Function and Evolution of the piRNA Pathway in the Amniote Gonad and Human Ovarian Cancer

by

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Contents

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List of Publications

Three publications will arise from this thesis. One has been submitted and two are in preparation. Another two research papers and a book chapter were published and a research paper has been submitted by the candidate during her PhD, but the research will not be discussed in this thesis.

Published:

1. Tsend-Ayush E., Lim S.L., Pask A.J., Hamdan D.D., Renfree M.B., Grutzner F. 2009. Characterisation of ATRX, DMRT1, DMRT7 and WT1 in the platypus (Ornithorhynchus anatinus). *Reproduction, Fertility, and Development* 21: 985-991.

2. Rowell D.M., Lim S.L., Grutzner F. 2011. Chromosome analysis in invertebrates and vertebrates. *Methods in Molecular Biology* 772: 13-35.

3. Tsend-Ayush, E., Kortschak, R.D., Bernard, P., Lim, S.L., Ryan, J., Rosenkranz, R., Borodina, T., Dohm, J.C., Himmelbauer, H., Harley, V.R., Grutzner, F. 2012. Identification of mediator complex 26 (Crsp7) gametologs on platypus X1 and Y5 sex chromosomes: a candidate testis-determining gene in monotremes? *Chromosome Research* 20: 127-138.

4. Hrdličková, R., Nehyba, J., Lim, S.L., Grützner, F., Bose, H.R.Jr. Platypus TERT preserves features of TERT genes of ancestral amniotes. *BMC Evolutionary Biology*.

Submitted manuscripts:

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Manuscript in preparation:

1. Lim, S.L., Ricciardelli, C., Oehler, M., Tan, M.D.D.A.I., Russell, D., Grutzner, F. Overexpression of piRNA pathway genes: a role in the progression of epithelial ovarian cancer. *Int J of cancer*.

2. Lim, S.L. and Grützner, F. Friend or foe? A role for the piRNA pathway in the mammalian ovary and ovarian cancers. *Developmental Biology*.

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List of Abbreviations

Aa	Amino acid
Actb	Beta actin
Ago	Argonaute
Ago3	Argonaute 3
Aldh-1	Aldehyde dehydrogenase-1
anti-5-MeC	Anti-5-methylcytosine antibody
Aub	Aubergine
BAC	Bacterial artificial chromosome
BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide BLAST
cDNA	Complementary DNA
Chr	Chromosome
Ck7	Cytokeratin 7
CL	Corpus luteum
Crem	cAMP-responsive element upmodulator
DEPC	Diethylpyrocarbonate
Dig	Digoxigenin-11-UTP
Dnmt3a	DNA-methyltransferase 3A
Dnmt3l	DNA-methyltransferase 3L
Dpp	Days post coitum
Dpp	Days post-partum
EMT	Epithelial to mesenchymal transition
EOC	Epithelial ovarian cancer
Ev	Empty vector
FBS	Fetal bovine serum
FISH	Fluorescence *in situ* hybridisation
Flam	Flamenco
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic DNA
Gon4l	Gon-4-like (C. elegans)
GSC	Germline stem cell
Gtsf1	Gametocyte-specific factor 1
H&E	Hematoxylin and Eosin
HCl	Hydrochloric acid
HMG	High mobility group
Hr	Hour
hTERT	Human telomerase reverse transcriptase
IAP	Intracisternal-A-particle
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISH	*In situ* hybridisation
Kb	Kilobase
L1	Long interspersed element-1/Line-1
LTR	Long terminal repeat
Mael	Maelstrom
Min	Minute
miRNA	MicroRNA
MMP	Matrix metalloproteinase

Muc1	Mucin 1
MUSCLE	Multiple Sequence Comparison by Log-Expectation
MYA	Million years ago
NBT/BCIP	Nitrobluetetrazolium chloride/ X-phosphate-5-bromo-4-chloro-3-indolylphosphate
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nrapiRNA	Non repeat associate piRNA
Nt	Nucleotide
OSE	Ovarian surface epithelium
PBS	Phosphate buffered saline
PDAC	Pancreas ductal adenocarcinomas
PFA	Paraformaldehyde
piNG body	piRNA nuage giant body
piRISC	piRNA-induced silencing complex
piRNA	Piwi-interacting RNA
Piwi	P-element induced wimpy testis
Piwil1	Piwi-like 1
Piwil2	Piwi-like 2
Piwil3	Piwi-like 3
Piwil4	Piwi-like 4
PL2L	PIWIL2-like
rapiRNA	repeat associate piRNA
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SC	Serous carcinoma
SC1	Serous carcinoma 1
SD	Segmental duplication
S	Sec
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
snoRNA	Small nucleolar RNA
SNP	Single nucleotide polymorphism
SSC	Saline-sodium citrate
Stat3	Signal transducer and activator of transcription 3
Ste	*Stellate*
Su(Ste)	*Suppressor of stellate*
Tdrd1	Tudor domain containing protein 1
TE	Transposable element
Vim	Vimentin
X-gal	Abbreviated BCIG for 5-bromo-4-chloro-indolyl-β-D-galactopyranoside
Yy1ap1	YY1-associated protein 1

Abstract

The Piwi-interacting RNA (piRNA) pathway is a RNA silencing pathway which represses the expression of gene and transposable elements (TE) in the gonads via the binding of piRNAs (26-30nt) to their complimentary RNA targets and by influencing the epigenetic makeup of chromatin via interacting with other proteins (e.g. HP1). piRNAs interact with PIWI (P-element induced wimpy testis) proteins and other components such as Maelstrom (Mael) for TE silencing. In addition, *Piwi-like (Piwil)* genes and *Mael* are important for germline stem cell (GSC) production from fly to mouse. The expression of these genes was reported exclusively in mammalian testis despite the presence of piRNAs in mouse testis and ovary. Although the pathway is essential for oogenesis in fly, fish and *Xenopus,* an important role in the mammalian ovary has been in doubt, as female knockout mice (*Piwil1, 2 and Mael*) genes are fertile. In addition *Piwil* genes in particular have undergone lineage specific changes leading to up to 4 *Piwil* genes in most eutherian mammals.

Work presented in this thesis investigates the expression of piRNA pathway genes in the amniote gonad. This confirmed the robust expression of *Piwil* genes and *Mael* in the mammalian testis. Importantly, specific expression of these genes in oocyte and growing follicles was detected in mammals. The extraordinary conservation of piRNA pathway gene expression in germ cells and ovarian somatic cells from fly to human suggests an important role in mammalian gonadal development. A comprehensive bioinformatics analysis of *Piwil* genes provided new aspects towards understanding the evolutionary trajectory of *Piwi like* genes in vertebrates. For example the correction of Xenopus *piwil3* as *piwil1* ortholog clearly showing that *Piwil3* evolved exclusively in eutherian mammals. Finally, based on the expression of piRNA pathway genes in the

ovary, we hypothesised that *PIWIL* genes and *MAEL* may play a part in the origin and progression of epithelial ovarian cancer (EOC). To test this hypothesis, expression of these genes was investigated in postovulatory tissues i.e. the corpus luteum (CL) and inclusion cysts. Preliminary data show that piRNA pathway genes are not expressed in the CL but transcripts are detected in the epithelial cells of inclusion cysts. This raises the possibility that piRNA pathway genes may be involved in the cancerous transformation of epithelial cells.

To test if the piRNA pathway plays a part in EOC progression, and is related to the activity of TEs, the expression of *PIWIL genes*, *MAEL* and *L1* (one of the most abundant TEs in human) was investigated in different types of EOC. Significant upregulation of these genes was found in malignant EOC when compared to benign ovarian tumours. This upregulation might be a result of increased *L1* activity in EOC, or may due to the stem cell like characteristic of malignant EOC. Analyses of the *PIWIL1* transcripts from a malignant EOC show that most of the *PIWIL1* transcripts contain premature stop codons. Therefore, although *PIWIL1* is overexpressed in malignant EOC, the function of PIWIL1 is likely to be compromised in these tumours.

To understand the effects of *PIWIL1* and *MAEL* overexpression on cancer cell invasiveness, *PIWIL1* and *MAEL* were transiently overexpressed in ovarian cancer SKOV3 cells. Overexpression of these genes decreases cell invasiveness, suggesting a repressive role in EOC progression. Hypomethylation of *L1* and chromosome instability was found in ovarian cancers. To understand the genome stability in EOC, primary ovarian cancer cells were established from patient ascites of different stages of

ovarian cancers. FISH analyses showed that 20%-40% of the cells are aneuploid. Thus, this is a good model for understanding aneuploidy in EOC development. Our research provides a better understanding of this ancient yet conserved piRNA pathway in mammalian gonads and ovarian cancers.

Chapter 1

Introduction

Friend or foe? A role for the piRNA pathway in the mammalian ovary and ovarian cancer

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Abstract

Piwi-interacting RNAs (piRNAs) belong to a group of small non-coding RNAs which interact with PIWI proteins for the regulation of transposable elements (TEs) in germline cells of animals. Besides TE repression, piRNA pathway genes have other functions such as germline stem cell maintenance and regulating gene transcripts during gametogenesis. The role of the piRNA pathway has been extensively investigated in the mammalian testes, but has been studied less in the ovary. In this review we discuss recent findings which suggest that the piRNA pathway is playing an important role in the mammalian ovary.

Increased TE activity, due to TE hypomethylation and overexpression of piRNA pathway genes, has also been implicated in cancer progression. Based on recent evidence about piRNA pathway activity in the mammalian ovary as well as increasing evidence of an involvement of this pathway in cancer, we discuss possible functions of this pathway in the origin and progression of ovarian cancer.

Small non-coding RNA mediated silencing

RNA silencing is a fundamental part of the epigenetic regulation of gene expression in almost all eukaryotic organisms. RNA mediated gene silencing is achieved by the recognition and binding of small non-coding RNAs (20–30nt) to their complimentary RNA targets which include both gene specific transcripts and TEs. A large number of small non-coding RNAs have been identified as part of a eukaryotic RNA silencing system. There are three main classes of functional small non-coding RNAs: microRNAs (miRNAs), small interfering RNAs (siRNAs) and piRNAs (Kim et al. 2009; Thomson and Lin 2009). In addition, there is another class of small RNAs called small nucleolar RNAs (snoRNAs) which are 60-300nt in size and predominantly found in the nucleolus and involved in postransciptional modification of ribosomal RNA and spliceosomal RNA maturation (Kiss-Laszlo et al. 1996; Tollervey and Kiss 1997).

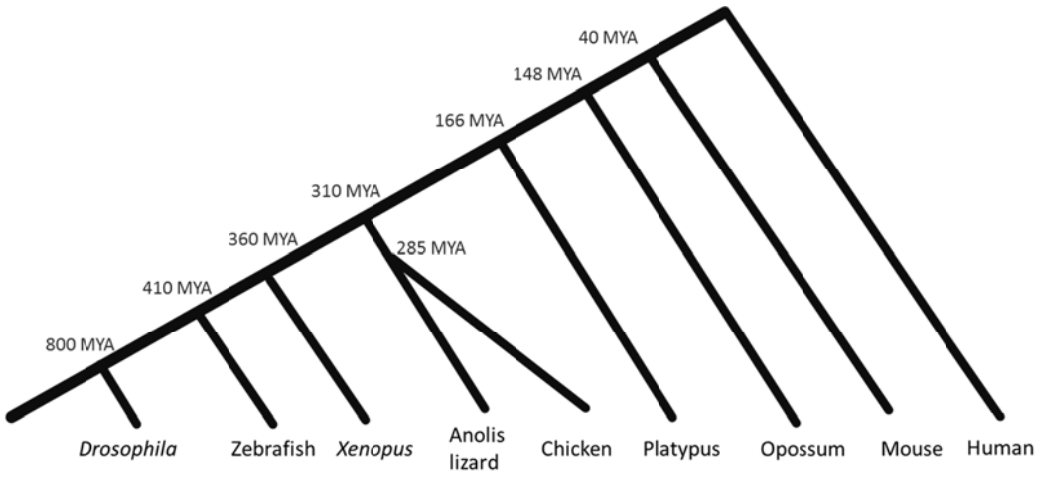
A distinct class of small RNAs are associated with PIWI/Argonaute family proteins. Based on different biogenesis mechanisms, they can be divided into either Dicer-dependent or independent. The Dicer-dependent groups include most microRNAs (miRNAs) and siRNAs. The precursors of miRNAs and siRNAs are processed by the Dicer endonuclease before associating with the Argonaute (AGO) protein to form the RNA-induced silencing complex (RISC). In the RISC, the small RNAs guide the RISC to the RNA targets, whereby AGO silences gene expression via cleavage or interaction with other proteins to block translation, modify chromatin structure or RNA stability (Ghildiyal and Zamore 2009; Kim et al. 2009; Malone and Hannon 2009).

Another group of small RNAs which belong to the Dicer-independent group are the piRNAs. Unlike the ubiquitously expressed siRNAs and miRNAs, piRNAs are only expressed in the germline and play essential roles during germ cell development (Aravin et al. 2006; Girard et al. 2006; Vagin et al. 2006; Saito et al. 2010). piRNAs interact specifically with PIWI protein/s and form piRNA-induced silencing complex (piRISC) which recognise and silence complementary TE transcripts during germline development. The presence and functional role of this pathway is conserved from fly to human (Cox et al. 1998; Qiao et al. 2002).

Evolution of *Piwi* genes

PIWI is the core component of the piRNA pathway. *Piwi* was first identified in *Drosophila* in a mutagenesis screen for genes controlling asymmetric cell division in the germline (Lin and Spradling 1997). There are three Piwi family proteins in the *Drosophila*: PIWI, Aubergine (AUB) and Argonaute 3 (AGO3). The *Drosophila Piwi* gene is orthologous to a number of Piwi-like (*Piwil*) genes in vertebrate lineages (Fig. 1), for example, three *Piwil* genes in mouse and four in human. Our recent work identified lineage specific gain/loss of *Piwi* genes based on sequence and synteny block analyses (Lim et al. submitted). *Piwil1* and *Piwil2* orthologs are present in all vertebrates investigated so far suggesting they were present before the divergence of fish (Fig. 1). However, synteny block and sequence analyses of *PIWIL2* in chicken and zebra finch support the presence of a new *PIWIL2* ortholog in birds. Eutherian mammals usually have four *Piwil* genes (*Piwil1, Piwil2, Piwil3, Piwil4*) (Sasaki et al. 2003), however, in mouse only three *Piwil* genes (*Piwil1, Piwil2 and Piwil4*) have been found (Kuramochi-Miyagawa et al. 2001; Carmell et al. 2007; Murchison et al. 2008). *Piwil4* has been reported in all mammalian lineages but is absent in birds and fish

suggesting that it evolved after the divergence of fish and has been lost in the bird lineage (Lim et al. submitted).



piRNAs in testis	+	+	?	?	+	+	+	+	+
piRNAs in ovary	+	+	+	+	?	?	?	+	?
piRNAs maturation	primary, ping-pong	primary, ping-pong	primary, ping-pong	?	?	?	? ping-pong	primary, ping-pong	primary, ping-pong
***Piwil* genes**	*Piwi, Aub, Ago3*	*piwil1, piwil2*	*piwil1, piwil2, piwil4*	*Piwil1, Piwil2, Piwil4*	*PIWIL1, PIWIL2B?*	*Piwil1, Piwil2, Piwil4*	*Piwil1, Piwil2, Piwil4*	*Piwil1, Piwil2, Piwil4*	*PIWIL1, PIWIL2, PIWIL3, PIWIL4*

Fig. 1. Summary of piRNA presence, piRNA maturation and the gain/loss of *PIWI* genes in different lineages. piRNAs have been identified in species ranging from *Drosophila* to human but no data are available in reptiles. *Piwil* genes are present in different vertebrate lineages. + indicates piRNAs have been identified in gonads; ? means "unknown".

piRNAs in different species

piRNAs were first characterised in *Drosophila* testis (Aravin et al. 2001). These piRNAs contain antisense sequence to the *Drosophila Stellate (Ste)* locus called the *suppressor of stellate (Su(Ste)).* Aravin and colleagues (2001) showed that the *Su(Ste)* locus encoded piRNAs which were homologous to *Ste* and able to silence *Ste* in trans, thus they were called *Su(ste)* piRNAs. Further characterisation such as deep sequencing showed that piRNAs belong to a highly complex family of small non-coding RNAs with millions of distinct species that are derived from specific genomic clusters (Aravin et al. 2006; Girard et al. 2006). There are three major piRNA sources; long RNAs derived from discrete genomic loci called piRNA clusters (normally enriched with TEs), transcripts from active TEs and endogenous genes (Siomi et al. 2010). *Drosophila* piRNAs are mostly TE rich (Brennecke et al. 2007). For example, piRNAs in *Drosophila* ovarian somatic cells are found to be transcribed exclusively from a single genomic strand at the *Flamenco* (*Flam*) locus (Girard et al. 2006; Brennecke et al. 2007). piRNAs derived from this cluster are antisense to the TEs (Malone and Hannon 2009).

piRNAs have also been identified in the gonads of various vertebrates. They were found in zebrafish adult testis and ovary (Houwing et al. 2007; Houwing et al. 2008). Similar to *Drosophila*, piRNAs in male and female zebrafish appear to be derived from different repeat element loci (Li et al. 2009; Malone et al. 2009; Zhou et al. 2010) suggesting that sex-specific organisation of piRNAs may be conserved from fly to vertebrates (Zhou et al. 2010). In *Xenopus*, piRNAs have only been identified in the oocytes. They are the most abundant small RNA population in the *Xenopus* oocyte being 26-31nt in length, similar to those found in mouse oocytes (Girard et al. 2006;

Lau et al. 2006), and mainly derived from TE sequences. Deep sequencing of individual *Xenopus* eggs showed that during oogenesis, multiple piRNA clusters will show similar expression patterns in different oocytes from the same female, but eggs from a different individual will have slightly different amounts of cluster expression, displaying their own oocyte expression signatures. The variation found in piRNA cluster expression could be due to distinct transcriptional regulation in different individuals. However, the exact mechanism of control causing this variation remains unclear (Lau et al. 2009).

In mammals, piRNAs have been found in mouse testis and ovary (Aravin et al. 2006; Girard et al. 2006; Watanabe et al. 2008). Similar to *Drosophila* and zebrafish, distinct size of piRNAs interact with different PIWIL proteins i.e. 26nt, 28nt and 30nt piRNAs interact with PIWIL2, PIWIL4 and PIWIL1 respectively, suggesting that PIWI proteins can act as indicators of the size of mature piRNAs (Siomi et al. 2011). Moreover, like in *Drosophila*, deep sequencing of piRNAs showed that there are nearly a Million copies of distinct piRNA species (Brennecke et al. 2007; Aravin et al. 2008). These piRNAs are derived from genomic clusters including retrotransposons, mRNAs, or intergenic regions (Gan et al. 2011). piRNA clusters range in size from 1 to ~130kb, and have distinct strand characteristics such that some piRNA sequences are mapped exclusively to a single strand, whilst others map to both strands. Thus, a large number of distinct piRNAs are derived from a small number of genomic piRNA clusters. The actual mechanism behind how a small number of clusters give rise to a diverse range of piRNAs is still currently unclear. However, studies proposed that certain mechanisms of piRNA biogenesis, such as ping-pong amplification, may contribute to the large

amount of distinct piRNAs found in the gonads (Brennecke et al. 2007; Aravin et al. 2008).

piRNA biogenesis

Although the mechanism of piRNA production remains to be elucidated, two different piRNA biogenesis pathways have recently been proposed in animals such as 'the 'primary processing pathway' and the 'ping-pong amplification pathway' (Fig. 2). Both pathways are essential to produce functional piRNAs for TE repression in different cell types in fly ovary but appear to operate at different stages during mouse spermatogenesis (Houwing et al. 2008; Kawaoka et al. 2009; Lau et al. 2009; R al. 2009).

Fig. 2. Biogenesis of piRNAs in *Drosophila* and mouse. (A) In *Drosophila* ovarian somatic cells, the primary piRNA pathway is exclusively involved in piRNA processing. Antisense piRNA precursors are transcribed from the piRNA cluster via uni/bidirectional transcription and further processed via unknown mechanisms which may involve PIWI. Mature piRNAs interact with PIWI so that it can exert its function over genomic processes such as epigenetic regulation and TE silencing. (B) Ping-pong amplification cycle in *Drosophila* germline cells. Mature antisense piRNAs are derived either from the primary processing pathway or are maternally inherited, and go on to interact with AUB. The AUB-piRNA complex targets TE transcripts or sense cluster transcripts, producing sense piRNAs which interact with AGO3. The AGO3-piRNA complex then degrades the antisense cluster transcripts and produces additional antisense piRNAs which are incorporated into AUB. (C) In mouse, the ping-pong amplification is involved in pre-pachytene piRNA biogenesis. Mature sense piRNAs, which are probably derived from bidirectionally transcribed precursors, bind to PIWIL2. The PIWIL2-piRNA complex degrades antisense cluster transcripts to produce antisense piRNAs, which are then loaded onto the PIWIL4 complex to target TE transcripts or sense cluster transcripts. Picture modified from Saxe and Lin (2012).

Evidence indicated that primary piRNAs are derived from the cleavage of long single stranded piRNAs precursors (Gunawardane et al. 2007; Saito et al. 2009) (Fig. 2). Although it is currently unclear of how primary piRNAs are processed from the piRNA precursors, lack of PIWI in *Drosophila* eliminates primary piRNAs, suggesting a role of PIWI proteins in primary piRNA processing (Fig. 2). Additional components have been identified for piRNA primary processing (reviewed by (Siomi et al. 2011)). However, how does these components and PIWI fit into the primary processing pathway remains unclear.

The primary piRNA processing pathway is active in the mouse testis during late spermatogenesis. In meiotic and post meiotic germ cells, primary piRNA biogenesis is essential for the maturation of pachytene piRNAs but not pre-pachytene piRNAs in the mouse testis (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Reuter et al. 2011). Pachytene piRNAs which represent the majority of piRNAs in mouse testis are highly expressed in round spermatids and disappear during spermiogenesis (Girard et al. 2006; Grivna et al. 2006). In mouse, PIWIL1 (MIWI) may be involved in primary piRNA processing (Beyret and Lin 2011; Siomi et al. 2011), however, the exact mechanism and nature of pachytene piRNA biosynthesis requires further investigation (Deng and Lin 2002; Girard et al. 2006; Ghildiyal and Zamore 2009).

The ping-pong amplification cycle is another mechanism of piRNA processing. It is essential for the repression of TEs in *Drosophila* ovary and testis germline cells, but not in the ovarian somatic cells (Malone and Hannon 2009; Nagao et al. 2010). In vertebrates, the ping-pong amplification pathway has been reported in mouse testis and

zebrafish ovary (Aravin et al. 2007; Houwing et al. 2007). It was proposed that ping-pong amplification cycle fine-tunes the piRNA population such that new and active TE can be supressed (Brennecke et al. 2007; Aravin et al. 2008). This is due to that the ping-pong amplification cycle only amplifying piRNAs that are present in the cell. In mouse, antisense piRNAs arise from piRNA clusters while sense piRNAs are derived from TE transcripts (Aravin et al. 2008). Thus, the presence of new TEs can lead to the generation of new piRNAs via the ping-pong amplification cycle.

The process of ping-pong amplification is well reviewed by others (Khurana and Theurkauf 2010; Saito and Siomi 2010). Briefly, in *Drosophila*, AGO3 and AUB are involved in this process (Fig. 2B). Primary piRNAs which are maternally inherited or produced from primary processing pathway (antisense to piRNA cluster) interact with AUB in the cytoplasm. The AUB-bound piRNAs then hybridise with TE transcripts or sense piRNA transcripts and cleave them to produced secondary piRNAs which are in sense orientation and then load onto AGO3. AGO3-bound secondary piRNAs then target antisense cluster transcript to produce more antisense piRNAs. These antisense piRNAs then interact with AUB for the cleavage of precursor primary piRNAs or TEs transcripts (Brennecke et al. 2007; Gunawardane et al. 2007).

Similarly, in mouse testis ping-pong amplification cycle is recruited for the synthesis of pre-pachytene piRNAs via PIWIL2 (MILI) and PIWIL4 (MIWI2) (Fig. 2C) (Aravin et al. 2007). During the amplification cycle, PIWIL2 binds to sense piRNAs which are likely derived from primary processing. The PIWIL2-bound piRNAs target antisense cluster transcripts for its degradation and produce antisense piRNAs. PIWIL4 binds to

antisense piRNAs and destructs the TE mRNAs and generates additional sense piRNAs which further interact with PIWIL2. In the *Piwil2* mutant, PIWIL4 associated piRNAs are not detected suggesting that PIWIL4 does not bind to piRNAs in the absent of PIWIL2. In contrast, in *Piwil4* mutant, PIWIL2 localisation and interaction with piRNAs are not affected, suggesting that PIWIL2 is required to initiate ping-pong amplification cycle in mouse testis (Aravin et al. 2008).

Function of the piRNA pathway in the vertebrate gonad

Silencing of transposable elements

In *Drosophila*, both germline and gonadal somatic cells are threatened by TEs activity. TEs are mobile DNA elements that are able to jump from one part of the genome to another via their own coded proteins or exploiting the host cellular machinery (Mc 1950). Some retroelements derived from ovarian somatic cells, such as *Gypsy,* can invade the oocyte via retroviral envelope-like particles (Pelisson et al. 1994; Chalvet et al. 1999; Leblanc et al. 2000). On the other hand, within the oocyte there are retrotransposons, such as non-LTR retrotransposons, which belong to the same class as mammalian long interspersed element-1 (Line-1/*L1*) and threaten genome stability (Chambeyron et al. 2008). piRNA pathways play an essential role in repressing TE activity in these situations (Saito et al. 2006; Vagin et al. 2006), even though different mechanisms have evolved in the germline cells and somatic cells (Saito et al. 2009). PIWI, AUB, and AGO3 are important components for the TE repression in *Drosophila* gonads. Loss-of-function mutations in these genes lead to vastly reduced piRNA levels and TE overexpression which results in severe defect in gametogenesis and male and female sterility (Malone et al. 2009).

In mammals, around 50% of the genome is composed of TEs (Lander et al. 2001). Unlike in plants and *Drosophila* where TEs reside in heterochromatic blocks, TEs in mammals are scattered throughout euchromatic regions (Goodier and Kazazian 2008). Without appropriate regulation, TEs present a threat to genomic integrity. DNA methylation plays a key role in TE silencing in mammals. DNA methylation occurs on cytosines of CpG dinucleotides. Methylation at the promoter region of TEs leads to transcriptional repression and induction of a repressive chromatin state where TEs reside (Rollins et al. 2006). Knockout of key enzymes DNA-methyltransferase 3A (DNMT3A) and DNA-methyltransferase 3L (DNMT3L) results in massively increased expression of TEs such as *L1* and Intracisternal-A-particle (*IAP*) in the male germline and complete sterility in males (Bourc'his and Bestor 2004). *L1* belongs to one of the most abundant TEs in the human genome. 17% of the mammalian genome is composed of *L1*. It is a group of TEs that integrate into the genome via RNA intermediates which encode their own reverse transcriptase (Lander et al. 2001; Ostertag and Kazazian 2001). Since *L1* is able to retrotranspose into the genome, it can negatively affect genome stability if not repressed. During early germline development, there is global DNA hypomethylation which results in TE derepression. Evidence showing that piRNA pathway is essential for TE repression under this situation in mouse testis (Aravin and Bourc'his 2008; Kuramochi-Miyagawa et al. 2008).

Distinct piRNAs and PIWI proteins are expressed at different stages of mouse spermatogenesis (Fig. 3). Functional studies of these proteins suggested important roles of distinct piRNA pathways in TE repression in fetal gonads and adult testis (Grivna et al. 2006; Aravin et al. 2007; Aravin et al. 2009; Reuter et al. 2011). There are three PIWIL proteins in mouse, PIWIL1, 2 and 4. PIWIL2 and PIWIL4 are expressed early

in the embryonic gonads during *de novo* DNA methylation and a period of TE derepression (Aravin et al. 2008) (Fig. 3). PIWIL2 and 4 are important for the maturation of pre-pachytene piRNAs which are enriched with TE sequences (Aravin et al. 2007). In addition, PIWIL2 and PIWIL4 work together in TE repression probably upstream of DNA methylation as piRNAs can still be found in *Dnmt3l* mutants (Aravin et al. 2008). The mechanism of piRNA-regulated DNA-methylation remains unclear. The nuclear localisation of PIWIL4 and reduction of DNA methylation which leads to TE derepression due to PIWIL4 mutation do support a role of the piRNA pathway in regulating DNA methylation; however, further experimental support is required such as directly interaction between PIWIL4 complex and DNMT3L or *de novo* methyltransferase. Nevertheless, mutations of *Piwil2* and *Piwil4* lead to the loss of TE methylation and increased expression of TE such as *L1* and *IAP* in the developing germline suggesting an important role of piRNA pathway in regulating *de novo* DNA methylation in these cells (Aravin et al. 2007; Carmell et al. 2007; Kuramochi-Miyagawa et al. 2008).

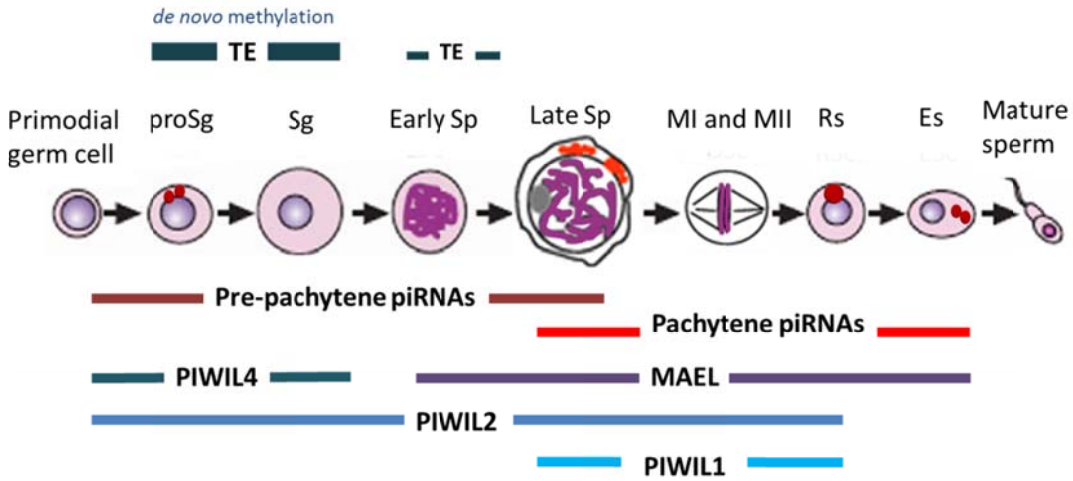


Fig. 3. The expression of piRNA pathway components in mouse spermatogenesis. Pre-pachytene piRNAs, PIWIL2 and PIWIL4 are found in early prenatal germ cells. The expression of these components coincides with TE activation and *de novo* DNA methylation. PIWIL4 expression ceases after birth, but this is not the case for PIWIL2 which continues to be expressed from germ cells until the round spermatid stage. MAEL is found in early meiotic prophase I, and is expressed from pachytene cells to elongated spermatid where its expression overlaps with PIWIL2 and PIWIL1. PIWIL1 expression is only found in the adult testis from the pachytene spermatocyte stage to the elongated spermatid stage, which coincides with the expression of pachytene piRNAs.

In adult testis, both PIWI proteins and piRNAs are found predominantly in both the nucleus and cytoplasm of the germline cell (Beyret and Lin 2011). After birth, PIWIL2 is continuously expressed in adult testis from mitotically arrested prenatal germline stem cells to the round spermatid stage (Kuramochi-Miyagawa et al. 2004; Unhavaithaya et al. 2009; Wang et al. 2009). PIWIL2 interacts with other components in adult round spermatids such as Tudor domain containing protein1 (TDRD1) protein for the repression of *L1*. Similar to PIWIl2, mutation of TDRD1 shows *L1* derepression. Furthermore, removal of the TDRD1 alters the PIWIL2 associated piRNA profile (Reuter et al. 2009), such that in the TDRD1 null mutant, there is increased transcript associated piRNAs but not TE associated piRNAs, suggesting that absence of TDRD1 affects PIWIL2 piRNAs production. Therefore, the interaction between PIWIL2 and TDRD1 is essential in maintaining the correct pool of piRNAs required for spermatogenesis (Reuter et al. 2009).

PIWIL1 expression is only found in the adult testis. PIWIL1 interacts with pachytene piRNAs and is co-expressed with these piRNAs during later stages of spermatogenesis from the meiosis I mid-pachytene stage to the elongating spermatid stage (Deng and Lin 2002; Girard et al. 2006; Grivna et al. 2006) (Fig. 3). The role of PIWIL1 and pachytene piRNAs in TE repression seems contradictory. On one hand mutations of *Piwil2* and *Piwil4,* but not *Piwil1,* lead to TE activation, meiotic arrest and male sterility in mouse (Aravin et al. 2007; Carmell et al. 2007; Kuramochi-Miyagawa et al. 2008) suggesting that PIWIL1 and pachytene piRNAs have roles in addition to TE repression in postnatal germ cells. Contrary to this, a recent study demonstrated that the catalytic (slicer) function of PIWIL1 is essential for TE suppression (Reuter et al. 2011). Mutation of a single amino acid from the PIWIL1 catalytic site leads to *L1* transcript

accumulation in the mutant germ cells and results in male sterility. This suggests a continuous role of PIWIL1 in TE repression at the post-transcriptional level after birth (Reuter et al. 2011).

Maelstrom (MAEL) is another evolutionary conserved protein that is important in TE repression in gonads. It was first identified in *Drosophila* ovary where it plays an important role in oocyte axis specification (Clegg et al. 1997). Furthermore, *Mael* is required for the maturation of repeat-targeting interfering RNAs in order to repress TEs (Klattenhoff et al. 2007; Lim and Kai 2007). In mouse, MAEL is essential for spermatogenesis and is expressed in early spermatocytes to round spermatids (Soper et al. 2008) (Fig. 3). MAEL mutants exhibit germ cell degradation and meiotic arrest at meiotic prophase I (Soper et al. 2008). In addition, *Mael* mutants were shown to have high levels of *L1* and *IAP* transcripts and loss of DNA methylation in the germ cells. MAEL interacts with PIWIL2 and co-localises in the same granules as PIWIL4 in the fetal testis (Costa et al. 2006; Aravin et al. 2009). These findings suggest that MAEL may play a similar role as PIWIL2 and PIWIL4 in TE repression via *de novo* DNA methylation.

mRNA regulation and translational control during spermiogenesis

PIWIL1 plays important roles in spermiogenesis (Deng and Lin 2002). Spermiogenesis is the final stage of spermatogenesis in which round spermatids are transformed into mature sperm. During spermiogenesis, PIWIL1 works in conjunction with the cAMP-responsive element upmodulator (CREM) pathway (Deng and Lin 2002). *Crem* has been proposed as the master switch gene that initiates spermiogenesis (Sassone-Corsi

2000). *Piwil1* mutants show down regulation of CREM targeted genes and has phenotypes similar to *Crem* mutants which show increased apoptosis and spermiogenic arrest (Blendy et al. 1996; Deng and Lin 2002). Furthermore, PIWIL1 and its associated piRNAs may be a component of polysomes which are involved in translational control during spermiogenesis (Grivna et al. 2006; Grivna et al. 2006). Moreover, PIWIL1 is required for miRNA synthesis despite the presence of Dicer (Grivna et al. 2006). This evidence suggests that PIWIL1 has divergent roles in initiating and maintaining spermiogenesis in the context of transcript stability and translational regulation (Deng and Lin 2001; Grivna et al. 2006).

Germline stem cell maintenance

Piwi was first identified in *Drosophila* in a mutagenesis screen for genes controlling asymmetric cell division in the germline (Lin and Spradling 1997). It is required for maintaining germline stem cell (GSCs) self-renewal in the gonads. In *Piwi* mutant flies, the amount of GSCs, eggs, and sperm bundles are greatly reduced, which leads to sterility in both sexes (Lin and Spradling 1997; Cox et al. 1998). Similarly *Ago3* mutant flies displayed a failure in GSC maintenance, which also results in sterility (Li et al. 2009). In zebrafish, null mutations of *piwil1* and *piwil2* lead to progressive loss of GSCs during larval development (Houwing et al. 2007; Houwing et al. 2008). In adult, GSCs with reduced function of piwil1 undergo increased levels of apoptosis (Houwing et al. 2007). In mammals, *PIWIL2* and *4* mutant mice have progressive loss of GSCs which leads to spermatogenesis arrest (Carmell et al. 2007). These findings strongly suggest a conserved role of PIWI in GSC maintenance in vertebrate ovary and testis.

A recent study demonstrated that the function of PIWI in GSC maintenance is independent to its function in TE control in *Drosophila* (Klenov et al. 2011). PIWI is usually loaded with piRNAs in the nucleus of GSCs and ovarian somatic cells (Brennecke et al. 2007; Malone and Hannon 2009; Olivieri et al. 2010; Saito et al. 2010; Qi et al. 2011). In this study, the nuclear localisation signal was deleted from PIWI, which resulted in exclusively cytoplasmic localisation of this protein. These mutant flies have normal GSC self-renewal in the ovary but have severe TE overexpression which led to female sterility. This result suggested that the presence of PIWI in the nucleus is required for TE silencing via the piRNA pathway but not GSC maintenance in the germline cells.

piRNA pathway in cancer

About 65 disease-causing mutations in human have been identified due to *L1*-mediated retrotransposition (Belancio et al. 2008; Goodier and Kazazian 2008). The retrotransposition event may directly disrupt exons (Kazazian et al. 1988) or regulatory regions in intronic or intergenic sequences (Belancio et al. 2008). Furthermore, the *de novo* insertion of *L1* into a genome can introduce premature polyadenylation which affects transcript elongation, possibly due to stalling of the RNA polymerase complex or premature dissociation of the transcriptional complex (Han et al. 2004). In addition, L1 endonuclease creates dsDNA breaks which may affect genome stability. Different defects including cancer, infertility and developmental defects were identified in mice with TE-induced insertions and rearrangements (Bannert and Kurth 2004). Therefore, repression of *L1* activity is critical in maintaining genome stability (Zamudio and Bourc'his 2010).

Global DNA and *L1* hypomethylation are commonly found in cancer cell genomes. *L1* hypomethylation has been reported in ovarian and other cancers and indicative of a poor prognosis for epithelial ovarian cancer (EOC) patients. Patients with greater hypomethylation have reduced mean survival times (Chalitchagorn et al. 2004; Widschwendter et al. 2004; Pattamadilok et al. 2008). In addition, increased *L1* hypomethylation is positively correlated with EOC progression (Pattamadilok et al. 2008).

piRNAs in cancer

piRNA expression appears to be affected in human cancers and cancer cell lines (Lu et al. 2010). Deep sequencing of small RNA libraries from HeLa cells following *Piwil2* overexpression show a decrease in *L1*-derived small RNAs, suggesting *Piwil2* represses *L1* activity in HeLa cancer cell line (Lu et al. 2010). Contrary to this, other studies showed that increased expression of specific piRNA may promote cancer cell growth. The expression of piR-651 is upregulated in various cancers including gastric, lung, colon, and breast cancers (Cheng et al. 2011). In gastric cancer cells, addition of a piR-651 inhibitor arrests the cell cycle at the G2/M phase, suggesting that piR-651 is needed for cancer cell growth (Cheng et al. 2011). At present information about piRNA profile from cancer tissues using deep sequencing is scarce. Further investigation in this direction is required to understand the role of piRNAs in cancers.

Expression and possible functions of PIWIL in cancers

Human *PIWIL1* (*HIWI*), is known as a testis cancer gene, and has been well investigated in various testicular cancers (Qiao et al. 2002). Increased expression of *PIWIL1* is found in malignant seminomas which originate from embryonic germ cells that retained their germ cell characteristics. Thus, *PIWIL1* overexpression may contribute to germ cell proliferation in cancers (Qiao et al. 2002). Furthermore, overexpression of *PIWIL1* maybe correlated to cancer progression. Higher expression of *PIWIL1* is found in malignant gastric and hepatocellular carcinomas compared to normal or benign tissues (Liu et al. 2006; Jiang et al. 2011) and *PIWIL1* overexpression is correlated with poorer prognosis and higher tumour related death. (Taubert et al. 2007; He et al. 2009). Furthermore, high expression of *PIWIL1,* together with other stem cell genes, such as human telomerase reverse transcriptase (*hTERT*) and survivin,

is shown to be correlated with an increased risk of tumour-related death in soft tissue sarcomas (Taubert et al. 2007).

The role of PIWIL1 in cancer growth remains controversial. Studies in gastric cancers demonstrated that *PIWIL1* may be a marker for cancer cell proliferation (Liu et al. 2006) as it is co-expressed with KI67, a reliable cell proliferation marker (Brown and Gatter 2002) in gastric cancer tissues. In addition, antisense or shRNA knockdown of *PIWIL1* in gastric cancer cells prevented the cells from entering mitosis. This data suggests that PIWIL1 may be participating in cancer cell proliferation. However, overexpression of PIWIL1 in KG1 myeloid cell line did not increase cell growth or even cause programmed cell death (Sharma et al. 2001). This raises the possibility of different roles in different cancers or cell line used and more work is needed in different *in vitro* and *in vivo* systems (e.g. transplantation of transfected cells) to understand the role of PIWIL1 in cancer growth and apoptosis.

Another *PIWIL* gene which has been widely investigated in cancers is *PIWIL2*. *PIWIL2* expression is found in a wide range of tumours including prostate, breast, gastrointestinal, ovarian and endometrial cancers (Lee et al. 2006), but is not present in human bladder cancer cell lines and early-stage bladder tumours (Nikpour et al. 2009). In breast and cervical cancers, *PIWIL2* is expressed from an early stage all the way through to the invasive metastatic stage, suggesting that it is a good biomarker for early diagnosis and prognosis of these cancers (He et al. 2010; Liu et al. 2010). There is increasing evidence that PIWIL2 takes part in the proliferation and anti-apoptotic pathways which drive cancer progression in seminomas and breast cancer stem cells

(Lee et al. 2006; Lee et al. 2010). This includes a role in cell death inhibition via interactions with cell death regulators, such as BCL-XL (Reed 1998; Lee et al. 2006) and its upstream regulator signal transducer and activator of transcription 3 (STAT3) (Umeda et al. 2003). STATs upregulate genes involved in apoptosis inhibition during tumourigenesis, such as *BCL-XL,* (Buettner et al. 2002). The cooperation of STAT3 and BCL-XL is important to regulate cell proliferation and survival (Umeda et al. 2003). The interaction of these pathways is found in both testicular germ cell tumour cell lines, Tera-1 and breast cancer stem cells (Lee et al. 2006; Lee et al. 2010). In *in vitro* studies, inhibition of *PIWIL2* expression decreases *STAT3* expression and induced apoptosis in embryonic fibroblast NIH-3T3 cells (Lee et al. 2006). Recent evidence shows that PIWIL2 interacts with STAT3 and helps in the activation of STAT3. Activated STAT3 translocates into nucleus and binds to the promoter region of tumour suppressor gene P53 which results in the repression of *P53* expression and thus promotes growth in cervical cancer HeLa cells (Lu et al. 2012). These findings provide a mechanism of *PIWIL2* promoting tumourigenesis via repression of anti-apoptosis in cancer.

Recently, several 5' truncated *PIWIL2* genes, called *PIWIL2-like* (*PL2L*), were identified. PL2L proteins are widely expressed in various types of tumour cell lines and tissues. In *PL2L* transfected cells, an increase in the amount of Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity was observed (Ye et al. 2010). NF-κB is a transcription factor involved in regulating cell survival, apoptosis and immune responses (Grandage et al. 2005; Dreesen and Brivanlou 2007). Thus, PL2L may promote tumour growth via an interaction with NF-κB which could further regulate other cell proliferation pathways, such as the STAT3/BCL-2 pathway (Ye et al. 2010).

In light of the previously discussed evidence, the piRNA pathway genes could be a regulator of cancer cell progression. Alteration of *PIWIL1* expression is associated with a higher risk of cancer-related deaths in some cancers. On the other hand, PIWIL2 or PL2L can promote tumour cell growth via interacting with the STAT3/BCL-2 apoptosis inhibition pathway. Also, PIWIL2 may be involved in the repression of *L1* via piRNA in human HeLa cells. Thus, the overexpression of piRNA pathway genes may be due to an increase in piRNA pathway activity triggered by TE derepression in certain cancers but the evidence presented indicates that piRNA pathways are involved in a range of aspects of cancer progression.

New possible roles of the piRNA pathway in mammalian gonadal somatic cells and ovarian cancers

Extensive evidence shows a vital role for the piRNA pathway genes in the gonadal somatic cells in the testis and ovaries of fly. By contrast in mammals much research has focused on the role of this pathway in particular in male germ cells. Initially expression of piRNA pathway genes was not found in mouse testicular Sertoli cells (Deng and Lin 2002) or ovarian granulosa cells respectively. More recently a role of this pathway and expression in supporting cells in the male and female gonad do indicate that this pathway has a conserved role in these cells in mammals as well.

In mammals, although *Piwil2* expression and presence of piRNAs have been reported in mouse ovary (Kuramochi-Miyagawa et al. 2001; Ro et al. 2007; Zhou et al. 2010), *Piwil* gene knockout females are fertile, and thus a role of this pathway in mammalian oogenesis was questioned (Thomson and Lin 2009; Siomi et al. 2011). Several recent

findings, together with our work, support a role for piRNA pathway genes in mouse testicular supporting cells, mammalian folliculogenesis, and in ovarian cancer initiation and progression.

Possible role of piRNA pathway in mammalian testicular somatic cells

Unlike in *Drosophila*, the expression and function of *Piwil* genes have not been reported in the testicular supporting cells, such as Sertoli cells in mouse. Northern blot analyses suggest that *Piwil1* and *Piwil2* expression is restricted to the germline, as no expression of these genes is found in testis that lack spermatogenesis (Loveland and Schlatt 1997; Kuramochi-Miyagawa et al. 2001). However, an increased number of Sertoli cells was found in *Piwil2* null mutant mice (Kuramochi-Miyagawa et al. 2004) and alteration of Sertoli cell appearance has been observed in *Mael* mutant mice (Soper et al. 2008) suggesting a role for *Piwil* genes in Sertoli cells. Furthermore, piRNAs have recently been found in Sertoli cell nucleoli. Loss of piRNA expression was reported in *Piwil1* null mice, suggesting that *Piwil1* may be responsible for the specifically loss of piRNAs from the nucleolus of Sertoli cells (Marcon et al. 2008). Furthermore, we recently showed that *Mael* and *Piwil2* mRNA is located in mouse Sertoli cell nuclei (Lim et al. submitted) and *Piwil4* is also expressed in mouse Sertoli cells (Carmell et al. 2007). Since the expression of *Piwil* genes is relatively low in Sertoli cells compared to meiotic cells, the expression of *Piwil* genes in the former cell type may have been overlooked. Further analysis such as deep sequencing of the small RNA population in isolated mouse Sertoli cells would allow a better understanding of piRNA activity in the mammalian supporting cells.

Role of piRNA pathway in mammalian oogenesis

Null mutations of many piRNA pathway genes, such as *Piwil1-4* and *Mael,* result in sterile male mice but fertile female mice (Deng and Lin 2002; Kuramochi-Miyagawa et al. 2004). Thus, studies concluded that piRNA pathway is vital for mammalian spermatogenesis but not for mammalian oogenesis (Thomson and Lin 2009). However so far no thorough analysis for female mutants has been published and recent evidence of piRNA activity in ovary does warrant a closer look at the role of the piRNA pathway in mammalian ovary (Watanabe et al. 2008; Zhou et al. 2010; Lim et al. submitted). piRNAs have been identified in new born and adult mouse ovaries (Ro et al. 2007; Watanabe et al. 2008; Ahn et al. 2010). A recent study showed that in the adult ovary, about 70% of the identified piRNAs are derived from repetitive sequences, called repeat associate piRNAs (rapiRNAs). The remaining 30% of piRNAs are derived from non-repetitive genomic regions termed nrapiRNAs. The ovarian rapiRNAs are longer (32-38nt) than the nrapiRNAs group (27-31nt). The presence of different sized piRNAs in the oocytes may suggest different PIWIL proteins are involved in the piRNA pathway. In addition, PIWIL2 associated piRNAs have been identified via immunoprecipitation of PIWIL2 in growing oocytes (Ro et al. 2007; Watanabe et al. 2008). Most of these piRNAs correspond to retrotransposons. In *Piwil2* mutant oocytes, there is a 3 fold increase in the *IAP* retrotransposons (Watanabe et al. 2008). This data suggests that the piRNA pathway is involved in TE suppression in mouse oocytes.

In mouse, the sterile male and fertile female phenotypes which arise from the previously discussed piRNA pathway gene knockouts, were also observed in mutants of a candidate gene in this pathway, the Gametocyte-specific factor 1 (*Gtsf1*)/*Cue110* gene (Yoshimura et al. 2007). However, further investigation of GTSF1 activity in

adult postnatal ovary suggests a function of this gene in ovarian germline development. *Gtsf1,* also known as *Cue110,* is expressed in adult gonads and is important for spermatogenesis in mouse testis. Null mutant mice have similar phenotype as the *Mael*, *Piwil1* and *Piwil2* mutants such as reduced TE methylation (Aravin et al. 2007; Carmell et al. 2007; Soper et al. 2008). Although the *Gtsf1*-null females are fertile, *Gtsf1* is expressed throughout oogenesis from the oocytes of primordial follicles to antral follicles (Krotz et al. 2009). GTSF1 acts together with NOBOX during oogenesis perhaps for the transition of primordial follicles to primary follicles (Choi et al. 2007; Krotz et al. 2009). Mutation of *Nobox* disrupts oocyte specific gene expression which leads to postnatal loss of oocytes and premature ovarian failure (Suzumori et al. 2002; Rajkovic et al. 2004). *Gtsf1* expression is regulated by NOBOX and it is expressed throughout folliculogenesis, this demonstrated that how a gene with a female fertile phenotype can still play a role in maintaining folliculogenesis.

Consistent with *Gtsf1*, other piRNA pathway genes are expressed in the mammalian ovary despite the fact that their mutants are fertile. *Mael*, *Piwil1* and *Piwil2* are essential for oogenesis from *Drosophila* to *Xenopus* (Deng and Lin 2002; Kuramochi-Miyagawa et al. 2004; Carmell et al. 2007; Houwing et al. 2007; Houwing et al. 2008). Recent research showed that *Mael*, *Piwil1* and *Piwil2* are expressed in the platypus and human ovaries, while only *Mael* and *Piwil2* expression is found in the mouse ovaries. *In situ* analyses showed that these genes are expressed in the ovarian supporting cells and oocytes at different stages of folliculogenesis, suggesting a role of these genes in oogenesis (Watanabe et al. 2008; Lim et al. submitted). In addition, *Piwil1* and *Piwil2* are expressed in porcine ovary (Zhou et al. 2010). The expression of piRNAs and piRNA pathway genes in the oocyte, similar to *Gtsf1*, may suggest a role for this

pathway in mammals, even though female mutant mice are fertile. A conserved role of this pathway in the vertebrate ovary is also supported by studies in fish and frog where expression has been found in both model organisms. Together this warrants further investigation into the quality and quantity of the oocytes in these mice for a better understanding of the role of the piRNA pathway in mammalian oogenesis.

piRNA pathway genes and the origin of ovarian cancer

At each menstrual cycle, a mature oocyte from an antral follicle is released from the ovarian surface epithelium. Ovarian somatic cells which line the same follicle, such as mural granulosa cells, are retained in the ovary. Previous findings have demonstrated that the piRNA pathway genes are expressed in the ovarian supporting cells and oocytes during mammalian folliculogenesis (Lim et al. submitted). In light of this it should be considered if this pathway plays a role in the origin of ovarian cancer. At present 90% of ovarian cancers have an unknown origin (Ricciardelli and Oehler 2009). Low expression of piRNA pathway genes, such as *PIWIL1*, *2* and *MAEL* and *L1* expression, was found in the epithelial cells of inclusion cysts, which has been proposed as a possible structure in which transformation of ovarian cancer stem cells occurs.

Possible role of piRNA pathway in epithelial ovarian cancer progression

piRNA pathway components are important in maintaining germline stemness in multiple species (Cox et al. 1998; Cox et al. 1998; Unhavaithaya et al. 2009), and increasing evidence suggests that the epigenetic profiles of cancer genomes is similar to that of stem cells. Upregulation of *PIWIL* genes has been found in various cancers

(Cheng et al. 2011). Similarly, our study has shown that *L1* and the piRNA pathway genes *PIWIL1* and *MAEL* are upregulated in the cancerous cells of malignant epithelial ovarian cancer (EOC). The increased expression of piRNA pathway genes in malignant cancers may be triggered by the overexpression of *L1* in the cancer genome, thereby increasing the protection of the genome against transposable element activity. *In vitro* studies have shown that overexpression of *PIWIL1* and *MAEL* have a repressive effect on ovarian cancer cell invasiveness (Chapter 3.1). In cancer tissues, transcript variants of *PIWIL1* were identified, suggesting that non-functional *PIWIL1* may be produced in the cancer tissues (Chapter 3.2). This may suggest a repressive role of the piRNA pathway genes in EOC is prevented by the production of aberrant PIWIL1.

Concluding remarks

Traditionally the piRNA pathway has been viewed as essential in protecting germline and gonadal somatic cells against the harmful expression of TEs (Kuramochi-Miyagawa et al. 2001; Kurth and Mochizuki 2009). A vast number of distinct piRNAs have been identified by deep sequencing, but not all of these piRNAs are derived from TE sequence. Furthermore, piRNA pathway genes have other functions such as mRNA regulation and translational control during spermiogenesis as well as changing the epigenetic makeup of chromatin (Grivna et al. 2006). So far, two different pathways in piRNA biogenesis have been identified *i.e.* the primary processing pathway and the ping-pong amplification pathway. These pathways are found in either germline cells or gonadal somatic cells and are highly conserved from fly to mammal (Houwing et al. 2008; Kawaoka et al. 2009; Lau et al. 2009; Robine et al. 2009).

In mammals, the piRNA pathway has been extensively investigated in mouse testis. Different classes of piRNAs are expressed in a coordinated manner during spermatogenesis, including the pre-pachytene and pachytene piRNAs. Knockouts of Piwi proteins result in male but not female sterility in mouse. Thus, the function of the piRNA pathway in the mammalian ovary remains unclear (Deng and Lin 2002; Kuramochi-Miyagawa et al. 2004). However, mounting evidence of piRNAs and piRNA pathway gene expression in the mammalian oocyte raises the possibility that this pathway may play a role in mammalian folliculogenesis, similar to that in *Drosophila*, zebrafish and *Xenopus*.

The majority of our knowledge about *PIWIL* genes in cancers is restricted to expression analyses so far. Although several roles have been suggested for piRNA pathway components in the progression of cancer, the function of these components in cancer progression remains controversial (Sharma et al. 2001; Lee et al. 2006; Liu et al. 2006; Lee et al. 2010; Li et al. 2010). Our findings suggest a repressive role of piRNA pathway genes in malignant EOC, such that the increased expression of piRNA pathway genes in malignant EOC may be triggered by the overexpression of *L1* in the cancer genome, therefore increasing genome protection against TE activity. Interestingly the production of aberrant PIWIL1 might interfere with this effect in ovarian cancer cells. Further experiments to test the repressive effect of piRNA pathway genes *in vitro* and *in vivo* will provide a better understanding of the effect of this pathway in *L1* regulation and cancer progression.

Chapter 2

Research paper

Conservation and expression of piRNA pathway genes in male and female adult gonad of amniotes suggest ancient roles in germ cell development

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Conservation and expression of piRNA pathway genes in male and female adult gonad of amniotes suggests ancient roles in germ cell development

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Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Abstract

PIWI/Argonaute protein family are essential for germline development and transposable element repression. PIWI proteins bind piRNAs and associate with other proteins including Maelstrom (*Mael*). In *Drosophila*, the Piwi-clade comprises three genes: *Piwi*, *Argonaute 3* (*Ago3*) and Aubergine (*Aub*), whereas up to four *Piwi* genes (*Piwil1* to *Piwil4*) have been reported in different vertebrate species. Genes associated with the piRNA pathway include *Mael* other genes. *Piwi* and *Mael* knockout mice feature aberrant DNA methylation, retrotransposon expression and spermatogenic arrest. PiRNAs have been identified in mouse testis and ovary but expression of *Piwil1, Piwil4* and *Mael* was reported exclusively in testis while in *Drosophila* it is known that they are active in testis and ovary. The lack of expression and absence of obvious phenotypic defects in female mouse knockouts has led to the view that this pathway may not be essential in the mammalian ovary. We investigated the evolution and expression of piRNA pathway genes in mammals and birds with sequence and synteny block analysis, RACE-PCR, RT-PCR and RNA *in situ* analysis. Our findings confirmed a high level of conservation and revealed lineage specific gain/loss of *Piwi* genes in chicken, frogs and eutherians. As expected *Piwi* and *Mael* orthologs are expressed in platypus and chicken testes. In contrast to mouse, *Piwil4* is expressed in platypus and human adult testis. Unlike earlier reports we found some evidence of *Mael* and *Piwil2* expression in mouse Sertoli cells. Importantly, we found expression of *Piwil2*, *Piwil4* and *Mael* in oocytes and supporting cells in human, mouse and platypus but no *Piwil1* expression in mouse and chicken ovary. These findings reveal lineage specific changes of piRNA pathway gene expression. Importantly, the extraordinary conservation of gene expression in somatic and germ cells in species that diverged over 800 million years ago suggests an important role in adult male and female gonad.

Introduction

Gametogenesis is central to sexual reproduction, and in mammals the development of oocytes and sperm involves epigenetic reprogramming and temporal erasure of DNA methylation, leading to increased retrotransposon expression. Small RNA regulatory pathways, the piRNA pathway, is particularly essential for genome integrity, retrotransposon repression and germ cell development (Siomi et al. 2011).

Major players of the piRNA pathway comprise the highly conserved PIWI/Argonaute protein family (Carmell et al. 2002). In *Drosophila* this includes PIWI, Aubergine (AUB) and Argonaute 3 (AGO3), which are expressed in germline tissues where they interact with piRNAs (Siomi et al. 2011). Orthologs of the *Drosophila Piwi* gene are highly conserved during evolution and have been identified in different metazoan lineages (Alie et al. 2011). Up to four *Piwi* related sequences have been reported in various vertebrates. Eutherian mammals usually have four *Piwil* genes (*Piwil1, Piwil2, Piwil3, Piwil4*) (Sasaki et al. 2003) but in mouse and rat only three *Piwi-like* genes (*Piwil1, Piwil2 and Piwil4*) have been identified (Kuramochi-Miyagawa et al. 2001; Carmell et al. 2007; Murchison et al. 2008).

An increasing number of piRNA pathway proteins and other proteins involved in RNA decay have been found to co-localize in granules (termed pi-bodies and piP-bodies) in the chromatoid body, a type of nuage in round spermatids in mammalian testis. The nuage is an electron dense perinuclear compartment that has been observed in germ cells in various animal species (Parvinen 2005; Lim and Kai 2007). Such proteins include mouse orthologs of *Maelstrom* (*Mael*), *Tudor* and *Mvh* (van der Heijden et al.

2010). The *Mael* gene was initially discovered in *Drosophila* where it is required for germline stem cell differentiation during ovarian development (Clegg et al. 1997; Pek et al. 2009). MAEL is an evolutionarily conserved protein which contains a DNA binding high mobility group (HMG) domain and a MAEL domain with unknown function (Zhang et al. 2008). MAEL is reported to interact and co-localize with other piRNA components such as PIWIL1, PIWIL2 in mouse adult testis (Costa et al. 2006) and PIWIL4 in the fetal testis (Aravin et al. 2009). Mutant analysis of mouse orthologs of *Drosophila Mael* and *Piwi* genes revealed similar phenotypes in males, confirming their conserved role in spermatogenesis (Costa et al. 2006; Soper et al. 2008).

Mael and *Piwi* are expressed in *Drosophila* testis where they have been localized in piRNA nuage giant body (piNG) of spermatocytes (Kibanov et al. 2011). In mice, *Piwi* genes are expressed during early spermatogenesis. Initially *Piwil4* is expressed in the mitotically arrested prenatal germline stem cells (GSCs) from 15.5 days post coitum (dpc) to 3 days post-partum (dpp) and in Sertoli cells (Carmell et al. 2007; Aravin et al. 2008). This is followed by *Piwil2* expression from mitotically arrested GSCs (3dpp) (Kuramochi-Miyagawa et al. 2004) to round spermatids. *Piwil1* expression is observed from mid-pachytene through to meiotic spermatocytes and in elongated spermatids (Deng and Lin 2002). In human *PIWIL1-4* expression has been reported in adult testis (Sasaki et al. 2003).

The expression and function of piRNA pathway genes in the ovary have mainly been investigated in *Drosophila*, zebrafish and *Xenopus*, and to a much lesser extent in mammals and birds. In *Drosophila*, *Piwi* mutant females are sterile due to deficiencies

in GSC maintenance, abnormal egg chamber polarity and reduced nurse cell numbers (Lin and Spradling 1997). *Drosophila Mael* mutants have defective oocyte development and show derepression of TEs (Clegg et al. 1997). In zebrafish, mutation of *piwil1* (*ziwi*) and *piwil2* (*zili*) results in no germ cell formation which leads to sterility (Houwing et al. 2007; Houwing et al. 2008). A missense mutation of the *piwil2* gene has been reported which allows the production of germ cells but leads to female but not male sterility, suggesting a conserved function of the piRNA pathway in the vertebrate ovary (Houwing et al. 2007; Houwing et al. 2008). In *Xenopus*, *piwil1* and *piwil2* orthologs (*xiwi* and *xili*) are expressed throughout oogenesis (Wilczynska et al. 2009). In mouse, previous studies reported no expression of *Mael* in the ovary (Costa et al. 2006). Despite the presence of piRNAs and *PIWIL2* in the mammalian ovary, it remains unclear whether other piRNA pathway genes are expressed and a role for this pathway in the mammalian ovary is questioned (Siomi et al. 2011).

Here, we provide a detailed analysis of the evolution and expression pattern of piRNA pathway genes in adult ovary and testis of mammals and birds. Overall, we find a high level of conservation of these genes as well as lineage specific changes in the presence or expression of piRNA pathway genes. We discovered conservation of piRNA pathway gene expression in testis of the basal monotremes and in chicken. In contrast to previous results, we find evidence that piRNA pathway genes are expressed in the adult ovary in birds and mammals including mouse and human. Conservation of the expression pattern in germ and somatic cells (granulosa and Sertoli cells) suggests an important evolutionarily conserved function of this pathway in mammalian gamete development.

Materials and methods

Sequence analyses

The transcripts or cDNA sequences of *Piwi* genes from different species were acquired from different sources (Table 1). The protein sequences of MAEL from species other than platypus were obtained from ENSEMBL release 65 (Table S1). Evolutionary trees were constructed by Bayesian inference phylogenetic method. Multiple alignments were performed using MUSCLE in Geneious v.4.6.5 (Drummond A et al. 2009). Bayesian analysis was performed using the Metropolis Coupled Markov Chain Monte Carlo (MCMC) simulation program MrBayes v3.1 (Ronquist and Huelsenbeck 2003) and the gamma-distributed rate variation across sites. Model parameters for each partition were estimated separately during the MCMC process. Bayesian analyses were run for 100,000 generations and sampling every 200 generations.

Table 1: *Piwil* genes for multiple alignment analyses

Species	Gene	Accession number
Drosophila (Dm)	*Aub*	FBtr0080165
	Ago3	FBtr0299882
	Piwi	FBtr0080166
zebrafish (Dr)	*piwil1*	ENSDART00000138019
Xenopus (Xi)	*piwil1*	XM_002942524.1
lizard (Ac)	*Piwil1*	XM_003229164.1
chicken (Gg)	*PIWIL1*	XM_415096.2
zebra finch (Tg)	*Piwil1*	ENSTGUT00000004991
platypus (Oa)	*Piwil1*	ENSOANT00000018940

opossum (Md)	*Piwil1*	ENSMODT00000019847
mouse (Mm)	*Piwil1*	CCDS19690
human (Hs)	*PIWIL1*	CCDS9268
zebrafish (Dr)	*piwil2*	ENSDART00000134274
Xenopus (Xi)	*piwil2*	ENSXETT00000023096
lizard (Ac)	*Piwil2*	XM_003227041.1
chicken (Gg)	*PIWIL2*	JN248386
zebra finch (Tg)	*Piwil2*	ENSTGUT00000014993
platypus (Oa)	*Piwil2*	ENSOANT00000017088
opossum (Md)	*Piwil2*	ENSMODT00000012071
mouse (Mm)	*Piwil2*	CCDS27252
human (Hs)	*PIWIL2*	CCDS6029
Xenopus (Xi)	*piwil3*	ENSXETT00000027524
human (Hs)	*PIWIL3*	CCDS33623
Xenopus (Xi)	*piwil4*	ENSXETT00000014018
lizard (Ac)	*Piwil4*	XM_003219341.1
platypus (Oa)	*Piwil4*	Sequence adapted from Murchison et al. (2008)
opossum (Md)	*Piwil4*	ENSMODT00000000262
mouse (Mm)	*Piwil4*	CCDS22824
human (Hs)	*PIWIL4*	CCDS31656

Synteny analyses

The information of the genomic regions surrounding *Piwil* genes from different species used in synteny analyses was obtained from ENSEMBL release 65. In cases where orthology was unclear such as cases with unclear identity of genes, BLASTn (Altschul et al. 1990) was used to determine sequence similarity. For genes that could not be found in the ENSEMBL database or BLASTn (example refers to Note S1), GENSCAN

(Burge and Karlin 1997) was performed to predict putative exons within this region between the known flaking genes. Then, the identity of these putative exons was identified with NCBI BLASTn (Altschul et al. 1990). In addition, pairwise local alignment between species within this region was conducted using SIM4 (Florea et al. 1998).

Tissue samples

Platypus tissues were obtained from an adult male or female platypus (Animal ethics permits AEEC R.CG.07.03 and AEC S-49-200 to F.G). Chicken tissues were obtained from adult testis and ovary. Adult mouse testis and ovary tissues were obtained from adult C57BL/6 animals. Normal human ovarian tissues were collected with approval from the RAH Human Ethics Committee (M.O. and C.R.). Testis total RNA was purchased from Stratagene (USA).

5' RACE PCR

The transcription start site of the platypus *Mael* was obtained by using the SMART RACE cDNA amplification kit (Clontech) following the manufacturer's protocol. The 5′ end of the platypus *Mael* was then amplified using a standard PCR protocol. Reaction products were analysed by 1.5% agarose gel electrophoresis and purified using the QIAquick gel extraction kit (Qiagen). A purified 5′ RACE fragment was cloned into the pGEM-T Easy vector (Promega) and sequenced.

BAC clones

The platypus *Mael* and other BAC clones were obtained from the CUGI BAC/EST resource center (Clemson) and purchased from the Children's Hospital Oakland Research Institute (CHORI). The BAC clones information is shown in Table 2. The platypus *Mael*-positive BAC clones were confirmed by PCR and sequencing.

Table 2: Platypus BAC clones for physical mapping

Chromosomal location	BACs ID
X2pY2p	Ch236-78K11
X3pY2p	Ch236-165F5
X4Y3	Ch236-639O23
18	Ch635-K18
19q	Ch236-42D22
20q	Ch236-26H19
21q	Ch236-103F20
Mael	Ch236-57B19

Preparation of chromosomes

Mitotic metaphase chromosomes were generated from established platypus fibroblast cell lines as described previously (Grutzner et al. 2004).

Fluorescence *in situ* hybridisation (FISH)

FISH was done as described previously (Grutzner et al. 2004). Briefly, 1µg of BACs DNA probes were directly labelled with either 0.3µl of SpectrumOrange or SpectrumGreen 2'-deoxyuridine-5'-trisphosphate (Vysis) using 5U of Klenow polymerase (New England Biolabs) and 5µg of 9-mer random primer (Geneworks). Probes were co-precipitated with 50µg of salmon sperm DNA and 10µg of sonicated platypus male gDNA and dissolved in deionised formamide and dextran sulfate.

Metaphase slide preparation and hybridisation were performed as described in (41). Images were taken with a Zeiss Axio Imager Z1 epifluorescence microscope equipped with a CCD camera and Zeiss Axio vision software.

RT-PCR and sequencing

2μg of total RNA was extracted from frozen tissues using TRIzol (Invitrogen) according to the manufacturer's instruction. RNA was treated with *DNase I* (New England Biolabs) and reverse transcribed using Random Hexamer and SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. cDNA expression was first normalized and checked for gDNA contamination by performing PCR with *Actb* or *Gapdh* primers from different species, respectively (Table S2). 27 cycles was performed in *Actb* or *Gapdh* PCR reaction while 32 cycles was performed for other gene PCR reactions. PCR products were confirmed by sequencing (Big Dye Terminator v3.1 cycle sequencing kit, Applied Biosystems). RT-PCR experiments were performed in triplicate for each primer pair.

RNA *in situ* hybridisation

Generally, cDNA fragments about 450-550bp are amplified with PCR (Table S2) and cloned into pGEM-T Easy vector (Promega). To obtain cRNA probes, T7 or Sp6 polymerases (Roche Diagnostics) were used. Probes were labelled with digoxigenin-11-UTP (Roche Diagnostics) according to the manufacturer's protocol. Testis and ovary tissues were fixed in 4% PFA and embedded in paraffin and sectioned by the IMVS anatomy service (SA Pathology, Australia). Sections were deparaffinised and

treated with 1.2µg of proteinase K (Roche Diagnostics) for 30 min at 37ºC, washed with 1x PBS and acetylated with 1M triethanolamin/0.178% HCL/0.25% Acetic anhydride (Sigma-Aldrich). Sections were prehybridized at 65ºC for two hours (50% deionized formamide/3xSSC/1x Denhardt's solution/0.005M Phosphate buffer/10% Dextran sulphate/ 1mg/ml Yeast total RNA/ 1mg/ml salmon sperm DNA) and hybridized with Dig-labelled probe overnight at 50ºC. Slides were washed with increasing stringency (2x SSC, 1x SSC, 0.5x SSC and 0.1x SSC) for 15 mins each at 50ºC and treated with 0.15 mg/ml of RNAse A for 30 min at 37ºC. Slide was blocked with 1% blocking solution (Roche Diagnostics). Detection was performed using anti-DIG-antibody coupled to alkaline phosphatase with nitroblue tetrazolium chloride/X-phosphate 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (Roche Diagnostics).

Results and Discussion

Conservation of piRNA pathway genes in amniotes

piRNA pathway genes are important for germline development and gametogenesis in invertebrates and vertebrates. Despite a high level of evolutionary conservation, this group of genes also contains gene lost or gained in specific lineages (Fig. 1A). High sequence conservation between *Piwi* genes has made it difficult to reconstruct the evolution of *Piwil* genes in animals. The presence of *Piwil1* and *Piwil2* orthologs in all species investigated so far suggests that one of these genes represent the original ortholog to the *Drosophila Piwi* gene. *Piwil2* is found in all vertebrates investigated (Kuramochi-Miyagawa et al. 2001; Murchison et al. 2008; Wilczynska et al. 2009). We identified sequence related to *PIWIL2* in another genomic region in chicken and zebra finch (Fig. 1B). Further analyses of the region which contains this putative avian *PIWIL2* gene revealed that this gene is located in a region in zebra finch and chicken that does not contain any *PIWIL* genes in mammals or other reptiles. Phylogenetic analyses showed that both chicken and zebra finch *PIWIL2* is the most diverged when compared to mammalian and lizard *Piwil2* (Fig. 1B). It is unclear at this point if this is due to an independent duplication or if this is an ortholog of *Piwil2* gene in other vertebrates that has been translocated to a different location. This may have been facilitated through multiple rearrangements in this region, which distributed genes in this region over several chromosomes in mammals (Fig. 1C).

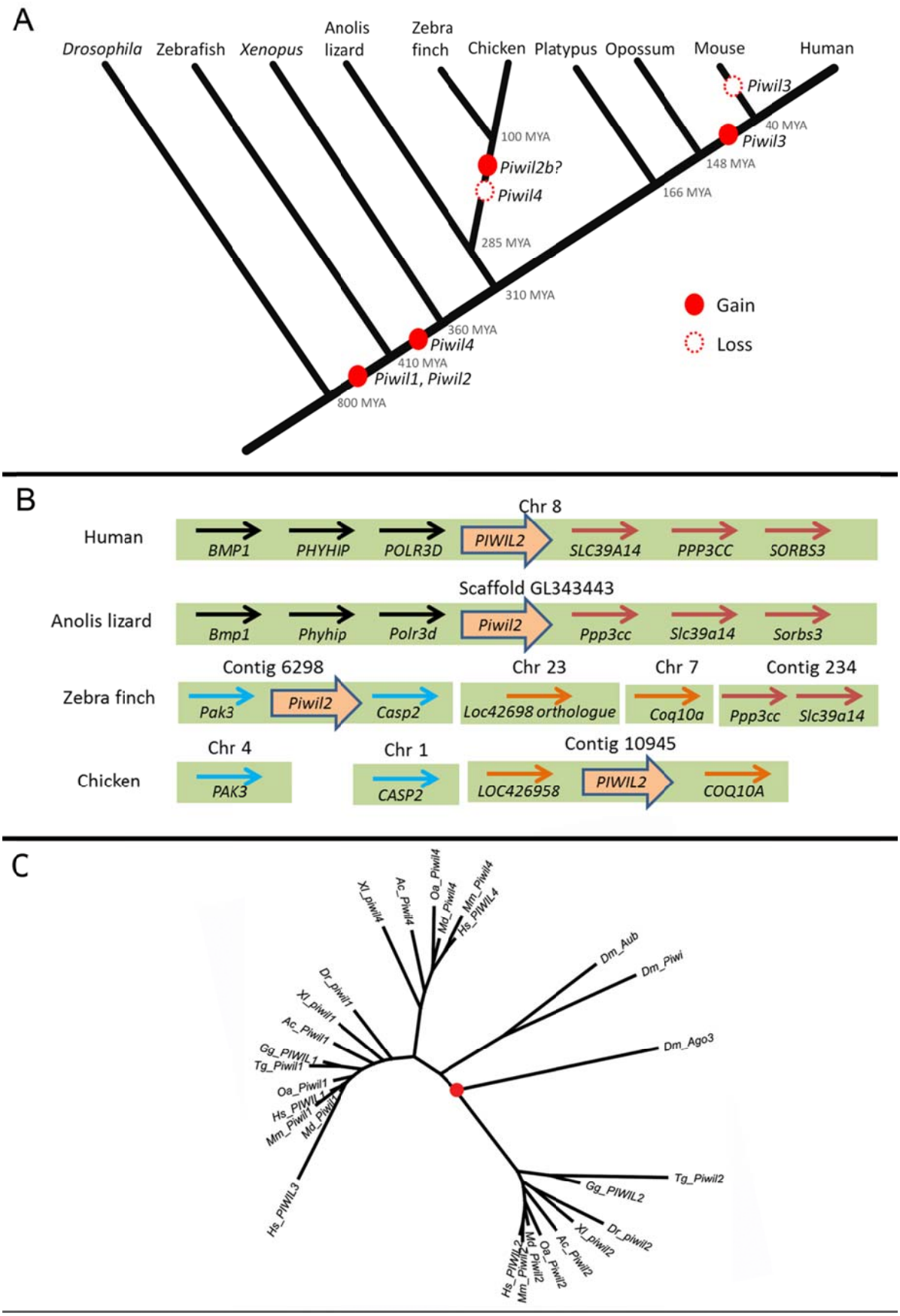
A
Drosophila
Zebrafish
Xenopus
Anolis lizard
Zebra finch
Chicken
Platypus
Opossum
Mouse
Human
Piwil3
40 MYA
Piwil3
148 MYA
100 MYA
Piwil2b?
Piwil4
166 MYA
285 MYA
310 MYA
360 MYA
Piwil4
410 MYA
Piwil1, Piwil2
800 MYA
Gain
Loss
B
Chr 8
Human
BMP1
PHYHIP
POLR3D
PIWIL2
SLC39A14
PPP3CC
SORBS3
Scaffold GL343443
Anolis lizard
Bmp1
Phyhip
Polr3d
Piwil2
Ppp3cc
Slc39a14
Sorbs3
Contig 6298
Chr 23
Chr 7
Contig 234
Zebra finch
Pak3
Piwil2
Casp2
Loc42698 orthologue
Coq10a
Ppp3cc
Slc39a14
Chr 4
Chr 1
Contig 10945
Chicken
PAK3
CASP2
LOC426958
PIWIL2
COQ10A
C
Xl_Piwil4
Ac_Piwil4
Oa_Piwil4
Md_Piwil4
Mm_Piwil4
Hs_PIWIL4
Dm_Aub
Dm_Piwi
Dm_Ago3
Dr_piwil1
Xl_piwil1
Ac_Piwil1
Gg_PIWIL1
Tg_Piwil1
Oa_Piwil1
Hs_PIWIL1
Mm_Piwil1
Md_Piwil1
Hs_PIWIL3
Tg_Piwil2
Gg_PIWIL2
Dr_piwil2
Xl_piwil2
Ac_Piwil2
Oa_Piwil2
Md_Piwil2
Hs_PIWIL2
Mm_Piwil2

Fig. 1. Evolutionary conservation of piRNA pathway genes in vertebrates and invertebrates. (A) Revised delineation of the evolution of *Piwi* genes spanning 800 million years of animal evolution. *Piwil1* and *Piwil2* are present in all vertebrates examined. *Piwil4* is gained in vertebrates after the divergence of fish but missing in birds based on currently available sequence. *Piwil3* is found in eutherian mammals but lost in rodents. (B) Synteny block analyses of *Piwil2* in vertebrates. *Piwil2* is located in a conserved synteny block in mammals and reptiles. *PIWIL2* in zebra finch and chicken are located on different chromosomal regions when compared to reptiles and mammals. Genes flanking zebra finch and chicken *PIWIL2* are located on different chromosomes in these two species, indicating that this region has been rearranged in birds. (C) Phylogenetic tree of *Piwi* genes in multiple species generated with MUSCLE (Drummond A et al. 2009). The posterior probability of all branches is 1 except branches with red dots which is 0.5. *Dm*, *Drosophila*; *Dr*, zebrafish; *Xl*, *Xenopus*; *Ac*, Anolis lizard; *Tg,* zebra finch; *Gg*, chicken; *Oa*, platypus; *Md*, opossum; *Mm*, mouse; *Hs*, human; Chr, chromosome.

Piwil3 has so far only been identified in eutherian mammals with the exception of mouse (Fig. 2A) (Sasaki et al. 2003; Zhou et al. 2010) and rat but not in marsupials or monotremes (Murchison et al. 2008). *Piwil3* synteny analysis in eutherian mamamals showed that this gene is located in an unstable region which may have undergone complex rearrangements (Fig. 2A). This may have resulted in the loss of *Piwil3* in mouse during evolution. In human, *PIWIL3* is flanked by *SGSM1* and *GGT1* but both of those genes are located on different chromosomes in mouse and only *Sgsm1* is found next to *Piwil3* in cow and dog. *Ggt1* seems to have translocated to a region downstream of *Piwil3* in cow and dog. Another gene flanking *Piwil3* gene in cow and dog, *Cmklr1,* is located on a different chromosome in human. Although both genes (*Sgsm1* and *Cmklr1*) that flank *Piwil3* in cow and dog are located on the same chromosome in mouse, there is no trace of *Piwil3* within this region on the mouse chromosome, suggesting loss of *Piwil3* in mouse. Surprisingly a *piwil3* ortholog has been annotated in the *Xenopus* genome assembly (ENSXETG00000012572). Four *piwil* genes (*piwil1a*, *piwil1b*, *piwil2* and *piwil4*) have previously been identified in *Xenopus* (Wilczynska et al. 2009). *Piwil1a* (*xiwi1a*) was suggested to be paralogous to *piwil1b* (*xiwi1b*) (Wilczynska et al. 2009). Sequence analyses show that the published *piwi1a* gene is identical with the annotated *piwil3* (ENSXETG00000012572, ENSEMBL release 65). The genes flanking *piwi1a* in *Xenopus* (*fimbp2* and *fzd10*) are located on human chromosome 12 and flanked human *PIWIL1* providing strong evidence that this is the ortholog of the mammalian *Piwil1* (Fig. 2B). Furthermore, BLASTn of *Xenopus piwil3* cDNA (ENSXETT00000027524) showed that 89% of the sequence aligned 100% with the predicted *Xenopus piwil1* (LOC100488735) and *Piwil1* from other species. Thus, synteny analysis suggested that *Xenopus piwil3* should be regarded as *piwil1* ortholog

in *Xenopus*. We found no evidence of a *piwil3* gene in *Xenopus* and concluded that this gene evolved exclusively in eutherian mammals.

Piwil4 has been reported in all mammalian lineages but not in birds and fish (Houwing et al. 2007). *Piwil4* orthologs are present in *Xenopus* and Anolis lizard but its absence in fish suggests evolution after the divergence of fish. In addition, *PIWIL4* orthologs seem to have been lost in birds (Fig. 2C, Note S1). It is currently unclear if the lineage specific gain and loss of *Piwil* genes has co-evolved with certain repeats (*L1* in particular) or the evolution of epigenetic reprogramming in the germline.

Another key gene in the piRNA pathway is *Maelstrom (Mael)* (Costa et al. 2006; Soper et al. 2008). *Mael* is highly evolution conserved and orthologs have been identified in various animal lineages and protists (Zhang et al. 2008). In amniotes, *Mael* orthologs have been identified in human, monodelphis (marsupial) and chicken (Zhang et al. 2008). For the third major mammalian lineage, the monotreme mammals, only incomplete sequence is available in the public database. We identified the complete cDNA sequence of the ortholog of *Mael* in platypus (accession no. JQ436565) and partial sequence in the short-beaked echidna (accession no. JQ446444). In order to physically map *Mael* in platypus, we identified a BAC clone containing the platypus *Mael* gene. FISH hybridisation shows that *Mael* maps to one of the small metacentric chromosomes, most likely chromosome 17 (Fig. S1). Multiple alignment of the amino acid sequence reveals overall high conservation of the platypus MAEL (Fig. S2). As in other vertebrates, the predicted platypus MAEL protein contains a High Mobility Group (HMG) domain at the amino-terminal end and a conserved MAEL domain at the

carboxy-terminal end. The HMG domain is a DNA-binding domain, but the function of the MAEL domain is currently unclear. A conserved MAEL motif is characterised by a set of six highly conserved residues (Glu-His-His-Cys-His-Cys) (Fig. S2B, red box). These residues are mostly unchanged in a wide range of species includin, vertebrates, invertebrates and protists (Zhang et al. 2008). This motif may represent an ancestral DnaQ-H 3'-5' exonuclease adopting a canonical RNaseH fold which catalyses unspecific hydrolytic cleavage of heteroduplex RNA (Zhang et al. 2008). The MAEL motif also associates with DNA-binding domains (HDAC-interaction domain) or RNA interacting domains (SR-25-like) in a variety of species (Zhang et al. 2008). According to the available sequence (ENSOANP00000006136), the first three residues of the motif are conserved in platypus. Surprisingly, alignment with the published MAEL sequence (ENSEMBL) showed changes in several important parts of the platypus MAEL domain (e.g. deletion of the 4th and 5th residues and mutation of the 6th residue from Cys to Leu), raising the possibility of structural and functional variations in the platypus protein. To further confirm the sequence changes in the platypus MAEL domain, we sequenced this region in an additional four different platypuses (including the platypus that was sequenced (Warren et al. 2008), and also echidna). This confirmed the conservation of those residues of the MAEL domain in monotremes (Fig. S2B, red boxes). The errors in the published sequence (ENSOANT00000006138) may be due to the high GC content (62%) of this region.

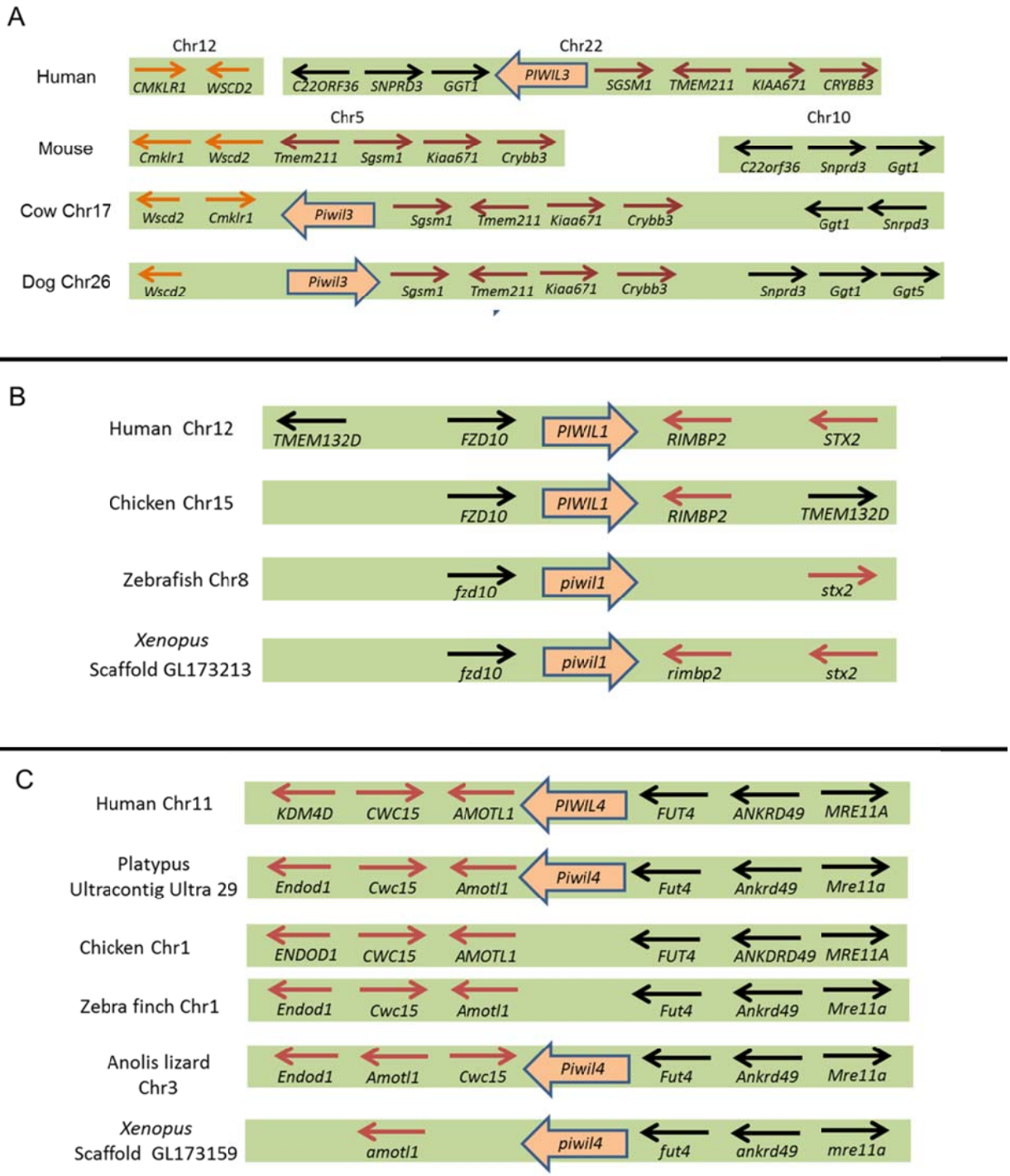
A
Chr12
Chr22
Human
CMKLR1
WSCD2
C22ORF36
SNPRD3
GGT1
PIWIL3
SGSM1
TMEM211
KIAA671
CRYBB3
Chr5
Chr10
Mouse
Cmklr1
Wscd2
Tmem211
Sgsm1
Kiaa671
Crybb3
C22orf36
Snprd3
Ggt1
Cow Chr17
Wscd2
Cmklr1
Piwil3
Sgsm1
Tmem211
Kiaa671
Crybb3
Ggt1
Snrpd3
Dog Chr26
Wscd2
Piwil3
Sgsm1
Tmem211
Kiaa671
Crybb3
Snprd3
Ggt1
Ggt5
B
Human Chr12
TMEM132D
FZD10
PIWIL1
RIMBP2
STX2
Chicken Chr15
FZD10
PIWIL1
RIMBP2
TMEM132D
Zebrafish Chr8
fzd10
piwil1
stx2
Xenopus
Scaffold GL173213
fzd10
piwil1
rimbp2
stx2
C
Human Chr11
KDM4D
CWC15
AMOTL1
PIWIL4
FUT4
ANKRD49
MRE11A
Platypus
Ultracontig Ultra 29
Endod1
Cwc15
Amotl1
Piwil4
Fut4
Ankrd49
Mre11a
Chicken Chr1
ENDOD1
CWC15
AMOTL1
FUT4
ANKDRD49
MRE11A
Zebra finch Chr1
Endod1
Cwc15
Amotl1
Fut4
Ankrd49
Mre11a
Anolis lizard
Chr3
Endod1
Amotl1
Cwc15
Piwil4
Fut4
Ankrd49
Mre11a
Xenopus
Scaffold GL173159
amotl1
piwil4
fut4
ankrd49
mre11a

Fig. 2. Synteny analyses of *Piwil1*, *Piwil3* and *Piwil4* in vertebrates. (A) Synteny of *Piwil3* in eutherian mamamals. *Piwil3* is located in a region which has undergone multiple rearrangements that interrupted synteny in human, dog and cow. The instability in this region may result in the loss of *Piwil3* in mouse during evolution. (B) The synteny block of *Piwil1* is conserved from zebrafish to human. *Xenopus piwil3* (ENSXETG00000012572) is located on scaffold GL173213 and flanked by *fzd10* and *rimbp2* and is suggested to be a *piwil1* ortholog. (C) Synteny block of *Piwil4* is highly conserved from *Xenopus* to human. However, the presence of *PIWIL4* cannot be determined within the region flanked by *AMOTL1* and *FUT4* in chicken and zebra finch based on synteny analysis, GENSCAN (Burge and Karlin 1997), NCBI BLAST (Altschul et al. 1990) and SIM4 program (Florea et al. 1998). Chr, chromosome.

Conserved testis expression of *Piwil1, Piwil2, Piwil4* and *Mael* in mammals

The role of PIWI and MAEL proteins in the repression of TEs during spermatogenesis has been investigated in detail in *Drosophila* and mouse. Strong testis expression of piRNA pathway genes including *Piwil1, 2, 4* and *Mael* (Fig. 3-5) is conserved from invertebrates to mammals (Cox et al. 1998; Kuramochi-Miyagawa et al. 2001), with the exception of mouse where *Piwil4* expression has not been detected in adult testis (Aravin et al. 2008). Using RT-PCR we investigated expression of these genes in species representing two major amniote lineages, birds and monotremes. As expected we found strong expression of *Piwil1* and *Mael* in adult testis of both species (Fig. 3, 4). In contrast to mouse we found weak expression of *Piwil4* in platypus testis. We were also able to confirm expression of *PIWIL4* in human adult testis (Sasaki et al. 2003). This suggests that *Piwil4* expression in testis may be common in mammals but has been reduced or lost altogether in adult mouse. We also detected low expression of platypus and mouse *Piwil1* (but not *Piwil2*) in kidney of both species (Fig. 4A, B), supporting recent reports showing piRNA expression in various somatic tissues in *Drosophila*, mouse and human (Yan et al. 2011).

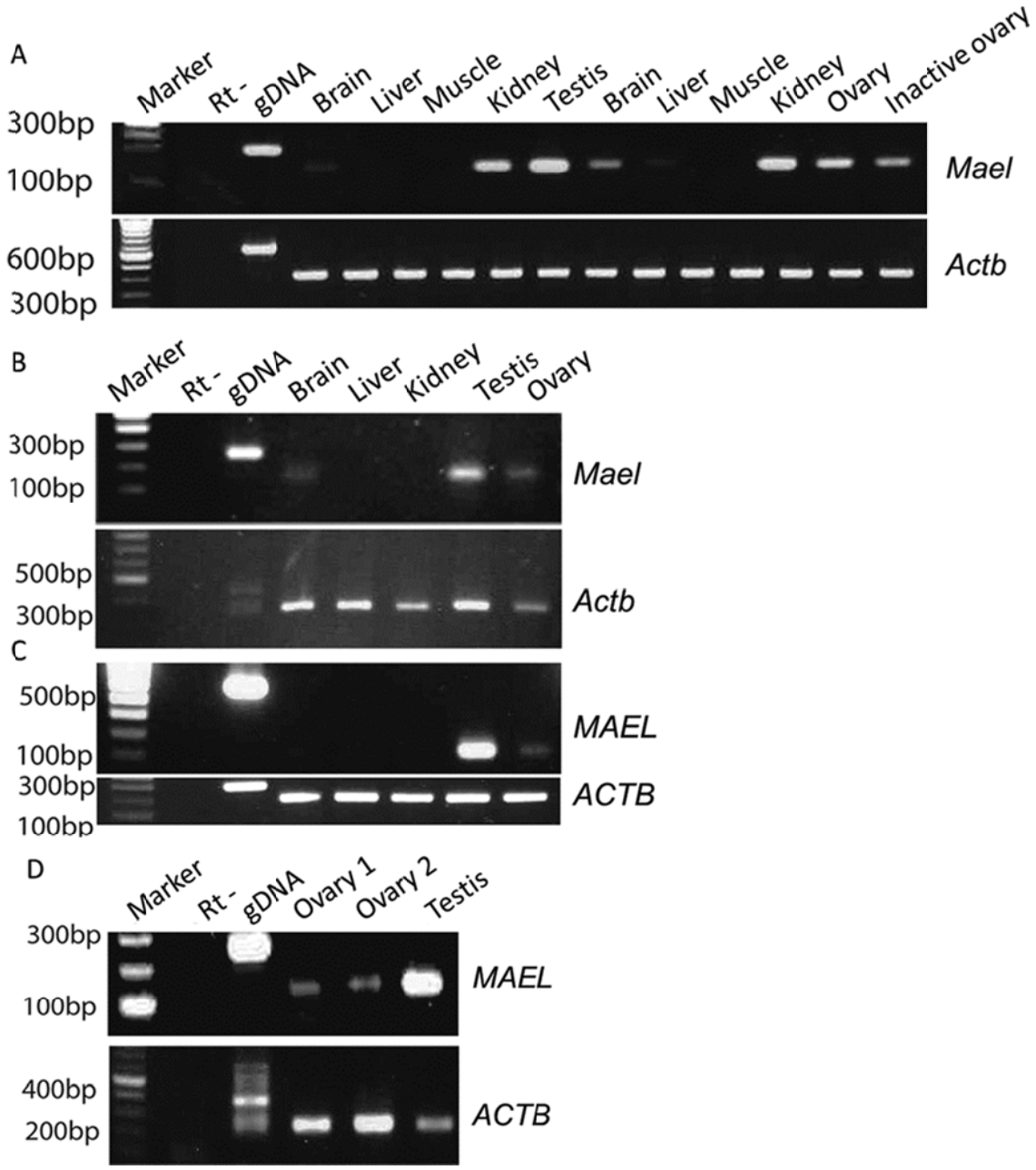

Fig. 3. Conserved expression of *Mael* in the adult testis and ovary in amniotes. RT-PCR showing *Mael* expression in multiple adult tissues in (A) platypus, (B) mouse, (C) chicken and (D) human. *Mael* is expressed in the ovary of human, mouse, platypus and chicken. Rt-, negative control.

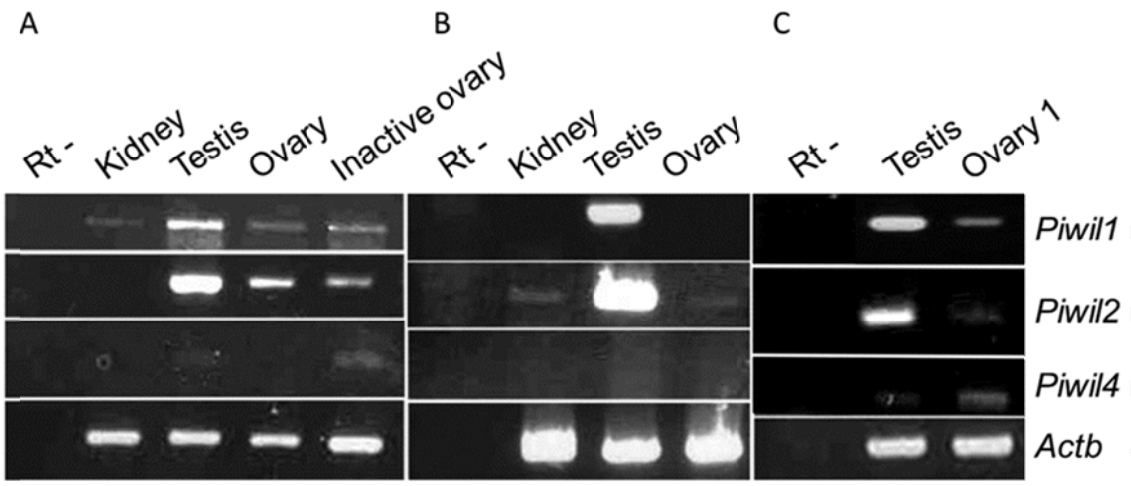

Fig. 4. *Piwil* gene expression in the mammalian adult ovary. RT-PCR of *Piwil1*, *Piwil2* and *Piwil4* in multiple adult tissues of (A) platypus, (B) mouse and (C) human. Unlike *Piwil2* which is expressed in the ovary of platypus, mouse and human, *Piwil1* and *Piwil4* is expressed in platypus and human ovary only. RT-, negative control.

More detailed analyses of the expression patterns of piRNA pathway genes revealed differential expression during spermatogenesis in mouse (Deng and Lin 2002; Kuramochi-Miyagawa et al. 2004; Carmell et al. 2007). In mouse PIWIL2 is expressed in different stages of spermatogenesis but not in mature sperm, while PIWIL1 is only expressed in pachytene-stage spermatocytes and round spermatids. MAEL is expressed in early meiotic cells to round spermatids in mouse testis (Soper et al. 2008). To investigate if this differential expression pattern is conserved in platypus, we performed RNA *in situ* hybridisation on adult platypus testis sections. This revealed widespread expression of *Mael* and *Piwil2* transcripts throughout spermatogenesis but not in mature sperm (Fig. 5A-B, H-I). *Piwil1* expression seems more restricted to early meiotic stages (Fig. 5O-P). Overall this expression pattern is similar to that described in mouse and shows conservation of the specific expression pattern in the mammalian testis (Fig. 5D-

F, K-M). In addition, we observed possible appearance of *Mael* and *Piwil2* mRNA in a specific region of the Sertoli cell nuclei in mouse testis (Fig. 5F, M). Germline cell specific expression of *Piwil1* and *Piwil2* has been suggested as Northern blot analyses showed no expression of these genes in mutant mice lacking spermatogenesis (Kuramochi-Miyagawa et al. 2001). However, increased number of Sertoli cells was observed in the seminiferous tubules of *Piwil2* mutants suggests a role for this gene in Sertoli cell proliferation (Kuramochi-Miyagawa et al. 2004). In *Mael* mutant mice, a subtle change in Sertoli cell appearance was described (Soper et al. 2008). In addition, more recently piRNAs were found to localise on the nucleolus of Sertoli cells in mouse (Marcon et al. 2008). Our result of *Piwil2* and *Mael* expression in Sertoli cell nuclei further supports a role for piRNA pathway genes in these supporting cells. Therefore the well-known importance of the piRNA pathway in supporting cells of the *Drosophila* ovary (Lin and Spradling 1997; Malone et al. 2009) may also apply to the corresponding somatic cells in testis.

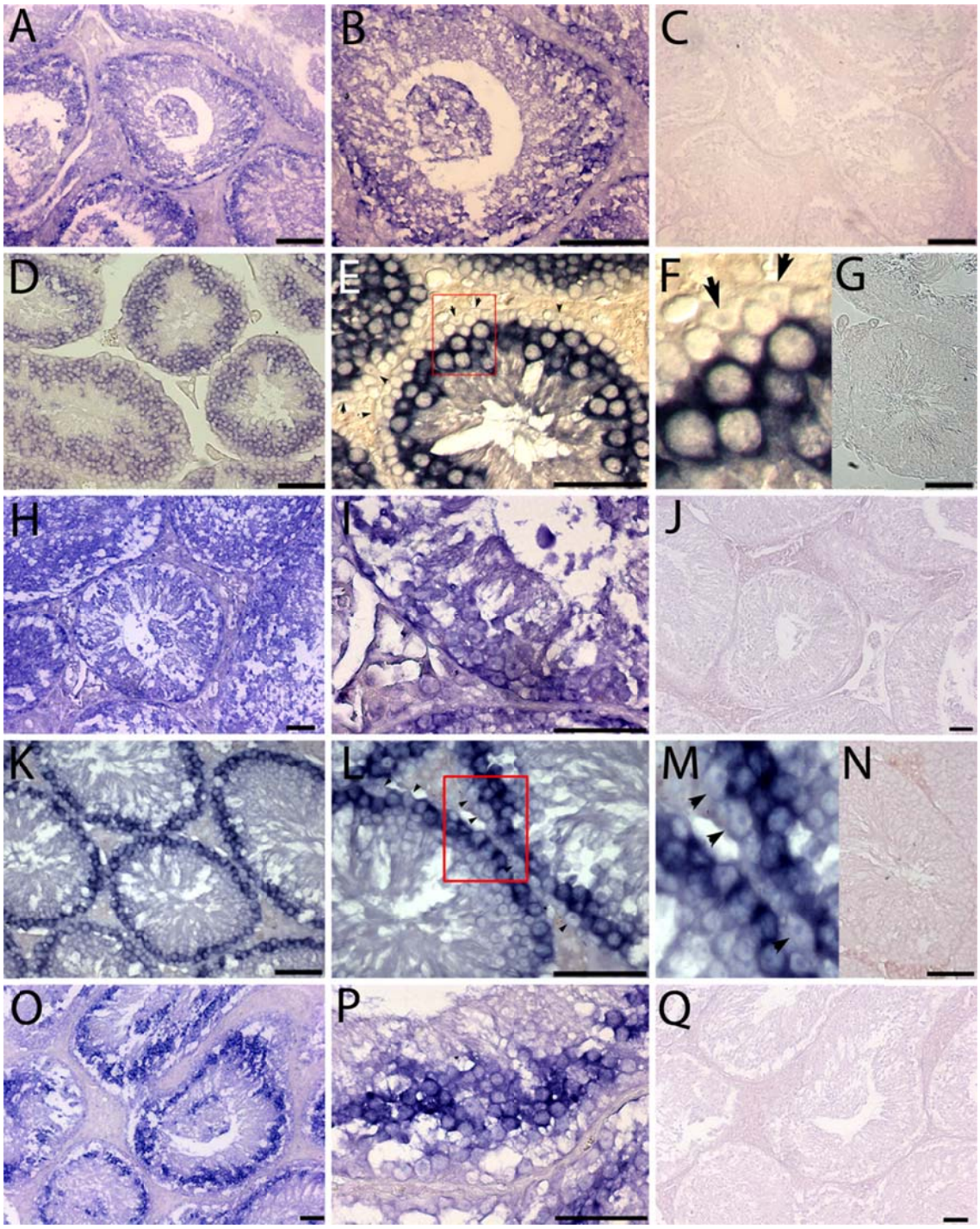
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Fig. 5. Expression pattern of piRNA pathway genes in the mammalian testis. RNA *in situ* hybridisation showing *Mael* expression in (A-B) platypus and (D-F) mouse, *Piwil2* expression in (H-I) platypus and (*K-M*) mouse, and *Piwil1* expression in (O-P) platypus adult testis. *Piwil1* expression is restricted to early meiotic cells in platypus testis. *Mael* and *Piwil2* are expressed from early meiotic cells to round spermatids with no expression in mature sperm in either species. Low level of (E, F) *Mael* and (L, M) *Piwil2* transcript appears to be expressed in the nuclei of Sertoli cells (arrows) in mouse testis. Box in E and L indicates regions which are enlarged in F and M respectively. Negative controls with sense probe in (C, J, Q) platypus and (G, N) mouse testis. Scale bar = 50μm.

Mael, Piwil1, Piwil2 and *Piwil4* are expressed in the mammalian adult ovary

In *Drosophila* the function of *Piwi* genes and *Mael* in ovary has been studied extensively. Expression of *Piwi* and *Mael* genes in the oocyte supporting nurse cells suggests an important function of these genes in oogenesis (Lin and Spradling 1997; Findley et al. 2003) and mutation of those genes leads to severe germline defects and sterility. *Mael* was initially discovered in the *Drosophila* ovary where it has an important function in establishing oocyte polarity and stem cell integrity (Clegg et al. 1997). Although the piRNA pathway is essential for oogenesis in *Drosophila*, there is almost no information about *Piwi* gene expression in the female mammalian germline. The presence of piRNAs has previously been shown in mouse oocytes (Ro et al. 2007), however, *Piwil1* and *Mael* were not detected in the adult mouse and human ovary by Northern blotting (Kuramochi-Miyagawa et al. 2001; Costa et al. 2006; Xiao et al. 2009). RT-PCR analyses have shown *Piwil2* expression in adult mouse ovary (Watanabe et al. 2008) and both *Piwil1* and *Piwil2* expression in adult porcine ovary (Zhou et al. 2010). Here we investigated in more detail the expression pattern of piRNA pathway genes in somatic and germ cells in the adult ovary in mammals. In contrast to previous findings, our experiments showed consistent and specific expression of *Mael* in adult mammalian and avian ovary (Fig. 3). *Piwil1* and *Piwil2* have conserved ovarian expression in platypus and human (Fig. 4). We also detected low levels of *Piwil4* expression in platypus and human ovary (Fig. 4A, C). These results demonstrate conservation of piRNA pathway gene expression in ovaries of birds and mammals, and suggest lineage specific changes indicative of different piRNA pathway activity in different species or stages of oogenesis as previously described in *Drosophila* (Malone et al. 2009).

Expression pattern of *Piwil1*, *Piwil2* and *Mael* during folliculogenesis in mammalian ovary

piRNA pathway genes are active in germ cells and their supporting cells (nurse cells) in the *Drosophila* ovary. To investigate if the supporting cells in the mammalian ovary show piRNA gene activity, we performed RNA *in situ* hybridisation in adult ovary of mouse (Fig. 6), human (Fig. 7) and platypus (Fig. 9). In mouse and human, we found *Mael* expression in oocytes and granulosa cells of primordial and preantral follicles during early folliculogenesis (Fig. 6A-C, 7A, D). In later stages of folliculogenesis, *Mael* is highly expressed in the cumulus cells of large antral follicles in mouse (Fig. 6B). *Piwil2* is expressed only in the supporting cells at different stages of follicular development in mouse ovary but not in the oocyte (Fig. 6D). In addition to *MAEL*, *PIWL1* and *PIWIL2* are expressed in the oocyte and granulosa cells of human primordial follicles (Fig. 7B, C). Consistent with mouse, expression of these genes is restricted to supporting cells of preantral follicles in human (Fig. 7D-F).

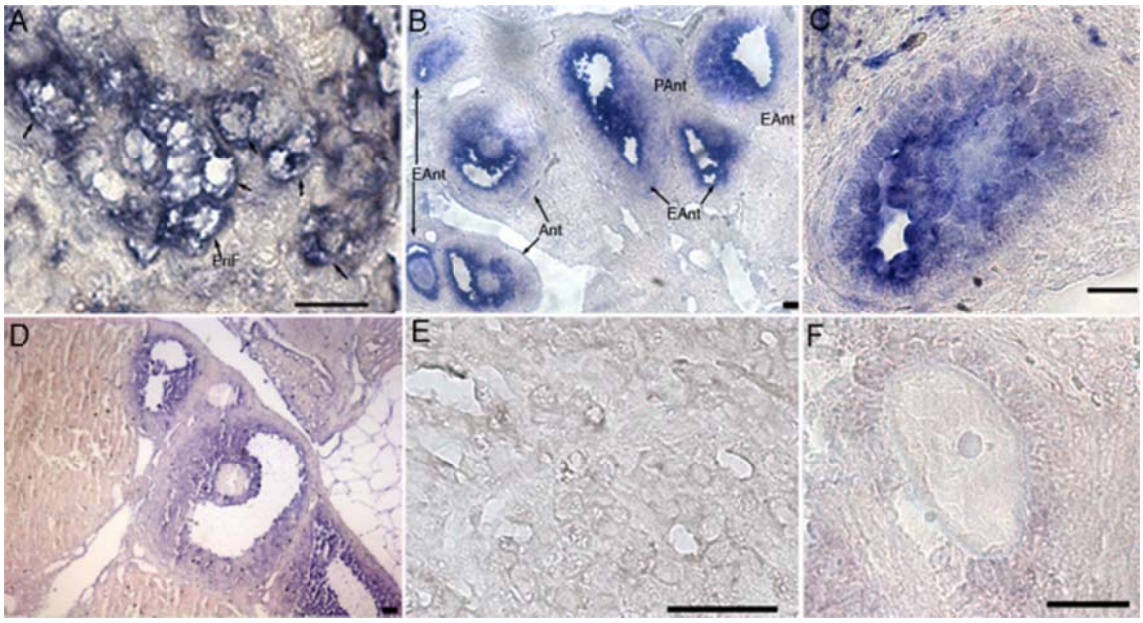

Fig. 6. Expression pattern of piRNA pathway genes in the mouse ovary. RNA *in situ* hybridisation of (A-C) *Mael* and (D) *Piwil2*. *Mael* is expressed in the granulosa cells and oocytes of (A) primordial, (B) preantral, early antral and antral follicles throughout folliculogenesis. (C) An early antral follicle showing strong expression of *Mael* in the granulosa cells and oocyte compared to its (F) sense negative control. In contrast to the dynamic expression of *Mael* in oocytes and supporting cells, *Piwil2* is expressed only in supporting cells throughout folliculogenesis. Negative controls with sense probe of (E) *Piwil2* and (F) *Mael*. PriF, primordial follicles; PAnt, preantral follicle; EAnt, early-antral follicle; Ant, antral follicle. Scale bar = 50μm.

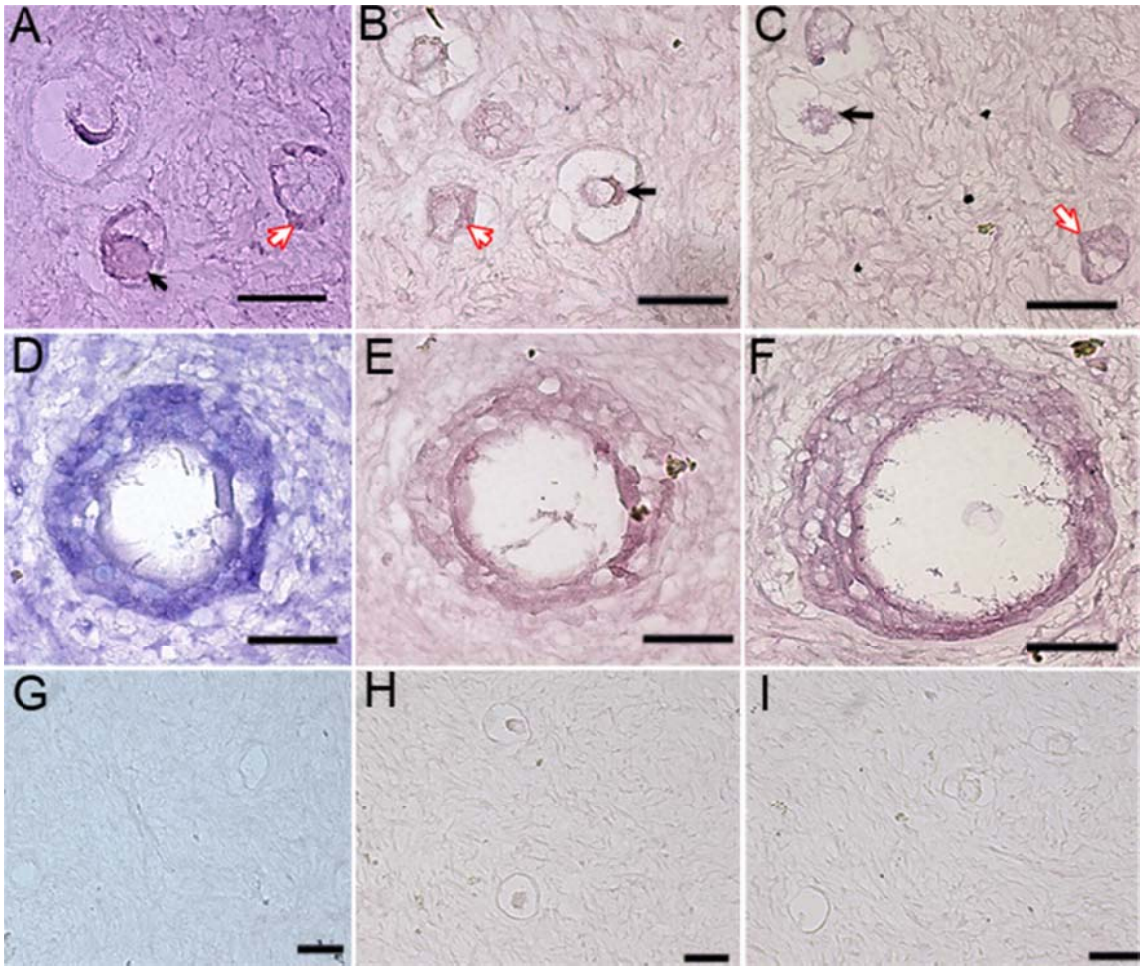

Fig. 7. *MAEL, PIWIL1 and PIWIL2* are expressed in ovarian somatic cells in human. RNA *in situ* hybridisation of (A, D) *MAEL*, (B, E) *PIWIL1* and (C, F) *PIWIL2* in human adult ovary. (A-C) piRNA pathway genes are expressed in the oocytes and supporting cells in primordial follicles. The closed arrows indicate oocytes and the open arrows indicate primordial follicle granulosa cells. (D-F) Similar to mouse, piRNA pathway genes are expressed in the ovarian supporting cell layer in preantral follicles. (G-I) Negative controls with sense probe of *MAEL*, *PIWIL1* and *PIWIL2* respectively. Scale bar = 50µm.

In platypus, we investigated the expression pattern of piRNA pathway genes in the active (left) and inactive ovaries (right). The comparison with the egg-laying platypus is particularly interesting as they share histological features with the avian ovary, including large oocytes, lack of an antrum and thin layers of granulosa cells (Grutzner et al. 2008) (Fig. 8). In addition, platypus ovulates only from the active ovary as in birds; however, the inactive ovary is more developed compared with chicken (Grutzner et al. 2008). The inactive platypus ovary contains numerous primordial follicles (Fig. 8D-F) and we observed no mature tertiary follicles, as reviewed previously (Grutzner et al. 2008).

Mael, Piwil1 and *Piwil2* are expressed in both active and inactive ovaries in platypus. *In situ* hybridisation shows that *Mael*, *Piwil1* and *Piwil2* are expressed in the oocytes of small primary follicles (Fig. 9) and granulosa cells (Fig. S3) in the active and inactive ovaries. *Mael* and *Piwil2* are always co-expressed in primary follicles in the active ovary (Fig. 9A-B). Interestingly we noticed that some follicles show exclusive expression of *Piwil1* but not *Mael* or *Piwil2* (Fig. 9A-C). The amorphous morphology of those primary oocytes with *Piwil1* expression (but not *Mael* or *Piwil2*) may indicate atretic follicles (Fig. 9C, F). Overall the expression pattern in platypus is similar to that in mouse and human. In the inactive ovary, *Mael*, *Piwil1* and *Piwil2* are expressed in primary follicles, which are abundant at the cortical parts of the ovary (Fig. 10A-C). In primordial follicles, *Piwil2* and *Mael* are expressed in granulosa cells and at a low level in the oocyte (Fig. 10D-F).

Overall this shows that *Mael* and *Piwil2* are expressed early during folliculogenesis. It is interesting that this expression pattern resembles observations in testis: in platypus and mouse testis, *Mael* and *Piwil2* are expressed in early meiotic cells while *Piwil1* expression is restricted to late spermatogenesis (Fig. 5) (Kuramochi-Miyagawa et al. 2001). Activity of different piRNA production pathways has been reported in *Drosophila* ovarian somatic cells and oocytes (Malone et al. 2009). In addition, the presence of *Piwi* genes has been linked to the maturation of different length piRNA products, such that in mouse, 26nt, 28nt and 30nt piRNAs are associated with PIWIL2, PIWIL4 and PIWIL1, respectively (Siomi et al. 2011). The lack of *Piwil1* and *Piwil4* expression in mouse ovary suggests the absence of 30nt and 28nt piRNAs in mouse. The 26nt piRNAs associated with PIWIL2 have been identified in mouse ovary (Watanabe et al. 2008). In contrast to mouse, the expression of *Piwil1* and *Piwil4* in human and platypus ovary would suggest the presence of 26-30nt piRNAs in ovary.

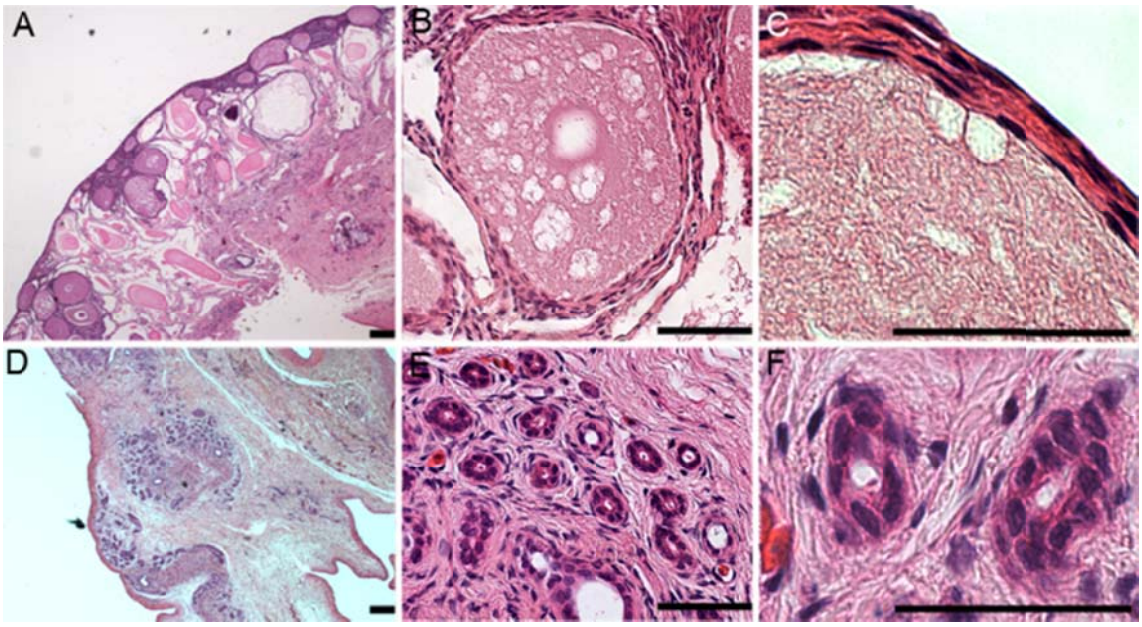

Fig. 8. Hematoxylin and Eosin (H&E) staining of platypus active and inactive ovaries. Platypus (A-C) active and (D-F) inactive ovaries. (A) Platypus active ovary is composed of a large number of primary follicles and some tertiary follicles. (B) A primary follicle. (C) The oocyte in a primary follicle is surrounded by a few layers of small flattened granulosa cells. (D) The inactive ovary contains a large number of primordial follicles. (E) In primordial follicles, the oocyte is surrounded by a single layer of granulosa cells. (F) Higher magnification of primordial follicles. Scale bar = 50µm.

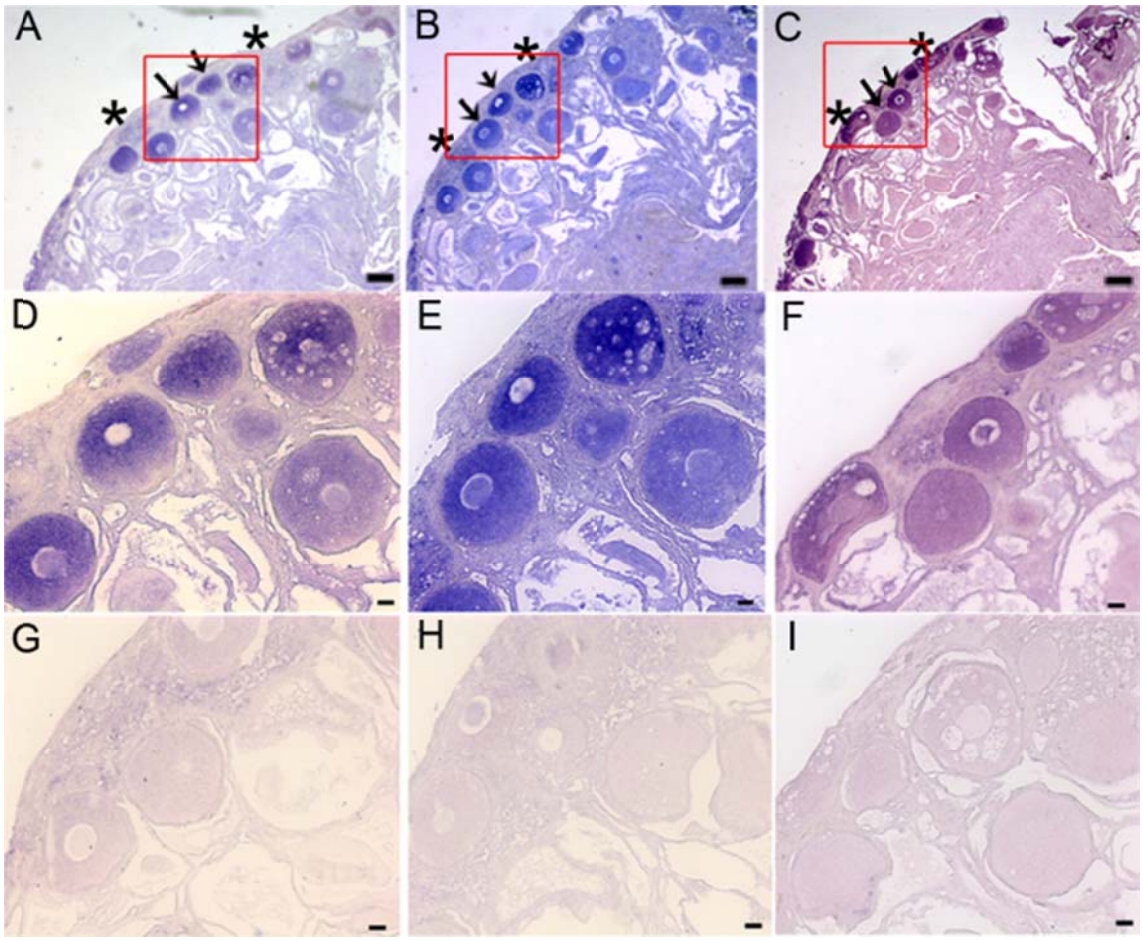

Fig. 9. *Piwil1, 2* and *Mael* expression in platypus active ovary. RNA *in situ* hybridisation of (A) *Mael*, (B) *Piwil2* and (C) *Piwil1* in adult ovary. (D-F) Enlarged version of *Mael*, *Piwil2* and *Piwil1* expression in the box from the top row respectively. *Mael* and *Piwil2* are co-expressed in the oocytes of all primary follicles examined but not *Piwil1* which is only co-expressed in some primary follicles. Asterisks indicate primary follicle that appears atretic and only expresses *Piwil1* but not *Mael* or *Piwil2*. Arrows show primary follicle that expresses all three genes. (G-I) Negative controls with a sense probe of *Mael*, *Piwil2* and *Piwil1* respectively. Scale bar = 50µm.

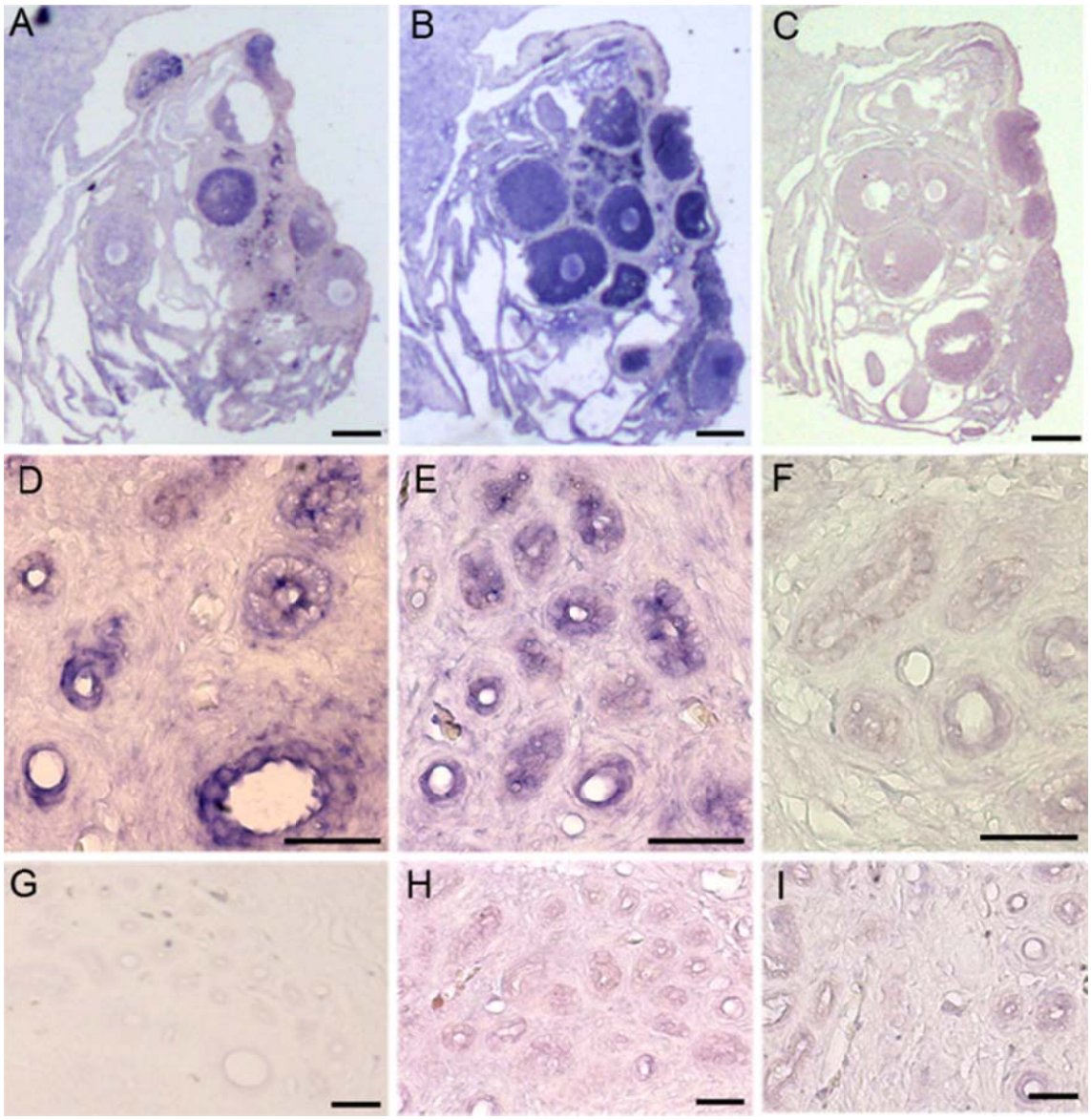

Fig. 10. *Piwil1, 2* and *Mael* expression in platypus inactive ovary. (A, D) *Mael*, (B, E) *Piwil2*, (C, F) *Piwil1* expression in the oocytes of primary follicles and granulosa cells of primordial follicles. (A-C) All three genes are expressed in the oocyte of primary follicles with strongest to weakest expression being *Piwil2*, *Mael* and *Piwil1*. (D-F) *Mael* and *Piwil2* but not *Piwil1* are expressed in the granulosa cells of primordial follicles, indicating early expression of these genes during folliculogenesis. (G-I) Negative controls with sense probe of *Mael*, *Piwil2* and *Piwil1* respectively. Scale bar = 50μm.

Conclusions

We have investigated the evolution and expression pattern of the piRNA pathway genes including *Piwil1*, *Piwil2*, *Piwil3, Piwil4* and *Mael* in mammals and birds. *Piwil1* is highly conserved in all species investigated. The presence of *Piwil1* and *Piwil2* in vertebrates is presumably the result of a duplication event of the original *Drosophila Piwi* gene. *Piwil3* is lost in mice but present in many eutherian mammals while *Piwil4* is present in all investigated mammals. It is interesting to note that in chicken two out of four *PIWI* genes seem to have been lost.

In mammals, the expression of piRNA pathway genes has been predominately studied in mouse testis. Here we confirmed the conserved expression pattern of piRNA pathway components, *Mael*, *Piwil1* and *Piwil2*, in the most basal mammalian group of egg-laying monotremes, suggesting the importance of this pathway during mammalian spermatogenesis. In addition, we observed possible expression of *Mael* and *Piwil2* in Sertoli cells providing further evidence for a role of piRNAs in supporting cells.

Importantly we report the expression of *Piwil1*, *Piwil2* and *Mael* in oocytes and supporting cells in mammals. In mouse, *Mael* expression shifts from oocytes to somatic cells but *Piwil2* expression is restricted to somatic cells during folliculogenesis. In addition, *Mael*, *Piwil1* and *Piwil2* are co-expressed in some but not all primary oocytes in platypus active and inactive ovaries. In human, *MAEL*, *PIWIL1* and *PIWIL2* are co-expressed in the ovarian somatic cells. These findings may provide first evidence that distinct piRNA pathways may be active in oocytes and supporting cells as previously

postulated in *Drosophila* (Malone et al. 2009). These results together suggest that piRNA pathways play an important role in folliculogenesis in amniotes.

Acknowledgments

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Supplementary materials

Supplementary materials include Note S1; Fig. S1, S2, S3 and Table S1, S2.

Note S1:

The information of *Piwil4* in chicken and zebra finch cannot be found via ENSEMBL and BLASTn of homologue sequences against the Gallus gallus (taxid: 9031) database. Thus, further analysis of the genetic region flanked by the conserved known genes in this case chicken and zebra finch *Amotl1* and *Fut4* was performed. Firstly, the genetic sequence between *Amotl1* and *Fut4* was obtained from ENSEMBL. GENSCAN was performed to predict putative exons within this region and the identity of these putative exons was identified with NCBI BLASTn. In addition pairwise local alignment between species within this region was conducted using SIM4 (Florea et al. 1998).

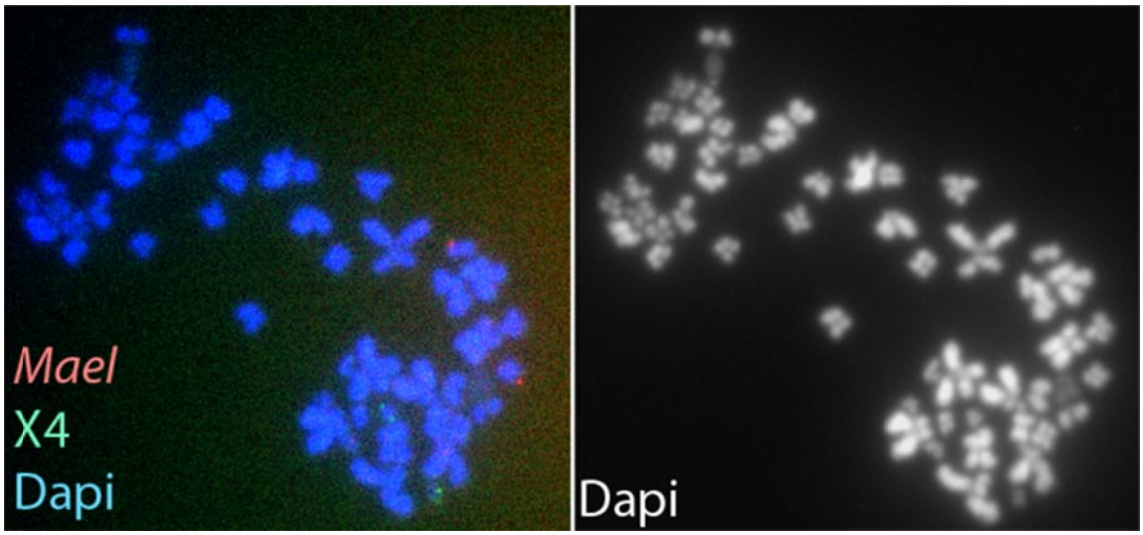

Fig. S1. Physical mapping of *Mael* in platypus. Two-colour Fluorescence *in situ* hybridisation shows that *Mael* is located on a small metacentric chromosome, most likely chromosome 17.

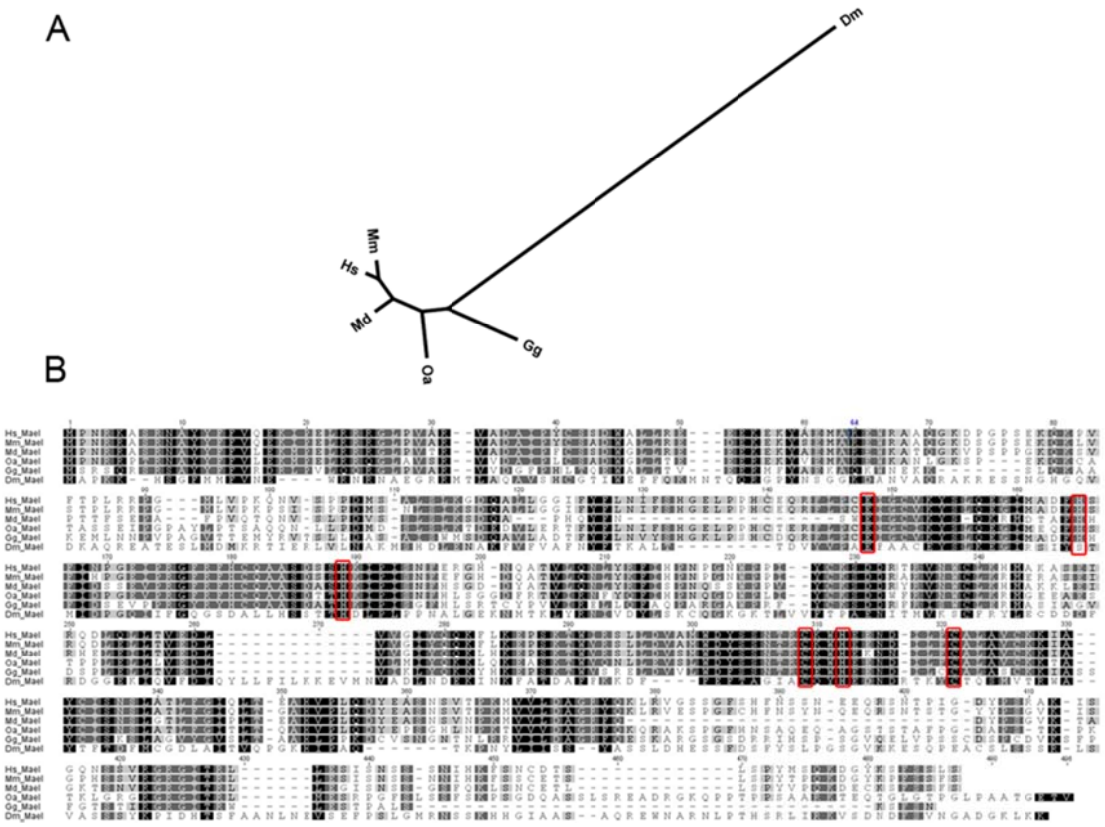

Fig. S2. Platypus MAEL motif is evolutionary conserved. (A) Phylogenetic tree of *Mael* indicates that this gene is conserved from fly to mammals. The posterior probability of all branches is 1. (B) Multiple peptide alignment of MAEL. Partial MAEL domain including the MAEL motif. Red boxes indicate the six highly conserved residues of the MAEL motif (Glu-His-His-Cys-His-Cys). Hs, human; Mm, mouse; Md, opossum; Oa, platypus; Gg, chicken; Dm, *Drosophila*.

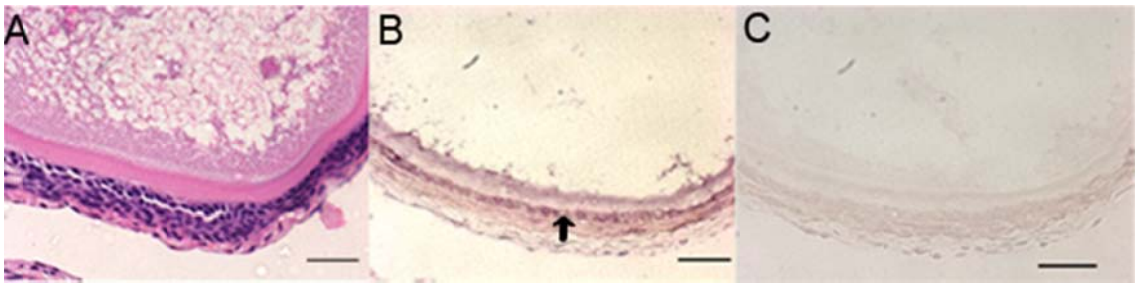

Fig. S3. *Piwil2* expression in the granulosa cell layer of a platypus primary follicle. (A) H&E staining of a platypus primary follicle. (B) RNA in *situ* hybridisation showing *Piwil2* expression in the granulosa cells of a platypus primary follicle. (C) Negative controls with sense probe. Arrow indicates granulosa cell layer which expresses *Piwil2*. Scale bar = 50µm.

Table S1: MAEL peptide sequences for multiple alignment analyses

Species	Accession number
Human	ENSP00000356846
Mouse	ENSMUSP00000045828
Opossum	ENSMODP00000003014
Chicken	ENSGALP00000024875
Drosophila	FBpp0088574

Table S2: Primers for RT-PCR and *in situ* hybridisation (ISH)

Primer	Sequence 5'-3'	Experiment
hMAEL ex7 forward	CTGATGATAGAACCAGAGTC	ISH
hMAEL ex8 reverse	GAATCCAAGTCTTAGAGGGC	RT-PCR
hMAEL ex11 reverse	TAGAAGAGTTGAAATGAGAGAA	ISH, RT-PCR
hPIWIL1 ex15 forward	CAAGTAATCGGAAGGACAAA	RT-PCR
hPIWIL1 ex18 reverse	CTACCAATGGATTTTAGACAA	RT-PCR
hPIWIL2 ex5 forward	TACCTTCAGCACACCGTCC	RT-PCR
hPIWIL2 ex6 reverse	GACACTGTATTTTGACGAGGT	RT-PCR
hPIWIL4 ex9 forward	TACTGTATCGGACCTGAATCAG	RT-PCR
hPIWIL4 ex10 reverse	TTCAGCCACAGCCTTCATCAG	RT-PCR
hACTB ex6 forward	GCCGTCTTCCCCTCCATCGT	RT-PCR
hACTB ex7 reverse	CCTCGGTCAGCAGCACGGGG	RT-PCR
mMael ex6 forward	CTGATGATAGAGCCAGAGTC	ISH
mMael ex7 reverse	GGAGGCTTCGCACCCAGG	RT-PCR
mMael ex11 reverse	TTCACCCCAGATGGATAATA	ISH, RT-PCR
mPiwil1 ex17 forward	CCATCGCAGGATTCGTCGCC	RT-PCR
mPiwil1 ex19 reverse	TTGTAACCTCTCCCGACTGA	RT-PCR
mPiwil2 ex2 forward	TTCAAAACGAGAAGTGCCT	ISH
mPiwil2 ex3 forward	AATGCTGGGAAGAGGTAGT	RT-PCR
mPiwil2 ex6 reverse	TGCTTCGTTATGACACTGGAT	ISH, RT-PCR
mPiwil4 ex9 forward	ATCACCTTATCTGACCTA	RT-PCR
mPiwil4 ex10 reverse	CTGCTGCTGCCTTCCCACA	RT-PCR
mActb ex4 forward	TGAGAGGGAAATCGTGCGTGACA	RT-PCR
mActb ex5 reverse	CCAGAGCAGTAATCTCCTTC	RT-PCR
chMAEL ex1 forward	CCTACTACTTCTTCGTGCGTGA	RT-PCR
chMAEL ex2 reverse	CCTCTCCTCCACCGTCAGCAG	RT-PCR
chGAPDH ex7 forward	GCTCATCTGAAGGGTGGTGCTA	RT-PCR
chGAPDH ex8 reverse	TAAGACCCTCCACAATGCCAA	RT-PCR

pMael ex6 forward	CAACTGGTGTCTGAAGCGCATGG	RT-PCR
pMael ex7 reverse	CCTCGTGTTGCTGGAATAATCCC	RT-PCR
pMael ex1 forward	GCTTACTATTTCTTCGTG	RACE PCR
pMael ex3 reverse	CCTCATCAGTCTTCAGAG	RACE PCR
pMael forward	GCACCTCAATCCCAAGACGG	ISH
pMael 3'UTR reverse	CAGCCAGGATGAAAAAGCGAC	ISH
pActb ex3 forward	GCCATCTACGAAGGTTACGC	RT-PCR
pActb ex4 reverse	AAGGTCGTTTCGTGGATACCAC	RT-PCR

h, human; *m*, mouse; *ch*, chicken; *p*, platypus; ISH, *in situ* hybridisation.

Chapter 3

Research paper 2

3.1 Overexpression of piRNA pathway genes: a role in the progression of epithelial ovarian cancer

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Text in manuscript

STATEMENT OF AUTHORSHIP

Overexpression of piRNA pathway genes: a role in the progression of epithelial ovarian cancer

Shu Ly Lim (Candidate)

Performed analyses on all samples, interpreted data and wrote manuscript

Certification that the statement of contribution is accurate

Carmela Ricciardelli (co-author)

Provided research materials, contributed to data interpretation and commented on manuscript

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Martin K. Oehler (co-author)

Provided research materials, contributed to data interpretation and commented on manuscript

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

Izza M.D. De Arao Tan

Contributed to interpretation of data and commented on manuscript

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Darryl Russell

Contributed to interpretation of data and commented on manuscript

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Frank Grützner (corresponding author)

Supervised development of work, helped in data interpretation and manuscript preparation

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Abstract

Genes of the Piwi-interacting RNA (piRNA) pathway are important for maintaining germ cells and for protecting genome integrity. In developing germ cells, the piRNA pathway represses transposable elements (TEs), such as *Line-1* (*L1*), via different mechanisms impacting on epigenetic modification like DNA methylation as well as post transcriptionally by binding repeat transcripts. Aberrant DNA methylation and retrotransposon activity are also hallmarks of many cancers and increasingly the importance of genes in the piRNA pathway in cancer biology is becoming apparent. We recently did a comprehensive expression analysis of piRNA pathway genes in the mammalian ovary and our finding of robust expression in oocytes and supporting cell led us to investigate whether the piRNA pathway could also play a role in epithelial ovarian cancer (EOC). We determined the expression of piRNA pathway genes, including *PIWIL1-4* and *MAEL,* in EOC. Semi-quantitative RT-PCR showed that *PIWIL1* and *MAEL* have significantly increased expression in malignant EOC compared to benign tissues and normal ovaries but the expression of *PIWIL3* is significantly lower in malignant and benign tissues when compared to normal ovary. *In situ* hybridization reveals that *L1*, *PIWIL1*, *2* and *MAEL* are overexpressed in the epithelial cells (cancerous cells) of EOC. Furthermore, *PIWIL2* and *MAEL* are co-expressed in the stromal cells in malignant EOC, suggesting a transformation of these cells perhaps to support the growth of cancer cells. Since *PIWIL1* and *MAEL* are upregulated in malignant EOC, we investigated if these two genes affect EOC invasiveness. *PIWIL1* and *MAEL* were transiently overexpressed in the ovarian cancer cell line SKOV3, followed by real-time measurements of cell invasiveness. *PIWIL1* and *MAEL* overexpression decreased the invasiveness of SKOV3 cells, suggesting that they have a repressive role in ovarian cancer cell invasiveness. In general these findings

suggest that the piRNA pathway is activated in EOC and play a role in tumour progression.

Introduction

Ovarian cancer is the most common cause of gynaecological cancer death, and the fifth leading cause of cancer-related death among women in the Western World (American-Cancer-Society 2012). The five-year relative survival rate for women with ovarian cancer is only around 40%. Ovarian cancers are heterogeneous tumours which exhibit distinct morphological characteristics, genetic mutations and origins. There are three major types of ovarian cancer - epithelial, germ cell and sex cord stromal tumours. Ovarian germ cell tumours and sex cord stromal tumours comprise 10% of ovarian cancers, and are derived from primitive ovarian germ cells or mesenchymal cells in the sex-cord derived tissue of the ovary, respectively (Chen et al. 2003; Jemal et al. 2005). EOC accounts for more than 90% of the ovarian malignancies. They represent a heterogeneous group of malignancies with distinct genetic and epigenetic properties. EOC are classified into four main histological subtypes including serous, mucinous, endometroid and clear cell carcinomas, with serous carcinomas (SCs) accounting for more than 70% of EOC cases (Ricciardelli and Oehler 2009).

Based on EOC morphology, molecular biology and cancer genetics two broad categories of EOC are classified as type I and II ovarian carcinomas (Shih Ie and Kurman 2004; Kurman and Shih Ie 2008). Type I EOC is derived from well-established precursor lesions, genetically stable and less malignant. They include transitional cell tumours, low grade serous, low grade endometriod, clear cell and mucinous carcinomas. Type II EOC tumours are rapidly growing, highly invasive, and chromosomally

unstable, with most having TP53 mutations (Ahmed et al. 2010). Type II EOC is comprised of high-grade serous, endometriod, malignant mesodermal tumours and undifferentiated carcinomas. Type I and II tumours both develop independently with distinct molecular pathways, and are probably derived from different ovarian tissues, and perhaps even extraovarian tissues such as the fallopian tube (Kurman and Shih Ie 2011). However, the exact origin of EOC is still controversial and a better understanding of the molecular pathways involved in tumor origin and progression is needed also to improve classification, diagnosis and ultimately treatment of this disease.

Alterations of epigenetic landmarks such as global DNA hypomethylation and gene specific DNA hypermethylation are frequently reported in cancer cells (Feinberg and Vogelstein 1983). Global DNA hypomethylation largely affects the intergenic and intronic regions of the genome, especially the repeat sequences and TEs. In normal somatic cells, DNA methylation is crucial for the repression of TEs which lead to genome instability when expressed. About 55% of the human genome is composed of TEs (Lander et al. 2001), including 17% *L1* repeats (Cordaux and Batzer 2009). During germ cell development, there is a transient period of global DNA hypomethylation which results in temporary activation of TEs (Smallwood and Kelsey 2012). The piRNA pathway specifically protects genome from the adverse effects of TE expression (Sarot et al. 2004; Aravin et al. 2008). The piRNA pathway consists of 21-26nt piRNAs which bind PIWI proteins to mediate posttranslational control of TE expression or epigenetic changes (such as DNA methylation) via interaction with other proteins (such as *Mael* or *HP1*) (Sarot et al. 2004; Aravin and Bourc'his 2008). Mutations of *Piwil2* and *Piwil4* in mouse lead to elimination of TE DNA methylation and male sterility similar to the phenotype in *Dnmt3L* null mutant males (Aravin et al. 2007; Carmell et al.

2007). Thus, piRNA directs *de novo* DNA methylation in the mammalian germline. However, the exact mechanism is still unclear (Aravin and Bourc'his 2008).

There are four human *PIWIL* genes which are *PIWIL 1-4* (Sasaki et al. 2003). In addition to these four genes, another gene, Maelstrom *(MAEL),* plays a key role in the piRNA maturation and TE silencing process (Soper et al. 2008). These five genes are expressed at different stages during germline development in mouse testis and *Drosophila* ovary (De Fazio et al. 2011; Wang and Elgin 2011). Mutation of *Piwil2* and *Mael* in mouse testis results in TE derepression which causes the failure of spermatogenesis (Kuramochi-Miyagawa et al. 2004; Soper et al. 2008). We have recently shown the evolutionary conserved expression of piRNA pathway genes in the mammalian ovary (Lim et al. submitted). Moreover, expression of *PIWIL1* and *PIWIL2* was found in a wide range of human cancers such as those from the stomach, breast, gastrointestinal tract and endometrium (Qiao et al. 2002; Lee et al. 2006; Liu et al. 2006; Liu et al. 2010). Increased expression of *PIWIL1* is associated with increased tumour grades in gliomas (Sun et al. 2011), poor diagnostic outcomes in adenocarcinomas of the pancreas (Grochola et al. 2008) and mortality in soft tissue sarcomas (Taubert et al. 2007). *PIWIL2* is found to be widely expressed in early-stage breast and cervical cancers and also in pre-cancerous stem cells involved in tumourigenesis (Lee et al. 2006). The expression pattern of *PIWIL3* and *4* has only been studied in colon cancers (Li et al. 2010). In advanced colonic tumours, upregulation of *PIWIL3* and *4* is found in cancerous tissues when compared to adjacent non-cancerous colon tissues (Li et al. 2010). Decreased DNA methylation of the *L1* promoter region is also associated with the progression of cervical and uterus cancers (Shuangshoti et al. 2007) and is believed to contribute to aggressive tumour behaviour in colorectal cancers (Kato et al. 2009). It

also predicts poor prognosis in a wide range of cancers such as ovarian clear cell carcinomas (Iramaneerat et al. 2011), colon cancers (Sunami et al. 2011) and chronic myeloid leukaemia (Roman-Gomez et al. 2005). Despite these expression studies there is little knowledge about what aspect of tumour biology the piRNA pathway might be involved in.

Here, we investigated the expression of *PIWIL1-4* and *MAEL* in EOCs (n=25), benign ovarian tumours (n=19), and normal ovarian tissues (n=8). Elevated expression of *PIWIL1* and *MAEL* in EOC compared to benign and normal ovarian tissues was found. *In vitro* analysis showed that overexpression of *PIWIL1* and *MAEL* in ovarian cancer cells decreases cancer cell invasiveness.

Materials and methods

Tissues and patient ovarian specimens

Human testis and pre-menopause ovarian tissue total RNA were purchased from Stratagene (USA). Malignant, benign and normal ovarian tissues were collected with informed written consent and approval by the Ethics Committee of the Royal Adelaide Hospital, Adelaide, South Australia.

Table 1: Age and cancer stage of EOC, benign tumours and normal ovaries

Tissue	n	Median of patient age in years (range)	Cancer stage
Serous carcinomas	25	60 (37-75)	FIGO stage 3c
Benign ovarian lesions	19	60 (36-88)	-
Normal ovaries	8	48 (44-76)	-

Cell lines

The human ovarian cancer cell lines SKOV3 and OVCAR3 were purchased from American type culture collection (ATCC, USA) while OVCAR5 was obtained from Dr. Stephen Williams (Fox Chase Cancer Center, Philadelphia, PA, USA). Cell lines were maintained in RPMI 1640 medium supplemented with L-glutamine (2mM), Penicillin-streptomycin (100U/ml, 100µg/ml) (Sigma). SKOV3 and OVCAR3 cells were supplemented with 5% FBS (Invitrogen) whilst OVCAR5 cells were supplemented with 10% FBS and 7.5 µg/ml insulin. All cell lines were maintained at 37˚C in a humid chamber with 5% CO_2.

RNA isolation and cDNA synthesis

RNA was isolated from tissues and cell lines using TRIzol reagent (Invitrogen) following the manufacturer's instructions. RNA was resuspended in nuclease free water, and stored at -80°C. RNA was treated with DNase I (New England Biolabs) before reverse transcription. 1µg of RNA was used to obtain cDNA using the Super Script III First-strand Synthesis System (Invitrogen) following the manufacturer's protocol. Briefly, RNA was incubated with 1µl of 50 µM oligo$(dT)_{20}$ and 1µl of 10mM dNTPs for 10 mins at 65°C. After incubation, 4µl of 5x RT buffer, 2µl of dithiothreitol (DTT, 0.1M), 1µl of RNaseOUT™ (40U/µl) and 1µl of Super Script III RT enzyme (200U/µl) were added and incubated for 50 mins at 50°C. The reaction was terminated at 85˚C for 5 mins. cDNAs were stored at -20^0C.

Gene expression analyses

RT-PCR was performed to determine the level of mRNA for the five piRNA pathway genes (Table S1) and Beta actin (*ACTB*) in 52 patient samples and 3 ovarian cancer cell lines. Each 25µl reaction contained 200ng of cDNA, 5µl of 5x Go Taq Green Master Mix (Promega), 1µl of 5mM dNTP solution (Roche), 0.5µl of each primer (20pmol/µl) and 0.5µl Taq DNA polymerase. The PCR conditions were the same for all genes, except the annealing temperature (Table S1), and were as follows: initial denaturation at 95˚C for 2 mins, 95˚C for 30 secs, annealing at gene specific temperature for 30 secs (Table S1), extension at 72 ˚C for 1 min, 32 cycles. PCR of *ACTB* control was performed with 27 cycles, followed by 5 mins of final extension at 72 ˚C. The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide. Band intensity was measured with the Quantity One program (Version 4, Bio-Rad), and the relative intensity of each gene was normalized to that of *ACTB*. PCR products were

confirmed by sequencing (Big Dye Terminator v3.1 cycle sequencing kit, Applied Biosystems). RT–PCR was performed in triplicate for each primer pair.

Statistical analyses of gene expression

Data are presented by depicting the median relative expression (first quartile to third quartile), and data points within 1.5 times interquartile range above or below the first and the third quartile. The transcript level of each gene was normalized to *ACTB* transcripts. Both the Kolmogorov-Smirnov and Shapiro-Wilk tests suggested that the expression level of each gene from all of the 52 samples is not normally distributed. Thus, a Kruskal-Wallis test, which uses a non-parametric method, was used to compare the gene expression from malignant, benign and normal groups. The Spearman's correlation test was performed to investigate the correlation between a patient's age and consequent gene expression. R-value closer to 1 indicates a stronger positive correlation, whereas an R-value closer to -1 shows a stronger negative correlation. In the Kruskal-Wallis and Spearman's tests, a P-value of <0.05 was considered statistically significant.

RNA *in situ* hybridisation

Generally, cDNA fragments about 450bp-550bp are amplified with PCR and cloned into pGEM-T Easy Vector (Promega). Probes were labelled with digoxigenin-11-UTP (Roche Diagnostics) according to the manufacturer's protocol. Formalin-fixed paraffin-embedded tissue sections were deparaffinised in xylene and subsequently washed in graded ethanol. All solutions were prepared in diethylpyrocarbonate (DEPC)-treated H_2O to inactivate RNase activity. mRNA-bound nucleoproteins were removed by proteinase K incubation (1.2μg/ml) (Roche Diagnostics) for 30 mins at 37 ˚C. Slides

were washed with 1x PBS and acetyled (1M triethanolamin/ 0.178% HCl/ 0.25% acetic anhydride). Slides were prehybridised at 65˚C for 2 hrs with prehybridisation buffer (50% deionized formamide/ 2x SSC/ 1x Denhardt's solution/ 0.005M phosphate buffer/ 10% dextran sulphate/ 1mg/ml yeast total RNA/ 1mg/ml salmon sperm DNA) and hybridised with about 200ng of cRNA probe in prehybridisation buffer overnight at 50˚C in a humid chamber. Slides were washed in SSC solutions of increasing stringency (2x SSC, 1x SSC, 0.5x SSC and 0.1x SSC) for 15 mins each at 50˚C. To further remove unbound cRNA probes, the tissue was subjected to RNase A (150µg/ml) digestion for 30 mins at 37˚C. Specifically bound cRNA probes were detected using an anti-DIG antibody coupled to alkaline phosphatase with nitrobluetetrazolium chloride/ X-phosphate-5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (Roche Diagnostics) as chromogenic substrate. After an incubation period of 6-18 hrs, the reaction was stopped with 1x TE buffer, dried and tissue sections were dehydrated with 100% ethanol and eventually mounted using Entellan mounting media (Merck).

Cloning and construct preparation for invasion analyses

The *MAEL* (CCDS1257) and *PIWIL1* (CCDS9268) cDNA sequences were obtained from the NCBI CCDS database. Full length cDNA sequence was amplified using the Expand High Fidelity PCR system (Roche) according to the manufacturer's protocol and cloned into a pcDNA3.1 mammalian expression vector. Plasmid DNAs including pcDNA 3.1 (Invitrogen) or pEGFP-N1 empty vectors (Clontech) were transfected into SKOV3 cells using Lipofectamine LTX (Invitrogen) following the manufacturer's instruction. SKOV3 cells were selected due to their initial endogenous *L1* expression levels and the fact they have no piRNA pathway gene expression (Fig. 1). For each

transfection, 8 X 10^4 SKOV3 cells were seeded onto a 24-well plate 14 hrs before transfection and were cultured in RPMI 1640/ 5% FBS without Penicillin-streptomycin. 500ng of plasmid DNA was diluted in 100µl of Opti-MEM medium (Invitrogen) and incubated with 0.5µl Plus reagent (Invitrogen) and 2µl Lipofectamine LTX (Invitrogen) for 30 mins before being added into each well. After 6 hrs, the cell culture medium was replaced with new RPMI 1640/ 5% FBS medium and cells were incubated for 24 hrs at 37˚C, 5% CO_2 before harvesting for *in vitro* invasion analyses. The transfection of pEGFP-N1 empty vectors into SKOV3 cells allowed the transfection efficiency to be measured, as successfully transfected cells expressed the GFP protein. The pEGFP-N1 vector was subjected to the same growth and transfection conditions as outlined above, to determine the transfection efficiency for each cell line, which was approximately 70% after 24 hrs.

In vitro invasion analyses with xCELLigence

Cell invasion was tested with real-time invasion assay monitoring using the CIM devices and the xCELLigence DP system (Roche Diagnostics) (Xi et al. 2008). Briefly, 4 hrs before the invasion assay, a CIM plate (Roche) was coated with 1:20 diluted Growth factor reduced Matrigel basement membrane matrix (BD Biosciences) (~450µg/ml). Then 40,000 cells, untransfected or transfected with empty vector (Ev) or *MAEL* or *PIWIL1* vectors, were seeded into each coated well. Cell activity was followed over a time period of 72 hrs by measuring the impedance signal in the CIM plate. The cell activity was recorded every minute in the first 12 hrs and every 5 mins for the following 12 hrs. Then from 24 hrs onwards until the end of the experiment, cell activity was recorded every 30 mins. In each CIM plate, triplicates of each group were

performed to obtain the mean and standard deviation. This experiment was repeated three times.

Results

Expression of piRNA pathway genes is increased in malignant EOC compared to benign tumours or normal ovarian tissues.

piRNA pathway genes are consistently expressed in the mammalian ovary (Lim et al. submitted). In order to investigate the role of this pathway in EOC, we performed semi-quantitative RT-PCR to investigate the expression of *PIWIL1*, *PIWIL2*, *PIWIL3*, *PIWIL4* and *MAEL* in advanced stage serous EOC (n = 25), benign ovarian tumours (n = 19) and normal ovarian tissue (n = 8) (Fig. 1). The relative expression levels of *PIWIL1* and *MAEL* were significantly higher in malignant EOC compared to benign and normal tissues (Fig. 2A, B). However, the relative expression levels of *PIWIL2* and *PIWIL4* in malignant groups were not significantly different to that in benign or normal ovarian tissues (Fig. 2C-E). The expression of *PIWIL2* and *PIWIL4* are significantly lower in benign tumours compared to normal ovarian tissue, suggesting an alteration of gene expression during the progression of EOC. The relative expression of *PIWIL3* is different to other *PIWIL* genes such that the expression of this gene is significantly higher in the normal ovary when compared to both malignant and benign tissues (Fig. 2C). Furthermore, the relative expression of *PIWIL3* in normal ovary is positively correlated with the age of patients despite the small sample size of normal ovary tissues (n = 8) (r = 0.750, P = 0.05) (Table 2). In contrast to *PIWIL3*, *PIWIL1* expression is negatively correlated with the age of patients (r = -0.737, P = 0.037). *PIWIL1* has previously been found to be expressed in growing follicles in pre-menopausal ovaries,

but in this study half of the ovaries tested were from individuals of less than 50 years old i.e. from donors with pre-menopausal ovary tissue.

Table 2: Correlation of the age of patients and piRNA pathway gene expression

Genes	R-value (M, n = 25)	P-value (M, n = 25)	R-value (B, n = 19)	P-value (B, n = 19)	R-value (N, n = 8)	P-value (B, n = 19)
PIWIL1	-0.376	0.064	-0.194	0.426	-0.737	0.037*
PIWIL2	-0.080	0.702	-0.442	0.058	0.491	0.217
PIWIL3	-0.082	0.695	0.595	0.009*	0.75	0.05
PIWIL4	-0.249	0.231	-0.376	0.113	0	1.0
MAEL	-0.087	0.678	0.251	0.457	-0.220	0.601

Spearman's correlation test; M = malignant; B = benign; N = normal; negative value = negative correlation; positive value = positive correlation; $P < 0.05$ and * indicates the correlation between the age of patients and piRNA pathway gene expression is significant. The R-value closer to ± 1 indicates the stronger correlation.

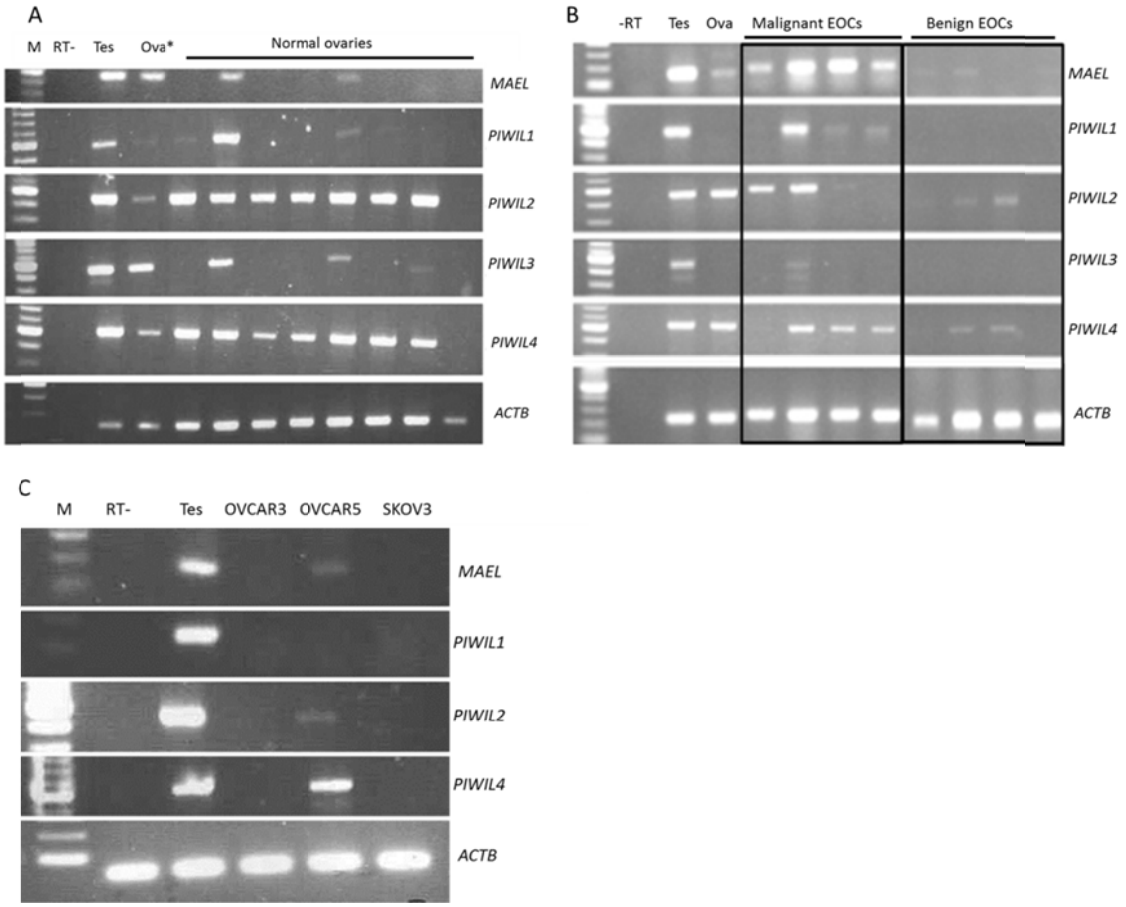
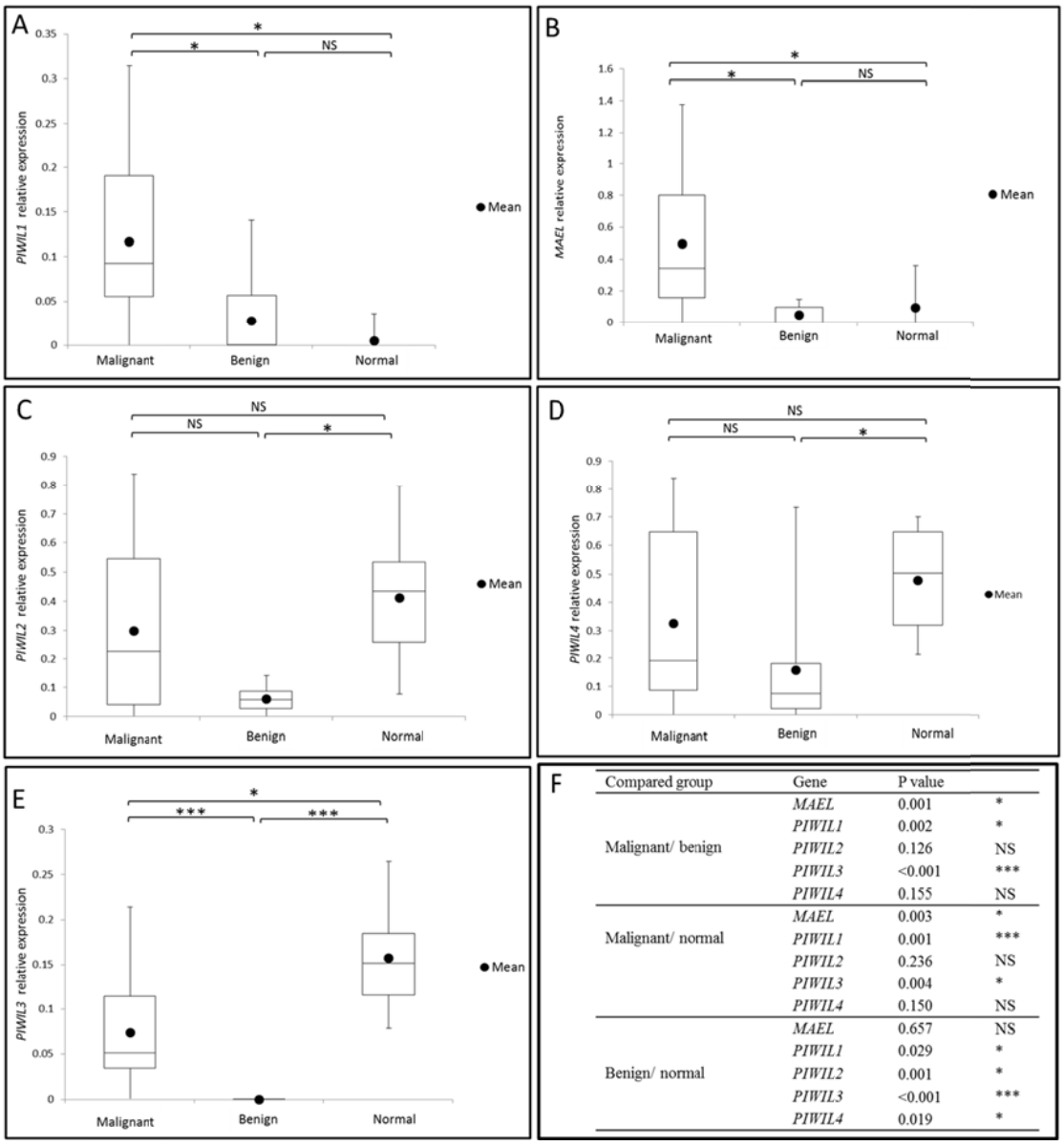


Fig. 1. piRNA pathway gene expression in human normal ovaries and ovarian cancers. RT-PCR analyses of *PIWIL1-4* and *MAEL* expression in (A) malignant EOC, benign ovarian cancers (B) normal ovaries and (C) ovarian cancer cell lines. This is a representation of 4 out of 25 malignant EOC and 19 benign ovarian cancer tissues. Increased expression of *PIWIL1, 2, 4* and *MAEL,* but not *PIWIL3,* are found in malignant cancers compared to benign tumours. All normal ovaries show *PIWIL2* and *PIWIL4* expression but only some have *PIWIL1, 3* and *MAEL* expression. No endogenous *PIWIL1, 2, 4* and *MAEL* expression is detected in OVCAR3 and SKOV3 cells. Ova* indicates tissue from a 20-year old pre-menopausal ovary while other ovaries are from individuals aged 44-76 years old.



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Compared group	Gene	P value	
Malignant/ benign	*MAEL*	0.001	*
	PIWIL1	0.002	*
	PIWIL2	0.126	NS
	PIWIL3	<0.001	***
	PIWIL4	0.155	NS
Malignant/ normal	*MAEL*	0.003	*
	PIWIL1	0.001	***
	PIWIL2	0.236	NS
	PIWIL3	0.004	*
	PIWIL4	0.150	NS
Benign/ normal	*MAEL*	0.657	NS
	PIWIL1	0.029	*
	PIWIL2	0.001	*
	PIWIL3	<0.001	***
	PIWIL4	0.019	*

Fig. 2. Box plot representing the expression of piRNA pathway genes in malignant EOC, benign ovarian cancer tissues and normal ovaries. Semi-quantitative RT-PCR analyses of mRNA expression (relative to *ACTB*) of (A) *PIWIL1*, (B) *MAEL*, (C) *PIWIL2*, (D) *PIWIL4* and (E) *PIWIL3*. (F) P-value of each comparison group. Malignant EOC (n = 25), benign ovarian cancer tissues (n = 19) and normal ovaries (n = 8). Kruskal-Wallis Test was performed to compare the gene expression level between two groups. Filled dot indicates mean of each group. * indicates $0.001 < P < 0.05$; *** indicates $P < 0.001$; NS, not significant.

***L1* and piRNA pathway genes are overexpressed in EOC cells**.

To analyse the expression pattern of piRNA pathway genes and *L1* in EOC, RNA *in situ* hybridisation was performed in malignant EOC (N = 5) and benign ovarian tumours (N = 2) (Table 3, Fig. S1). We found strong expression of *L1* in the epithelial cells but not stromal cells in all samples (Fig. 3A, E). Also it appeared that there was variable expression of *L1* between epithelial cells (Fig. 3A'). Similarly, strong expression of *PIWIL1* was found in the epithelial cells but not stromal cells in all EOC tissues (Fig. 3B, F) while *MAEL* and *PIWIL2* showed strong expression in the epithelial cells and stromal cells of all EOC tested (Fig. 3C, G and D, H respectively).

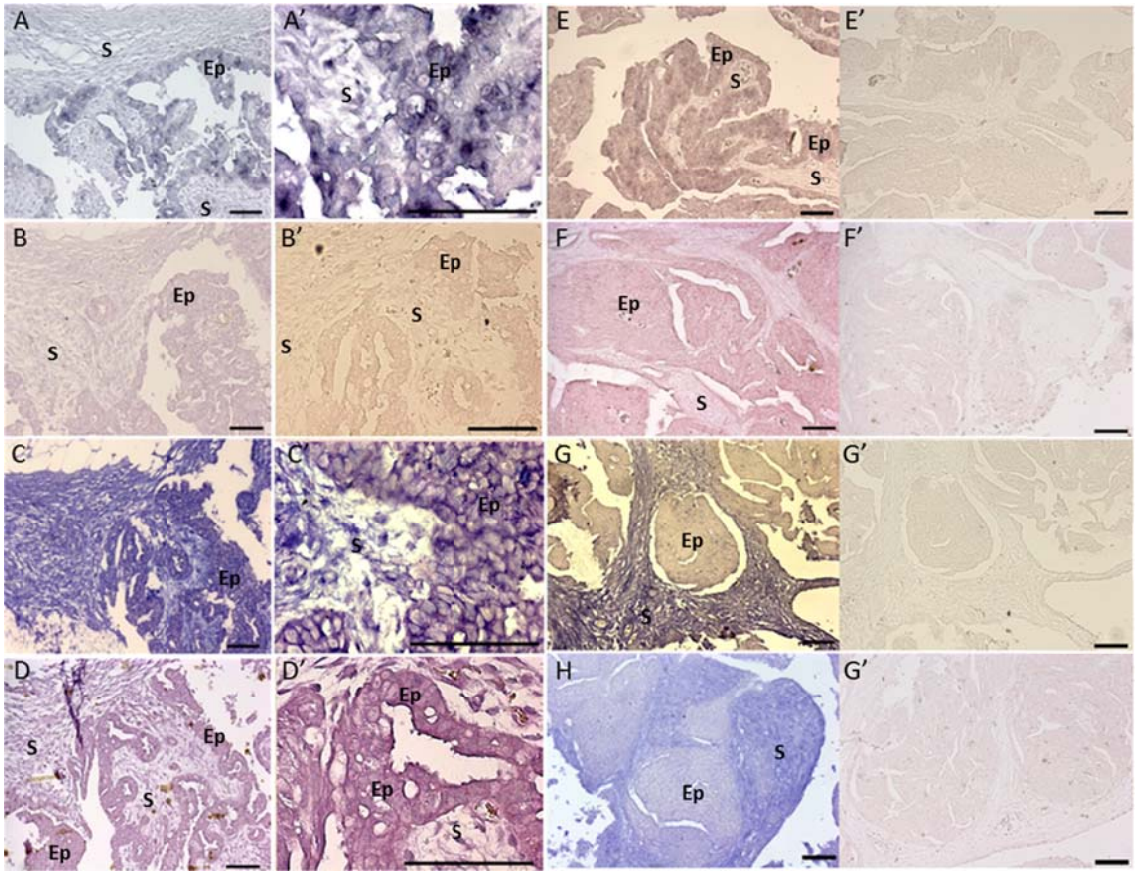

Fig. 3. *L1*, *PIWIL1*, *MAEL* and *PIWIL2* are expressed in the epithelial cells of malignant EOC. *In situ* analyses of (A, A', E) *L1*, (B, B', F) *PIWIL1*, (C, C', G) *MAEL*, (D, D', H) *PIWIL2* in two malignant EOC (A-D from SC3 while E-H from SC2). (A'-D') Amplification images of A-D respectively. Strong expression of piRNA pathway genes and *L1* was found in the squamous-to-cuboidal like epithelial cells of both malignant EOC except *PIWIL1* which only expressed in SC2 but not SC3. *L1* has patchy expression in some EOC such that some epithelial cells seem to have stronger *L1* expression compared to others. *MAEL* and *PIWIL2* but not *PIWIL1* are also expressed in the stromal cells in both EOC. (E'-H') Negative controls with sense probe of *L1*, *PIWIL1*, *MAEL* and *PIWIL2* respectively. Ep = epithelial cells; S = stromal cells. Scale bar = 50μm

Table 3: Expression trends of piRNA pathway genes and *L1* in the EOC.

Patient	Gene	Epithelial cells	Stromal cells
SC1	*MAEL*	weak	strong
	PIWIL2	weak	strong
	PIWIL1	strong	-
	L1	strong	-
SC2	*MAEL*	weak	strong
	PIWIL2	weak	strong
	PIWIL1	weak	-
	L1	strong	-
SC3	*MAEL*	strong	strong
	PIWIL2	strong	strong
	PIWIL1	-	-
	L1	strong	weak
SC4	*MAEL*	-	-
	PIWIL2	strong	-
	PIWIL1	strong	-
	L1	strong	-
SC5	*MAEL*	weak	weak
	PIWIL2	weak	-
	PIWIL1	weak	-
	L1	weak	-
BSC1	*MAEL*	weak	weak
	PIWIL2	weak	weak
	PIWIL1	-	-
	L1	-	-
BSC2	*MAEL*	weak	-
	PIWIL2	weak	-
	PIWIL1	-	-
	L1	-	-

- undetectable expression.

Overexpression of piRNA pathway genes reduces invasiveness *in vitro.*

In order to investigate the possible role of piRNA pathway genes in cancer cell progression, full length *PIWIL1* or *MAEL* transcripts were cloned and transfected transiently into the ovarian cancer cell line SKOV3 and assayed for invasiveness after 24 hrs of transfection. RT-PCR was performed to validate the insertion of plasmids into these cells (Fig. S2A). RT-PCR demonstrated that only transfected cells have *GFP*, *PIWIL1* or *MAEL* expression which suggested that cells were successful transfected and transcripts are overexpressed in these cells (Fig. S2). Expression of *MAEL* and *PIWIL1* constructs remains high in the transfected cells (and not in the untransfected cells) at least 24 hrs until 74 hrs post-transfection. *MAEL*, *PIWIL1* and empty vector (Ev) transfected cells and untransfected SKOV3 cells were applied to the xCELLigence system to investigate their effect on cell invasiveness (Eisenberg et al. 2011).

Surprisingly we observed a decrease in the invasiveness of *MAEL* and *PIWIL1* transfected cells is lower than untransfected cells (Fig. 4). Cell invasiveness from individual experiments can be found in Fig. S3. Cell invasiveness from each group started to reach a plateau after 30 hrs, thus only cell activity of the first 30 hrs is shown. *MAEL* and *PIWIL1* transfected cells showed decreased invasiveness compared to that of wild type cells (untransfected and Ev transfected). RT-PCR demonstrated that the expression of *MAEL* and *PIWIL1* in transfected cells remained at a high level throughout the *in vitro* study (Fig. S2A). However, endogenous *L1* expression was lost after several cell passages. RT-PCR with *L1* proved to be challenging due to the high level of heterogeneity in *L1* sequence within an individual. Our results suggest that *PIWIL1* and *MAEL* have a repressive effect on SKOV3 cell invasiveness.

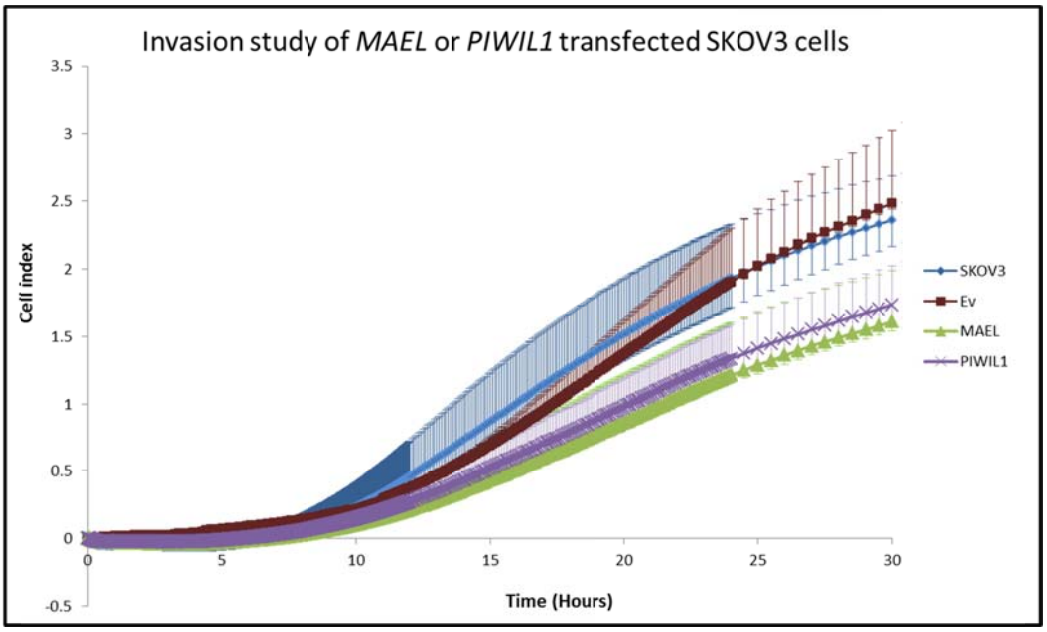

Fig. 4. Invasion assay of *MAEL* or *PIWIL1* transfected SKOV3 cells. *PIWIL1* and *MAEL* transfected cells have lower invasiveness compared to untransfected cells and *Ev* transfected cells. In each experiment, each group was performed in triplicate and this experiment was repeated three times. This is a combined data plot of three independent experiments for all groups. Error bars represent standard deviation. *WT* indicates untransfected SKOV3 cells; empty vector (Ev), *MAEL* and *PIWIL1* indicate cells transfected with *MAEL* or *PIWIL1* vectors. *None* indicates well without cells.

Discussion

The piRNA pathway is important in RNA silencing and stem cell self-renewal in a wide range of organisms (Cox et al. 1998). In addition, it is required for the repression of TEs in animal germ cells (Siomi et al. 2010). Global DNA hypomethylation and TE derepression, such as *L1,* is a common feature of cancer genome (Roman-Gomez et al. 2005; Piskareva et al. 2011). *Piwi* has been implicated in tumour growth in *Drosophila,* and human *PIWIL1-2* overexpression has been observed in tumours from various tissues (Qiao et al. 2002; Lee et al. 2006; Liu et al. 2006; Taubert et al. 2007; Grochola et al. 2008; Liu et al. 2010; Sun et al. 2011). However, it is still unclear whether the piRNA pathway is active in ovarian cancer tissues and has a role in ovarian tumour progression. We examined the expression of piRNA pathway genes *PIWIL1-4*, *MAEL* and *L1* in malignant EOC, benign tumours and normal ovary tissues, and also investigated possible roles of *PIWIL1* and *MAEL* in ovarian cancer cell lines.

Expression of *PIWIL1-2* has been investigated in various cancerous tissues (Qiao et al. 2002; Lee et al. 2006; Liu et al. 2006; Taubert et al. 2007; Grochola et al. 2008; Liu et al. 2010; Sun et al. 2011), but not *MAEL*, *PIWIL3* and *PIWIL4*. In this study we provide evidence that expression of *PIWIL1* and *MAEL* is significantly increased in malignant EOC when compared to benign tumours. Although the expression of *PIWIL2* and *4* appeared high this was not significant, likely due to the fact that *PIWIL2* and *PIWIL4* were strongly expressed in normal ovarian tissues (Fig. 1B) and their expression among malignant tissues is highly variable. *PIWIL1* and *MAEL,* but not *PIWIL2* and *4,* were significantly upregulated when compared to normal ovaries. In contrast to *PIWIL2* and *4* which are expressed in all normal ovarian tissues tested, the expression of *PIWIL1* and *MAEL* is found only in a subset of normal ovarian tissues from patients who were

less than 50 years old. In normal ovary, *PIWIL1* and *MAEL* are expressed in the cumulus cells of growing follicles in human (Lim et al., submitted). The normal ovarian tissues tested are from individuals aged 44-76 years old, and half of these samples were derived from individuals less than 50 years old. Thus, the number of growing follicles which express piRNA pathway genes varied across the samples, which could explain the variable expression of *PIWIL1* and *MAEL* in the normal ovaries.

The expression pattern of *PIWIL3* has only been studied in colon cancers (Li et al. 2010). In advanced colon tumours, upregulation of *PIWIL3* was found in cancerous tissues when compared to adjacent non-cancerous tissues (Li et al. 2010). In this study, we showed significantly higher expression of *PIWIL3* in normal tissues compared to malignant and benign tissues, using RT-PCR. No expression of *PIWIL3* was detected in benign tissues. Unlike *PIWIL1* and *MAEL* which are expressed in the growing follicles of pre-menopausal individuals, our finding showed that the expression of *PIWIL3* is positively correlated with age in normal ovary, suggesting that it may play a role in aged ovary.

MAEL is known as a cancer/testis gene as it is expressed in testis and a number of cancer cell lines (Xiao et al. 2010). Here we identified for the first time the increased expression of *MAEL* in malignant EOC and benign ovarian tumours. Similar to PIWI, MAEL is essential to ensure proper germline stem cell differentiation in *Drosophila* and other vertebrates (Cox et al. 1998; Pek et al. 2009). Overexpression of piRNA pathway genes in EOC may indicate that some stem cell characteristics are present in tumour cells or that the piRNA pathway has been activated for example by activity of retrotransposons. The expression of piRNA pathway genes in normal ovary and certain

types of EOC, suggests that there might be a link between the normal function of *PIWIL* genes and *MAEL* in the maturing follicles and EOC development. The expression of *PIWIL* genes and *MAEL* in both normal ovary and malignant EOC raises the possibility that the piRNA pathway genes may be involved in the origin of ovarian cancer.

In situ analyses revealed that *L1*, *PIWIL1*, *PIWIL2* and *MAEL* are overexpressed in the epithelial cells but not in the stromal cells in malignant EOC. Hypomethylation of the *L1* promoter region, which is correlated with increased *L1* mRNA expression, was found in malignant breast cancer tissues and cell lines (Alves et al. 1996; Asch et al. 1996). Thus, the overexpression of *L1* in EOC indicates that *L1* is derepressed in epithelial cells and these epithelial cells. Expression of other piRNA pathway genes in the same cells could be explained by the activation of piRNA pathway as a consequence of TE derepression. Interestingly, we also observed upregulation of *MAEL* and *PIWIL2* in the stromal cells in malignant EOC. *MAEL* and *PIWIL2* expression is restricted to the granulosa cells of early follicles and the cumulus cells of antral follicles (Lim et al., submitted), but not in either stromal or epithelial cells in normal ovaries. Therefore, the expression of these two genes in stromal cells from malignant EOC indicates a change in gene expression in the stromal cells surrounding cancerous cells. Several lines of evidence have demonstrated that alteration of gene expression in stromal cells may create a microenvironment which facilitates tumour growth, playing a role in cancer progression (Gilles et al. 1996; Stahtea et al. 2007; Cirri and Chiarugi 2011). For example, changes of gene expression in the stromal cells surrounding colon cancer cells have been shown to produce matrix metalloproteinases (MMPs) which increase tumour invasion capacity *in vitro* and *in vivo* (Kunju et al. 2011).

The role of *PIWIL1* and *MAEL* in cancer is controversial and remains elusive. Recent studies suggested that *PIWIL1* may be a marker for cancer cell proliferation as it is co-expressed with KI67 (Liu et al. 2006), a reliable proliferating cell marker (Brown and Gatter 2002). In addition, studies in a *Drosophila* brain tumour model suggested that inactivation of *Piwi* suppressed tumour growth and thus *Piwi* may promote cancer progression in this system (Janic et al. 2010). However, another study in humans showed that overexpression of *PIWIL1* did not increase cell growth but caused programmed cell death in myeloid leukaemia KG1 cells. This result suggests that PIWIL1 may prevent tumour development (Sharma et al. 2001). Thus, the role of *PIWIL1* in cancer proliferation is controversial. The role of *MAEL* in cancer is equally unclear (Xiao et al. 2010). Our experiments have shown that both *PIWIL1* and *MAEL* transfected cells have decreased cell invasiveness compared to wild type and empty vector controls. Although *PIWIL1* transcript level was significantly increased in malignant EOC compared to benign and normal tissues, cloning and sequencing of these transcripts suggested that these transcripts may produce aberrant and non-functional PIWIL1. A range of mutations were found in *PIWIL1* transcripts including unspliced introns and loss of exons. These mutations introduced premature stop codons in almost all of the clones tested, suggesting that although *PIWIL1* expression is high in malignant tissues, the corresponding protein (if any) would be truncated and is predicted to result in a loss of function. The functions of the PIWI domain in RNA splicing and small RNA binding are highly conserved from fission yeast to human[57]. Mutations in the PIWI domain in malignant EOC may affect the proper functioning of this vital part of the PIWI protein. At the gDNA level, no somatic mutations were observed in the malignant EOC, indicating that mutations were introduced post-

transcriptionally and most likely due to impairment of pre-mRNA splicing. Defects in pre-mRNA splicing have been found in liver cancer and may lead to liver cancer progression (Berasain et al, 2010).

Conclusions

Overexpression of piRNA pathway genes and *L1* elements suggests an active role of this pathway in EOC. Expression of *PIWIL1* and *MAEL* is significantly upregulated in malignant EOC when compared to benign lesions and normal ovaries. *In situ* analyses revealed that *L1*, *PIWIL1*, *PIWIL2* and *MAEL* are strongly expressed in the cancerous cells but surprisingly *MAEL* and *PIWIL2* expression was also found in the stromal cells lining tumour tissues, suggesting a change in the tissue surrounding the cancer cells. Analysis of PIWIL1 transcript revealed that, although high level of *PIWIL1* expression was found in malignant EOC, due to defects in splicing, non-functional PIWIL1 proteins may be produced. *In vitro* real-time invasion assay showed that overexpression of piRNA pathway components such as *PIWIL1* and *MAEL* has a repressive effect on cancer cell invasiveness. Together, these results suggest a role of the piRNA pathway in the progression of EOC. It remains possible that piRNA pathway gene expression in malignant EOC may be triggered by the overexpression of *L1* in the cancer genome, and increased expression of piRNA pathway genes may have a repressive effect on ovarian cancer cell invasiveness.

Acknowledgments

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Supplementary materials

Supplementary materials include Fig. S1, S2, S3 and Table S1.

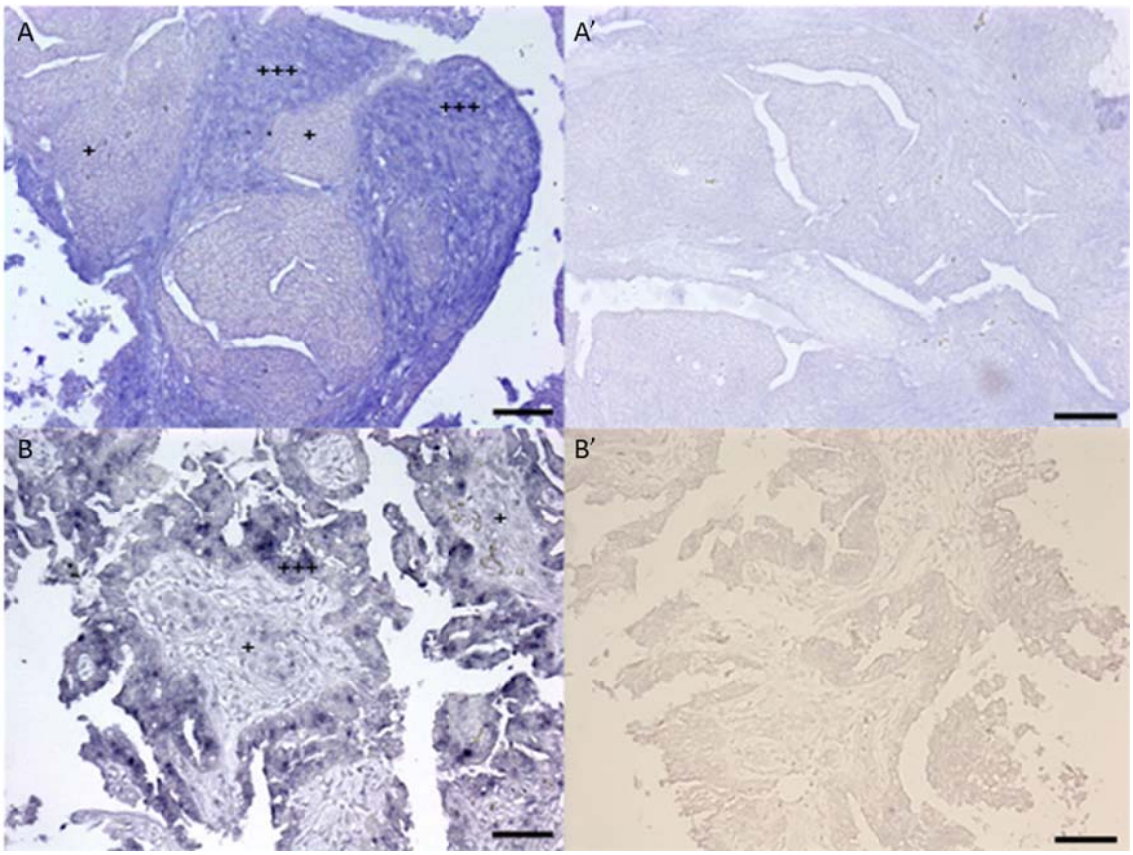


Fig. S1. Expression trends of piRNA pathway genes and *L1* in malignant EOC. (A) *MAEL* antisense from SC2 which strong expression in stromal cells (+++) compared to epithelial cells (+). (B) *L1* expression in SC3. Epithelial cells have patchy strong expression (+++) of *L1* while weak expression was observed in the stromal cells (+). +++ strong expression; + weak expression. (A'-B') Negative controls with a sense probe of *MAEL* and *L1* respectively. Scale bar = 50µm

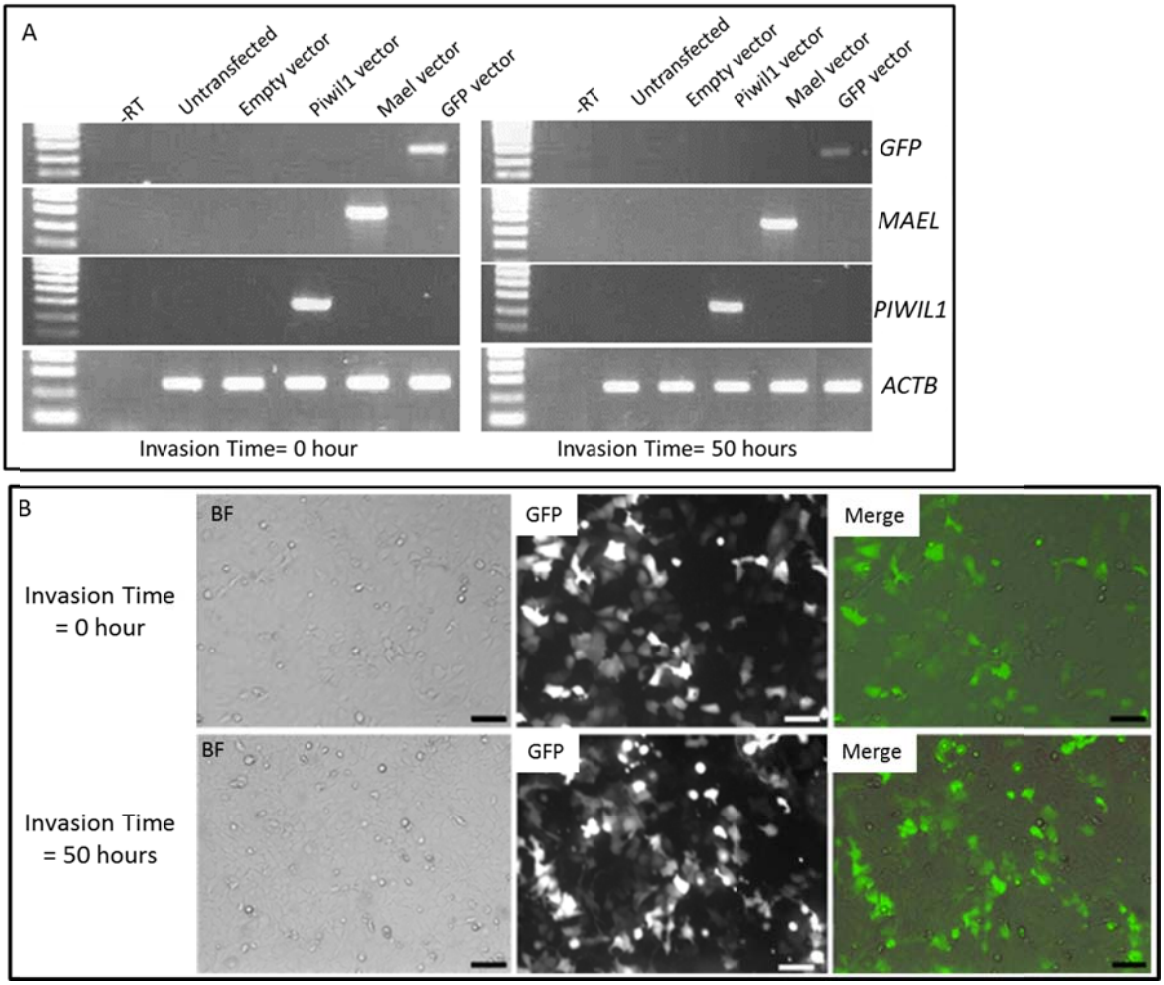
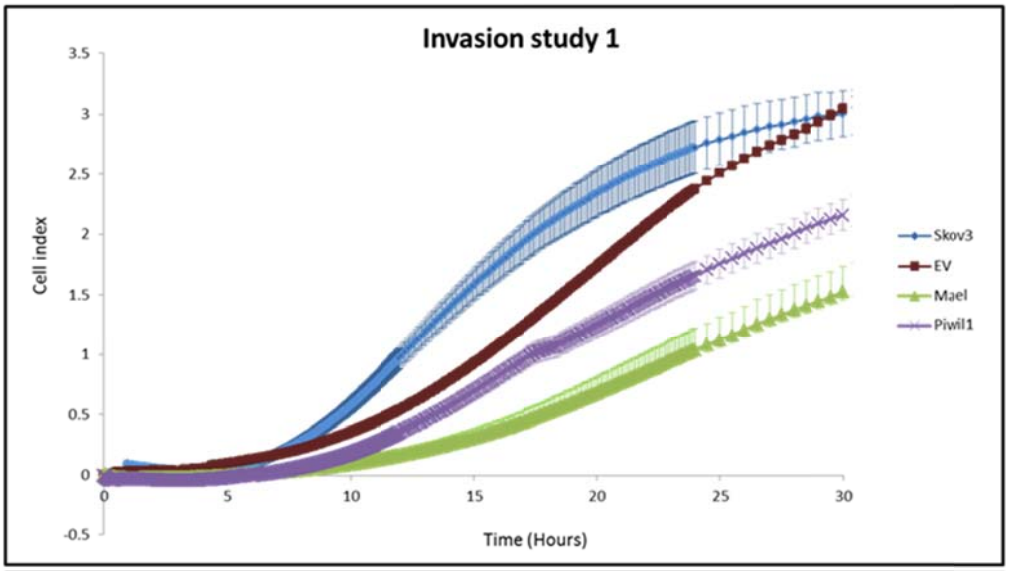
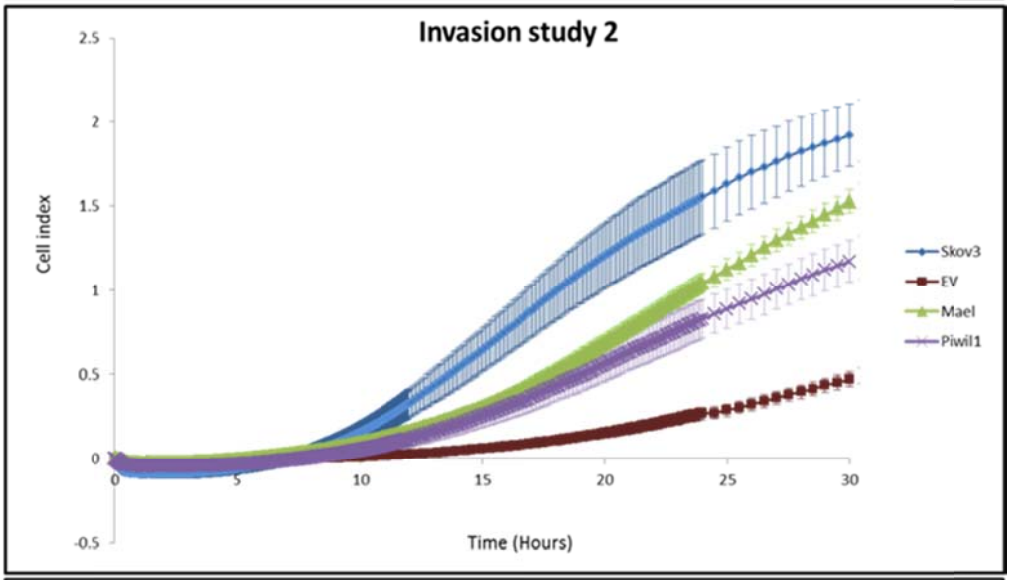


Fig. S2. Expression of *PIWIL1* and *MAEL* is maintained in transfected cells throughout the invasion study. (A) RT-PCR showing the expression of *GFP*, *MAEL* and *PIWIL1* in transfected cells (left panel) at the start of invasion study (24 hrs post-transfection) and (right panel) the end of invasion study (74 hrs after transfection). The expression of *GFP, MAEL* or *PIWIL1* can only be detected in specific vector transfected cells but not empty vector transfected or wildtype cells. (B) A high number of GFP positive cells were still observed after 74 hrs of transfection. Scale bar = 10µm.

A


Invasion study 1
Cell index
Time (Hours)
Skov3
EV
Mael
Piwil1

B


Invasion study 2
Cell index
Time (Hours)
Skov3
EV
Mael
Piwil1

C

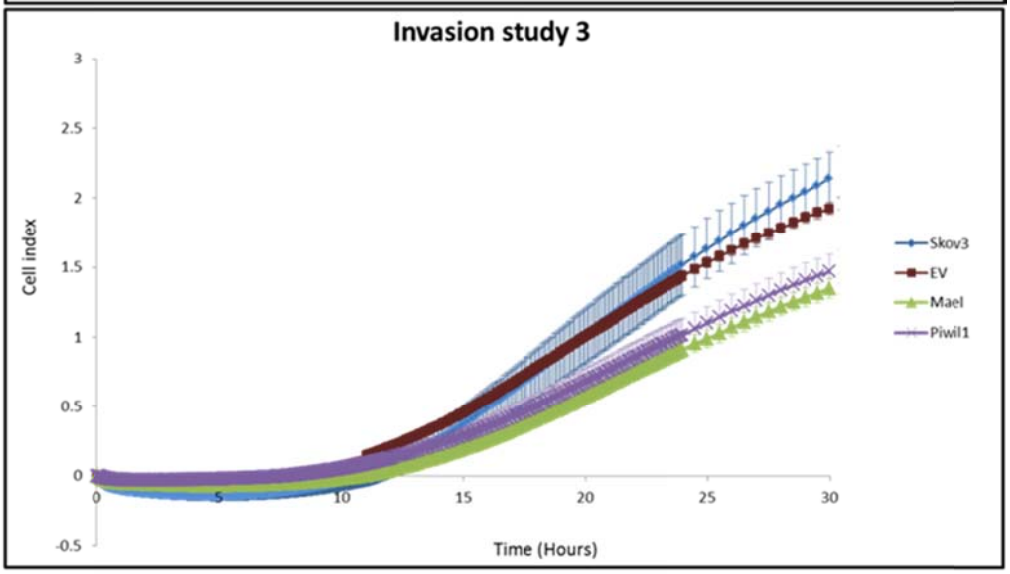

Invasion study 3
Cell index
Time (Hours)
Skov3
EV
Mael
Piwil1

Fig. S3. Three independent invasion studies of *MAEL* and *PIWIL1* transfected SKOV3 cells. (A-C) Invasion study 1 to 3 respectively. The invasiveness of untransfected SKOV3 cells, *PIWIL1* and *MAEL* transfected cells are similar in the three independent experiments. However, the cell activity of the empty vector was similar to that of untransfected cells only in experiments 1 and 3 but not in experiment 2, which could be due to technical error such that fewer cells were loaded onto wells at the beginning of the second invasion study.

Table S1: Primers for RT-PCR and *in situ* hybridisation (ISH).

Primer	Sequence 5'-3'	Tm	Experiment
MAEL ex7 forward	CTGATGATAGAACCAGAGTC	55˚C	ISH
MAEL ex8 reverse	GAATCCAAGTCTTAGAGGGC	55˚C	RT-PCR
MAEL ex11 reverse	TAGAAGAGTTGAAATGAGAGA	55˚C	ISH, RT-PCR
PIWIL1 ex15 forward	CAAGTAATCGGAAGGACAAA	52˚C	ISH, RT-PCR
PIWIL1 ex18 reverse	CTACCAATGGATTTTAGACAA	52˚C	ISH, RT-PCR
PIWIL2 ex3 forward	AACAGTTTCTAAGACCCCTC	55˚C	ISH
PIWIL2 ex5 forward	TACCTTCAGCACACCGTCC	55˚C	RT-PCR
PIWIL2 ex6 reverse	GACACTGTATTTTGACGAGGT	55˚C	RT-PCR
PIWIL3 ex15 forward	GGTGATTTGTATCCTGCCCA	55˚C	RT-PCR
PIWIL3 ex18 reverse	TGACCATCTCCCACTCCATC	55˚C	RT-PCR
PIWIL4 ex9 forward	TACTGTATCGGACCTGAATCA	55˚C	RT-PCR
PIWIL4 ex10 reverse	TTCAGCCACAGCCTTCATCAG	55˚C	RT-PCR
ACTB ex6 forward	GCCGTCTTCCCCTCCATCGT	55˚C	RT-PCR
ACTB ex7 reverse	CCTCGGTCAGCAGCACGGGG	55˚C	RT-PCR
L1 forward	CTCAAAGGAAAGCCCATCAG	52˚C	ISH, RT-PCR
L1 reverse	CGTGAGATGGGTTTCCTGA	52˚C	ISH, RT-PCR

Chapter 3

3.2 *PIWIL1* transcript variants may produce non-functional PIWIL1 in malignant epithelial ovarian cancer

Introduction

Argonaute (AGO) proteins are well-characterised RNA interference (RNAi) components (Langdon et al. 1988). Humans have eight AGO proteins with four being derived from the eIF2D/AGO subfamily (hAGO1-4) and four from the PIWI subfamily (PIWIL1-4) (Sasaki et al. 2003). In contrast to the ubiquitous expression of AGO proteins in somatic tissues, PIWIL proteins are specifically expressed in the gonads (Sasaki et al. 2003). *PIWIL* genes are highly conserved across evolution (Kurth and Mochizuki 2009; Siomi et al. 2010), especially *Piwil1* which has orthologs in all species investigated despite lineage specific loss or gain occurring to other *PIWIL* genes (Chapter 2). PIWIL1 is important in the biosynthesis of piRNAs from fish to mouse (Kuramochi-Miyagawa et al. 2001; Houwing et al. 2007; Kuramochi-Miyagawa et al. 2008) and is essential for the repression of TEs in order to maintain genome integrity (Reuter et al. 2011). The role of PIWIL1 in development and germ cell maintenance has also been shown in fish ovary and mouse testis (Kuramochi-Miyagawa et al. 2001; Houwing et al. 2007; Kuramochi-Miyagawa et al. 2008). In human, overexpression of *PIWIL1* is found in a broad range of cancers (Qiao et al. 2002), suggesting that it may be important in TE repression as well as for maintaining the stem cell like characteristic of cancer cells. Increased *PIWIL1* expression is associated with increased tumour grade in glioma (Sun et al. 2011), poor prognosis in pancreatic adenocarcinoma (Grochola et al. 2008) and soft tissues sarcoma (Taubert et al. 2007). However, the precise nature of the role played by PIWIL1 in cancer remains elusive.

Human *PIWIL1* is located on chromosome 12q24.3312 and has 21 exons which produce a 861-amino acid (aa) protein (AAC97371.2) with a molecular weight of

98.5kDA (Qiao et al. 2002). Alternative splicing produces three PIWIL1 isoforms (Fig. 1). Similar to other AGO proteins, PIWIL1 has three protein motifs: the PAZ, MID, and PIWI domains. The PAZ domain (277-391 aa) is located at the N-terminus which specifically recognizes and binds to the 2'-O-methylated 3'-end of piRNAs (Jin et al. 2012). The central MID domain is important for the binding of 5' phosphate RNA. The PIWI domain (555-847 aa) is positioned at the C-terminal end. The peptide folds as an RNAse-H endonuclease structure and (Zeng et al. 2011) incorporates a slicer catalytic site for the cleavage of target RNA during RNAi (Parker et al. 2004). In fission yeast, the PIWI domain pocket binds to the 5'end of siRNAs and other regulators to affect transcriptional silencing (Till et al. 2007). Mutation of this pocket significantly decreases the affinity of PIWI binding to siRNA (Parker et al. 2004). Piwi and Piwil proteins differ in the N-terminal region but are conserved at the C-terminal PIWI box (43 aa) region. (Cox et al. 1998).

Data presented in Chapter 3.1 shows that there is significant upregulation of PIWIL1 in malignant EOC cells compared to benign and normal tissues. However, whether these overexpressed transcripts are functional is currently unknown. To address this question, *PIWIL1* transcripts from the malignant EOC which contain the PIWI domain (from 560 aa onwards, Fig. 1) were amplified, cloned, sequenced and subjected to *in silico* analysis. Multiple transcript variants were obtained from single malignant EOC tissue. A number of transcript variants were identified including unspliced introns (intron 15, 16) and deletion of an entire exon (exon 17). These changes introduced a premature stop codon in the PIWI domain, suggesting the production of non-functional PIWIL1 proteins or proteins that function in a dominant-negative fashion in malignant EOC.

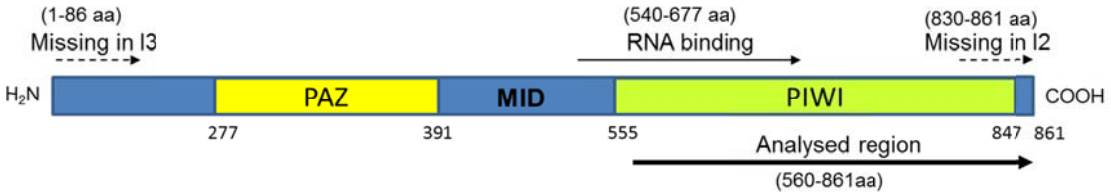


Fig. 1. Diagram of human PIWIL1 protein. Similar to other AGO proteins, PIWIL1 has a PAZ domain at the N-terminal and a PIWI domain at the C-terminal. There are three PIWIL1 isoforms. Amino acid (aa) residues 830-861 are missing in isoform 2 and aa residues 1-86 are not found in isoform 3. In this study PCR primers were designed to amplify transcripts corresponding to the PIWI domain from residue 560 to the stop codon.

Materials and methods

cDNA preparation and RT-PCR

cDNAs from malignant EOC were prepared as previously described in Chapter 3.1 (page 82). RT-PCR was performed with *PIWIL1* primers which amplified from the partial or entire PIWI domain to the stop codon from SC1. Primers were designed based on the *PIWIL1* cDNA sequence (CCDS9268). Primer details are listed in Table 1. PCR were performed using Expand High Fidelity PCR system (Roche), with proofreading DNA Taq Polymerase.

Table 1: Primers for the characterisation of *PIWIL1* transcript variants

Primer	Sequence 5'-3'	Tm
PIWIL1 ex15 forward	CAAGTAATCGGAAGGACAAA	52˚C
PIWIL1 ex18 reverse	CTACCAATGGATTTTAGACAA	52˚C
PIWIL1 stop reverse	TTAGAGGTAGTAAAGGCGGTT	55˚C
ACTB ex6 forward	GCCGTCTTCCCCTCCATCGT	55˚C
ACTB ex7 reverse	CCTCGGTCAGCAGCACGGGG	55˚C
SP6 primer	ATTTAGGTGACACTATAGAA	52˚C
T7 primer	TAATACGACTCACTATAGGG	52˚C

Genomic DNA (gDNA) isolation

gDNAs from serous carcinoma 1 (SC1) (tissue from which transcript variants were found) were isolated using conventional phenol/chloroform extraction to obtain high molecular weight DNA (Wolf et al. 1987). Briefly, frozen tissue was crushed into powder in liquid nitrogen, treated with 30µl of Proteinase K (20mg/ml) (Roche) and homogenised in lysis buffer (10mM Tris-Cl/ 1mM EDTA/ 0.1% SDS, PH8) by incubating overnight at 55ºC. After treating the mixture with 3µl of 4mg/ml DNase-free RNase A (Roche) for 1 hr at 37ºC, proteins and other impurities were removed by

phenol:chloroform extraction and centrifugation at 14,000 rpm. Further removal of protein was performed by mixing the sample with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Invitrogen) and centrifugation at 14,000 rpm. gDNAs were then precipitated with 3 vol of 100% ETOH and 0.3 vol of 7.5M ammonium acetate. DNA pellet was washed with 70% ETOH and dried pellet was dissolved in sterile water.

Cloning of *PIWIL1* transcripts

PIWIL1 PCR products from testis, malignant SC1 (two different sizes) and gDNAs were gel purified using a Qiaquick gel extraction kit (Qiagen) and then cloned into pGEM-T easy vector (Promega) according to the manufacturer's protocols. Briefly, ligation reactions contained 20-50ng of purified PCR products with 5ul of 2x rapid ligation buffer, 3U T4 DNA ligase and 50ng of pGEM-T easy vector. After 2-3 hrs of incubation at RT, 5ul of ligation reaction was added to the chemically competent DH5alpha cells and incubated on ice for 20 mins. Cells were transformed by incubation with plasmid at 42ºC for 42s and plated on 1% agar containing 64µg/ml X-gal, 0.5mM IPTG and 100µg/ml Ampicillin. Plates were left overnight at 37ºC to obtain cell colonies.

Sequencing of clones

A total of 29 white colonies were selected from each ligation reaction and plasmid DNAs were purified using standard alkaline lysis methods (Wolf et al. 1987). 1µg of plasmid DNA was digested with 5U of EcoR1 (NEB) or 100ng of plasmid DNA was

used for PCR amplification with *PIWIL1* primers. Clones with correct inserts were then sequenced (Big Dye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems) using SP6 and T7 universal primers.

Sequence analyses

Sequencing results were analysed using Geneious v.4.6.5 (Drummond A et al. 2009). Multiple alignments were performed on different clones, testis *PIWIL1* transcripts and published *PIWIL1* cDNA sequence (CCDS9268) using MUSCLE in Geneious (Drummond A et al. 2009). Clone sequences were translated and aligned using MUSCLE in Geneious.

Results

Multiple *PIWIL1* transcript variants in malignant EOC

PIWIL1 is known as a cancer/testis gene which is highly expressed in normal testis and multiple cancers (Sharma et al. 2001; Brown and Gatter 2002; Liu et al. 2006). We have identified overexpression of *PIWIL1* in malignant EOC when compared to benign ovarian tissues and normal ovaries. Furthermore, PCR amplification of the PIWI domain from EOC cDNAs produced two distinct bands compared to cDNAs from normal testis or ovary (Fig. 2A). PCR products from testis and malignant EOC were extracted (bands b and c in malignant EOC, Fig 2A, top panel) and subjected to cloning. A total of 29 clones (inserts from bands b and c, Fig 2A top panel, b and c) were selected for further PCR analysis. Different *PIWIL1* PCR product sizes were obtained from clones B and C (Fig. 2B), which may indicate the presence of multiple *PIWIL1* transcript variants in malignant EOC. This was confirmed by sequencing. Multiple alignments of clone sequences showed 42 changes (Fig. 3A, Table S1) within the PIWI domain at the nucleotide level when compared to the testis and published *PIWIL1*, suggesting the presence of different transcripts. These changes include 30 single nucleotide polymorphisms (SNPs), 2 unspliced introns and 10 different deletions (Table S1). Among these, a 73-nt deletion which comprised the entire exon 17 was present in more than one third of the clones (11 out of 29) (Fig. 3B). Furthermore, 2 clones showed partial splicing of introns 15 and 16 (Fig. 3B) such that 17bp and 20bp from 5' end of the introns 15 and 16, respectively, are unspliced. In order to confirm that the transcript variants are due to splicing defect, *PIWIL1* was sequenced at the gDNA level. No mutations were identified from exons 15-18 (where splicing errors occurred) in the genomic *PIWIL1* sequence from SC1 tissue (Fig. 4).

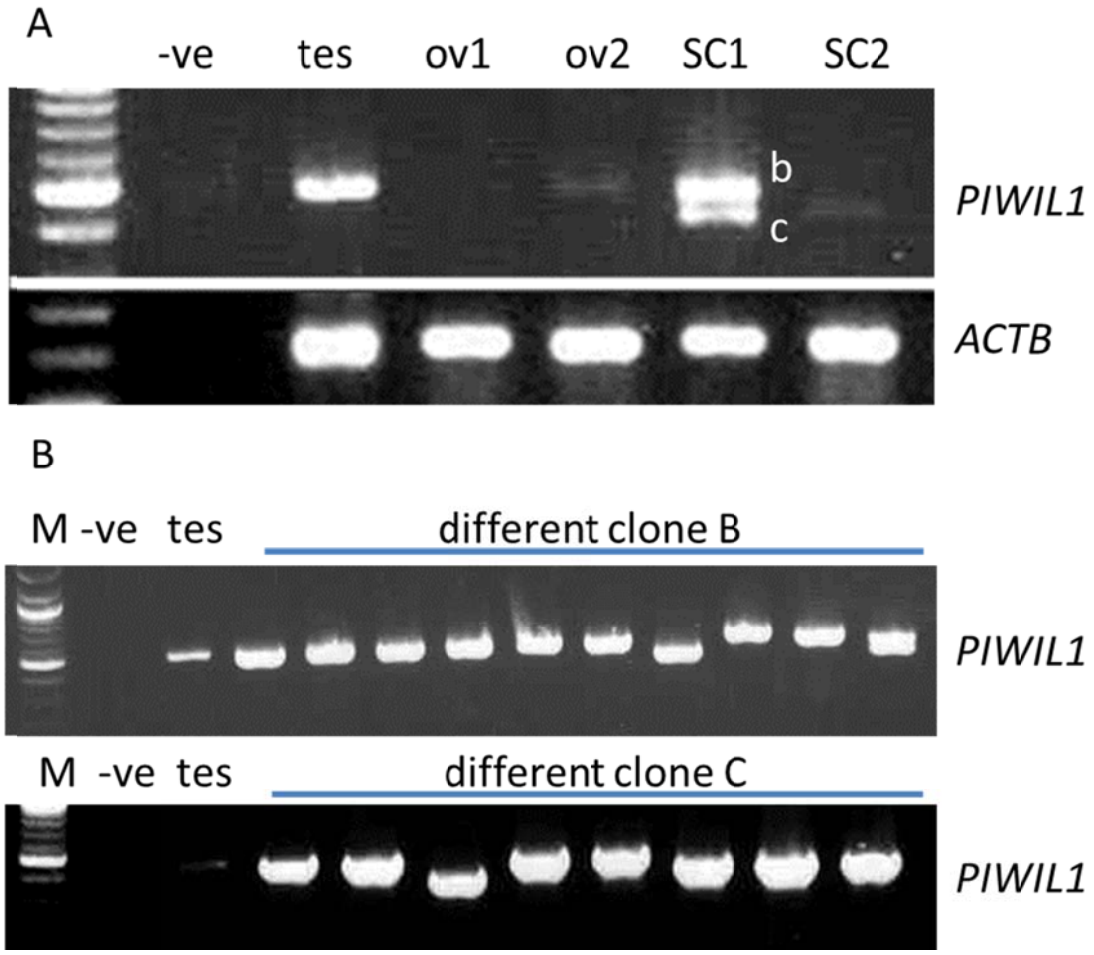


Fig. 2. *PIWIL1* transcript variants in serous carcinoma 1. (A) *PIWIL1* expression in control tissues (human testis, normal ovary (ov) 1, 2) and SC1 and 2. In the positive controls, one ~500bp band was amplified, but in malignant SC**1**, two bands were obtained i.e. bands b and c. (B) PCR with *PIWIL1* primers on 20 clones, 10 clones from bands b and c respectively. PCR products with different sizes were observed in different clones from bands b and c.

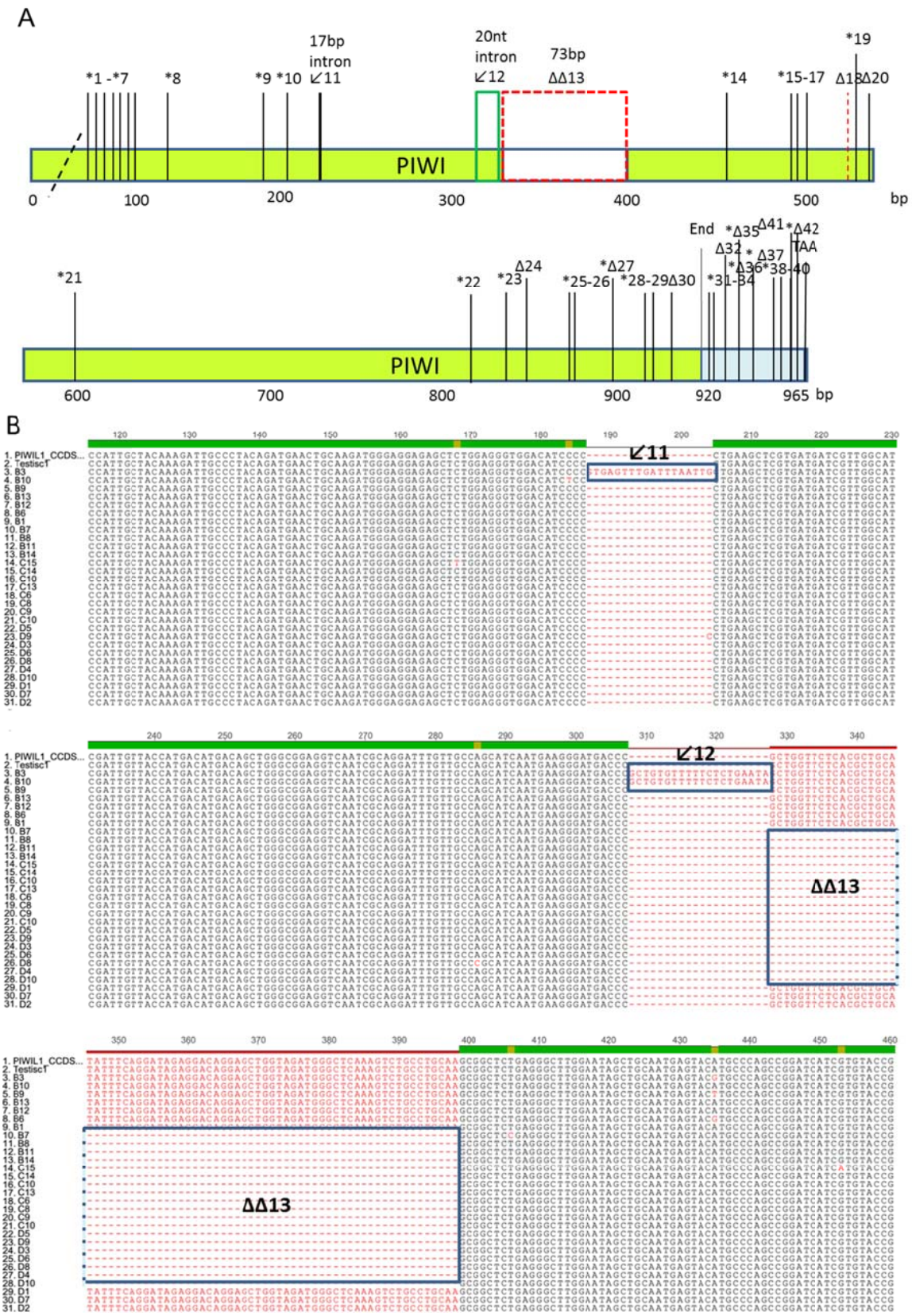

A
17bp intron ↙11
20nt intron ↙12
73bp ΔΔ13
*1 -*7
*8
*9 *10
*14
*15-17
Δ18Δ20
*19
PIWI
0
100
200
300
400
500
bp
*21
*22
*23
Δ24
*25-26
*Δ27
*28-29Δ30
*31-34
End
*Δ35
Δ41
*Δ42
Δ32
TAA
*Δ37
*Δ36
*38-40
PIWI
600
700
800
900
920
965 bp
B
↙11
↙12
ΔΔ13
ΔΔ13

Fig. 3. *PIWIL1* transcript variants from serous carcinoma 1. (A) Diagram showing the position and type of mutations in the PIWI domain until the end of the gene. A total of 42 changes were found in the transcript variants including SNPs (*), unspliced introns (↙) and deletions (Δ). (B) cDNA multiple alignment (partial) showing that most of the clones have a deletion of exon 17 (ΔΔ13) and 3 clones have partial splicing of intron 15 and 16 (↙11, ↙12). PIWIL1_ccds: published cDNA of *PIWIL1* (CCDS9268); Testis C1: testis transcript clone 1; B1-3, B6, B9-B14, C6, C8, C9, C10-C15, D1-D10: clones with *PIWIL1* transcripts from SC1.

Fig. 4. No mutation was found at the gDNA level in serous carcinoma 1. SC1 gDNA was aligned with published *PIWIL1* gDNA (ENSG00000125207) sequence from exon 15 to exon 18. All exon sequences and partial intron sequences were shown. Green bar indicates 100% conservation between the aligned sequences.

Possible non-functional *PIWIL1* variants

In order to predict if the above post-transcriptional alterations affect PIWIL1 function, clone sequences were translated and compared to the testis control and published peptide sequence (AAC97371.2) (Fig. 5A). Loss of the entire exon 17 (73-nt deletion) in 11 clones introduced a premature stop codon in the PIWI domain (Table 2). Similarly, stop codons were found in clones that have unspliced introns. SNPs resulted in the formation of stop codons and peptide substitutions in some clones (Fig. 5A, Table 2). 79.30% (23 out of 29 clones) of the sequenced clones contain premature stop codon in the PIWI domain (Fig. 5B) which might result in the production of non-functional and/or truncated PIWIL1 protein with unknown function. 14% of the clones (4 out of 29 clones) have single aa substitutions which in half of these clones, the substituted aa has less than 60% similarity to the original aa. Only 7% of the clones (2 out of 29 clones) have a wild type PIWI domain, indicating large numbers of transcript variants in malignant SC1 might be non-functional. Cloning data presented was from one malignant EOC and exon 17 deletion was confirmed in other ovarian cancer tissues (data not shown). In addition, double bands which might indicate the presence of non-functional *PIWIL1* variants were also observed in other malignant EOC tissues by RT-PCR (Fig. 6). Furthermore, *PIWIL1* transcript variants might also found in other EOC which PCR amplified only a single band as several transcript variants were found in band B which are ~500bp (same size as the testis band) from SC1.

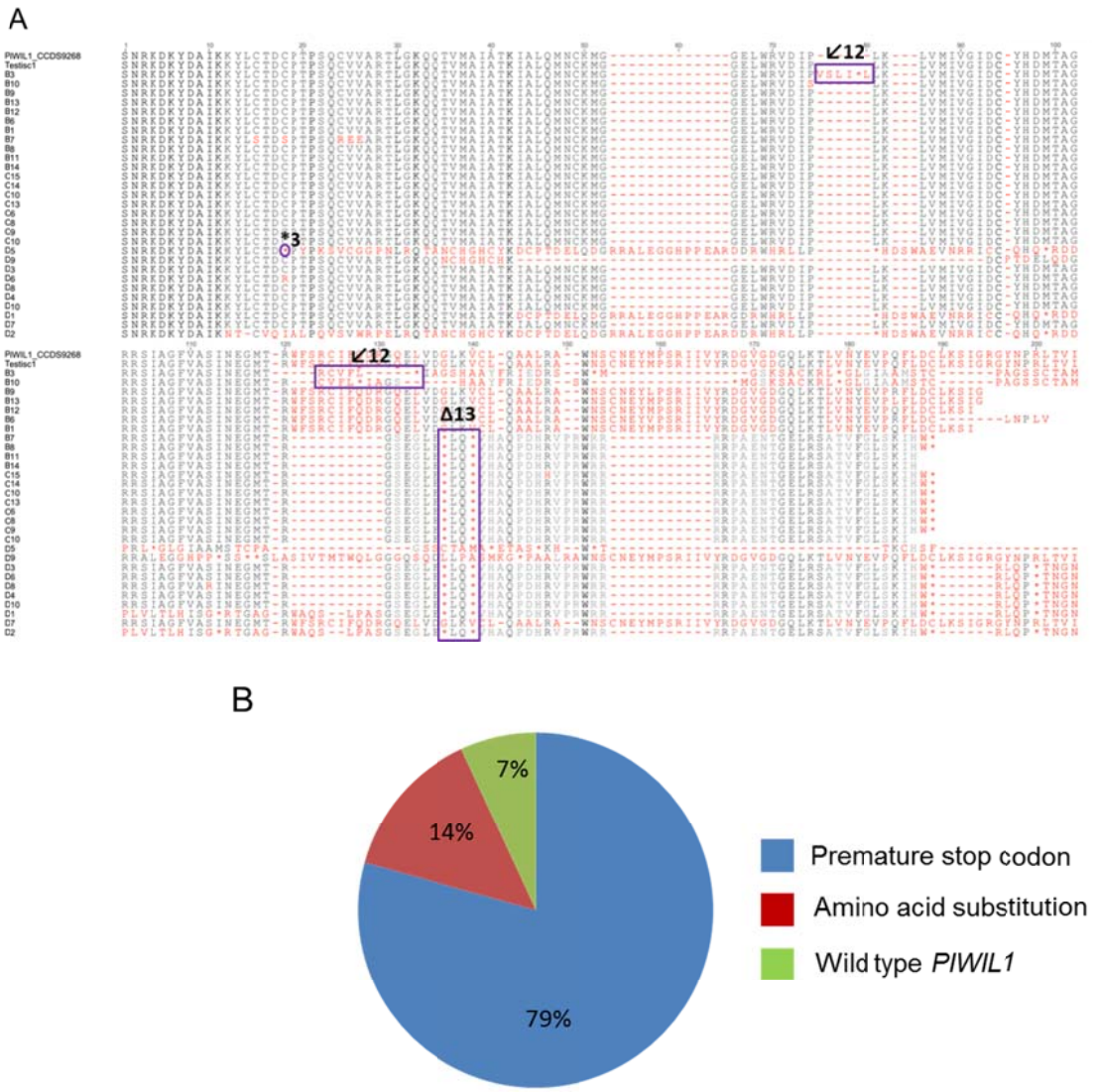


Fig. 5. *PIWIL1* transcript variants encoded premature stop codon. (A) 19 clones with exon 17 (ΔΔ13) deletions, 3 clones with unspliced introns and 1 clone with a SNP resulted in the introduction of premature stop codons. (B) Pie chart showed the percentage of clones with premature stop codons, aa substitutions and wild type PIWIL1. PIWIL1_ccds9268: published PIWIL1 peptide sequence (AAC97371.2); Testis C1: translated testis clone 1; B1-3, B6, B9-B14, C6, C8, C9, C10-C15, D1-D10: clones with PIWIL1 translated sequence. * inside the boxes in panel A indicates premature stop codon.

Table 2: Summary of possible transcript variants in PIWIL1 from serous carcinoma 1

Peptide changes	Corresponding changes in transcript variant	Clone
Premature stop codon (23/29 clones)	SNP (*3)	D3
	17-nt unspliced intron 15 (↙11)	B3
	20-nt unspliced intron 16 (↙12)	B3, B10
	73-nt deletion (ΔΔ13)	B7, B8, B11, B14, C15, C14, C10, C13, C6, C8, C9, C10, D3, D6, D8, D4, D10, D1, D2
Amino acid substitution (< 60% similarity) (2/29 clones)	SNP (*15)	B13
	SNP (*28)	D7
Amino acid substitution (80-100% similarity) (2/29 clones)	SNP (*21)	B9, B6

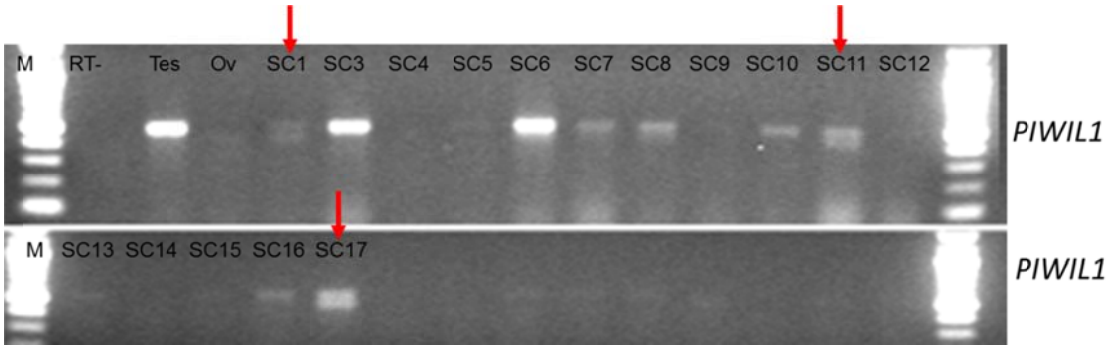


Fig. 6. RT-PCR of *PIWIL1* in malignant serous carcinomas. Double bands of *PIWIL1* transcript were observed in more than one malignant SC tissues. Red arrow indicates SC tissues with double bands, suggesting the presence of multiple *PIWIL1* transcripts. SC: serous carcinoma.

Discussion

Human *PIWIL1* is located on chromosome 12q24.3312. Genome-wide association studies indicate that this is a breast cancer and estrogen receptor related disease susceptible region, accounting for about 8% of the disease (Antoniou et al. 2012; Ghoussaini et al. 2012). Overexpression of *PIWIL1* has been widely reported in seminomas (Qiao et al. 2002), gastric and pancreatic cancers (Liu et al. 2006), soft-tissue sarcomas (Taubert et al. 2007), gliomas (Sun et al. 2011) and malignant EOC (Chapter 3.1). However, as yet somatic mutations of *PIWIL1* have not been identified in cancers. This study is the first to analyse *PIWIL1* transcripts in malignant EOC. Although *PIWIL1* transcript level was significantly increased in malignant EOC compared to benign and normal tissues, cloning and sequencing of these transcripts suggested that transcript variants may not produce functional PIWIL1. A range of changes at the transcript levels were found in *PIWIL1* including unspliced introns and loss of exons. These nucleotide changes may introduce premature stop codon in almost all of the clones tested (23/29), suggesting that although *PIWIL1* expression is high in malignant tissues, the corresponding protein (if any) would be truncated and is predicted to result in a loss of function or may have dominant-negative effect on PIWIL1 function. The functions of the PIWI domain in RNA splicing and small RNA binding are highly conserved from fission yeast to human (Till et al. 2007). Nucleotide changes at the transcript level in PIWI domain in malignant EOC suggest that these proteins may not be functional. At the gDNA level, no somatic mutations were observed in the malignant EOC, indicating that nucleotide changes were introduced post-transcriptionally and most likely due to impairment of pre-mRNA splicing. Defects in pre-mRNA splicing have been found in liver cancer and may lead to liver cancer progression (Berasain et al. 2010).

Overexpression of PIWIL1 in ovarian cancer SKOV3 cells results in decreased cell invasiveness *in vitro* (Chapter 3.1), suggesting a repressive role of PIWIL1 in cancer invasion. In the *in vivo* situation, although high level of *PIWIL1* expression was found in malignant EOC (Chapter 3.1), due to defects in splicing, non-functional PIWIL1 proteins may be produced, and thus, the repressive effect of PIWIL1 may not be exerted.

To further confirm if splicing defects lead to the production of non-functional PIWIL1 proteins, future experiments analysing *PIWIL1* at the transcript and protein level need to be performed. Sequencing of *PIWIL1* transcripts from malignant EOC which have double bands such as SC11 and SC17 (Fig. 6) may allow confirmation of the splicing defects in *PIWIL1* transcripts. Immunohistochemistry and Western blot experiments are needed to assess levels and variants of PIWIL1 in malignant EOC. Further investigation on splicing control mechanism and post-transcriptional regulation of PIWIL1 may provide insight into the defects in post-transcriptional control in cancers.

Supplementary materials

Supplementary material include Table S1

Table S1: Nucleotide changes in PIWI domain in *PIWIL1* transcripts

Symbol	Mutant locus	Mutation	Clone
*1	64	A→T	B7
*2	69	A→G	B7
*3	73	A→T	B7
*4	91	T→A	B7
*5	93	T→A	B7
*6	95	T→A	B7
*7	98	T→A	B7
*8	109	T→C	C14
*9	186	C→T	C15
*10	202	C→T	B3
↙11	205	17-nt unspliced intron 15	B3
↙12	326	20-nt unspliced entire intron 16	B3, B10
ΔΔ13	327	73-nt deletion	B7-B8, B11, B14, C6, C8-C10, C13-C15
*14	452	C→T	B3, B6, B9
*15	499	C→T	C15, B13
*16	509	C→T	B3
*17	529	C→T	B9
*18	530	A→T	B9, B10
*19	532	A→G/T	B3, B6, B9, B10, B13
Δ20	548	Single deletion	B3, B6
*21	555	A→G	B9, B6
*22	849	T→C	D2
*23	849	A→G	D3, D9

Δ24	856	Single deletion	D3
*25	877	A→G	D3
*26	887	A→T	D9
*/Δ27	889	A→G or single deletion	D9, D3
*28	897	T→C	D7
**28	899	TC→CT	D3
Δ*29	901	2-nt deletion and T→G	D3
*30	910	A→G	D8, D3
Δ31	915	Single deletion	D3, D9
*32	921	A→G	D3
*33	923	A→G	D6
Δ+**34	931	2-nt deletion and CTG→TCT	D3
Δ35	935	Single deletion	D3, D9
Δ36	941	Single deletion	D3, D9
*37	952	T→A	D8

* Single nucleotide change; ↙ unspliced intron; ΔΔ large deletion; Δ single deletion.

Chapter 3

3.3 piRNA pathway genes and the origin of epithelial ovarian cancer

Introduction

Ovarian cancer is the most lethal type of gynaecological malignancy among women from the Western world according to the 2012 report from National Institute of Health. In 2012, it has been estimated that 22,280 new cases will be diagnosed and 15,500 deaths will be due to ovarian cancer (American-Cancer-Society 2012). Due to the lack of early detection, the 5-year survival rate of this disease is less than 30% (Ricciardelli and Oehler 2009). Whilst all human ovarian cells, including epithelial, stromal and germ cells, may transform into cancer, around 90% of ovarian cancers are derived from the single layer of the epithelial cells covering the ovary. Epithelial ovarian cancer (EOC) is divided into four main histotypes which are serous, mucinous, endometrioid and clear cells. Molecular profiling of the different EOC subtypes has been conducted previously (Farley et al. 2008; Kurman and Shih Ie 2008). However, so far, no common genetic mutations have been identified in terms of the initiation and progression of ovarian cancer (Ricciardelli and Oehler 2009).

The origin of EOC is currently unknown. Studies of ovarian cancer etiology have proposed several hypotheses for EOC pathogenesis (Ricciardelli and Oehler 2009). One of the widely accepted models is the incessant ovulation hypothesis (Fathalla 1971; Casagrande et al. 1979). In this hypothesis, a wound is created on the ovarian surface epithelium (OSE) after the rupture of follicles during each ovulation. Follicular fluid released during ovulation can induce inflammation as it contains reactive oxygen species which may change the microenvironment of the OSE cells (Kurman and Shih Ie 2011). Other inflammation conditions, such as endometriosis or pelvic inflammatory disease, also increase the risk of ovarian cancer development (Parazzini et al. 1996; Melin et al. 2006). On the other hand, the wound created during ovulation is repaired

by the OSE cells by increasing cell proliferation (Okamura and Katabuchi 2001). The resulting high mitosis rate, due to the increase in cell proliferation, may increase the chance of DNA damage and lead to oncogenic mutations (Auersperg et al. 1991). The OSE cells are able to migrate to cover the ovulatory defect to repair the wound (Kurman and Shih Ie 2011). However, epithelial cells on the surface epithelium lack this mobility. To acquire cell mobility, OSE cells differentiate into fibroblast type mesenchymal cells via the epithelial to mesenchymal transition (EMT) (Gubbels et al. 2010). EMT helps OSE cells to move via changes in the extracellular matrix (Ahmed et al. 2006). A higher number of ovulation events indicate that more EMT and more inflammation at the ovarian surface epithelium are occurring, which increases the risk of ovarian cancer development. Studies in rats have shown that continuous subculturing of OSE cells *in vitro* results in chromosome aberrations and cellular transformation into cancerous cells (Godwin et al. 1992; Testa et al. 1994). In addition, organelles and nuclei in the OSE cells are destroyed after ovulation, suggesting that there may be an accumulation of mutations and genetic alterations in these cells which may lead to cancer progression (Okamura et al. 1980).

Ovarian inclusion cysts may be formed after ovulation. After each ovulation event, OSE cells proliferate and migrate to repair the wound on the surface epithelium. Then the healing sites are disconnected from the surface epithelium and the resulting invagination of ovarian surface epithelium leads to the formation of inclusion cyst which enters the stroma (Okamura and Katabuchi 2001) (Fig. 1). Exposure of the epithelium lining, around the inclusion cyst, to the ovarian stromal microenvironment may create changes in the epithelial cells from the cyst (Feeley and Wells 2001). Distinct level of gene expression were identified in the epithelial cells (originally from

the surface epithelium) which cover the inclusion cyst. Furthermore, increased secretion of estrogen and progesterone receptors were found in epithelial cells from inclusion cyst, but not from surface epithelium cells (Okamura and Katabuchi 2001). In addition, different gene expression profiles, such as increased expression of genes involved in proliferation and decreased amount of apoptosis, are observed in epithelial cells from benign inclusion cyst, when compared to their surface epithelium cell counterparts (Pothuri et al. 2010). Aneuploidy is also found in the epithelial cells from inclusion cyst but not in surface epithelium cells (Pothuri et al. 2010). Histological studies of early *de novo* ovarian cancers suggested that a subset of EOC arises from OSE cells (Bell and Scully 1994; H et al. 1995). In addition, the *in vivo* transition of epithelial cells lining the inclusion cyst to abnormal cells, and eventually to cancerous cells, was observed in advanced ovarian carcinomas (Okamura and Katabuchi 2001). Moreover, other research has shown that some high grade SC may also derive from ovarian inclusion cyst (Pothuri et al. 2010).

The corpus luteum (CL) derives from an ovarian follicle after ovulation. The release of a mature oocyte from the follicle results in the collapse and folding of the follicular wall, which consequently forms a CL. CL is thus derived from granulosa and thecal cells after ovulation. As an important hormonal structure, CL produces both progesterone and estrogen hormones, and is maintained for 14 days in the stroma if no fertilization occurs. Previous chapters have shown robust expression patterns of piRNA pathway genes in mammalian ovaries. Both the *MAEL* and *PIWI* transcripts are found in the follicular granulosa and cumulus cells before ovulation. However, it is unknown as to whether CL, which is derived from granulosa cells, continues to express the piRNA pathway genes after its formation.

Fig. 1. Hypothesised origin of EOC from an inclusion cyst. (1) An oocyte with cumulus cells is released from the ovary after each ovulation event. (2) The mural granulosa cells lining the follicle remain in the ovary and later develop into corpus lutuem. (3) After the release of the oocyte, a wound on the ovarian surface epithelium is generated. OSE cells increase proliferation and migrate to repair the wound. (4) Invagination of the surface epithelium cells from around the wound site into the stroma results in the formation of an inclusion cyst. (5) Exposure of the epithelial cells lining the inclusion cyst to the stromal microenvironment causes the sequential accumulation of genetic alterations in the epithelial cells which may lead to tumour development from the inclusion cyst. Modified from Katabuchi and Okamura (2003).

DNA methylation is an essential epigenetic modification for normal cell function, and its alteration may result in malignant tumour transformation (Bork et al. 2010; Houshdaran et al. 2010). Methylation occurs at the cytosine of CpG dinucleotides. After DNA replication, DNA methyltransferase I establishes the same DNA methylation on the newly synthesized DNA strand. Thus, the daughter cells inherit the same methylation status as the parental cells. Changes in DNA methylation is a hallmark of cancer genomes. In colon cancer, global DNA hypomethylation is correlated with genomic damage and cancer development (Kamiyama et al. 2012). In advanced ovarian cancer, DNA hypermethylation has been found in genomic regions which regulate gene associated with DNA damage response (Teodoridis et al. 2005). A recent study predicts that DNA demethylation in normal colon tissue may result in the formation of multiple cancers (Kamiyama et al. 2012). However, it is currently unclear whether there are any changes in global DNA methylation levels in the inclusion cyst of benign ovarian lesion and the corpus luteum cells of normal ovary.

PIWI genes are well known for their role in stem cell maintenance in animals (Cox et al. 1998; Deng and Lin 2002). Overexpression of *PIWIL1* and *PIWIL2* is found in a large number of tumours (Qiao et al. 2002; Lee et al. 2006; Liu et al. 2006; Taubert et al. 2007; Grochola et al. 2008; Liu et al. 2010; Sun et al. 2011) and in malignant EOC (Chapter 3.1), suggesting a role for the piRNA pathway genes in maintaining the stem cell-like characteristic of these cancers. This research links piRNA pathway gene expression in ovarian somatic cells to ovarian cancer, which may support the idea that OSE cells can be reprogrammed into potential cancer cells via their contacts with granulosa and cumulus cells, during the rupturing of an oocyte and later in the inclusion cyst. In human and mouse, the expression of piRNA pathway genes and *L1* was tested

using RNA *in situ* hybridisation on the inclusion cyst and corpus luteum, which contains post-ovulatory granulosa cells. Furthermore, to understand the global DNA methylation status of the epithelial cells in corpus luteum and inclusion cyst, immunohistochemistry with an anti-methylcytosine antibody was performed.

Materials and methods

Tissues and patient ovarian specimens

Benign and normal post-menopausal ovarian tissues were collected with the approval of the Ethics Committee during surgery at the Royal Adelaide Hospital, Adelaide, South Australia by A/Prof. Martin K. Oehler.

RNA *in situ* hybridisation

See method in Chapter 2 (page 43).

Hematoxylin and Eosin Staining

Formalin-fixed paraffin-embedded tissue sections were deparaffinised in Xylene and subsequent washes in graded ethanol. Nuclei were stained with Mayer's haematoxylin (Invitrogen) for 2-5 mins and the slide was washed under running tap water for 2-3 mins. The slide was then rinsed in 0.3% acid-alcohol for a few seconds and cytoplasm was stained with Eosin for 2 mins. Slides were dehydrated in 70%, 90% and 100% ETOH for 3 mins, each then cleaned in Xylene and covered with Entellan mounting media (Merck).

Immunohistochemistry

Immunohistochemistry with an anti-5-methylcytosine antibody (anti-5-MeC) (Calbiochem) was modified from other study (Piyathilake et al. 2001). 5-μm thick tissue sections were cut and put on superfrost plus slides. The negative control was

carried through the same procedure as the experimental slide, but without incubation with the primary antibody. Formalin-fixed, paraffin-embedded tissue sections were deparaffinised in xylene and subsequently washed in graded ethanol. Sections were subjected to antigen retrieval by boiling in 0.01mol/L citric acid, pH 6.0, in a microwave oven set at full potency for 15 mins, and then immersed in 3.5N HCL for 15 mins at RT to expose the CpG. Sections were treated with 3.0% H_2O_2 for 4 mins to quench endogenous peroxidase activity, blocked with goat serum (3%) for 1 hr at RT to suppress nonspecific staining, and incubated with anti-5-MeC antibody (1:100 dilutions) for 1 hr at RT or overnight at 4˚C. The slides were then incubated with a secondary antibody (i.e. anti-mouse-HRP) in 3% goat serum/PBS for 1 hr at 37˚C in a humid chamber and developed to visualise for 10 mins with Diaminobenzidine tetrahydrochloride (DAB) solution (1ml of DAB+ 9ml 1xPBS+ 12.5ul of H_2O_2). Finally, slides were counterstained with haematoxylin, dehydrated in 100% ETOH and Xylene, and then mounted using Entellan mounting media (Merck).

Results

Increased global DNA methylation but no expression of piRNA pathway genes and *L1* in the CL of mouse and human

Global DNA methylation was investigated in mouse ovarian follicles and CL. Increased DNA methylation was found in mouse CL (Fig. 2A, B) when compared to preantral, early antral and antral follicles (Fig. 2C, D). RNA *in situ* hybridisation was performed to investigate the expression of *PIWIL1*, *2*, *MAEL* and *L1* in the CL of mouse and human. The expression of *Mael* and *Piwil2* is only observed in the growing follicles but not in the CL in mouse ovary (Fig. 3A, B). Human has much larger CL structures. Similar to mouse, no expression of the piRNA pathway genes and *L1* was found in the human CL (Fig. 3C-F).

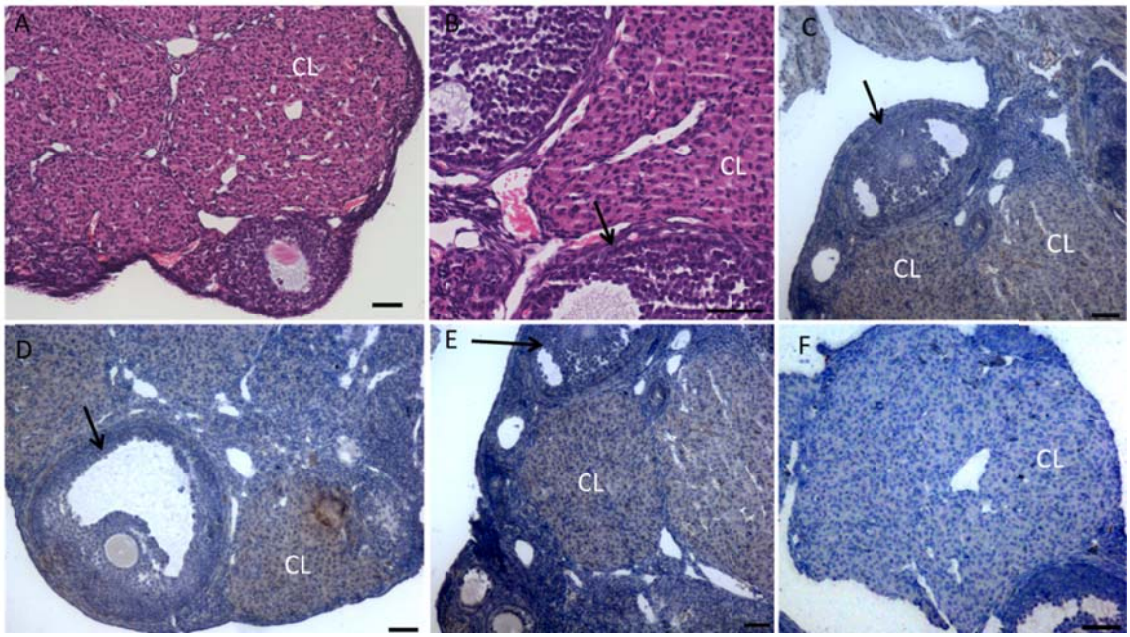


Fig. 2. Possible increased global DNA methylation in the corpus luteum. Immunohistochemistry with anti-5-MeC on mouse ovary. (A-B) H&E staining on mouse CL. (C) Early antral follicle and (D) antral follicle are less methylated compared to (E) CL. (F) Negative control for immunohistochemistry. Arrow indicates different stages of follicles. CL: corpus luteum. Scale bar = 50μm.

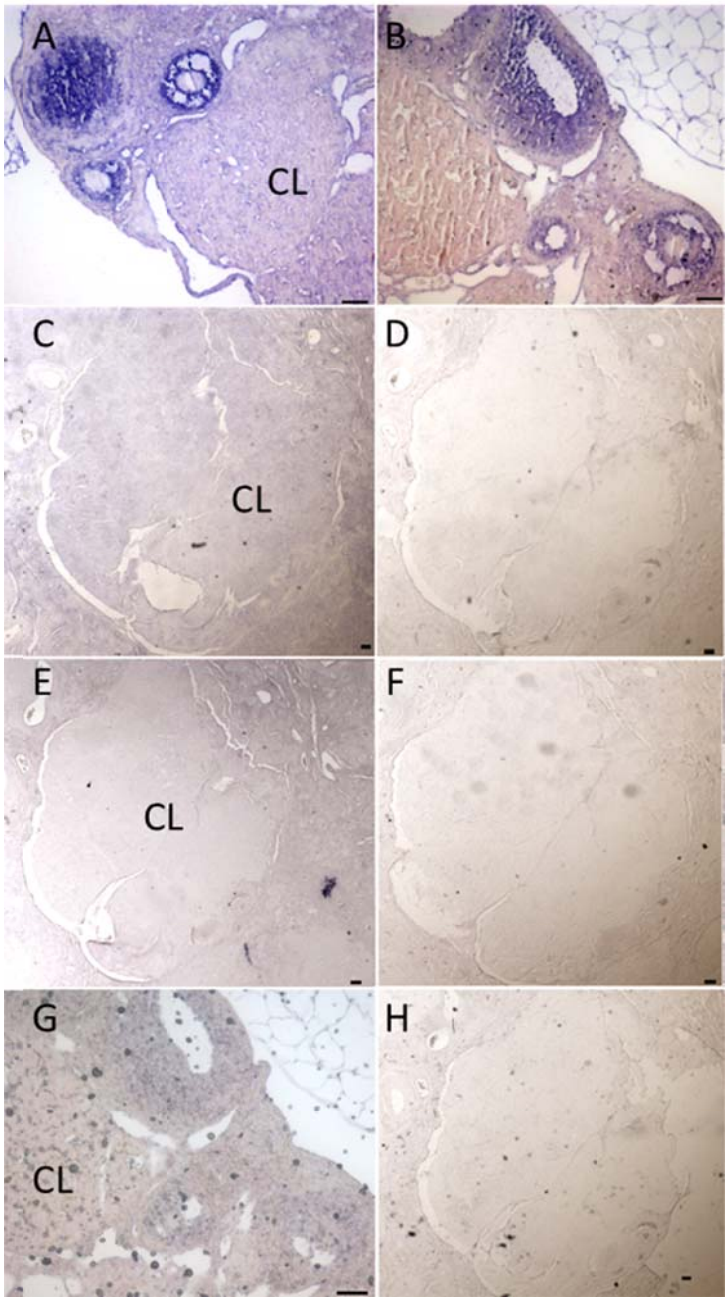


Fig. 3. piRNA pathway genes are not expressed in the mouse and human corpus luteum. RNA *in situ* hybridisation of (A) *Mael* and (B) *Piwil2* on mouse CL. *Mael* and *Piwil2* are expressed in the granulosa and cumulus cells in the mouse follicles, but no expression was found in the CL. *In situ* hybridisation of (C) *MAEL*, (D) *PIWIL1*, (E) *PIWIL2* and (F) *L1* on human CL. Sense probes were used as negative controls in (G) mouse and (H) human CL. CL: corpus luteum. Scale bar = 50µm.

L1 is expressed in the epithelial cells lining the inclusion cysts but alteration of global DNA methylation in these cells has not been detected

Inclusion cysts were obtained from benign serous carcinoma samples. Fig. 4A shows an inclusion cyst which is composed of stromal cells and covered by a layer of squamous-cuboidal epithelial cells. The global DNA methylation staining in the lining epithelial cells is similar to the negative control, indicating no increase in global DNA methylation in these cells. Interestingly, expression of *L1* was found in a subpopulation of epithelial cells in the inclusion cysts (Fig. 4D-E). The expression level of *L1* was much lower than that observed in the malignant EOC (Chapter 3.1, Fig. 3), but higher than normal epithelial cells. In addition, the spatial expression pattern of *L1*, i.e. not all epithelial cells have *L1* expression, is similar in the epithelial cells in both inclusion cysts and malignant tumours. Expression of *PIWIL1*, *PIWIL2* and *MAEL* were also examined in the inclusion cysts (Fig. 5). Very low expression of these genes was found in the epithelial cells lining inclusion cysts. Interestingly, expression of *PIWIL2* was found in the stromal cells of the inclusion cysts, similar to the stromal expression of this gene in EOC (Chapter 3.1, Fig. 3).

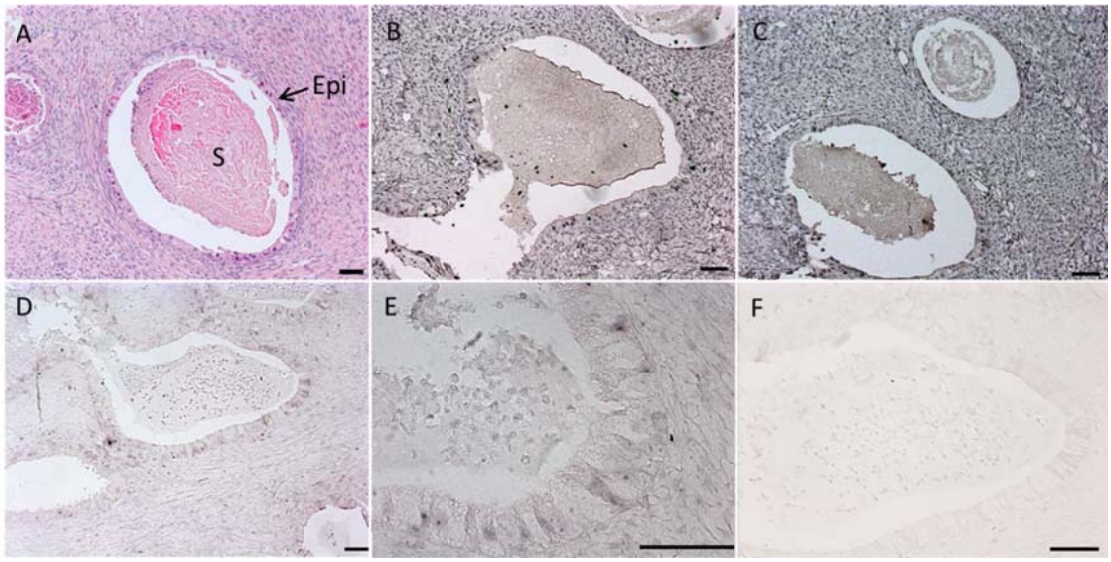


Fig. 4. Global DNA methylation and *L1* expression in human inclusion cysts. (A) H&E staining showing the morphology of an inclusion cyst from a benign serous tumour. Stromal cells can be found within the inclusion cysts surrounded by a single layer of squamous-cuboidal epithelial cells. (B) Immunohistochemistry with anti-5-MeC antibody to detect global DNA methylation in inclusion cysts. (C) Negative control for immunohistochemistry. No detectable difference in staining between experiment and control. (D-E) Increased expression of *L1* in some epithelial cells in the inclusion cysts. (F) *L1* sense negative control. S indicates stromal cells. Epi and arrow indicate epithelial cells. Scale bar = 50μm.

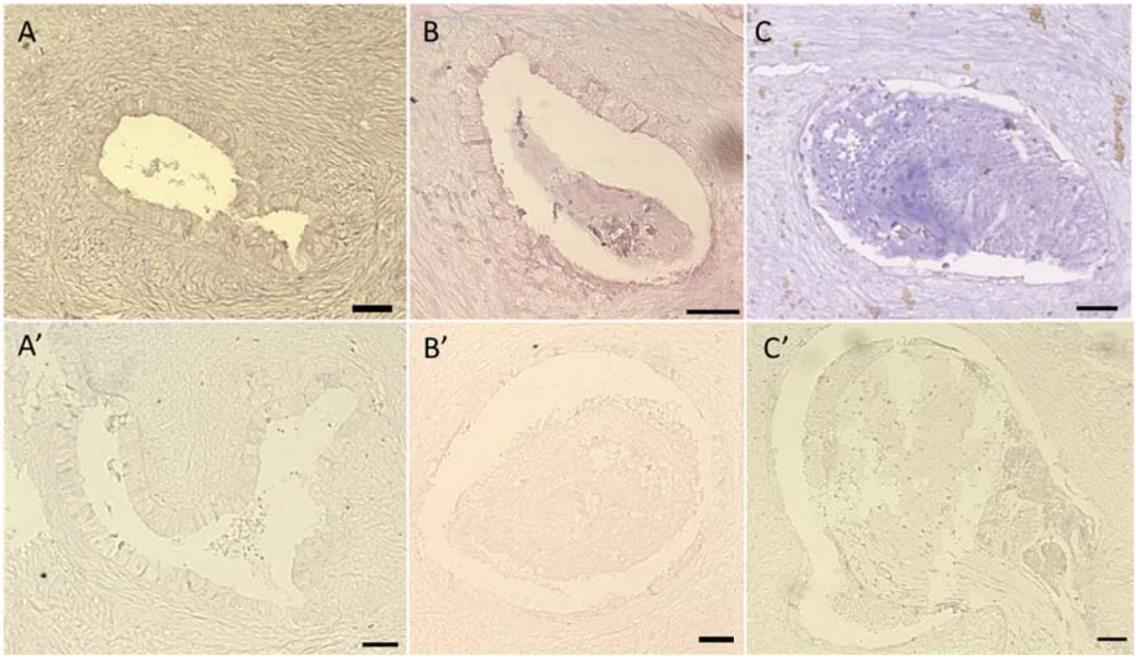


Fig. 5. Expression of *PIWIL1*, *2* and *MAEL* in human inclusion cysts. Low expression of (A) *MAEL*, (B) *PIWIL1* and (C) *PIWIL2* in the epithelial cells lining the inclusion cyst. Stronger expression of *PIWIL2* was found in the stromal cells. (A'-C') Negative controls with sense probe of *MAEL*, *PIWIL1* and *PIWIL2* respectively. Scale bar = 50μm.

Discussion

piRNA pathway genes such as *PIWIL1* and *PIWIL2* are required for stem cell maintenance in animals (Cox et al. 1998; Deng and Lin 2002). *PIWIL2* and *MAEL* expression was found in the granulosa and cumulus cells of developing follicles (Chapter 2), suggesting a role for the piRNA pathway in folliculogenesis. To understand the possible roles of piRNA pathway in the ovary, expression of piRNA pathway genes and *L1* was investigated in postovulatory structures such as the CL (which develops from granulosa cells after ovulation) and inclusion cysts. Unlike developing follicles, no expression of piRNA pathway genes was observed in human and mouse CL, suggesting changes in gene expression after ovulation. This is not surprising as distinct gene expression patterns and epigenetic regulation were commonly found in granulosa cells before and after ovulation (Seneda et al. 2008). Interestingly, by using an antibody targeted to the 5-methyl-cytosine, we observed possible higher DNA methylation in the mouse CL compared to the growing follicles. This further supports the hypothesis that epigenetic changes are occurring in the post-ovulatory granulosa cells. Similarly, reduced methylation of the H3-K4 histone mark has been demonstrated in granulosa cells before and after ovulation in porcine (Seneda et al. 2008). So far, global DNA methylation levels have not been investigated in the mouse CL. The consequences of possible higher global DNA methylation levels in the CL, compared to other follicles, are currently unclear. However, we do know that the piRNA pathway is recruited to repress *L1* due to DNA hypomethylation in animal germ cells (Kuramochi-Miyagawa et al. 2004; Ku and McManus 2008; Kuramochi-Miyagawa et al. 2008). Lack of the piRNA pathway genes and *L1* expression was expected in CL with possible high DNA methylation level. Nevertheless, these

preliminary results suggested an alteration in gene expression and possible changes of DNA methylation profiles in granulosa cells before and after ovulation.

Evidence suggests that advanced ovarian cancers may derive from benign epithelial inclusion cysts (Okamura and Katabuchi 2001; Pothuri et al. 2010). piRNA pathway genes such as *PIWIL1* and *PIWIL2* are overexpressed in various cancers (Qiao et al. 2002; Lee et al. 2006; Liu et al. 2006; Taubert et al. 2007; Grochola et al. 2008; Liu et al. 2010; Sun et al. 2011). In Chapter 3.1, we report overexpression of *L1*, *PIWIL1*, *2* and *MAEL* in the epithelial cells of malignant EOC, but not in the OSE of normal ovaries. Here, we showed that *PIWIL1*, *2* and *MAEL* are expressed at very low levels in the epithelial cells of inclusion cysts. The low expression of these genes is consistent with the RT-PCR analyses in 19 benign lesions (Chapter 3.1). This expression pattern is similar to that of some EOC biomarker genes, *Claudin 3* and *DDR1*, of which low expression was found in benign inclusion cysts but overexpression was found in malignant EOC (Heinzelmann-Schwarz et al. 2004). Knockdown of *Claudin 3* suppressed ovarian cancer growth and metastasis (Huang et al. 2009). Similarly, knockout of *Piwi* in *Drosophila* reduced brain tumour size, suggesting that *Piwi* may promote cancer growth in this system (Janic et al. 2010). The co-expression of piRNA pathway genes and *L1* in the epithelial cells may suggest that these genes are potential EOC markers which may be involved in reprogramming of epithelial cells in the inclusion cyst for a possible first step in EOC initiation.

Similar to malignant EOC, we also found increased expression of *PIWIL2* in the stromal tissues of inclusion cysts. No piRNA pathway gene expression was found in the

stromal cells of normal ovary. Thus, increased expression of *PIWIL2* may suggest an alteration in the gene expression of stromal cells surrounded by potentially cancerous epithelial lining cells. *PIWIL2* expression was found in early stage breast and cervical cancers, suggesting that it is a good biomarker for diagnosis and prognosis for these cancers (He et al. 2010; Liu et al. 2010).

Global DNA hypomethylation together with *L1* derepression and overexpression have been reported in various cancers, including advanced ovarian cancers (Ting et al. 2011; Woloszynska-Read et al. 2011). Interestingly, *L1* expression is increased in some epithelial cells of inclusion cysts. Normally, *L1* is repressed by DNA methylation; therefore, the expression of *L1* in epithelial cells may suggest global DNA hypomethylation in these cells. However, immunohistochemistry studies, with an anti-5-MeC antibody, are not sensitive enough to demonstrate DNA hypomethylation in the inclusion cysts. More sensitive approaches will be needed to measure the differences in DNA methylation levels between the epithelial and stromal cells from inclusion cysts and malignant EOC. Perhaps in the future, microdissection, bisulfite conversion and pyrosequencing methods can be used to detect global DNA hypomethylation in distinct cell types. Furthermore, analysis of *L1* methylation will shed light on genome stability in the epithelial cells of inclusion cysts, which may help to understand the origin of EOC.

Chapter 4

Analysis of genome instability and aneuploidy in ovarian cancer derived primary cells

Introduction

Human EOC cell lines allow the investigation of tumour biology at different stages and in different types of cancer (Wolf et al. 1987; Langdon et al. 1988; Briers et al. 1989). Manipulation of cancer cells, for example, by overexpressing or knocking down protein-coding genes or non-coding RNA sequences that are thought to be involved in tumour formation (Jin et al. 2012) followed by careful examination of the cancer cell characteristics, allows greater understanding of many aspects of tumour biology (Fan et al. 2011; Huo et al. 2011) (Chapter 3.1). Tumour cell culturing can be divided into two categories, primary and secondary. In primary cell culture, establishment is initiated from tissues or organs taken directly from an individual, and continued growth and division results in primary cell lines (Paul 1970). Secondary cultures are subpopulations of primary cells that have progressed through quiescent periods (in which most primary cells die) and successfully maintained growth to acquire immortality. Most of the commercially available ovarian cancer cell lines are secondary cell lines. Since cancer cells are adaptive, the *ex vivo* removal and culture alters their growth characteristics (Barnes et al. 1984) away from their *in vivo* behaviour. Thus, compared to secondary cultures, primary cells are cultured for much shorter periods *ex vivo* which diminishes culture-induced changes in the cells.

Genetic instability, such as aneuploidy, is a hallmark of cancer. Aneuploidy is the presence of an abnormal number of chromosomes, either more or less than the diploid number, in a cell. Gain or loss of chromosomes have been observed in many different types of human cancers (Gordon et al. 2012). Mutations in genes or pathways that are involved in regulating the fundamental cellular processes, such as kinetochore-microtubule attachment, spindle assembly, checkpoint integrity, chromosome cohesion

and centrosome number, result in aneuploidy (reviewed by Thompson et al. 2010; Compton 2011). Organism-wide aneuploidy is generally lethal in plants, insects, mice and humans (Torres et al. 2008). A recent study which introduced chromosomes 1, 13 or 16 into mouse embryonic fibroblasts found that the production of trisomic embryos resulted in lethality or *in utero* death (Williams et al. 2008). However, aneuploidy may offer an advantage to cancer cells. Although the role of aneuploidy in cancer pathogenesis is currently unknown, studies have suggested that it may provide an advantage to cancer cells by changing the expression of multiple oncogenes (Rancati et al. 2008; Sheltzer et al. 2011). For example, trisomy of chromosome 4 increases the expression of *C-KIT* which promotes survival, proliferation, differentiation and migration in myeloid leukaemia (Beghini et al. 2000; Langabeer et al. 2003). In addition, aneuploidy has been implicated in lung cancer relapse and recurrence (Sotillo et al. 2010). Aneuploidy has also been shown to promote haematologic cancers or inhibit solid tumour growth in human, evidenced by individuals with Down's syndrome (trisomy 21) who have a higher risk of haematologic cancers but a lower risk of solid tumours (Malinge et al. 2009). Current evidence supports a role for aneuploidy in tumour progression, however, the underlying mechanism remains unclear (Schvartzman et al. 2010). Understanding the role of aneuploidy in cancer progression is therefore of critically important to further develop cancer treatments (Gordon et al. 2012).

Chromosomal gain or loss have been observed in early and malignant EOC and therefore are implicated in the initiation of malignancy (Tapper et al. 1997; Patael-Karasik et al. 2000; Capo-chichi et al. 2009). Loss of chromosomes 17, 20q and 18p and gains of chromosomes 12p13 and q23 are found in borderline SC which is a cancer

placed between benign and malignant EOC (Osterberg et al. 2006). In malignant EOC, chromosomes 1, 2, 7, 8, 9, 13 and 17 are affected (Tapper et al. 1997). Chromosomal gain is more frequently identified in serous, mucinous and endometroid EOC. A high level of amplification is found in chromosome 1q22 in serous and mucinous EOC (Tapper et al. 1997; Patael-Karasik et al. 2000). The amplification of chromosome 1q22 centred on *RAB25* is found in approximately 50% of tested advanced serous EOC (n = 50), resulting in *RAB25* upregulation (Cheng et al. 2004). In addition, there is an unstable region, located on chromosome 1q22, containing a large segmental duplication (SD) (240kb) which occurred about 37 MYA and has resulted in the formation of paralogous segment (Kuryshev et al. 2006). This SD event has created a new gene from Gon-4-like (C. elegans) (*GON4L*) called YY1-associated protein 1 (*YY1AP1*). *YY1AP1* is known as an oncogene in ovarian cancer (Hall et al. 2010; Zhang et al. 2011) and other tumours (Zeng and Hong 2008). Thus, chromosomal instability and amplification of 1q22 may promote cancer development in ovarian and other cancers.

DNA hypomethylation is involved in cancer progression through chromosomal instability, reactivation of TEs, aneuploidy and mutations (Esteller and Herman 2002; Eden et al. 2003). Global *L1* hypomethylation which leads to increased *L1* activity has been found in many cancers including ovarian cancers (Menendez et al. 2004). *L1* hypomethylation and overexpression may result in chromosomal instability, translocation and DNA breaks (Zamudio and Bourc'his 2010). A recent study showed that *L1* hypomethylation is statistically correlated with the degree of aneuploidy in ovarian cancers (Zeimet et al. 2011). However, if increased *L1* activity is found in aneuploid ovarian cancer cells remains unclear.

In order to further understand the chromosomal alteration especially at human 1q22 in ovarian cancer, primary ovarian cancer cells from ascites has been cultured and the 1q22 region was investigated using DNA FISH with BACs which cover *GON4L* and *YY1AP1*. Ovarian cancer primary cells were cultured from five patient peritoneal ascites with different types and stages of ovarian cancer. All primary cell cultures expressed EOC markers such as Vimentin (*VIM*) and Cytokeratin 7 (*CK7*). However, the expression of another EOC marker, Mucin 1 (*MUC1*), was only found in one culture. FISH analyses showed that all of the primary cancer cells have significantly higher number of aneuploid cells compared to the normal cells. Furthermore, to understand the role of piRNA pathway genes and *L1* in ovarian cancer, the expression of these genes and *L1* was examined in the primary ovarian cancer cell cultures with distinct origins and stages. Expression analysis showed that only cells from one out of five primary cultures co-expressed *PIWIL2, 4* and *MAEL* but not *L1* expression. Overall short term culture and analysis of cells derived from ascites provide a useful tool to investigate aneuploidy in relation to repeat expression and piRNA pathway activity.

Materials and methods

EOC ascites

Peritoneal ascites of ovarian cancer patients was provided by A/Prof. Martin K. Oehler (Robinson Institute, University of Adelaide). Cancer type, cancer stage and patient age are listed in Table 1.

Table 1: Origin of primary ovarian cancer cell lines

Ovarian cancer cell line	Cancer type	Stage	Age
OC1	Ovarian endometrioma	0	37
OC2	Malignant mixed müllerian tumour ovary	3c	63
OC3	Serous carcinoma	3c	64
OC4	Poorly differentiated ovarian carcinoma	1c	69
OC5	Serous carcinoma	3c	79

Establishing primary cultures

Ascites drained from the peritoneum of an ovarian cancer patient was freshly collected and transferred to a sterile laminar flow hood for further processing. The fluid was centrifuged for 10 mins at 3000 rpm at RT to obtain a cell pellet. The cell pellet was washed twice by resuspending with 1xPBS (sterile) and centrifuging at 276g at RT. Cells were then resuspended in culture medium (RPMI-1640 supplemented with 10%FBS, 1% Penicillin streptomycin and 1% Glutamine (Gibco)) and grown in a T25 ($25cm^2$) flask at 37˚C, under 100% humidity and 5% CO_2. To allow cancer cell attachment, medium was changed after 4 days followed by 2-3 times a week. Subculturing of cells was performed at ~70% cell confluence. The period of time taken

for the first subculturing varied among patients within a range of 2-3 weeks. During subculturing, culture medium was removed and cells were detached with Trypsin/ EDTA (0.25%, 2.5g/l) (Gibco) for 5-10 mins at 37˚C. Cells were resuspended with fresh medium and transferred to a T75 (75cm^2) flask. Cells were grown for several passages to remove fibroblasts, lymphocytes and mesothelial cells (Langdon and Lawrie 2000).

Culturing of secondary cell line OVCAR3

See methods in Chapter 3.1 (page 81).

Characterisation of cultured cells

Cell pellets were obtained around passage 3 or 4 and subjected to RT-PCR analyses with EOC markers including *CK7*, *Vim* (Ceausu et al. 2008) and *MUC1* (Van Elssen et al. 2010).

Normal interphase cells from whole blood

9ml of complete medium (RPMI1640 supplemented with 15% FBS, 1% Penicillin streptomycin and 1% Glutamine) with 2.5% Phytohemagglutinin was added into 1ml of whole blood donated by a normal individual. Blood was transferred to a T25 flask and cultured for 70 hrs at 37˚C, under 100% humidity and 5% CO_2. Colchicine (0.1mg/ml) was added into culture medium for 1 hr before harvesting the cells. Cells were spun at 150g for 10 mins. Supernatant was removed and cells were incubated in hypotonic

solution (75mM KCL) at 37˚C for 25 mins. Cells were fixed with ice-cold fixative (3:1 v/v Methanol: Acetic acid) overnight at -20˚C.

BAC clones

Two human BAC clones, RP11-96F12 and RP11-586I23 which contain *GON4L* and *YY1AP1* genes (from 1q22 region) respectively, were purchased from Empire Genomics (USA). The BAC clones were confirmed by PCR with *GON4L* and *YY1AP1* primers (Table 2, Page 152) and sequencing.

Preparation of metaphase chromosomes

Mitotic metaphase chromosomes were harvested from primary cell lines at passage 3-4 as described previously (Rowell DM et al. 2011). Briefly, Colchicine (0.1mg/ml) was added into medium about 1.5 hrs before harvesting the cells. Cells were harvested with Trypsin/ EDTA (0.25%, 2.5g/l) (Gibco) and treated with hypotonic solution (75mM KCL) for 20 mins at 37˚C. Cells were fixed overnight at -20˚C in fixative (3:1 v/v Methanol: Acetic acid). Cell suspension was dropped onto a glass slide under an environment with 70-80% humidity and dried at RT.

Fluorescence *in situ* hybridisation (FISH)

FISH was performed similar to previously described in Chapter 2 (page 42).

Cell counting and statistical analysis

For each primary culture, 50 nuclei were recorded in three independent counts. For each, the ploidy of a cell was determined by the number of BAC signals for each locus. Cells with two signals that are separated enough to not represent a duplex signal (a duplex signal occurs after DNA replication of a locus (Fig.1)) were counted as diploid, three signals as triploid, four signals as tetraploid and more than four signals as polyploid. All triploid and tetraploid cells were classified as aneuploid cells. From the numbers of cells which are diploid or aneuploid, mean, standard deviation and standard error mean were calculated with Microsoft Excel 2010. Two-tailed Student's *t*-tests were performed to compare the numbers of diploid or aneuploid cells between each of the cancerous cultures and normal cells. $P<0.05$ was considered statistically significant.

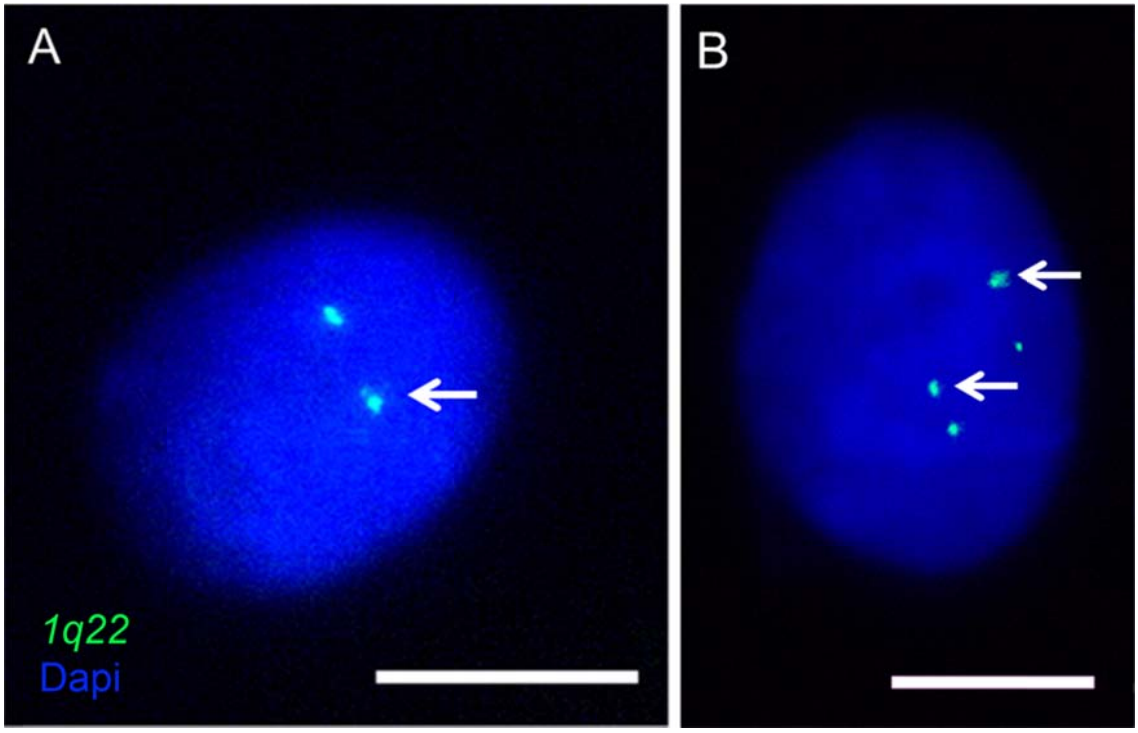


Fig. 1. Assessment of the cell ploidy level in primary ovarian cancer cells. (A) An interphase cell with two duplex signals. This is counted as two signals and the cell is diploid. (B) An interphase cell with four signals which are composed of two duplexes and two singlets. This cell is counted as tetraploid. Arrows indicate a duplex signal at the 1q22 locus. Scale bar = 50μm.

cDNA preparation and RT-PCR

cDNA was generated from cell pellet of each line. Details of cDNA synthesis can be found in Chapter 3.1 (page 82). RT-PCR was performed to amplify piRNA pathway genes (*PIWIL1, 2, 4* and *MAEL)* and EOC markers *(CK7, Vim* and *MUC1*). Primers were designed to amplify across an intron (Table S1). Primer information for piRNA pathway genes are listed in Table S1 in Chapter 3.1 (page 104) and that for EOC markers can refer to Table 2. SC tissue isolated previously (Chapter 3.2) was used as a positive control for RT-PCR.

Table 2**:** Primers to assess primary ovarian cancer cell characteristics

Primer	Sequence 5'-3'	Tm
MUC1 ex2 forward	TCTACCCCAGGTGGAGAAAAG	58˚C
MUC1 ex3 reverse	AGAAGTGCTGTGATTGGAGGAG	58˚C
CK7 ex5 forward	GACAACAGTCGCTCCCTG	54˚C
CK7 ex6 reverse	CTGGTTCTTGATGTTGTCG	52˚C
VIM ex5 forward	GGAACAATGACGCCCTGC	59˚C
VIM ex6 reverse	CTTCCTGTAGGTGGCAATCTC	56˚C
GON4L ex30 forward	TAGAGGGGGTTTGGCTAAAGACA	55˚C
GON4L ex31 reverse	CTCAGCAGGGGTCTTATTTC	52˚C
YY1AP1 forward	TGGAGACTGGGAGCCTTCACTT	58˚C
YY1AP1 reverse	TTTATTGAAGCAAAAGTAT	50˚C
L1 forward	CTCAAAGGAAAGCCCATCAG	52˚C
L1 reverse	CGTGAGATGGGTTTCCTGA	52˚C

Results

Establishment and characterisation of primary EOC cultures

In this experiment, five primary ovarian cancer cultures were established from 50ml ascites. The ascites was drained from patients with a median age of 64 years old (range from 37-79) with different stages of ovarian cancer (Table 1). The ovarian cancer cells normally reach confluence after ~2 weeks and can be further expanded to yield ~$1x10^7$ cells by passage 3 or 4, however, this varies between patient samples. The present culturing method was modified from the work of Shepherd and his colleagues (Shepherd et al. 2006). Unlike other research (Hirte et al. 1992; Evangelou et al. 2000), no additional supplement was added to the culture medium besides the standard supplements added to the complete medium. Additional supplements may increase cell growth but affect cell characteristics (Salamanca et al. 2004). Cell morphology of OC3 suggested the presence of ovarian cancer cells as it was similar to the morphology of other primary malignant ovarian cancer cells (Shepherd et al. 2006; Kurbacher et al. 2011) (Fig. 2A). Binucleated cells were observed in OC3 culture (Fig. 2B). A lower population of ovarian cancer cells were found in other primary culture compared to OC3. High number of fibroblast cells were found in OC5 (Fig. 2C) and lower numbers in OC1, OC2, OC4 and the least in OC3 (Fig. 2D). The growth rate of all primary cultures was slower than the commercially available OVCAR3 cells. During subculturing, a 1:3 dilution of OVCAR3 cells reached confluence after two days, however, the primary ovarian cancer cells generally took about seven days to reach confluence.

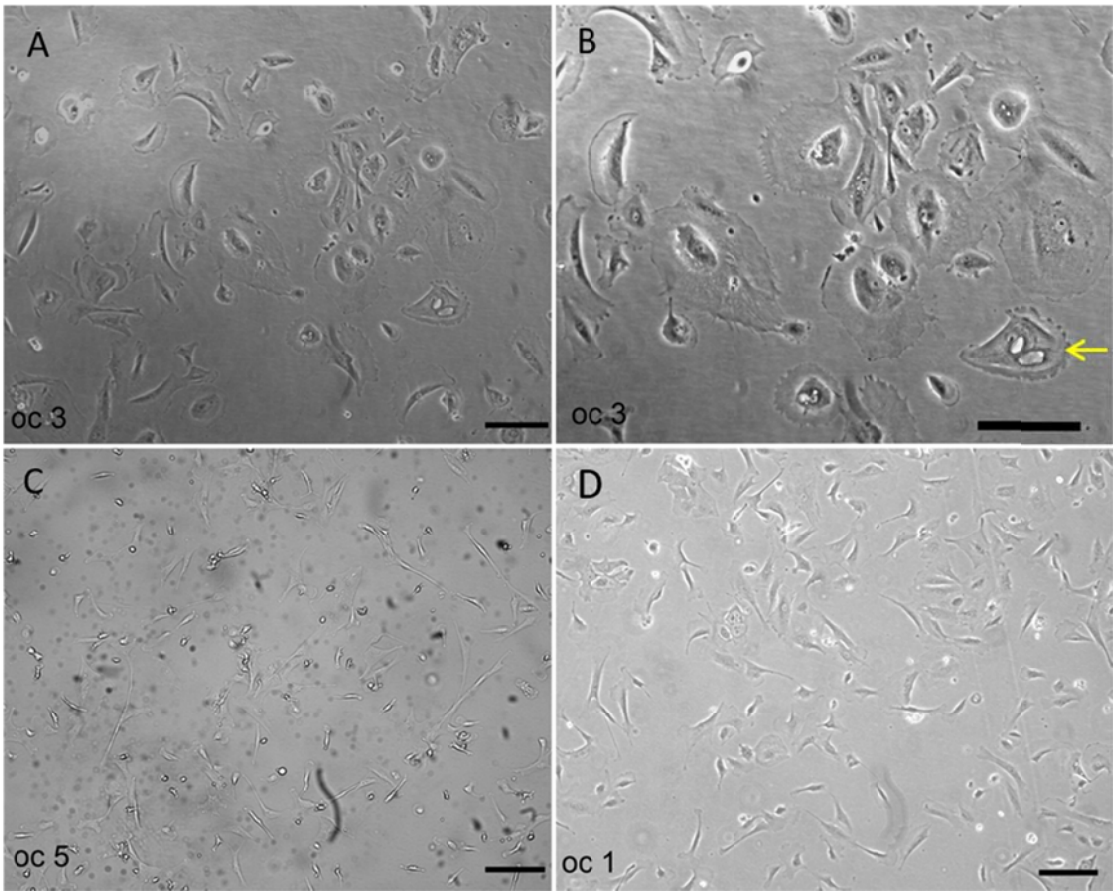


Fig. 2. Primary culture of human ovarian cancer cells from patient ascites. (A-B) Cells in OC3 have irregular shape similar to the published ovarian cancer cell morphology, suggesting the presence of ovarian cancer cells. (C) OC5 and (D) OC1 have both fibroblasts and ovarian cancer cells. More fusiform cells (i.e. fibroblast cells) are found in OC5 compared to OC1 and there are more ovarian cancer cells in OC1 than OC5. Yellow arrow indicates a binucleated ovarian cancer cell in OC3. Scale bar =10μm.

Three EOC markers, *VIM*, *CK7* and *MUC1*, were tested to confirm the presence of EOC cells in different cell cultures (Fig. 3). *VIM* is a mesenchymal cell marker that is upregulated in ovarian cancer cells during the EMT (Thiery 2003), a key step in ovarian cancer metastasis (Radisky 2005). *CK7* distinguishes ovarian cancer from malignant colon cancer (Shin et al. 2010) while *MUC1* is expressed in primary ovarian cancers

and metastatic lesions but not in normal ovary (Van Elssen et al. 2010). *VIM* and *CK7* expression was detected in all primary cultures while *MUC1* was only amplified in OC3 which also expressed *PIWIL2* and *PIWIL4*. However, *L1* expression was not detected in these cells using RT-PCR. Nevertheless, the expression of the EOC markers suggests that the cultured cells are ovarian cancer cells.

piRNA pathway genes were upregulated in malignant EOC compared to benign tissues and normal ovary (Chapter 3.1). Thus, expression of piRNA pathway genes *PIWIL1, 2, 4* and *Mael* was investigated in primary ovarian cancer cells using semi-quantitative RT-PCR (Fig. 3). A stage-3 malignant SC was used as a positive control as all genes tested showed expression. Among the primary ovarian cancer cultures, only OC3, had expression of piRNA genes. The expression of *PIWIL2* was similar to the positive control while *PIWIL4* and *MAEL* expression was lower than the SC tissues. *Piwil* genes and *Mael* are important in maintaining stem cell function in vertebrates (Carmell et al. 2002; Houwing et al. 2007; Houwing et al. 2008) and thus expression of these genes in OC3 suggests the presence of ovarian cancer stem cells.

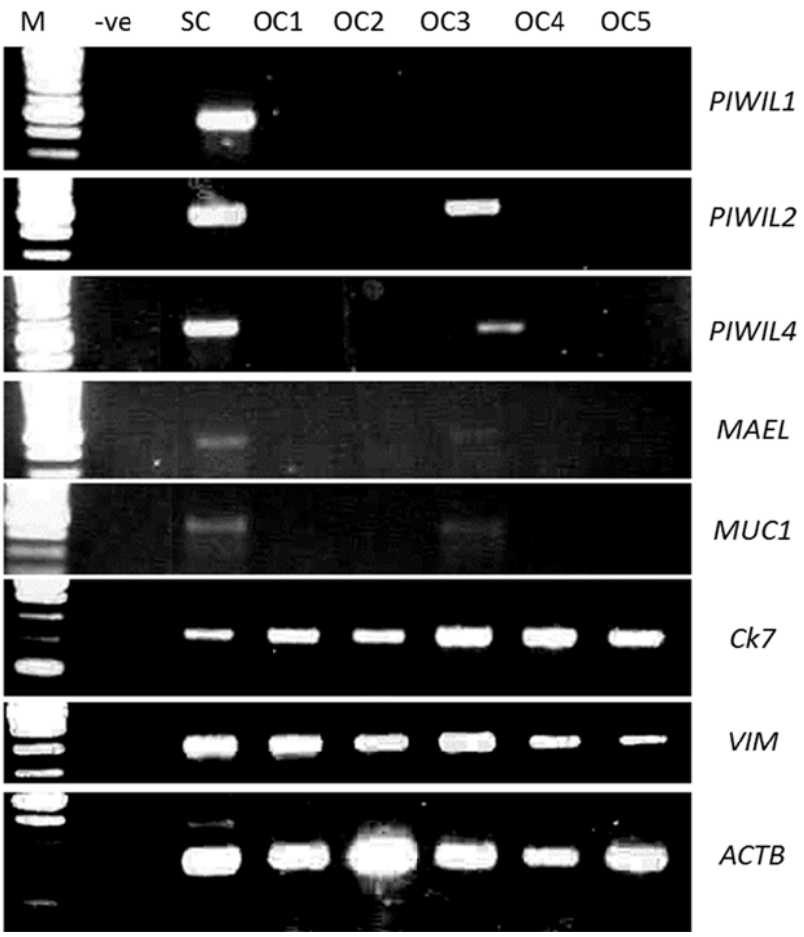


Fig. 3. Expression of EOC markers and piRNA pathway genes in human primary ovarian cancer cultures. All ovarian cancer cells have *CK7* and *VIM* expression but only OC3 has *MUC1* expression. *PIWIL2*, *PIWIL4* and *MAEL* were expressed in OC3. SC: a third stage serous carcinoma tissue used as positive control.

Aneuploidy in cultured primary ovarian cancer cells

Characterisation of ploidy levels using DNA FISH is one of the tools for routine clinical diagnosis of cancer cells (Capo-chichi et al. 2011). Nuclear morphology and ploidy are common assessment tools to determine the degree of malignancy and disease prognosis (Zink et al. 2004). In order to determine ovarian cancer cell ploidy, we employed DNA FISH using the BACs from human 1q22 (Fig. 4A). The controls for this experiment were the cells obtained from normal whole blood. Typically, two signals were found in one cell, indicating a diploid state (Fig. 4C). Rarely, three signals were observed in normal cells (1.5%, 3/200 nuclei) (Fig. 4C). In contrast, nuclei with more than 2 signals (Fig. 4B, D) were more frequently observed in primary ovarian cancer cells. Approximately 21%-43% of ovarian cancer cells were aneuploid (Fig. 5). The proportion of aneuploid cells was significantly higher in all primary ovarian cancer cells compared to normal cells (Table 3). More than 40% of cells in OC1 and OC2 were aneuploid. The number of aneuploid cells appears to be independent of the tumour malignancy. For example, OC1 and OC2, which have high aneuploid levels (~40% of total cells), are derived from a stage-0 ovarian endometrioma and a stage-3 malignant mixed Müllerian tumour, respectively. Furthermore, less aneuploid cells were found in stage-3 SC derived cells (OC3 and OC5) compared to OC1. In the secondary ovarian cancer cell line OVCAR3, ~80% of the cells were aneuploid. Nevertheless, all primary ovarian cancer cell cultures had levels of aneuploidy similar to the OVCAR3 (Capo-chichi et al. 2011). Aneuploidy was also associated with increased nuclear size in cancers (Maffini et al. 2001). One of the explanations is that aneuploid cells such as triploid or tetraploid, have increased nuclear content which therefore increased nuclear size (Lu and Kang 2010). Consistently, the nuclei of ovarian cancer cells were much larger than that of normal cells (Fig. 4).

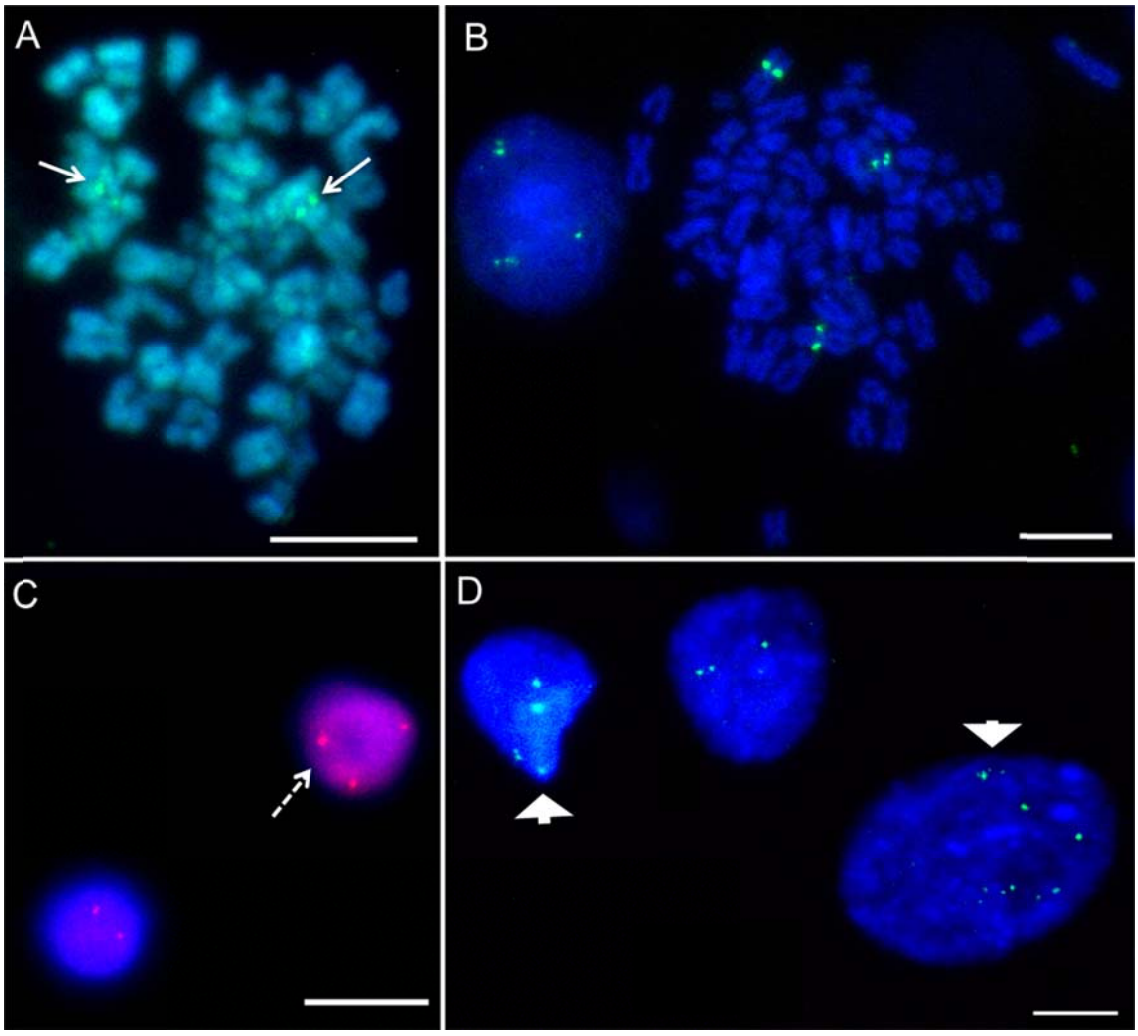


Fig. 4. Ovarian cancer cells with increased nuclear size and ploidy level. DNA FISH using 1q22 probes was performed on normal and ovarian cancer cells. (A) Metaphase chromosomes showing hybridisation of BAC DNA to the short arm of chromosome 1 from a normal cell (Arrow indicates chromosome 1q22). (B) An interphase cell from an ovarian cancer patient with 3 signals and metaphase chromosomes with chromosome 1 trisomy. (C) Two interphase cells from a normal individual. One cell is diploid, the other triploid (indicated by broken arrow). (D) Aneuploid ovarian cancer cells with nuclei showing two, four and multiple signals, arrow heads indicate nuclei with multiple signals. The nuclei of normal cells are smaller compared to that of aneuploid ovarian cancer cells. Scale bar = 10μm.

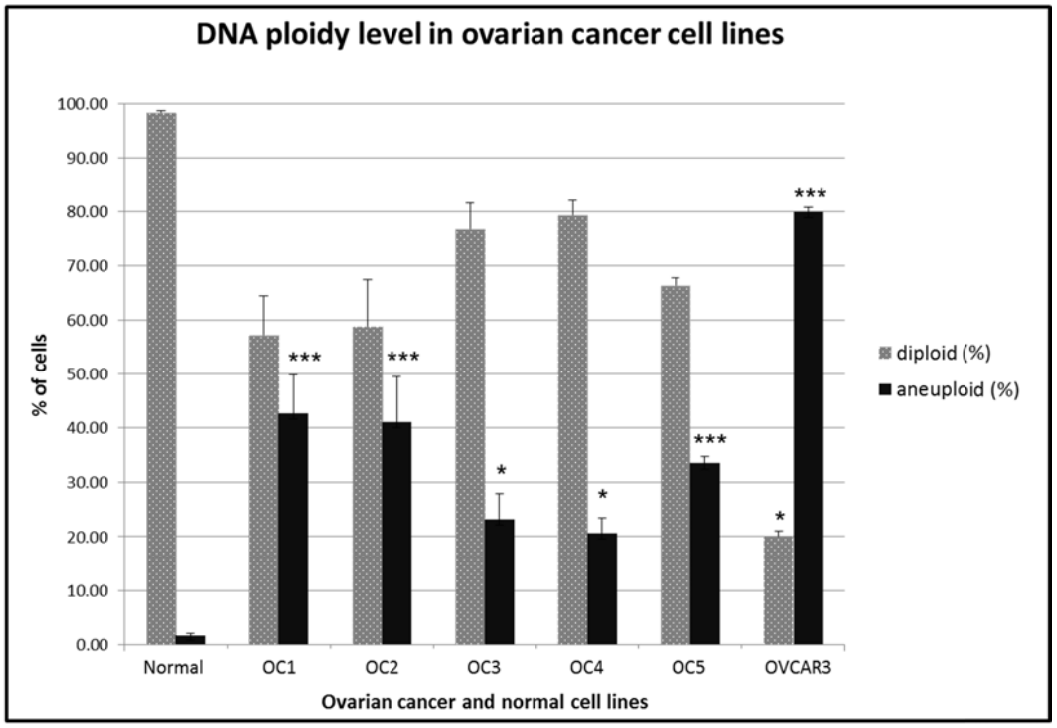


Fig. 5. DNA ploidy level in primary and secondary ovarian cancer cells. The percentage of aneuploid nuclei is significantly higher in all primary ovarian cancer cells than normal cells while the secondary culture OVCAR3 has the highest number of aneuploid nuclei. Two-tailed Student's *t*-tests were performed to compare the number of diploid and aneuploid cells between normal and ovarian cancer cell lines. At least 50 nuclei were counted in triplicate. * indicates $0.001 < P < 0.05$; *** indicates $P < 0.001$. Error bars represent the standard error mean. OC1-5: primary ovarian cancer cultures; OVCAR3: secondary cancer cell line.

Table 3: P-value of the DNA ploidy level comparison between OC cultures and normal cells

Ploidy	Primary cultures	P-value	Significance
Diploid	OC1	0.15	NS
	OC2	0.13	NS
	OC3	0.26	NS
	OC4	0.18	NS
	OC5	0.18	NS
	OVCAR3	0.035	*
Aneuploid	OC1	0.00085	***
	OC2	0.0054	***
	OC3	0.011	*
	OC4	0.011	*
	OC5	4.62E-05	***
	OVCAR3	0.000327628	***

P-value was calculated via two-tailed Student's *t*-test. * indicates $0.001 < P < 0.05$; *** indicates $P < 0.001$; NS = not significant.

Discussion

Aneuploidy of cells results in the amplification of oncogenes and loss of tumour suppressor genes which can lead to malignant transformation and tumour development (Fearon and Vogelstein 1990; Hanahan and Weinberg 2000). In this study, we have established five primary ovarian cancer cultures from patient ascites and analysed the expression of EOC markers, piRNA pathway genes and *L1* as well as the ploidy levels in these cells. All cells express the EOC markers *VIM* and *CK7* but not *MUC1*. Similarly, the expression of *MUC1* is found in some but not all of the malignant EOC tested previously (Chapter 3.1). No expression of this gene was found in the benign and normal ovarian tissues examined. This result suggests that *MUC1* is expressed in some malignant EOC tissues and cells such as OC3 which is derived from a stage-3 SC. Expression of piRNA pathway genes *PIWIL2, 4* and *MAEL* was only found in OC3 but not in the other primary cells. The lack of detectable *MUC1, PIWIL1, 2*, *4* and *MAEL* expression in other ovarian cancer cells could be due to the low fraction of ovarian cancer cells relative to OC3 and the ovarian tissue masses used for RT-PCR analyses. In addition, the expression of *L1* was not found in the primary ovarian cancer cells. The *L1* primers in this study were designed from the published consensus *L1* sequence (Smit et al. 1995). Since *L1* sequence is highly heterogeneous within an individual, designing a primer to amplify all *L1* transcripts using RT-PCR is challenging. Thus, the lack of *L1* expression in ovarian cancer cells could be due to non-specificity of the primers. Further analysis to look at the expression of *L1* can be performed using degenerate L1 primer and RNA *in situ* hybridisation. Normally, a *L1* probe of more than 500bp is used for *in situ* hybridisation; this may allow a better detection of *L1* expression. Therefore, to understand the correlation between *L1* expression and DNA ploidy level in the primary ovarian cancer cell lines, further experiments such as

examination of *L1* expression using RNA *in situ* hybridisation combined with DNA FISH on the ovarian cancer cells which are aneuploid can be performed.

In order to determine the ploidy level of the primary ovarian cancer cells, FISH was performed with the BAC DNAs from human 1q22 region. FISH detection with BACs from one chromosome cannot represent whole chromosome ploidy levels but rather changes in chromosome material restricted to 1q22 only. Thus, in this study, aneuploid cells are referred to those cells that have more than two chromosome 1 signals at interphase. FISH with more probes from other chromosomes would provide better understanding of the whole genome ploidy level changes in primary ovarian cancer cell cultures.

From DNA FISH analyses, we observed that nuclei of aneuploid cells are larger than normal diploid cell nuclei, presumably due to the increase in genome size. Aneuploid cells that can be induced by the loss of nuclear envelope protein Lamin A/C also feature larger nuclei (Capo-chichi et al. 2011). Furthermore, in yeast and mouse, aneuploid cells have distinct growth rate, metabolism and cell size when compared to normal cells (Torres et al. 2007) and increased nuclear size is found in trisomic mouse embryonic fibroblasts (Williams et al. 2008).

Despite being derived from different sources, all primary ovarian cancer cells have significantly higher numbers of aneuploid cells compared to normal cells. ~1.5% (3/200 nuclei) of normal cells are triploid, however, this is a normal observation as normal

diploid cells have been shown to segregate a chromosome incorrectly approximately once every 100 cell divisions (Thompson and Compton 2008). A large number of aneuploid cells have been detected in early stage of ovarian cancer, for example, in OC1. Although OC1 is derived from stage-0 ovarian endometrioma, more than 40% of OC1 cells are aneuploid, suggesting that aneuploidy may be an early event in cancer or plays a role in ovarian cancer initiation. Aneuploidy induced in cultured ovarian surface epithelial cells leads to neoplastic transformation of these cells (Cai et al. 2009; Capo-chichi et al. 2009). In addition, Pothuri and collegues showed that aneuploidy was often found during the early stages of ovarian cancer, suggesting that it contributes to cancer progression (Pothuri et al. 2010). The number of aneuploid cells in the secondary ovarian cancer cell line OVCAR3 is much higher compared to the primary ovarian cancer cells suggesting accumulation of genetic mutations in secondary culture.

In summary, aneuploidy and chromosome instability promote loss of tumour suppressor genes and loss of oncogenes which contributes to malignant transformation, suggesting that aneuploidy plays important roles in tumour initiation and development (Michor et al. 2005). The primary ovarian cancer cells derived from ascetic fluid can be cultured relatively easily and are useful system in which to investigate of the effects of aneuploidy on ovarian cancer initiation and progression.

Chapter 5

Conclusions and future directions

Conclusions

RNA silencing is an important epigenetic regulator of gene expression in almost all eukaryotic organisms. One of the small RNA pathways, the piRNA pathway, is essential for silencing transposable elements (TEs) in gonads but increasingly other functions of genes in this pathway are being discovered. piRNA pathway genes are highly conserved and have been found in all animals studied (Kuramochi-Miyagawa et al. 2001; Kurth and Mochizuki 2009). piRNA binding PIWI proteins are one of the core components of the piRNA pathway (Malone and Hannon 2009; Senti and Brennecke 2010) and various numbers of *Piwil* genes have been identified in animal lineages. Thorough bioinformatics analyses presented in Chapter 2 revealed additional duplications and deletions indicating lineage specific changes have arisen in the pathway.

In mammals, piRNA pathway genes such as *PIWIL* and *MAEL* have been extensively investigated in mouse testis, however, despite the presence of piRNAs in oocytes a role for piRNAs in the mammalian ovary has remained controversial. Work presented in Chapter 2 provides a comprehensive expression analysis of piRNA pathway genes in gonads of amniote species including chicken, platypus, mouse and human. We confirmed the conserved expression of *Piwil* genes and *Mael* in the most basal mammal lineage, the platypus, suggesting functional importance during mammalian spermatogenesis. Importantly, we discovered the robust expression of these genes in mammalian ovary. Moreover, expression of *PIWIL* genes and *MAEL* is found in the oocytes and ovarian supporting cells in developing follicles of platypus, mouse and human, suggesting a conserved role of this pathway in ovaries from flies to mammals.

The extraordinary conservation of gene expression in germ cells and gonadal supporting cells in species that diverged over 800 million years ago suggests an important role in adult male and female gonad.

One of the widely accepted hypotheses is that ovarian cancer may originate from the epithelial cells in inclusion cysts (Okamura and Katabuchi 2001; Pothuri et al. 2010). Based on the expression of piRNA pathway genes in the ovary and EOC, a role of these genes in the origin and progression of ovarian cancer has been hypothesised. Since the expressions of *PIWIL1* and *MAEL* are found in the granulosa cells during folliculogenesis and in malignant epithelial cells of EOC, it was examined if these genes are expressed in the inclusion cyst surface epithelial cells and post-ovulated granulosa cells in corpus luteum. Low expression of *L1, PIWIL1, 2* and *MAEL* was found in epithelial cells of inclusion cysts but not post-ovulated granulosa cells (Chapter 3). These preliminary data suggest that piRNA pathway may be involved in the initiation of EOC derived from inclusion cysts.

Next, the expression was investigated in a variety of ovarian cancer samples. *L1* is well known to be expressed in cancers and correlated with poor prognosis and survival. In addition, components of the piRNA pathway, such as *PIWIL1, 2, 4* and *MAEL*, are overexpressed in many cancer tissues (Qiao et al. 2002). Several roles have been suggested for the piRNA pathway components in the cancer progression (Sharma et al. 2001; Lee et al. 2006; Liu et al. 2006; Lee et al. 2010; Li et al. 2010). However, whether these components are involved in the repression of TEs in cancers is currently unclear. We showed that piRNA pathway genes *PIWIL1, 2, 4* and *MAEL* are

upregulated in malignant EOC. *In situ* analyses revealed *MAEL* and *PIWIL2* expression in the stromal cells lining cancer tissues, suggesting cancerous transformation of the stromal cells. In addition, *L1*, *PIWIL1, 2* and *MAEL* are strongly expressed in the cancerous cells. The co-expression of *L1* and piRNA pathway genes in the cancerous cells may indicate activation of piRNA pathway genes due to increased *L1* activity in these cells. In addition, real-time *in vitro* assay suggested that *PIWIL1* and *MAEL* may have a repressive effect on ovarian cancer cell invasiveness. In addition, although *PIWIL1* is upregulated in the malignant tissues, non-functional PIWIL1 proteins may be produced as a result of splicing defects. Perhaps due to the repressive effect of PIWIL1 in cancer cell invasiveness, this gene is more prone to produce non-functional products in malignant cancers. Together, these findings suggested that upregulation of piRNA pathway genes especially *MAEL* and *PIWIL1* might be triggered by increased TE activity in order to protect genome stability and repress EOC progression.

Increased *L1* activity is also associated with chromosomal instability. Chromosome instability is correlated with aneuploidy which promotes the gain of oncogenes and loss of tumour suppressor genes that consequently contributes to malignant transformation and development (Michor et al. 2005). In order to further understand the chromosomal alteration especially at human 1q22 region, I established primary ovarian cancer cell lines from ascites and investigated this region using DNA FISH. FISH analyses showed that all of the cancer cell lines have significantly higher number of aneuploidy cells compared to normal cells, providing a good model to understand chromosomal instability in ovarian cancers.

Future directions

The piRNA pathway is crucial for oogenesis in *Drosophila* and fish (Cox et al. 1998; Houwing et al. 2007). Distinct piRNA pathways have been identified in *Drosophila* ovarian somatic tissues and cell lines (Malone et al. 2009; Saito et al. 2009). In these tissues and cells, different piRNA pathway components and distinct types of piRNAs are expressed (Saito et al. 2009). Our data demonstrated conserved ovarian expression of piRNA pathway genes in the mammalian ovary, however, if different piRNA pathways are involved in the oocytes and ovarian somatic cells requires further investigation. Deep sequencing of piRNAs and mRNAs from mouse oocytes and ovarian somatic cells such as cumulus and granulosa cells may provide better understanding of the distinct piRNA pathways (if any) in mammalian ovary. In addition, understanding of the global DNA methylation level in the ovary and its supporting cells will provide insights into *L1* regulation as *L1* is normally repressed by DNA methylation. To understand the global DNA methylation, bisulfite conversion and pyrosequencing can be performed. From this data, further analysis at the promoter region of *L1* allow the understanding of *L1* methylation in different tissues. Furthermore, analysis of *Piwil* genes and *Mael* mutant in mouse will provide insight into the effect of these genes on the ovary beyond folliculogenesis. Studies showed that young female mutant mice are fertile (Deng and Lin 2002; Kuramochi-Miyagawa et al. 2004), however, it is still possible that effects of the lack of piRNA pathway genes might only become apparent at a later stage. Therefore, thorough examination of the phenotype of mutants such as follicle development, number and quality of oocytes, reproductive age, and expression of PIWI proteins and *L1* in the mutant mice will shed light on the function of piRNA pathway in female reproduction.

Upregulation of *PIWIL1* was found in malignant EOC compared to benign and normal tissues. However, evidence suggested that, due to defects in mRNA splicing, non-functional PIWIL1 proteins may be produced. Future experiments analysing *PIWIL1* at the transcript and protein level in a larger number of EOC samples need to be performed to confirm this finding. Sequencing of *PIWIL1* transcripts may allow confirmation of the splicing defect in *PIWIL1* transcripts. Immunohistochemistry or Western blot to assess levels and variants of PIWIL1 in malignant EOC using an antibody raised against the PIWI domain will give insight into whether truncated PIWIL1 proteins have been produced. Further investigation on splicing control mechanism and post-transcriptional regulation of PIWIL1 may provide insight into post-transcriptional control defects in cancers.

Preliminary data demonstrated expression of *L1* and piRNA pathway genes in the epithelial cells of inclusion cysts, suggesting that the pathway may be activated at early EOC development. To further test this hypothesis, studies to examine the *PIWIL* genes, *MAEL* and *L1* expression in a larger number of benign inclusion cysts can be conducted. Microdissection of epithelial cells from inclusion cysts that have positive piRNA gene expression and further analysis of its global DNA and *L1* methylation levels will test our hypothesis of ongoing *L1* derepression in the inclusion cyst and its consequence of activating the piRNA pathway as a signature of cancer development.

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