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Evidence that active demethylation mechanisms maintain the genome of carcinoma *in situ* cells hypomethylated in the adult testis

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Background: Developmental arrest of fetal germ cells may lead to neoplastic transformation and formation of germ cell tumours via carcinoma *in situ* (CIS) cells. Normal fetal germ cell development requires complete erasure and re-establishment of DNA methylation. In contrast to normal spermatogonia, the genome of CIS cells remains unmethylated in the adult testis. We here investigated the possible active and passive pathways that can sustain the CIS genome hypomethylated in the adult testis.

Methods: The levels of 5-methyl-cytosine (5mC) and 5-hydroxy-methyl-cytosine (5hmC) in DNA from micro-dissected CIS cells were assessed by quantitative measurements. The expression of TET1, TET2, APOBEC1, MBD4, APEX1, PARP1, DNMT1, DNMT3A, DNMT3B and DNMT3L in adult testis specimens with CIS and in human fetal testis was investigated by immunohistochemistry and immunofluorescence.

Results: DNA from micro-dissected CIS cells contained very low levels of 5hmC produced by ten eleven translocation (TET) enzymes. CIS cells and fetal germ cells expressed the suggested initiator of active demethylation, APOBEC1, and the base excision repair proteins MBD4, APEX1 and PARP1, whereas TETs – the alternative initiators were absent. Both maintenance and *de novo* methyltransferases were detected in CIS cells.

Conclusion: The data are consistent with the presence of an active DNA de-methylation pathway in CIS cells. The hypomethylated genome of CIS cells may contribute to phenotypic plasticity and invasive capabilities of this testicular cancer precursor.

In most western countries, testicular germ cell cancer (TGCC) is the most common cancer encountered in men aged 15–45 years (Huyghe *et al*, 2003; Chia *et al*, 2010). Although TGCC shows a strong familial risk compared to other cancers, recent association studies (Chung *et al*, 2013; Ruark *et al*, 2013) have only been able to explain a genetic cause in less than a quarter of cases leaving still a major part of the cases to be caused by unknown gene variants and by environmental factors (Turnbull and Rahman, 2011). The causal influence of environmental factors is substantiated by a rapid increase in incidence of TGCC in recent decades and differences in prevalence between first- and second-generation immigrant populations (Hemminki and Li, 2002).

The pathogenic model for TGCC suggests that the tumours develop from a precursor cell; the carcinoma *in situ* (CIS) cell (Skakkebaek, 1972). Carcinoma *in situ* is also described in the literature as intratubular germ cell neoplasia unclassified or testicular intraepithelial neoplasia. The core event in the pathogenesis of CIS is the developmental arrest of primordial germ cells (PGCs) or gonocytes, which remain locked in an immature state as 'dormant' or pre-CIS cells during fetal and postnatal life. At puberty, CIS cells proliferate and gain invasive capacity, eventually resulting in the development of a seminoma, a non-seminoma or a combined tumour (Rajpert-De Meyts, 2006).

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Morphological and immunohistochemical studies have indicated that CIS cells resemble fetal germ cells (Nielsen *et al*, 1974; Berthelsen *et al*, 1979; Albrechtsen *et al*, 1982; Skakkebaek *et al*, 1987) and more recent gene expression analyses have provided further evidence for this similarity. In analogy to PGCs and gonocytes, CIS cells express transcription factors associated with embryonic stem cell pluripotency, including *POU5F1 (OCT-3/4), NANOG, T1A-2, MYCL1, GDF3, DPPA4, DPPA5, KIT* and *TFAP2C* (*AP-2γ*) (Looijenga *et al*, 2003; Sperger *et al*, 2003; Almstrup *et al*, 2004; Hoei-Hansen *et al*, 2004, 2005; Skotheim *et al*, 2005; Almstrup *et al*, 2007; Biermann *et al*, 2007; Sonne *et al*, 2009a). In addition, recent studies have shown that the overall epigenetic features of CIS cells, including the miRNA profile, shows similarities with the pattern found in fetal germ cells (Almstrup *et al*, 2010; Novotny *et al*, 2012). The CIS cells had an open and permissive chromatin structure similar to observations in fetal germ cells (Almstrup *et al*, 2010) and in particular very low levels of DNA methylation were detected (Netto *et al*, 2008). Interestingly, despite the very low DNA methylation level, high levels of the maintenance DNA methyltransferase DNMT1 were found in CIS, indicating that maintenance of DNA methylation has to be on-going (Netto *et al*, 2008) in the highly proliferating CIS cells (Almstrup *et al*, 2010).

Upon fertilisation, the maternal and paternal genomes undergo epigenetic reprogramming, mainly characterised by genome-wide loss of DNA methylation. As the only cell type during mammalian development, the PGCs undergo a second round of epigenetic reprogramming where DNA methylation is erased genome-wide. In mice, PGCs at E11.5, which have colonized the gonads, are completely devoid of DNA methylation and remain unmethylated until E13.5 (Hajkova *et al*, 2002; Seki *et al*, 2005; Hajkova *et al*, 2008; Popp *et al*, 2010; Guibert *et al*, 2012). DNA methylation of the germ cell genome, including methylation of paternal imprints, is progressively established from E14.5 throughout embryonic development until birth by the *de novo* DNA methyltransferases (DNMTs) (Kato *et al*, 2007). Even though less studied, human fetal germ cells seem to show similar re-methylation patterns, where gonocytes, which are un-methylated, at gestational week (GW) 7–17 begin to regain methylation gradually as they mature to pre-spermatogonia (Wermann *et al*, 2010; Gkountela *et al*, 2013).

In mammals, the genome-wide DNA demethylation occurs through several interconnected mechanisms, both passive and active. Passive DNA demethylation can occur due to absence or reduction of DNMTs responsible for establishment and maintenance of DNA methylation. Accordingly, *Dnmt3a*, *Dnmt3b* and *Dnmt1* are significantly downregulated in murine PGCs compared with the neighbouring somatic cells (Seki *et al*, 2005; Kurimoto *et al*, 2008). However, the rapid genome-wide DNA demethylation in murine PGCs, within a narrow developmental time window (one day), suggests that additional active demethylation mechanisms exist (Hajkova *et al*, 2002, 2008).

Two different pathways for active DNA demethylation in PGCs have been proposed. One suggested initiator is the ten eleven translocation (TET) protein family that convert 5mC to hydroxymethylated cytosine (5hmC) (He *et al*, 2011; Ito *et al*, 2011). Murine Tet1 and Tet2 are expressed in PGCs, coinciding with the time of rapid 5mC erasure (Hajkova *et al*, 2010; Hackett *et al*, 2013; Kagiwada *et al*, 2013). Another suggested initiating pathway is the direct deamination of 5mC to T by AID/APOBEC1. This introduces a T:G mismatch in the DNA. High expression of *Aid* and *Apobec1* is found in murine PGCs at E10.5–E12.5 (Morgan *et al*, 2004; Guibert *et al*, 2012), and $Aid^{-/-}$ PGCs were found to be less demethylated than the wild-type PGCs (Popp *et al*, 2010). Both of the proposed initiating pathways (TETs and AID/APOBEC1) result in the activation of the base excision repair (BER) machinery, which subsequently to TDG and MBD4 glycosylase activity entails the action of APEX1, PARP1 and XRCC1 (Hegde *et al*, 2008; Zharkov, 2008). Apex1, Parp1 and Xrcc1 were all found in murine PGCs isolated at E10.5–12.5 where demethylation occurs (Hajkova *et al*, 2010; Kagiwada *et al*, 2013).

In contrast to mice, very little is known about DNA demethylation pathways operating in human fetal germ cells. Even less is known about neoplastic germ cells, where this knowledge is of primary importance for understanding the timing of the neoplastic transformation. In the present study, we addressed these gaps in knowledge with focus on CIS. We investigated whether CIS cells, located within the niche of an adult testis, use the same DNA demethylation mechanisms as fetal germ cells, to maintain the low methylation level.

MATERIAL AND METHODS

Tissues. The Regional Committee for Medical Research Ethics in Denmark approved the use of human adult and fetal tissue samples from the archives of Copenhagen University Hospital. Adult testicular tissue samples were obtained from testis cancer patients undergoing orchiectomy and from biopsies of the contralateral testis. Tissue samples of normal human fetal testes at GW 11 (where only hypomethylated gonocytes are present), at GW 21 (where a mixture of gonocytes and methylated pre-spermatogonia is present) and at GW 33 (where predominantly pre-spermatogonia are present) were obtained from induced and spontaneous abortions. Gestational age was estimated by the measurement of foot length and sex determined by staining for anti-Müllerian hormone. For the purpose of DNA purification, adult testicular tissue samples were snap-frozen and stored at $-80\,^{\circ}C$. For immunohistochemistry (IHC) and immunofluorescence (IF) investigations, tissue samples were fixed in formalin or paraformaldehyde at $4\,^{\circ}C$ overnight immediately after surgery, and subsequently embedded in paraffin.

Testicular-germ-cell-cancer-derived cell lines, NTera2 and TCam-2, were obtained from Dr PW Andrews (UK), and Dr J Shipley (UK), respectively, after permission from Dr S Kitazawa (Japan). Cell lines were cultured according to standard conditions as previously described (Jorgensen *et al*, 2013). In brief, cells were grown at $37\,^{\circ}C$ in a 5% CO_2 atmosphere, in DMEM (NTera2 cells) or RPMI 1640 (TCam-2 cells) supplemented with 10% fetal calf serum, glutamine ($58.5\,mg\,ml^{-1}$), penicillin ($100\,U\,ml^{-1}$) and streptomycin ($100\,mg\,ml^{-1}$). Cell media and reagents were from Gibco (Naerum, Denmark) and cell culture flasks were from NUNC (Roskilde, Denmark).

RT–PCR. Total RNA was isolated with NucleoSpin RNA II purification kit with DNase I treatment as described by the manufacturer (Macherey-Nagel, Düren, Germany).

The isolated RNA was reverse transcribed with AMV reverse transcriptase (USB, Cleveland, OH, USA) using a dT20 primer and random hexamers and PCR performed using Taq polymerase (GE Healthcare Life Sciences, Freiburg, Germany). The following primers were used:

TET1 (Entrez Gene: 80312) fwd 5′-CACACCAGCTCCACT GAAGA-3′ and rev 5′-CTCCATCACAGGAGCAGACA-3′; TET2 (Entrez Gene: 54790) fwd 5′-CCCACTTACCTGCGTTTCAT-3′ and rev 5′-ACTGTGACCTTTCCCCACTG-3′;

TET3 (Entrez Gene: 200424) fwd 5′-CACCAAGAGTCTGCTG-GACA-3′ and rev 5′-CTCCTTCCCCGTGTAGATGA-3′;

PCR conditions were: one cycle of 5 min at $95\,^{\circ}C$; 40 cycles of 30 s at $95\,^{\circ}C$, 1 min at $62\,^{\circ}C$, 1 min at $72\,^{\circ}C$ and one cycle of 5 min at $72\,^{\circ}C$. Products were run on 2% agarose gels and visualised by ethidium bromide staining. Representative bands from each primer combination were excised and sequenced for verification (Eurofins MWG, Ebersberg, Germany).

Laser micro-dissection of CIS. Cryosections of adult testicular tissue samples were prepared, subjected to direct histochemistry and subsequently micro-dissected as previously described (Sonne *et al*, 2009b; Jorgensen *et al*, 2011). In brief, tissue samples were embedded in optimum cutting temperature compound (O.C.T. compound, Tissue-Tek, Sakura Finetek Europe, Netherlands), immediately frozen in −78 °C isopentane and stored at −80 °C until use. Sections of 20 μm were cut on a Shandon SME cryotome (Thermo Scientific, Hvidovre, Denmark) and collected on nuclease- and nucleic acid-free membrane slides (Molecular Machines & Industries, Glattbrugg, Switzerland), immediately fixed in 75% RNAse free ethanol and stored in absolute ethanol at −80 °C. To visualise CIS, sections were stained for the activity of alkaline phosphatase with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution. Stained CIS cells were micro-dissected from sections within 2 h using the MMI SmartCut System (Olympus). Only tubules with classical appearance of CIS cells along the periphery of tubules were micro-dissected to avoid unspecific stained cells and intratubular tumour cells. For each sample 500–5000 cells were micro-dissected and collected on the adhesive cap of collection tubes (Molecular Machines & Industries, Glattbrugg, Switzerland). Lysis buffer was immediately added to micro-dissected cells and stored at −80 °C.

DNA purification. DNA was purified from tissue samples of embryonal carcinoma (EC; a highly methylated TGCC), tissue samples with CIS situated among normal tubules (CIST), and from micro-dissected CIS cells (MCIS) using the QIAamp DNA Micro Kit (QIAGEN, Copenhagen, Denmark) according to the manufacturer's protocol. As very little DNA was extracted from samples of micro-dissected CIS, purified DNA was pooled for later application in ELISA. DNA concentration was measured on a NanoDrop ND-1000 Spectrophotometer (Saveen Werner, Limhamn, Sweden).

ELISA. For relative quantification of global content of 5mC and 5hmC, ELISA was performed on DNA purified from EC, CIST and MCIS. ELISA was carried out using the fluorometric assays MethylFlash Methylated DNA Quantification Kit (Colorimetric) and MethylFlash Hydroxymethylated DNA Quantification Kit (Colorimetric) (Epigentek Group Inc., Farmingdale, NY, USA) according to the manufacturer's protocol. Two hundred nanogram DNA was applied to microtiter plates in three technical replicates and colorimetrical quantification carried out on a microplate spectrophotometer (Tecan Sunrise, Männedorf, Switzerland). The content of 5mC and 5hmC was calculated relative to positive and negative controls included in the kit (containing 50% 5mC and 5hmC, respectively). The assays showed a linear response in the range of 0.1–1% 5mC and 0.01–0.1% 5hmC (at least) and in both cases R^2 were >0.9.

Immunohistochemistry. 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). Sections were counterstained with Mayers Hematoxylin.

Table 1. List of primary antibodies

				Dilution		
Antigen	**Product name and supplier**	**Host species and clonality**	**Buffer**	**IHC**	**IF**	**Fetal IF**
TET1	TET1 (Y-14), sc-163446, Santa Cruz Biotechnologies, Inc.	Goat polyclonal	TEG	1 : 100	1 : 100	–
TET2	TET2 (S-13), sc-136926, Santa Cruz Biotechnologies, Inc.	Rabbit polyclonal	CIT	1 : 2000	1 : 1500	–
APOBEC1	APOBEC1 anti-Human (N-Terminus) Antibody, LS-C98890, LifeSpan BioSciences, Inc., Seattle, WA, USA	Rabbit polyclonal	TEG	1 : 100	1 : 150	1 : 50
MBD4	MBD4 antibody, NBP1-91189, Novus Biologicals, Cambridge, UK	Rabbit polyclonal	CIT	1 : 1000	1 : 800	1 : 200
APEX1	APEX1 / APE1 Goat anti-Human Polyclonal (N-Terminus) Antibody, LS-B3924, LifeSpan BioSciences, Inc.	Goat polyclonal	CIT	1 : 100	1 : 100	1 : 50
PARP1	Anti-PARP antibody, ab6079, Abcam, Cambridge, UK	Rabbit polyclonal	CIT	1 : 150	1 : 150	1 : 75
XRCC1	Anti-XRCC1 antibody, ab1838, Abcam	Mouse monoclonal	CIT	1 : 50	1 : 50	–
5mC	Anti-5-Methylcytosine Mouse mAb (162 33 D3), NA81, Calbiochem, Damstadt, Germany	Mouse monoclonal	-MBO	1 : 200	–	–
5hmC	5-hmC monoclonal antibody (Mab-31HMC), Diagenode, Liege, Belgium	Mouse monoclonal	-MBO	1 : 1000	–	–
DNMT1	DNMT1 Rabbit anti-Human Polyclonal (aa1603-1616) Antibody, LS-B800, LifeSpan BioSciences, Inc.	Rabbit polyclonal	CIT	1 : 3000	1 : 3000	–
DNMT3A	Dnmt3a (H-295), sc-20703, Santa Cruz Biotechnologies, Inc.	Rabbit polyclonal	CIT	1 : 600	1 : 600	–
DNMT3B	Dnmt3b (Q-25), sc-130740, Santa Cruz Biotechnologies, Inc.	Rabbit polyclonal	CIT	1 : 75	1 : 50	–
DNMT3L	A kind gift from Dr. Okamoto, Tokyo, Japan	Rabbit polyclonal	TEG	1 : 5000	1 : 5000	–
CIS and fetal germ cell markers						
D2-40 (CIS)	Anti-Human D2-40, M3619, Dako	Mouse monoclonal	CIT	1 : 100	1 : 100	–
PLAP (CIS)	Anti-Human Placental Alkaline Phosphatase, M7191, Dako	Mouse monoclonal	TEG	–	1 : 100	–
MAGE-A4 (fetal GC)	Kind gift from Dr. Guilio Spagoli, Basel, Switzerland	Mouse monoclonal	TEG + CIT		–	1 : 250
OCT 3/4 (fetal GC)	Oct-3/4 Antibody (C-10), sc-5279, Santa Cruz Biotechnologies, Inc.	Mouse monoclonal	CIT	–	–	1 : 50

Abbreviations: CIS = carcinoma *in situ*; CIT = citrate buffer (10 mM, pH 6); GC = germ cells; IF = Immunofluorescence; IHC = immunohistochemistry; -MBO = no buffer and microwave treatment; TEG = TEG buffer (10 mM Tris, 0.5 mM EDTA, pH 9).
Antibodies were used at indicated dilutions and buffers.

Primary antibodies were used at indicated dilutions and buffers for microwave treatment as listed in Table 1. 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 (Supplementary Figure 1). Positive controls were samples of intestine (for APOBEC1), prostate (for TET1), EC (for DNMTs) and normal testis (the remaining antibodies). In all cases controls stained positive (data not shown).

Sections were scanned on NanoZoomer (Hamamatsu Photonics, Herrsching am Ammersee, Germany) and NDP.view software (Hamamatsu Photonics) was used for analysis. Tubules containing CIS cells were identified in serial sections by staining with classical CIS markers D2-40/M2A/PDPN (Marks *et al*, 1999; Sonne *et al*, 2006b) or PLAP (Giwercman *et al*, 1991). For each tissue sample, the intensity of the staining of CIS cells, was evaluated and scored as negative (neg), faint staining (+), moderate staining (++) and strongly positive staining (+++). The general staining pattern observed across the investigated tissue samples was subsequently summarised, by collectively describing all the staining intensities observed in the included samples. If staining was observed only in a few (1–2) of the included tissue samples, this was noted (few). Tissue samples were excluded from the summary if the following was observed: abnormal morphology (probably due to bad tissue preservation), lack of staining in a positive control and increased background staining.

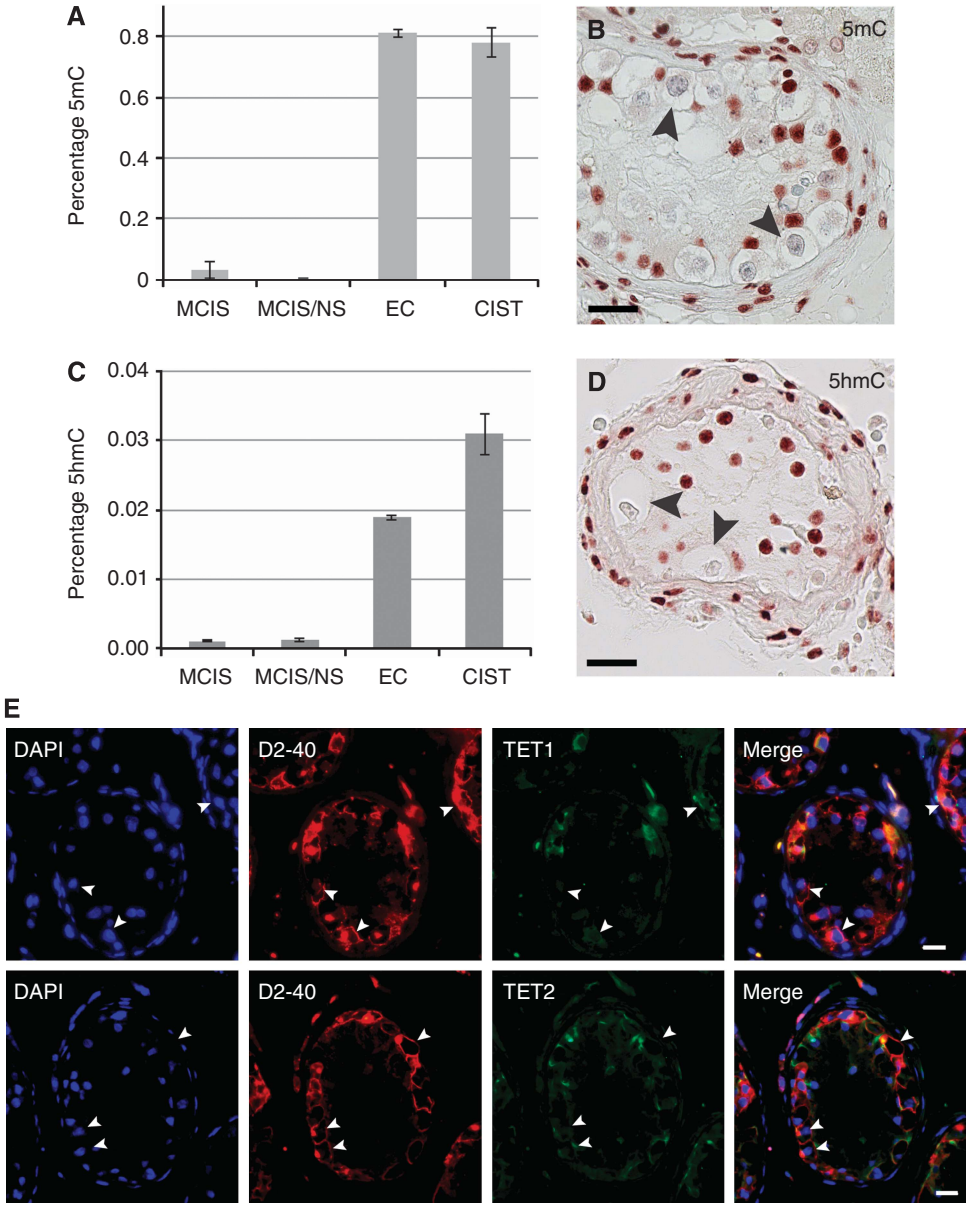

Figure 1. Assessment of 5mC and 5hmC levels in CIS cells and IF detection of TET proteins. (**A**, **C**) Quantitative measurements of respectively the 5mC and 5hmC levels in DNA isolated from micro-dissected CIS cells (MCIS and MCIS/NS), whole tissue section of testicular tissue containing CIS (CIST) and tissue containing embryonal carcinoma (EC). The content of 5mC and 5hmC was calculated relative to positive and negative controls included in the kit (containing 50% 5mC and 5hmC, respectively). Bars represent mean ± std. (**B**, **D**) Immunohistochemical staining for, respectively, 5mC and 5hmC in testicular tissue with CIS cells (arrowheads). Bars represent 25 microns. (**E**) Immunofluorescence detection of TET1 (upper row of images) and TET2 (the bottom row) is shown in green. CIS cells (arrowheads) are marked with D2-40 (red) and DAPI (blue) stains in nuclei. Bars represent 10 μm. Negative control samples are shown in Supplementary Figure 1. TET1 and TET2 expression in normal testis are shown in Supplementary Figure 2B.

Immunofluorescence double staining. Immunofluorescence double staining was performed on adult and fetal testicular tissue samples. The protocol described above for IHC was used for IF with only minor adjustments. Primary antibodies were applied overnight at 4 °C and left for 1 h at room temperature (RT) the following day. Fetal gonocytes were detected by OCT3/4, pre-spermatogonia were detected by a spermatogonial marker MAGE-A4 (Aubry *et al*, 2001; Gaskell *et al*, 2004) and CIS cells identified by the specific markers D2-40 or PLAP as described above. Primary antibodies were used at indicated dilutions and buffers as listed in Table 1. For negative controls, dilution buffer without the primary antibody was applied to serial sections. No staining was observed in negative control samples (Figure 1 and Supplementary Figure 1). The following secondary antibodies were applied in pairs according to the respective primary antibodies: Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 488 donkey anti-goat, Alexa Fluor 568 donkey anti-mouse (Life Technologies, Naerum, Denmark), all diluted 1 : 600, and incubation took place for 45 min at RT. Sections were counterstained with DAPI (2 $\mu g\,ml^{-1}$ in TBS), mounted with anti-fade mounting solution (Prolong Gold anti-fade reagent, P36930, Life Technologies) and left to dry for 24 h. In between incubation steps, slides were washed in TBS. Finally, cover glasses were sealed with nail polish. Fluorescence microscopy analysis was carried out on Olympus BX61 (Olympus Ltd., Southend-on-Sea, UK) coupled to the fluorescent light source X-Cite series 120 PC Q (Lumen Dynamics Group Inc., Mississauga, ON, Canada). Image processing, acquisition and photography were performed with the software cellSens Dimensions version 1.6 (Olympus Ltd.).

Western blotting. Western blotting (WB) was performed in order to validate the specificity of antibodies used in this study (Table 1). Protein was extracted from frozen testicular tissue samples containing CIS cells in all tubules (CIS + NT), normal adult testis (NT), seminoma (s.e.m.), EC and from the cell lines TCam2 and NT2. Samples were prepared and WB performed as previously described (Sonne *et al*, 2006a), except that HRP-conjugated secondary antibodies were used and protein signal detected by chemiluminescent detection with Pierce ECL detection (No. 34095, Thermo Scientific). Ten microgram of protein was loaded in each lane and primary antibodies were diluted 1 : 200 in 1% milk powder with TBS Tween (0.1%), except TET1 which was diluted 1 : 100. β-actin was used as loading control and the primary antibody diluted 1 : 200 (β-actin, Santa Cruz Sc-47778, Santa Cruz, CA, USA). Secondary antibodies were HRP-conjugated rabbit anti-mouse (P0260, Dako, Glostrup, Denmark), HRP-conjugated rabbit anti-goat (P0160, Dako) and HRP-conjugated swine anti-rabbit (P0217, Dako), all diluted at 1 : 1000. For all antibodies investigated protein bands of expected size were detected (see Supplementary Figure 2).

RESULTS

Are CIS cells hydroxymethylated by TET proteins? The TET family proteins are responsible for the conversion of 5mC to 5hmC. TET proteins and 5hmC have been identified in PGCs and may have a pivotal role in demethylation of their genome during development. Owing to the close resemblance of CIS to fetal germ cells (Almstrup *et al*, 2004; Sonne *et al*, 2009a; Almstrup *et al*, 2010), we asked whether TET proteins and 5hmC might be present in CIS cells. We therefore performed IHC of 5hmC and 5mC (Figure 1B and D) and found no staining in the nuclei of CIS cells, whereas intense staining was present in the nuclei of the adjacent Sertoli cells. To address the question further, we isolated CIS cells from testicular tissue sections by laser micro-dissection and purified the DNA. By ELISA, we assessed the amount of 5hmC and 5mC in DNA obtained from two different micro-dissected samples (one with CIS next to a non-seminoma: MCIS/NS; and one with CIS without tumour: MCIS) as well as from a whole tissue sample containing testicular tissue with CIS (CIST) and a sample with EC (which is highly methylated). Both of the micro-dissected samples showed low levels of 5hmC and 5mC (Figure 1A and C), in accordance with our findings by IHC. We therefore conclude that CIS cells retain low levels of both 5mC and 5hmC. The EC sample and the sample with CIS from a whole tissue section showed substantially higher levels of both 5mC and 5hmC.

RT–PCR of RNA from biopsies containing CIS and different TGCTs, as well as TGCT-derived cell lines, showed that *TET1* and *TET2* were found in nearly all samples, whereas *TET3* was absent from tissue containing CIS cells (Supplementary Figure 3A). We hence focused on TET1 and TET2, which are also described to be the TET proteins mostly engaged in 5mC hydroxylation (Koh *et al*, 2011). As shown in Figure 1E, we neither detected TET1 nor TET2 in the nucleus of CIS cells by IF. A slight staining in the cytoplasm of CIS cells could however not be excluded. TET1 and TET2 were, however, detected in spermatogonia, spermatocytes and Leydig cells (Supplementary Figure 3B), which explains the presence of detectable messenger RNA by RT–PCR in tissue blocks containing various kinds of testicular cells. Interrogation of earlier microarray data on micro-dissected cell populations (Sonne *et al*, 2009a), also indicated a low expression of all TET proteins (Supplementary Figure 3C). We conclude that the TET proteins are not expressed in the nucleus of CIS cells and that only very low levels of 5hmC can be detected in the genome of CIS cells.

Are CIS cells engaged in active DNA demethylation? We next asked whether proteins that have been described to be engaged in active DNA demethylation in PGCs also were present in CIS cells. By IHC, we investigated APOBEC1, MBD4, APEX1 and PARP1 and found all to be present in the nuclei of CIS cells (Table 2). XRCC1 was not found in CIS cells (Supplementary Figure 4). As shown in Figure 2, APOBEC1, MBD4, APEX1 and PARP1 all co-localised to CIS cells marked by either PLAP or D2-40. APOBEC1 localised mainly to the cytoplasm, but less intense staining was also found in the nucleus. MBD4, APEX1 and PARP1 were all localised to the nuclei of CIS cells. We conclude that

Table 2. Staining intensities of potential demethylating proteins and DNA methylases observed in CIS by immunohistochemistry

Antigen	Nucleus	Cytoplasm
TET1 $N=6$	neg, few +	neg, few +
TET2 $N=6$	neg, few +	neg, few +
APOBEC1 $N=8$	neg, +	+ + +
MBD4 $N=8$	+, + +, few neg	neg
APEX1 $N=8$	+ +, + + +, few neg	neg
PARP1 $N=8$	+, + +, few neg, few + + +	neg
DNMT1 $N=8$	+, + +, few neg	neg
DNMT3A $N=8$	neg	neg
DNMT3B $N=8$	neg, few +	neg
DNMT3L $N=8$	neg and + +	neg

For each tissue sample, the intensity of the staining of CIS cells, was evaluated and scored as negative (neg), faint staining (+), moderate staining (+ +) and strong staining (+ + +). For each antigen, the general staining pattern observed across the investigated tissue samples was subsequently summarised, by collectively describing all the staining intensities observed in the included samples (*N*). If a staining intensity was observed only in a few (1–2) of the included tissue samples, this was noted (few).

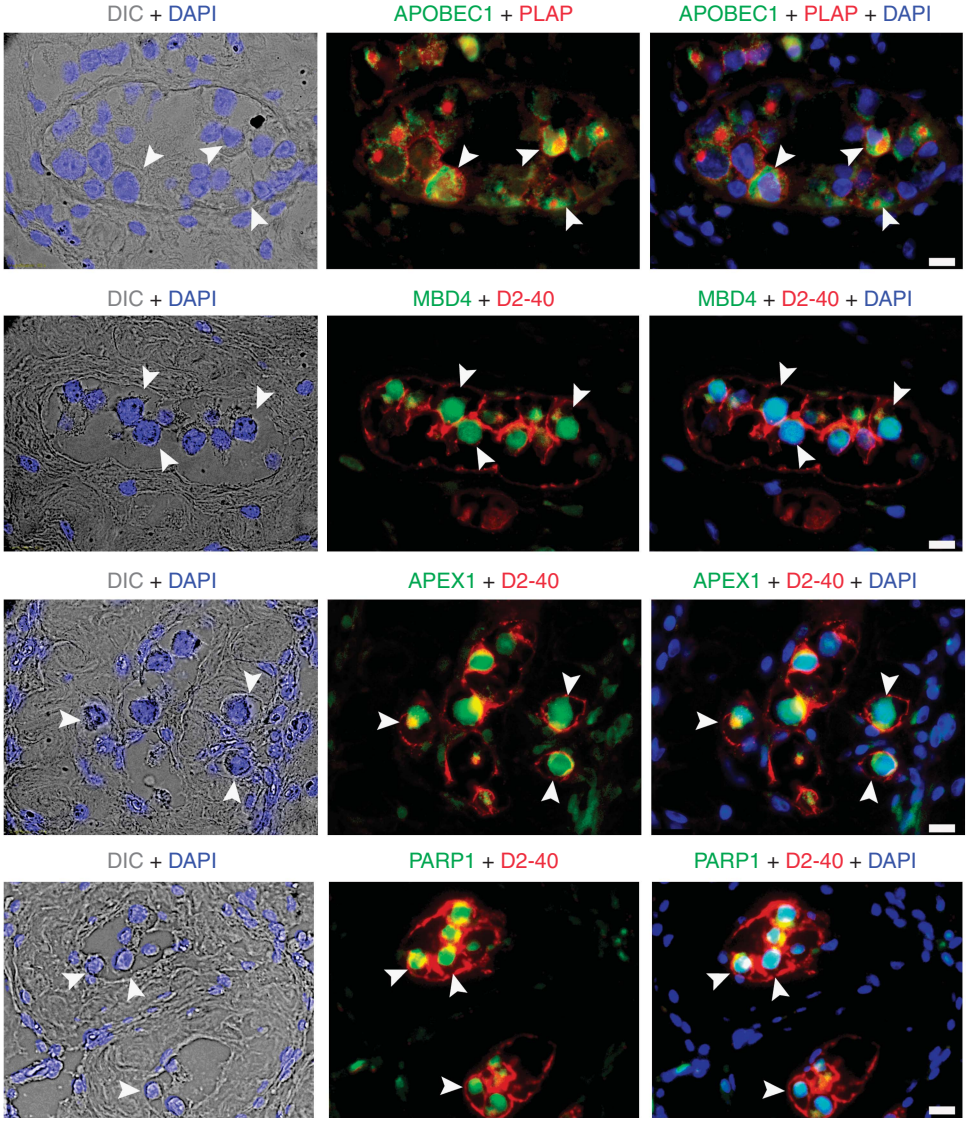

Figure 2. Immunofluorescence detection of proteins involved in active DNA demethylation in adult testis samples with CIS. On the left-hand side differential interference contrast (DIC) images display morphology of each section and are merged with DAPI staining (blue) to reveal nuclei. CIS cells (arrowheads) are marked by the classical CIS markers PLAP or D2-40 (red). The CIS cells show expression of the markers of active demethylation APOBEC1, MBD4, APEX1 and PARP1 (green). Bars represent 10 μm.

CIS cells seem to express proteins that facilitate active DNA demethylation by AID/APOBEC1 and BER.

Do human fetal germ cells express the same DNA demethylation markers as CIS cells? As a high degree of similarity between gonocytes and CIS cells has been described (recently reviewed in Kristensen *et al*, 2013), we next asked whether human fetal germ cells express the same DNA demethylation proteins as CIS cells. By IF, we investigated fetal germ cells at GW 11, 21 and 33 for the co-localisation of APOBEC1, MBD4, APEX1 and PARP1. Fetal germ cells were identified by OCT3/4 at GW 11 and by MAGE-A4 at GW 21 and 33, both specifically mark fetal germ cells at the developmental stages corresponding to gonocytes and pre-spermatogonia, respectively (Gaskell *et al*, 2004). At GW 21, we found MBD4 and PARP1 to be highly expressed in the nucleus of fetal germ cells (Figure 3), as high intensities of fluorescent signals were observed. APOBEC1 and APEX1 localised to the cytoplasm of fetal germ cells, and were expressed at a lower level compared with MBD4 and PARP1, as lower intensities of fluorescent signals were observed. APOBEC1, MBD4, APEX1 and PARP1 also localised to MAGE-A4 negative cells within the tubules, which could be fetal germ cells at an earlier developmental stage, most likely gonocytes. In addition, some variation was observed for MBD4 expression, as germ cells from the same section could be found both positive and negative. The same observations were found for fetal testis samples at GW 11 and GW 33 (Supplementary Figure 5), except that APOBEC1 also localised to the nuclear membrane of fetal germ cells at GW 33, in addition to the previously described cytoplasmic localisation. We conclude that human fetal germ cells express many of the proteins that facilitate active DNA demethylation by AID/APOBEC1 and BER, but at an apparent lower level than CIS cells and with some variation.

Are CIS cells also engaged in passive DNA demethylation? Lastly we asked whether the genome of CIS cells might also be demethylated passively as a consequence of absent or down-regulated DNMTs. By IHC, we investigated the expression of DNMT1, DNMT3A, DNMT3B and DNMT3L. We found DNMT1, DNMT3B and DNMT3L localised in the nucleus of CIS cells, whereas DNMT3A was absent (Table 2). By IF,

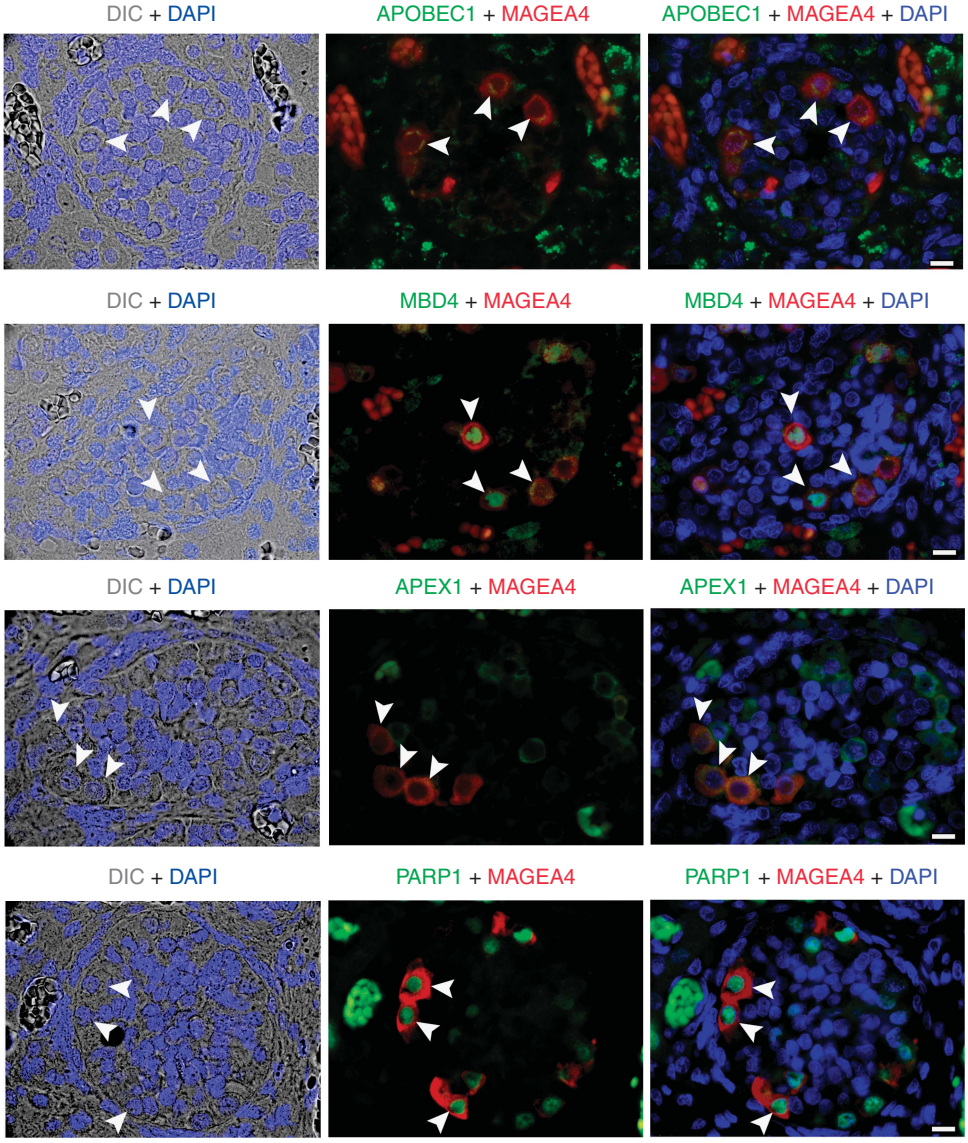

Figure 3. Immunofluorescence detection of proteins involved in active DNA demethylation in a human fetal testis sample at 21 weeks of gestation (GW). A differential interference contrast (DIC) image displaying morphology of each section merged with DAPI (blue) staining to reveal nuclei, is shown to the left. Human fetal germ cells (arrowheads) marked by MAGE-A4 (red) show expression of most of the markers of active demethylation APOBEC1, MBD4, APEX1 and PARP1 (green). Bars represent 10 μm.

we showed that DNMT1 co-localised to the nuclei of CIS cells. DNMT3B and its co-enzyme DNMT3L were also found to co-localise to CIS cells, but were expressed at a lower level and, very interestingly, in small foci within the nuclei (Figure 4 and Supplementary Figure 6). Some variation was observed among CIS cells from the same section with regard to expression of DNMT3B and L (Figure 4). DNMT3A was not observed in CIS, but was present in the nuclei of Sertoli cells. We conclude that the maintenance methyl-transferase DNMT1 is expressed in CIS cells and that *de novo* methyl-transferases DNMT3B and 3L also could be detected in the nucleus of CIS cells; however, the level seemed lower and some variation was observed.

DISCUSSION

We have previously shown that CIS cells retain an open chromatin and gonocyte-like epigenetic modifications (Almstrup *et al*, 2010). In addition to the permissive chromatin observed in CIS cells, one of the most noteworthy epigenetic features of CIS cells is the complete absence or extremely low level of 5mC (Smiraglia *et al*, 2002; Netto *et al*, 2008). Human fetal germ cells are also hypomethylated during the embryonic period, but gradually become methylated (5mC positive immunostaining) and by the time of birth most germ cells are methylated (Wermann *et al*, 2010). A hypomethylated genome promotes proliferation and may cause genomic instability thereby contributing to tumorigenesis (Eden *et al*, 2003). DNA hypomethylation may also facilitate aberrant expression of pluripotency genes, like *POU5F1*, *NANOG* and *TFAP2C*, which indeed are highly expressed in CIS cells (Looijenga *et al*, 2003; Hoei-Hansen *et al*, 2004; Rajpert-De Meyts *et al*, 2004; Hoei-Hansen *et al*, 2005). Aberrant expression of other epigenetic modifiers in CIS cells, like BLIMP/PRMT5 (Eckert *et al*, 2008), might also add to abnormal epigenetic reprogramming and thereby genomic instability.

In this study, we show that CIS cells express APOBEC1, MBD4, APEX1 and PARP1, proteins that are involved in active removal of

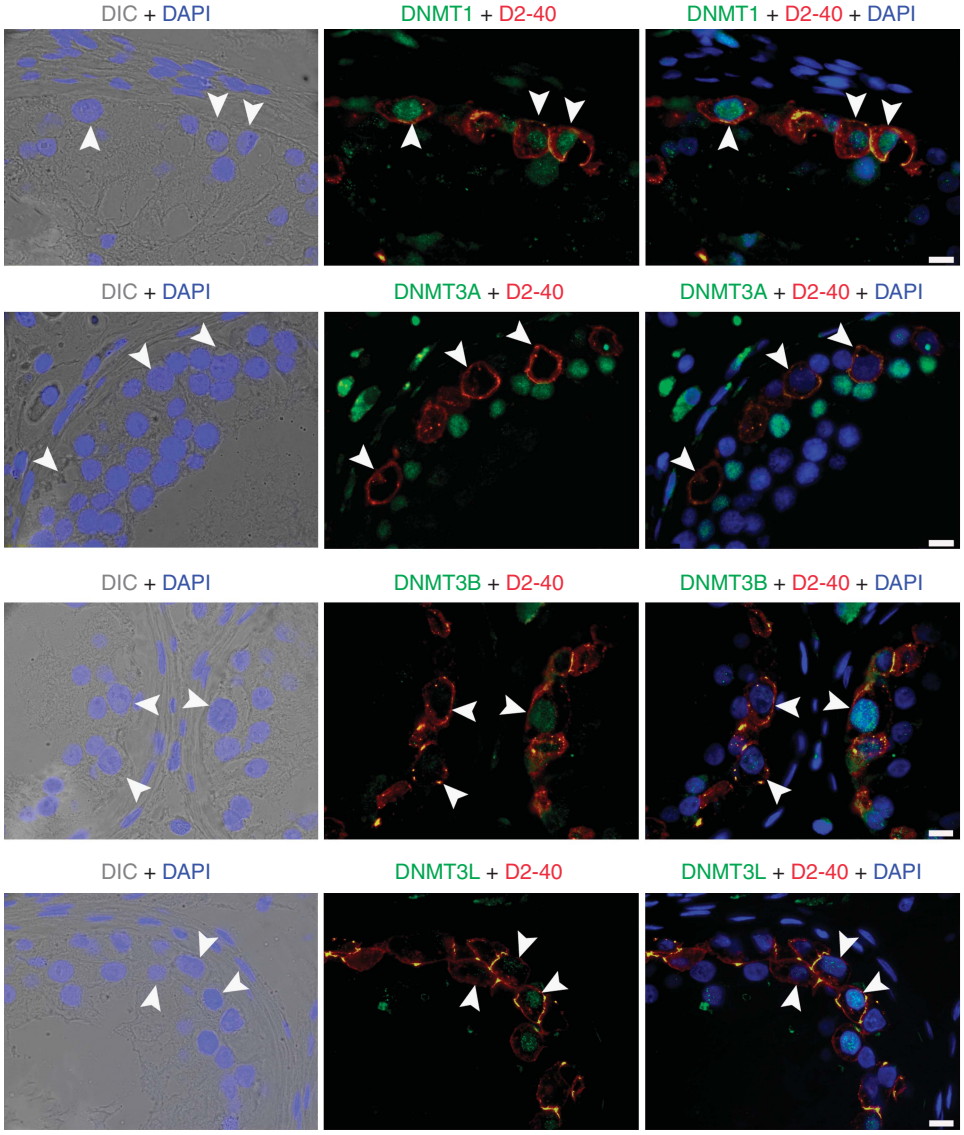

Figure 4. Adult testis samples with CIS displaying IF detection of DNMT proteins (green) involved in generation of 5mC. On the left-hand side differential interference contrast (DIC) images display morphology of each section and are merged with DAPI staining (blue) to reveal nuclei. CIS cells are marked with D2-40 (arrowheads). CIS cells showed a substantial expression of DNMT1. DNMT3B and L show a faint expression in small foci in the nucleus of CIS cells (high-power magnification is shown in Supplementary Figure 6). DNMT3A showed no expression in CIS cells. Bars represent 10 μm.

5mC. A possible pitfall is however that many of these proteins have dual or multiple roles. MBD4, APEX1 and PARP1 are part of the BER complex, which functions also in repair of DNA besides being engaged in active removal of 5mC. The presence of BER components in CIS cells does not *per se* prove that they participate in retaining the genome in a hypomethylated state. However, we also showed that the known alternative demethylation pathway via TET proteins and the generation of 5hmC was absent in CIS cells, as we observed very low levels of 5hmC and no expression of TET1 and TET2 in the nucleus of CIS cells. This stands in contrast to studies in mice, where TETs are suggested to be the main proteins involved in the demethylation of the genome in PGCs, based on observations of an initial increase (E10.5–E11.5) and a subsequent decrease (E13.5) in 5hmC levels, coinciding with decreasing 5mC levels (Hackett *et al*, 2013). However, knock out of Tet1 in mouse did not impair fertility and only resulted slightly increased methylation levels (Yamaguchi *et al*, 2012). Hence, even though it seems likely that 5hmC and TET proteins have an important role during fetal germ cell development, Tet1 is dispensable in the development of normal murine germ cells.

Our results tend to suggest an active demethylation pathway initiated by the deaminase APOBEC1 and not TETs, as CIS cells expressed APOBEC1 and the downstream BER components MBD4, APEX1 and PARP1, where the latter indeed have been demonstrated to be involved in active DNA demethylation in murine PGCs (Morgan *et al*, 2004; Hajkova *et al*, 2010; Popp *et al*, 2010; Hashimoto *et al*, 2012; Nabel *et al*, 2012). However, contradictory results regarding the level of expression of APOBEC1 in murine PGCs have been reported (Hajkova *et al*, 2010; Kagiwada *et al*, 2013).

Inhibition of DNMTs is yet another alternative pathway to hypomethylate a genome. Replication-coupled passive DNA demethylation has been suggested to be sufficient for demethylation of the genome in murine PGCs, as they were found to divide more rapidly than previously thought and to have a low expression of DNMTs, leading to dilution of 5mC over time

(Kagiwada *et al*, 2013). Even though CIS cells are highly proliferative and express BLIMP1 (Eckert *et al*, 2008; Almstrup *et al*, 2010), we did find a substantial expression of DNMT1, which is in line with a previous report (Netto *et al*, 2008) and low expression of DNMT3B and L in CIS cells. Hence, both maintenance and *de novo* methyltransferases seem to be present in CIS cells, but it is yet unknown whether they are post-translationally inhibited or whether the low level of *de novo* DNMTs is sufficient to drive re-methylation of the CIS genome.

In any case, multiple mechanisms are likely to operate in concert to demethylate the genome of CIS cells and fetal gonocytes. As in CIS cells, we found APOBEC1, MBD4, APEX1 and PARP1 to be expressed in human male fetal germ cells at GW11, GW21 and GW33, when a transition from hypomethylated gonocytes to methylated pre-spermatogonia is in progress (Wermann *et al*, 2010; Gkountela *et al*, 2013). The localisation of APOBEC1 to the cytoplasm of fetal germ cells at GW11 and GW21, could indicate that it may not be involved in active DNA demethylation. The appearance of APOBEC1 in the nuclear membrane of fetal germ cells at GW33, could indicate a function of APOBEC1 in active DNA demethylation in the nucleus at this later time point. The presence of APOBEC1 in human gonocytes stands in contrast to the absence of APOBEC1 observed in murine PGCs (Hajkova *et al* 2010). However, epigenetic cues in human and murine fetal germ cells may differ (Almstrup *et al*, 2010; Gkountela *et al*, 2013). In particular, a more prolonged process of demethylation is evident in human fetal germ cells compared with the rapid demethylation in murine PGCs (Wermann *et al*, 2010; Guibert *et al*, 2012).

In this study, we found that PARP1 was expressed in the nuclei of CIS cells. In addition to its role in BER, PARP1 participates in regulating the activity of a wide range of proteins involved in epigenetic modulation (Quenet *et al*, 2009; Gibson and Kraus, 2012). When bound to its target, PARP1 synthesizes a negatively charged polymer of poly-(ADP-ribose) units, which can be added to Glu, Asp or Lys residues of target proteins. Thereby, PARP1 performs posttranscriptional modification of its target proteins – a process also called PARylation. PARP1 can thereby affect the chromatin structure and has been shown to impair chromatin remodelling, histone demethylation and nucleosome–nucleosome interaction facilitating decondensation of chromatin (Gibson and Kraus, 2012). It is hence possible, that PARP1 itself can contribute to an unstable chromatin structure in CIS and fetal germ cells, in addition to its role in active demethylation. The high expression of PARP1 in CIS is supported by our earlier results on the CIS chromatin, which we found to be in a highly open and permissive conformation (Almstrup *et al*, 2010).

Epigenetic modifications occurring during embryonic development are well recognised as being susceptible to alterations by exposure to environmental factors. Indeed, animal studies show that certain exposures (e.g. to endocrine disrupting chemicals) during fetal development cause changes in epigenetic patterns with a phenotypic outcome, including alterations in the development of male germ cells (Feil and Fraga, 2011). Studies in humans have shown that e.g. prenatal starvation can be traced in the adult epigenome of peripheral blood mononuclear cells affecting, for example, the insulin promoter (Heijmans *et al*, 2008; Tobi *et al*, 2009). The important role of epigenetics during development and experimental evidence that environmental factors can influence the epigenetic programming indicate a role for epigenetics in the pathogenesis of TGCC. It is speculative at this point but likely that *in utero* environmental exposure can affect the reprogramming of the epigenome in developing fetal germ cells and thereby contribute to their neoplastic transformation into CIS. The present study substantiates this viewpoint by indicating developmental failure to end active DNA demethylation processes in CIS cells. Cues to inhibit demethylation processes in fetal germ cells could indeed be promoted by maturation of surrounding Sertoli cells and hence we speculate that the lack of this function may be a consequence of testicular dysgenesis or an under-virilised gonad as suggested earlier by our group (Skakkebaek *et al*, 2001).

In conclusion, the common precursor of TGCC – CIS – was shown to be devoid of TET proteins and with low levels of both 5mC and 5hmC. In contrast, the hypomethylated CIS cells expressed a range of proteins that have been implicated in active demethylation of fetal germ cells. We therefore suggest that the CIS genome is subjected to active DNA demethylation. The hypomethylated state is likely associated with a continuous expression of pluripotency genes and may facilitate increased proliferation of CIS cells exposed to a high hormonal activity of the post-pubertal testis, which are features that undoubtedly contribute to the invasive progression.

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