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Carcinoma *in situ* testis displays permissive chromatin modifications similar to immature foetal germ cells

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BACKGROUND: The majority of testicular germ cell cancers develop through a pre-invasive carcinoma *in situ* (CIS) stage. The CIS cell is a neoplastic counterpart of foetal germ cells. During their development, foetal germ cells undergo extensive and essential epigenetic modifications, but little is known about epigenetic patterns in CIS cells.

METHODS: Immunohistochemistry was used to investigate epigenetic patterns in CIS, germ cell tumours, normal adult and foetal testicular tissue.

RESULTS: CIS cells show low levels of DNA methylation and repressive histone modifications H3K9me2 and H3K27me3, but high levels of H3K9 acetylation, H3K4 methylation and H2A.Z, which all are associated with an activated and accessible chromatin structure. Collectively this renders a permissive chromatin structure and in accordance high levels of RNA polymerase II activity and proliferation (Ki-67 and mitotic index) is observed in CIS cells. Epigenetic patterns similar to that of CIS cells were observed in human gonocytes present within sex cords in foetal testes but correspond to migrating primordial germ cell in mice. Development of overt tumours involves epigenetic repression of the chromatin.

CONCLUSION: CIS cells have a permissive and foetal-like chromatin structure, which is associated with a high transcriptional and proliferative activity, likely empowering neoplastic transformation. Developmental epigenetic cues in foetal germ cells are substantially different between humans and mice.

Testicular cancer is the most common cancer in young men and the incidence is increasing (Huyghe *et al*, 2003). Genetic predisposition is indicated by familial cases and ethnic differences in incidence rates but the increasing trend and major geographic differences in incidence are consistent with environmental influence. Increased use of endocrine disruptors has been suggested to be one of the environmental factors responsible for the increasing incidence of testicular cancer, which is regarded as one of the outcomes of the testicular dysgenesis syndrome, a group of reproductive disorders linked together by developmental origins (Skakkebaek *et al*, 2001).

The vast majorities of testicular tumours are derived from germ cells and are traditionally divided into a morphologically homogeneous group of seminomas and a very heterogeneous group of non-seminomas. Even though seminomas and non-seminomas histologically are very different, they both originate from the same precursor called carcinoma *in situ* (CIS), intratubular germ cell neoplasia or testicular intraepithelial neoplasia. Thus, CIS has a central role in pathogenesis of testicular cancer.

Evidence from morphological (Nielsen *et al*, 1974; Albrechtsen *et al*, 1982; Skakkebaek *et al*, 1987), epidemiological (Hemminki and Li, 2002; Hemminki *et al*, 2002) immunohistochemical (Rajpert-De Meyts *et al*, 2003; Honecker *et al*, 2004; Pauls *et al*,

2006), and gene expression profiling studies (Almstrup *et al*, 2004; Sonne *et al*, 2009) indicate that the CIS cell is derived from a gonocyte or primordial germ cell (PGC). We have earlier shown that CIS cells are indistinguishable from gonocytes in terms of gene expression profiles (Sonne *et al*, 2009) but the exact developmental time point and the mechanisms of malignant transformation remain unknown. It is possible that a slight delay of gonadal differentiation, under-stimulation of the developing testis or physical misplacement of PGC during migration or colonisation of the gonad might be triggering factors. This will imply that immature germ cells would later become neoplastic due to their undifferentiated nature in the changed microenvironment of the adult testis.

The PGCs and gonocytes are known to undergo extensive epigenetic reprogramming. Seki *et al* (2007) observed that migrating murine PGCs progressively erase H3K9me2 and subsequently establish H3K27me3 concurrently with erasure of genome-wide DNA methylation. Both histone marks are repressive and in the transition between erasure of H3K9me2 and establishment of H3K27me3 PGCs most probably display a transient period with possible hyper-transcription. Hyper-transcription seems however to be prevented as PGCs were shown to enter G2 arrest and repress RNA polymerase II-dependent transcription in this transition period (Seki *et al*, 2007). Hajkova *et al* (2008) showed that mice PGCs upon gonadal colonisation (E11.5) lose most of their linker histone H1, H3K9ac, H3K9me3, H3K27me2 and H4/H2A R3me2.

Very little is known about the epigenetic status of CIS cells, probably because it has not been possible to cultivate CIS cells.

Genetics and Genomics

Immunohistochemical investigations have shown that CIS cells have arginine 3 of histone H2A and H4 dimethylated (H4/H2A R3me2), which are thought to repress – among others – the *HOX* genes, involved in somatic differentiation programs (Ohinata *et al*, 2005). Most seminomas also have high levels of H4/H2A R3me2, whereas low levels are observed in non-seminomas (Eckert *et al*, 2008). It is also known that the genome of CIS cells contains very little DNA methylation (Netto *et al*, 2008), in contrast to highly methylated non-seminomas, whereas seminomas display variable but mainly low levels of DNA methylation (Smiraglia *et al*, 2002; Netto *et al*, 2008). This is in accordance with our findings of high expression of the DNA methylation enzymes DNA methyl transferase 3A and DNA methyl transferase 3L in the undifferentiated non-seminoma component, embryonal carcinoma (EC), when compared with seminoma and CIS (Almstrup *et al*, 2005).

As developmental epigenetic cues seem a prerequisite for proper germ cell development in mice, we, in this study, addressed the question whether early human neoplastic germ cells – the CIS cells – that seem developmentally arrested, indeed could have an epigenetic status similar to immature germ cells. We also hypothesised that epigenetic modifications may be associated with differences in transcriptional activity between normal and malignant germ cells. To answer that, we systematically investigated the pattern of DNA methylation and histone modifications, which are known to be differentially set during embryonic development of the germ line, in CIS cells and derived cancers and compared them to the epigenetic patterns of the same modifications in normal human germ cells in the adult testis and in foetal gonocytes. We also assessed the transcriptional activity and proliferation index in the CIS cells.

MATERIALS AND METHODS

Tissues

The regional Committee for Medical Research Ethics in Denmark approved the use of human tissues stored in the tissue archives of Copenhagen University Hospital. The tissues were obtained after orchidectomy for testicular cancer (adult testis and testicular tumours) from spontaneous abortions/miscarriages (foetal tissues) and from control biopsies to monitor for relapses of acute leukaemia (pre-pubertal testis samples). Tissue samples were fixed overnight at 4°C in buffered formalin or 4% paraformaldehyde. Histological diagnosis was established by experienced pathologists and confirmed by immunohistochemical staining for tumour markers (as described below).

Immunohistochemical evaluation of DNA methylation and histone modifications

Immunohistochemistry was performed as previously described (Rajpert-De Meyts *et al*, 2003). A standard indirect peroxidase method was used and development was done primarily with 3-amino-9-ethylcarbazole (yielding a red colour). For double staining, colour development with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium was in addition applied (yielding a deep blue colour) as previously described (Jørgensen *et al*, 2009).

The following antibodies were used at indicated dilutions and buffers for microwave treatment: anti-5-methylcytosine, 1:400, (TEG buffer) TRIS 1.21 g/l, EGTA 0.19 g/l, pH 9.0 (5-meC; Calbiochem, Darmstadt, Germany, NA81), rabbit anti-human placental alkaline phosphatase, 1:300, TEG buffer (placental-like alkaline phosphate; Thermo Fisher Scientific, Waltham, MA, USA, RM-9115-S0), anti-dimethyl-histone H3 (Lys9) 1:250, TEG buffer (H3K9me2; Millipore, Billerica, MA, USA, 07–441), anti-trimethyl-Histone H3 (Lys27), 1:500, citrate buffer (H3K27me3; Millipore, 07–449); anti-histone H3 (mono methyl Lys4), 1:6000, TEG buffer

(H3K4me1; Abcam, Cambridge, MA, USA, ab8895), anti-histone H3 (di + tri methyl Lys4), 1:2000, TEG buffer (H3K4me2/3; Abcam, ab6000), anti-EZH2, 1:2500, urea buffer (Millipore, 07–689), anti-UTX, 1:50, urea buffer (Abcam, ab36938), anti-JMJD3, 1:75, citrate buffer (Millipore, 07–1533), anti-histone H3 (acetyl Lys9), 1:10000, citrate buffer (H3K9ac; Abcam, ab10812), anti-histone H2A.Z 1:3000, citrate buffer (Abcam, ab4174), anti-RNA polymerase II (N-20), 1:500, citrate buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-899), anti-RNA polymerase II Ser-2 phosphorylated, 1:200, TEG buffer (H5; Covance, Princeton, NJ, USA, MMS129R), anti-OCT4 (C-10), 1:250, urea buffer (Santa Cruz Biotechnology, sc5279). For negative controls, serial sections were processed with the primary antibody replaced by the dilution buffer alone. None of the control slides showed any staining (data not shown). Stained slides were scanned on a NanoZoomer (Hamamatsu Photonics, Herrsching am Ammersee, Germany) and analysed using the software NDPview (Hamamatsu Photonics). For each antibody at least three different samples of the same histology were investigated. Intensity of the staining, in cells from the histology in question, was evaluated and scored from negative (neg) to strong positive (+ + +) as outline in Table 1.

Proliferation index

A total of 13 tissue samples containing CIS (no overt tumours) and four normal adult testes (patients with obstructive forms of infertility) were included. The proliferation rate was assessed by two methods: the mitotic index and the Ki-67-labelling index and proliferation rates of CIS cells were compared with those of normal spermatogonia. Biopsies were fixed in formalin or Stieve's fixative and stained with haematoxylin and eosin or by immunohistochemistry for placental alkaline phosphatase (mouse anti-human placental alkaline phosphatase; placental-like alkaline phosphate; Dako, Glostrup, Denmark, M7191) to identify CIS cells, and Ki-67 (Dako, M7189) to mark proliferating cells.

Statistical analysis was conducted by Mann–Whitney *U*-test to compare the median of two unmatched groups and Kruskal–Wallis test to compare averages of more than two samples.

RESULTS

DNA methylation levels in CIS cells

We first verified results from earlier work on the level of DNA methylation as measured by cytosine methylation (Smiraglia *et al*, 2002; Netto *et al*, 2008). The classical cytoplasmic CIS marker, placental-like alkaline phosphate, was used simultaneously with staining for 5-methyl-cytosine and indeed verified low levels of DNA methylation in CIS cells (Figure 1B). We also confirmed that non-seminomas have high levels of DNA methylation, whereas seminomas show variable but predominantly low DNA methylation levels (data not shown).

Histone modifications in CIS cells

Immunohistochemical staining for the repressive chromatin modifications H3K9me2 and H3K27me3 revealed low levels of these modifications in CIS cells, whereas Sertoli cells in CIS-containing tubules showed high levels of both modifications (Figure 1C and D).

Activating modifications H3K4me1 and H3K4me2/3 were observed abundant in CIS cells, whereas a weak staining was observed in Sertoli cells (Figure 1E and F). Furthermore, H3K9ac as well as H2A.Z were strongly positive in CIS cells but present at a much lower level in Sertoli cells (Figure 1G and H).
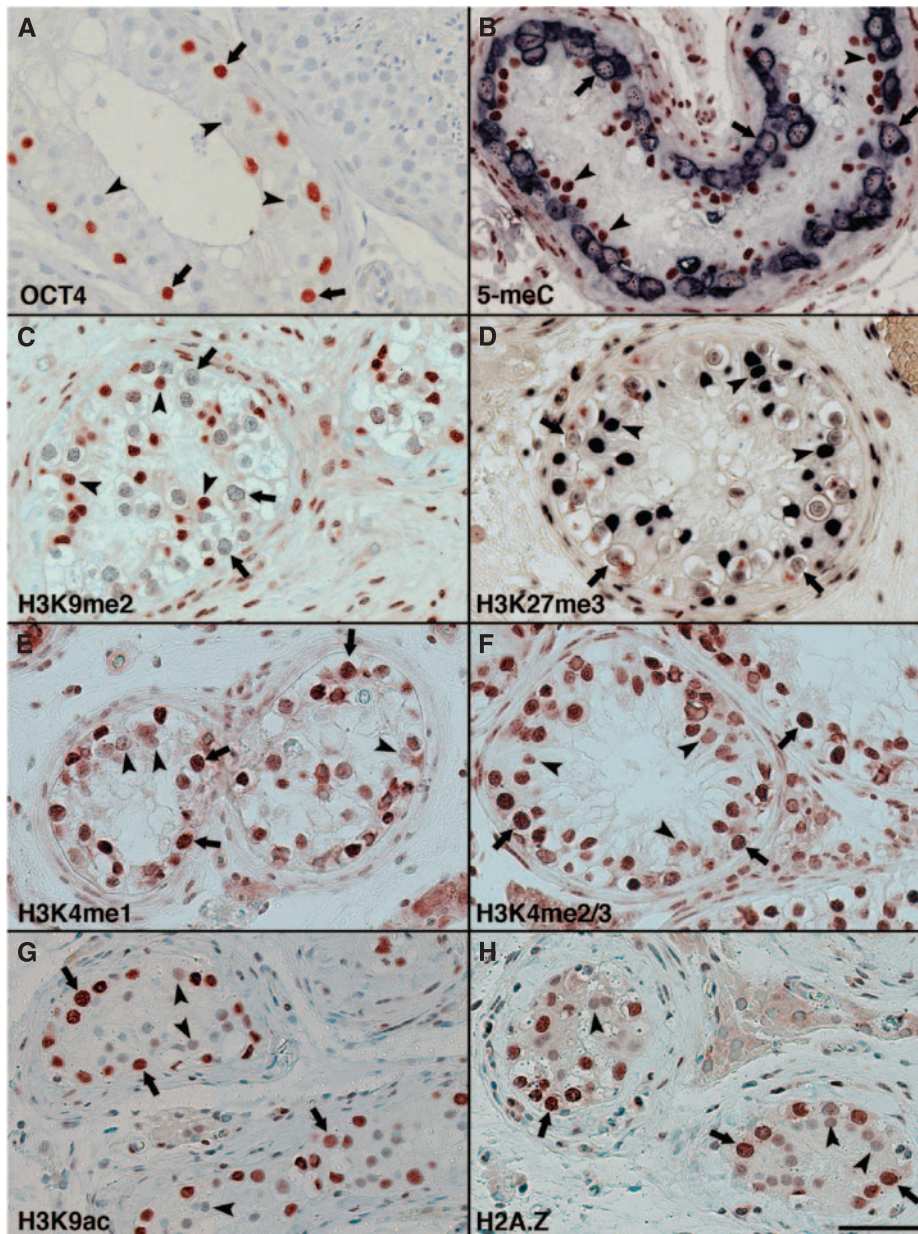
Figure 1 Epigenetic patterns in carcinoma *in situ* (CIS) testis visualised by immunohistochemistry. (**A**) A typical tubule with CIS cells marked by one of the classical markers, OCT4 (POU5F1) (de Jong *et al*, 2005; Rajpert-De Meyts *et al*, 2004). Note that CIS cells are bigger than unstained spermatogonia (visible in the tubule on the right) and have large irregular nuclei with coarse chromatin clumps. (**B**) Double staining for 5-methyl-cytosine (reddish brown) and the classical CIS marker placental-like alkaline phosphate (PLAP, dark blue). (**C**) H3K9me2. (**D**) Double staining for H3K27me3 (deep blue) and PLAP (reddish brown). (**E**) H3K4me1, (**F**) H3K4me2/3, (**G**) H3K9ac and (**H**) H2A.Z. Arrows denote CIS cells and arrowheads Sertoli cells. Bar represent 50 microns.

Histone modifications in overt germ cell tumours

Seminomas showed high levels of H3K9me2, H3K27me3, H3K4me1 and H2A.Z, whereas low levels of H3K4me2/3 and H3K9ac were observed (Supplementary Figure S1). Some variation was observed for the H3K9me2 mark between seminoma samples (from a faint staining to strongly positive).

The undifferentiated component of non-seminomas, EC, showed no H3K27me3 and H3K9me2 staining but moderate levels of H3K4me2/3 methylation. A weak staining was observed for H3K4me1, H3K9ac and H2A.Z in EC (Supplementary Figure S1).

Histone modifications in normal adult testis

In normal testicular tissue H3K9me2 showed a very specific signal in a subset of spermatogonia, probably type A, and in round and elongating spermatids, whereas Sertoli cells was only faintly stained (Supplementary Figure S1), in contrast to the strongly stained Sertoli cells in CIS tubules (Figure 1C).

H3K27me3 by contrast was present in a broader set of germ cells with a low level in most spermatogonia and higher expression in round to elongating spermatids. Sertoli cells show high levels of H3K27me3 in both normal and CIS-containing tubules (Supplementary Figure S1 and Figure 1D).

H3K4me1 was observed in high levels in a subset of spermatogonia, probably type B but was also weakly present in other types of spermatogonia and Sertoli cells. Expression was again observed in late round spermatids but not in elongated spermatids. However, H3K4me2/3 was observed in a broad range of germ cells including elongating spermatids and in Sertoli cells (Supplementary Figure S1).

H3K9ac was abundant in spermatogonia, present at lower levels in spermatocytes but was absent from late spermatids. A low level was observed in Sertoli cells. Expression of H2A.Z was similar to the observed levels of H3K9ac, except that H2A.Z was highly expressed in round spermatids (Supplementary Figure S1).

Histone modifications in human foetal and pre-pubertal germ cells

Samples of foetal gonad tissue ranging from gestation week 17 to 41 were investigated to explore the level of histone modifications in human foetal germ cells. Foetal germ cells around GW 21–24 showed no DNA methylation and absence of repressive H3K9me2, H3K27me3 modifications (Figure 2B–D). In contrast, H3K9ac and H2A.Z were very abundant in foetal germ cells (Figure 2G and H). Surprisingly, H3K4me1 was observed in the cytoplasm of foetal germ cells and H3K4me2/3 was observed in high levels in only a

subset of the foetal germ cells (Figure 2E and F). It was roughly estimated that 20% of the foetal germ cells around GW 21 were marked with H3K4me2/3 with slight variations in the amount of positive foetal germ cells observed at earlier and later time points. However, no definitive trend could be identified.

The level of the investigated histone modifications did not differ significantly between foetal and adult Sertoli cells except for H3K9me2, which was found in high levels in Sertoli cells surrounding CIS and in foetal germ cells but not in normal Sertoli cells of any age (Figures 1C, 2C and supplementary Figure S1).

All results are outlined in Table 1.

Regulation of the H3K27me3 mark in CIS cells

Demethylation of di- and tri-methylated histone H3 at K27 can be carried out by the Jumonji proteins UTX and JMJD3 (Agger *et al*, 2007), whereas methylation of H3K27 can be carried out by
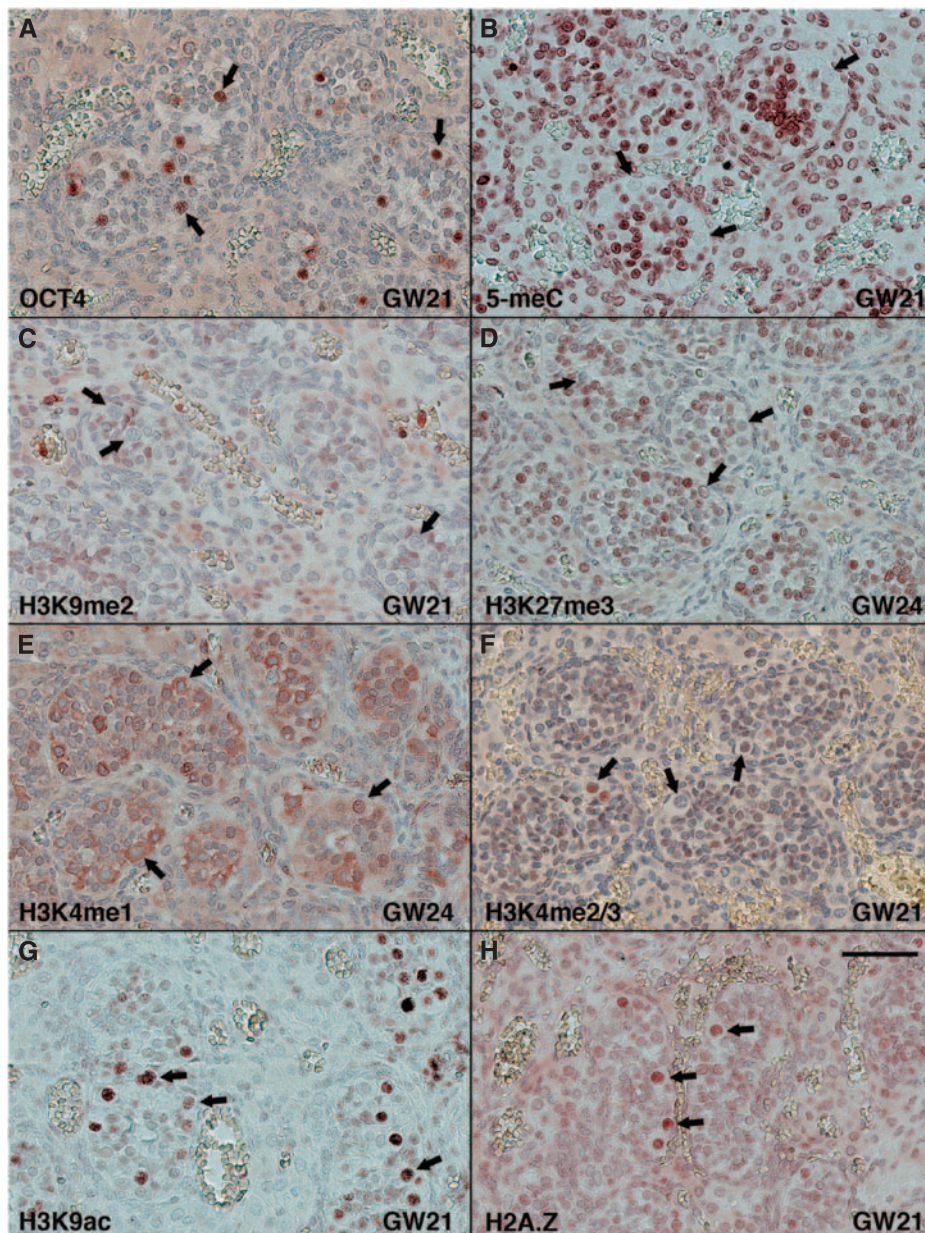

Figure 2 Histone modifications in human foetal gonads. Fetal testis tissues at GW 21–24 marked by immunohistochemical staining for (**A**) OCT4 (POU5F1) to visualise gonocytes, (**B**) 5-methylcytosine (5-meC), (**C**) H3K9me2, (**D**) H3K27me3, (**E**) H3K4me1, (**F**) H3K4me2/3, (**G**) H3K9ac and (**H**) H2A.Z. Arrows indicate foetal germ cells. Bar represents 50 microns.

Table 1 Summary of epigenetic patterns in normal and neoplastic testicular tissues

	Putative function	CIS	Gonocytes[a]	SEM	NonSEM (EC)	Normal adult testis
5-methyl-cytosine	Chromatin repression	neg	neg	neg/++	+++	Sp.gonia: all, Sp.cytes: all, Sp.tids: all
		Sertoli: ++	Sertoli: ++			Sertoli: ++
H3K9me2	Chromatin repression	neg	neg	+/+++	neg	Sp.gonia: A +++, Sp.cytes: neg, Sp.tids: round and elongated ++/neg
		Sertoli: +++	Sertoli: ++			Sertoli: neg/+
H3K27me3	Chromatin repression	neg/+	neg/+	+++	neg	Sp.gonia: neg/+, Sp.cytes: neg, Sp.tids: round to elongating +++/+
		Sertoli: +++	Sertoli: ++			Sertoli: +++
H3K4me1	Chromatin activation	+++	neg/+ (cytoplasma)	++/+++	neg/+	Sp.gonia: B, Sp.cytes: neg, Sp.tids: late round ++
		Sertoli: +	Sertoli: neg			Sertoli: +
H3K4me2/3	Chromatin activation	++/+++	–/++	neg/+	+/++	Sp.gonia: ++/+++, Sp.cytes: +/+++, Sp.tids: round +/++
		Sertoli: +	Sertoli: –/+			Sertoli: +/++
H3K9ac	Chromatin activation	+++	+++	neg/+	neg/+	Sp.gonia: +++/+, Sp.cytes: ++/neg, Sp.tids: neg
		Sertoli: neg/+	Sertoli: neg/+			Sertoli: +
H2A.Z	Chromatin activation	+++	+++	++/+++	neg/+	Sp.gonia: +/+++, Sp.cytes: ++, Sp.tids: round +++
		Sertoli: neg/+	Sertoli: neg/++			Sertoli: +
EZH2	H3K27 tri-methylation	+++ (cytoplasma)	Neg	neg/+	neg	Sp.gonia: neg (+ cytoplasma), Sp.cytes: neg, Sp.tids: neg
		Sertoli: neg	Sertoli: neg			Sertoli: neg
UTX	H3K27me3 demethylation	neg	Neg	neg/+	neg/+	Sp.gonia: neg, Sp.cytes: neg/+, Sp.tids: neg
		Sertoli: +	Sertoli: neg			Sertoli: neg
JMJD3	H3K27me3 demethylation	neg	neg/+	neg/+	neg/+	Sp.gonia: neg/+, Sp.cytes: neg/++, Sp.tids: neg/+
		Sertoli: neg/+	Sertoli: neg			Sertoli: neg/+ (cytoplasma)

Abbreviations: EC; embryonal carcinoma; EZH2, Enhancer of Zeste homologue 2; neg, negative; NonSEM, non-seminoma; SEM; seminoma; Sp.tid, spermatid; Sp.cytes, spermatocytes; Sp.gonia, spermatogonia. +, faint staining; ++, apparent; +++, strong positive. [a]Gestational week 20–40.

Enhancer of Zeste homologue 2 (EZH2), the catalytically active component of the Polycomb repressive complex 2 (Cao *et al*, 2002; Czermin *et al*, 2002).

We observed that UTX and JMJD3 were not expressed in CIS cells (Supplementary Figure S2B and C) but surprisingly, EZH2 was highly expressed in the cytoplasm of CIS cells (Supplementary Figure S2A). JMJD3 was weakly expressed in Sertoli cells, whereas only a faint EZH2 staining was observed in the cytoplasm of Sertoli cells. In overt tumours, faint staining of both proteins was observed, except for EZH2 expression in EC, which was totally absent (data not shown).

Re-evaluation of earlier genome-wide studies of gene expression in CIS cells (Almstrup *et al*, 2004; Skotheim *et al*, 2005; Sonne *et al*, 2009) did not indicate that any of the Polycomb genes or other transcripts encoding for proteins known to be involved in modifying histones were differentially expressed in CIS cells when compared with normal testicular tissue or gonocytes.

Transcriptional activity and proliferation of CIS cells

Methylation of histone H3K9 demarcates heterochromatin, whereas H3K4 methylation demarcates euchromatin (Fischle *et al*, 2003; Li *et al*, 2008) and collectively the above histone modifications observed in CIS cells could indicate an 'open' and permissive chromatin structure in CIS cells. We thus investigated the levels of RNA polymerase II and its Ser2-phosphorylated variant as measured by the H5 antibody. Ser2 phosphorylation is tightly associated with transcriptional elongation (Phatnani and Greenleaf, 2006) and is a marker of the global transcriptional

activity. Both RNA polymerase II and its Ser2-phosporylated variant were present in CIS cells in high levels (Figure 3A and B) indicating that transcription is highly active in CIS cells.

The RNA polymerase II was also highly expressed in a subset of spermatocytes and spermatogonia, in seminomas, but was, surprisingly, lower expressed in EC than CIS. High expression of RNA polymerase II, however, did not always correlate with a strong H5 antibody staining. In seminomas, the H5 level was low and expression of RNA polymerase II was high, whereas both RNA polymerase II itself and its active Ser2 form were observed in moderate levels in EC (data not shown).

These data suggested that CIS cells are highly transcriptionally active, which could reflect a high proliferation rate. Assessment of the proliferation rate of CIS cells was thus investigated by counting the mitotic index and the Ki-67-labelling index on 13 tissue sections containing CIS. Comparison of both mitotic index and Ki-67-labelling index of CIS cells and spermatogonia indeed indicated that CIS cells are highly proliferative. A statistically significant difference ($P = 0.001$) was observed between the mitotic index and Ki-67-labelling index of normal spermatogonia and those of the CIS cells (Table 2). No significant differences in the proliferation ratios were observed between the CIS cells adjacent to seminomas compared with CIS cells adjacent to non-seminomas (data not shown).

DISCUSSION

In this study, we performed a detailed analysis of epigenetic status of germ cell neoplasia and show that the precursor for the majority
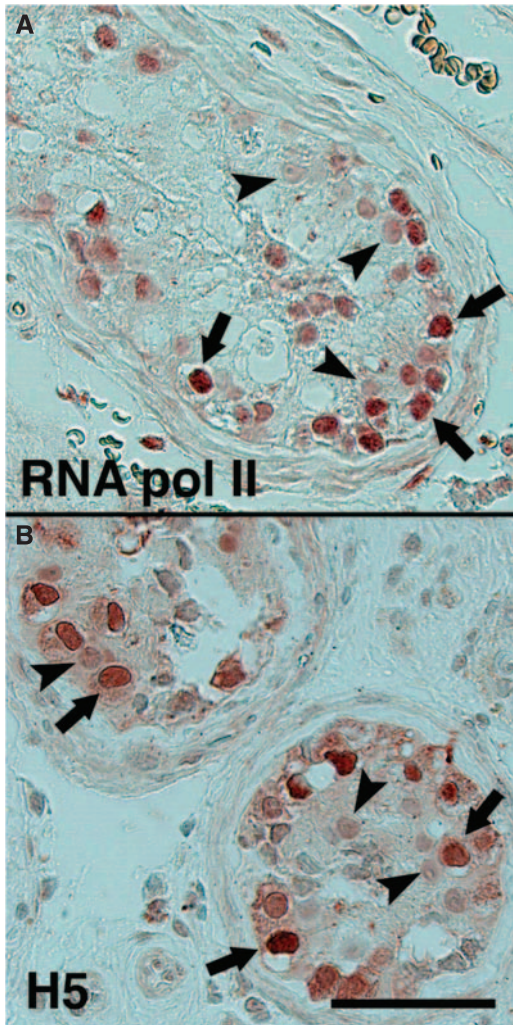
Genetics and Genomics

Figure 3 RNA polymerase II activity in CIS cells. Immunohistochemical detection of RNA polymerase II (**A**) and its Ser2-phosphorylated variant as measured by the H5 antibody (**B**). Arrows denotes CIS cells and arrowheads Sertoli cells. Bar represent 50 microns.

Table 2 Proliferation index

	MI (median)	Ki-67 (mean)
CIS	1.58% ($n = 13$)	17.42% ($n = 4$)
Spermatogonia	0.25% ($n = 4$)	6.75% ($n = 4$)
CIS *vs* spermatogonia	$P = 0.001$	$P < 0.001$

Abbreviations: CIS, carcinoma in situ; MI, mitotic index.

of germ cell cancers – carcinoma *in situ* – possesses low levels of repressive chromatin modifications (H3K9me2 and H3K27me3) concurrently with low DNA methylation and a range of activating chromatin modifications (H3K4me, H3K9ac, and H2A. Z). We also show that RNA polymerase II seems to be very active in CIS cells, and that this fits with a high proliferation rate of CIS cells.

On the basis of a high similarity between gene expression profiles of CIS cells and gonocytes we have earlier suggested that the CIS cell is a transformed gonocyte (Sonne *et al*, 2009). The present data further substantiate that hypothesis as the epigenetic profiles of gonocytes and CIS are highly similar but markedly differ from those of adult germ cells. The only exception was the level of H3K4me1 and H3K4me2/3 that were both abundantly present in the CIS nucleus but had only a limited presence in foetal germ cells. H3K4me2/3 was observed only in a subset of foetal germ cells, most likely gonocytes, which at the gestational age in the samples available for the study constitute only a very small minority of foetal germ cells. Identification of histone variants in the cytoplasm often indicates that they are bound for degradation by chaperones but the importance of the apparent discrepancy between foetal germ cells and CIS is currently unknown. Methylation of histone H3 at Lys4 is, however, known to be tightly associated with methylation of DNA. During embryonic development RNA polymerase II binds to CpG islands and directs H3K4 methylation by recruitment of H3K4 methyltransferases (Guenther *et al*, 2007). Such CpG regions are consequently methylated at Lys4 of histone H3, whereas the rest of the genome contains unmethylated H3K4. In fact, DNA methyl transferase 3L is known to interact directly with histone H3 when unmethylated at Lys4 and facilitate *de novo* methylation. Consequently, DNA methylation may be prevented at such CpG islands primed by RNA polymerase II due to methylation of H3K4. This is in line with the correlation between the high levels of H3K4 methylation and the low DNA methylation levels observed in CIS cells. In addition, the high RNA polymerase II levels could be a consequence of the correlation between RNA polymerase occupancy, H3K4 methylation and low DNA methylation. Similarly, it is known that H2A.Z protects genes from DNA methylation (Zilberman *et al*, 2008), so the high H2A.Z expression in CIS cells is consistent with their low DNA methylation. Loci containing H2A.Z have been observed predominantly at sites occupied by RNA polymerase II along with enhancer regions (Barski *et al*, 2007) and again this seems to fit with the observed high levels of elongating RNA polymerase II in CIS cells. However, we do not know whether the DNA methylation in CIS cells may be hydroxylated and thus unrecognised by the currently used antibodies (Tahiliani *et al*, 2009; Huang *et al*, 2010). Low DNA methylation in the gene body and out of a CpG context has recently been suggested to be of great importance in embryonic stem cells (Ramsahoye *et al*, 2000; Lister *et al*, 2009).

In mice, during PGC migration a progressive erasure of H3K9me2 and establishment of H3K27me3 occurs concurrently with genome-wide erasure of DNA methylation (Seki *et al*, 2007). In addition, as the PGC colonise the gonad in mice, a range of other epigenetic events occurs, which includes erasure of both H2A.Z and H3K9ac (Hajkova *et al*, 2008). One of the important findings of our study is that the regulation of these chromatin marks is substantially different in humans. Migration of human PGCs takes place during GW 4–6 and after GW 6 germ cells are observed in the gonadal anlage and begin to be surrounded by immature Sertoli cells. From that point of time, the term gonocyte is used to describe the germ cells in the male gonad (Fujimoto *et al*, 1977). At GW 21–24, human gonocytes are definitively post-migrational germ cells but show low levels of H3K27me3 and high levels of H3K9ac and H2A.Z, which is comparable with migrating mouse PGC. In addition, human foetal germ cells at GW 16–24, show absence, or very low levels, of H3K9me3 (Bartkova *et al*, 2010). High levels of H4/H2A R3me2s have also been reported in human GW 19 gonocytes (Eckert *et al*, 2008), matching the epigenetic profile of migrating PGCs in mice (Seki *et al*, 2007; Hajkova *et al*, 2008). There is a possibility that all of these marks are transiently reverted in mouse gonocytes at a later foetal age, however, it is not likely, given that the subset of murine germ cells, recognised later as stem spermatogonia, do retain some features of PGC, including high expression of pluripotency genes.

Presumably there is a tight connection between the epigenetic and DNA repair machinery (Hajkova *et al*, 2008) and previous studies has demonstrated that CIS cells and foetal germ cells use different machinery of cell cycle and DNA repair than that operating in post-pubertal germ cells (for review, see Bartkova *et al*, 2007). This is clearly linked to the post-pubertal switch to meiotic division, which requires double breaks of DNA and their

subsequent repair in the process of homologous recombination. The similarities between the epigenetic profile of CIS and foetal germ cells are in concert with these data.

Taken together our results clearly indicate that the CIS genome seems to have a open and foetal-like chromatin structure, which potentially could lead to chromosomal instability and subsequently a malignant phenotype, as observed in some somatic cancers, for example, colorectal cancer (Rodriguez *et al*, 2006). High proliferation rates of CIS cells could be a consequence of the open chromatin in CIS cells but stimulation by gonadotropins and androgens, which normally induce proliferation and maturation of germ cells in the post-pubertal testis, may also have a role. It is currently unknown whether the epigenetic profile observed in CIS remains the same or dynamically changes during malignant progression.

Enhancer of Zeste homologue 2 is one of the key enzymes involved in creating the H3K27me3 mark, and we observed it to be highly expressed in the cytoplasm of CIS cells. This enzyme is known to be amplified in a large range of cancers (Bracken *et al*, 2003) and in prostate cancer it is involved in promoting proliferation and invasiveness (Bryant *et al*, 2007). In benign prostate epithelial cells, EZH2 is expressed at low levels in the cytoplasm but becomes overexpressed in prostate cancer cells, in which its putative function is regulation of actin polymerisation (Su *et al*, 2005; Bryant *et al*, 2008). However, it is not known whether EZH2 is involved in actin regulation in CIS cells or whether the cytoplasmic location is a result of a defective translocation to the nucleus, but EZH2 is most probably not engaged in its 'normal' function as part of polycomb or swi/snf complexes as none of the genes encoding co-enzymes were observed overexpressed in CIS.

In contrast to CIS, overt germ cell tumours derived from CIS cells seem to have acquired variable epigenetic modifications. Seminomas show high levels of selected repressive modifications, exemplified by H3K9me2 and H3K27me3. Non-seminomas, however, show high methylation levels (Smiraglia *et al*, 2002; Netto *et al*, 2008), but the embryonal carcinoma, which is the most undifferentiated component of non-seminomas, nevertheless retains a very open and foetal-like histone profile. We observed some heterogeneity, but many more samples need to be investigated to deduce whether, for example, there are discrete subsets of seminomas with different modification patterns. Heterogeneity of DNA methylation was indeed observed in seminomas in which 6 out of 33 samples were positive (Netto *et al*, 2008).

Concepts of epigenetic plasticity in cells, which upon niche disturbances stochastically select for epigenetic heterogeneity and consequently, facilitate the ability of cells to acquire abnormal characteristics, have been put forward (Ushijima and Asada, 2009; Feinberg and Irizarry, 2010). These data are in line with this concept and the current views on the pathogenesis of germ cell tumours, which suggest that developmental disturbances of the somatic niche could result in improper maturation of foetal germ cells (Skakkebaek *et al*, 2001). We can add to this hypothesis that developmental delay of germ cell maturation results in preservation of the permissive foetal epigenetic profile, which on hormone stimulation during puberty leads to an aberrant induction of transcription and proliferation, ultimately leading to germ cell cancer later in life.

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