Expression patterns of HENMT1 and PIWIL1 in human testis: implications for transposon expression

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Abstract

This study aimed to define the expression patterns of HENMT1 and PIWI proteins in human testis and investigate their association with transposon expression, infertility sub-type or development of testicular germ cell tumours (TGCTs). Testis biopsies showing normal spermatogenesis were used to identify normal localisation patterns of HENMT1 and PIWIL1 by immunolocalisation and RT-PCR after laser microdissection. 222 testis biopsies representing normal spermatogenesis, hypospermatogenesis, spermatogenic arrests, Sertoli cell-only (SCO) tumours and TGCTs were analysed by RT-qPCR for expression of *HENMT1/PIWIL1/PIWIL2/PIWIL3/PIWIL4* and *LINE-1*. Additionally, *HENMT1*-overexpressing Tcam2 seminoma cell lines were analysed for the same parameters by RT-qPCR. We found that *HENMT1* and *PIWIL1* are coexpressed in pachytene spermatocytes and spermatids. Expression of *HENMT1*, *PIWIL1* and *PIWIL2* was mainly dependent on germ cell content but low levels of expression were also detected in some SCO samples. Levels of *HENMT1*, *PIWIL1* and *PIWIL2* expression were low in TGCT. Samples with *HENMT1*, *PIWIL2* and *PIWIL4* expression showed significantly ($P < 0.05$) lower transposon expression compared to samples without expression in the same histological group. *HENMT1*-overexpressing Tcam2 cells showed lower *LINE-1* expression than empty vector-transfected control lines. Our findings support that the transposon-regulating function of the piRNA pathway found in the mouse is conserved in adult human testis. *HENMT1* and *PIWI* proteins are expressed in a germ-cell-specific manner and required for transposon control.

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Introduction

Human male infertility and testis cancer show a growing incidence and are increasingly considered to be interconnected as part of a 'testicular dysgenesis syndrome' (Skakkebaek 2016). Despite this, the mechanisms underlying both pathologies are not well understood, thus hampering prevention and treatment. Testis cancer is the most common form of cancer in young men; while it responds well to treatment if discovered early, significant side effects and treatment-resistant cases pose a problem (Nitzsche et al. 2012). On the other hand, many infertility patients will never find out why their spermatogenesis is impaired and will never be able to father their own biological offspring. One essential prerequisite for successful spermatogenesis is the control of mobile genomic elements called retrotransposons in germ cells

by methylation or post-transcriptional repression (Kuramochi-Miyagawa et al. 2008, Reuter et al. 2011a, Zamudio et al. 2015). Retrotransposons are mobile DNA elements which are able to move around in the genome by a 'copy and paste' mechanism using enzymes encoded in their own, or other retrotransposons', sequence. Failure to suppress retrotransposon replication frequently results in damage to the genome, such as double strand breaks and mutations, leading to infertility or birth defects in offspring. This is especially important during the phase of genome-wide hypomethylation and the zygotene–pachytene transition where other transposon control mechanisms, like DNA methylation and repressive histone marks, are reduced or temporarily inactive (Bourc'his & Bestort 2004, Webster et al. 2005, Di Giacomo et al. 2013).

The PIWI/piRNA pathway is an ancient pathway first identified in *Arabidopsis* (Chen et al. 2002, Yu et al. 2005)

and is recognised as an important mechanism that protects the germ cell genome from retrotransposon insertional mutagenesis.

piRNAs are short noncoding RNAs that bind to PIWI proteins (Girard *et al.* 2006), a subfamily of the PIWI/Argonaute protein family with a distinct RNA cleaving activity (Lau 2006). Primary piRNAs are encoded as long precursor transcripts transcribed from piRNA genes (Williams *et al.* 2015). They are exported into the cytoplasm, where the endonuclease MITOPLD/PLD6 generates the 5' ends of what will ultimately be piRNAs (Watanabe *et al.* 2011). These are subsequently loaded onto PIWIL1 or PIWIL2 (Kawaoka *et al.* 2011, Vourekas *et al.* 2012). The 3' end of the piRNA is generated by an unknown endonuclease, and subsequently methylated by HENMT1 (HUA Enhancer 1) (Kirino & Mourelatos 2007, Saito *et al.* 2007, Plotnikova *et al.* 2013), producing a mature primary piRNA. This piRNA can then direct PIWI proteins to target complementary mRNA sequences which are subsequently cleaved.

Secondary piRNAs are produced via the ping-pong cycle (Brennecke *et al.* 2007, Aravin *et al.* 2008), a feed-forward mechanism involving PIWIL2 and PIWIL4, which is recognised as an essential 'adaptive immune system' to destroy transposon transcripts in foetal germ cells and spermatogonia and has been proposed to exist in adult germ cells as well (Ha *et al.* 2014, Goh *et al.* 2015, Lim *et al.* 2015, Rounge *et al.* 2015). In addition to this post-transcriptional mechanism, piRNAs are thought to build an epigenetic defence layer against transposon expression by directing the methylation of CpG islands and histones (Carmell *et al.* 2007, Di Giacomo *et al.* 2013, Guo & Wu 2013).

Mammals express two distinct populations of piRNAs, a more transposon-enriched group in pre-pachytene germ cells and a transposon-depleted pachytene piRNA population, more enriched for intergenic regions (Aravin *et al.* 2007, Brennecke *et al.* 2007, Lim *et al.* 2015). At least a portion of the latter is thought to be involved in controlling the spermatogenic expression programme, as well as transposon expression, and the removal of excess mRNA in the last steps of spermatogenesis (Gou *et al.* 2014, Lim *et al.* 2015).

A recent paper investigating a *Henmt1* mouse mutant demonstrated that the methyltransferase is essential for mouse spermatogenesis (Lim *et al.* 2015). Lack of HENMT1 led to piRNA instability due to reduced methylation of the piRNA 3' end, the de-repression of retrotransposons in meiotic and haploid germ cells, and the precocious expression of the haploid germ cell programme in meiotic cells, resulting in malformed spermatids and male infertility.

HENMT1 was shown to be produced in all germ cells of the seminiferous epithelium in the mouse with highest expression in late pachytene spermatocytes and spermatids (Lim *et al.* 2015).

The mouse PIWIL1 homolog, MIWI, is detected specifically in the cytoplasm of spermatocytes and spermatids and is essential for spermatogenesis (Deng & Lin 2002). *Miwi* mutant mice show an arrest of spermatogenesis during the round spermatid period. In addition to MIWI, mice also express the PIWI proteins MILI (PIWIL2) and MIWI2 (PIWIL4). MILI is produced in the cytoplasm of germ cells from the primordial germ cell stage to the round spermatids stage and mutants are male sterile, with normal primordial germ cell development but an arrest at the zygotene–pachytene transition (Kuramochi-Miyagawa *et al.* 2004, Aravin *et al.* 2008). MIWI2 is the only mouse PIWI protein localizing to both nucleus and cytoplasm. It is only detected in primordial germ cells and prospermatogonia between E12.5 and P3 (Aravin *et al.* 2008, Bao *et al.* 2014), corresponding to the period of *de novo* DNA methylation. *Miwi2* knockout in mice resulted in demethylation of LINE-1 (L1) elements, a pre-pachytene arrest of spermatogenesis, as well as a progressive loss of germ cells that ultimately resulted in tubules containing only Sertoli cells (Carmell *et al.* 2007).

In summary, the localisation and function of HENMT1 and PIWI proteins in mouse spermatogenesis has been investigated extensively while the spatio-temporal expression of their human homologues is still largely unknown. Additionally, an association of Piwi proteins has also been discovered in different cancer types (Suzuki *et al.* 2012, Wang *et al.* 2015, Iliev *et al.* 2016).

To improve understanding of the PIWI/piRNA pathway in human testis, this study aimed to define the expression patterns of HENMT1 and PIWIL1 in the human testis and investigate whether changes in their expression were associated with transposon de-repression, particular types of human infertility or TGCTs.

Materials and methods

Testis biopsies

This study was approved by the ethics committee of the Medical Faculty of the Justus Liebig University Giessen (Decision 187b/09) and the Monash University human ethics committee. Testicular biopsies were taken, after obtaining written informed consent, at the Department for Clinical Andrology, Centre for Reproductive Medicine and Andrology in the University of Muenster or at the Department of Urology in the University Hospital Giessen (UKGM).

Testis samples were obtained from men, aged 15–74 years, who underwent diagnostic testicular biopsies as described previously (Bergmann & Kliesch 2010). Testicular biopsies were fixed by immersion in Bouin's solution and embedded in paraffin. For histological evaluation, slides were prepared and evaluated following score count analysis as described previously (Bergmann & Kliesch 2010). Testicular biopsies showed normal spermatogenesis (Nsp, $n=21$), hypospermatogenesis (hyp, score 1–10, $n=49$), hypospermatogenesis with focal Sertoli cell-only (SCO) (hyp+fsc, $n=11$), tumours (TIN/Seminoma/

Carcinoma, $n=22$), arrest at the level of primary spermatocytes or spermatogonia (Arrests, $n=55$) as well as SCO syndrome ($n=65$) (Bergmann & Kliesch 2010).

Laser assisted microdissection PCR

Microdissection was performed as described previously (Fietz *et al.* 2011). Samples representing the whole seminiferous epithelium, and the seminiferous epithelium without spermatogonia, were microdissected from normal spermatogenesis. Samples representing spermatocytes or spermatogonia were dissected using tissue showing arrests at the level of spermatocytes or spermatogonia, respectively. A pure Sertoli cell population was obtained from SCO testes.

RNA extraction was accomplished with the RNeasy FFPE kit (Qiagen) according to the 'Purification of Total RNA from Microdissected FFPE Tissue Sections' protocol. After DNase treatment, RNA was reverse transcribed with Roche Superscript III (Roche) according to manufacturer's instructions. A control PCR with *GAPDH* primers was performed to control for gDNA contamination using AmpliTaq Gold (Applied Biosystems) according to the manufacturer's instructions. Primer pairs used on cDNA from microdissected material were HENMT1V2 for CCAGAATGGAGTTTCAGACC and HENMT1V2 rev GATTCTGTTGCCTTCCTCC and PIWIL1 for ACTGGTGAACCTACGAAGTGC and PIWIL1 rev GGATTCTGAAGTCTTCCTCC.

Immunohistochemistry

The immunofluorescent labelling of proteins was conducted as described previously (Jamsai *et al.* 2011). The well-established primary antibodies (HENMT1 (specificity proven on knockout in (Lim *et al.* 2015)) and PIWIL1 (Abcam ab12337); both 5 µg/mL) were incubated at 4°C overnight. Secondary antibodies anti-goat AF 455 and anti-rabbit 455 (Life Technologies; 4 µg/mL) were incubated 1 h at room temperature in the darkness. Sections were counterstained with DAPI and coverslipped with fluorescence mounting media (DAKO). Pictures were taken on a Leica SP8 confocal microscope (Leica).

For immunohistochemistry sections were labelled as described previously (Fietz *et al.* 2014) using the PIWIL1 antibody

(Abcam ab12337; 7 µg/mL). For a negative control, the primary antibody was pre-incubated with an excess of the immunizing peptide ab13827 (Abcam). Pictures were taken with a Leica ICC50 HD Microscope Camera on a Leica DM750 light microscope (Leica).

qPCR

RNA extraction from whole Bouins-fixed tissue sections was accomplished with the RNeasy FFPE kit (Qiagen) following the 'Purification of Total RNA from FFPE Tissue Sections' protocol. cDNA was produced and a control PCR was performed as described above. 1 µL cDNA was then used with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) in real-time PCRs with TaqMan gene expression assays (Table 1) on a Biorad CFX384 Touch cycler (Biorad). Input was normalised to the housekeeping gene 18S (Table 1).

For detection of actively transposing L1 sequences, we designed TaqMan assays interrogating the active human consensus L1 sequence *L1HS* (Table 1). These assays were tested for specificity in an ePCR (<https://www.ncbi.nlm.nih.gov/projects/e-pcr/reverse.cgi>; parameters: max. three mismatches, no gap allowed) that returned one target amplification site, which is the expected sequence across the whole human genome.

Samples were analysed in triplicate and excluded from analysis if the standard deviation between the replicates exceeded 0.6 Cq. Cq values >38 were considered as no expression/no detection. The $2^{-D_{Cq}}$ value was used for further analysis in GraphPad Prism.

Cell culture conditions and transfection

TCam2 cells were cultured in RPMI 1640 medium with 10% FBS and 1% penicillin–streptomycin at 37°C. Cells were transfected with the PiggyBac Transposon Vector system (SBI System Biosciences, Palo Alto, USA) using the NEON transfection system (Thermo Fisher Scientific) according to the manufacturer's instructions with a single pulse of 1000V for 40 ms. Three cell lines were transfected with a PiggyBac vector (6.5 µg PiggyBac vector+2.25 µg transposase vector/12×10⁵ cells) containing the human *HENMT1* full

Table 1 TaqMan gene expression assays.

Assay name	Assay ID	RefSeq/GenBank/Rebase ID
<i>HENMT1</i>	Hs00989130_m1	NM_144584.2592.1
<i>PIWIL1</i>	Hs01041737_m1	NM_001102592.1
<i>PIWIL2</i>	Hs00216263_m1	NM_001190971.1
<i>PIWIL3</i>	Hs00908825_m1	NM_004764.4
<i>PIWIL4</i>	Hs00381509_m1	NM_001135721.1
<i>18S</i>	Hs99999901_s1	NM_018068.3
<i>HL1A</i> Custom TaqMan	SM AIGJQ74 P140602-000G08	NM_001008496.3
<i>HL1B</i> Custom TaqMan	SM AIHSPIC P140602-000G09	NM_001255975.1
		NR_045648.1
		NR_045649.1
		NM_152431.2
		X03205.1
		L1HS Consensus 5'UTR
		L1HS Consensus ORF1

length sequence (NM_144584.2, verified by sequencing) and three cell lines with an empty PiggyBac vector (6.5 µg PiggyBac vector + 2.25 µg transposase vector/ 12×10^5 cells) as a control. Additionally, one reaction was transfected with a pEGFP vector (4.4 µg pEGFP vector/ 6×10^5 cells) as a transfection efficiency control. The PiggyBac vector carried a puromycin resistance gene, so transfected TCam2 cells were cultured in the medium described above plus 2 µg/mL puromycin to select successfully transfected cells.

RNA was purified using Trizol, and DNase-treated and reverse-transcribed with Superscript III (Thermo Fisher Scientific). *HENMT1* overexpression was confirmed by real-time PCR as described in the section 'qPCR'.

Statistics

For the cell line qPCR data, the mean of three repetitions and three technical replicates per cell line (exception Hen1 #3: only 1 repetition) and then the mean of three cell lines for empty vector and *HENMT1*-transfected cells respectively were calculated and the final mean compared with an unpaired, two-tailed *t*-test.

Results

Localisation of *HENMT1* and *PIWIL1*

The localisation and cell-specific production of *HENMT1* within the human seminiferous epithelium has not been defined before. While *PIWIL1* expression has been studied in more depth, a detailed description of *PIWIL1* stage specific localisation in the human testis has not been published. We showed expression of *HENMT1* and *PIWIL1* in human testis using

RT-PCR following laser microdissection of human testis sections and pinpointed their cell-specific expression and subcellular localisation using immunolocalisation.

Using RT-PCR on laser microdissected cell populations (Fig. 1), *HENMT1* expression was found in all germ cell types (Fig. 1A, B, C and D) and isolated Sertoli cells (Fig. 1E). *PIWIL1* expression was found in fractions containing spermatocytes (Fig. 1A, B and C), and spermatids (Fig. 1A and B), and in isolated spermatocytes (Fig. 1C), but not in spermatogonia (Fig. 1D) or Sertoli cells (Fig. 1E). *HENMT1* protein was shown to be localised in the cytoplasm of all stages of germ cells (spermatogonia, spermatocytes and spermatids) as well as Sertoli cells, with highest levels in spermatocytes (Fig. 2A and C).

PIWIL1 protein production was first seen in the cytoplasm of late pachytene cells in stage IV, where it was strongest, and continued on a lower level in the cytoplasm of spermatids until it disappeared in elongated spermatids of stage II (Fig. 2B, E and K). This resulted in co-localisation of *HENMT1* and *PIWIL1* in late pachytene until elongated spermatids (Fig. 2C and E). *PIWIL1* was not detected in spermatogonia or Sertoli cells at RNA or protein level (Figs 1D, E and 2F, K). In pachytene cells, the strongest staining for *PIWIL1* was observed in irregularly formed structures in the cytoplasm (Fig. 2I inset), which likely represents the mitochondrial cement (Fawcett *et al.* 1970, Takebe *et al.* 2013). In spermatids, the strongest staining was limited to one round structure in the cytoplasm, fitting the description of the chromatoid body as a changing but clear structure in spermatids (Fawcett *et al.* 1970, Takebe *et al.* 2013).

Figure 1 *HENMT1* mRNA is expressed in all types of germ cells and Sertoli cells. *PIWIL1* mRNA is expressed in spermatocytes and spermatids, but not in spermatogonia or Sertoli cells. *HENMT1* and *PIWIL1* expression analysed by RT-PCR after laser microdissection of different cell populations from paraffin sections. (A) Whole seminiferous epithelium from normal spermatogenesis. (B) Seminiferous epithelium without spermatogonia from normal spermatogenesis. (C) Spermatocytes from testis showing arrest at the stage of spermatocytes. (D) Spermatogonia from testis showing arrest at the stage of spermatogonia. (E) Sertoli cells from SCO. Arrows point from section to the respective PCR band, and the PCR bands have been labelled with matching letters. The last two PCR bands show the positive (+) and no template control (-) respectively. The first lane represents the DNA marker (bands 100bp and 200bp). PCR product sizes are 159bp for *HENMT1* and 143bp for *PIWIL1*.

Figure 2 HENMT1 and PIWIL1 are produced in human testis and colocalise in pachytene spermatocytes and spermatids. (A) HENMT1 localisation in all cells of the seminiferous epithelium (red). (B) PIWIL1 localisation (green) in late pachytene spermatocytes and spermatids. (C) Colocalisation of HENMT1 and PIWIL1 in pachytene spermatocytes and spermatids (merge). (D) Negative control (secondary antibodies AF 555 + 488 only) merge. Scale bars = 50 μ m. (E) Stages I–VI corresponding to F–K with cells showing PIWIL1 immunoreactivity marked by green, and cells showing HENMT1 immunoreactivity marked by red boxes. (F, G, H, I and J) Immunohistochemistry: PIWIL1 localisation in different steps (pachytene to step 7) and stages (I (F), II (G), III (H), IV (I), V (J)) of the human seminiferous epithelium, (Inset I) pachytene spermatocyte with irregular structure in cytoplasm (arrow). (K) Negative control (pre-incubation of antibody with peptide). Scale bars = 50 μ m.

piRNA pathway components are differentially expressed in histological groups

To get a picture of the expression of the piRNA pathway and retrotransposon sequences in human testis and how they relate to each other, we quantified the expression of *HENMT1*, *PIWIL1*, *PIWIL2*, *PIWIL3* and *PIWIL4*, and *L1* (*LINE-1*) in human testis biopsies. *L1* was selected as the retrotransposon family of choice as they are significantly de-repressed in numerous mouse models of piRNA pathway dysfunction (Carmell *et al.* 2007, Fazio *et al.* 2011, Lim *et al.* 2015) and retrotransposition events due to *L1* and *Alu* are most common in human genomes. DNA transposons and LTR retrotransposons do not play as significant a role in generating human mobile element insertions, and *Alu* retrotransposons are dependent on *L1* ORF2 (International Human

Genome Sequencing Consortium 2001). *L1*s make up ~17% of the human genome (International Human Genome Sequencing Consortium 2001) and another ~17% of the genome are comprised of *Alu* elements (10%) and processed pseudogenes mobilised with the help of *L1* (Esnault *et al.* 2000, Symer *et al.* 2002). Most *L1* copies are ancient/inactive and/or 5' truncated and thus inactive so *L1* expression is a function of younger, actively transposing ('hot') *L1* A and B.

HENMT1 was detected in 81% of Nsp samples, 82% of hypospermatogenesis (Hyp) samples, 80% of arrest samples, 55% of tumour samples and 25% of SCO samples. Taken as a group, expression was highest in Nsp. Expression was halved in samples containing Hyp and became progressively lower in Hyp+fsc and arrest groups (Fig. 3). The lowest expression in all groups was found in SCO samples. These data suggest that

Figure 3 Expression of piRNA pathway genes and TEs decreases with reduced germ cell content in samples. Relative expression (per cent of mean Nsp expression) of HENMT1, L1A, L1B, PIWIL1 and PIWIL2 in the different histological groups. Only samples expressing HENMT1 included. Nsp $n=17$; Hyp $n=40$; Hyp+fsc $n=7$; Arrests $n=40$; Tumour $n=12$; SCO $n=16$.

HENMT1 is predominantly produced by germ cells and is consistent with germ cells being the primary site of piRNA actions in mice.

PIWIL1 was expressed in all Nsp and Hyp samples and 96% of arrest samples, but only in 5% of SCO samples. Expression was detected in 70% of tumour tissues which was unexpected as *PIWIL1* is not normally expressed in gonocytes or spermatogonia, from which germ cell tumours are thought to be derived (Jorgensen *et al.* 1995). The highest expression was again found in Nsp samples, with Hyp samples showing less than half of the Nsp expression and expression in Hyp+fsc and arrest groups being below 20% and 10% respectively (Fig. 3). Expression in tumour and SCO groups was very low.

PIWIL2 was expressed in all Nsp/Hyp, 98% of arrest samples and 95% of tumour samples, but also in 56% of SCO samples, suggesting less strict germ-cell-specific expression than *PIWIL1*. However, *PIWIL2* levels were very low in SCO samples (Fig. 3). Expression was highest in Nsp samples and reduced to a similar degree as *PIWIL1* in Hyp and Hyp+fsc groups (Fig. 3). In the arrest group, however, *PIWIL2* levels were higher than in Hyp samples. In arrest and tumour samples, we found a reduction compared to Nsp expression for *PIWIL2*, but it is less pronounced than that for *PIWIL1*. Within the tumour group, we could not identify significant differences in expression of transposon sequences or piRNA pathway components between TIN, carcinoma and seminoma (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

PIWIL3 was not detected in any of the 94 human testis biopsies examined. *PIWIL4* expression was exclusively

analysed in the arrest group and detected in 91% of samples.

L1 (A and B) transcripts were expressed in all patients. Surprisingly, their mean expression was highest in Nsp and reduced in Hyp, Hyp+fsc and arrest samples in a pattern similar to the piRNA pathway components. The high expression in some Nsp samples was unexpected, as retrotransposons are not thought to be significantly expressed during normal spermatogenesis. In contrast to the other groups where *L1* and piRNA components were reduced in a similar manner, the mean *L1* expression in the tumour samples seemed to be higher in relation to all three piRNA pathway components.

HENMT1 expression is inversely correlated with *L1* expression in human testis

Since *HENMT1* expression was not detectable in all testis biopsies, we investigated an association between *HENMT1* expression and the expression of transposons and other piRNA pathway components in human testis by comparing the *L1*, *PIWIL1*, *PIWIL2* and *PIWIL4* expression in samples with and without *HENMT1* expression. The *L1* expression was significantly higher in samples without *HENMT1* expression in all six histological groups, suggesting a suppressive effect of *HENMT1* expression on transposon expression in human testis (Fig. 4A).

Interestingly, *PIWIL1* levels were also higher in samples without *HENMT1* expression in Nsp/Hyp and in the arrest group (Fig. 4A). The correlation between *PIWIL1* and *L1* expression was very strong in the Nsp/Hyp group (Spearman $r=0.94$). It was much weaker in all other groups, probably because they contained much lower quantities of the cell types normally expressing *PIWIL1*.

Surprisingly, the expression associations of *L1* and piRNA pathway components in SCO testes were quite similar to samples containing germ cells, albeit at much lower levels (Fig. 3). *L1s* were expressed at significantly higher levels in SCO samples without *HENMT1* expression (Fig. 4A).

As with *PIWIL1* and *L1*, *PIWIL2* expression was increased in the Nsp/Hyp group lacking *HENMT1* expression (Fig. 4A). The correlation between *PIWIL2* and *L1* expression was very strong in Nsp/Hyp ($r=0.95$). It was slightly weaker ($r=0.9$) in the arrest group and dropped to 0.6 in the tumour samples.

We also compared retrotransposon expression in SCO samples with ($n=37$) or without ($n=28$) *PIWIL2* expression. Samples without *PIWIL2* expression were found to have a significantly higher *L1A* expression than samples which had detectable *PIWIL2* expression (Fig. 4B). However, the majority of these samples also had no *HENMT1* expression so we compared samples lacking both *PIWIL2* and *HENMT1* with samples lacking only one piRNA component. We found that

Figure 4 Loss of HENMT1 correlates with higher L1, PIWIL1 and PIWIL2 expression within the same histological group. (A) Mean ($\pm$ s.d.) expression (bar) of L1A, L1B, PIWIL1 and PIWIL2 (columns) in patient samples (dots) with no detected HENMT1 expression (left bar) and with detected HENMT1 expression (right bar) in the histological groups (rows). Nsp $n=4/17$, Hyp $n=9/40$, Hyp+fsc $n=4/7$, Arrests $n=11/44$, Tumour $n=10/12$, SCO $n=49/16$. P values: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. (B) Mean ($\pm$ s.d.) expression (bar) of L1A in patient samples (dots) with no detected PIWIL2 or PIWIL4 expression (left bar) and with detected PIWIL2 or PIWIL4 expression (right bar) in the histological groups SCO $n=49/16$ (left graph) and arrests (right graph) $n=28/37$. P values: * $P \leq 0.05$.

Figure 5 Transposon overexpression occurs least when both *HENMT1* and *PIWIL2/4* are expressed in SCO, arrest and tumour groups. Mean expression $\pm$ s.d. (lines) and values they are derived from (dots). Legends: No HENMT1/PIWIL1/2/4 = HENMT1/PIWIL1/2/4 not detected; but HENMT1/PIWIL1/2/4 = HENMT1/PIWIL1/2/4 detected.

L1B (ORF1) expression was lowest when both *HENMT1* and *PIWIL2/4* were expressed (Fig. 5) while no obvious differences in *L1A* (5'UTR) expression were found between these subgroups.

Along the same line we wanted to know if *PIWIL4* expression had an effect on the level of *L1* expression. This investigation was performed in the arrest group. Samples without detectable *PIWIL4* expression ($n=7$) showed a higher *L1* expression than samples with detectable *PIWIL4* ($n=49$) (Fig. 4B). Samples lacking both *HENMT1* and *PIWIL2*, or 4, showed a slightly (not significantly) higher *L1* expression than samples lacking *HENMT1* alone (Fig. 5).

To investigate whether similar associations could be found in the tumour context, we plotted tumour samples with and without *HENMT1*, *PIWIL1* and *PIWIL2* expression against each other (Fig. 5). Samples lacking both *HENMT1* and *PIWIL1*, but expressing *PIWIL2*, had only a slightly higher *L1* expression compared to samples expressing *HENMT1*, *PIWIL1* and *PIWIL2* which had a very low expression of *L1*. In contrast, all samples expressing *PIWIL1* and *PIWIL2* without *HENMT1* had an elevated expression of *L1*.

In summary, patient samples lacking expression of a piRNA pathway component showed an elevated expression of *L1*. Additionally, elevated *L1* expression coincided with significantly increased *PIWIL1* and *PIWIL2* expression in qualitatively complete spermatogenesis.

HENMT1 overexpression leads to changes in *L1* expression in a seminoma cell line

To investigate the effect of *HENMT1* on transposon expression in a controlled environment, we overexpressed the full-length *HENMT1* sequence in the seminoma cell line TCam2. Overexpression was performed in three independent TCam2 cell lines, and three independent TCam2 cell lines were transfected with the empty vector. *HENMT1* was on average 11-fold overexpressed in the *HENMT1*-transfected cell lines compared to the wild-type TCam2 cells (Fig. 6A) with expression increased 15-fold (cell line Hen1 #3), 13-fold (Hen1 #4) and 8-fold (Hen1 #5) respectively (Supplementary Fig. 2). Empty-vector-transfected cells showed no significant increase in *HENMT1* expression compared to wild-type TCam2 (fold change = 1, empty vector #3; 1.6, empty vector #4; 1.2, empty vector #5). There was no significant change in the expression of *PIWIL1*, *PIWIL2* or *L1B* in the *HENMT1*-overexpressing cells (Fig. 6A). However, the expression of the *L1A* sequence was significantly lower in the cell lines overexpressing *HENMT1*, indicating a moderating effect of *HENMT1* on the expression of *L1* sequences, similar to what we observed in the human testis biopsies and what has been published for mouse testis (Lim et al. 2015).

Figure 6 *HENMT1* overexpression results in decreased *L1A* expression in a seminoma cell line. Expression of piRNA pathway components (*HENMT1*, *PIWIL1*, *PIWIL2*) and transposon sequences (*L1A*, *L1B*) in empty-vector-transfected (black) and *HENMT1*-transfected (grey) TCam2 cell lines relative to wild-type expression. Mean $\pm$ S.E.M. ($n=3$ cell lines for both empty-vector-transfected and *HENMT1*-transfected cells with three technical replicates). * $P \leq 0.05$.

Discussion

We have for the first time defined the exact localisation and co-localisation of *HENMT1* and *PIWIL1* in human testis. They were found to be co-expressed in late-pachytene spermatocytes and round and elongating spermatids. *HENMT1* was additionally found in spermatogonia, early spermatocytes, and at lower levels in Sertoli cells. The co-expression of *HENMT1* and *PIWIL1* also coincided with the expression of the pachytene piRNA population (Aravin *et al.* 2007, Brennecke *et al.* 2007, Lim *et al.* 2015) and matched data from the mouse (Deng & Lin 2002, Lim *et al.* 2015).

A germ-cell-dominated expression pattern was supported by data gained from human testis biopsies. Namely, expression of *HENMT1*, *PIWIL1*, *PIWIL2* and *L1* was highest in complete spermatogenesis and progressively reduced consistent with reduced germ cell content in hypospermatogenesis, germ cell arrests, tumours and SCO samples. The high *L1* expression in some Nsp samples was unexpected considering *L1* overexpression is thought to cause abnormal spermatogenesis in mammals (Yang & Wang 2016).

Low levels of expression could be detected for *HENMT1* and *PIWIL2* in the SCO group. However, *Piwi2* expression in Sertoli cells has previously been reported in the mouse (Lim *et al.* 2013) and the *PIWIL2* promoter methylation level in human SCO samples has been reported to be lower than in other somatic cell types (Heyn *et al.* 2012).

While the expression of *HENMT1* and *PIWIL2* in tumours was not unexpected, as they are normally

expressed in spermatogonia or PGCs, the cells TGCT are thought to derive from, a surprising 70% of tumour samples also expressed *PIWIL1* which is not normally expressed in premeiotic germ cells. This indicates an aberrant expression of *PIWIL1* in many germ cell tumours. Overexpression of *PIWIL1* in the TGCT context has previously been reported (Qiao *et al.* 2002).

The reduction of *PIWIL2* expression in the arrest and tumour groups compared to Nsp was less pronounced than that for *PIWIL1*. This pattern is consistent with the mouse data, if we assume the *PIWIL2* protein localisation is similar to the mouse where *PIWIL2* is expressed from 12.5dpc until the round spermatid stage (Kuramochi-Miyagawa *et al.* 2004, Aravin *et al.* 2008, Manakov *et al.* 2015) while *PIWIL1* is mainly produced in spermatocytes and spermatids which are reduced in arrests and tumour samples. Nevertheless, *PIWIL2* expression was reduced in the arrest and tumour groups. *PIWIL2* has been found to be hypermethylated in patients with spermatogenic failure, leading to lower *PIWIL2* expression, defective piRNA biogenesis and *L1* hypomethylation (Heyn *et al.* 2012). This could be an explanation for the reduction we observed, in addition to the changes in cell population.

PIWIL3 could not be detected in human testis. It has previously been reported to be expressed in the human ovary (Lim *et al.* 2014) and in some cancers (Li *et al.* 2010, Chen *et al.* 2013), but has not been seen in the testis. While it has been reported that an SNP in the coding region of *PIWIL3* correlates with oligozoospermia in Chinese patients (Gu *et al.* 2010), expression of *PIWIL3* in the testis was not investigated. While *PIWIL3* might be involved in testis development and not expressed in the adult testis, this could not be tested in our samples.

PIWIL4 was expressed in 91% of the samples analysed. This is not unexpected as it has been shown to be essential for prospermatogonial development in the mouse (Bao *et al.* 2014) and thus should be present in all samples containing spermatogonia.

L1 was expressed in all samples examined, with slight differences between *L1A* and *L1B*. Highest *L1* expression was found in complete spermatogenesis, and expression levels were lower in all spermatogenesis-impaired groups. In the tumour samples however, *L1* expression was higher in relation to the piRNA pathway components than in any other group. High *L1* expression in tumours could be explained considering seminoma cells are thought to be gonocyte-like cells (Sperger *et al.* 2011), wherein global demethylation takes place and retrotransposons may be de-repressed. In addition, cancer cells in general are prone to abnormal DNA methylation patterns and *L1* upregulation (Carreira *et al.* 2014, Koukoura *et al.* 2014, Rodic *et al.* 2014). To further complicate the story, it has been shown that some piRNA pathway components are epigenetically disrupted in a subset of human TGCTs (Ferreira *et al.* 2014),

which could additionally affect retrotransposon control in the tumour context.

Unexpectedly, we discovered that *L1s* could be overexpressed in individual human testes, even in those showing normal spermatogenesis. This was surprising, considering that retrotransposon control is thought to be a prerequisite for normal spermatogenesis (Kuramochi-Miyagawa *et al.* 2008, Reuter *et al.* 2011a, Zamudio *et al.* 2015). These data indicate that a certain level of retrotransposon expression is tolerated without obviously affecting spermatogenesis at a histological level. In support of this idea, in parallel studies where we have assessed *L1* expression in mice, animals heterozygous for the *Henmt1* mutation also display significantly elevated *L1* expression (unpublished, MKOB). This is potentially a very significant finding as it indicates that sperm from fertile men with unrecognised *L1* overexpression could transmit active *L1* elements, and a potentially abnormal epigenome, to offspring with serious consequences for long-term health.

Consistent with *HENMT1* having an important role in TE suppression in the human testis, mean *L1* levels were higher in testis biopsies without *HENMT1* expression, regardless of the histological group. This association was confirmed in a controlled system provided by TCam2 seminoma cells overexpressing *HENMT1*, which showed significantly reduced *L1* expression, and is consistent with overexpression of retrotransposons in germ cells of the *Henmt1* mouse mutant due to impaired piRNA stability (Lim *et al.* 2015).

Our finding that *L1s* were overexpressed in samples lacking expression of one or more *Piwis* fits well with the established models which show PIWI proteins are essential for retrotransposon control at an epigenetic and post-transcriptional levels (Kuramochi-Miyagawa *et al.* 2004, Reuter *et al.* 2011b, Manakov *et al.* 2015). Although this cannot be directly tested in human testis, our data supports a requirement of PIWIL1 and PIWIL2 for complete spermatogenesis in humans as their encoding mRNA was detected in all samples with complete spermatogenesis. Again, this supports a conserved function of the piRNA pathway within mammals, as both proteins have been shown to be required for mouse spermatogenesis (Deng & Lin 2002, Kuramochi-Miyagawa *et al.* 2004).

Somewhat surprisingly, elevated *L1* expression in samples with low *HENMT1* coincided with significantly increased *PIWIL1* and *PIWIL2* expression in qualitatively complete spermatogenesis. This increased expression of *PIWIL1* and *PIWIL2* is suggestive of a feedback loop wherein the instability of piRNAs, due to less methylation by *HENMT1*, and increased retrotransposon expression leads to the upregulation of other piRNA pathway members (Lim *et al.* 2015). The upregulation of *PIWIL1* and *PIWIL2* was only significant in the Nsp and Hyp groups, so it might be impaired in severely affected spermatogenesis, arrest and tumour

samples. Alternatively, *PIWIL1/PIWIL2* upregulation might occur mainly in spermatids, which are present in large numbers only in normal spermatogenesis and hypospermatogenesis.

Overall, our data supports both the presence of the PIWI/piRNA pathway in the human testis, and functions in the regulation of retrotransposon expression. In addition, we show that the effectiveness of this protective mechanism could be affected in a neoplastic context and in severely affected non-malignant spermatogenesis. Furthermore, we found that *L1* retrotransposons were overexpressed in some human testes.

Further functional studies and a definition of the piRNA populations present in normal and abnormal spermatogenesis are needed to determine the exact mechanisms of the piRNA pathway in human testis and how they can be understood to improve clinical outcomes.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0586>.

Declaration of interest

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

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