside the testis, type II GCTs are found mainly along the body mid-line, the thymus and the brain (Utz & Buscemi 1970, Johnson *et al.* 1976, Bassetto *et al.* 1995, Oosterhuis & Looijenga 2005).

Common to the vast majority of GCTs is an amplification of the short arm on chromosome 12 (12p gain), mainly caused by isochromosome formation (Rosenberg *et al.* 1998, Oosterhuis & Looijenga 2005) (Fig. 1B). So far, 12p gain was detected predominantly in invasive GCT samples. Therefore, it is believed that amplification of this chromosomal region occurs during invasive growth of GCTs (Rosenberg *et al.* 2000, Summersgill *et al.* 2001). Nevertheless, amplification of 12p was also detected in some GCNIS cases in tubules adjacent to tumors, when the invasive process likely has begun (Ottesen *et al.* 2003). Since the amplified region on chromosome 12 encodes for the pluripotency- and PGC-related genes *NANOG*, *GDF3* and *DPPA3* (Ezech *et al.* 2005), gain of these genes might contribute to the invasive capacities of GCTs.

Type III GCTs, the spermatocytic tumor are detected in the 5th–6th decade of life and are a rare GCT (<1% of testicular tumors) (Looijenga & Oosterhuis 1999, Oosterhuis & Looijenga 2005, Raiss *et al.* 2011) (Fig. 1A). It is hypothesized that spermatocytic tumors originate from germ cells that are capable of maturing at least to spermatogonia/pachytene spermatocytes, since these GCTs express typical pachytene spermatocyte markers like SCP1, XPA and SSX (Aggarwal & Parwani 2009). Screening for oncogenic driver mutations in spermatocytic tumors revealed genetic aberrations in the *FGFR3* and *HRAS* gene (Goriely *et al.* 2009). Goriely and coworkers demonstrated by parallel sequencing of sperm DNA that occurrence of these mutations increases with paternal age, arguing for a category of the so-called paternal age effect mutations (Goriely *et al.* 2009). These mutations are thought to occur in spermatogonia. Owing to the proliferative capacities of the *FGFR3*- and *HRAS*-involved signaling pathways, it is believed that these mutations then selectively become enriched during clonal expansion of spermatogonia (Goriely *et al.* 2009). Spermatocytic tumors typically consist of three cell types: small, medium and large size, which morphologically resemble spermatogonia or

spermatocytes with round and sometimes multinucleated nuclei (Oosterhuis & Looijenga 2005). As in type I GCTs, GCNIS cells are not present in spermatocytic tumors (Aggarwal & Parwani 2009). Spermatocytic tumors are negative for classical seminoma or EC markers, like PLAP, OCT3/4 and CD30, but sometimes positive for cKIT and VASA (Oosterhuis & Looijenga 2005, Aggarwal & Parwani 2009). In contrast to classical seminomas and other type II GCTs, spermatocytic tumors display gain of chromosome 9 or X, but show no isochromosome 12p formation (Rosenberg *et al.* 1998, Verdorfer *et al.* 2004, Reuter 2005).

Type I and type II GCTs: results of a flawed licensing

As mentioned, after specification, PGCs initiate the expression of germ cell and pluripotency markers. After arrival of the PGCs in the genital ridge, they prepare for gametogenesis; this stage is called 'licensing' (Gill *et al.* 2011). During licensing, the genes *Dazl*, *Ddx4* and *Dmrt1* are induced and repress the PGC-specific genes and the pluripotency markers. This is evident in *Dazl* mutants, where expression of pluripotency markers persists and the germ cells eventually initiate apoptosis (Lin & Page 2005). Interestingly some of the gonadal cells in *Dmrt1* mutants, as well as in animals heterozygous for *Tfap2c* and *Nanos3* also fail to downregulate pluripotency markers after arrival in the genital ridge in the 129 genetic background (Krentz *et al.* 2013, Schemmer *et al.* 2013). In these mutants, the persistence of *Nanog* and *Oct3/4* as well as other PGC and pluripotency marker genes like *Dnd1*, *Prdm14*, *Lin28*, *cKit* and *Dppa3* counteracts apoptosis leading to rapid development of a germ cell tumor equivalent to a type I GCT, an early childhood tumor (Kehler *et al.* 2004, Western 2009, Pirouz *et al.* 2012). These data in mice suggest that interference with genes involved in downregulation of the pluripotency genes after arrival in the genital ridge is a critical (and error-prone) step. According to these findings, human type I germ cell tumors might be initiated during licensing, when arriving PGCs downregulate the PGC-specific genes but fail to downregulate the pluripotency markers. A similar mechanism might apply to extragonadal GCTs (germinomas and non-germinomas). There, misrouting of the cells prevents them from entering the genital ridge. The cells eventually initiate apoptosis, since they are deprived of their growth and guidance factors. The misrouted GCs are not confronted with the somatic cells of the genital ridge and never encounter the crosstalk leading to initiation of the licensing cascade. If they survive and persist, they might give rise to extragonadal GCTs.

Similar to Type I GCTs, the pre-GCNIS and the GCNIS of the type II GCTs is also initiated at licensing. Here, the arriving PGCs not only maintain expression of the

pluripotency genes, but also retain expression of *SOX17*, *PRDM1* and *PRMT5* (Eckert *et al.* 2008). Recent data indicate that in humans, *SOX17* is a critical determinant of PGC fate transactivating expression of *PRDM1* (Irie *et al.* 2015). In turn, *PRDM1* and *PRMT5* proteins cooperate to establish a symmetric dimethylation at arginine 3 of histones H2a and H4 (H2a/H4R3me2s), leading to suppression of somatic differentiation (Ohinata *et al.* 2005, Ancelin *et al.* 2006). Since such cells remain as undifferentiated GCNIS in the developing testicular environment for years, we speculate that the *SOX17*–*PRDM1* cascade not only blocks further differentiation along the germ cell fate but also maintains a survival signal and renders the cells in proliferative dormancy.

Therefore, these data indicate that ‘licensing’ is a critical step in germ cell biology where the pluripotency and the PGC program must be suppressed for proper germ cell development to proceed.

Germinoma: misrouting of germ cells by defects in cKIT or CXCR4

Type II GCTs can also be found in thymus and brain, where they are termed germinoma (Oosterhuis & Looijenga 2005). It is assumed that intracranial germinomas, which are highly similar to the histology of seminomas, arise from PGCs that failed to enter the genital ridges during embryogenesis. PGCs positive for the receptor tyrosine kinase cKIT migrate along an SCF gradient to the genital ridges (Runyan *et al.* 2006, Gu *et al.* 2009). After arrival at the developing gonadal site, cKIT expression is downregulated. Therefore, it is conceivable that auto-activating mutations of the cKIT gene (exon 17: D816V, D816H, N822K, Y823D; exon 11: L576P, W557C), rendering cKIT expression independent of SCF, might cause erroneous migration of PGCs, eventually leading to formation of intracranial germinomas (Przygodzki *et al.* 2002, Kemmer *et al.* 2004, Willmore-Payne *et al.* 2006, Biermann *et al.* 2007). Sakuma and coworkers and Kamakura and coworkers demonstrated cKIT expression in all germinomas or germinomatous components of mixed non-germinomas (Sakuma *et al.* 2004, Kamakura *et al.* 2006). Additionally, Sakuma and coworkers found that 25% of all germinomas analyzed harbored an autoactivating cKIT mutation at exon 11 or 17, while Kamakura and co-workers detected mutated cKIT in 23% of germinomas (exon 2 (E73K, T96M), 11 (V560D), 13 (A636V), 17 (D816Y)) (Sakuma *et al.* 2004, Kamakura *et al.* 2006). These mutation rates are very similar to the cKIT mutation rates found in classical seminoma (24.1%) (Sakuma *et al.* 2003, Kemmer *et al.* 2004). Thus, autoactivating cKIT mutations might lead to an SCF-independent migrational path of the PGCs, leading to formation of intracranial germinomas.

An alternative mechanism represents the chemokine-mediated CXCR4 pathway, which is activated by

selective binding of CXCL12. Increased activity of the CXCR4 pathway correlates to an increased risk to develop metastases and stimulates tumor proliferation, invasiveness as well as angiogenesis (Guan *et al.* 2015, Ma *et al.* 2015). CXCR4-positive PGCs migrate along somatic sites expressing CXCL12 (Ara *et al.* 2003, Molyneaux *et al.* 2003, Gilbert *et al.* 2009, Takeuchi *et al.* 2010). CXCR4-positive GCTs mainly metastasize to sites with CXCL12 expression. Gilbert and coworkers demonstrated that expression of CXCR4 is high in seminomas and in the seminoma cell line TCam-2 compared with normal testis (Gilbert *et al.* 2009). They demonstrated further that TCam-2 cells start to migrate in response to CXCL12, while CXCR4-negative 2102EP cells do not. Since it has been demonstrated that TCam-2 cells cluster close to human PGC-like cells, it seems reasonable to assume that human PGCs use both cKIT and CXCR4/CXCL12 for migration (Irie *et al.* 2015, Tang *et al.* 2015).

GCT cell lines as model to study germ cell development and malignancy

Several human GCT cell lines have been established in order to generate suitable model systems for *in vitro* studies: Most of them are derived from ECs and, therefore, share pluripotent or nullipotent characteristics (i.e. 2102EP, NCCIT; Table 1). In addition, cell lines have been established from choriocarcinomas (JEG-3, JAR and BeWo; Table 1), resembling differentiated non-seminomas. From seminoma patients, three cell lines have been derived so far, namely TCam-2, JKT-1 and SEM-1 (Table 1) (Mizuno *et al.* 1993, Kinugawa *et al.* 1998, Russell *et al.* 2013). Thorough analyses revealed that TCam-2, but not JKT-1 cells, resemble seminoma *in vitro* (de Jong *et al.* 2008, Eckert *et al.* 2008, Nettersheim *et al.* 2012), while SEM-1 cells show intermediate characteristics between seminoma and nonseminoma (Russell *et al.* 2013).

EC cell lines have long been used as a proxy for embryonic stem cell (ESC) research, owing to their similarities in marker expression, their extended self-renewal capacities and their ability to differentiate into a wide range of cell types (Thomson 1998, Chaerkady *et al.* 2010, Pera *et al.* 2000). Today EC cell lines still represent a valuable *in vitro* model for studying pluripotency and stem cell regulatory networks particularly in testicular tumors, in order to identify novel targets for GCT therapy.

Seminomas and the seminoma-derived cell line TCam-2 closely resemble PGCs. Like PGCs, TCam-2 cells express pluripotency markers *LIN28*, *NANOG*, *OCT3/4* and PGC markers *PRDM1*, *TFAP2C*, *DMRT1* as well as cKIT (Eckert *et al.* 2008, Nettersheim *et al.* 2011a, 2012) (Fig. 1B). In humans, PGCs express *SOX17* (Campolo *et al.* 2013), which is also detected in GCNIS/seminoma and TCam-2 cells (Irie *et al.* 2015) (Fig. 1B).

Table 1 Summary of available GCT cell lines.

Cell line	Origin	Growth <i>in vitro</i> as...	Growth <i>in vivo</i> as...	Reference
TCam-2	Seminoma	Seminoma	EC (Flank), CIS/Seminoma (Testis)	Mizuno <i>et al.</i> (1993)
JKT-1	Seminoma, EC	Sem/EC-intermediate	Seminoma	Kinugawa <i>et al.</i> (1998)
SEM-1	Seminoma	Sem/EC-intermediate	mediastinal GCC	Russell <i>et al.</i> (2013)
1411HP	Seminoma, EC, Teratoma, YST	EC	EC, YST	Vogelzang <i>et al.</i> (1985)
169A/218A/228A/240A	EC	EC	n.d.	Houldsworth <i>et al.</i> (1997)
1777N	Retroperitoneal metastasis of non-sem.	EC	EC	Bronson <i>et al.</i> (1983)
2102EP	EC, Teratoma	EC	EC	Andrews (1982)
833Ke	Seminoma, EC, Teratoma, CC	EC	EC	Bronson <i>et al.</i> (1980)
GCT27	EC, Teratoma	EC	EC, Teratoma, YST	Pera <i>et al.</i> (1987)
GCT35	EC, Teratoma	EC	EC, YST	Pera <i>et al.</i> (1987)
GCT44/46/72	EC, Teratoma	EC	YST	Pera <i>et al.</i> (1987)
GCT48	EC, Teratoma	EC	EC	Pera <i>et al.</i> (1987)
H12.1/.5/.7	Seminoma, EC, Teratoma, CC	EC	EC	Casper <i>et al.</i> (1987)
NCCIT	EC, Teratoma	EC	EC, Teratoma	Damjanov <i>et al.</i> (1993)
NEC-8/-14/-15	EC, YST, CC	EC	EC, Teratoma	Sekiya <i>et al.</i> (1985)
SuSa	EC, Teratoma	EC	n.d.	Hogan <i>et al.</i> (1977)
TERA1/2	EC, Teratoma	EC	EC	Andrews <i>et al.</i> (1984)
577MF/L/RPLN	Metastasis of non-sem.	Undifferentiated carcinoma	Teratoma	Wang <i>et al.</i> (1980)
BeWo	CC	CC	CC	Pattillo & Gey (1968)
JAR	CC	CC	CC	Hochberg <i>et al.</i> (1992)
JEG-3	CC	CC	CC	Kohler & Bridson (1971)

Using a murine system allowing for differentiation of PGC-like cells from embryonic stem cells (ESCs), Nakaki and coworkers defined a set of 75 genes as 'core' PGC genes (Nakaki *et al.* 2013). Meta-analysis of expression microarray analyses demonstrated that many of these genes are also expressed in human GCNIS, seminoma, EC and teratoma tissues as well as TCam-2 cells (Fig. 2A, B, C and D) (Biermann *et al.* 2007, Eckert *et al.* 2008). A Venn diagram illustrates that 14 of these 'core' PGC genes are commonly expressed in these samples with an expression intensity threshold $\geq \text{Log}_2 10$, namely *cKIT*, *KLF2*, *DPPA3*, *PYGL*,

TCL1, *CKB*, *BRPF3*, *SORBS1*, *KCTD15*, *HEY1*, *RGS16*, *AP3B2*, *GRHL3* and *TDH* (Fig. 2E and F).

Furthermore, a meta-analysis of genome-wide gene expression between TCam-2 and murine male/female PGCs at embryonic developmental days 11.5, 12.5 and 13.5 (data from (Jameson *et al.* 2012) (expression intensity threshold $\geq \text{Log}_2 10$)) demonstrates that the majority of genes expressed in male or female PGCs are also detectable in TCam-2 cells ($n=1022$) (Fig. 3A, B and C). A comparison of these 1022 genes with the gene expression data of GCNIS, seminomas and ECs shows that 690 genes are also expressed in these GCT entities

Figure 2 (A, B, C and D) Microarray expression analysis of 'core' PGC genes as defined by Nakaki and coworkers (2013) in GCNIS ($n=3$), seminoma ($n=4$) and EC ($n=3$) tissues as well as TCam-2 cells ($n=1$). Standard deviation is given above the graphs. (E) Venn diagram of commonly expressed 'core' PGC genes. Cut-off = expression intensity $< \text{Log}_2 10$. (F) Expression intensity of the 14 commonly expressed 'core' PGC genes in indicated GCT tissues and cell lines. Standard deviation is given above the graphs.

Figure 3 (A and B) Venn diagrams illustrating commonly expressed genes in TCam-2 and male (A) as well as female (B) PGCs at different developmental time points (E11.5, E12.5, E13.5). (C) A Venn diagram summarizes commonly expressed genes (1022) between TCam-2 and both male and female PGCs. (D) 690 of these 1022 are also expressed in GCNIS, seminomas and ECs. Venn diagrams were created with Venny 2.1 (www.bioinformatics.org/venny).

(expression intensity threshold $\geq \text{Log}_2 10$) (Fig. 3D). Recently, Irie and coworkers reported that human ES-derived PGC-like cells share a transcriptional profile with TCam-2 seminoma-like cells (Irie *et al.* 2015). These data point out that PGCs, GCC tissues (GCNIS, seminomas, ECs) and TCam-2 cells are closely related to each other and that TCam-2 cells clearly represent a suitable PGC model *in vitro*.

In mice, development of GCNIS and seminomas are not observed, but the orthotopical transplantation of TCam-2 cells into the murine seminiferous tubules results in the formation of a GCNIS/seminoma. Thus, this approach represents a valuable strategy for the study of human seminomas *in vivo* (Nettersheim *et al.* 2012) (Fig. 1A).

Taken together, GCT-derived cell lines display a wide variety of phenotypes, from being close to PGC (TCam-2), close to ESC (EC lines) or differentiated in a variety of tissues. Expression of the pluripotency network is detected in seminomas, ECs and derived cell lines. Thus, regulation and timely repression of genes involved in pluripotency appears to be the most critical step in germ cell development.

Regulation of PRDM1 by LIN28 in PGCs and GCTs

In addition to SOX17, PRDM1 levels seem to be controlled by LIN28. Lin28 is a repressor of miRNA let7 (miR-let7) and miR-let7 in turn represses Prdm1. Thus, expression of Lin28 is essential for proper murine PGC development (Newman *et al.* 2008, West *et al.* 2009). LIN28 expression is also detectable in GCNIS, seminomas (including TCam-2) and ECs (West *et al.* 2009, Nettersheim *et al.* 2011b). TCam-2 cells differentiate *in vitro* into a mixed nonseminoma upon stimulation with FGF4, EGF and TGF- β 1 (Nettersheim *et al.* 2011b)

(Fig. 1A). During this process, LIN28A, but not LIN28B, and PRDM1 are downregulated, accompanied by reduced H2a/H4R3me2s. Knocking down LIN28A in TCam-2 by siRNA suppresses PRDM1 expression, but reduction of PRDM1 leaves LIN28 unaffected (Nettersheim *et al.* 2011b). This suggests that in human PGCs, PRDM1 levels are fine-tuned by miR-let7, which in turn is suppressed by LIN28.

DNA (de)methylation in GCs and GCTs

PGCs undergo global DNA demethylation during migration, although running through several rounds of replication in the presence of DNMT1. Additionally, later during migration, they arrest in G2-phase. Thus, an active DNA demethylation process has been assumed (Hajkova *et al.* 2002, Saitou & Yamaji 2012, Saitou *et al.* 2012). Hackett and coworkers demonstrated that this active DNA demethylation is driven by members of the Tet hydroxylase family (TET1/2) (Hackett *et al.* 2013). TET enzymes oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), resulting in activation of the base excision repair (BER) mechanism and in excision as well as replacement of a modified cytosine by an unmodified one (Hajkova *et al.* 2002, 2010). Expression of the *TETs* (1–3) and the co-keyplayer of active DNA demethylation *TDG* was detected in GCNIS, seminomas, ECs and corresponding cell lines (Nettersheim *et al.* 2013). In general, 5mC levels are low in GCNIS and seminomas, but high in non-seminomas (Smiraglia *et al.* 2002, Netto *et al.* 2008, Almstrup *et al.* 2010, Wermann *et al.* 2010). Nevertheless, the 5mC oxidation products 5hmC, 5fC and 5caC were detectable in all GCT cell lines analyzed and EC tissues (Nettersheim *et al.* 2013). In GCNIS and seminoma

tissues, 5hmC correlated to the presence or absence of 5mC levels, while 5fC and 5caC were detectable in all GCNIS/seminoma samples independent of 5mC/5hmC status (Nettersheim *et al.* 2013). In contrast to these data, Kristensen and coworkers found only very low levels of 5hmC and TET1 in GCNIS cells (Kristensen *et al.* 2014). Furthermore, they found high expression of APOBEC1, a key player of the deamination pathway of active DNA demethylation and of BER key players like MBD4, APEX1 and PARP (Branco *et al.* 2012, Kristensen *et al.* 2014). At the end of both pathways of active DNA demethylation, the oxidation and deamination pathway, the BER mechanism catalyzes replacement of a modified cytosine by an unmodified cytosine. Thus, expression of BER keyplayers in GCNIS/GCTs is consistent between published data, but future studies will show which (or if both) pathway(s) of active DNA demethylation is/are active in GCTs.

In adult human testis tissue, persisting 5mC marks were detected throughout all cell types of spermatogenesis, while 5hmC, 5fC and 5caC marks were detectable in spermatogonia, but steadily decreased with progression of spermatogenesis (Nettersheim *et al.* 2013). These findings point at an active DNA demethylation in spermatogonia, which is turned off upon initiation of spermatogenesis. The authors assume that 5hmC, 5fC and 5caC marks are diluted out with each round of mitosis/meiosis – similarly to a process already described during mouse preimplantation development (Inoue *et al.* 2011). Thus, the ability to actively demethylate DNA is maintained during maturation of PGCs to spermatogonia (but not further), development of GCTs and in GCT cell lines.

The pluripotency factor NANOG maintains self-renewal of undifferentiated cells in concert with OCT3/4 and SOX2 (Chambers *et al.* 2003). Human spermatogonia (fetal and adult) as well as seminomas and TCam-2 cells display hypomethylation of the NANOG promoter (0–10% 5mC), which corresponds to high levels of NANOG expression (Nettersheim *et al.* 2011a). Hypomethylation of the *Nanog* promoter was also found in murine PGCs (Seisenberger *et al.* 2012). In murine testis tissue and sperm as well as in adult human testis tissue and mature sperm, the NANOG promoter is found hypermethylated (>60–100% 5mC) (Imamura *et al.* 2006). At present, we can only speculate that, similar to the murine situation, human PGCs display hypomethylation of the NANOG promoter as well. Therefore, epigenetic silencing of NANOG expression via DNA methylation seems to occur at later stages of germ cell development. In line, expression of NANOG can be detected in human PGCs, but not in mature GCs (Kerr *et al.* 2008, Sugawa *et al.* 2015).

We speculate that in GCNIS cells, the methylation of the NANOG promoter fails to occur, keeping the gene locus open and accessible for transcription factors, thereby promoting pluripotency and self-renewal.

While failure to methylate (and silence expression) is clearly correlated to malignant aberration, in normal GCs downregulation of NANOG transcription clearly precedes the DNA methylation. Therefore, it seems that failure to methylate the NANOG promoter is not the cause of the malignancy, but rather the consequence.

Screening for GC- and GCT-specific expression of noncoding RNAs

Short noncoding RNAs (sncRNAs) like micro-RNAs (miRNAs) and PIWI-interacting RNAs (piRNAs) play important regulatory roles in many developmental processes, including germ cell development. De Jong and coworkers, Gillis and coworkers and Novotny and coworkers screened for the expression of miRNAs in GCNIS and invasive GCTs as well as fetal and adult testis tissue. In these studies, different groups of miRNAs that specifically distinguish among GCNIS, GCTs and (fetal, adult) testis tissue were identified (Gillis *et al.* 2007, de Jong *et al.* 2008, Novotny *et al.* 2012). For example, the miR146 is underexpressed in seminoma compared with normal testis tissue and putatively targets the PGC marker cKIT (He *et al.* 2005, Gillis *et al.* 2007). Furthermore, the embryonic germ cell-specific miRNA cluster 371–373 was upregulated in TCam-2 and GCT tissues, acting in conjunction with RAS downstream of P53. Additionally, miRNA cluster 302–367 is expressed in GCNIS, GCTs and fetal gonads (Gillis *et al.* 2007, Novotny *et al.* 2012). Novotny and coworkers conclude from these results that the miRNA expression profile changes during testis development and that the miRNA profile of adult testis with GCNIS cells shares characteristic similarities with the expression in fetal gonocytes (Novotny *et al.* 2012). Mclver and coworkers screened for changes in miRNA expression between postnatal gonocytes and spermatogonia to gain insight into the miRNA processes that influence maturation of gonocytes and might cause GCNIS development (Mclver *et al.* 2012). They found seven differentially expressed miRNAs (upregulated in spermatogonia vs gonocytes: miR136, -743q and -463; downregulated miR290-5p, -291a-5p, -293 and -294) that are involved in regulation of PTEN and WNT signaling and CyclinD1 (Mclver *et al.* 2012). The miRNA cluster 290–295 is highly expressed in ESCs and promotes proliferation and pluripotency by maintaining OCT3/4 expression (Zheng *et al.* 2011). Therefore, Mclver and coworkers believe that downregulation of miRNA cluster 290–295 marks initiation of gonocyte differentiation. Failure in downregulating this miRNA cluster might contribute to GCNIS formation (Mclver *et al.* 2012).

piRNAs are key players of genome integrity in the developing germ line as they inhibit the activity of transposable elements (Siomi *et al.* 2011). Ferreira and coworkers and Rounge and coworkers analyzed the

expression of piRNAs and interacting PIWI proteins in GCTs compared with normal testis tissue. These studies revealed a strong downregulation of piRNAs in seminomatous and non-seminomatous GCTs (Ferreira *et al.* 2014, Rounge *et al.* 2015) and silencing of the PIWI genes PIWIL-1, -2, and -4 by DNA methylation (Ferreira *et al.* 2014). Since PIWIL-2 is highly expressed in the fetal gonad and during early spermatogenesis to inhibit germline transposons like LINE-1 in concert with mature piRNAs, Ferreira and coworkers believe that the loss of the PIWI/piRNA machinery might play an important role in GCT development (Ferreira *et al.* 2014).

The alternative explanation is that the miRNA and piRNA profile detected in GCTs (persistence of expression of miRNAs from early gonocytes and lack to upregulate the piRNAs, which are indicative for germ cell differentiation) are yet another indicator for the arrested GC origin of GCTs as put forward by Skakkebaek (Skakkebaek *et al.* 1987).

The somatic microenvironment determines the fate of TCam-2 cells

TCam-2 cells xenotransplanted orthotopically into the seminiferous tubules of nude mice grow GCNIS/seminoma-like, while growth in a somatic microenvironment, like the flank or brain, promotes transition into an EC-like state (Nettersheim *et al.* 2012, 2013) (Fig. 1A). Additionally, cultivation of TCam-2 under culture conditions mimicking a somatic environment, forces differentiation into a mixed non-seminoma (Nettersheim *et al.* 2011b) (Fig. 1A). This transition of seminoma to an EC-like phenotype is accompanied by a number of epigenetic and molecular changes: the initial step in this process is the inhibition of BMP signaling by the microenvironment, resulting in de-repression of NODAL signaling (Nettersheim *et al.* 2015). During the so-called maturation phase, active NODAL signaling further represses intrinsic BMP signaling by activating *LEFTY1/2*, *CRIPTO*/*CRYPTIC* and *ZIC3* (Nettersheim *et al.* 2015). This phase is accompanied by an overall increase in *de novo* DNA methylation and the upregulation of pluripotency-associated genes (Nettersheim *et al.* 2015). Over time, a slow feedback loop re-establishes BMP signaling by activation of *WNT3/5B*, *BMP4/BMP7* and *FGF2/19*, which stabilizes the acquired EC-like cell fate and results in a balance between NODAL and BMP signaling pathways (Nettersheim *et al.* 2015). *SOX2* is essential for initiation of the NODAL signaling loop during this reprogramming. Lack of *SOX2* results in failure to activate the NODAL signaling key-players *LEFTY1/2* and *CRIPTO*, suggesting that *SOX2* is downstream of BMP inhibition but upstream of NODAL activation (Nettersheim *et al.* 2016).

This demonstrates that the cellular microenvironment has a profound impact on the cell fate of TCam-2 cells. Since it was demonstrated that TCam-2 closely

resemble PGCs (Irie *et al.* 2015, Tang *et al.* 2015), we speculate that proper PGC development also depends on a crosstalk with the somatic cells from the gonadal microenvironment, i.e. BMP signaling regulating NODAL activity. Factors leading to alterations of this crosstalk might interfere with the tightly controlled repression of pluripotency factors. In this context, the roles of endocrine disruptors might also be important.

Endocrine disruptors as a risk factor for GCT development and testicular dysgenesis syndrome

In Western countries, human sperm count is steadily declining, while the incidence of testicular dysgenesis syndrome (TDS), involving testicular cancer, cryptorchidism and hypospadias, is rising – both indications might be explained by the exposure to hormonally active substances found in our environment (Jensen *et al.* 1995, Nilsson 2000, Skakkebaek *et al.* 2001, Nohynek *et al.* 2013, Hauser *et al.* 2015). Endocrine disruptors (EDs), i.e. phthalates, polychlorinated biphenyls or alkylphenols are small molecules acting like hormones, which can cause adverse health effects (toxicity) via endocrine-mediated mechanisms (Nohynek *et al.* 2013). Several studies suggest their negative influence on reproduction by mimicking/modulating of sex hormones that are secreted by the ovaries or testes (Nilsson 2000). As reviewed by Del-Mazo and coworkers, EDs may adversely affect gene expression levels in PGCs and developing testis (i.e. oxidative stress response) or epigenetic processes in sperm (i.e. by deregulation of epigenetic enzymes) (Wu *et al.* 2010, Del-Mazo *et al.* 2013). Therefore, exposure of PGCs to EDs may interfere with the downregulation of the PGC- and pluripotency program, leading to increased development of GCNIS or type I GCTs.

Further, Ohlson and coworkers demonstrated a six-fold increased risk for developing seminomas among men exposed to polyvinyl chloride (PVC), the third-most commonly used plastic polymer in the environment (Ohlson & Hardell 2000).

So, it seems that exposure to EDs *in utero* could lead to increased rate of PGCs transformed to GCNIS cells, which in adulthood are further triggered by e.g. PVC to progress into a GCT. In this respect, *in vitro* studies using GCT cell lines, especially TCam-2 could help to get insights into the effects of EDs on gene expression, protein composition and epigenetics of GCs (Del-Mazo *et al.* 2013).

Does poised chromatin render germ cells prone to transformation?

According to the theory first described by Skakkebaek, a PGC/gonocyte has failed to differentiate further and remains silent until puberty, where it starts to proliferate

giving rise to GCNIS and subsequently seminomas or non-seminomas (Skakkebaek 1972, Skakkebaek *et al.* 1987). Molecular analyses of the past decades in part reviewed here further substantiated this claim.

Recently, several groups found that through the germ line, a very characteristic pattern of histone modifications, the presence of repressive marks (H3K27me3) and active marks (H3K4me3) is detected at key developmental promoters (Hammoud *et al.* 2009, Sachs *et al.* 2013, Lesch & Page 2014). This so-called 'poised' chromatin is also detected in pluripotent ESCs and thought to be instrumental for pluripotency (Azuara *et al.* 2006, Bernstein *et al.* 2006, Mikkelsen *et al.* 2007). As a consequence, the mammalian germ line has been described as a pluripotency cycle featuring an epigenetic status, which is primed to rapidly re-activate toti- and pluripotency upon fertilization (Leitch & Smith 2013).

In light of these findings, we would like to extend the hypothesis of the origin of type II GCTs from Skakkebaek (Fig. 1A). While several lines of evidence indicate that a germ cell tumor originates from a 'misinstructed' PGC or early gonocyte, we propose that germline cells from adult mice and humans, which have not initiated meiosis, are also able to give rise to a type II GCT, because these cells are epigenetically prepared to rapidly re-activate totipotency. Therefore, exposure to EDs interfering with the mechanisms and stabilizing this poised state might lead to re-initiation of the pluripotency program, resulting in the formation of a GCNIS or a type I GCT. In fact, stochastic re-activation of the pluripotency program has been demonstrated for gonadal cells from adult mice and humans. They convert to induced pluripotent cells solely by application of appropriate media conditions (Kanatsu-Shinohara *et al.* 2008, Ko *et al.* 2009, Kossack *et al.* 2009, Liu *et al.* 2011). Therefore, it seems that germ cells harboring 'poised' histone marks in conjunction with reduced DNA methylation are more than other somatic cells at risk to re-activate the PGC and pluripotency program and, as such, initiate the formation of GCTs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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