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# Epigenetic features of testicular germ cell tumours in relation to epigenetic characteristics of foetal germ cells

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**ABSTRACT Foetal development of germ cells is a unique biological process orchestrated by cellular specification, migration and niche development in concert with extensive epigenetic and transcriptional programs. Many of these processes take place early in foetal life and are hence very difficult to study in humans. However, the common precursor of testicular cancers – the carcinoma *in situ* (CIS) cell – is thought to be an arrested foetal germ cell. Therefore studies of CIS cells may leverage information on human foetal germ cell development and, in particular, when neoplastic transformation is initiated. In this review, we will focus on current knowledge of the epigenetics of CIS cells and relate it to the epigenetic changes occurring in early developing germ cells of mice during specification, migration and colonization. We will focus on DNA methylation and some of the best studied histone modifications like H3K9me2, H3K27me3 and H3K9ac. We also show that CIS cells contain high levels of H3K27ac, which is known to mark active enhancers. Proper epigenetic reprogramming seems to be a pre-requisite of normal foetal germ cell development and we propose that alterations in these programs may be a pathogenic event in the initiation of testicular germ cell cancer. Even though only sparse information is available on epigenetic cues in human foetal germ cells, these indicate that the developmental patterns differ from the findings in mice and emphasize the need for further studies of foetal germ cell development in humans.**

KEY WORDS: *primordial germ cell, gonocyte, epigenetics, carcinoma in situ testis*

## Introduction

The natural history of germ cells is characterised by several developmental milestones which are characterised by early embryonic pluripotency, followed by gradual increase in germ-cell specific factors and a post-pubertal onset of meiotic division required to produce haploid gametes. During early foetal development, migrating primordial germ cells (PGCs) as well as gonocytes undergo extensive epigenetic modifications, which include erasure and re-establishment of DNA methylation and exchange of histone modifications. These sequential changes in epigenetic modifications, occurring within a narrow developmental time window, is termed epigenetic reprogramming and seems to be a prerequisite of normal germ cell development. It is well recognized that epigenetic modifications are susceptible to alterations when exposed to environmental factors (Reviewed in Feil and Fraga, 2011). Indeed, animal studies show that certain exposures during foetal development cause epigenetic alterations with a phenotypic outcome, including alterations in the development of male germ cells.

There is growing evidence that testicular germ cell-derived tumours (TGCTs) in humans profoundly differ in their gene expression profiles and epigenetic regulation from normal germ cells residing in the adult testis. Epigenetic reprogramming of TGCTs is sensitive to environmental exposures, hence etiological factors seem to be linked to environmental cues. This pinpoints that the epigenetic layer of information in foetal germ cells is detrimental in the pathogenesis of testicular cancer (TC). In this article we shall review the current knowledge on the epigenetics of TC, with focus on the precursor lesion, carcinoma *in situ* (CIS) and relate these findings to the epigenetic changes occurring in early developing germ cells.

*Abbreviations used in this paper:* CIS, carcinoma *in situ*; PGCs, primordial germ cells; TGCTs, testicular germ cell-derived tumours; TC, testicular cancer; TDS, Testicular Dysgenesis Syndrome; ITGCN, intratubular germ cell neoplasia unclassified; TIN, testicular intraepithelial neoplasia; GW, gestational week; 5mC, DNA methylation at the 5$^{th}$ position of cytosine; 5hmC, hydroxymethylation at the 5$^{th}$ position of cytosine; EC, embryonal carcinoma; TSGs, tumour suppressor genes; SS, spermatocytic seminoma.

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## Testicular cancer: epidemiology and pathology

In most western countries TGCTs are the most frequent malignancy found among young males. In Norway and Denmark they account for approximately 1% of all cancers in men. Worldwide, TC has more than doubled in the last 40 years, but the incidence varies considerably in different geographical areas and also between ethnic groups (Huyghe *et al.*, 2003). TGCTs is 4.5 times more common among white men than black men in the USA (Moul *et al.*, 1994) and twice more frequent in Denmark than Sweden (Chia *et al.*, 2010). Interestingly, analysis of incidence rates among immigrants to Sweden (including men from Chile and Finland) has revealed that the first-generation immigrants have similar rates to the men in their country of origin, whereas the second-generation men acquire the Swedish risk irrespectively of the parental geographical origin, indicating that the place of birth determines the risk of TC and implying that *in utero* environmental exposures play a large role as risk factor (Hemminki and Li, 2002a, Hemminki and Li, 2002b, Hemminki *et al.*, 2002). Recently Norway and Denmark were reported to have the highest and second highest incidence of TC in the world (Chia *et al.*, 2010). Chile was reported to have the third highest incidence, which indicates that high TC incidence is not confined to the closely related environment found in Nordic European countries.

Clinical risk factors for TC include male infertility (RR=3) (Mancini *et al.*, 2007, Walsh *et al.*, 2009) and a history of cryptorchidism (RR=2-8) (Dieckmann and Pichlmeier, 2004, Wood and Elder, 2009). Some cases of cryptorchidism, male infertility, and most TGCTs share the common feature of foetal developmental origins. Furthermore, histological investigations showed that TGCTs often coincides with dysgenetic tubules presenting spermatogenic arrest, microcalcifications, poorly differentiated Sertoli cells, Sertoli-cell-only pattern etc. (Hoei-Hansen *et al.*, 2003). These observations, among others, linked TGCTs to other disorders of male reproduction, and led to the proposed grouping of these disorders within the so-called Testicular Dysgenesis Syndrome (TDS) (Skakkebaek *et al.*, 2001). Growing evidence suggests that the majority of cases of TDS are caused predominantly by external factors, which may act directly on numerous pathways involved in testis development. The factors remain to be identified and proven causative, but factors related to modern lifestyle, including endocrine disruptors have been under growing scrutiny as possible culprits. Indeed, evidence from animal models indicates that perinatally administered endocrine disrupters, like phthalates, can induce dysgenic lesions in the testes of rats and rabbits (Fisher *et al.*, 2003, Foster *et al.*, 2001, Higuchi *et al.*, 2003), similar to those frequently observed in patients with TGCTs (Hoei-Hansen *et al.*, 2003, Skakkebaek *et al.*, 2003). Interestingly, primarily somatic cells, in particular Leydig cells, and not directly germ cells, seem to be affected (Mahood *et al.*, 2005). The effect on germ cells thus seems to be secondary but nevertheless adversely affecting their perinatal development. Increased and widespread use of industrial products which act like endocrine disrupters (e.g. phthalates) combined with an increase in endocrine-related diseases, which may cause dysgenesis of the testis (e.g. obesity), have thus raised growing concerns of possible association between exposure of endocrine disrupting chemicals and human adverse effects. Indeed, elevated levels of chemicals, such as polychlorinated biphenyls, hexachlorobenzene and chlordanes have been detected in blood from the mothers of men with TC (Hardell *et al.*, 2003). The dramatic increase in TC observed over few generations suggests that the effect on the cellular level should be either a direct (affecting transcription factors directly) or an epigenetic effect inflicting foetal germ cell differentiation.

The vast majority of TGCT occur in young men and manifest either as a seminoma or non-seminoma. These are different both in terms of histology and treatment regimes but appear equally frequent. Seminoma is a homogeneous tumour composed of mitotically dividing germ cells, whereas non-seminomas are heterogeneous tumours that may contain varying proportions of undifferentiated embryonal carcinoma, partially differentiated somatic tissues (teratoma), and extra-embryonic elements such as choriocarcinoma and yolk sac tumour (Ulbright *et al.*, 1999). Both of these tumour types are preceded by a symptom-less pre-invasive stage, the carcinoma *in situ* (CIS) cell (Skakkebaek, 1972) also known as intratubular germ cell neoplasia unclassified (ITGCN) or testicular intraepithelial neoplasia (TIN). CIS cells are often found in usually normally arranged seminiferous tubules where these cells are situated along the peritubular membrane, as shown in Fig. 1A.

According to our hypothesis, under-stimulation or dysregulation of primordial germ cells (PGCs) or gonocytes during early development is a key event leading to an arrest in germ cell differentiation and hence formation of "dormant" CIS cells, that later in life progress to invasiveness and TGCTs. In similarity to embryonic stem cells and early foetal germ cells, CIS cells retain a high expression of pluripotency genes (Almstrup *et al.*, 2004) and virtually no difference was found between gene expression profiles of microdissected gonocytes and CIS cells (Sonne *et al.*, 2009b). These observations substantiate our hypothesis that the formation of CIS cells is a result of impaired differentiation and developmental arrest of foetal germ cells. We further hypothesize that the developmental arrest is caused by disruption of the endocrine environment of the testis niche. The niche is comprised of somatic cells; mainly Sertoli- and Leydig cells as well as peritubular cells, which secrete hormones and paracrine factors that stimulate male-specific germ cell maturation into spermatogonia (Rajpert-De Meyts, 2006). The stimulation of "dormant" CIS cells in the pubertal testis is thought to increase proliferation and accumulation of genomic changes, which at some point trigger a malignant phenotype.

## Epigenetic cues in developing foetal germ cells

In order to understand the epigenetic disruptions on germ cells leading to development of CIS it is a prerequisite to understand the reprogramming of normal foetal germ cells. Most of our current knowledge on foetal germ cell development comes from studies in mice, probably due to difficulties in obtaining sufficient material for human studies. The subsequent sections will hence focus on epigenetic cues in foetal mouse germ cells followed by a section summarizing the sparse knowledge of epigenetics in human germ cell development.

### *Specification*

During embryonic development, PGCs are specified from a subset of posterior proximal epiblast cells which are first observed at embryonic day E7.25 (mice) in the posterior end of the primitive

streak. *Blimp1* positive cells are however already seen at E6.25 and mark founder PGCs, which prior to E6.25 are indistinguishable from somatic neighbours (Ohinata *et al.*, 2005). *Blimp1* is crucial for normal germ cell specification and is thought to be engaged in suppression of somatic programs by *Hox* genes (i.e. *HoxA1* and *HoxB1*) (Ohinata *et al.*, 2005), which already have been initiated at E6.25. In the few founder (approximately 40 PGCs) and migrating PGCs Blimp1 is found in the nucleus in complex with Prmt5, where it mediates symmetrical di-methylation of histones H4 and H2A at arginine 3 (H4/H2AR3me2). When murine PGCs arrive in the genital ridge, the complex translocates to the cytoplasm, leading to reduced levels of H4/H2AR3me2 (Ancelin *et al.*, 2006). Translocation of Blimp1/Prmt5 to the cytoplasm leads to down-regulation of Blimp1, which in turn coincides with re-expression of pluripotency-associated genes, including Nanog and Sox2

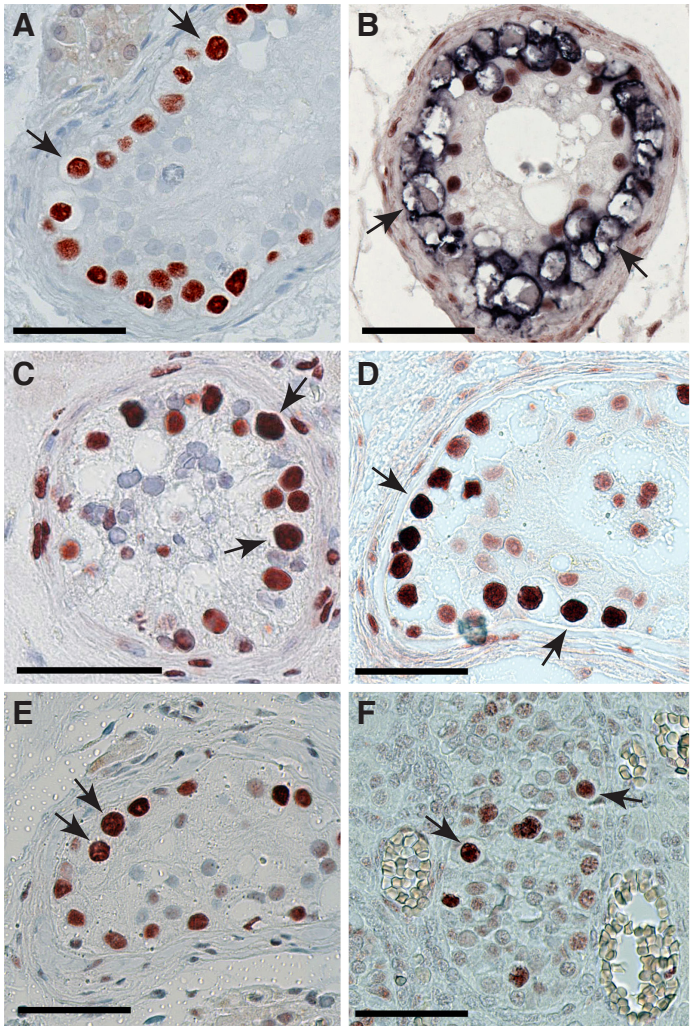


**Fig. 1. Examples of immunohistochemical stainings of tissue containing carcinoma *in situ* (CIS) cells or gonocytes. (A)** *CIS cells (arrows) express the transcription factor AP-2γ gamma (TFAP2C; Hoei-Hansen* et al.*, 2004).* **(B)** *Double staining with PLAP, a classical CIS marker, in blue and 5-methylcytosine in red. CIS cells (arrows) show no staining with 5-methylcytosine, whereas somatic Sertoli cells stain intensively. CIS cells (arrows) display the following histone modifications:* **(C)** *H3K27ac,* **(D)** *H3K4me1 and* **(E)** *H3K9ac.* **(E)** *Gestational week 21 gonocytes (arrows), which like CIS cells, display staining for H3K9ac. All bars represent 50 microns.*

(Ohinata *et al.*, 2005, Yabuta *et al.*, 2006). In this particular stage, there is a significant difference between species: human PGCs do not express SOX2 but retain high expression of NANOG and POU5F1 (Perrett *et al.*, 2008).

### *Migration*

Approximately at embryonic day E8 (mice) PGCs start to migrate towards the genital ridge and colonize the primitive gonad, which is tightly associated with the mesonephros. The primitive gonad is still sexually bipotential and sex-determination first takes place between E10.5 and E11.5.

During specification and migration PGCs undergo epigenetic reprogramming, including genome-wide DNA de-methylation (starting at E8), progressive erasure of H3K9me2 (starting at E7.5) and subsequently establishment of H3K27me3 (starting at E8.25) in a progressive, cell-by-cell manner, presumably depending on their developmental maturation (Seki *et al.*, 2005, Seki *et al.*, 2007). DNA methylation and the above-mentioned histone modifications are repressive epigenetic marks associated with DNA silencing. The loose chromatin structure would allow massive transcription, but in this period transcription in PGCs is kept silent by transient repression of RNA polymerase II-dependent transcription (Seki *et al.*, 2007). In addition, prior to H3K9 de-methylation PGCs repress the histone methyltransferase, GLP, which mediates H3K9 methylation (Tachibana *et al.*, 2008).

The genome-wide DNA de-methylation is suggested to occur due to the downregulation of the DNA methyltransferase Dnmt1, which maintains methylation patterns of mother strands after DNA replication. Dnmt1 is transiently downregulated at least in a portion of PGCs after the time of their fate determination, and is subsequently re-expressed by the time of their migration (E8.25) (Seki *et al.*, 2005).

In humans, migration of PGCs takes place during gestational week (GW) 4-6 and after GW 6 germ cells are found in the gonadal region and immature Sertoli cells begin to surround them. From that point of time, the term gonocyte is used to describe the germ cells in the male gonad (Fujimoto *et al.*, 1977).

### *Colonization*

Arrival in the genital ridge seems to set a critical milestone in the germ cells development, because at this stage a cascade of epigenetic events takes place, leading to differentiation of the surrounding niche into male or female characteristics. In murine PGCs the colonization of the genital ridge (E11.5) is associated with extensive chromatin and epigenetic reprogramming involving linker histone H1, H3K9ac, H3K9me2, H3K27me3, DAPI chromocenters and H4/H2A R3me2 (Hajkova *et al.*, 2008) and a second wave of DNA de-methylation (the first wave occurs during pre-implantation), where parental imprints are erased (Hajkova *et al.*, 2002). Some parental imprints are already set again starting at E14.5, while others are first acquired at the new-born stage (Li *et al.*, 2004). By FACS analysis of dissected urogenital ridges of an *Oct4-GFP* transgenic mouse, Hajkova *et al.,* (2008) identified two Oct4+ (GFP) populations of PGCs at E11.5. These two populations were shown to reflect a developmentally primitive and a more advanced PGC population. The advanced population showed low levels of H2A.Z, linker H1, H3K9ac, H3K9me2 and H3K27me3 as well as H2A/H4R3me2, when compared to the primitive population showing higher levels of these marks. Accordingly,

the primitive population showed variations in the levels of DNA methylation (5-methyl-cytosine levels), whereas the advanced population was practically devoid of DNA methylation (Hajkova *et al.*, 2008). A rapid erasure of DNA methylation has been found to occur between E10.5 to E12.5 at imprinting control regions (ICRs) in murine PGCs colonized in the gonadal ridge (Hajkova *et al.,* 2002). However, a recent study investigated the methylation status of gene promoters in PGCs by MeDIP-sequencing, and showed that de-methylation was already initiated during migration at E9.5, with a complete erasure of DNA methylation occurring between E11.5 and E12.5 (Guibert *et al.*, 2012). This indicates that different kinetics of de-methylation exist, as non-imprinted regions are de-methylated with an earlier onset compared to de-methylation of ICRs. In line with these observations Popp *et al.,* (Popp *et al.*, 2010) found that only 15% of the mouse PGC genome at E13.5 was methylated. Interestingly, in mice with knock-out of the DNA deaminase *(Aid)* a significant increase in PGC-methylation was observed indicating implication of AID in the de-methylation of PGCs. Deamination of a methylated C (5mC) by AID results in conversion of the C into T and hence a T-G mismatch, which is recognized and repaired by the base-excision repair (BER) machinery. In line with these observations, Hajkova *et al.,* (Hajkova *et al.*, 2010) found an upregulation of genes involved in BER, including *Parp1*, *Ape1*, and *Xrcc1*, in PGCs. However, other parallel mechanisms of active DNA de-methylation exist, which among other possibilities, implicate conversion of 5mC into hydroxymethylated-C (5hmC). Conversion of 5mC into 5hmC is catalysed by the ten-eleven translocation (TET) family of proteins. 5hmC can subsequently be converted into other derivatives of 5hmC to end as an unmethylated C. Indeed, Tet1 expression was found at high levels in 11.5 dpc PGCs (Hajkova *et al.*, 2010) and it remains to be deduced whether several lines of DNA de-methylation can efficiently substitute each other in order to achieve complete de-methylation. Complete de-methylation seems to be a pre-requisite of achieving pluripotency as the first events in the reprogramming of somatic cells into induced pluripotent stem cells depend on the action of Parp1 and Tet2 (Doege *et al.*, 2012).

Besides the above-mentioned active DNA de-methylation mechanisms passive DNA de-methylation may also occur. This could be facilitated by inhibition of DNA methyl-transferases (both de novo enzymes DNMT3A and B and maintenance enzyme DNMT1). Indeed, PARP1 has been suggested to be implicated in regulation of DNMT1 (Zampieri *et al.*, 2012, Zampieri *et al.*, 2009) together with UHRF1 (Bostick *et al.*, 2007).

Due to extensive epigenetic reprogramming, PGCs that colonize the early gonads attain low levels of DNA methylation and low levels of both active (H2A.Z, H3K9ac) and repressive histone marks (H3K9me2, H3K27me3, H2A/H4R3me2) as well as low expression of linker H1.

During the colonization of the gonadal ridges, sex-determination of developing germ cells also takes place. Expression of the gene *Sry* in precursors of Sertoli cells is thought to be one of the most important sex-determination events. If *Sry* is expressed, this subsequently triggers expression of *Sox9* and several other masculinising genes, which are essential for proper Sertoli cell differentiation and formation of cord structures in mice at E12 (reviewed in Koopman, 2010).

***Testicular development***

After colonization of the genital ridges, PGCs proliferate and when a sufficient number is reached female germ cells enter meiotic prophase while male germ cells (gonocytes) arrest in the G0/G1-phase of the cell cycle until after birth. After birth, gonocytes give rise to primitive spermatogonia (spermatogonial stem cells) that start to proliferate and develop into spermatocytes at days 10-14 post natal in mice.

Recently, it was demonstrated in mice that the mitosis-meiosis switch is controlled by Dmrt1 in a sex-specific manner (Krentz *et al.*, 2011, Matson *et al.*, 2010). The sex-dimorphic expression pattern of DMRT1 in human gonads indicates that DMRT1 may play a similar role in regulation of meiotic entry in humans (Jorgensen *et al.*, 2012).

Murine PGCs in the genital ridge are virtually devoid of DNA methylation (Reik and Walter, 2001) but *de novo* methylation is initiated in male germ cells at E14.5 and thereafter in female germ cells, and mature gametes of both sexes will eventually become highly methylated. Additional DNA de-methylation and histone modifications take place in pubertal and adult male germ cells after meiosis during spermatogenesis (Bernardino *et al.*, 2000, del Mazo *et al.*, 1994, Norris *et al.*, 1994). Histones are exchanged with transition proteins and finally with protamines. It has been suggested that re-methylation and possible further chromatin modifications may occur when spermatozoa undergo final maturation in the epididymis (Xie *et al.*, 2002).

***Human germ cell development***

Very little is known about epigenetics in human foetal germ cells; the sparse information indicates that epigenetic cues may be different between human and mouse (Almstrup *et al.*, 2010).

We have shown that human foetal gonocytes in a developing testis around gestational week 24 (GW24), show high levels of the histone variant H2A.Z and histone modifications H3K9ac and H3K4me2/3, but low levels of H3K9me2 and H3K27me3 (Almstrup *et al.*, 2010). In addition, gonocytes at GW19 have been shown to contain H4/H2AR3me2 (Eckert *et al.*, 2008). Even though detailed developmental analysis of histone marks in human germ cells is missing, the above mentioned histone marks of human gonocytes indicates that epigenetic cues between human and mice are different. In mice, H3K27me3 is acquired during migration (Seki *et al.*, 2007) but seems absent in human gonocytes (Almstrup *et al.*, 2010). H3K9ac and H4/H2AR3me2 become less abundant after migration in mice (Hajkova *et al.*, 2008) but seem still present in human gonocytes (Almstrup *et al.*, 2010, Eckert *et al.*, 2008).

Similar to mice, DNA methylation is slowly re-established in foetal gonocytes from GW15 (earliest time point investigated) until around birth where DNA methylation seems similar to their somatic neighbours (Almstrup *et al.*, 2010, Wermann *et al.*, 2010). The re-methylation period in human gonocytes/pre-spermatogonia however seems to be somewhat prolonged compared to what is observed in mice.

Taken together, the sparse information from human foetal germ cells indicates that some differences exist between mice and human epigenetic cues. By contrast, epigenetic patterns of porcine foetal germ cells seem to be similar to those observed in mice (Hyldig *et al.*, 2011a, Hyldig *et al.*, 2011b) and further

investigation is needed to thoroughly map human epigenetic developmental cues in foetal germ cells.

## Epigenetics in the pathogenesis of testicular germ cell cancer

CIS cells were first described in 1972 by Niels E. Skakkebæk (Skakkebaek, 1972). Probably due to a similar intratubular localization as early spermatogonia, many scientists did not acknowledge this new finding and believed that these cells were abnormal spermatogonia. However, growing evidence indicated that CIS cells indeed were neoplastic cells (Akhtar and Sidiki, 1979, Berthelsen *et al.*, 1979).

Already a few years after the initial discovery of CIS cells morphological studies indicated that the CIS cells were very similar to foetal germ cells (Albrechtsen *et al.*, 1982, Nielsen *et al.*, 1974, Skakkebaek *et al.*, 1987). Later, the immunohistochemical markers for CIS cells; placental-like alkaline phosphatase (PLAP) and the

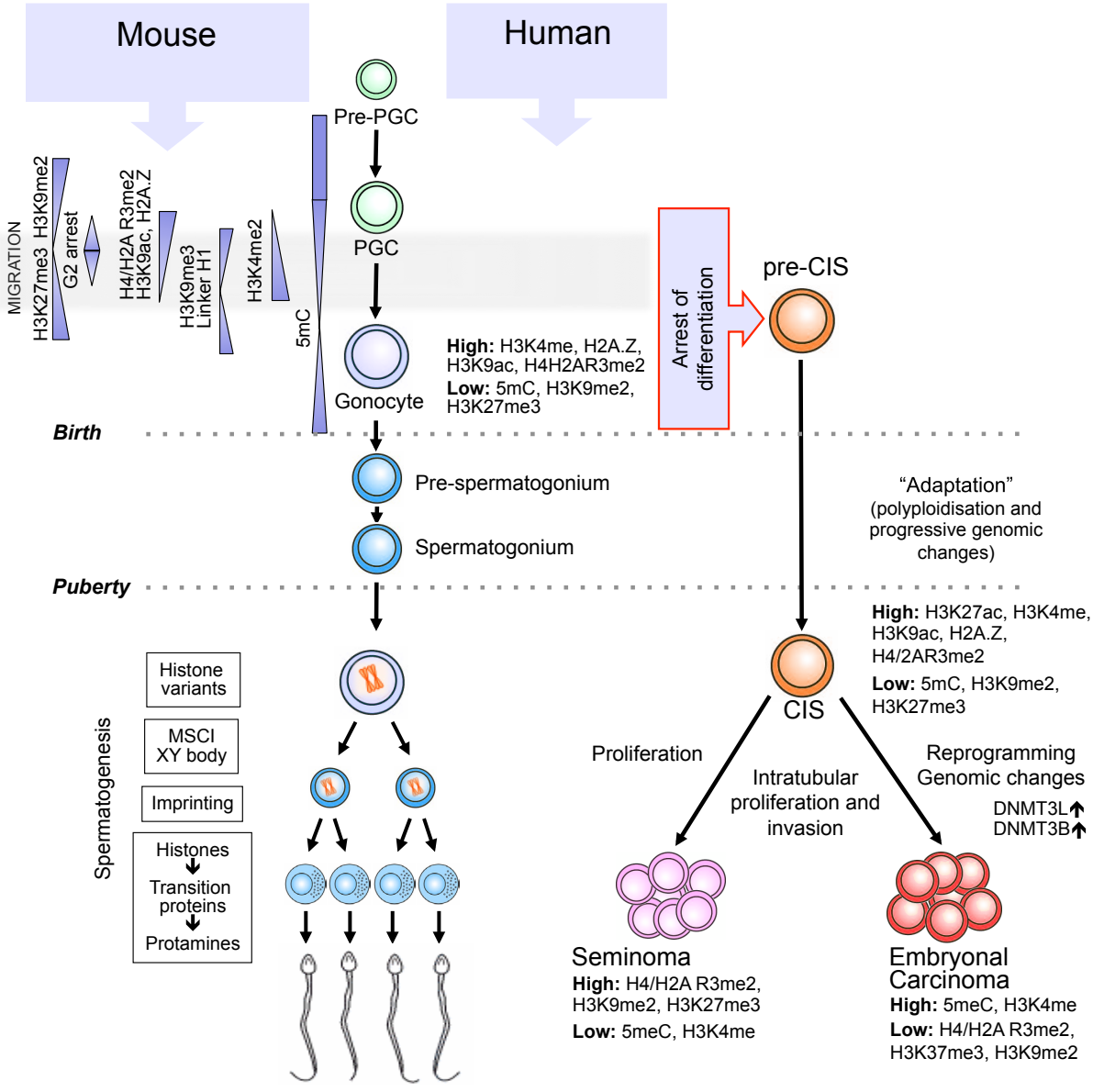


**Fig. 2. Schematic drawing of germ cell development in human and mouse from specification of foetal primordial germ cells (PGCs) until pubertal onset of spermatogenesis and development of spermatozoa.** *After puberty only human germ cell development (left) is illustrated and neoplastic transformation of foetal germ cells into carcinoma* in situ *(CIS) is only observed in humans (right). In contrast to mice, very little is known about epigenetic patterns during human foetal germ cell development. DNA methylation patterns are greatly simplified as several waves of DNA demethylation occur and different categories of sequence (CpG, non-CpG, repeat elements, X chromosome, imprinted genes etc.) may be independently regulated. Accordingly, represented regulations are not exact but are intended as an overview. Data on H3K27me3 (Hajkova* et al.*, 2008, Seki* et al.*, 2007) is somewhat divergent and the depicted pattern is adapted from Seki* et al.*, 2007. The only non-seminoma depicted is the embryonal carcinoma as this is the most investigated non-seminoma subtype. MSCI; Meiotic sex chromosome inactivation, CIS; carcinoma in situ, PGC; primordial germ cell.*

protooncogene c-kit protein were also found to be highly expressed in human foetal germ cells (Jørgensen *et al.*, 1995). Subsequently several comparative studies of protein markers (reviewed in Rajpert-De Meyts *et al.*, 2003) and global gene expression profiles of CIS cells (Almstrup *et al.*, 2004, Almstrup *et al.*, 2007, Sonne *et al.*, 2009a) further substantiated that CIS cells are indeed very similar to gonocytes. Moreover, we have recently shown that miRNA profile of CIS cells partially overlaps with that of foetal gonocytes (Novotny *et al.*, 2012).

Our current hypothesis stipulates that foetal germ cells (gonocytes or PGCs) are arrested during perinatal development, probably due to an under-virilisation of the developing somatic niche, and hence give rise to dormant pre-CIS cells. Further transformation of pre-CIS cells, most likely occurs as a response to the changed endocrine environment during the peri- and post-pubertal testicular development and ultimately leads to malignant transformation and proliferation. As pre-CIS cells develop into "real" neoplastic CIS cells they undergo polyploidisation (Oosterhuis *et al.*, 1989) and acquire a characteristic pattern of genomic aberrations, including gain of chromosome 12p (Ottesen *et al.*, 2003, Rosenberg *et al.*, 2000), often seen as the isochromosome 12p (Atkin and Baker, 1982). In analogy to PGCs and gonocytes, CIS cells express transcription factors associated with embryonic stem cell pluripotency, including e.g., POU5F1/OCT-3/4, NANOG, T1A-2, MYCL1, GDF3, LIN28-A, DPPA4, DPPA5, KIT and AP-2$\gamma$ (Almstrup *et al.*, 2004, Biermann *et al.*, 2007, Gillis *et al.*, 2011, Hoei-Hansen *et al.*, 2005, Hoei-Hansen *et al.*, 2004, Looijenga *et al.*, 2003, Skotheim *et al.*, 2005, Sperger *et al.*, 2003).

In accordance with the expression pattern of genes and transcription factors associated with pluripotency and foetal expression patterns, the epigenetic pattern of CIS is associated with an open and permissive chromatin structure (Almstrup *et al.*, 2010). CIS cells contain very low levels of DNA methylation when detected by immunohistochemical staining for 5-methyl-cytosine (Netto *et al.*, 2008) as shown in Fig. 1B. Histone modifications H3K9me2 and H3K27me3, both associated with a restrictive chromatin structure, were absent in CIS cells but H3K4me1 (Fig. 1D), H3K4me2/3, H3K9ac (Fig. 1E) and the histone variant H2A.Z, which are all modifications associated with active and permissive chromatin structure, were abundantly present (Almstrup *et al.*, 2010). This epigenetic pattern, in accordance to the epigenetics of mouse PGCs (see above), indicates that the CIS cells are arrested in the transition between H3K9me2 erasure (E7.5) and establishment of H3K27me3 (E8.25). However, in comparison to human foetal germ cells the CIS pattern reflects a much larger developmental time span, as human foetal gonocytes still show low levels of H3K9me2 and H327me3 in GW24 (Almstrup *et al.*, 2010). The permissive and foetal-like epigenetic state of CIS will also dictate massive transcription, and we have shown that, unlike mouse PGCs, the CIS cells have an active RNA polymerase II and show a great proliferation rate (Almstrup *et al.*, 2010). In accordance to this observation we showed that also H3K27ac is abundantly present in CIS cells (Fig. 1C). The H3K27ac mark has been found to co-localize to a proportion of H3K4me1-marked enhancer regions, but has also been found to separate active enhancers (H3K27ac+) from poised enhancers (H3K27ac-) and hence more truly reflects transcriptional activity. Active enhancers marked with both H3K27ac and H3K4me1 were found to be associated with developmental genes typical of multipotent stem cells (Creyghton *et al.*, 2010). Interestingly both of these marks are abundant in CIS cells (Fig. 1C). In addition to the above-mentioned epigenetic marks in CIS cells, H3K9me3, HP1$\alpha$ and $\gamma$ (Bartkova *et al.*, 2011) as well as H4/H2AR3me2 (Eckert *et al.*, 2008) have also been found at high levels in CIS cells.

The epigenetic patterns of normal foetal germ cell development and derivation of human foetal germ cells into neoplastic CIS cells are illustrated in Fig. 2.

## Epigenetic patterns in overt germ cell tumours

The permissive and foetal-like epigenetic profile of CIS cells is lost once the CIS cells differentiate into an overt tumour. The overt tumour manifests either as a seminoma or non-seminoma, with very distinct histology as described above. It is yet elusive which mechanisms are responsible for the development of the two very different tumour types from the same cell of origin (CIS). Seminomas and non-seminomas have different epigenetic profiles and epigenetics may play an important role in their tumourigenesis. The epigenetic pattern of seminomas is characterized by high levels of H3K9me2, H3K27me3, H3K4me1 and H2A.Z, but erasure of H3K4me2/3 and H3K9ac compared to CIS. On the other hand, the undifferentiated component of non-seminomas, embryonal carcinoma (EC), displays high levels of DNA methylation, but shows similar levels of histone modifications to CIS cells (Netto *et al.*, 2008, Smiraglia *et al.*, 2002) (see Fig 2).

It is well recognized that DNA methylation plays a crucial role in tumourigenesis of somatic cancers. The genome of somatic cancer cells is characterized by global hypomethylation, which has been associated with genomic instability (Eden *et al.*, 2003). In this way the hypomethylated genome of seminomas resembles that of somatic cancers, whereas the non-seminomatous germ cell tumour differs as it retains high levels of DNA methylation (Netto *et al.*, 2008, Smiraglia *et al.*, 2002). Furthermore, TGCTs has been found to differ from somatic cancers in the degree of hypomethylation of DNA repetitive elements, where hypomethylation of LINE1 and Alu repeats is more pronounced in seminomas compared to somatic cancers (Ushida *et al.*, 2012). Non-seminomas show more pronounced hypomethylation of LINE1 as well, but are observed to be methylated at Alu repeats underlining the differential methylation pattern between the two types of TGCTs. Hypomethylated areas in the genome of the germline have in addition been ascribed to a much higher mutation rate than highly methylated areas (Li *et al.*, 2012).

In parallel to global hypomethylation, hypermethylation of gene promoters, resulting in silencing and inactivation of tumour suppressor genes (TSGs) is a hallmark of somatic cancers (reviewed in Herman, 1999). As in somatic cancers, TSGs are hypermethylated in both seminomas and non-seminomas (Brait *et al.*, 2012); however distinct promoter methylation profiles between the two types of TGCTs exist, possibly explaining how the two tumour types are able to develop from the same cell of origin.

We have recently investigated the expression of a broad range of epigenetic marks in the rare spermatocytic seminoma (SS) that represents 1-2% of all seminomas (Kristensen *et al.*, 2012). In contrast to the common TGCTs, SS occurs in elder men at 45-80 years of age and is usually benign. Most importantly SS originates from spermatogonia – and not from CIS, and not from foetal gonocytes. However, the epigenetic pattern of SS does not seem to match the

pattern found in spermatogonia, e.g. a low level of DNA methylation is found in SS, whereas spermatogonia are highly methylated. In general, a very heterogeneous epigenetic pattern is observed in SS, in contrast to the more uniform pattern observed in seminoma and non-seminoma. The histone modifications H2A.Z and H3K9me2 are the most consistently expressed epigenetic marks, whereas H3K4me1, H3K4me2/3, H3K9ac and H3K27me3 are expressed at various levels within the same tumour. SS thereby displays a markedly heterogeneous epigenetic landscape, implying that each cell comprising the tumour might be able to individually modify its epigenetic state. We have suggested that this phenomenon may be a consequence of the loss of contact to the somatic niche, which under normal circumstances regulates the maturation of the surrounding germ cells including their epigenetic state.

## Concluding remarks

Testicular cancer originates from foetal germ cells at the time where massive epigenetic reprogramming is taking place. Epigenetic reprogramming is a pre-requisite of proper germ cell development and it is hence likely that alterations in the epigenetic modifications may cause testicular cancer. The epigenetic reprogramming is vulnerable to external environmental stimuli and this is consistent with environmental factors being predominantly responsible in aetiology of testicular cancer. However only scarce information exists on human epigenetic cues in foetal germ cells and further studies are needed to deduce which epigenetic modifications may be affected and in which part of the genome.

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