The role of the piRNA pathway genes in ovarian and prostate cancer progression

by

Eunice Hsiu Yee Lee
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PUBLICATIONS

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ABBREVIATIONS

ADT	Androgen deprivation therapy
AR	Androgen receptor
BSA	Bovine serum albumin
CAM	Chicken chorioallantoic membrane
CRPC	Castrate resistant prostate cancer
DAB	3,3'-diaminobenzidine
DSBs	Double-stranded breaks
DDX4	DEAD-Box Helicase 4
DMSO	Dimethyl sulfoxide
EMT	Epithelial to mesenchymal transition
ENZ	Enzalutamide
EOC	Epithelial ovarian cancer
FBS	Fetal bovine serum
FCS	Fetal calf serum
FFPE	Formalin-fixed paraffin-embedded
FIGO	Fédération Internationale de Gynécologie et d'Obstétrique
FSH	Follicle stimulating hormone
GUSB	Glucuronidase beta
HGSOC	High grade serous ovarian cancer
HENMT1	HEN methyltransferase 1
HR	Hazard ratio
KM	Kaplan-Meier
LH	Luteinizing hormone
LINE-1	Long-interspersed nuclear element-1

MAEL	Maelstrom
mRNA	Messenger RNA
ncRNA	Non-coding RNA
OC	Ovarian cancer
OS	Overall survival
PBS	Phosphate-buffered saline
PCa	Prostate cancer
PFS	Progression free survival
piRNA	PIWI-interacting RNA
PIWIL (1-4)	P-element induced wimpy testis-like (1-4)
PLD6	Phospholipase D family member 6
PPFS	Post-progression free survival
PSA	Prostate specific antigen
TBP	TATA-box binding protein
TCGA	The cancer genome atlas
TDRD (1,9)	Tudor domain containing (1,9)
TE	Transposable element

ABSTRACT

The PIWI-interacting RNA (piRNA) pathway consists of small non-coding RNAs (piRNAs) and pathway genes that play an intricate role in maintaining genomic stability through repressing transposable elements (TEs). The pathway genes are involved in piRNA biogenesis and TE repression through primary and secondary pathways. More recently, deregulated expression and implication in pro-cancer pathways were reported for several piRNA pathway genes (*PIWIL1-4*, *DDX4*, *MAEL* and *TDRD1*) in various cancers. However, discrepancies in expression changes and effects from manipulating piRNA pathway gene expression in different cancers were observed. Here, we investigated expression of a broader set of piRNA pathway genes and their effects on migration and invasion in two hormone-sensitive cancers, high grade serous ovarian (HGSOC) and prostate (PCa) cancer.

We showed dissimilar expression of 10 piRNA pathway genes (*PIWIL1-4*, *DDX4*, *HENMT1*, *MAEL*, *PLD6*, *TDRD1* and *TDRD9*) in clinical HGSOC and PCa samples. In HGSOC, we discovered that genes involved in the primary and secondary pathway were differentially expressed, suggesting differential regulation of these pathways in HGSOC. Notably, their expression change in PCa occurred between benign and malignant samples indicating potential contribution to PCa development. Additionally, their different expression profile in low grade ovarian cancer, HGSOC and PCa demonstrated possible cancer-specific expression.

Aberrant expression of certain piRNA pathway genes showed significant association with progression-free and overall survival when clinical

data and samples from HGSOC and PCa patients were analysed. This hints at their clinical relevance and potential as novel cancer biomarkers. Preliminary studies on chemoresistant HGSOC and relapsed PCa patient samples also revealed dissimilar trends in expression patterns of the 10 piRNA pathway genes. In particular, we observed *PIWIL3* expression in chemosensitive but not chemoresistant primary HGSOC cells. This points towards their potential involvement in the development of resistance in HGSOC and PCa.

Addressing the hormone-sensitive aspect of HGSOC and PCa, HGSOC cells treated with high dose follicle stimulating hormone showed significantly upregulated *PIWIL2* expression. PCa cells engineered to have reduced androgen receptor activity showed different trends in piRNA pathway gene expression. These pilot studies demonstrated possible hormonal regulation of piRNA pathway genes.

Next, we assessed the motility and invasion of HGSOC and PCa cells overexpressing piRNA pathway genes *in vitro* and *in vivo*. In contrast to pro-cancer effects observed in other cancers, we demonstrated that their overexpression decreased motility and invasion of HGSOC and PCa. We propose that this discrepancy may be related to the unusually high (~100%) *TP53* mutation in HGSOC and the prevalent *TMPRSS2:ERG* fusion in PCa. Therefore, the piRNA pathway genes may have a protective effect on HGSOC and PCa through maintaining genomic stability. The previously reported *PIWIL1* and *PIWIL2* mutants with truncated domains postulated to have impaired endonuclease activity and piRNA binding respectively raised

questions about heightened effects compared to wildtype. Surprisingly, overexpression of *PIWIL1* and *PIWIL2* mutants showed increased motility (HGSOC and PCa) and invasion (HGSOC) compared to wildtype.

Altogether, this work broadened our understanding of the piRNA pathway genes in cancer and provided novel perspectives on how they may be functioning in different cancers. It is clear from the literature and this study that the piRNA pathway genes play a role in cancer development. This research reinforces the role of the piRNA pathway genes but also highlights the possibility of opposing effects depending on the malignancy.

THESIS DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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CHAPTER 1: INTRODUCTION

1.1 Protector of genome stability: the piRNA pathway

Non-coding RNAs (ncRNAs) are known as transcripts that are not translated into proteins. ncRNAs are expressed from a wide range of sequences in the genome (repeats, introns and intergenic regions) and have been classified in terms of length or mode of action. These ncRNAs are increasingly recognized as key regulators of genomes as they can bring about epigenetic changes on DNA and chromatin, and silence genes post-transcriptionally through degradation and repression of messenger RNAs (mRNAs) (1-3). A rapidly increasing body of evidence uncovers the roles ncRNAs play in human diseases in particular, cancer (4-6).

The 23-36 nucleotide piRNAs, originally characterized in *Drosophila*, are a more recently discovered class of ncRNA in the family of small ncRNAs (7-9). Thus far, there has been 173 million piRNA sequences annotated on the piRNA database, piRBase. piRNAs are expressed from clusters located in both intergenic and genic regions of the genome (10). Interestingly, these piRNA clusters often harbor inactive transposon insertions (11). This feature allows piRNAs produced from these clusters to target and silence transposable elements (TEs) (11). Mobile TEs are threats to genome integrity as they induce genomic instability by causing mutations and double-stranded breaks (DSBs) through transposition and insertion of its repetitive sequence (12, 13). Therefore, piRNAs act to keep active TEs at bay through CpG island DNA methylation and chromatin modifications (14, 15). piRNAs are especially important during spermatogenesis when global demethylation results in TE

activation and piRNA mediated TE silencing ensures proper germ cell development and fertility of the organism (14, 15).

1.2 piRNA biogenesis pathways

A growing number of genes have been identified to contribute to the production of fully functional, mature piRNAs including the *P-element induced wimpy testis-like 1-4* (*PIWIL1-4*) genes which directly bind piRNAs and associated genes (16). Together, they form the piRNA pathway. There are two pathways involved in the biogenesis of piRNAs, namely the primary and secondary processing pathway (Figure 1) (16). piRNA biogenesis starts with its transcription in the nucleus and subsequently, piRNAs are transported into the cytoplasm for further processing either through the primary or secondary pathways or both (17).

In the primary pathway, piRNA precursors are first cleaved by phospholipase D 6 (PLD6) which typically produce a signature uridine at the 5' end (18, 19). These piRNAs are then loaded into PIWIL1 which can form a complex with Tudor domain containing 1 (TDRD1) for further cleavage followed by 2'-O-methylation by HEN methyltransferase 1 (HENMT1) at the 3' end (20-23). The 2'-O-methylation of piRNAs ensures piRNA stability and prevents them from being degraded (24). piRNAs bound to the PIWIL1/ TDRD1/ Maelstrom (MAEL) complex can then re-enter the nucleus to execute TE repression (25, 26). The presence of TDRD1 has been proposed to act as a molecular scaffold for the pathway and correct localisation of PIWIL proteins (27-29). Association of MAEL in the pathway aids in repressing TEs such as LINE-1 and intra-cisternal A-type particle (25, 26). After cleavage by PLD6, if

piRNA precursors are loaded into PIWIL2, piRNAs then enter the secondary processing pathway (16, 30).

These PIWIL2 bound piRNAs can act as the guide strand for cleavage of newly transcribed piRNAs from the nucleus (17). To make sense and anti-sense piRNAs, DEAD-box helicase 4 (DDX4) aids in the transfer of piRNAs from PIWIL2 to PIWIL4 or vice versa, forming a loop (16, 31). Within this loop, TEs that are present in the cytoplasm can be loaded into PIWIL4 for cleavage and be 2'-O-methylated by HENMT1 (11, 22, 23, 32). Association of TDRD9 to PIWIL4 has been proposed to aid in the loading process of piRNAs and repression of TEs (33, 34). TEs that have been cleaved and methylated then become mature piRNAs (11). The secondary pathway is also known as the ping-pong amplification cycle due to the interchange of piRNAs between PIWIL2 and PIWIL4 for the destruction of TEs by processing them into piRNAs and through this, generate a multitude of piRNAs (11, 14, 18, 35).

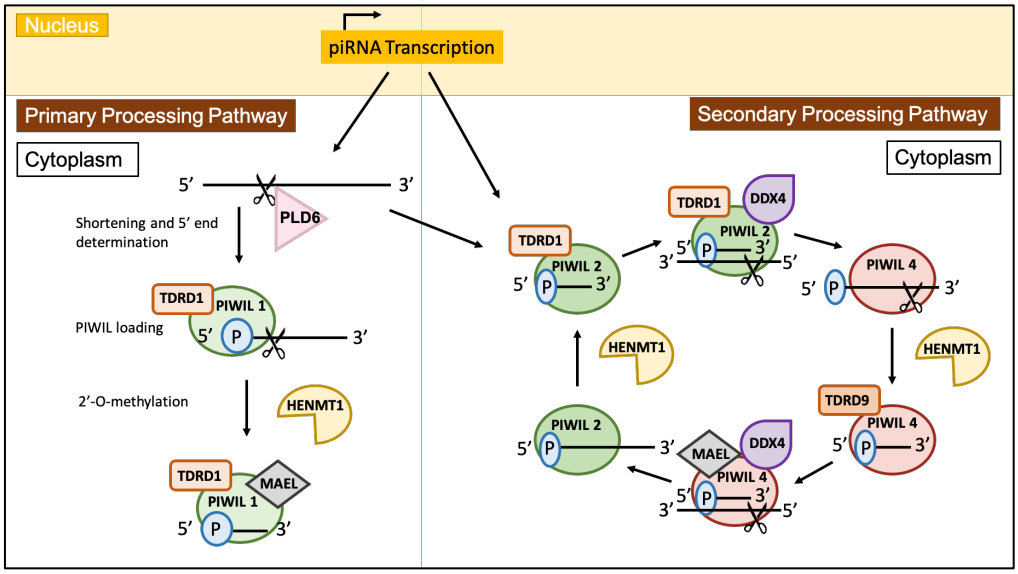

Figure 1. Simplified diagram of the primary and secondary processing pathways for the production of mature and functional piRNAs. After the transcription of precursor piRNAs

in the nucleus, they are transported into the cytoplasm for processing either by PLD6 or PIWIL2. Loading of piRNAs into PIWIL1 and PIWIL2 triggers the start of the primary and secondary processing pathways respectively. piRNAs are cleaved by PLD6, PIWIL1, PIWIL2 and PIWIL4 to produce 23-36 nucleotide piRNAs. Subsequently, HENMT1 is recruited to 2'-O-methylate 3' ends of piRNAs for piRNA stability. Association of TDRD1 and TDRD9 with the PIWIL proteins have been postulated to ensure proper loading of piRNAs, localisation of PIWIL proteins and act as molecular scaffolds for the processing pathways. MAEL is vital in these pathways to execute transposon repression while DDX4 aids in the transfer of piRNAs between PIWIL2 and PIWIL4.

The piRNA pathway was initially thought to be exclusively expressed in germ cells. It is now known to also be present in somatic supporting cells of gonads (36, 37). Interestingly, it was found in *Drosophila* ovaries where both the primary and secondary pathways are active in germ cells (37, 38). However, only the primary pathway is active in somatic cells (38). This produces different populations of piRNAs, indicating that it is possible for these pathways to function independently of each other (38, 39).

1.3 piRNA pathway genes are expressed in cancer

Mounting evidence indicates that the piRNA pathway can play a role in cancer. While the piRNA pathway genes work intricately together for piRNA biogenesis, they can function independently of piRNAs in cancer (40). A gain in interest on the roles of piRNA pathway genes in various cancers has led to expression and functional studies where the more prominent genes studied have been *PIWIL1*, *PIWIL2* and *MAEL* (Table S1). Interestingly, they demonstrated dissimilar trends in expression when comparing normal or benign tissues to various cancer tumours (41). This was observed for *PIWIL2* where its expression was higher in colon cancer tumours compared to normal

colon tissue but was lower in breast cancer tumours compared to normal breast tissue. Similarly, gene manipulation studies *in vitro* have shown opposing effects in different cancers, summarised in Table S1 (42-45). Therefore, it remains unclear how piRNA pathway gene expression affects the progression of different cancers and if they have cancer specific effects.

Further investigations into piRNA pathway genes led to the discovery of mutants in cancer. The mutants that have been described are *PIWIL1* transcripts with an exon 17 deletion (*P1Δ17*) and a mutant protein termed PL2L60 where it is 60kDa instead of 110kDa which is the normal size of PIWIL2 (43, 46). While *P1Δ17* was shown to be present only in malignant epithelial ovarian cancer but not ovarian tissue, its function has yet to be elucidated (43). It is postulated to have a premature stop codon which may cause a truncation in the PIWI domain and hinder its endonuclease activity (Figure 2) (43). PL2L60 was found in a range of cancer cell lines and has been linked to cell-cycle progression (46). This mutant was shown to have a truncated PAZ domain which may impair its binding with piRNAs (Figure 2) (46). Clearly, more research is needed to identify the roles of these mutants in cancer.

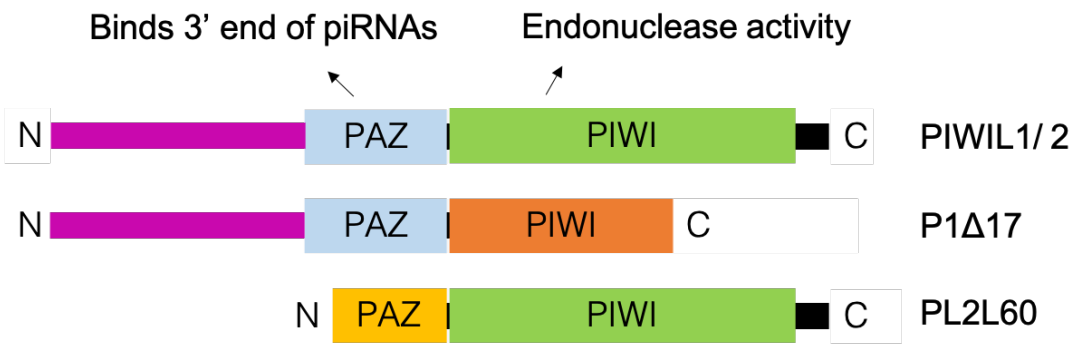

Figure 2. Schematic of domains (PAZ and PIWI) and their function in wildtype and mutant PIWIL proteins. Intact PAZ and PIWI domains are able to bind 3' end of piRNAs and cleave piRNAs, respectively. PIWIL1 mutant (P1Δ17) with a deletion in exon 17 was proposed to have a truncated PIWI domain. PIWIL2 mutant (PL2L60) was discovered to have a truncated PAZ domain.

1.4 piRNA pathway genes in reproductive cancers

As the piRNA pathway has expression in reproductive organs, it raises the possibility of a function in malignancies derived from these tissues (36, 43, 47). Currently, there are limited studies on the piRNA pathway genes and cancers of the reproductive system, OC and PCa as compared to breast, colon and liver cancer. Here, we explore the current literature of OC and PCa and their relationship with the piRNA pathway genes.

1.4.1 Ovarian cancer

Ovarian cancer (OC) is one of the most lethal gynaecologic malignancy with low 5-year survival rates of 26-42% which could be attributed by the prevalent problem of resistance to chemo drug treatment (48, 49). It was projected that 21,750 new cases will arise and 13,940 deaths will occur from OC in 2020 in the United States (50). Benign tissues can have ovarian and non-ovarian origin where they do not necessarily become malignant (51). These benign tissues can be classified and managed as a separate disease from OC (51). Epithelial OC accounts for 90% of OC. It consists of subtypes serous, mucinous, clear cell and endometroid (52). Low and high grade serous OC are two of the subtypes of epithelial OC (53). Of note, these two grades of

serous OC are different diseases with very different molecular pathology (Table 1).

Table 1. Molecular pathology of low grade and high grade serous ovarian cancer (54).

Molecular characteristics	**Low grade**	**High grade**
Proliferative activity	Low	High
Progression	Slow	Fast
Chromosomal stability	High	Low
DNA repair system	Generally normal	Always defective
TP53 mutation	Low	High
Chemotherapy response	Fair	Good but relapse

Diagnosis of OC is difficult as its symptoms, such as fatigue and bloating, are non-specific and commonly experienced in women with premenstrual syndrome (55, 56). Additionally, due to lack of early detection, women diagnosed with OC are often already in an advanced Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) stage where the cancer has spread to other parts of the body (57, 58). Women with a family history of OC are at a higher risk of developing OC as they may be pre-disposed to *BRCA* and *RAD51C* mutations which can impair DNA damage response in cells (59-61). The number of ovulation cycles has also been hypothesised to correlate with increasing risk of OC (62, 63). Hormones involved in ovulation include follicle stimulating hormone and luteinising hormone where they have been shown to cause OC proliferation and migration (64-67).

Expression analysis of the piRNA pathway genes in OC has only been performed on *PIWIL1-4* and *MAEL* where their aberrant expression has been reported. For example, Chen, Liu (68) demonstrated increased *PIWIL1-4* expression in malignant ovarian tissues. Singh, Roy (69) found no *PIWIL3* expression in both normal and malignant ovarian samples however, reported increased expression for *PIWIL1*, *PIWIL2* and *PIWIL4* in malignant tissues. Interestingly, Lim, Ricciardelli (43) demonstrated that only *PIWIL1* and *MAEL* had increased expression in malignant ovarian tissues while *PIWIL3* had higher expression in normal ovarian tissue compared to benign and malignant samples. *PIWIL2* and *PIWIL4* had no change in expression. Comparing these expression studies, inconsistencies in the trend of expression of *PIWIL1-4* can also be observed. A possible explanation for these differences could be the investigations were being carried out on epithelial OC in a broader context rather than specific subtypes and grades as they can have different molecular pathology as shown in Table 1.

Further work using functional assays revealed that overexpression of *PIWIL1* and *MAEL* resulted in decreased invasion for the OC cell line, SKOV-3 (43). This was surprising as overexpression of *PIWIL1* and *MAEL* in other cancers such as colorectal, endometrial and liver cancer promoted cell proliferation, cell growth and increased expression of mesenchymal markers (Vimentin, Fibronectin and N-cadherin) which are characteristic of tumour malignancy (45, 70, 71). This raises the possibility that the piRNA pathway genes could have cancer-specific effects. A study on chronic myeloid leukemia had a similar observation with OC where overexpression of *PIWIL1* induced decreased K562 cell proliferation and migration (44). Therefore, more studies

are needed to validate their expression and functional roles in a broader range of cancer to address the possibility of cancer-specific effects.

1.4.2 Prostate cancer

Prostate cancer (PCa) is one of the most commonly diagnosed cancer in men and is postulated to originate either from luminal or basal epithelial cells, or a combination of both (72, 73). In the United States, it was estimated that there will be 191,930 new PCa cases and 33,330 deaths in 2020 (50). Like ovarian cancer, *BRCA* mutations account for a risk in developing PCa (74, 75). Symptoms of PCa include painful urination and the appearance of blood in the urine (76). Monitoring the levels of prostate specific antigen (PSA) is a known detection method where a higher level of PSA may indicate the presence of PCa. This method, however, is controversial as it has limited specificity and has contributed to over-diagnosis of clinically insignificant prostate cancer patients, reducing their quality of life (77, 78).

One of the roles of the prostate is converting androgens, testosterone to dihydrotestosterone (DHT) (79, 80). DHT is a more potent form of androgen as it has 5-fold higher affinity to androgen receptors (ARs) compared to testosterone (81). PCa cells were discovered to be dependent on androgen for growth and survival as androgen is able regulate proliferation and apoptosis of normal prostate cells through the AR (81). Therefore, the first line of treatment for PCa is androgen deprivation therapy (ADT) (82). While ADT is an effective treatment for PCa, a proportion of cancer patients develop resistance to ADT, known as castrate resistant prostate cancer (CRPC) (81, 83).

CRPC cells are able to continue growth and proliferation at low levels or castrate levels of androgen. This led to the hypothesis that CRPC is androgen independent (84). Through a paradigm shift, ARs are now key therapeutic targets understood to have a role in CRPC development (81, 85, 86). The existence of AR variants such as AR-V7 has been hypothesised to contribute to CRPC. AR-V7 can override the function of wildtype AR and be constitutively active, triggering downstream cell growth and proliferation pathways for PCa (87). To investigate CRPC with AR-V7, the use of cell lines such as 22RV1 would provide a more accurate representation of the disease as it contains genomic alterations seen in PCa patients (84, 87).

A recent report indicated that androgen was able to affect piRNA expression levels in PCa cell lines, LNCaP and PC-3 (88). It is currently unclear if the piRNA pathway genes are affected by hormone levels in cancer. Hence, investigating the relationship of the piRNA pathway and ARs will enable further understanding of their mechanisms in PCa and potentially, CRPC. Thus far, two piRNA pathway genes, *PIWIL2* and *TDRD1*, have been investigated in PCa (89, 90). It was found that *PIWIL2* had significantly higher expression in PCa tumours compared to normal adjacent tissue. Further work revealed that *PIWIL2* knockdown in PCa cell line, PC-3, decreased invasion and mesenchymal markers (90). With a small sample size, Xiao, Lanz (89) demonstrated that a majority of PCa tumours had *TDRD1* expression while normal prostate tissue had none. *TDRD1* was also discovered to be a target gene of transcription factor, ETS-related gene (ERG). *ERG* is frequently seen to fuse with *transmembrane serine protease 2* (*TMPRSS2*) from cancer related genomic rearrangements where this fusion has been proposed to play a role

in the early development of PCa (91, 92). These factors led to the proposal of *TDRD1* being a potential biomarker for prostate cancer (89).

1.5 Thesis Objectives

piRNA pathway genes have emerged as new players in the field of cancer due to significantly deregulated expression in normal versus cancer tissues. While the piRNA pathway genes are known to execute functions beyond piRNA biogenesis, there is still much to be elucidated. In particular, contrasting outcomes from preliminary work in various cancers showed the complexity of the piRNA pathway where it can potentially act in a cancer-specific manner. Furthermore, there are limited to no studies on a broader range of piRNA pathway genes in cancer such as *DDX4*, *HENMT1*, *PLD6* and *TDRD9*. These genes, in addition to *PIWIL1-4*, *MAEL* and *TDRD1*, are integral to the functionality of the piRNA pathway (30, 39, 93, 94). As current studies have linked their involvement to the development of key cancer characteristics, they were selected for further investigation (95-97).

A new and exciting prospect of the piRNA pathway is its clinical relevance to patient survival, links to chemoresistance and possible regulation by hormones. Here, we hypothesised in OC and PCa that the piRNA pathway genes:

1. Will have cancer-specific expression profiles when comparing OC and PCa (*PIWIL1-4*, *DDX4*, *HENMT1*, *MAEL*, *PLD6*, *TDRD1* and *TDRD9*)
2. Can affect the progression free, post-progression free and overall survival of patients
3. Are responsive to hormone treatment

4. Will decrease motility and invasion of cancer cells overexpressing wildtype and mutant piRNA pathway genes *in vitro* and *in vivo*.

References

1. Ghildiyal M, Zamore PD. Small silencing RNAs: an expanding universe. Nature Reviews Genetics. 2009;10(2):94-108.
2. Schmitz SU, Grote P, Herrmann BG. Mechanisms of long noncoding RNA function in development and disease. Cellular and Molecular Life Sciences. 2016;73(13):2491-509.
3. Ramat A, Simonelig M. Functions of PIWI Proteins in Gene Regulation: New Arrows Added to the piRNA Quiver. Trends in Genetics. 2020.
4. Esteller M. Non-coding RNAs in human disease. Nature Reviews Genetics. 2011;12(12):861-74.
5. Song E. The long and short non-coding RNAs in cancer biology: Springer; 2016.
6. Slack FJ, Chinnaiyan AM. The role of non-coding RNAs in oncology. Cell. 2019;179(5):1033-55.
7. Aravin AA, Lagos-Quintana M, Yalcin A, Zavolan M, Marks D, Snyder B, et al. The Small RNA Profile during Drosophila melanogaster Development. Developmental Cell. 2003;5(2):337-50.
8. Aravin A, Gaidatzis D, Pfeffer S, Lagos-Quintana M, Landgraf P, Iovino N, et al. A novel class of small RNAs bind to MILI protein in mouse testes. Nature. 2006;442(7099):203-7.
9. Aravin AA, Naumova NM, Tulin AV, Vagin VV, Rozovsky YM, Gvozdev VA. Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the D. melanogaster germline. Current Biology. 2001;11(13):1017-27.
10. Chirn G-w, Rahman R, Sytnikova YA, Matts JA, Zeng M, Gerlach D, et al. Conserved piRNA Expression from a Distinct Set of piRNA Cluster Loci in Eutherian Mammals. PLOS Genetics. 2015;11(11):e1005652.
11. Czech B, Hannon GJ. One Loop to Rule Them All: The Ping-Pong Cycle and piRNA-Guided Silencing. Trends in Biochemical Sciences. 2016.
12. Kazazian HH. Mobile Elements: Drivers of Genome Evolution. Science. 2004;303(5664):1626-32.
13. Zamudio N, Bourc'his D. Transposable elements in the mammalian germline: a comfortable niche or a deadly trap? Heredity. 2010;105(1):92.
14. Siomi MC, Sato K, Pezic D, Aravin AA. PIWI-interacting small RNAs: the vanguard of genome defence. Nature Reviews Molecular Cell Biology. 2011;12(4):246-58.
15. Kuramochi-Miyagawa S, Watanabe T, Gotoh K, Totoki Y, Toyoda A, Ikawa M, et al. DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. Genes & Development. 2008;22(7):908-17.

16. Iwasaki YW, Siomi MC, Siomi H. PIWI-Interacting RNA: Its Biogenesis and Functions. Annual Review of Biochemistry. 2015;84:405-33.
17. Czech B, Munafò M, Ciabrelli F, Eastwood EL, Fabry MH, Kneuss E, et al. piRNA-guided genome defense: from biogenesis to silencing. Annual Review of Genetics. 2018;52:131-57.
18. Gunawardane LS, Saito K, Nishida KM, Miyoshi K, Kawamura Y, Nagami T, et al. A Slicer-Mediated Mechanism for Repeat-Associated siRNA 5' End Formation in Drosophila. Science. 2007;315(5818):1587-90.
19. Mohn F, Handler D, Brennecke J. piRNA-guided slicing specifies transcripts for Zucchini-dependent, phased piRNA biogenesis. Science. 2015;348(6236):812-7.
20. Chen C, Nott TJ, Jin J, Pawson T. Deciphering arginine methylation: Tudor tells the tale. Nature Reviews Molecular Cell Biology. 2011;12(10):629-42.
21. Siomi MC, Mannen T, Siomi H. How does the Royal Family of Tudor rule the PIWI-interacting RNA pathway? Genes & Development. 2010;24(7):636-46.
22. Saito K, Sakaguchi Y, Suzuki T, Suzuki T, Siomi H, Siomi MC. Pimet, the Drosophila homolog of HEN1, mediates 2′-O-methylation of Piwi-interacting RNAs at their 3′ ends. Genes & Development. 2007;21(13):1603-8.
23. Horwich MD, Li C, Matranga C, Vagin V, Farley G, Wang P, et al. The Drosophila RNA Methyltransferase, DmHen1, Modifies Germline piRNAs and Single-Stranded siRNAs in RISC. Current Biology. 2007;17(14):1265-72.
24. Lim SL, Qu ZP, Kortschak RD, Lawrence DM, Geoghegan J, Hempfling A-L, et al. HENMT1 and piRNA Stability Are Required for Adult Male Germ Cell Transposon Repression and to Define the Spermatogenic Program in the Mouse. PLOS Genetics. 2015;11(10):e1005620.
25. Soper SFC, van der Heijden GW, Hardiman TC, Goodheart M, Martin SL, de Boer P, et al. Mouse Maelstrom, a component of nuage, is essential for spermatogenesis and transposon repression in meiosis. Developmental Cell. 2008;15(2):285-97.
26. Sato K, Siomi MC. Functional and structural insights into the piRNA factor Maelstrom. FEBS letters. 2015;589(14):1688-93.
27. Mathioudakis N, Palencia A, Kadlec J, Round A, Tripsianes K, Sattler M, et al. The multiple Tudor domain-containing protein TDRD1 is a molecular scaffold for mouse Piwi proteins and piRNA biogenesis factors. RNA 2012;18(11):2056-72.
28. Chuma S, Hosokawa M, Kitamura K, Kasai S, Fujioka M, Hiyoshi M, et al. Tdrd1/Mtr-1, a tudor-related gene, is essential for male germ-cell differentiation and nuage/germinal granule formation in mice. Proceedings of the National Academy of Sciences. 2006;103(43):15894-9.
29. Hosokawa M, Shoji M, Kitamura K, Tanaka T, Noce T, Chuma S, et al. Tudor-related proteins TDRD1/MTR-1, TDRD6 and TDRD7/TRAP: domain composition, intracellular localization, and function in male germ cells in mice. Developmental biology. 2007;301(1):38-52.
30. Ozata DM, Gainetdinov I, Zoch A, O'Carroll D, Zamore PD. PIWI-interacting RNAs: small RNAs with big functions. Nature Reviews Genetics. 2019;20(2):89-108.

31. Xiol J, Spinelli P, Laussmann MA, Homolka D, Yang Z, Cora E, et al. RNA clamping by Vasa assembles a piRNA amplifier complex on transposon transcripts. Cell. 2014;157(7):1698-711.
32. Aravin AA, Sachidanandam R, Bourc'his D, Schaefer C, Pezic D, Fejes Toth K, et al. A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. Molecular Cell. 2008;31(6):785-99.
33. Wenda JM, Homolka D, Yang Z, Spinelli P, Sachidanandam R, Pandey RR, et al. Distinct Roles of RNA Helicases MVH and TDRD9 in PIWI Slicing-Triggered Mammalian piRNA Biogenesis and Function. Developmental Cell. 2017;41(6):623-37.e9.
34. Shoji M, Tanaka T, Hosokawa M, Reuter M, Stark A, Kato Y, et al. The TDRD9-MIWI2 complex is essential for piRNA-mediated retrotransposon silencing in the mouse male germline. Developmental Cell. 2009;17(6):775-87.
35. Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, et al. Discrete Small RNA-Generating Loci as Master Regulators of Transposon Activity in Drosophila. Cell. 2007;128(6):1089-103.
36. Lim SL, Tsend-Ayush E, Kortschak RD, Jacob R, Ricciardelli C, Oehler MK, et al. Conservation and Expression of PIWI-Interacting RNA Pathway Genes in Male and Female Adult Gonad of Amniotes. Biology of Reproduction. 2013;89(6):136, 1-13.
37. Malone CD, Brennecke J, Dus M, Stark A, McCombie WR, Sachidanandam R, et al. Specialized piRNA Pathways Act in Germline and Somatic Tissues of the Drosophila Ovary. Cell. 2009;137(3):522-35.
38. Sato K, Siomi MC. The piRNA pathway in Drosophila ovarian germ and somatic cells. Proceedings of the Japan Academy, Series B. 2020;96(1):32-42.
39. Hirakata S, Siomi MC. piRNA biogenesis in the germline: From transcription of piRNA genomic sources to piRNA maturation. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms. 2016;1859(1):82-92.
40. Genzor P, Cordts SC, Bokil NV, Haase AD. Aberrant expression of select piRNA-pathway genes does not reactivate piRNA silencing in cancer cells. Proceedings of the National Academy of Sciences. 2019;116(23):11111-2.
41. Lee E, Jacob R, Grützner F. PIWI-Interacting RNAs (piRNAs) and Cancer. Cancer and Noncoding RNAs: Elsevier; 2018. p. 131-50.
42. Wang D-W, Wang Z-H, Wang L-L, Song Y, Zhang G-Z. Overexpression of hiwi promotes growth of human breast cancer cells. Asian Pacific Journal of Cancer Prevention. 2013;15(18):7553-8.
43. Lim SL, Ricciardelli C, Oehler MK, De Arao Tan IMD, Russell D, Grützner F. Overexpression of piRNA Pathway Genes in Epithelial Ovarian Cancer. PLOS One. 2014;9(6):e99687.
44. Wang Y, Jiang Y, Ma N, Sang B, Hu X, Cong X, et al. Overexpression of Hiwi Inhibits the Growth and Migration of Chronic Myeloid Leukemia Cells. Cell Biochemistry and Biophysics. 2015;73(1):117-24.
45. Liu L, Dai Y, Chen J, Zeng T, Li Y, Chen L, et al. Maelstrom promotes hepatocellular carcinoma metastasis by inducing epithelial‐mesenchymal

transition by way of Akt/GSK-3β/Snail signaling. Hepatology. 2014;59(2):531-43.
46. Ye Y, Yin D-T, Chen L, Zhou Q, Shen R, He G, et al. Identification of Piwil2-Like (PL2L) Proteins that Promote Tumorigenesis. PLOS One. 2010;5(10):e13406.
47. Martinez VD, Vucic EA, Thu KL, Hubaux R, Enfield KS, Pikor LA, et al. Unique somatic and malignant expression patterns implicate PIWI-interacting RNAs in cancer-type specific biology. Scientific Reports. 2015;5.
48. Torre LA, Trabert B, DeSantis CE, Miller KD, Samimi G, Runowicz CD, et al. Ovarian cancer statistics, 2018. CA: A Cancer Journal for Clinicians. 2018;68(4):284-96.
49. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians. 2018;68(6):394-424.
50. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA: A Cancer Journal for Clinicians. 2020;70(1):7-30.
51. RCOG/BSGE. Management of suspected ovarian masses in premenopausal women. 2011.
52. Matulonis UA, Sood AK, Fallowfield L, Howitt BE, Sehouli J, Karlan BY. Ovarian cancer. Nature Reviews Disease Primers. 2016;2(1):1-22.
53. Prat J. Ovarian carcinomas: five distinct diseases with different origins, genetic alterations, and clinicopathological features. Virchows Archiv. 2012;460(3):237-49.
54. Kurman RJ, Shih I-M. The Dualistic Model of Ovarian Carcinogenesis: Revisited, Revised, and Expanded. The American Journal of Pathology. 2016;186(4):733-47.
55. Australia H. Ovarian cancer symptoms. 2017.
56. Olson SH, Mignone L, Nakraseive C, Caputo T, Barakat R, Harlap S. Symptoms of ovarian cancer. Obstetrics & Gynecology. 2001;98(2):212-7.
57. Li J, Fadare O, Xiang L, Kong B, Zheng W. Ovarian serous carcinoma: recent concepts on its origin and carcinogenesis. Journal of Hematology & Oncology. 2012;5(1):8.
58. Prat J, Oncology FCoG. Staging classification for cancer of the ovary, fallopian tube, and peritoneum. International Journal of Gynecology & Obstetrics. 2014;124(1):1-5.
59. Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nature Reviews Cancer. 2012;12(1):68-78.
60. Cunningham JM, Cicek M, Larson N, Davila J, Wang C, Larson M, et al. Clinical characteristics of ovarian cancer classified by BRCA1, BRCA2, and RAD51C status. Scientific Reports. 2014;4.
61. Pharoah PD, Ponder BA. The genetics of ovarian cancer. Best Practice & Research Clinical Obstetrics & Gynaecology. 2002;16(4):449-68.
62. Mertens-Walker I, Baxter RC, Marsh DJ. Gonadotropin signalling in epithelial ovarian cancer. Cancer Letters. 2012;324(2):152-9.
63. Gharwan H, Bunch KP, Annunziata CM. The role of reproductive hormones in epithelial ovarian carcinogenesis. Endocrine-Related Cancer. 2015;22(6):R339-R63.

64. Zheng W, Lu JJ, Luo F, Zheng Y, Feng Y-j, Felix JC, et al. Ovarian Epithelial Tumor Growth Promotion by Follicle-Stimulating Hormone and Inhibition of the Effect by Luteinizing Hormone. Gynecologic Oncology. 2000;76(1):80-8.
65. Feng D, Zhao T, Yan K, Liang H, Liang J, Zhou Y, et al. Gonadotropins promote human ovarian cancer cell migration and invasion via a cyclooxygenase 2-dependent pathway. Oncology Reports. 2017;38(2):1091.
66. Mertens-Walker I, Bolitho C, Baxter RC, Marsh DJ. Gonadotropin-induced ovarian cancer cell migration and proliferation require extracellular signal-regulated kinase 1/2 activation regulated by calcium and protein kinase Cδ. Endocrine-Related Cancer. 2010;17(2):335.
67. Choi J-H, Choi K-C, Auersperg N, Leung PC. Gonadotropins activate proteolysis and increase invasion through protein kinase A and phosphatidylinositol 3-kinase pathways in human epithelial ovarian cancer cells. Cancer Research. 2006;66(7):3912-20.
68. Chen C, Liu J, Xu G. Overexpression of PIWI proteins in human stage III epithelial ovarian cancer with lymph node metastasis. Cancer Biomarkers. 2013;13(5):315-21.
69. Singh G, Roy J, Rout P, Mallick B. Genome-wide profiling of the PIWI-interacting RNA-mRNA regulatory networks in epithelial ovarian cancers. PLOS One. 2018;13(1):e0190485.
70. Yang L, Bi L, Liu Q, Zhao M, Cao B, Li D, et al. Hiwi Promotes the Proliferation of Colorectal Cancer Cells via Upregulating Global DNA Methylation. Disease Markers. 2015;2015:10.
71. Chen Z, Che Q, He X, Wang F, Wang H, Zhu M, et al. Stem cell protein Piwil1 endowed endometrial cancer cells with stem-like properties via inducing epithelial-mesenchymal transition. BMC Cancer. 2015;15(1):811.
72. Park JW, Lee JK, Phillips JW, Huang P, Cheng D, Huang J, et al. Prostate epithelial cell of origin determines cancer differentiation state in an organoid transformation assay. Proceedings of the National Academy of Sciences. 2016;113(16):4482-7.
73. Goldstein AS, Huang J, Guo C, Garraway IP, Witte ON. Identification of a Cell of Origin for Human Prostate Cancer. Science. 2010;329(5991):568.
74. Gallagher DJ, Gaudet MM, Pal P, Kirchhoff T, Balistreri L, Vora K, et al. Germline BRCA mutations denote a clinicopathologic subset of prostate cancer. Clinical Cancer Research. 2010;16.
75. Cavanagh H, Rogers KMA. The role of BRCA1 and BRCA2 mutations in prostate, pancreatic and stomach cancers. Hereditary Cancer in Clinical Practice. 2015;13(1):16.
76. Miller DC, Hafez KS, Stewart A, Montie JE, Wei JT. Prostate carcinoma presentation, diagnosis, and staging. Cancer. 2003;98(6):1169-78.
77. De Angelis G, Rittenhouse HG, Mikolajczyk SD, Blair Shamel L, Semjonow A. Twenty Years of PSA: From Prostate Antigen to Tumor Marker. Reviews in Urology. 2007;9(3):113-23.
78. Lomas DJ, Ahmed HU. All change in the prostate cancer diagnostic pathway. Nature Reviews Clinical Oncology. 2020:1-10.

79. Kumar V, Majumder P. Prostate gland: structure, functions and regulation. International Urology and Nephrology. 1995;27(3):231-43.
80. Sandberg AA. Endocrine control and physiology of the prostate. The Prostate. 1980;1(2):169-84.
81. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nature Reviews Cancer. 2001;1(1):34-45.
82. Sartor O, de Bono JS. Metastatic prostate cancer. New England Journal of Medicine. 2018;378(7):645-57.
83. Harris WP, Mostaghel EA, Nelson PS, Montgomery B. Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion. Nature Clinical Practice Urology. 2009;6(2):76-85.
84. Watson PA, Arora VK, Sawyers CL. Emerging mechanisms of resistance to androgen receptor inhibitors in prostate cancer. Nature Reviews Cancer. 2015;15(12):701.
85. Karantanos T, Corn PG, Thompson TC. Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate-resistance and novel therapeutic approaches. Oncogene. 2013;32(49):5501.
86. Teo MY, Rathkopf DE, Kantoff P. Treatment of advanced prostate cancer. Annual Review of Medicine. 2019;70:479-99.
87. Li Y, Chan SC, Brand LJ, Hwang TH, Silverstein KA, Dehm SM. Androgen receptor splice variants mediate enzalutamide resistance in castration-resistant prostate cancer cell lines. Cancer Research. 2013;73(2):483-9.
88. Öner Ç, Turgut Coşan D, Çolak E. Estrogen and Androgen Hormone Levels Modulate the Expression of PIWI Interacting RNA in Prostate and Breast Cancer. PLOS One. 2016;11(7):e0159044.
89. Xiao L, Lanz RB, Frolov A, Castro PD, Zhang Z, Dong B, et al. The germ cell gene TDRD1 as an ERG target gene and a novel prostate cancer biomarker. The Prostate. 2016;76(14):1271-84.
90. Yang Y, Zhang X, Song D, Wei J. Piwil2 modulates the invasion and metastasis of prostate cancer by regulating the expression of matrix metalloproteinase-9 and epithelial-mesenchymal transitions. Oncology Letters. 2015;10(3):1735-40.
91. Mani R-S, Tomlins SA, Callahan K, Ghosh A, Nyati MK, Varambally S, et al. Induced chromosomal proximity and gene fusions in prostate cancer. Science. 2009;326(5957):1230-.
92. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun X-W, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science. 2005;310(5748):644-8.
93. Tan M, van Tol HT, Rosenkranz D, Roovers EF, Damen MJ, Stout TA, et al. PIWIL3 forms a complex with TDRKH in mammalian oocytes. Cells. 2020;9(6):1356.
94. Hempfling A-L, Lim SL, Adelson D, Evans J, O'Connor AE, Qu Z, et al. Expression patterns of HENMT1 and PIWIL1 in human testis-Implications for transposon expression. Reproduction. 2017:REP-16-0586.
95. Kim KH, Kang Y-J, Jo J-O, Ock MS, Moon SH, Suh DS, et al. DDX4 (DEAD box polypeptide 4) colocalizes with cancer stem cell marker CD133 in

ovarian cancers. Biochemical and Biophysical Research Communications. 2014;447(2):315-22.
96. Guijo M, Ceballos-Chávez M, Gómez-Marín E, Basurto-Cayuela L, Reyes JC. Expression of TDRD9 in a subset of lung carcinomas by CpG island hypomethylation protects from DNA damage. Oncotarget. 2017;9(11):9618-31.
97. Liu Y, Dou M, Song X, Dong Y, Liu S, Liu H, et al. The emerging role of the piRNA/piwi complex in cancer. Molecular Cancer. 2019;18(1):123.
98. Li C, Zhou X, Chen J, Lu Y, Sun Q, Tao D, et al. PIWIL1 destabilizes microtubule by suppressing phosphorylation at Ser16 and RLIM-mediated degradation of stathmin1. Oncotarget. 2015;6(29):27794-804.
99. Liu X, Sun Y, Guo J, Ma H, Li J, Dong B, et al. Expression of hiwi gene in human gastric cancer was associated with proliferation of cancer cells. International Journal of Cancer. 2006;118(8):1922-9.
100. Xie Y, Yang Y, Ji D, Zhang D, Yao X, Zhang X. Hiwi downregulation, mediated by shRNA, reduces the proliferation and migration of human hepatocellular carcinoma cells. Molecular Medicine Reports. 2015;11:1455+.
101. Liang D, Fang Z, Dong M, Liang C, Xing C, Zhao J, et al. Effect of RNA interference-related HiWi gene expression on the proliferation and apoptosis of lung cancer stem cells. Oncology Letters. 2012;4(1):146-50.
102. Siddiqi S, Terry M, Matushansky I. Hiwi mediated tumorigenesis is associated with DNA hypermethylation. PLOS One. 2012;7(3):e33711.
103. Lee JH, Jung C, Javadian-Elyaderani P, Schweyer S, Schütte D, Shoukier M, et al. Pathways of Proliferation and Antiapoptosis Driven in Breast Cancer Stem Cells by Stem Cell Protein Piwil2. Cancer Research. 2010;70(11):4569-79.
104. Liu JJ, Shen R, Chen L, Ye Y, He G, Hua K, et al. Piwil2 is expressed in various stages of breast cancers and has the potential to be used as a novel biomarker. International Journal of Clinical and Experimental Pathology. 2010;3(4):328-37.
105. Zhang H, Ren Y, Xu H, Pang D, Duan C, Liu C. The expression of stem cell protein Piwil2 and piR-932 in breast cancer. Surgical Oncology. 2013;22(4):217-23.
106. Lu Y, Zhang K, Li C, Yao Y, Tao D, Liu Y, et al. Piwil2 Suppresses P53 by Inducing Phosphorylation of Signal Transducer and Activator of Transcription 3 in Tumor Cells. PLOS One. 2012;7(1):e30999.
107. Jiang S, Zhao L, Lu Y, Wang M, Chen Y, Tao D, et al. Piwil2 inhibits keratin 8 degradation through promoting p38-induced phosphorylation to resist Fas-mediated apoptosis. Molecular and Cellular Biology. 2014;34(21):3928-38.
108. Li D, Sun X, Yan D, Huang J, Luo Q, Tang H, et al. Piwil2 modulates the proliferation and metastasis of colon cancer via regulation of matrix metallopeptidase 9 transcriptional activity. Experimental Biology and Medicine. 2012;237(10):1231-40.
109. Li J, Xu L, Bao Z, Xu P, Chang H, Wu J, et al. High expression of PIWIL2 promotes tumor cell proliferation, migration and predicts a poor prognosis in glioma. Oncology Reports. 2017;38(1):183-92.

110. Qu X, Liu J, Zhong X, Li X, Zhang Q. PIWIL2 promotes progression of non-small cell lung cancer by inducing CDK2 and Cyclin A expression. Journal of Translational Medicine. 2015;13(1):1.
111. Wang Q-E, Han C, Milum K, Wani AA. Stem cell protein Piwil2 modulates chromatin modifications upon cisplatin treatment. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2011;708(1–2):59-68.
112. Xiao L, Wang Y, Zhou Y, Sun Y, Sun W, Wang L, et al. Identification of a novel human cancer/testis gene MAEL that is regulated by DNA methylation. Molecular Biology Reports. 2010;37(5):2355-60.
113. Kim S-H, Park E-R, Cho E, Jung W-H, Jeon J-Y, Joo H-Y, et al. Mael is essential for cancer cell survival and tumorigenesis through protection of genetic integrity. Oncotarget. 2017;8(3):5026-37.
114. Yuan L, Xiao Y, Zhou Q, Yuan D, Wu B, Chen G, et al. Proteomic analysis reveals that MAEL, a component of nuage, interacts with stress granule proteins in cancer cells. Oncology Reports. 2014;31(1):342-50.

Supplementary Table

Table S1. Roles of selected piRNA pathway genes and mutants in cancer.

piRNA pathway genes	Effects in cancer		Ref
	Cancer type	**Observations**	
PIWIL1	**Breast**	• Overexpression: o Increased cell growth	(42)
	Cervical	• Binds stathmin1, causing: o Inhibited microtubule polymerization o Enhanced proliferation, migration and invasion	(98)
	Chronic Myeloid Leukemia	• Overexpression: o Decreased cell proliferation o Induced apoptosis o Decreased migration o Inhibited expression and activity of matrix metalloproteinase-2 and -9	(44)
	Colorectal	• Overexpression: o Increased proliferation	(70)

		○ Increased global DNA methylation	
	Endometrial	• Overexpression: ○ Increased mesenchymal markers; suppression of epithelial markers ○ Augmented expression of stem cell markers ○ Maintenance of stem-like characteristics	(71)
	Gastric	• Suppression: ○ Inhibited cell growth ○ Induced cell cycle arrest at G_2/ M phase	(99)
	Liver	• Binds stathmin1, causing: ○ Inhibited microtubule polymerization ○ Enhanced proliferation, migration and invasion	(98)
		• Suppression: ○ Decreased proliferation, migration and invasion	(100)
	Lung	• Silencing: ○ Decreased proliferation ○ Increased apoptosis	(101)
	Ovarian	• Overexpression: ○ Decreased invasion	(43)
	Sarcoma	• Suppression: ○ Inhibited cell growth ○ Decreased DNA methylation	(102)
PIWIL2	**Breast**	• Silencing: ○ Decreased *STAT3* expression ○ Decreased proliferation and cell survival	(103)

		• Expression pattern associated with Ki67 (proliferation marker)	(104)
		• Decreased Latexin (tumor suppressor) expression via CpG methylation of promoter	(105)
	Cervical	• Suppressed *P53* expression via c-Src phosphorylation of STAT3	(106)

		• Suppression:
		  ○ Increased FAS-mediated apoptosis
		  ○ Decreased P53 phosphorylation via P38
			(107)

	Colon	• Silencing:
		  ○ Decreased proliferation
		  ○ Increased apoptosis *in vitro*
		  ○ Inhibited tumor growth *in vivo*
		  ○ Decreased migration and invasion via regulation of matrix metalloproteinase-9
			(108)

	Glioma	• Suppression:
		  ○ Induced cell cycle arrest
		  ○ Increased apoptosis
		• Silencing:
		  ○ Suppressed migration
			(109)

	Liver	• Suppression:
		  ○ Increased FAS-mediated apoptosis
		  ○ Decreased P53 phosphorylation via P38
			(107)

	Lung	• Correlated expression with CDK2 and Cyclin A (cell cycle proteins) • Suppression: ○ Decreased cell proliferation	(110)

		○ Arrested cells at G2/ M phase	
	Ovarian	• Suppression in cisplatin-resistant cells: ○ Increased sensitivity to cisplatin	(111)
	Prostate	• Suppression: ○ Decreased cell migration and invasion ○ Decreased matrix metalloproteinase-9 expression ○ Decreased expression of mesenchymal markers ○ Increased expression of epithelial markers	(90)
MAEL	**Breast**	• Decreased DNA methylation with increased *MAEL* expression	(112)
		• Silencing: • Decreased proliferation	(113)
		• Interacted and localized with stress granules	(114)
	Colorectal		
	Cervical	• Suppression: ○ Cell cycle arrest at G_2/ M phase ○ Increased DNA damage ○ Generated intracellular reactive oxygen species ○ Induced ATM sensing of DNA damage	(113)
	Liver		
		• Overexpression: ○ Cell growth and migration ○ Tumor formation *in vivo* ○ Increased AKT activity, GSK-3β phosphorylation and Snail stabilization	(45)

		○ Decreased expression of epithelial markers ○ Increased expression of: a) mesenchymal markers b) stemness markers c) cancer stem cell markers d) multidrug resistance genes	
	Ovarian	• Overexpression: ○ Decreased invasion	(43)

CHAPTER 2: PIRNA PATHWAY

GENES AND OVARIAN CANCER

A published article in *Cancers*

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Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	16/2/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Noor A Lokman		
Contribution to the Paper	Provided guidance on experiments and data analysis, supervision and manuscript editing.		
Signature		Date	17/2/2021

Name of Co-Author	Martin K Oehler		
Contribution to the Paper	Provided benign and ovarian cancer tumours, supervision and manuscript editing.		
Signature		Date	17/2/2021

Name of Co-Author	Carmela Ricciardelli		
Contribution to the Paper	Provided guidance on data analysis, supervision and manuscript editing.		
Signature		Date	17/2/2021

Name of Co-Author	Frank Grutzner		
Contribution to the Paper	Conceived the study, provided supervision and manuscript editing.		
Signature		Date	17/2/2021

Please cut and paste additional co-author panels here as required.

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Article

A Comprehensive Molecular and Clinical Analysis of the piRNA Pathway Genes in Ovarian Cancer

Eunice Lee [1], Noor A. Lokman [2,3], Martin K. Oehler [2,3,4], Carmela Ricciardelli [2,*,†] and Frank Grutzner [1,*,†]

[1] Department of Molecular and Biomedical Sciences, Robinson Research Institute, University of Adelaide, Adelaide, SA 5000, Australia; eunicehsiuyee.lee@adelaide.edu.au
[2] Discipline of Obstetrics and Gynaecology, Robinson Research Institute, Adelaide Medical School, University of Adelaide, Adelaide, SA 5000, Australia; noor.lokman@adelaide.edu.au (N.A.L.); martin.oehler@adelaide.edu.au (M.K.O.)
[3] Future Industries Institute, University of South Australia, Mawson Lakes, SA 5095, Australia
[4] Department of Gynaecological Oncology, Royal Adelaide Hospital, Adelaide, SA 5005, Australia
* Correspondence: carmela.ricciardelli@adelaide.edu.au (C.R.); frank.grutzner@adelaide.edu.au (F.G.); Tel.: +61-8-8313-8255 (C.R.); +61-8-8313-4812 (F.G.)
† Authors contributed equally to the work.

Simple Summary: Although ovarian cancer (OC) is one of the most lethal gynecological cancers, its development and progression remain poorly understood. The piRNA pathway is important for transposon defense and genome stability. piRNA maturation and function involve a number of genes known as the piRNA pathway genes. These genes have recently been implicated in cancer development and progression but information about their role in OC is limited. Our work aimed to provide a better understanding of the roles of piRNA pathway genes in OC. Through analyzing changes in the abundance of 10 piRNA pathway genes, we discovered gene expression differences in benign vs. cancer, chemosensitive vs. chemoresistant and post hormone treatment in OC samples and cells. Furthermore, we observed the differential effects of these genes on patient survival and OC cell invasion. Overall, this work supports a role of the piRNA pathway genes in OC progression and encourages further study of their clinical relevance.

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Abstract: Ovarian cancer (OC) is one of the most lethal gynecological malignancies, yet molecular mechanisms underlying its origin and progression remain poorly understood. With increasing reports of piRNA pathway deregulation in various cancers, we aimed to better understand its role in OC through a comprehensive analysis of key genes: *PIWIL1-4*, *DDX4*, *HENMT1*, *MAEL*, *PLD6*, *TDRD1,9* and mutants of *PIWIL1* (*P1Δ17*) and *PIWIL2* (*PL2L60*). High-throughput qRT-PCR (n = 45) and CSIOVDB (n = 3431) showed differential gene expression when comparing benign ovarian tumors, low grade OC and high grade serous OC (HGSOC). Significant correlation of disparate piRNA pathway gene expression levels with better progression free, post-progression free and overall survival suggests a complex role of

this pathway in OC. We discovered *PIWIL3* expression in chemosensitive but not chemoresistant primary HGSOC cells, providing a potential target against chemoresistant disease. As a first, we revealed that follicle stimulating hormone increased *PIWIL2* expression in OV-90 cells. *PIWIL1*, *P1Δ17*, *PIWIL2*, *PL2L60* and *MAEL* overexpression in vitro and in vivo decreased motility and invasion of OVCAR-3 and OV-90 cells. Interestingly, *P1Δ17* and *PL2L60*, induced increased motility and invasion compared to *PIWIL1* and *PIWIL2*. Our results in HGSOC highlight the intricate role piRNA pathway genes play in the development of malignant neoplasms.

Keywords: ovarian cancer; piRNA pathway; patient survival; invasion; follicle stimulating hormone; chemoresistance; therapeutic targets

1. Introduction

Ovarian cancer (OC) is one of the leading causes of death in women worldwide where 184,799 deaths were projected in 2018. This was the second highest mortality among all gynecological cancers [1]. Due to unspecific symptoms and lack of early detection, OC is most commonly diagnosed at an advanced stage and subsequently has very poor prognosis [2,3]. It has been hypothesized that the number of ovulation cycles correlates with the risk of developing OC and that hormones such as follicle stimulating hormone (FSH) and luteinizing hormone (LH) might be involved in the process and induce malignant transformation [4,5]. While incessant ovulation is considered a risk factor, this theory is still controversial [6,7].

OC consists of epithelial ovarian cancers (EOC) (90%) which include subtypes serous, mucinous, clear cell and endometroid. Nonepithelial ovarian cancers (10%) include small-cell carcinomas and sarcomas and malignancies originating from germ or sex cord-stromal cells [8,9]. HGSOC is known for its highly heterogeneous nature, chromosomal instability and high rates of chemoresistance where mutations in the *TP53* gene occur in a majority of HGSOC samples, more than any other cancer [10–14]. While mutations in tumor suppressor *TP53* have been postulated as the driver of HGSOC, the etiology, subsequent progression and development of chemoresistance are poorly understood [15,16]. OC is classified through FIGO staging (stages I–IV) and grading (grades 1–3). In the serous OC context, low grade (grade 1) and high grade (grades 2 and 3) are classified as two separate diseases with different underlying molecular pathology and clinical behavior [11,17,18]. Tumor malignancies are often linked to a large number of genes and their associated mutants as seen in OC [12,19,20]. With the origin and development of OC still under much discussion, the emergence of deregulated piRNA pathway in cancer has led to a few studies investigating their involvement in OC development [6,21–26].

The piRNA pathway consists of piRNAs (small noncoding RNAs (ncRNAs)) and a growing list of associated pathway genes which are involved in the biogenesis of piRNAs [27–29]. The core biogenesis pathway genes, the *PIWIL* genes (*PIWIL1-4*), are essential in ensuring the maturation of piRNAs through their involvement in either the primary or secondary biogenesis pathway [30]. To do this, PIWILs utilize their three main domains known as PAZ, MID and PIWI. These domains mainly function in binding the 3′ end of piRNAs, 5′ end of piRNAs and having endonuclease activity, respectively [31–33]. On top of the *PIWIL* genes, there is a myriad of pathway genes including *DDX4*, *HENMT1*, *MAEL*, *PLD6*, *TDRD1* and *TDRD9*. A more detailed list of pathway genes and their respective functions in piRNA biogenesis was recently reviewed by Ozata, Gainetdinov, Zoch, O'Carroll and Zamore [28]. The piRNA pathway was originally discovered in gonads as a mechanism to control expression of transposable elements (TEs) [34,35]. In addition to having crucial roles in the production of mature piRNAs, the pathway genes can also work in tandem or independently of each other to aid piRNAs in the execution of TE repression [36–39]. One method is through the fascinating secondary biogenesis pathway where PIWIL2 and PIWIL4 convert TEs into

piRNAs, generating a multitude of piRNA species while destroying TEs [36,40,41]. Since the discovery of the piRNA pathway, its roles beyond TE repression has steadily increased especially after reports of its deregulation in cancer [42–44].

piRNAs and piRNA pathway genes are increasingly discovered to be involved in various aspects of cancer development and progression [43]. Some of these pathway genes (*PIWIL1*, *PIWIL2*, *TDRD1* and *MAEL*) are categorized as cancer/testis genes due to their restricted expression in testis but in recent years, have been observed to be aberrantly expressed in multiple cancers [43–46]. The functions of these genes go beyond the piRNA pathway and include regulating cell motility, invasion, proliferation and apoptosis which are hallmarks of cancer progression and malignancy [19,24,43,47–49]. Additionally, cancer specific mutations have been revealed including a *PIWIL1* mutant (*P1Δ17*) which lacks an exon 17 and a PIWIL2 mutant (PL2L60) with a truncated PAZ domain [25,50]. The *P1Δ17* transcript was only present in malignant HGSOC and was proposed to have a premature stop codon with a truncated PIWI domain in its protein form [25]. Upon discovery of PL2L60 in mouse testis, it was then found to be the predominant form of *PIWIL2* in precancerous stem cells where its expression associates with the antiapoptotic *STAT-3/BCL-2* pathway [50].

Here, we present extensive expression screening of piRNA pathway genes in early and late stage HGSOC tumors, benign serous cystadenoma as well as low grade OC tumors (workflow diagram, Figure 1). We assessed their potential link to treatment response (chemosensitivity versus chemoresistance), progression-free survival (PFS), post progression-free survival (PPFS) and overall survival (OS) in HGSOC patients. We also determined if there was a change in *PIWIL2* expression after OC cells were treated with FSH and LH, individually and in combination. Finally, we assessed whether overexpression of the pathway genes and mutants (*P1Δ17* and *PL2L60*) affected the motility and invasion of HGSOC in vitro and in vivo. This work highlights that the piRNA pathway may function differently in different cancers and provides novel insights into its role in OC.

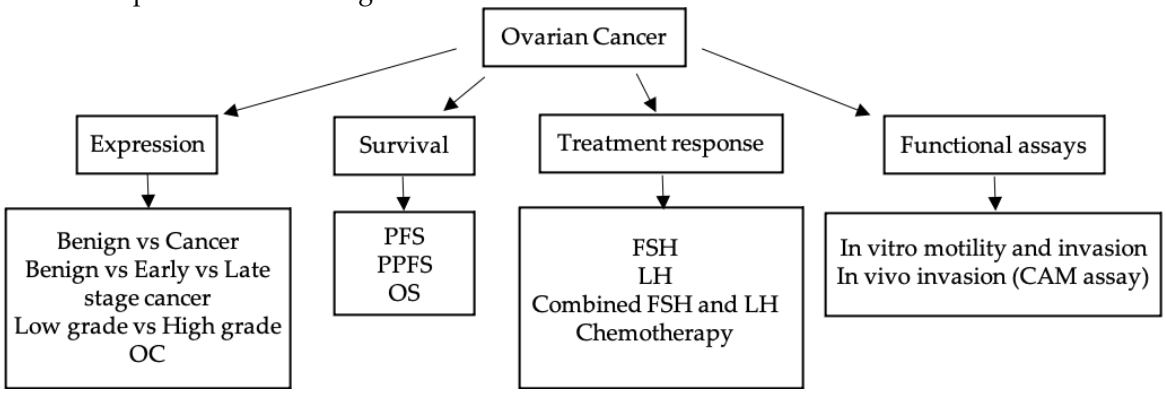


Figure 1. Workflow diagram of this study showing analysis performed for investigating expression differences and effects of the piRNA pathway genes in multiple aspects of OC.

2. Results

2.1. Differential Expression of piRNA Pathway Genes Occurs between Benign and Malignant HGSOC Tissue Samples

A growing list of piRNA pathway genes are being implicated in cancer but only limited studies address their potential role in OC. We profiled the mRNA expression levels of 10 piRNA pathway genes in benign serous cystadenoma (n = 16), early and late stage HGSOC tissue samples (n = 29) (Figure 2). Interestingly, we observed that the *PIWIL* genes had dissimilar trends of expression in benign and cancerous tumors. *PIWIL1* had significantly higher expression (Figure 2a), while *PIWIL2* expression was significantly lower in cancerous

tumors (early and late stage) as compared to benign tumors (Figure 2b). Most tumors had no *PIWIL3* expression (Figure 2c) whereas *PIWIL4* had significantly lower expression in early stage HGSOC samples but not late stage when compared to benign tumors (Figure 2d).

RNA helicase *DDX4* had no significant difference in expression when comparing benign to cancerous tumors but there was an increasing trend in expression between early and late stage HGSOC samples (Figure 2e) [51]. RNA methyltransferase *HENMT1* (Figure 2f) and *spindle* class gene *MAEL* (Figure 2g) both had significantly increased expression in HGSOC tumors compared to benign tumors [52,53]. While *HENMT1* expression was significantly increased between benign and early and late stage tumors, *MAEL* expression was only significantly increased between benign and late stage tumors. Phospholipase *PLD6* had similar expression levels in benign and cancerous tumors (Figure 2h) [54]. Methylarginine binding genes *TDRD1* (Figure 2i) and *TDRD9* (Figure 2j), had overall low expression in HGSOC and benign tumors [55]. *TDRD1* expression was reduced in HGSOC compared to benign tumors and expression was significantly lower in late stage tumors compared to benign tumors.

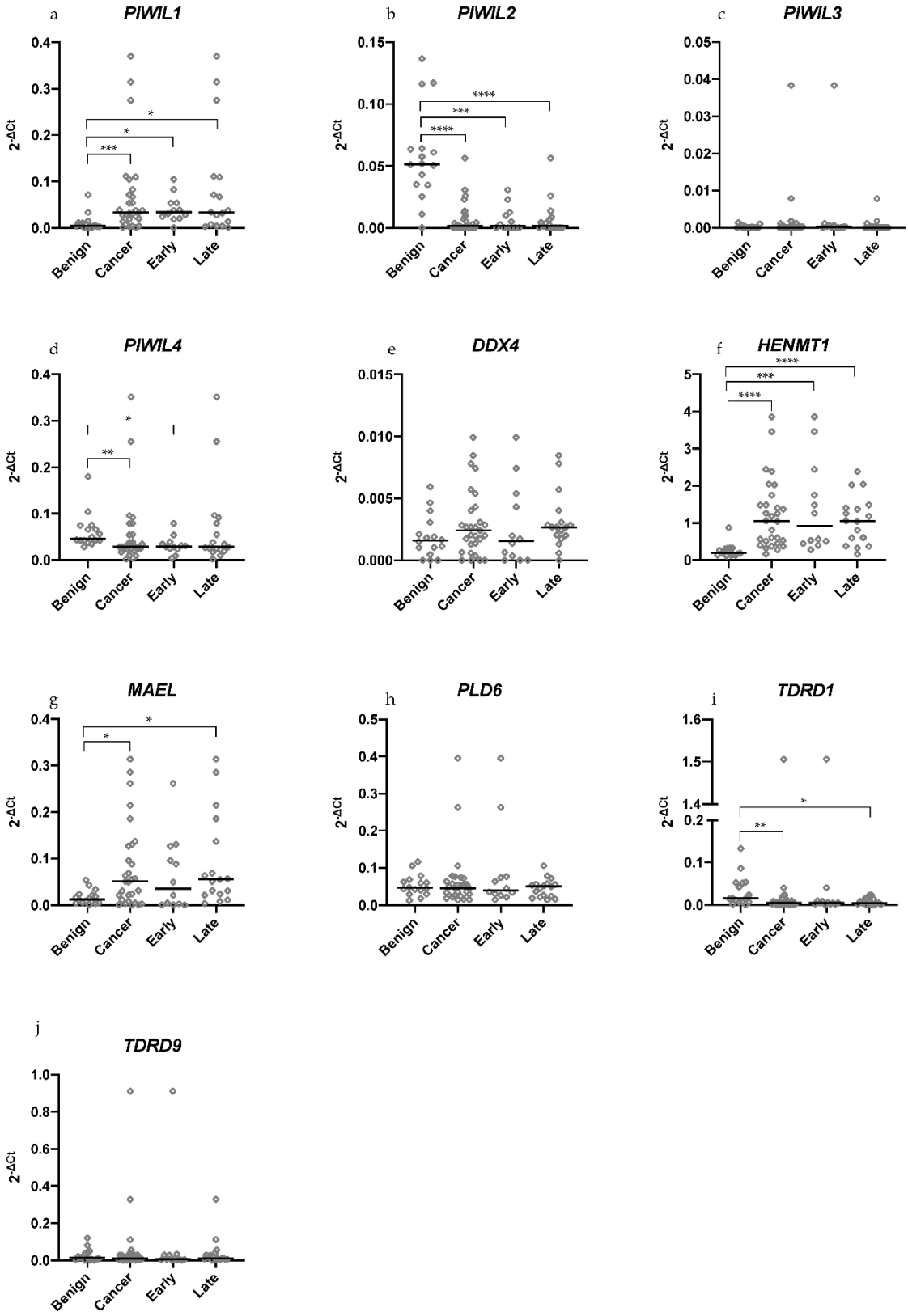


Figure 2. piRNA pathway gene expression in benign serous cystadenoma and high grade serous ovarian cancer (HGSOC) tumors. (**a**–**d**) Among the 4 *PIWIL* genes, only *PIWIL3* had no significant difference between benign and cancerous tumors. *PIWIL1* had increased expression while *PIWIL2* and *PIWIL4* had decreased expression between benign and cancerous samples. (**e**–**j**) Comparing expression of benign to cancerous tumors, *HENMT1* and

MAEL had significantly increased expression while *TDRD1* expression was significantly decreased. *DDX4*, *PLD6* and *TDRD9* presented similar expression levels across benign and cancerous tumors. All Ct scores were normalized to the geomean of housekeepers, *TBP* and *GUSB*. A median line was used to plot the respective dataset. Benign (n = 16), cancer (early and late HGSOC, n = 29), early HGSOC (stages 1,2, n = 12) and late HGSOC (stage 3, n = 17). Statistical tests Mann–Whitney U was used to analyze benign versus cancer while Kruskal–Wallis (non-normal distribution) or one-way ANOVA (normal distribution) were used accordingly when comparing expression differences in benign, early and late stage HGSOC. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$.

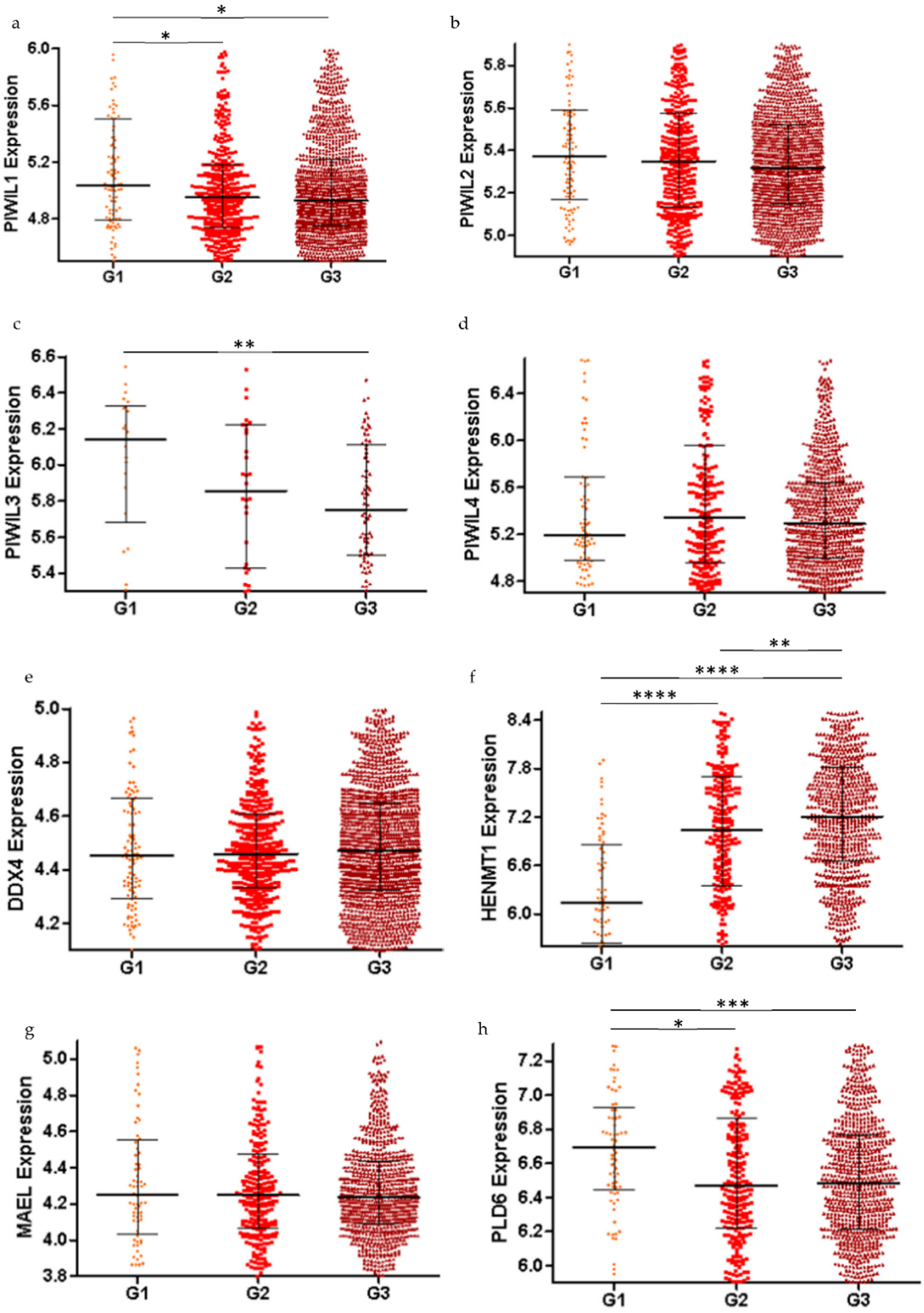
2.2. piRNA Pathway Genes Are Differentially Expressed in Low Grade and High Grade Ovarian Cancer (All Subtypes)

Following the differential expression observed in HGSOC staging, we next investigated if the piRNA pathway genes had distinct expression levels in low grade (G1) versus high grade (G2, G3) disease in OC (Figure 3). *PIWIL1* and *PIWIL3* had significantly lower expression in high grade compared to G1 (Figure 3a,c). *PIWIL2* and *PIWIL4* expression was not different between low grade and high grade (Figure 3b,d). We also observed similar expression levels for *DDX4*, *MAEL* and *TDRD9* across low and high grade OC (Figure 3e,g,j). In contrast, *HENMT1* expression was significantly higher in G2 and G3 compared to G1 (Figure 3f). Of note, *HENMT1* expression was significantly increased from G2 to G3 (Figure 3f). *PLD6* and *TDRD1* expression were significantly lower in G2 and G3 compared to G1 (Figure 3h,i).

2.3. The Level of piRNA Pathway Gene Expression Is Associated with HGSOC Patient Overall and Progression Free Survival

Next, we correlated expression of piRNA pathway genes with early and advanced stage patient outcome using the Kaplan–Meier (KM) online plotter which is based on data from large public microarray databases (Table 1). With the exception of *HENMT1* which did not significantly correlate with PFS, PPFS or OS, all other genes were associated with HGSOC patient outcome. High *PIWIL1* expression was significantly associated with improved PPFS and OS in both early (p = 0.034; p = 0.017) and late (p = 0.0002; p = 0.00033) stage HGSOC patients. Conversely, low *PIWIL2* and *PIWIL4* expression were significantly associated with increased PFS in late stage (p = 0.0051; p = 0.015) patients. HGSOC patients with low *PIWIL2* expression also had significantly increased PPFS (p = 0.048).

Although high expression of *DDX4* was associated with longer PFS (p = 0.046), low *DDX4* was associated with improved OS (p = 0.035) in early stage HGSOC patients. Low *MAEL* expression in both early (p = 0.0037) and late (p = 0.048) stage HGSOC patients was associated with increased PFS whereas high expression was associated significantly with increased OS (p = 0.041) in only late stage HGSOC patients. Low *PLD6* expression was significantly associated with increased OS (p = 0.027) in late stage patients. Early stage HGSOC patients with high *TDRD1* expression had very significantly longer PFS (p = 0.000042) while late stage patients with low *TDRD9* expression had significantly better PFS (p = 0.05).


a
*
*
PIWIL1 Expression
6.0
5.6
5.2
4.8
G1
G2
G3
b
PIWIL2 Expression
5.8
5.6
5.4
5.2
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G1
G2
G3
c
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PIWIL3 Expression
6.6
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d
PIWIL4 Expression
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HENMT1 Expression
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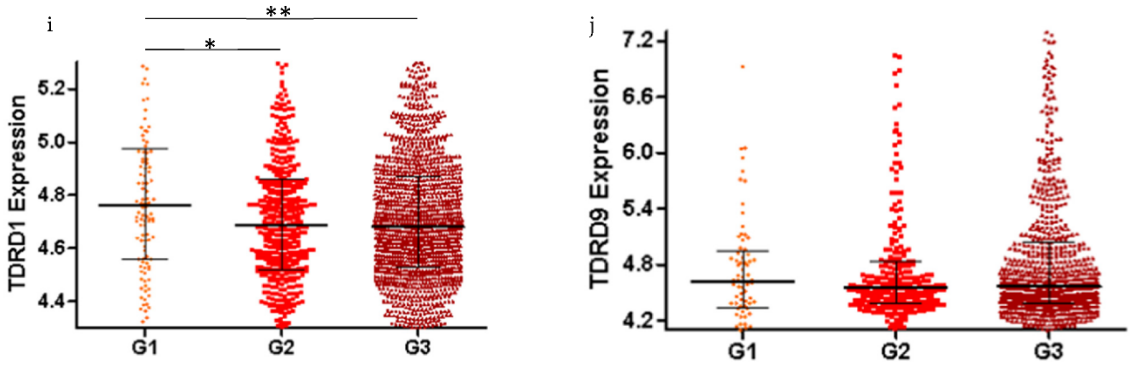

Figure 3. Using the CSIOVDB public microarray database (n = 3431) to determine piRNA pathway gene expression in grades 1, 2 and 3 (G1, G2 and G3) of all ovarian cancer subtypes. (**a**–**d**) Among the 4 *PIWIL* genes, *PIWIL1* and *PIWIL3* showed significantly lower expression in the high grade (G2/G3) disease as compared to low grade (G1). *PIWIL2* and *PIWIL4* had similar expression levels in G1, G2 and G3 ovarian cancer samples. **(e**, **g**–**j**) *PLD6* and *TDRD1* also had significantly decreased expression when comparing low grade to high grade. *DDX4*, *MAEL* and *TDRD9* had no significant difference in expression across G1, G2 and G3. **(f)** *HENMT1*, in contrast, had significantly higher expression in G2 and G3 as compared to G1. Mann–Whitney U test was used to assess significance. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$.

2.4. PIWIL3 Is Expressed in Chemosensitive Primary HGSOC Cells But Not in Chemoresistant Primary HGSOC Cells

Recognizing that chemoresistance is a major problem for treatment in OC, we evaluated piRNA pathway gene expression in chemosensitive and chemoresistant HGSOC patient-derived primary cells in search of a potential chemoresistant treatment target [2]. We found that *PIWIL1* and *PIWIL2* had a similar expression pattern in chemosensitive and chemoresistant primary HGSOC cells (Figure 4a,b). Only *PIWIL3* expression was observed to be significantly increased in chemosensitive primary cells (Figure 4c). *PIWIL4*, *DDX4*, *MAEL*, *TDRD1* and *TDRD9* expression decreased in chemoresistant primary cells, but those changes were not significant (Figure 4d,e,g,i,j). *HENMT1* showed no difference in expression while *PLD6* expression had a slight increase in chemoresistant cells compared to chemosensitive cells, albeit without significance (Figure 4f,h).

Table 1. Kaplan–Meier survival analysis of high grade serous ovarian cancer (HGSOC) patients according to piRNA pathway gene expression levels.

	a) Early Stage HGSOC Patients (FIGO Stage I and II)								
piRNA Pathway Genes	**Progression Free Survival**			**Post Progression Survival**			**Overall Survival**		
	HR	**95% CI**	***p*-Value**	**HR**	**95% CI**	***p*-Value**	**HR**	**95% CI**	***p*-Value**
PIWIL1	0.66	0.31–1.4	0.27	**0.33**	**0.11–0.96**	**0.034**	**0.37**	**0.16–0.86**	**0.017**
PIWIL2	0.55	0.25–1.21	0.13	3.64	0.47–28.09	0.18	2.03	0.87–4.78	0.097
PIWIL4	2	0.74–5.4	0.16	0.17	0.02–1.48	0.07	0.66	0.21–2.1	0.48
DDX4	**0.45**	**0.2–1.01**	**0.046**	0.41	0.09–1.88	0.24	**3.51**	**1.02–12.09**	**0.035**
HENMT1	2.79	0.96–8.15	0.051	4.47	0.81–24.55	0.059	2.33	0.7–7.72	0.15
MAEL	**4.75**	**1.5–15.08**	**0.0037**	2.78	0.32–24.36	0.34	2.54	0.74–8.69	0.12
PLD6	0.42	0.14–1.29	0.12	0.3	0.04–2.23	0.21	3.4	0.74–15.61	0.094

TDRD1	**0.24**	**0.11–0.5**	**0.000042**	0.44	0.14–1.38	0.15	0.49	0.21–1.17	0.1
TDRD9	0.6	0.2–1.84	0.37	3.11	0.59–16.26	0.16	0.6	0.17–2.08	0.42
		b)	**Late Stage HGSOC Patients (FIGO Stage III)**						
PIWIL1	0.91	0.76–1.08	0.27	**0.68**	**0.55–0.83**	**0.0002**	**0.7**	**0.58–0.85**	**0.00033**
PIWIL2	**1.32**	**1.09–1.61**	**0.0051**	**1.27**	**1–1.6**	**0.048**	0.88	0.73–1.06	0.18
PIWIL4	**1.35**	**1.06–1.72**	**0.015**	1.25	0.94–1.68	0.13	1.3	0.98–1.74	0.067
DDX4	0.91	0.76–1.09	0.3	1.17	0.96–1.43	0.11	0.88	0.72–1.07	0.19
HENMT1	1.17	0.9–1.52	0.23	1.2	0.87–1.66	0.27	1.28	0.93–1.74	0.13
MAEL	**1.27**	**1–1.62**	**0.048**	0.77	0.56–1.05	0.093	**0.72**	**0.53–0.99**	**0.041**
PLD6	1.19	0.94–1.51	0.15	1.18	0.88–1.59	0.27	**1.38**	**1.04–1.84**	**0.027**
TDRD1	0.89	0.74–1.08	0.24	0.84	0.67–1.05	0.14	0.83	0.69–1.01	0.061
TDRD9	**1.27**	**1–1.61**	**0.05**	0.82	0.6–1.11	0.2	1.18	0.89–1.56	0.24

Sample size of early stage HGSOC patients assessed for PFS (n = 84), PPFS (n = 32) and OS (n = 87); late stage HGSOC patients for PFS (n = 807), PPFS (n = 573) and OS (n = 836). HR > 1: low expression confers better outcome; HR < 1: high expression confers better outcome. HR = hazard ratio; 95% CI = 95% confidence interval; $p < 0.05$ = significant. HR, 95% CI and p-values in bold are significant.

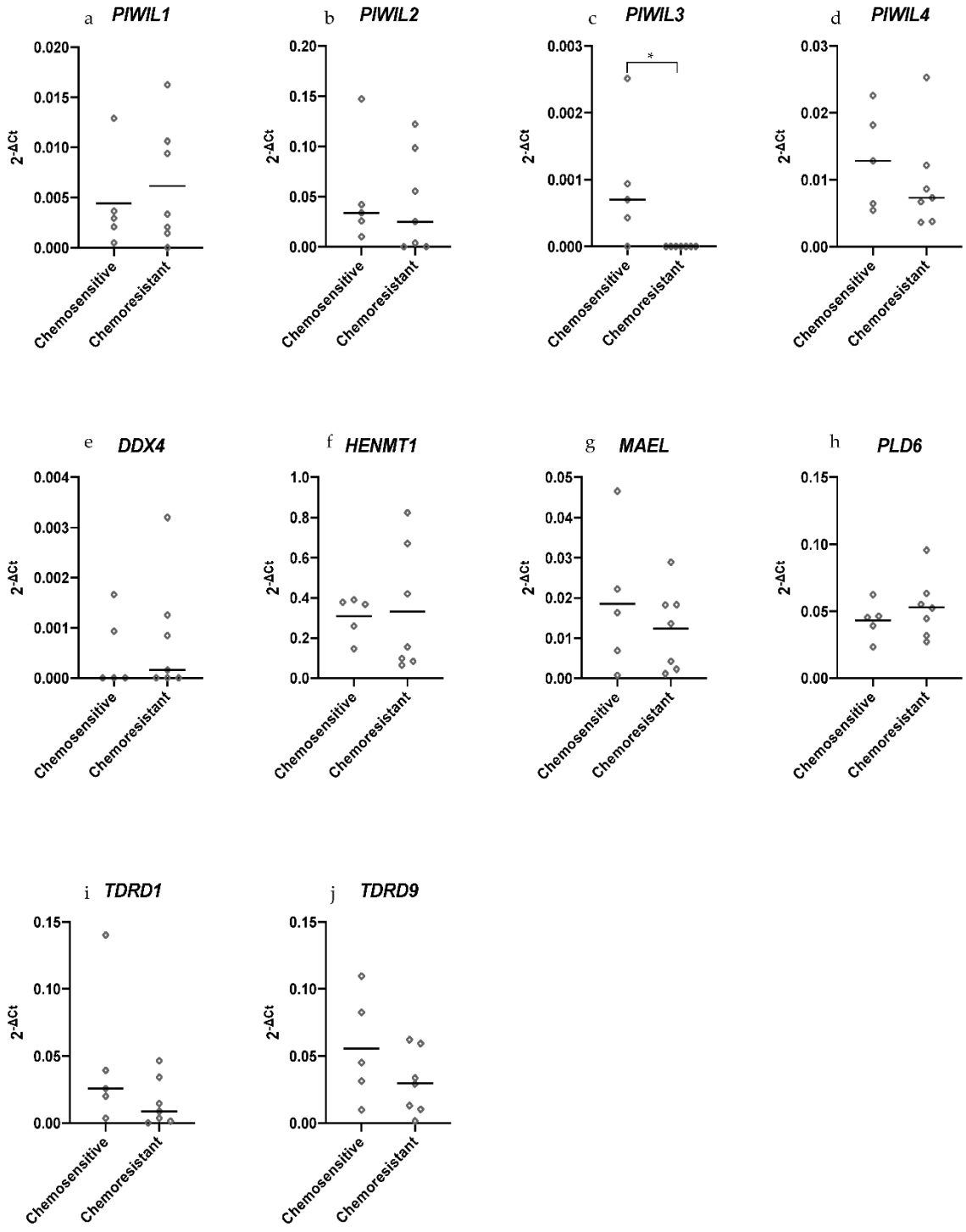

Figure 4. piRNA pathway gene expression in chemosensitive (n = 5) and chemoresistant (n = 7) primary HGSOC cells. (**c**) *PIWIL3* was not expressed in chemoresistant cells but was expressed in most of the chemosensitive cells (n = 4). (**a**,**b**,**d**–**j**) While the other genes had no significant change in expression, it was observed that they had varying degrees of differential expression. All Ct scores were normalized to the geomean of housekeepers, *TBP* and *GUSB*. Either a median (non-normal distribution) or mean (normal distribution) line was plotted. Statistical tests Mann–Whitney U (non-normal distribution) or unpaired *t*-test (normal distribution) were used accordingly. * $p < 0.05$.

2.5. PIWIL2 Expression Increased upon FSH Treatment in OV-90 Cells

As OC development has been linked to hormones, we were interested to see if the piRNA pathway genes are affected by hormones. RT-PCR of OV-90 cells after treatment with different concentrations of gonadotropins, FSH and LH, showed increased *PIWIL2* expression (Figure 5a). Further analysis using qRT-PCR showed specifically a higher dose of FSH (100 mIU/mL) significantly increased *PIWIL2* expression (Figure 5b). While both treatment doses of LH (25 and 50 mIU/mL) elevated *PIWIL2* expression, this change was not significant (Figure 5b). Interestingly, *PIWIL2* expression after treatment with both LH and FSH (in doses 25 and 50 mIU/mL and 50 and 100 mIU/mL) were lower (not significant) than OV-90 cells treated with 100 mIU/mL FSH alone.

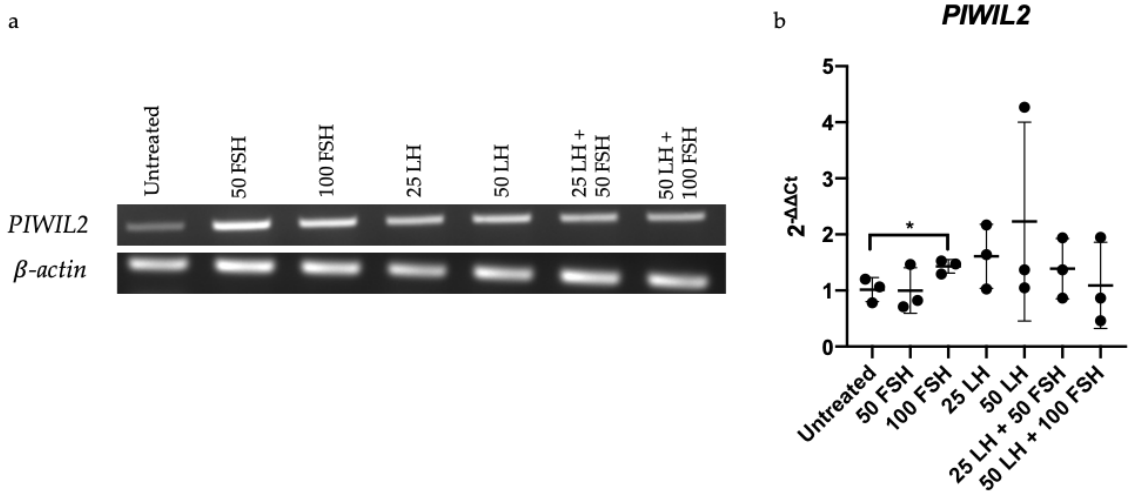

Figure 5. *PIWIL2* expression in OV-90 cells before and after a 24-h follicle stimulating hormone (FSH) and luteinizing hormone (LH) treatment. (**a**) RT-PCR: *PIWIL2* expression increased upon individual and combination treatment of FSH and LH. (**b**) qRT-PCR: *PIWIL2* had a significant increase in expression after 100 mIU/mL FSH treatment. Ct scores were normalized to housekeeper, *HPRT1* and the average of untreated cells for $2^{-\Delta\Delta CT}$. Each colored circle represents one biological replicate with technical duplicate where three biological replicates were carried out per treatment group. Labels with numerical values indicate concentration (mIU/mL) of hormone used. Mean with standard deviation was plotted. Unpaired *t*-test was used to assess significance. * $p < 0.05$.

2.6. Cells Overexpressing Wildtype and Mutant piRNA Pathway Genes Have Altered Motility and Invasion In Vitro

In several cancer models, knockdown of piRNA pathway genes result in decreased motility and invasion [56–58]. Initial work in ovarian cancer indicated an opposite trend where overexpression induced decreased motility and invasion [25]. Here, we assessed the effects of overexpression of *PIWIL1*, *PIWIL2* and *MAEL* as well as known mutants of *PIWIL1* (*P1Δ17*) and *PIWIL2* (*PL2L60*) on motility and invasion in vitro. Overexpression of *PIWIL1*, *PIWIL2* and *PL2L60* in OVCAR-3 had significantly decreased motility compared to the empty vector control (Figure 6a). In OV-90, overexpression of the same set of genes showed decreased motility, however, only *PIWIL2* overexpression significantly reduced motility compared to the control (Figure 6b). *P1Δ17* overexpression in OVCAR-3 resulted in a more motile phenotype (nonsignificant) while in OV-90 there was no change in cell motility as compared to their respective empty vector control (Figure 6a,b). Comparing the overexpression of wildtype and mutant *PIWIL1* and *PIWIL2* motility in both cell lines, only cells overexpressing *P1Δ17* were significantly more motile than *PIWIL1* in OVCAR-3 (Figure

6a). *MAEL* overexpressing OVCAR-3 and OV-90 cells displayed decreased cell motility (nonsignificant) compared to the empty vector control.

Furthermore, OVCAR-3 cells overexpressing *PIWIL1*, *PIWIL2*, *PL2L60* and *MAEL* and OV-90 cells overexpressing *PIWIL2* showed significantly decreased invasion compared to their respective empty vector control (Figure 6c,d). OVCAR-3 cells overexpressing *P1Δ17* had increased invasion (nonsignificant) compared to the empty vector control (Figure 6c). Comparison of OVCAR-3 overexpressing wildtype and mutant *PIWIL1* demonstrated that this mutant was more invasive (nonsignificant) (Figure 6c). OV-90 cells overexpressing *PIWIL1*, *P1Δ17*, *PL2L60* and *MAEL* were not significantly different in invasiveness compared to empty vector (Figure 6d). While there was no significant difference in invasion between *PIWIL1* and *P1Δ17* overexpression in OV-90 cells, overexpression of *PL2L60* was significantly more invasive than *PIWIL2* (Figure 6d).

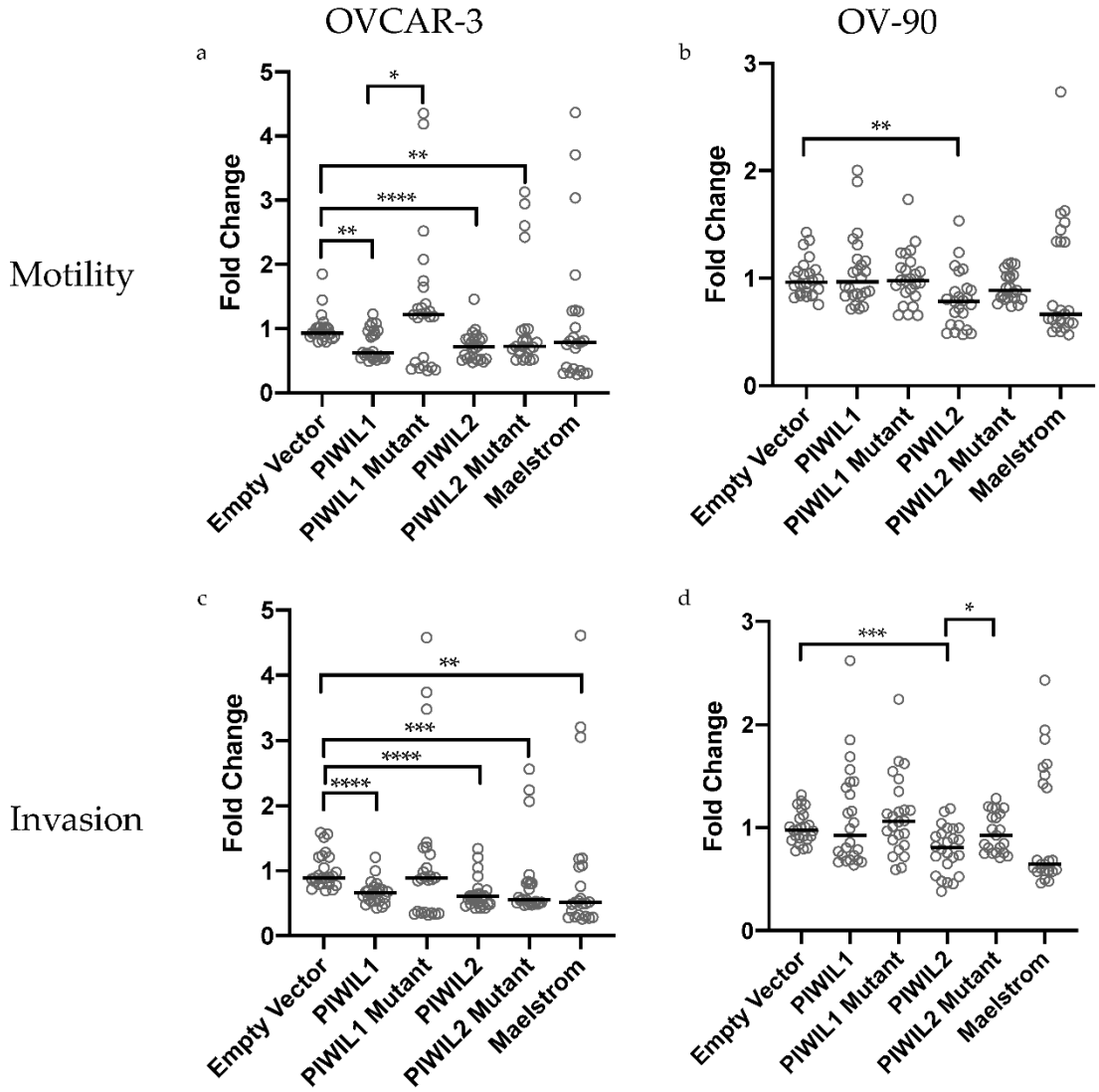

Figure 6. In vitro motility and invasion of OVCAR-3 and OV-90 cells after overexpressing piRNA pathway genes and its mutants (n = 21–24 per cell line per transfection). (**a**,**b**) Motility: *PIWIL1* and *PL2L60* (*PIWIL2* mutant) overexpression significantly decreased OVCAR-3 motility while *PIWIL2* overexpression significantly decreased motility in both OVCAR-3 and OV-90. OVCAR-3 cells overexpressing *P1Δ17* (*PIWIL1* mutant) had increased motility compared to empty vector and was significantly more motile than *PIWIL1* overexpression. (**c**,**d**) Invasion: overexpression of *PIWIL1*, *PIWIL2*, *PL2L60* and *MAEL* significantly decreased

OVCAR-3 invasion while *PIWIL2* overexpression significantly decreased OV-90 invasion. OV-90 cells overexpressing *PL2L60* had significantly increased invasion compared to *PIWIL2* overexpression. All fold changes were calculated relative to empty vector. Three biological replicates were carried out per cell line per transfection group. Bar represents median values plotted for all the datasets. Statistical tests Mann–Whitney U (non-normal distribution) or unpaired *t*-test (normal distribution) were used accordingly. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$.

2.7. OV-90 Cells Overexpressing Mutant PIWIL1 Are More Invasive than Wildtype In Vivo

The functional roles of *PIWIL1*, *PIWIL2*, *MAEL*, *P1Δ17* and *PL2L60* were further assessed in vivo using the chicken chorioallantoic membrane (CAM) invasion assay. We quantified the number of OV-90 cells that successfully invaded into the ectoderm and beyond as a measure of invasion (Figure 7a–f). We found that *P1Δ17* overexpressing OV-90 resulted in a significantly more invasive phenotype as compared to the empty vector control and *PIWIL1* (Figure 7g). *PL2L60*, on the other hand, was more invasive than the empty vector control but not significantly more invasive than *PIWIL2* (Figure 7g). OV-90 cells overexpressing *PIWIL1*, *PIWIL2* and *MAEL* showed a slightly more invasive phenotype (nonsignificant) compared to empty vector in vivo (Figure 7g).

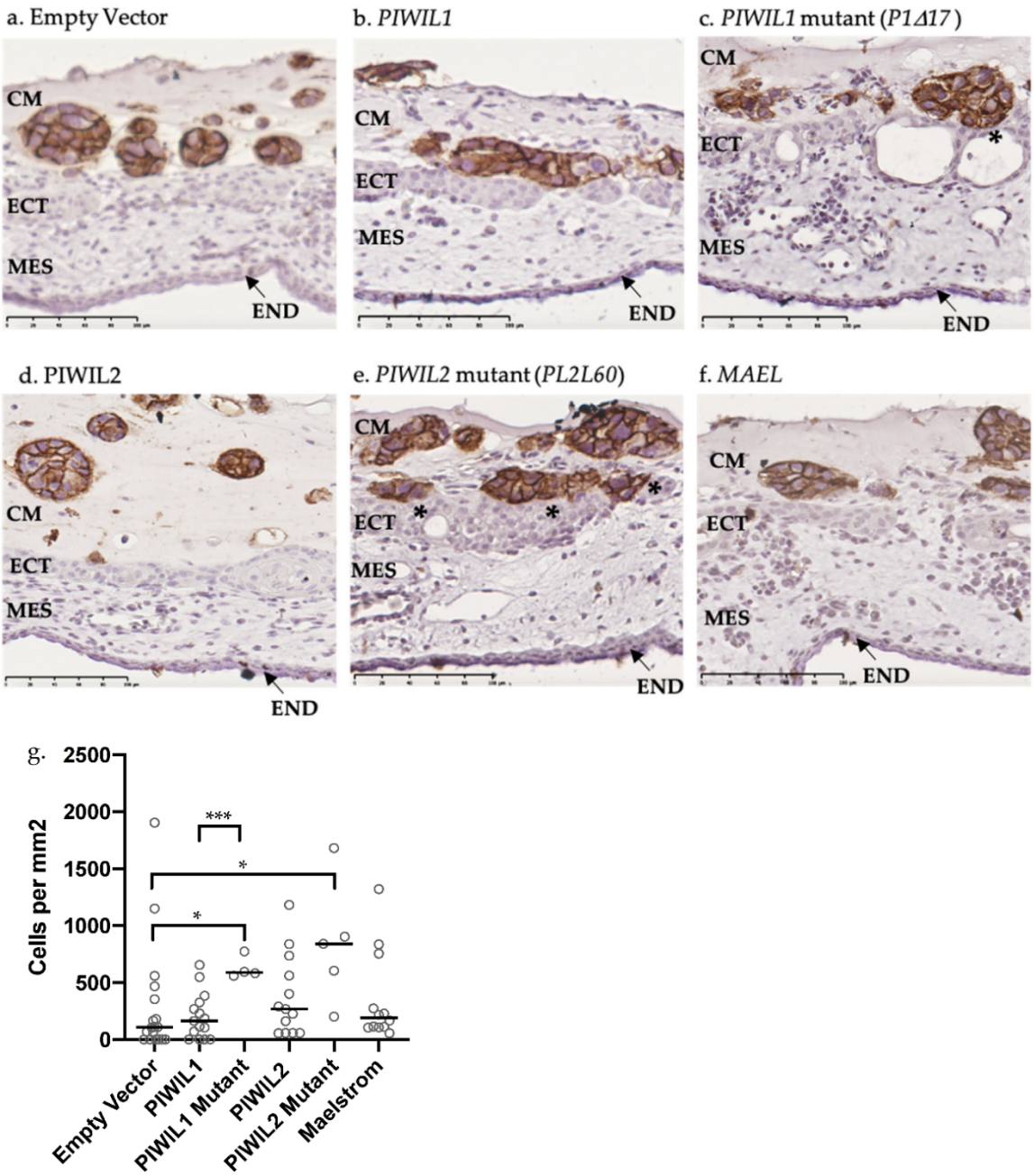

Figure 7. The in vivo invasion analysis using the chicken chorioallantoic membrane (CAM) assay. (**a**–**f**) CD44 stained OV-90 cells transfected with (**a**) empty vector (pcDNA3.1(+)) and overexpression constructs, (**b**) *PIWIL1*, (**c**) *P1Δ17*, (**d**) *PIWIL2*, (**e**) *PL2L60* and (**f**) *MAEL*. CAMs were counterstained with haematoxylin. Invasion was quantified by the number of OV-90 cells that successfully breached the ECT and beyond (* marked next to breached area). CM = cancer cells mixed with matrigel, ECT = ectoderm, MES = mesoderm, END = endoderm. Scale bar = 100 μm. (**g**) Significantly more CAM area was invaded by *P1Δ17* and *PL2L60* overexpressing OV-90 cells. Both mutants were more invasive than its respective wildtype form where only overexpression of *P1Δ17* was significant. n= 4–18 per construct transfected and carried out in biological duplicates. Statistical tests Mann–Whitney U (non-normal distribution) or unpaired t-test (normal distribution) were used accordingly. * $p < 0.05$; *** $p < 0.0005$.

3. Discussion

The piRNA pathway is increasingly recognized as an important aspect of cancer development and progression [42,43]. Given the limited information of this pathway in OC, we investigated its expression and effects on OC progression. Expression analysis of 10 selected piRNA pathway genes revealed dynamic changes in relation to OC malignancy, patient survival, and chemoresistance. Additional experiments revealed that FSH treatment on HGSOC cells increased *PIWIL2* expression. Furthermore, overexpression studies on the motility and invasion of HGSOC cells demonstrated that *PIWIL1* or *PIWIL2* mutants can induce a more aggressive phenotype as compared to wildtype in vitro and in vivo.

A number of studies reported that increased expression of piRNA pathway genes contributed to the metastatic progression of various cancers, including OC [56,59–62]. Limited information is available on OC and while knockdown of pathway genes has been linked to decreased malignancy in vitro, overexpression analysis in OC surprisingly revealed decreased invasion [25,56–58]. Our comprehensive analysis revealed a more differentiated pattern where *PIWIL1*, *MAEL* and *HENMT1* had increased expression, while *PIWIL2*, *PIWIL4* and *TDRD1* had decreased expression when comparing benign and malignant tumor samples. This indicates that different aspects of the pathway may be turned up or down as the cancer progresses. For example, PIWIL1 is known to be involved in the primary piRNA biogenesis pathway while PIWIL2 and PIWIL4 participate in the secondary biogenesis pathway. MAEL, HENMT1 and TDRD1 function in both pathways [28,30]. This may be showing that the secondary piRNA biogenesis pathway is specifically downregulated in HGSOC. This also suggests that the pathway genes, having functions beyond piRNA biogenesis, may be behaving independently in malignant tumors. Another possibility is that HGSOC tumors which harbor a high percentage of *TP53* mutations and are very genetically unstable may contribute to why our results contrast other cancers [4–6,11,16]. Downregulation of piRNA pathway genes in cancer may be uncommon but have been reported in testicular germ cell tumor (*PIWIL1*, *PIWIL2*, *PIWIL4* and *DDX4)* and renal cell carcinoma (*PIWIL1*, *PIWIL2* and *PIWIL4*) and now, also in our expression study for *PIWIL2*, *PIWIL4* and *TDRD1* [63,64]. Further evidence that the role of this pathway is more complicated comes from piRNAs where the same piRNA species (piRNA-823) can have a suppressive effect in gastric cancer while oncogenic in multiple myeloma [65,66]. Similarly, within gastric cancer, it was observed that different piRNA species can react to support (piRNA-651) and inhibit (piRNA-823) cancer cell growth [65,67]. The rapidly increasing body of work highlights the importance of ncRNAs in terms of oncogenicity or tumor suppression in various cancers including OC. For example, different species of microRNAs (miRNAs) can also have oncogenic or tumor suppressive roles in OC [68]. This can be linked to the piRNA pathway and miRNAs regulating transcription factors ZEB1,2 and SNAI1-3 which affect cell epithelial to mesenchymal transition (EMT), enabling the promotion or repression of cell invasion and metastasis [47,68,69]. Such piRNA and miRNA species involved in the molecular regulation of cancer progression could be potential targets for OC treatment. Hence, if piRNAs, as part of the piRNA pathway, could function to promote and suppress cancer, it is possible that the pathway genes act in a similar fashion. Together, our findings add to the mounting evidence that the piRNA pathway can act in different ways in different cancers or even different diseases of the same cancer as we demonstrated in OC.

It is accepted that low grade and high grade OC are considered different diseases. Interestingly, this is also reflected in the piRNA pathway expression profile demonstrated by the significantly lower expression of *PIWIL1*, *PIWIL3*, *PLD6* and *TDRD1* in high grade as compared to low grade. *HENMT1*, on the other hand, had elevated expression in high grade compared to low grade OC. The molecular action for PIWIL3 and PLD6 in low grade OC could be involvement in growth regulation such as in glioma and mediating downstream pathways

for proto-oncogene, MYC, as seen in breast cancer, respectively [70,71]. At present, there are no studies of *TDRD1* in OC however, *TDRD1* is now considered a potential biomarker for prostate cancer as it strongly associates with expression of the frequently mutated transcription factor, *ERG* [23,49]. Thus, *TDRD1* may also be a biomarker for low grade OC. Implications of aberrant *HENMT1* expression in cancer are unknown but it has known roles in maintaining piRNA stability by 2′-O-methylation of piRNAs and ensuring TE repression [39,72]. This analysis suggests that piRNA pathway genes may have expression profiles specific to the histotype of OC. Interestingly, a microarray study demonstrated similar OC histotype-specific miRNA signatures, supporting the diversity of ncRNA expression in different OC histotypes and highlighting the need for further studies [73].

Next, we assessed whether piRNA pathway gene expression levels were associated with HGSOC progression and patient survival using the KM plotter. High *PIWIL1* expression was linked to better PPFS and OS. In contrast, patients with low *PIWIL2* and *PIWIL4* expression had better PFS. With limited patient data in our gene expression study, we were unable to compare if the lower expressing *PIWIL2* and *PIWIL4* samples remained progression free as seen using the KM online plotter. However, the difference in expression level for *PIWIL1* versus *PIWIL2* and *PIWIL4* contributing to better patient outcome was expected given the difference we observed when comparing their expression in benign and malignant tumors as well as low grade OC versus high grade OC. This supports the possibility that the *PIWIL* genes could be behaving differently in HGSOC. *DDX4* and *MAEL* had conflicting results as they both had low and high expression significantly linked to better prognosis of PFS and OS. The current literature on *DDX4* suggests that it plays a role in OC progression due to its influence on DNA damage checkpoints and association with cancer stem cell marker, CD133 [59,60]. Our results showed higher *MAEL* expression with increasing tumor stage. The association of low *MAEL* expression with longer PFS in early and late stage HGSOC patients may be related to its putative role in other cancers of promoting EMT and preventing apoptosis and DNA damage in cancer cells [22,47]. However, we observed that high *MAEL* expression was associated with increased OS in late stage HGSOC. This is consistent with our previous and current work where *MAEL* overexpression in EOC decreased invasion [25]. High *TDRD1* expression was strongly correlated with better PFS but it is currently unknown what its functional role is in cancer other than strong association with *ERG* expression in prostate cancer [49,74]. On the other hand, low *TDRD9* expression was associated with longer PFS. This is consistent with a study where *TDRD9* knockdown impaired proliferation of two lung cancer cell lines [61].

The prevalence of chemoresistance in OC and late diagnosis have contributed to its concerningly low 5-year survival rates (26–42%) and label as one of the most lethal gynecologic malignancies [2]. The mechanisms leading to the development of resistance has yet to be elucidated. Here, we discovered that *PIWIL3* was not expressed in chemoresistant HGSOC cells but, interestingly, was expressed in chemosensitive cells. This finding agrees with previous work demonstrating lower *PIWIL3* expression in benign and malignant EOC as compared to in a normal ovary which points towards reduced *PIWIL3* expression possibly being involved in the progression of OC [25]. In contrast, *PIWIL3* had increased expression in more malignant tumors of gastric cancer and melanoma wherein overexpression leads to proliferation and invasion [56,75]. Further conflicting results showed *PIWIL3* overexpression induced glioma regression whereas knockdown of *PIWIL3* in pancreatic cancer conferred sensitivity to resistant cells [70,76]. Here, we reason that *PIWIL3* may be silenced in HGSOC cells as it may play a role in genomic stability through its putative role in piRNA biogenesis [77]. Extensive genomic instability can confer sensitivity to chemotherapy [78,79]. Additional work will be needed to address its role in sensitizing HGSOC cells and the mechanism of action in the piRNA pathway. Keeping in mind that our sample size was small, it may be

worthwhile exploring *PIWIL4*, *MAEL*, *PLD6*, *TDRD1* and *TDRD9* as potential targets in addition to *PIWIL3* for overcoming chemotherapy resistance.

The effects of hormones on the piRNA pathway have been reported in mice, frogs and fish with only one study in breast and prostate cancer [80–84]. Exploring this novel avenue in OC, we exposed a HGSOC cell line, OV-90, with varying concentrations of FSH and LH. We found that a higher concentration of FSH was able to stimulate increased *PIWIL2* expression but none of the treatments stimulated a change in *PIWIL1* or *MAEL* expression [85] *PIWIL2* had no change in expression when treated with a high dose of combined FSH and LH. This could be explained by their antagonistic nature where FSH induced cell proliferation was blocked when LH was introduced in EOC cell lines [86]. We postulate that PIWIL2 may be a downstream target of FSH as they both affect proteins involved in cell proliferation and EMT pathways namely, STAT3 and MMP-9 [57,87,88]. Another set of proteins that PIWIL2 and FSH are involved in regulating are stem cell markers, OCT4, NANOG and SOX-2 [89–91]. In addition, induction of *PIWIL2* was also reported to endow cancer stem cell-like properties to human fibroblasts with ectopic expression of these stemness markers [90]. While there are many papers on FSH causing OC proliferation and migration [4,92–95], there is a study claiming FSH as having protective properties against cancer [96]. Therefore, further investigation is needed to identify the relationship between FSH and PIWIL2 and whether they contribute to or against the development of HGSOC.

To establish the effects of increased piRNA pathway gene expression on HGSOC motility and invasion in vitro and in vivo, we assessed this in 2 HGSOC cell lines, OVCAR-3 and OV-90, both harboring a *TP53* missense mutation [97]. Our previous work on SKOV-3 overexpressing *PIWIL1* and *MAEL* showed a decrease in invasion in vitro [25]. Here, we expanded the overexpression study to include *PIWIL2*, *P1Δ17* and *PL2L60* in cell lines, OVCAR-3 and OV-90. As well as demonstrating similar results for *PIWIL1* and *MAEL* as SKOV-3 cells, we revealed that OVCAR-3 and OV-90 cells overexpressing *PIWIL2*, *P1Δ17* and *PL2L60* also had decreased motility and invasion compared to empty vector transfected cells. This is unexpected as in most cancers, the overexpression of these wildtype and mutant piRNA pathway genes were associated with a more invasive phenotype [50,98–100]. Key cancer-related pathways and proteins that these genes can regulate include the AKT/GSK-3*β*/SNAIL pathway, MMP-9, CD44 and STMN1 which are involved in EMT and metastasis [47,57,88,89,99]. There is a possibility that these pathways and proteins react differently in HGSOC due to genomic instability and the presence of TP53 mutants that could either have loss-of-function, gain-of-function or dominant negative mutations [16]. More importantly, TP53 mutants are more resistant to degradation than the wildtype form, hence could cause different outcomes regardless of the presence of wildtype TP53 [16]. There is evidence that PIWIL2 can form a complex with STAT3 and c-SRC through interaction with its PAZ domain to repress *TP53* transcription [101,102]. Could PIWIL2 have a protective role by downregulating mutant *TP53* in HGSOC? It is interesting that OVCAR-3 and OV-90 cells overexpressing *P1Δ17* and *PL2L60* were more invasive as compared to cells overexpressing *PIWIL1* and *PIWIL2*. This may be attributed to the truncation of their PIWI and PAZ domains, respectively [25,50]. It is possible that truncation of these domains could lead to impaired piRNA binding, endonuclease activity and interaction with the growing list of oncogenes and tumor suppressors.

4. Materials and Methods

4.1. Patient Cohort

Patient tissue samples were obtained with written consent and approval by the Central Adelaide Local Health Network Human Research Ethics Committee (RAH Protocol #140201).

Clinicopathological characteristics of the patient samples used in the Fluidigm qRT-PCR expression analysis are listed in Tables S1 and S2. Ethics Committee approval was obtained on 13 January 2014. Formalin-fixed paraffin-embedded (FFPE) tissue sections of patients were stained with hematoxylin and eosin to confirm cancer content [85] RNA extractions were then carried out on frozen patient tissues which FFPE sections had at least 50% cancer content.

4.2. RNA Extraction, cDNA Synthesis and Quantification

RNA extractions and cDNA syntheses were carried out on all samples using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) and iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturers' protocol. RNA samples were quantified using Qubit (Invitrogen, Waltham, MA, USA) where 400 ng was cDNA synthesized and 2 μL of cDNA was used in the Fluidigm high throughput qRT-PCR. For the RT-PCRs and other qRT-PCRs, RNA samples were quantified using NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). A 1 μg sample of RNA was used for each cDNA synthesis reaction and diluted 1 in 5 after the reaction. A sample of 5 μL was then used in the RT-PCRs and 2 μL in the other qRT-PCRs.

4.3. Primary and Established Ovarian Cancer Cell Lines

Chemosensitive (n = 5) and chemoresistant (n = 7) primary cells were obtained from ascites of high grade OC patients before and after chemotherapy treatment as previously described [103]. Chemoresistance was determined when patients relapse and no longer respond to chemotherapy. Chemosensitivity was classified as patients responding to chemotherapy and not progressing within 6 months of completing their treatment. These primary cells were grown in Advanced RPMI 1640 media (Gibco, Waltham, MA, USA, cat no. 12633-020) containing 10% fetal calf serum (FCS) (Scientifix, Clayton, VIC, Australia) and 1% each of penicillin/streptomycin (Life Technologies, Mulgrave, VIC, Australia), fungizone (Sigma−Aldrich, St. Louis, MO, USA) and glutamax (Life Technologies, Mulgrave, VIC, Australia) and maintained at 37 °C in a 6% CO_2 environment.

Human HGSOC cell lines, OVCAR-3 and OV-90, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were cultured in RPMI 1640 (Gibco, Waltham, MA, USA, cat no. 11875-093) with FCS (5% for OVCAR-3, 10% for OV-90), 1% penicillin/streptomycin and 1% glutamax.

4.4. Quantitative Real-Time PCR (qRT-PCR)

To assess piRNA pathway gene expression in HGSOC tumors, chemosensitive and chemoresistant primary HGSOC cells, a high throughput gene expression qRT-PCR (Fluidigm, South San Francisco, CA, USA) was performed at the Australian Cancer Research Foundation Cancer Genomics Facility using the 96.96 Dynamic Array integrated fluidic circuit (IFC) (Integrated Sciences, Chatswood, NSW, Australia). The 20X Taqman assays (listed in Table S3), 2 μL each, were pooled and cDNA preamplified under cycling conditions: 95 °C for 2 min then 14 cycles of 95 °C for 15 s lastly, 60 °C for 4 min on the C1000 cycler (Bio-Rad Laboratories, Hercules, CA, USA). IFC Controller MX (Fluidigm, South San Francisco, CA, USA) was used to prime the IFC with control line fluid (Fluidigm, South San Francisco, CA, USA) using the Prime (136×) script. Load Mix (136×) script was utilized to load samples and assays. A premix containing preamplified cDNA, 2X Quanta PerfeCTa qPCR Fast Mix, low ROX (Quanta BioSciences, Beverly, MA, USA) and 20X GE Sample Loading Reagent (Fluidigm, South San Francisco, CA, USA) was made. The premix and Taqman assays with 2X Assay Loading Reagent (Fluidigm, South San Francisco, CA, USA) were loaded into individual inlets. Thermal cycling then started in the BioMark HD System (Fluidigm, South

San Francisco, CA, USA) with 45 °C for 2 min, thermal mix: 70 °C for 40 min and 60 °C for 30 s, hot start: 98 °C for 1 min, followed by 35 cycles of 97 °C for 5 s and lastly, 60 °C for 20 s. Gene expression data was collected using the BioMark HD Data Collection software (Fluidigm, South San Francisco, CA, USA). Visualization and exportation of data were performed using the Fluidigm Real-Time PCR Analysis software (Fluidigm, South San Francisco, CA, USA). The $2^{-\Delta CT}$ method was used to normalize the Ct values to the geomean of the Ct of housekeepers, *TBP* and *GUSB*.

To confirm *PIWIL2* expression after hormone treatment on OV-90 cells, qRT-PCR was carried out using Taqman assays *HPRT1* and *PIWIL2* (Table S3). Technical duplicates of Taqman gene expression master mix, respective Taqman assays and cDNA were loaded in the MicroAmp Fast Optical 96-well plate (Applied Biosystems, Waltham, MA, USA) for thermal cycling in the StepOnePlus System (Applied Biosystems, Waltham, MA, USA). Cycling conditions were: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The $2^{-\Delta\Delta CT}$ method was used to normalize *PIWIL2* Ct values to housekeeper, *HPRT1*.

GraphPad Prism 8 was used for all statistical analysis. Normality of data was tested using the Shapiro–Wilk test followed by either Kruskal–Wallis (non-normal distribution) or one-way ANOVA (normal distribution) for comparing expression in benign, early and late stage HGSOC samples and Mann–Whitney U (non-normal distribution) or unpaired *t*-test (normal distribution) for comparing expression of chemosenstive and chemoresistant primary cells in the Fluidigm qRT-PCR. Mann–Whitney U test (non-normal distribution) was used to analyze the expression differences between benign and cancerous samples. Data from the other qRT-PCRs were subjected to the Shapiro–Wilk test followed by unpaired *t*-test as the data was normally distributed.

4.5. Public Microarray Online Databases

Expression profiles of the piRNA pathway genes across OC grades 1–3 were evaluated using CSIOVDB (http://csiovdb.mc.ntu.edu.tw/CSIOVDB.html), a microarray gene expression database n = 3431 [104]. The OC subtypes analyzed consisted of clear cell, endometrioid, mucinous, and serous. The Kaplan–Meier plotter (http://kmplot.com/analysis/) was used to examine the relationship with PFS, PPFS and OS for OC (n = 2190) patients and expression levels of the piRNA pathway genes (Table S4) [105]. There was no microarray data available for *PIWIL3*. Parameters were set in accordance with the Fluidigm samples of HGSOC (grades 2 and 3). Early stage consisted of stage 1 and 2 while the late stage consisted of only stage 3 HGSOC patients. Patients were split by the best cut-off selected by the online plotter tool.

4.6. Overexpression Transfections

Transfections on OVCAR-3 and OV-90 were carried out using Attractene (Qiagen, Chadstone, VIC, Australia) according to the manufacturer's protocol. To ensure better survival and transfection efficiency, the cells were seeded 48 h pre-transfection at 40–50% confluency and the culture media was replaced with Opti-MEM (Gibco, Waltham, MA, USA, cat no. 11835-030) for DNA and Attractene complex formation. The passage number was kept below 10 post-thaw for all transfections to reduce variability in experiments. Cells were harvested 60–65 h post transfection for subsequent assays. pcDNA3.1 (+) plasmid (Invitrogen, Waltham, MA, USA) was used as the empty vector control. Overexpression constructs were *PIWIL1* (oHu24048), *PIWIL2* (oHu26193) and *MAEL* (oHu11219) inserted in pcDNA3.1 (+) plasmids (GenScript, Piscataway, New Jersey, USA), respectively. Overexpression mutant constructs, *P1Δ17* and *PL2L60* (GenScript, Piscataway, New Jersey, USA), were made by removing exon 17 from *PIWIL1* cDNA (ENST00000245255.7) and a readily available *PL2L60* cDNA sequence (AK027497.1) inserted in pcDNA3.1 (+) plasmids.

4.7. RT-PCR

RT-PCRs were performed on a 94 °C preheated C1000 Touch thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) with cycling conditions: 94 °C for 30 s, 35 cycles of 94 °C for 30 s, 55 °C (*β-actin*)/59 °C (*PIWIL2*) for 30 s, 68 °C for 1 min followed by a final extension of 68 °C for 5 min. The composition of each 25 µL reaction using One*Taq* DNA polymerase (New England Biolabs, Ipswich, MA, USA) were following manufacturer's protocol. The ChemiDoc MP Imager (Bio-Rad Laboratories, Hercules, CA, USA) was used to visualize PCR products on 2% agarose gels stained with ethidium bromide and generate images. *β-actin* and *PIWIL2* primers (Table S5) were purchased from Integrated DNA Technologies.

4.8. In Vitro Motility and Invasion Assay

The ChemoTx® 96-well plate (Neuroprobe, Gaithersburg, MD, USA) was used to assess motility of transfected OVCAR-3 and OV-90 cells. Addition of an even spread of dried 0.6 µL Geltrex (Gibco, Waltham, MA, USA) diluted 1:1 with media (RPMI1640 + 0.1% BSA) on the filter membrane was used to determine invasion. Briefly, cells were labelled with calcein AM (Life Technologies, Mulgrave, VIC, Australia) after 30 min of incubation in the dark on a nutator. Excess calcein AM were removed by washing the cells twice with media (RPMI1640 + 0.1% BSA). Portions of 4×10^4 cells were then pipetted onto each pore of the filter above a microplate containing wells prefilled with chemoattractant (10% FCS) and media (RPMI1640 + 0.1% BSA). Reverse pipetting was employed at every step to prevent bubble entrapment. After a 6-hour, 37 °C incubation, cells that had migrated or invaded the filter were measured using the Triad series multimode detector (Dynex Technologies, Chantilly, VA, USA) at 485–520 nm. Assays were carried out in biological triplicate and had technical replicates to a total of n = 21–24 per cell line per construct transfected. Statistical analysis was carried out on GraphPad Prism 8 first by Shapiro–Wilk test of normality followed by either Mann–Whitney U test (non-normal distribution) or unpaired *t*-test (normal distribution).

4.9. Chicken Chorioallantoic Membrane (CAM) Assay and Immunohistochemistry

The CAM assays for OV-90 cells were carried out as described previously [106]. CAM harvested from each embryo had 1–2 separate onplants consisting of a mixture of 90,000 cells and matrigel (n = 4–18 per construct transfected) for analysis. Paraffin serial sections (5 µm) were stained with hematoxylin and eosin to determine the best paired section for immunohistochemistry (IHC). Paraffin sections for IHC were firstly placed on a 60 °C heat plate for a minimum of 1 h then dewaxed with 100% xylene and ethanol and washed in 1X PBS. After incubating for 5 min in 1% H_2O_2 and 1X PBS washes, citrate buffer antigen retrieval was performed in a steam microwave (Sixth Sense, Whirlpool, Dandenong South, VIC, Australia) for 10 min. Sections were incubated for 30 min in blocking buffer (5% goat serum in 1X PBS) before an overnight incubation with 1:800 CD44 antibody (Thermo Fisher Scientific, Waltham, MA, USA, cat no. #MA5-13890) in blocking buffer. With 1X PBS washes in between, sections underwent an hour incubation of 1:400 goat anti-mouse antibody (Dako, Agilent, Santa Clara, CA, USA, cat no. #E0433) in blocking buffer, an hour incubation of 1:500 streptavidin (Dako, Agilent, Santa Clara, CA, USA, cat no. #P0397) in 1X PBS and 6 min of 1:1 DAB and H_2O_2 mixture. Lastly, sections were counterstained with hematoxylin, dipped in 70% ethanol, 100% ethanol, 100% xylene and mounted with Pertex mounting medium (HD Scientific, Ringwood, VIC, Australia). After drying, slides were scanned with the Nanozoomer Digital Pathology System (Hamamatsu Photonics, Hamamatsu City, Shizuoka Prefecture, Japan). The NDP scan software v2.2 (Hamamatsu Photonics, Hamamatsu City, Shizuoka Prefecture, Japan) was used to collect slide images and quantify cells that crossed the ectoderm as a measure of invasion. CAM assays were carried out on biological duplicates. Statistical

analysis was carried out on GraphPad Prism 8 first by Shapiro–Wilk test of normality followed by either Mann–Whitney U test (non-normal distribution) or unpaired *t*-test (normal distribution). Ethical approval was obtained on 5 October 2018 from the University of Adelaide Animal Ethics Committee (#33109).

4.10. FSH and LH Treatment

OV-90 cells (1×10^6) were seeded in T25 flasks and cultured for at least 24 h in normal culture media. Next, the flasks were rinsed with phenol red-free RPMI 1640 (Gibco, Waltham, MA, USA, cat no. 11835-030) and cultured in "complete" media for a minimum of 24 h. "Complete" media consisted of phenol red-free RPMI 1640, 1% each of AlbuMAX II 20% solution (Life Technologies, Mulgrave, VIC, Australia, cat no. E003000PJ), SPITE 100X (Sigma–Aldrich, St. Louis, MO, USA, cat no. S5666), penicillin/streptomycin, glutamax and 0.2% fungizone. Hormone treatment media then replaced the "complete" media for 24 h before RNA was harvested for subsequent RT-PCR and qRT-PCR. The untreated control was maintained in "complete" media. Hormone treatments were made up in "complete" media: 50 mIU/mL FSH, 100 mIU/mL FSH, 25 mIU/mL LH, 50 mIU/mL LH, 50 mIU/mL FSH + 25 mIU/mL LH and 100 mIU/mL FSH + 50 mIU/mL LH. FSH (AFP-7298A) and LH (AFP-4395A) were purchased from the National Hormone and Peptide Program (Torrance, CA, USA).

5. Conclusions

In summary, our extensive analysis of piRNA pathway genes in OC adds to the growing knowledge base about the role of this pathway in cancer. Differential expression of these piRNA pathway genes in OC depicts a more complex association of their expression with various aspects of malignancy, patient survival, chemoresistance, gonadotropin treatment and invasion. The observation that piRNA pathway genes may act differently in the HGSOC context compared to other cancers is possibly due to its exceptionally high proportion of *TP53* mutations, genomic instability and highly heterogeneous nature. Here, we present an expression profile of 10 piRNA pathway genes in benign ovarian tumors, low grade OC and HGSOC and their effects on patient survival. We identified *PIWIL3* as a potential target for chemoresistance in HGSOC. Further, we made a novel discovery that FSH can mediate *PIWIL2* expression. Together with in vitro and in vivo studies, we revealed that overexpression of *PIWIL1*, *P1Δ17*, *PIWIL2*, *PL2L60* and *MAEL* caused a decrease in motility and invasion. However, mutants *P1Δ17* and *PL2L60* surprisingly induced a more invasive phenotype compared to wildtype. While providing a deeper understanding, this work also raises a number of questions about the complicated relationship between piRNAs, the pathway genes and their functions in OC pathogenesis. Additional work is required to further explore the clinical relevance of the variability in piRNAs and pathway genes in OC patients.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6694/13/1/4/s1, Table S1: Clinicopathological characteristics of benign and high grade serous ovarian cancer cohort used in the Fluidigm qRT-PCR. Table S2: Clinicopathological characteristics of chemosenstitive and chemoresistant ovarian cancer patient cohort used in the Fluidigm qRT-PCR. Table S3: Taqman assays used in qRT-PCR. Table S4: Affymetrix ID of piRNA pathway genes assessed in the Kaplan–Meier plotter. Table S5: Primers used in RT-PCR.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Central Adelaide Local Health Network Human Research (RAH Protocol #140201, date approved 13 January 2014) and Animal Ethics Committee of the University of Adelaide (#33109, date approved 5 October 2018).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available in this article and supplementary materials.

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Abbreviations

BSA	Bovine serum albumin
CAM	Chicken chorioallantoic membrane
CSIOVDB	Ovarian cancer database of Cancer Science Institute Singapore
DAB	3,3′-diaminobenzidine
DDX4	DEAD-Box Helicase 4
EMT	Epithelial to mesenchymal transition
EOC	Epithelial ovarian cancer
FFPE	Formalin-fixed paraffin-embedded
FIGO	Fédération Internationale de Gynécologie et d'Obstétrique
FSH	Follicle stimulating hormone
GUSB	Glucuronidase Beta
HGSOC	High grade serous ovarian cancer
HENMT1	HEN Methyltransferase 1
HR	Hazard ratio
KM	Kaplan-Meier
LH	Luteinizing hormone
MAEL	Maelstrom
miRNA	MicroRNA
ncRNA	Noncoding RNA
OC	Ovarian cancer
OS	Overall survival
PBS	Phosphate-buffered saline
PFS	Progression free survival
piRNA	PIWI-interacting RNA
PIWIL (1-4)	P-element induced wimpy testis-like (1-4)
PLD6	Phospholipase D Family Member 6
PPFS	Post-progression free survival
SNAI (1-3)	Snail family zinc finger (1-3)
TBP	TATA-Box Binding Protein
TDRD (1:9)	Tudor Domain Containing (1,9)
TE	Transposable element
ZEB (1,2)	Zinc finger E-box binding homeobox (1,2)

References

1. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA A Cancer J. Clin.* **2018**, *68*, 394–424.
2. Torre, L.A.; Trabert, B.; DeSantis, C.E.; Miller, K.D.; Samimi, G.; Runowicz, C.D.; Gaudet, M.M.; Jemal, A.; Siegel, R.L. Ovarian cancer statistics, 2018. *CA A Cancer J. Clin.* **2018**, *68*, 284–296.
3. Kurman, R.J.; Visvanathan, K.; Roden, R.; Wu, T.C.; Shih, I.-M. Early Detection and Treatment of Ovarian Cancer: Shifting from Early Stage to Minimal Volume of Disease Based on a New Model of Carcinogenesis. *Am. J. Obstet. Gynecol.* **2008**, *198*, 351–356, doi:10.1016/j.ajog.2008.01.005.
4. Mertens-Walker, I.; Baxter, R.C.; Marsh, D.J. Gonadotropin signalling in epithelial ovarian cancer. *Cancer Lett.* **2012**, *324*, 152–159, doi:10.1016/j.canlet.2012.05.017.
5. Gharwan, H.; Bunch, K.P.; Annunziata, C.M. The role of reproductive hormones in epithelial ovarian carcinogenesis. *Endocr. Relat. Cancer* **2015**, *22*, R339–R363.
6. Kurman, R.J.; Shih, I.-M. The Dualistic Model of Ovarian Carcinogenesis: Revisited, Revised, and Expanded. *Am. J. Pathol.* **2016**, *186*, 733–747, doi:10.1016/j.ajpath.2015.11.011.
7. Webb, P.M.; Jordan, S.J. Epidemiology of epithelial ovarian cancer. *Best Pract. Res. Clin. Obstet. Gynaecol.* **2017**, *41*, 3–14, doi:10.1016/j.bpobgyn.2016.08.006.
8. Prat, J. Ovarian carcinomas: Five distinct diseases with different origins, genetic alterations, and clinicopathological features. *Virchows Arch.* **2012**, *460*, 237–249.
9. Boussios, S.; Moschetta, M.; Zarkavelis, G.; Papadaki, A.; Kefas, A.; Tatsi, K. Ovarian sex-cord stromal tumours and small cell tumours: Pathological, genetic and management aspects. *Crit. Rev. Oncol. Hematol.* **2017**, *120*, 43–51.
10. Lheureux, S.; Braunstein, M.; Oza, A.M. Epithelial ovarian cancer: Evolution of management in the era of precision medicine. *CA A Cancer J. Clin.* **2019**, *69*, 280–304.
11. Lisio, M.-A.; Fu, L.; Goyeneche, A.; Gao, Z.-H.; Telleria, C. High-Grade Serous Ovarian Cancer: Basic Sciences, Clinical and Therapeutic Standpoints. *Int. J. Mol. Sci.* **2019**, *20*, 952.
12. Flaum, N.; Crosbie, E.J.; Edmondson, R.J.; Smith, M.J.; Evans, D.G. Epithelial ovarian cancer risk: A review of the current genetic landscape. *Clin. Genet.* **2020**, *97*, 54–63.
13. Olivier, M.; Hollstein, M.; Hainaut, P. TP53 mutations in human cancers: Origins, consequences, and clinical use. *Cold Spring Harb. Perspect. Biol.* **2010**, *2*, a001008.
14. Vang, R.; Levine, D.A.; Soslow, R.A.; Zaloudek, C.; Shih, I.-M.; Kurman, R.J. Molecular alterations of TP53 are a defining feature of ovarian high-grade serous carcinoma: A rereview of cases lacking TP53 mutations in the Cancer Genome Atlas Ovarian Study. *Int. J. Gynecol. Pathol. Off. J. Int. Soc. Gynecol. Pathol.* **2016**, *35*, 48.
15. Kim, S.; Han, Y.; Kim, S.I.; Kim, H.-S.; Kim, S.J.; Song, Y.S. Tumor evolution and chemoresistance in ovarian cancer. *Npj Precis. Oncol.* **2018**, *2*, 1–9.
16. Zhang, Y.; Cao, L.; Nguyen, D.; Lu, H. TP53 mutations in epithelial ovarian cancer. *Transl. Cancer Res.* **2016**, *5*, 650–663, doi:10.21037/tcr.2016.08.40.
17. Telloni, S.M. Tumor Staging and Grading: A Primer. In *Molecular Profiling: Methods and Protocols*; Espina, V., Ed.; Springer: New York, NY, USA, 2017; pp. 1–17, doi:10.1007/978-1-4939-6990-6_1.
18. Slomovitz, B.; Gourley, C.; Carey, M.S.; Malpica, A.; Shih, I.-M.; Huntsman, D.; Fader, A.N.; Grisham, R.N.; Schlumbrecht, M.; Sun, C.C. Low-grade serous ovarian cancer: State of the science. *Gynecol. Oncol.* **2020**, *156*, 715–725.
19. Hanahan, D.; Weinberg, R.A. Hallmarks of Cancer: The Next Generation. *Cell* **2011**, *144*, 646–674, doi:10.1016/j.cell.2011.02.013.
20. Shibue, T.; Weinberg, R.A. EMT, CSCs, and drug resistance: The mechanistic link and clinical implications. *Nat. Rev. Clin. Oncol.* **2017**, *14*, 611.
21. Han, Y.N.; Li, Y.; Xia, S.Q.; Zhang, Y.Y.; Zheng, J.H.; Li, W. PIWI Proteins and PIWI-Interacting RNA: Emerging Roles in Cancer. *Cell. Physiol. Biochem.* **2017**, *44*, 1–20.
22. Kim, S.-H.; Park, E.-R.; Cho, E.; Jung, W.-H.; Jeon, J.-Y.; Joo, H.-Y.; Lee, K.-H.; Shin, H.-J. Mael is essential for cancer cell survival and tumorigenesis through protection of genetic integrity. *Oncotarget* **2017**, *8*, 5026–5037, doi:10.18632/oncotarget.13756.
23. Xiao, L.; Lanz, R.B.; Frolov, A.; Castro, P.D.; Zhang, Z.; Dong, B.; Xue, W.; Jung, S.Y.; Lydon, J.P.; Edwards, D.P. The germ cell gene TDRD1 as an ERG target gene and a novel prostate cancer biomarker. *Prostate* **2016**, *76*, 1271–1284.
24. Schudrowitz, N.; Takagi, S.; Wessel, G.M.; Yajima, M. Germline factor DDX 4 functions in blood-derived cancer cell phenotypes. *Cancer Sci.* **2017**, *108*, 1612–1619.

25. Lim, S.L.; Ricciardelli, C.; Oehler, M.K.; De Arao Tan, I.M.D.; Russell, D.; Grützner, F. Overexpression of piRNA Pathway Genes in Epithelial Ovarian Cancer. *PLoS ONE* **2014**, *9*, e99687, doi:10.1371/journal.pone.0099687.
26. Wang, Q.-E.; Han, C.; Milum, K.; Wani, A.A. Stem cell protein Piwil2 modulates chromatin modifications upon cisplatin treatment. *Mutat. Res. Fundam. Mol. Mech. Mutagenesis* **2011**, *708*, 59–68, doi:10.1016/j.mrfmmm.2011.02.001.
27. Hirakata, S.; Siomi, M.C. piRNA biogenesis in the germline: From transcription of piRNA genomic sources to piRNA maturation. *Biochim. Biophys. Acta (Bba) Gene Regul. Mech.* **2016**, *1859*, 82–92, doi:10.1016/j.bbagrm.2015.09.002.
28. Ozata, D.M.; Gainetdinov, I.; Zoch, A.; O'Carroll, D.; Zamore, P.D. PIWI-interacting RNAs: Small RNAs with big functions. *Nat. Rev. Genet.* **2019**, *20*, 89–108.
29. Aravin, A.; Gaidatzis, D.; Pfeffer, S.; Lagos-Quintana, M.; Landgraf, P.; Iovino, N.; Morris, P.; Brownstein, M.J.; Kuramochi-Miyagawa, S.; Nakano, T. A novel class of small RNAs bind to MILI protein in mouse testes. *Nature* **2006**, *442*, 203–207.
30. Iwasaki, Y.W.; Siomi, M.C.; Siomi, H. PIWI-Interacting RNA: Its Biogenesis and Functions. *Annu. Rev. Biochem.* **2015**, *84*, 405–433, doi:10.1146/annurev-biochem-060614-034258.
31. Cora, E.; Pandey, R.R.; Xiol, J.; Taylor, J.; Sachidanandam, R.; McCarthy, A.A.; Pillai, R.S. The MID-PIWI module of Piwi proteins specifies nucleotide- and strand-biases of piRNAs. *RNA* **2014**, *20*, 773–781, doi:10.1261/rna.044701.114.
32. Bamezai, S.; Rawat, V.P.S.; Buske, C. Concise Review: The Piwi-piRNA Axis: Pivotal Beyond Transposon Silencing. *Stem Cells* **2012**, *30*, 2603–2611, doi:10.1002/stem.1237.
33. Simon, B.; Kirkpatrick, J.P.; Eckhardt, S.; Reuter, M.; Rocha, E.A.; Andrade-Navarro, M.A.; Sehr, P.; Pillai, R.S.; Carlomagno, T. Recognition of 2′-O-methylated 3′-end of piRNA by the PAZ domain of a Piwi protein. *Structure* **2011**, *19*, 172–180.
34. Aravin, A.A.; Sachidanandam, R.; Girard, A.; Fejes-Toth, K.; Hannon, G.J. Developmentally Regulated piRNA Clusters Implicate MILI in Transposon Control. *Science* **2007**, *316*, 744–747.
35. Brennecke, J.; Aravin, A.A.; Stark, A.; Dus, M.; Kellis, M.; Sachidanandam, R.; Hannon, G.J. Discrete Small RNA-Generating Loci as Master Regulators of Transposon Activity in Drosophila. *Cell* **2007**, *128*, 1089–1103, doi:10.1016/j.cell.2007.01.043.
36. Siomi, M.C.; Sato, K.; Pezic, D.; Aravin, A.A. PIWI-interacting small RNAs: The vanguard of genome defence. *Nat. Rev. Mol. Cell Biol.* **2011**, *12*, 246–258.
37. Soper, S.F.C.; van der Heijden, G.W.; Hardiman, T.C.; Goodheart, M.; Martin, S.L.; de Boer, P.; Bortvin, A. Mouse Maelstrom, a component of nuage, is essential for spermatogenesis and transposon repression in meiosis. *Dev. Cell* **2008**, *15*, 285–297, doi:10.1016/j.devcel.2008.05.015.
38. Sienski, G.; Dönertas, D.; Brennecke, J. Transcriptional Silencing of Transposons by Piwi and Maelstrom and Its Impact on Chromatin State and Gene Expression. *Cell* **2012**, *151*, 964–980, doi:10.1016/j.cell.2012.10.040.
39. Lim, S.L.; Qu, Z.P.; Kortschak, R.D.; Lawrence, D.M.; Geoghegan, J.; Hempfling, A.-L.; Bergmann, M.; Goodnow, C.C.; Ormandy, C.J.; Wong, L.; et al. HENMT1 and piRNA Stability Are Required for Adult Male Germ Cell Transposon Repression and to Define the Spermatogenic Program in the Mouse. *PLoS Genet.* **2015**, *11*, e1005620, doi:10.1371/journal.pgen.1005620.
40. Mani, S.R.; Juliano, C.E. Untangling the web: The diverse functions of the PIWI/piRNA pathway. *Mol. Reprod. Dev.* **2013**, *80*, 632–664, doi:10.1002/mrd.22195.
41. Sato, K.; Siomi, M.C. The piRNA pathway in Drosophila ovarian germ and somatic cells. *Proc. Jpn. Acad. Ser. B* **2020**, *96*, 32–42.
42. Ng, K.W.; Anderson, C.; Marshall, E.A.; Minatel, B.C.; Enfield, K.S.S.; Saprunoff, H.L.; Lam, W.L.; Martinez, V.D. Piwi-interacting RNAs in cancer: Emerging functions and clinical utility. *Mol. Cancer* **2016**, *15*, 5, doi:10.1186/s12943-016-0491-9.
43. Liu, Y.; Dou, M.; Song, X.; Dong, Y.; Liu, S.; Liu, H.; Tao, J.; Li, W.; Yin, X.; Xu, W. The emerging role of the piRNA/piwi complex in cancer. *Mol. Cancer* **2019**, *18*, 123, doi:10.1186/s12943-019-1052-9.
44. Tan, Y.; Liu, L.; Liao, M.; Zhang, C.; Hu, S.; Zou, M.; Gu, M.; Li, X. Emerging roles for PIWI proteins in cancer. *Acta Biochim. Biophys. Sin.* **2015**, *47*, 315–324, doi:10.1093/abbs/gmv018.
45. Xiao, L.; Wang, Y.; Zhou, Y.; Sun, Y.; Sun, W.; Wang, L.; Zhou, C.; Zhou, J.; Zhang, J. Identification of a novel human cancer/testis gene MAEL that is regulated by DNA methylation. *Mol. Biol. Rep.* **2010**, *37*, 2355–2360.
46. Babatunde, K.A.; Najafi, A.; Salehipour, P.; Modarressi, M.H.; Mobasheri, M.B. Cancer/Testis genes in relation to sperm biology and function. *Iran. J. Basic Med. Sci.* **2017**, *20*, 967–974, doi:10.22038/IJBMS.2017.9259.

47. Liu, L.; Dai, Y.; Chen, J.; Zeng, T.; Li, Y.; Chen, L.; Zhu, Y.H.; Li, J.; Ma, S.; Xie, D. Maelstrom promotes hepatocellular carcinoma metastasis by inducing epithelial-mesenchymal transition by way of Akt/GSK-3β/Snail signaling. *Hepatology* **2014**, *59*, 531–543.
48. Fite, K. *Dysregulation of Phospholipase D (PLD) Isoforms Increases Breast Cancer Cell Invasion*; Wright State University: Dayton, OH, USA, 2017.
49. Boormans, J.L.; Korsten, H.; Ziel-van der Made, A.J.; van Leenders, G.J.; de Vos, C.V.; Jenster, G.; Trapman, J. Identification of TDRD1 as a direct target gene of ERG in primary prostate cancer. *Int. J. Cancer* **2013**, *133*, 335–345.
50. Ye, Y.; Yin, D.-T.; Chen, L.; Zhou, Q.; Shen, R.; He, G.; Yan, Q.; Tong, Z.; Issekutz, A.C.; Shapiro, C.L.; et al. Identification of Piwil2-Like (PL2L) Proteins that Promote Tumorigenesis. *PLoS ONE* **2010**, *5*, e13406, doi:10.1371/journal.pone.0013406.
51. Linder, P. Dead-box proteins: A family affair—Active and passive players in RNP-remodeling. *Nucleic Acids Res.* **2006**, *34*, 4168–4180, doi:10.1093/nar/gkl468.
52. Horwich, M.D.; Li, C.; Matranga, C.; Vagin, V.; Farley, G.; Wang, P.; Zamore, P.D. The Drosophila RNA Methyltransferase, DmHen1, Modifies Germline piRNAs and Single-Stranded siRNAs in RISC. *Curr. Biol.* **2007**, *17*, 1265–1272, doi:10.1016/j.cub.2007.06.030.
53. Findley, S.D.; Tamanaha, M.; Clegg, N.J.; Ruohola-Baker, H. Maelstrom, a Drosophila spindle-class gene, encodes a protein that colocalizes with Vasa and RDE1/AGO1 homolog, Aubergine, in nuage. *Development* **2003**, *130*, 859–871.
54. Gomez-Cambronero, J. Phospholipase D in cell signaling: From a myriad of cell functions to cancer growth and metastasis. *J. Biol. Chem.* **2014**, *289*, 22557–22566.
55. Chen, C.; Nott, T.J.; Jin, J.; Pawson, T. Deciphering arginine methylation: Tudor tells the tale. *Nat. Rev. Mol. Cell Biol.* **2011**, *12*, 629–642.
56. Jiang, L.; Wang, W.-J.; Li, Z.-W.; Wang, X.-Z. Downregulation of Piwil3 suppresses cell proliferation, migration and invasion in gastric cancer. *Cancer Biomark.* **2017**, *20*, 499–509.
57. Yang, Y.; Zhang, X.; Song, D.; Wei, J. Piwil2 modulates the invasion and metastasis of prostate cancer by regulating the expression of matrix metalloproteinase-9 and epithelial-mesenchymal transitions. *Oncol. Lett.* **2015**, *10*, 1735–1740.
58. Wang, X.; Tong, X.; Gao, H.; Yan, X.; Xu, X.; Sun, S.; Wang, Q.; Wang, J. Silencing HIWI suppresses the growth, invasion and migration of glioma cells. *Int. J. Oncol.* **2014**, *45*, 2385–2392.
59. Kim, K.H.; Kang, Y.-J.; Jo, J.-O.; Ock, M.S.; Moon, S.H.; Suh, D.S.; Yoon, M.S.; Park, E.-S.; Jeong, N.; Eo, W.-K. DDX4 (DEAD box polypeptide 4) colocalizes with cancer stem cell marker CD133 in ovarian cancers. *Biochem. Biophys. Res. Commun.* **2014**, *447*, 315–322.
60. Hashimoto, H.; Sudo, T.; Mikami, Y.; Otani, M.; Takano, M.; Tsuda, H.; Itamochi, H.; Katabuchi, H.; Ito, M.; Nishimura, R. Germ cell specific protein VASA is over-expressed in epithelial ovarian cancer and disrupts DNA damage-induced G2 checkpoint. *Gynecol. Oncol.* **2008**, *111*, 312–319, doi:10.1016/j.ygyno.2008.08.014.
61. Guijo, M.; Ceballos-Chávez, M.; Gómez-Marín, E.; Basurto-Cayuela, L.; Reyes, J.C. Expression of TDRD9 in a subset of lung carcinomas by CpG island hypomethylation protects from DNA damage. *Oncotarget* **2017**, *9*, 9618–9631, doi:10.18632/oncotarget.22709.
62. Li, J.; Xu, L.; Bao, Z.; Xu, P.; Chang, H.; Wu, J.; Bei, Y.; Xia, L.; Wu, P.; Cui, G. High expression of PIWIL2 promotes tumor cell proliferation, migration and predicts a poor prognosis in glioma. *Oncol. Rep.* **2017**, *38*, 183–192.
63. Gainetdinov, I.V.; Kondratieva, S.A.; Skvortsova, Y.V.; Zinovyeva, M.V.; Stukacheva, E.A.; Klimov, A.; Tryakin, A.A.; Azhikina, T.L. Distinguishing epigenetic features of preneoplastic testis tissues adjacent to seminomas and nonseminomas. *Oncotarget* **2016**, *7*, 22439.
64. Iliev, R.; Stanik, M.; Fedorko, M.; Poprach, A.; Vychytilova-Faltejskova, P.; Slaba, K.; Svoboda, M.; Fabian, P.; Pacik, D.; Dolezel, J. Decreased expression levels of PIWIL1, PIWIL2, and PIWIL4 are associated with worse survival in renal cell carcinoma patients. *Oncotargets Ther.* **2016**, *9*, 217.
65. Cheng, J.; Deng, H.; Xiao, B.; Zhou, H.; Zhou, F.; Shen, Z.; Guo, J. piR-823, a novel non-coding small RNA, demonstrates in vitro and in vivo tumor suppressive activity in human gastric cancer cells. *Cancer Lett.* **2012**, *315*, 12–17.
66. Yan, H.; Wu, Q.; Sun, C.; Ai, L.; Deng, J.; Zhang, L.; Chen, L.; Chu, Z.; Tang, B.; Wang, K. piRNA-823 contributes to tumorigenesis by regulating de novo DNA methylation and angiogenesis in multiple myeloma. *Leukemia* **2015**, *29*, 196–206.
67. Cheng, J.; Guo, J.-M.; Xiao, B.-X.; Miao, Y.; Jiang, Z.; Zhou, H.; Li, Q.-N. piRNA, the new non-coding RNA, is aberrantly expressed in human cancer cells. *Clin. Chim. Acta* **2011**, *412*, 1621–1625, doi:10.1016/j.cca.2011.05.015.

68. Palma Flores, C.; García-Vázquez, R.; Gallardo Rincón, D.; Ruiz-García, E.; Astudillo de la Vega, H.; Marchat, L.A.; Salinas Vera, Y.M.; López-Camarillo, C. MicroRNAs driving invasion and metastasis in ovarian cancer: Opportunities for translational medicine. *Int. J. Oncol.* **2017**, *50*, 1461–1476.
69. Law, P.T.-Y.; Qin, H.; Ching, A.K.-K.; Lai, K.P.; Co, N.N.; He, M.; Lung, R.W.-M.; Chan, A.W.-H.; Chan, T.-F.; Wong, N. Deep sequencing of small RNA transcriptome reveals novel non-coding RNAs in hepatocellular carcinoma. *J. Hepatol.* **2013**, *58*, 1165–1173.
70. Liu, X.; Zheng, J.; Xue, Y.; Yu, H.; Gong, W.; Wang, P.; Li, Z.; Liu, Y. PIWIL3/OIP5-AS1/miR-367-3p/CEBPA feedback loop regulates the biological behavior of glioma cells. *Theranostics* **2018**, *8*, 1084–1105, doi:10.7150/thno.21740.
71. von Eyss, B.; Jaenicke, L.A.; Kortlever, R.M.; Royla, N.; Wiese, K.E.; Letschert, S.; McDuffus, L.-A.; Sauer, M.; Rosenwald, A.; Evan, G.I.; et al. A MYC-Driven Change in Mitochondrial Dynamics Limits YAP/TAZ Function in Mammary Epithelial Cells and Breast Cancer. *Cancer Cell* **2015**, *28*, 743–757, doi:10.1016/j.ccell.2015.10.013.
72. Hempfling, A.-L.; Lim, S.L.; Adelson, D.; Evans, J.; O'Connor, A.E.; Qu, Z.; Kliesch, S.; Weidner, W.; O'Bryan, M.; Bergmann, M. Expression patterns of HENMT1 and PIWIL1 in human testis-Implications for transposon expression. *Reproduction* **2017**, *154*, 363–374.
73. Iorio, M.V.; Visone, R.; Di Leva, G.; Donati, V.; Petrocca, F.; Casalini, P.; Taccioli, C.; Volinia, S.; Liu, C.-G.; Alder, H. MicroRNA signatures in human ovarian cancer. *Cancer Res.* **2007**, *67*, 8699–8707.
74. Kacprzyk, L.A.; Laible, M.; Andrasiuk, T.; Brase, J.C.; Börno, S.T.; Fälth, M.; Kuner, R.; Lehrach, H.; Schweiger, M.R.; Sültmann, H. ERG Induces Epigenetic Activation of Tudor Domain-Containing Protein 1 (TDRD1) in ERG Rearrangement-Positive Prostate Cancer. *PLoS ONE* **2013**, *8*, e59976, doi:10.1371/journal.pone.0059976.
75. Gambichler, T.; Kohsik, C.; Höh, A.-K.; Lang, K.; Käfferlein, H.U.; Brüning, T.; Stockfleth, E.; Stücker, M.; Dreißigacker, M.; Sand, M. Expression of PIWIL3 in primary and metastatic melanoma. *J. Cancer Res. Clin. Oncol.* **2017**, *143*, 433–437.
76. Li, W.; Martinez-Useros, J.; Garcia-Carbonero, N.; Fernandez-Aceñero, M.J.; Orta, A.; Ortega-Medina, L.; Garcia-Botella, S.; Perez-Aguirre, E.; Diez-Valladares, L.; Celdran, A.; et al. The Clinical Significance of PIWIL3 and PIWIL4 Expression in Pancreatic Cancer. *J. Clin. Med.* **2020**, *9*, 1252, doi:10.3390/jcm9051252.
77. Tan, M.; van Tol, H.T.; Rosenkranz, D.; Roovers, E.F.; Damen, M.J.; Stout, T.A.; Wu, W.; Roelen, B.A. PIWIL3 forms a complex with TDRKH in mammalian oocytes. *Cells* **2020**, *9*, 1356.
78. Salomon-Perzyński, A.; Salomon-Perzyńska, M.; Michalski, B.; Skrzypulec-Plinta, V. High-grade serous ovarian cancer: The clone wars. *Arch. Gynecol. Obstet.* **2017**, *295*, 569–576.
79. Birkbak, N.J.; Eklund, A.C.; Li, Q.; McClelland, S.E.; Endesfelder, D.; Tan, P.; Tan, I.B.; Richardson, A.L.; Szallasi, Z.; Swanton, C. Paradoxical relationship between chromosomal instability and survival outcome in cancer. *Cancer Res.* **2011**, *71*, 3447–3452.
80. Öner, Ç.; Turgut Coşan, D.; Çolak, E. Estrogen and Androgen Hormone Levels Modulate the Expression of PIWI Interacting RNA in Prostate and Breast Cancer. *PLoS ONE* **2016**, *11*, e0159044, doi:10.1371/journal.pone.0159044.
81. Wang, H.; Wang, B.; Liu, J.; Li, A.; Zhu, H.; Wang, X.; Zhang, Q. Piwil1 gene is regulated by hypothalamic-pituitary-gonadal axis in turbot (Scophthalmus maximus): A different effect in ovaries and testes. *Gene* **2018**, *658*, 86–95, doi:10.1016/j.gene.2018.03.016.
82. Zhang, D.; Duarte-Guterman, P.; Langlois, V.S.; Trudeau, V.L. Temporal expression and steroidal regulation of piRNA pathway genes (mael, piwi, vasa) during Silurana (Xenopus) tropicalis embryogenesis and early larval development. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2010**, *152*, 202–206.
83. Zhou, Y.; Zhong, H.; Liu, S.; Yu, F.; Hu, J.; Zhang, C.; Tao, M.; Liu, Y. Elevated expression of Piwi and piRNAs in ovaries of triploid crucian carp. *Mol. Cell. Endocrinol.* **2014**, *383*, 1–9, doi:10.1016/j.mce.2013.11.019.
84. Pan, Y.; Hu, M.; Liang, H.; Wang, J.-J.; Tang, L.-J. The expression of the PIWI family members miwi and mili in mice testis is negatively affected by estrogen. *Cell Tissue Res.* **2012**, *350*, 177–181.
85. Lee, E. (University of Adelaide, Adelaide, South Australia, Australia). Personal communication, 2020.
86. Zheng, W.; Lu, J.J.; Luo, F.; Zheng, Y.; Feng, Y.-J.; Felix, J.C.; Lauchlan, S.C.; Pike, M.C. Ovarian Epithelial Tumor Growth Promotion by Follicle-Stimulating Hormone and Inhibition of the Effect by Luteinizing Hormone. *Gynecol. Oncol.* **2000**, *76*, 80–88, doi:10.1006/gyno.1999.5628.

87. Syed, V.; Ulinski, G.; Mok, S.C.; Ho, S.-M. Reproductive hormone-induced, STAT3-mediated interleukin 6 action in normal and malignant human ovarian surface epithelial cells. *J. Natl. Cancer Inst.* **2002**, *94*, 617–629.
88. Li, D.; Sun, X.; Yan, D.; Huang, J.; Luo, Q.; Tang, H.; Peng, Z. Piwil2 modulates the proliferation and metastasis of colon cancer via regulation of matrix metallopeptidase 9 transcriptional activity. *Exp. Biol. Med.* **2012**, *237*, 1231–1240, doi:10.1258/ebm.2012.011380.
89. Lee, J.H.; Jung, C.; Javadian-Elyaderani, P.; Schweyer, S.; Schütte, D.; Shoukier, M.; Karimi-Busheri, F.; Weinfeld, M.; Rasouli-Nia, A.; Hengstler, J.G.; et al. Pathways of Proliferation and Antiapoptosis Driven in Breast Cancer Stem Cells by Stem Cell Protein Piwil2. *Cancer Res.* **2010**, *70*, 4569–4579, doi:10.1158/0008-5472.can-09-2670.
90. Zhang, D.; Wu, X.; Liu, X.; Cai, C.; Zeng, G.; Rohozinski, J.; Zhang, Y.; Wei, G.; He, D. Piwil2-transfected human fibroblasts are cancer stem cell-like and genetically unstable. *Oncotarget* **2017**, *8*, 12259.
91. Zhang, Z.; Zhu, Y.; Lai, Y.; Wu, X.; Feng, Z.; Yu, Y.; Bast, R.C.; Wan, X.; Xi, X.; Feng, Y. Follicle-stimulating hormone inhibits apoptosis in ovarian cancer cells by regulating the OCT4 stem cell signaling pathway. *Int. J. Oncol.* **2013**, *43*, 1194–1204.
92. Gera, S.; Kumar, S.S.; Swamy, S.N.; Bhagat, R.; Vadaparty, A.; Gawari, R.; Bhat, R.; Dighe, R.R. Follicle-Stimulating Hormone Is an Autocrine Regulator of the Ovarian Cancer Metastatic Niche Through Notch Signaling. *J. Endocr. Soc.* **2018**, *3*, 340–357, doi:10.1210/js.2018-00272.
93. Feng, D.; Zhao, T.; Yan, K.; Liang, H.; Liang, J.; Zhou, Y.; Zhao, W.; Ling, B. Gonadotropins promote human ovarian cancer cell migration and invasion via a cyclooxygenase 2-dependent pathway. *Oncol. Rep.* **2017**, *38*, 1091, doi:10.3892/or.2017.5784.
94. Mertens-Walker, I.; Bolitho, C.; Baxter, R.C.; Marsh, D.J. Gonadotropin-induced ovarian cancer cell migration and proliferation require extracellular signal-regulated kinase 1/2 activation regulated by calcium and protein kinase Cδ. *Endocr. Relat. Cancer* **2010**, *17*, 335.
95. Song, K.; Dai, L.; Long, X.; Wang, W.; Di, W. Follicle-stimulating hormone promotes the proliferation of epithelial ovarian cancer cells by activating sphingosine kinase. *Sci. Rep.* **2020**, *10*, 1–13.
96. Ivarsson, K.; Sundfeldt, K.; Brännström, M.; Hellberg, P.R.; Janson, P.O. Diverse effects of FSH and LH on proliferation of human ovarian surface epithelial cells. *Hum. Reprod.* **2001**, *16*, 18–23.
97. Bourgeois, D.L.; Kabarowski, K.A.; Porubsky, V.L.; Kreeger, P.K. High-grade serous ovarian cancer cell lines exhibit heterogeneous responses to growth factor stimulation. *Cancer Cell Int.* **2015**, *15*, 112–112, doi:10.1186/s12935-015-0263-4.
98. Chen, Z.; Che, Q.; He, X.; Wang, F.; Wang, H.; Zhu, M.; Sun, J.; Wan, X. Stem cell protein Piwil1 endowed endometrial cancer cells with stem-like properties via inducing epithelial-mesenchymal transition. *BMC Cancer* **2015**, *15*, 811, doi:10.1186/s12885-015-1794-8.
99. Li, C.; Zhou, X.; Chen, J.; Lu, Y.; Sun, Q.; Tao, D.; Hu, W.; Zheng, X.; Bian, S.; Liu, Y.; et al. PIWIL1 destabilizes microtubule by suppressing phosphorylation at Ser16 and RLIM-mediated degradation of stathmin1. *Oncotarget* **2015**, *6*, 27794–27804.
100. Qu, X.; Liu, J.; Zhong, X.; Li, X.; Zhang, Q. PIWIL2 promotes progression of non-small cell lung cancer by inducing CDK2 and Cyclin A expression. *J. Transl. Med.* **2015**, *13*, 301, doi:10.1186/s12967-015-0666-y?optIn=true.
101. Jiang, S.; Zhao, L.; Lu, Y.; Wang, M.; Chen, Y.; Tao, D.; Liu, Y.; Sun, H.; Zhang, S.; Ma, Y. Piwil2 inhibits keratin 8 degradation through promoting p38-induced phosphorylation to resist Fas-mediated apoptosis. *Mol. Cell. Biol.* **2014**, *34*, 3928–3938.
102. Lu, Y.; Zhang, K.; Li, C.; Yao, Y.; Tao, D.; Liu, Y.; Zhang, S.; Ma, Y. Piwil2 Suppresses P53 by Inducing Phosphorylation of Signal Transducer and Activator of Transcription 3 in Tumor Cells. *PLoS ONE* **2012**, *7*, e30999, doi:10.1371/journal.pone.0030999.
103. Lokman, N.A.; Price, Z.K.; Hawkins, E.K.; Macpherson, A.M.; Oehler, M.K.; Ricciardelli, C. 4-Methylumbelliferone Inhibits Cancer Stem Cell Activation and Overcomes Chemoresistance in Ovarian Cancer. *Cancers* **2019**, *11*, 1187.
104. Tan, T.Z.; Yang, H.; Ye, J.; Low, J.; Choolani, M.; Tan, D.S.P.; Thiery, J.-P.; Huang, R.Y.-J. CSIOVDB: A microarray gene expression database of epithelial ovarian cancer subtype. *Oncotarget* **2015**, *6*, 43843.
105. Győrffy, B.; Lánczky, A.; Szállási, Z. Implementing an online tool for genome-wide validation of survival-associated biomarkers in ovarian-cancer using microarray data from 1287 patients. *Endocr. Relat. Cancer* **2012**, *19*, 197–208.

106. Lokman, N.A.; Elder, A.S.F.; Ricciardelli, C.; Oehler, M.K. Chick Chorioallantoic Membrane (CAM) Assay as an In Vivo Model to Study the Effect of Newly Identified Molecules on Ovarian Cancer Invasion and Metastasis. *Int. J. Mol. Sci.* **2012**, *13*, 9959–9970, doi:10.3390/ijms13089959.

Supplementary Tables

Table S1. Clinicopathological characteristics of benign and high grade serous ovarian cancer cohort used in the Fluidigm qRT-PCR

Benign serous cystadenomas (n=17)		
Age	Median (range)	60 (25-75)
High grade serous ovarian carcinomas (n=29)		
Age at Diagnosis	Median (range)	59 (38-84)
Histological Grade	High	29
FIGO stage	Stage I Stage II Stage III	4 8 17

Table S2. Clinicopathological characteristics of chemosenstitive and chemoresistant ovarian cancer patient cohort used in the Fluidigm qRT-PCR.

Chemoresponse	Patient	Age at Diagnosis	Diagnosis	Stage	Grade
Sensitive	1	66	Serous carcinoma of ovary/ peritoneum	3c	3
	2	72	Serous papillary carcinoma of the ovary	3c	3
	3	58	Serous carcinoma of the ovary	3c	3
	4	85	Serous papillary carcinoma of the peritoneum	3c	3
	5	58	Serous papillary carcinoma of the peritoneum	1c	3

Resistant	6	85	Recurrent serous carcinoma of the ovary	3c	3
	7	47	Recurrent serous carcinoma of the ovary	3c	3
	8	47	Recurrent serous papillary carcinoma of the peritoneum	3c	3
	9	81	Recurrent serous papillary carcinoma of the peritoneum	4	3
	10	69	Recurrent serous papillary carcinoma of the ovary	3a	3
	11	46	Recurrent serous papillary carcinoma of the ovary	3c	3
	12	N/A	Recurrent serous carcinoma of the ovary	1c	3

Table S3. Taqman assays used in qRT-PCR

Gene	Assay ID
PIWIL1	Hs01041737_m1
PIWIL2	Hs01032720_m1
PIWIL3	Hs00908825_m1
PIWIL4	Hs00381509_m1
DDX4	Hs00987125_m1
HENMT1	Hs00989130_m1
MAEL	Hs00262601_m1
PLD6	Hs00381651_m1
TDRD1	Hs00229805_m1
TDRD9	Hs00403678_m1
GUSB	Hs00939627_m1
TBP	Hs00427620_m1

Table S4. Affymetrix ID of piRNA pathway genes assessed in the Kaplan-Meier plotter

Gene	Affymetrix ID
PIWIL1	214868_at
PIWIL2	220686_s_at
PIWIL4	230480_at
DDX4	221630_s_at
HENMT1	225841_at
MAEL	229475_at
PLD6	227037_at
TDRD1	221018_s_at
TDRD9	228285_at

Table S5. Primers used in RT-PCR

Gene	Sense	Anti-sense
β-actin	5′-GACGACATGGAGAAAATCTG-3′	5′-ATGATCTGGGTCATCTTCTC-3′
PIWIL2	5′-TATGAGGTGAACCACTGTCTAC-3′	5′-GTTGAGAACACAGACATAATGC-3′

CHAPTER 3: PIRNA PATHWAY

GENES AND PROSTATE CANCER

Text in manuscript prepared for submission to *BMC Cancer*

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Name of Principal Author (Candidate)	Eunice Hsiu Yee Lee
Contribution to the Paper	Conceived the study, carried out cell culture, RNA extractions, cDNA synthesis, Fluidigm qRT-PCR, data analysis of relapsed patients, generation of survival plots, overexpression transfections, *in vitro* motility and invasion experiments, statistical analysis and writing the manuscript.
Overall percentage (%)	60%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 30/11/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Chui Yan Mah
Contribution to the Paper	Performed transcriptome data analysis and statistical analysis
Signature	Date 30/11/2020

Name of Co-Author	Natalie Ryan
Contribution to the Paper	Organised tumour samples and some RNA extractions
Signature	Date 30/11/2020

Name of Co-Author	Noor A Lokman		
Contribution to the Paper	Provided guidance on experiments and data analysis, supervision and manuscript editing.		
Signature		Date	30/11/2020

Name of Co-Author	Carmela Ricciardelli		
Contribution to the Paper	Provided guidance on data analysis, supervision and manuscript editing.		
Signature		Date	30/11/2020

Name of Co-Author	Lisa M Butler		
Contribution to the Paper	Provided transcriptome and clinical data, benign and prostate cancer samples, guidance on data analysis and manuscript editing		
Signature		Date	30/11/2020

Name of Co-Author	Frank Grutzner		
Contribution to the Paper	Conceived the study, provided supervision and manuscript editing.		
Signature		Date	30/11/2020

Please cut and paste additional co-author panels here as required.

piRNA pathway genes are altered during prostate cancer development and progression and can affect migration and invasion properties

Eunice Lee[1], Chui Yan Mah[2], Natalie Ryan[2], Noor A Lokman[3], Carmela Ricciardelli[3], Lisa M Butler[2], Frank Grutzner[1]*

[1]Department of Molecular and Biomedical Sciences, Robinson Research Institute, University of Adelaide, Adelaide, 5000, South Australia, Australia

[2]School of Medicine and Freemasons Foundation Centre for Men's Health, University of Adelaide, and South Australian Health and Medical Research Institute

[3]Discipline of Obstetrics and Gynaecology, Adelaide Medical School, Robinson Research Institute, University of Adelaide, Adelaide, 5000, South Australia, Australia

*Correspondence: frank.grutzner@adelaide.edu.au

Abstract

Background: A growing number of studies link deregulation of piRNA pathway gene expression with functional changes in multiple cancers. However, limited investigation of the piRNA pathway has been performed in the development and progression of prostate cancer (PCa) and advanced castrate resistant prostate cancer (CRPC). Here, we perform the first characterisation of this pathway in PCa cell lines and clinical tissues.

Methods: To quantitatively profile the expression of *PIWIL1-4*, *DDX4*, *HENMT1*, *PLD6*, *TDRD1* and *TDRD9* across normal, benign and PCa tissues, we performed qRT-PCR (Fluidigm) and compared this to publicly available transcriptome data. Disease-free survival of PCa patients with altered piRNA pathway gene expression was analysed using cBioPortal. Differential expression of the piRNA pathway genes between tumours from longer-term disease-free and relapsed patients, and in LNCaP cells after inhibition of androgen receptor (AR) activity was investigated using clinical data and RNA sequencing. *In vitro* motility and invasion assays were performed on CRPC cells (22RV1) engineered to overexpress *PIWIL1*, *PIWIL1* mutant, *PIWIL2*, *PIWIL2* mutant and *MAEL*.

Results: *PIWIL1-4* and *MAEL* had lower expression while *DDX4*, *HENMT1*, *PLD6*, *TDRD1* and *TDRD9* had higher expression levels in normal and benign samples compared to PCa tumours. Unaltered piRNA pathway genes associated significantly with better disease-free survival for PCa patients. We discovered higher *DDX4* and *PLD6* expression but lower *HENMT1* expression between tumours of non-relapsed versus relapsed PCa patients. LNCaP cells

showed increased *HENMT1* and *PLD6* expression in response to AR inhibition, while *TDRD1* expression decreased. Overexpression of *PIWIL2*, *PIWIL2* mutant and *MAEL* decreased motility and invasion of 22RV1 cells. However, cells overexpressing *PIWIL2* mutant had increased motility compared to those overexpressing *PIWIL2*.

Conclusion: This work suggests that altered expression of piRNA pathway genes could be an early event for PCa, associates with poorer prognosis and relapse of patients. Furthermore, we observed that the expression profile here differed from other cancers indicating potential cancer-specific expression or role of the piRNA pathway genes. This may be due to the hormone-sensitive aspect of PCa where changed piRNA pathway gene expression was observed after reduced AR activity. Motility and invasion assays indicated protective effects of the piRNA pathway genes and opposing effects of overexpressing wildtype and mutant *PIWIL2*. Altogether, our work demonstrated possible roles of the piRNA pathway genes in various aspects of PCa and the differential behaviour of *PIWIL2* mutant.

Keywords: Prostate cancer; Castrate resistant prostate cancer; piRNA pathway; invasion; patient survival; expression profile; androgen receptor

Background

Prostate cancer (PCa) is one of the most commonly diagnosed cancer in men where over 1 million new cases and 358,989 deaths were projected to occur worldwide in 2018 (49). The proliferation and growth of PCa cells can be stimulated by androgen through androgen receptor (AR) signalling (115, 116).

AR can trigger downstream transcription of genes such as prostate specific antigen (PSA) (117). Due to the androgen dependency of PCa cells, androgen deprivation therapy (ADT) was developed (83). While ADT is an effective first line treatment, a portion of PCa patients develop resistance, known as castrate resistant prostate cancer (CRPC) (83, 85). CRPC development has been hypothesised to involve modified AR signalling and the presence of AR variants (81, 84). One of the most prominent AR variants is the constitutively active AR-V7 which does not require ligand binding for activity (118). Therefore, new targeted drugs such as enzalutamide were developed to target and inhibit AR signalling through multiple modes of action (119). However, these are only temporarily effective (119). A better understanding of the key drivers of prostate cancer development and progression is necessary to develop alternative therapeutic strategies.

Genomic aberrations are frequently observed in PCa and CRPC where fusion of androgen responsive promoter *transmembrane serine protease 2* (*TMPRSS2*) and transcription factor *ETS-related gene* (*ERG*) is one of the most prevalent genomic rearrangement (92, 120, 121). Studies suggest that the *TMPRSS2:ERG* fusion enables the activation of NOTCH and WNT/TGFβ pathways which play roles in PCa cell invasion and epithelial-to-mesenchymal transition (EMT) respectively (122-125). Due to the prominence of *ERG* overexpression in PCa, its gene targets are being investigated to further elucidate its mechanism in PCa development and progression. One of the gene targets identified is cancer-testis gene *TDRD1*, which is part of the PIWI-interacting RNA (piRNA) pathway (27, 126, 127).

The piRNA pathway consists of small non-coding RNAs known as piRNAs and pathway genes which includes its direct interactors, *PIWIL1-4* and associated genes *DDX4*, *HENMT1*, *PLD6*, *TDRD1* and *TDRD9*. These genes play a role in piRNA biogenesis and repress transposable elements (TEs) such as LINE-1 and intracisternal A-type particle to maintain genomic stability (17, 25, 128). More recently, the piRNA pathway genes have shown aberrant expression in various cancers and roles in the regulation of EMT, cell invasion, growth, proliferation, and apoptosis (45, 96, 97, 107). Further investigation revealed the presence of piRNA pathway gene mutants in ovarian cancer (*PIWIL1* mutant) and precancerous stem cells (PIWIL2 mutant) (43, 46). The *PIWIL1* mutant has a deletion in its exon 17 and was only found in malignant ovarian cancer tumours (43). The *PIWIL2* mutant, with a truncated domain, was identified as the predominant form of *PIWIL2* expressed in precancerous stem cells and was shown to associate with the anti-apoptotic *STAT3/ BCL-2* pathway (46). Although studies investigating piRNA pathway genes in cancer have been increasing rapidly, very little is known about their expression and function in PCa.

Here, we provide an expression analysis of 10 piRNA pathway genes *PIWIL1-4*, *DDX4*, *HENMT1*, *PLD6*, *TDRD1* and *TDRD9* in normal, benign and PCa samples. We revealed that these genes have varying expression profiles, indicating possible roles in PCa. Patient survival plots generated showed that alteration in certain genes conferred shorter disease-free survival. Further, these 10 genes showed different trends in expression in non-relapsed versus relapsed patient tumours and LNCaP cells after reduced AR activity using enzalutamide treatment and AR knockdown. Lastly, we demonstrated that the

overexpression of *PIWIL2*, *PIWIL2* mutant and *MAEL* can decrease the motility and invasion of CRPC cells where cells overexpressing *PIWIL2* mutant had increased motility compared to *PIWIL2* overexpression. Together, these results suggest a possible involvement of the piRNA pathway genes in PCa progression and CRPC development.

Methods

Patient cohort

The histopathological features of clinical patient tissues used in the Fluidigm qRT-PCR study are listed in Table S1. Ethics were obtained from the Human Research Ethics Committees of the University of Adelaide (H-2018-222).

RNA extraction, cDNA synthesis and quantification

RNA extractions from frozen tissue samples were carried out using TRIzol (Thermo Fisher) and subsequently treated with the DNA-free kit (Invitrogen) to degrade any remaining DNA. The RNA samples were quantified using Qubit (Invitrogen). iScript cDNA synthesis kit (Bio-Rad) was then used to synthesise cDNA from 400ng of RNA and 2μL of each sample was used in the qRT-PCR experiment. All steps were performed according to the manufacturers' protocol.

qRT-PCR (Fluidigm)

For expression profiling of benign prostate tissues (n=9) and PCa tumours (n=28; early stage n=10; late stage n=18), a gene expression qRT-PCR was performed at the Australian Cancer Research Foundation Cancer Genomics

Facility. Firstly, 2μL of each 20X Taqman assay (Table S2) were pooled to pre-amplify cDNA on the C1000 cycler (Bio-Rad) with cycling conditions: 95°C for 2 minutes, 14 cycles of 95°C for 15 seconds and 60°C for 4 minutes. The 96.96 Dynamic Array™ integrated fluidic circuit (IFC) (Integrated Sciences) was then primed with control line fluid (Fluidigm) using the Prime (136x) script on the IFC Controller MX (Fluidigm). Taqman assays with 2X Assay Loading Reagent (Fluidigm) and a pre-mix of pre-amplified cDNA, 2X Quanta PerfeCTa qPCR Fast Mix, low ROX (Quanta BioSciences) and 20X GE Sample Loading Reagent (Fluidigm) were loaded in their respective inlets on the IFC. Thermal cycling was performed on the BioMark™ HD System (Fluidigm) with cycling conditions: 45°C for 2 minutes, thermal mix: 70°C for 40 minutes and 60°C for 30 seconds, hot start: 98°C for 1 minute, 35 cycles of 97°C for 5 seconds and 60°C for 20 seconds. BioMark™ HD Data Collection software was used to collect gene expression data. Fluidigm Real-Time PCR Analysis software was used to visualise and export data. Ct values were normalised to the Ct of housekeeper, *HPRT1* using the $2^{-\Delta CT}$ method.

Clinical datasets

Transcriptomic data for expression analysis of piRNA pathway genes was retrieved from Ding, Wu (129) (GSE89194), Grasso, Wu (130) (GSE35988) and The Cancer Genome Atlas (TCGA; https://www.cancer.gov/tcga). The GSE89194 (n=90) and TCGA (n=86) datasets consisted of matched normal and PCa tissues. The normal and PCa tissues from GSE35988 were unmatched with differing number of tissues included in the analysis of the piRNA pathway genes: *PIWIL2* normal n= 12, cancer n= 48; *PIWIL4* normal

n= 12, cancer n= 49; *DDX4* normal n= 2, cancer n= 10; *MAEL* normal n= 12, cancer n= 47; *TDRD1* normal n= 5, cancer n= 40; *TDRD9* normal n= 12, cancer n= 49.

The cBioPortal (131, 132) (http://cbioportal.org) TCGA Firehose Legacy dataset (n=499) was used to generate PCa patient disease-free and overall survival plots (z-score = 2) and their respective statistical outcome. The number of patients analysed with the selected gene signature consisting *PIWIL1-4*, *DDX4*, *HENMT1*, *MAEL*, *PLD6*, *TDRD1* and *TDRD9* were: disease-free survival unaltered n= 364, altered n= 128; overall survival unaltered n= 368, altered n= 130. The number of patients included in the disease-free survival data of each gene differed and are as follows: *PIWIL1* unaltered n= 478, altered n= 14; *PIWIL2* unaltered n= 409, altered n= 83; *PIWIL3* unaltered n= 486, altered n= 6; *PIWIL4* unaltered n= 487, altered n= 5; *DDX4* unaltered n= 463, altered n= 29; *HENMT1* unaltered n= 489, altered n= 3; *MAEL* unaltered n= 480, altered n= 12; *PLD6* unaltered n= 487, altered n= 5; *TDRD1* unaltered n= 483, altered n= 9; *TDRD9* unaltered n= 492, altered n= 8. Patient tissues found to have any genetic alterations on the gene(s) selected were classified as altered.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 8. Shapiro-Wilk test of normality was used to select statistical tests Mann Whitney U (unmatched samples; non-normal distribution), paired t-test (matched samples; normal distribution) or unpaired t-test (unmatched samples; normal distribution) for comparison of expression differences between normal, benign and cancer

tissues in the Fluidigm qRT-PCR, GSE89194, TCGA and GSE35988 datasets. To compare expression differences across benign, early and late stage samples in the Fluidigm study, either the Kruskal Wallis (non-normal distribution) or one-way ANOVA (normal distribution) was used after the Shapiro-Wilk test of normality. The Shapiro-Wilk test of normality followed by Mann Whitney U (non-normal distribution) or unpaired t-test (normal distribution) were used for analysing expression differences between control and relapsed PCa patient tumours; transcriptomic data of DMSO and enzalutamide treatments and, siControl and siAR transfections in LNCaP cells.

To assess statistical significance of the motility and invasion assay, the Shapiro-Wilk test of normality was used followed by either the Mann Whitney U (non-normal distribution) or unpaired t-test (normal distribution).

Cell culture

Human PCa cell lines LNCaP and 22RV1 were obtained from the American Type Culture Collection (ATCC). The cells were cultured in RPMI 1640 (Gibco, cat no. 11875-093) containing 10% fetal bovine serum (FBS) (Corning) and maintained at 37^{o}C with 5% CO_2 in a humidified environment.

RNA sequencing

LNCaP cells were seeded at density 5x10^5 cells in 6-well dishes (Corning) and treated with 1 or 10 μM enzalutamide (Sapphire Bioscience) (or 0.1% DMSO control) or transfected with 12.5nM AR siRNA (or scrambled siRNA control) with 3-4 replicates each. After 24 hours, cells were collected in Trizol (for RNA analysis) or RIPA Buffer + protease inhibitors (for protein analysis). RNA

extractions were performed using RNeasy Mini spin columns (Qiagen) according to the manufacturer's protocol and RNA eluted in 40µl RNase-free H_2O. Western blot was performed to verify the expected response of known AR-regulated protein, PSA (Fig. S1). Subsequently, libraries were generated using 800ng of RNA and NEXTflex Rapid Illumina Directional RNA-Seq Library Prep Kits (Bio Scientific) according to the manufacturer's instructions. Sequencing was carried out at the South Australian Health and Medical Research Institute Genomics Facility using the Illumina NextSeq 500 (single read 75bp v2 sequencing chemistry). The quality and number of reads for each sample were assessed with FastQC v0.11.3. Adaptors were trimmed from reads, and low-quality bases (Phred scores <28) were trimmed from ends of reads, using Trimgalore v0.4.4. Trimmed reads of <20 nucleotides were discarded. Reads passing all quality control steps were aligned to the hg38 assembly of the human genome using TopHat v2.1.1 allowing for up to two mismatches. Reads not uniquely aligned to the genome were discarded. HTSeq-count v0.6.1 was used with the union model to assign uniquely aligned reads to Ensembl Hg38.86-annotated genes. Data were normalized across libraries by the trimmed mean of M-values normalization method, implemented in the R v3.5.0 using Bioconductor v3.6 EdgeR v3.20.9 package. Only genes expressed at count-per-million value greater than 10 in at least 2 samples per group were retained for further analysis. Differentially expressed genes were selected based on the robust version of the quasi-likelihood negative binomial generalized log-linear model, with false discovery rate set at 0.05. RNA-seq data are available through NCBI's Gene Expression Omnibus (GSE152254).

Overexpression transfections

Overexpression transfections on 22RV1 cells were carried out 48 hours post-seeding using Attractene (Qiagen) following the manufacturer's protocol. Opti-MEM (Gibco, cat no. 11835-030) was used for DNA and Attractene complex formation. 22RV1 cells were harvested 60-65 hours post transfection for downstream assays. Transfection constructs used were pcDNA3.1(+) plasmid (Invitrogen) as the empty vector control, genes *PIWIL1* (oHu24048), *PIWIL2* (oHu26193) and *MAEL* (oHu11219) inserted in pcDNA3.1(+) plasmid (GenScript) for overexpression. Deletion of exon 17 from *PIWIL1* cDNA (ENST00000245255.7) was used to make the *PIWIL1* mutant gene while the *PIWIL2* mutant gene sequence was retrieved online (AK027497.1) and inserted in pcDNA3.1(+) plasmid (GenScript) for overexpression.

***in vitro* motility and invasion assay**

Chemtrex® plates with 96 wells (Neuroprobe) were used to assess motility of transfected 22RV1 cells. Invasion assays were performed with the addition of 0.6μL diluted Geltrex (Gibco) 1:1 with media (RPMI1640 + 0.1% BSA) per filter pore. Briefly, transfected cells labelled with calcein AM (Life Technologies) after a 30-minute incubation in the dark were washed twice in media to remove excess calcein AM. Each filter pore had $4x10^4$ cells (50μL) placed above the wells containing chemoattractant (10% FBS) and media. Cells that successfully migrate or invade into the wells below after a 6-hour incubation were measured at 485-520nm on the Triad series multimode detector (Dynex Technologies). Assays were replicated to a total of n=36-42 per construct transfected.

Results

PIWIL genes have low expression in prostate cancer tumours

To identify the expression profile of direct interactors of piRNAs, we analysed the mRNA expression of the *PIWIL* genes in normal, benign and PCa samples across four independent studies: Fluidigm qRT-PCR (our study), and transcriptome studies from Ding, Wu (129) (GSE89194), Grasso, Wu (130) (GSE35988) and TCGA. *PIWIL1* was observed to have lower expression in cancer compared to benign and normal samples in both Fluidigm and GSE89194 studies (Fig. 1a,b). Interestingly, *PIWIL2* and *PIWIL3* had no expression in benign and a majority of tumour samples in the Fluidigm study (Fig. 1c,g). While *PIWIL2* had lower expression between normal and cancer samples across other datasets (GSE89194, GSE35988 and TCGA), only the GSE89194 ($p=0.0139$) and TCGA ($p<0.0001$) datasets showed this as a significant change (Fig. 1d-f). *PIWIL3* had significantly lower expression in cancer compared to normal samples ($p=0.0209$) in the GSE89194 study (Fig. 1h). Similar to *PIWIL1*, *PIWIL4* had lower expression between benign and cancer tumours in the Fluidigm study (Fig. 1i). *PIWIL4* expression was similar in normal and cancer samples for GSE89194 and GSE35988 but significantly lower in the TCGA study ($p<0.0001$) (Fig. 1j-l). Of note, there was no difference in *PIWIL* expression between PCa samples with low and high Gleason score in the Fluidigm study however, a change in expression was observed to occur between benign and early stage tissues (Fig. 1a,c,g,i).

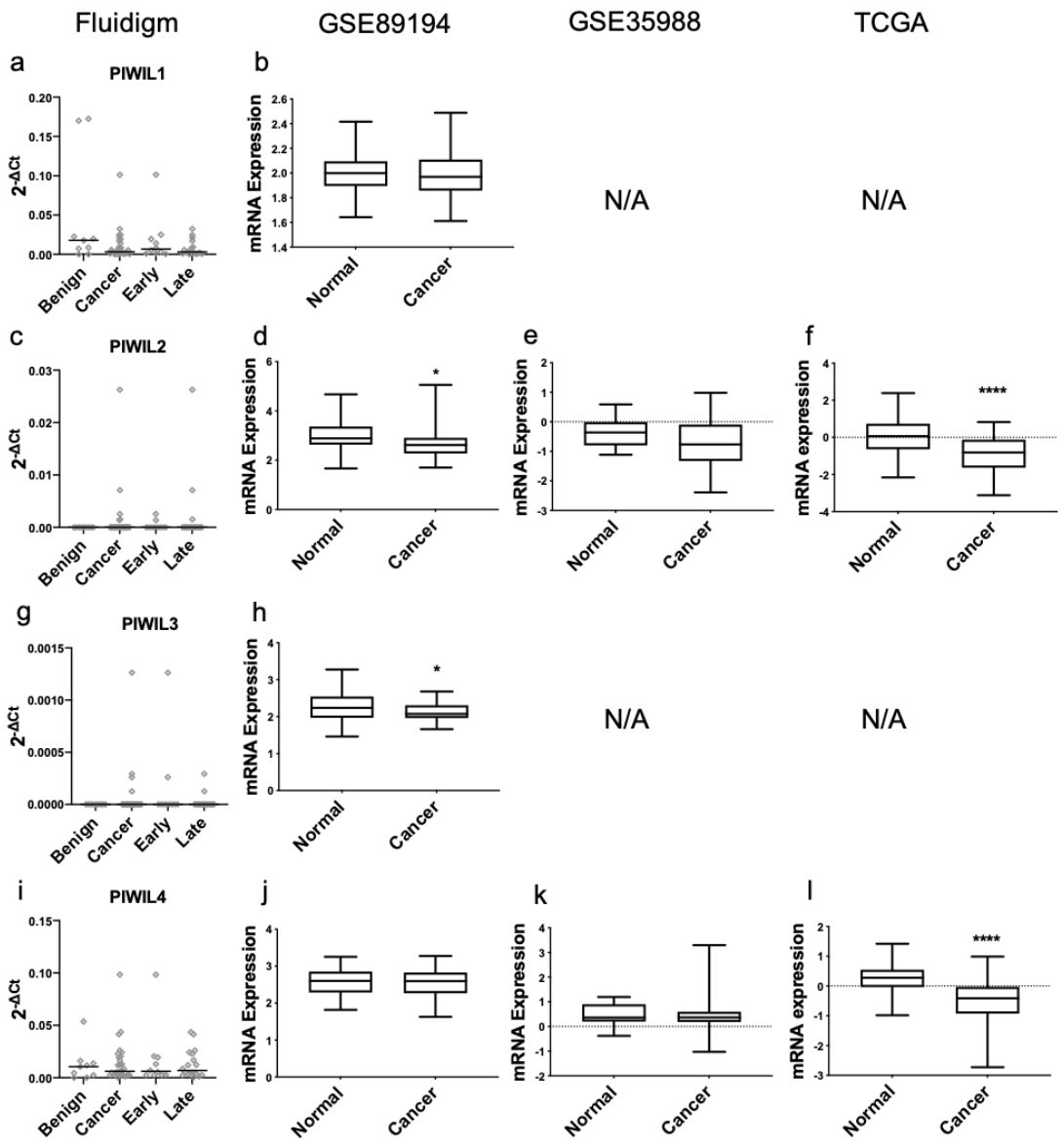


Fig. 1 Analysis of *PIWIL* expression in normal, benign and prostate cancer samples across four independent datasets. **a,c,g,i** In the Fluidigm study, prostate cancer tumors with Gleason score of 3+4 were classified as early stage while 4+3, 4+4, 4+5 and 5+4 were classified as late stage. Cancer consists of both early and late stage samples. A median line was used to plot each group in the Fluidigm study. **b,d-f,h,j-l** GSE89194, GSE35988 and TCGA are publicly available transcriptome datasets. N/A represents genes that were not detected. *p<0.05; ****p<0.0001.

Accessory piRNA pathway genes are differentially expressed in normal, benign and malignant prostate tissues

To determine if accessory piRNA pathway genes also have deregulated expression in PCa, we expanded our study to include a further 6 piRNA pathway genes. We found significantly increased *DDX4* expression in PCa tumours compared to benign tissue (p=0.0364) in the Fluidigm study. While the GSE89194 study (Fig. 2b) showed significantly increased expression of *DDX4* in cancer samples (p=0.0093), there was no significant difference in expression for the GSE35988 study (Fig. 2c). *HENMT1* expression was significantly higher in both early (p=0.0045) and late (p=0.0131) stage PCa tumours as well as all cancer samples (p=0.0011) compared to benign samples in the Fluidigm study (Fig. 2d). For *HENMT1*, we observed a more tightly distributed expression among the benign samples as compared to cancerous tumours, likely due to the heterogeneous nature of PCa samples (Fig. 2d). Although *MAEL* had similar expression between normal, benign and cancer tissues in the Fluidigm (Fig. 2e) and GSE89194 (Fig. 2f) studies, the GSE35988 study showed significantly decreased *MAEL* expression in PCa tumours compared to normal tissue (p=0.0046) (Fig. 2g). *PLD6* expression was higher in cancer tumours compared to benign samples in the Fluidigm study (Fig. 2h) but had similar expression in the GSE89194 study (Fig. 2i). The TCGA study on the other hand, showed significantly increased *PLD6* expression (p<0.0001) in cancer samples (Fig. 2j). *TDRD1* is the most studied piRNA pathway gene in PCa and has been shown to be expressed in prostate tumours but not in adjacent normal prostate tissue (89). Indeed, we observed *TDRD1* expression in prostate tumours while only half of the benign samples

expressed *TDRD1* in the Fluidigm study (Fig. 2k). Similar to *HENMT1*, *TDRD1* expression, was significantly higher in prostate tumours (p=0.0007) as well as in both early (p=0.0092) and late (p=0.0128) stage cancer samples compared to benign samples (Fig. 2k). Interestingly, the two late stage PCa tumours which had the highest expression of *TDRD1* were the only ones with a primary Gleason score of 5 (Fig. 2k). Consistently, *TDRD1* expression was significantly upregulated in PCa tumours of the GSE89194 (p<0.0001), GSE35988 (p=0.0047) and TCGA (p<0.0001) studies (Fig. 2l-n). Most benign samples showed no *TDRD9* expression but it was expressed in PCa tumours (Fig. 2o). Both GSE89194 and GSE35988 studies showed similar *TDRD9* expression across normal and PCa tissues (Fig. 2p,q). Notably, the difference in expression of *DDX4*, *HENMT1*, *PLD6* and *TDRD1* occurred only between benign and early stage tissues in the Fluidigm study.

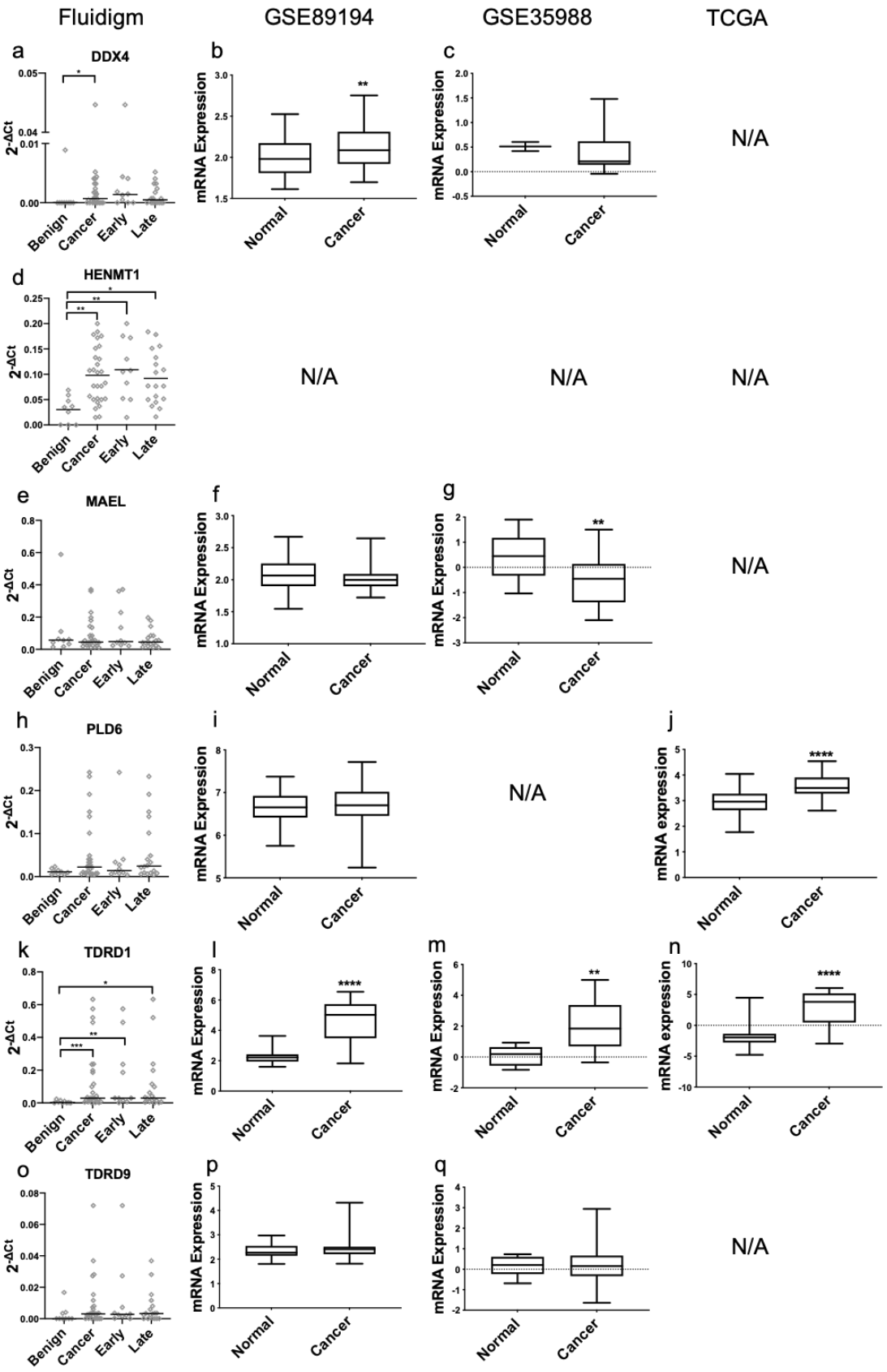
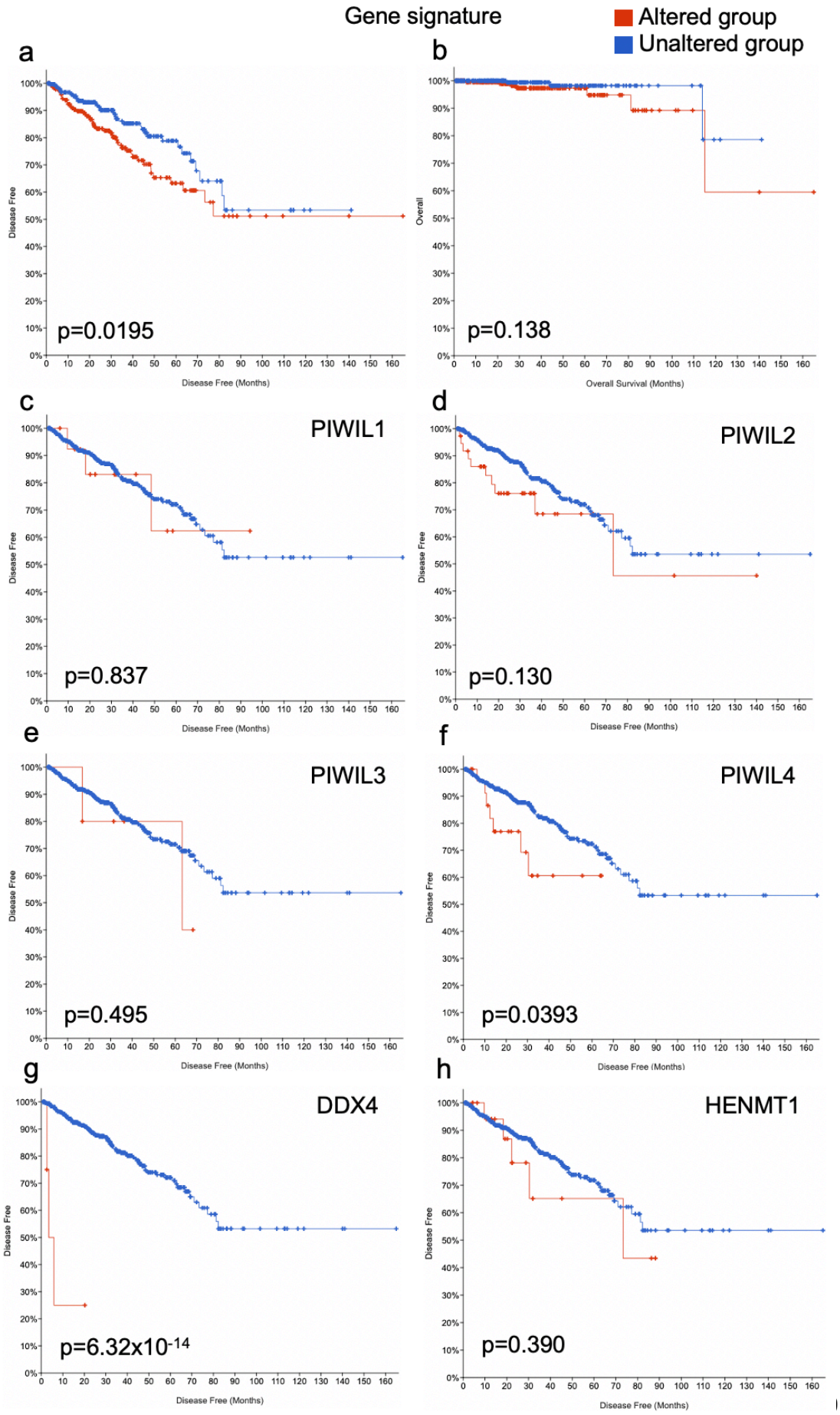

Fluidigm
GSE89194
GSE35988
TCGA
a
DDX4
b
c
N/A
d
HENMT1
N/A
N/A
N/A
e
MAEL
f
g
N/A
h
PLD6
i
N/A
j
k
TDRD1
l
m
n
o
TDRD9
p
q
N/A
2-ΔCt
mRNA Expression
mRNA expression
Benign
Cancer
Early
Late
Normal
Cancer

Fig. 2 piRNA pathway gene expression profile in normal, benign and prostate cancer samples across four datasets. Expression of these genes were analysed through our Fluidigm study and publicly available transcriptome datasets, GSE89194, GSE35988 and TCGA. Cancer consists of both early and late stage samples. A median line was used to plot each group in the Fluidigm study. N/A represents genes that were not detected. N/A represents genes that were not detected. *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001.

Altered piRNA pathway gene expression associates with poorer disease-free survival in prostate cancer patients

To further assess the clinical relevance of aberrant piRNA pathway gene expression in PCa, we investigated the effects of altered and unaltered piRNA pathway genes in the disease-free and overall survival of PCa patients. Using a piRNA pathway gene signature of *PIWIL1-4*, *DDX4*, *HENMT1*, *MAEL*, *PLD6*, *TDRD1* and *TDRD9*, we showed that aberrant expression of this gene signature was significantly associated with worse disease-free survival in the TCGA dataset (p=0.0195) (Fig. 3a) while there was no significant difference in overall survival of PCa patients (Fig. 3b). Looking at individual genes, altered expression of *PIWIL1-3* had no significant effect on the disease-free survival of PCa patients (Fig. 3c-e). However, altered *PIWIL4* expression showed significantly poorer disease-free survival (p=0.0393) (Fig. 3f). Subsequently, among the 6 piRNA pathway genes, only aberrant expression of *DDX4* ($p=6.32x10^{-14}$) and *TDRD1* ($p=6.971x10^{-3}$) were observed to significantly associate with worse disease-free survival (Fig. 3g-l). This demonstrates that altered piRNA pathway gene expression is associated with disease-free survival outcomes of PCa patients.


Gene signature
Altered group
Unaltered group
a
b
c
d
e
f
g
h
Disease Free
Overall
Disease Free (Months)
Overall Survival (Months)
p=0.0195
p=0.138
PIWIL1
p=0.837
PIWIL2
p=0.130
PIWIL3
p=0.495
PIWIL4
p=0.0393
DDX4
p=6.32x10-14
HENMT1
p=0.390

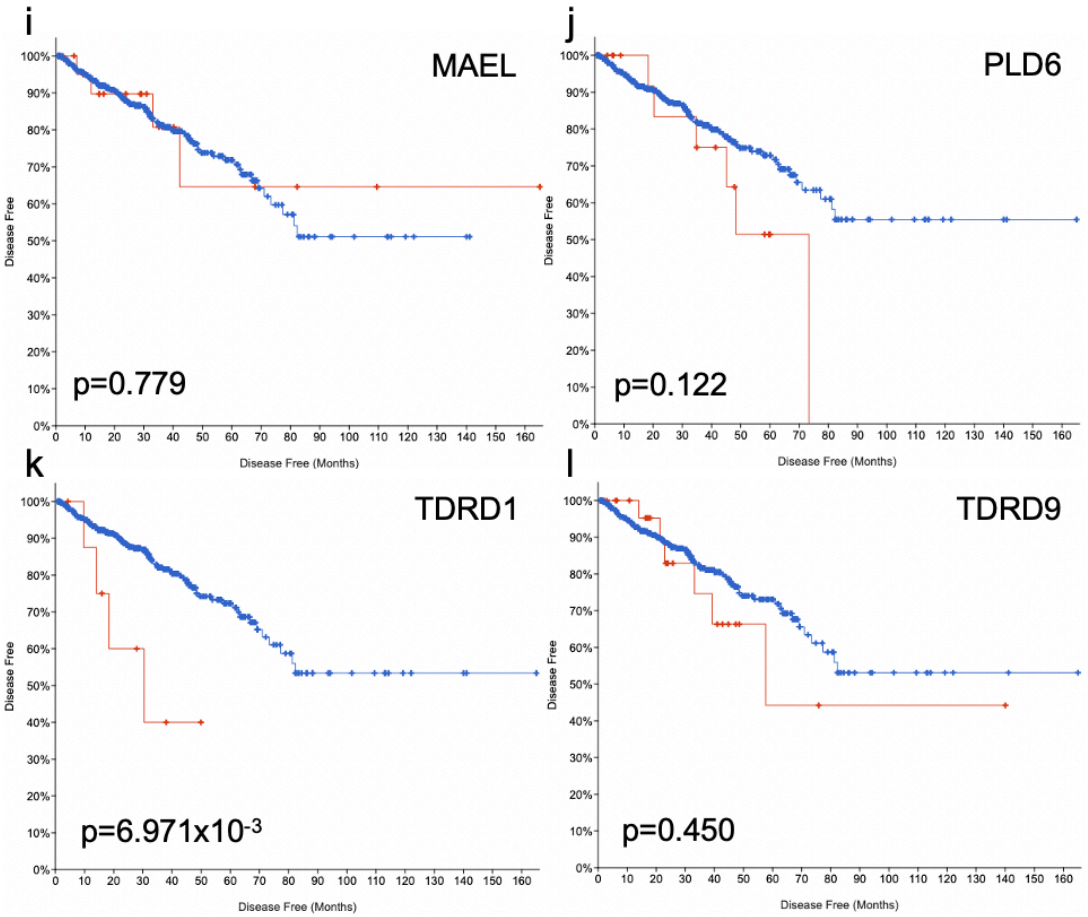


Fig. 3 Effects of unaltered and altered piRNA pathway genes on prostate cancer patient survival. cBioPortal analysis comparing a piRNA pathway gene signature consisting of *PIWIL1-4*, *DDX4*, *HENMT1*, *MAEL*, *PLD6*, *TDRD1* and *TDRD9* in (**a**) disease-free and (**b**) overall survival. (**c-l**) Disease-free survival analysis of individual genes. Altered group consists of aberrant expression of any piRNA pathway gene. $p<0.05$ represents significant difference.

Comparison of piRNA pathway gene expression in non-relapsed (control) and relapsed prostate cancer patients

Next, we investigated if piRNA pathway gene expression associates with relapse of PCa patients. The *PIWIL* genes, *PIWIL1-4*, showed similar expression between tumours of control and relapsed patients (Fig. 4a-d). We

identified trends in expression for the remaining genes however, they did not reach significance. *DDX4* had slightly increased expression in tumours of patients that have relapsed while *HENMT1* showed decreased expression (Fig. 4e,f). As observed with the *PIWIL* genes, *MAEL* had similar expression between tumours of control and relapsed patients (Fig. 4g). While *PLD6* showed increased expression in tumours of relapsed patients (Fig. 4h), *TDRD1* and *TDRD9* had similar expression across tumours of control and relapsed patients (Fig. 4i-j).

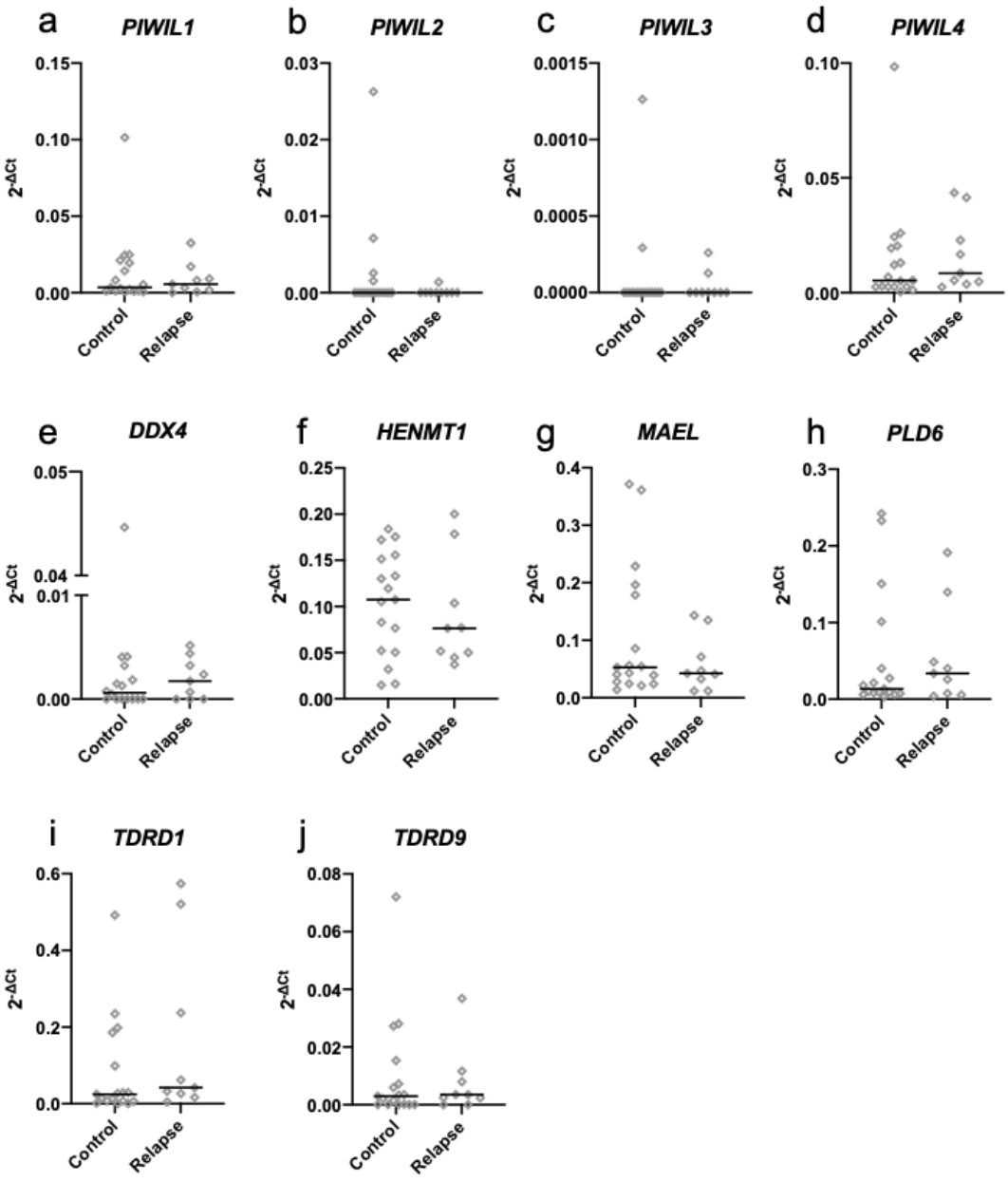


Fig. 4 piRNA pathway gene expression in non-relapsed (control) and relapsed prostate cancer patients. Prostate cancer tumours from the Fluidigm study categorised as control (n=17) and relapse (n=9) based on clinical information obtained from the Australian Prostate Cancer BioResource. Patients are only classified as control after a minimum of 5 years without relapse. Median was plotted for all control and relapse groups.

piRNA pathway genes have different trends of expression in prostate cancer cells after enzalutamide treatment and androgen receptor knockdown

To test the effects of inhibiting AR activity or expression on piRNA pathway genes, we treated LNCaP cells with different concentrations of enzalutamide or knocked down AR expression using siRNA. Reduced AR activity (indicated by decreased PSA levels) for both treatments and almost a complete knockdown of AR by siRNA was confirmed through Western blot (Fig. S1). From our transcriptomic analysis of the 10 piRNA pathway genes, only *HENMT1*, *PLD6* and *TDRD1* expression were of detectable levels in LNCaP cells. We found different trends in expression for these genes, albeit without statistical significance. Compared to the control treatment (DMSO), *HENMT1* had no change in expression when LNCaP cells were treated with 1μM enzalutamide but had an increasing trend in expression with 10μM treatment (Fig. 5a). Knockdown of AR had no effect on *HENMT1* expression (Fig. 5a). *PLD6* on the other hand, showed considerable variation in expression for the control replicates (Fig. 5b). While *PLD6* showed a slight increase in expression in 1μM enzalutamide treated cells, its expression from the 10μM enzalutamide treatment was similar to the control treatment (Fig. 5b). There was a trend of increased *PLD6* expression observed in response to AR knockdown (Fig. 5b). *TDRD1* expression showed a consistent decreasing trend in expression after either 1μM or 10μM enzalutamide treatment (Fig. 5c). *TDRD1* also had a non-significant decrease in expression after AR knockdown (Fig. 5c).

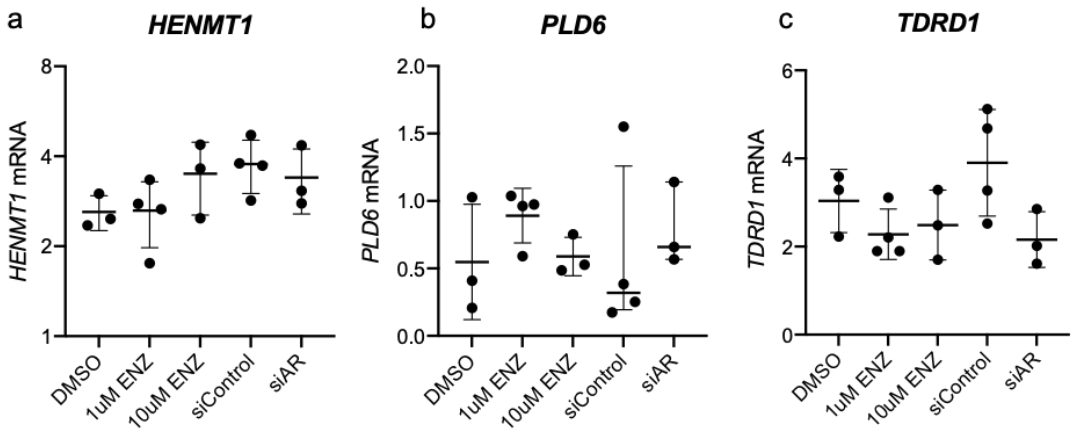


Fig. 5 Transcriptome analysis of piRNA pathway genes after enzalutamide treatment and AR knockdown in LNCaP cells (n=3-4 per group). DMSO and siControl are treatment and transfection controls respectively. ENZ represents enzalutamide treatment. Mean with standard deviation was plotted for all groups except siControl and siAR for *PLD6* where these two groups were plotted using median with interquartile range.

Overexpression of piRNA pathway genes decreased motility and invasion of 22RV1 cells

The piRNA pathway genes *PIWIL1*, *PIWIL2* and *MAEL* are known for having roles in fundamental processes associated with malignancy including EMT and migration which have been reported in a variety of cancers (45, 98, 108). Here, *in vitro* motility and invasion assays were carried out using a CRPC cell line, 22RV1, which harbours the prominent AR-V7 mutant commonly found in CRPC patient tumours (133, 134). There was an overall trend of decreased motility (Fig. 6a) and invasion (Fig. 6b) after overexpression of *PIWIL1*, *PIWIL2* and *MAEL* compared to empty vector. However, only *PIWIL2* (motility $p=0.0003$; invasion $p=0.001$) and *MAEL* (motility $p<0.0001$; invasion $p<0.0001$) were significant.

Next, we investigated if the overexpression of *PIWIL1* and *PIWIL2* mutants amplified the effects of their wildtype counterpart (43, 46). Therefore, we overexpressed them in CRPC cells and compared their effects in relation to the empty vector control as well as their respective wildtype form. We observed that cells overexpressing *PIWIL1* mutant had a slight decrease in motility but similar invasion when compared to the empty vector control. Overexpression of *PIWIL2* mutant significantly decreased motility and invasion of CRPC cells compared to empty vector (motility p=0.03; invasion p=0.0003) (Fig. 6a,b). Surprisingly, cells overexpressing *PIWIL2* mutant had significantly higher motility (p=0.04) compared to *PIWIL2* (Fig. 6a).

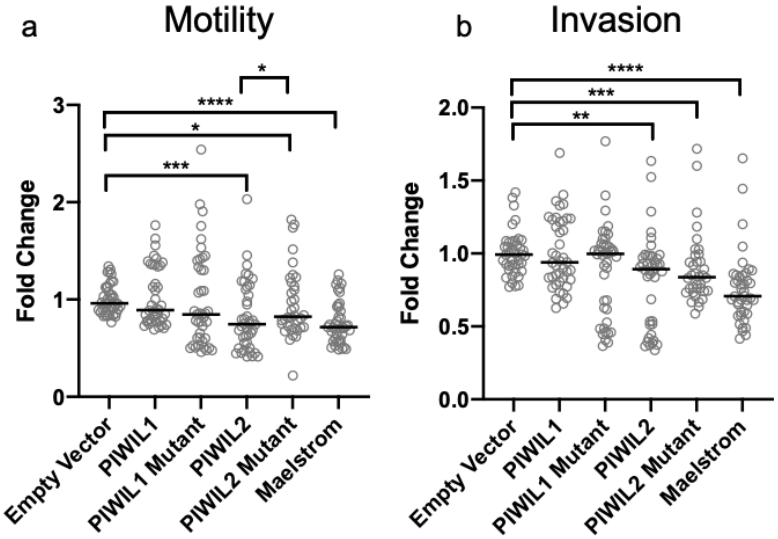


Fig. 6 Motility and invasion of CRPC cell line, 22RV1 after overexpression of piRNA pathway genes. Fold change of *in vitro* (**a**) motility and (**b**) invasion of 22RV1 overexpressing piRNA pathway genes were measured relative to empty vector. Motility and invasion of cells overexpressing mutant genes were also compared to their respective wildtype gene. A median line was plotted for each group analysed. *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001.

Discussion

A rapidly growing body of work supports an important role of the piRNA pathway in cancer development and progression. However, very limited work has been done on piRNA pathway genes in PCa. Our investigation in PCa provided a first look into the expression profile of a set of 10 piRNA pathway genes across normal, benign and PCa samples and links to prognosis of PCa patients. Furthermore, we showed trends in piRNA pathway gene expression between tumours of patients with and without relapse where a common set of genes was also affected by reduced AR activity. Overexpression of wildtype and mutant piRNA pathway genes decreased motility and invasion of CRPC cells *in vitro*.

With only few previous reports of piRNA pathway gene expression (*PIWIL2* and *TDRD1*) in PCa tumours, we characterised the expression of 10 piRNA pathway genes including *PIWIL2* and *TDRD1* (89, 90, 135, 136). It was noted that some genes were not available in certain transcriptome datasets likely due to low abundance of the gene and hence, they were below the limit of detection. This discrepancy in transcriptomic detection may also be attributed to the heterogeneity of PCa tumours and contribution from different ethnic groups with varying genetic profiles. Therefore, the Fluidigm study, using qRT-PCR, provided another form of validation for the quantitative expression of these genes in benign and PCa tissues where we showed little to no expression of *PIWIL2* and *PIWIL3*. Despite the different techniques used and variability in samples, we observed similar trends in expression across these studies (Fluidigm, GSE89194, GSE35988, TCGA). Based on the four

studies, an overall lowered expression was observed for the *PIWIL* genes between normal and benign to cancer. This was surprising as higher *PIWIL* gene expression in other cancers has predominantly been associated with poorer prognosis and malignancy (70, 137, 138). However, low PIWIL4 protein expression has been linked with poor prognosis in pancreatic cancer (139). Therefore, it is possible that *PIWIL* gene expression is different in different cancers.

With the exception of *MAEL* also having lowered expression, *DDX4*, *HENMT1*, *PLD6*, *TDRD1* and *TDRD9* had higher expression when comparing normal or benign to cancer samples. A possible explanation for low *PIWIL* and *MAEL* expression is their role in maintaining genomic stability through the biogenesis of piRNAs and repression of TEs (17, 25, 140). It was shown in PCa that *PIWIL1* and *PIWIL2* overexpression was able to reduce the fusion formation and expression of *TMPRSS2:ERG* likely through suppression of LINE-1 *ORF2* expression (141). Thus, a decrease in *PIWIL* and *MAEL* expression could potentially contribute to the formation of more oncogenic *TMPRSS2:ERG* fusion events. *TDRD1* on the other hand was shown to have no influence on LINE-1 *ORF2* expression and in addition, been proposed to not play a role in the proliferation of *TMPRSS2:ERG* positive PCa cells (136). The *TMPRSS2:ERG* fusion is known to happen as an early event and is prevalent (~50% frequency) in PCa and CRPC tumours (92, 120, 121, 142). ERG regulation of *TDRD1* promoter suggests that changes in *TDRD1* expression could be an early event in PCa (135, 136). While *TDRD1* might not have proliferative function for PCa cells, it may contribute to other hallmarks of cancer such as cell invasion or reprogramming cellular metabolism (136, 143).

We acknowledge that our current study has a limitation whereby it does not contain the fusion status of clinical samples used. Association of exceptionally high *TDRD1* expression to tumours with a primary Gleason score of 5 may inform future work looking into markers for aggressive PCa to improve patient management. Like *TDRD1*, changes in *DDX4*, *HENMT1*, *PLD6* and *TDRD9* expression may also contribute to early PCa progression. This was demonstrated by their change in expression occurring between benign and early stage PCa tumours in the Fluidigm study. As benign samples could become precursor lesions to PCa tumours, changes in expression from benign samples to early stage tumours are indicative of a possible role of these piRNA pathway genes in PCa development (144, 145).

Next, we assessed if altered piRNA pathway gene expression affected the prognosis of PCa patients. Using a gene signature of the 10 piRNA pathway genes (*PIWIL1-4*, *DDX4*, *HENMT1*, *MAEL*, *PLD6*, *TDRD1* and *TDRD9*), we demonstrated that their altered expression significantly associated with shorter disease-free survival but had no effect on overall survival. Analysing the effects of individual genes revealed that changes in *PIWIL4*, *DDX4* and *TDRD1* expression significantly reduced disease-free survival of PCa patients. While survival analysis of altered piRNA pathway gene expression has been done in other cancers, there have been no studies in PCa. As seen in glioma and kidney cancer, altered expression of *PIWIL1*, *PIWIL2* and *PIWIL4* was linked to poorer prognosis and have been proposed to be prognostic markers (109, 146). Therefore, the association of altered piRNA pathway gene expression with PCa patient prognosis requires further development to explore them as prognostic markers for PCa.

Despite ADT being an effective treatment, a proportion of PCa patients do relapse and develop the lethal CRPC. Cai, Wang (121) discovered that although ADT downregulates *TMPRSS2:ERG* expression, reactivation of AR in CRPC restored its expression. With links of the *TMPRSS2:ERG* fusion with piRNA pathway genes (*PIWIL1*, *PIWIL2* and *TDRD1*), we explored their potential involvement in the relapse of PCa patients (89, 135, 136, 141). Although *PIWIL1-4*, *MAEL*, *TDRD1* and *TDRD9* showed similar expression between tumours of non-relapsed and relapsed patients, we observed changes in *DDX4*, *HENMT1* and *PLD6* expression. The highly conserved germ cell factor *DDX4* has been found in leukaemia, myeloma and ovarian cancer to be involved in cancer cell cycle regulation, proliferation and migration (147, 148). There are currently no studies of phospholipase *PLD6* and methyltransferase *HENMT1* in cancer. Our results suggest a possible involvement of *DDX4*, *HENMT1* and *PLD6* in CRPC progression which warrants further investigation.

Androgen has been shown to affect piRNA expression in the androgen dependent PCa cell line, LNCaP (88). Here, we investigated the possible effects of reducing AR signalling on piRNA pathway gene expression in LNCaP cells. We identified different trends in expression where *HENMT1* increased after a higher dose (10μM) of enzalutamide treatment, *PLD6* increased after AR knockdown and *TDRD1* decreased after both doses (1μM and 10μM) of enzalutamide treatment and AR knockdown. It is unknown how reduced AR activity can affect *HENMT1* and *PLD6* hence, this could provide a connection to their changed expression between control and relapsed patients

shown in this study. A reduction in *TDRD1* expression from decreased AR activity may be from its regulation by ERG where ERG has known interactions with AR in PCa (135, 149). Therefore, it would be interesting to explore these genes in relation to CRPC where aberrant AR activity frequently occurs.

We then investigated if overexpressing *PIWIL1*, *PIWIL2* and *MAEL in vitro* would have a negative effect on CRPC cell line, 22RV1. Here, we showed that overexpression of *PIWIL2* and *MAEL* decreased motility and invasion of 22RV1 cells. Our results contrast what was seen previously where knockdown of *PIWIL2* in CRPC cell line, PC-3 decreased migration and invasion (90). However, this could be due to the use of different cell lines as PC-3 cells do not harbour any AR where else, 22RV1 cells express AR variants (84, 118, 150). Interestingly, 22RV1 cells express AR variants which harbours intragenic rearrangements containing insertion of the TE, LINE-1 (118, 134). *MAEL* is known to be essential in TE repression through the piRNA pathway (25, 140). Decreased motility and invasion of 22RV1 cells overexpressing *MAEL* could be contributed by its role in maintaining genomic stability. It has been shown previously that MAEL depletion increased expression and stability of TEs in cervical, breast, liver and bone cancer cell lines which may be related to its role in the piRNA pathway (113). Hence, the overexpression of *MAEL* could protect the genomic integrity of CRPC cells and prevent the formation of oncogenic mutations such as the *TMPRSS2:ERG* fusion possibly through LINE-1 suppression as seen previously with *PIWIL1* and *PIWIL2* (141).

We also assayed the overexpression of mutants *PIWIL1* and *PIWIL2* in 22RV1 cells. Interestingly, cells overexpressing *PIWIL2* mutant had increased

motility compared to cells overexpressing *PIWIL2*, almost restoring the original phenotype of 22RV1 cells. This could be attributed to its truncated PAZ domain which is likely to affect binding of piRNAs (46, 151). The PIWIL2 mutant may have a loss of function effect, impairing the piRNA pathway, potentially increasing genomic instability where chromosomal translocations such as the *TMPRSS2:ERG* fusion may be more prevalent as TEs could be repressed less efficiently.

Conclusions

The role of the piRNA pathway in PCa and CRPC is still largely unknown. Our analysis of the piRNA pathway genes revealed marked differences in expression and function in PCa and CRPC. Here, the expression study in PCa contrasts what is observed in other cancers suggesting that the piRNA pathway genes may have cancer-specific expression profiles. With a prior study demonstrating links between *PIWIL1* and *PIWIL2* with *TMPRSS2:ERG* fusion, we proposed that the piRNA pathway genes may have protective effects on PCa through TE suppression. This was supported by altered piRNA pathway gene expression associating with shorter disease-free survival. We also demonstrated piRNA pathway genes potentially being involved in the relapse of PCa patients and affected by AR activity. We suggested a connection between decreased motility and invasion of CRPC cells overexpressing *PIWIL2*, *PIWIL2* mutant and *MAEL* with their roles in maintaining genomic stability. More importantly, the increase in motility of cells overexpressing *PIWIL2* mutant may be attributed to impaired binding of

piRNAs raising questions of how they may affect piRNA biogenesis and TE repression. Therefore, this work highlights potential cancer-specific aspects of the piRNA pathway genes and links to their protective role in PCa and CRPC through TE repression.

Abbreviations: ADT: androgen deprivation therapy; AR: androgen receptor; CRPC: castrate resistant prostate cancer; DDX4: DEAD-Box helicase 4; DMSO: dimethyl sulfoxide; EMT: epithelial to mesenchymal transition; ENZ: enzalutamide; ERG: ETS-related gene; FBS: fetal bovine serum; HENMT1: HEN methyltransferase 1; LINE-1: long-interspersed nuclear element-1; MAEL: maelstrom; PCa: prostate cancer; PIWI-interacting RNA: piRNA; PIWIL: P-element induced wimpy testis-like; PLD6: phospholipase D family member 6; PSA: prostate specific antigen; TCGA: The Cancer Genome Atlas; TDRD1/9: tudor domain containing protein 1/9; TE: transposable elements TMPRSS2: transmembrane serine protease 2.

Declarations

Ethics approval and consent to participate: Ethics approval was obtained from the Human Research Ethics Committees of the University of Adelaide (H-2018-222) and samples were collected by the St Andrew's Hospital.

Consent for publication: Written consent for patient tissues was obtained through the Australian Prostate Cancer BioResource.

Availability of data and materials: All data generated or analysed during this study are included in this published article.

Competing Interests: The authors declare no conflicts of interest.

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Authors' contributions: EL and FG conceived the study, LMB provided cell lines, tumour samples and RNA sequencing data, EL and NR performed experiments, EL and CYM analysed the data, EL and CYM carried out statistical analysis with participation from NAL and CR, EL wrote the manuscript, EL, NAL, CR, LMB and FGR revised the manuscript, LMB and FGR supervised the project. All authors read and approved the final manuscript.

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References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians. 2018;68(6):394-424.
2. Xu Y, Chen S-Y, Ross KN, Balk SP. Androgens induce prostate cancer cell proliferation through mammalian target of rapamycin activation and post-transcriptional increases in cyclin D proteins. Cancer Research. 2006;66(15):7783-92.
3. Wang Q, Li W, Liu XS, Carroll JS, Jänne OA, Keeton EK, et al. A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. Molecular Cell. 2007;27(3):380-92.
4. Holzbeierlein J, Lal P, LaTulippe E, Smith A, Satagopan J, Zhang L, et al. Gene expression analysis of human prostate carcinoma during hormonal therapy identifies androgen-responsive genes and mechanisms of therapy resistance. The American Journal of Pathology. 2004;164(1):217-27.
5. Harris WP, Mostaghel EA, Nelson PS, Montgomery B. Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion. Nature Clinical Practice Urology. 2009;6(2):76-85.
6. Karantanos T, Corn PG, Thompson TC. Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate-resistance and novel therapeutic approaches. Oncogene. 2013;32(49):5501.
7. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nature Reviews Cancer. 2001;1(1):34-45.
8. Watson PA, Arora VK, Sawyers CL. Emerging mechanisms of resistance to androgen receptor inhibitors in prostate cancer. Nature Reviews Cancer. 2015;15(12):701.
9. Dehm SM, Schmidt LJ, Heemers HV, Vessella RL, Tindall DJ. Splicing of a novel androgen receptor exon generates a constitutively active androgen

receptor that mediates prostate cancer therapy resistance. Cancer Research. 2008;68(13):5469-77.
10. Linder S, van der Poel HG, Bergman AM, Zwart W, Prekovic S. Enzalutamide therapy for advanced prostate cancer: efficacy, resistance and beyond. Endocrine-Related Cancer. 2019;26(1):R31-R52.
11. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun X-W, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science. 2005;310(5748):644-8.
12. Holcomb IN, Young JM, Coleman IM, Salari K, Grove DI, Hsu L, et al. Comparative analyses of chromosome alterations in soft-tissue metastases within and across patients with castration-resistant prostate cancer. Cancer Research. 2009;69(19):7793-802.
13. Cai C, Wang H, Xu Y, Chen S, Balk SP. Reactivation of Androgen Receptor–Regulated TMPRSS2: ERG Gene Expression in Castration-Resistant Prostate Cancer. Cancer Research. 2009;69(15):6027-32.
14. Kron KJ, Murison A, Zhou S, Huang V, Yamaguchi TN, Shiah Y-J, et al. TMPRSS2–ERG fusion co-opts master transcription factors and activates NOTCH signaling in primary prostate cancer. Nature Genetics. 2017;49(9):1336.
15. Brase JC, Johannes M, Mannsperger H, Fälth M, Metzger J, Kacprzyk LA, et al. TMPRSS2-ERG-specific transcriptional modulation is associated with prostate cancer biomarkers and TGF-β signaling. BMC Cancer. 2011;11(1):1-8.
16. Hafeez BB, Adhami VM, Asim M, Siddiqui IA, Bhat KM, Zhong W, et al. Targeted knockdown of Notch1 inhibits invasion of human prostate cancer cells concomitant with inhibition of matrix metalloproteinase-9 and urokinase plasminogen activator. Clinical Cancer Research. 2009;15(2):452-9.
17. Gupta S, Iljin K, Sara H, Mpindi JP, Mirtti T, Vainio P, et al. FZD4 as a mediator of ERG oncogene–induced WNT signaling and epithelial-to-mesenchymal transition in human prostate cancer cells. Cancer Research. 2010;70(17):6735-45.
18. Babatunde KA, Najafi A, Salehipour P, Modarressi MH, Mobasheri MB. Cancer/Testis genes in relation to sperm biology and function. Iran J Basic Med Sci. 2017;20(9):967-74.
19. Reuter M, Chuma S, Tanaka T, Franz T, Stark A, Pillai RS. Loss of the Mili-interacting Tudor domain–containing protein-1 activates transposons and alters the Mili-associated small RNA profile. Nature Structural & Molecular Biology. 2009;16(6):639.
20. Mathioudakis N, Palencia A, Kadlec J, Round A, Tripsianes K, Sattler M, et al. The multiple Tudor domain-containing protein TDRD1 is a molecular scaffold for mouse Piwi proteins and piRNA biogenesis factors. RNA 2012;18(11):2056-72.
21. Czech B, Munafò M, Ciabrelli F, Eastwood EL, Fabry MH, Kneuss E, et al. piRNA-guided genome defense: from biogenesis to silencing. Annual Review of Genetics. 2018;52:131-57.
22. Soper SFC, van der Heijden GW, Hardiman TC, Goodheart M, Martin SL, de Boer P, et al. Mouse Maelstrom, a component of nuage, is essential for spermatogenesis and transposon repression in meiosis. Developmental Cell. 2008;15(2):285-97.

23. Kuramochi-Miyagawa S, Watanabe T, Gotoh K, Takamatsu K, Chuma S, Kojima-Kita K, et al. MVH in piRNA processing and gene silencing of retrotransposons. Genes & Development. 2010;24(9):887-92.
24. Liu Y, Dou M, Song X, Dong Y, Liu S, Liu H, et al. The emerging role of the piRNA/piwi complex in cancer. Molecular Cancer. 2019;18(1):123.
25. Liu L, Dai Y, Chen J, Zeng T, Li Y, Chen L, et al. Maelstrom promotes hepatocellular carcinoma metastasis by inducing epithelial‐mesenchymal transition by way of Akt/GSK‐3β/Snail signaling. Hepatology. 2014;59(2):531-43.
26. Jiang S, Zhao L, Lu Y, Wang M, Chen Y, Tao D, et al. Piwil2 inhibits keratin 8 degradation through promoting p38-induced phosphorylation to resist Fas-mediated apoptosis. Molecular and Cellular Biology. 2014;34(21):3928-38.
27. Guijo M, Ceballos-Chávez M, Gómez-Marín E, Basurto-Cayuela L, Reyes JC. Expression of TDRD9 in a subset of lung carcinomas by CpG island hypomethylation protects from DNA damage. Oncotarget. 2017;9(11):9618-31.
28. Ye Y, Yin D-T, Chen L, Zhou Q, Shen R, He G, et al. Identification of Piwil2-Like (PL2L) Proteins that Promote Tumorigenesis. PLOS One. 2010;5(10):e13406.
29. Lim SL, Ricciardelli C, Oehler MK, De Arao Tan IMD, Russell D, Grützner F. Overexpression of piRNA Pathway Genes in Epithelial Ovarian Cancer. PLOS One. 2014;9(6):e99687.
30. Ding Y, Wu H, Warden C, Steele L, Liu X, Iterson Mv, et al. Gene expression differences in prostate cancers between young and old men. PLOS Genetics. 2016;12(12):e1006477.
31. Grasso CS, Wu Y-M, Robinson DR, Cao X, Dhanasekaran SM, Khan AP, et al. The mutational landscape of lethal castration-resistant prostate cancer. Nature. 2012;487(7406):239-43.
32. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. AACR; 2012.
33. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Science Signaling. 2013;6(269):pl1-pl.
34. Xiao L, Lanz RB, Frolov A, Castro PD, Zhang Z, Dong B, et al. The germ cell gene TDRD1 as an ERG target gene and a novel prostate cancer biomarker. The Prostate. 2016;76(14):1271-84.
35. Li C, Zhou X, Chen J, Lu Y, Sun Q, Tao D, et al. PIWIL1 destabilizes microtubule by suppressing phosphorylation at Ser16 and RLIM-mediated degradation of stathmin1. Oncotarget. 2015;6(29):27794-804.
36. Li D, Sun X, Yan D, Huang J, Luo Q, Tang H, et al. Piwil2 modulates the proliferation and metastasis of colon cancer via regulation of matrix metallopeptidase 9 transcriptional activity. Experimental Biology and Medicine. 2012;237(10):1231-40.
37. Guo Z, Yang X, Sun F, Jiang R, Linn DE, Chen H, et al. A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion–resistant growth. Cancer Research. 2009;69(6):2305-13.

38. Li Y, Alsagabi M, Fan D, Bova GS, Tewfik AH, Dehm SM. Intragenic rearrangement and altered RNA splicing of the androgen receptor in a cell-based model of prostate cancer progression. Cancer Research. 2011;71(6):2108-17.
39. Yang Y, Zhang X, Song D, Wei J. Piwil2 modulates the invasion and metastasis of prostate cancer by regulating the expression of matrix metalloproteinase-9 and epithelial-mesenchymal transitions. Oncology Letters. 2015;10(3):1735-40.
40. Boormans JL, Korsten H, Ziel‐van der Made AJ, van Leenders GJ, de Vos CV, Jenster G, et al. Identification of TDRD1 as a direct target gene of ERG in primary prostate cancer. International Journal of Cancer. 2013;133(2):335-45.
41. Kacprzyk LA, Laible M, Andrasiuk T, Brase JC, Börno ST, Fälth M, et al. ERG Induces Epigenetic Activation of Tudor Domain-Containing Protein 1 (TDRD1) in ERG Rearrangement-Positive Prostate Cancer. PLOS One. 2013;8(3):e59976.
42. Yang L, Bi L, Liu Q, Zhao M, Cao B, Li D, et al. Hiwi Promotes the Proliferation of Colorectal Cancer Cells via Upregulating Global DNA Methylation. Disease Markers. 2015;2015:10.
43. Zeng G, Zhang D, Liu X, Kang Q, Fu Y, Tang B, et al. Co-expression of Piwil2/Piwil4 in nucleus indicates poor prognosis of hepatocellular carcinoma. Oncotarget. 2017;8(3):4607.
44. Gambichler T, Kohsik C, Höh A-K, Lang K, Käfferlein HU, Brüning T, et al. Expression of PIWIL3 in primary and metastatic melanoma. Journal of Cancer Research and Clinical Oncology. 2017;143(3):433-7.
45. Li W, Martinez-Useros J, Garcia-Carbonero N, Fernandez-Aceñero MJ, Orta A, Ortega-Medina L, et al. The Clinical Significance of PIWIL3 and PIWIL4 Expression in Pancreatic Cancer. Journal of Clinical Medicine. 2020;9(5):1252.
46. Sienski G, Dönertas D, Brennecke J. Transcriptional Silencing of Transposons by Piwi and Maelstrom and Its Impact on Chromatin State and Gene Expression. Cell. 2012;151(5):964-80.
47. Lin C, Yang L, Tanasa B, Hutt K, Ju B-g, Ohgi KA, et al. Nuclear receptor-induced chromosomal proximity and DNA breaks underlie specific translocations in cancer. Cell. 2009;139(6):1069-83.
48. Perner S, Mosquera J-M, Demichelis F, Hofer MD, Paris PL, Simko J, et al. TMPRSS2-ERG fusion prostate cancer: an early molecular event associated with invasion. The American Journal of Surgical Pathology. 2007;31(6):882-8.
49. Hanahan D, Weinberg Robert A. Hallmarks of Cancer: The Next Generation. Cell. 2011;144(5):646-74.
50. Bostwick DG, Cheng L. Precursors of prostate cancer. Histopathology. 2012;60(1):4-27.
51. Furusato B, Gao C-L, Ravindranath L, Chen Y, Cullen J, McLeod DG, et al. Mapping of TMPRSS2–ERG fusions in the context of multi-focal prostate cancer. Modern Pathology. 2008;21(2):67-75.
52. Li J, Xu L, Bao Z, Xu P, Chang H, Wu J, et al. High expression of PIWIL2 promotes tumor cell proliferation, migration and predicts a poor prognosis in glioma. Oncology Reports. 2017;38(1):183-92.

53. Iliev R, Stanik M, Fedorko M, Poprach A, Vychytilova-Faltejskova P, Slaba K, et al. Decreased expression levels of PIWIL1, PIWIL2, and PIWIL4 are associated with worse survival in renal cell carcinoma patients. OncoTargets and Therapy. 2016;9:217.
54. Schudrowitz N, Takagi S, Wessel GM, Yajima M. Germline factor DDX 4 functions in blood‐derived cancer cell phenotypes. Cancer Science. 2017;108(8):1612-9.
55. Hashimoto H, Sudo T, Mikami Y, Otani M, Takano M, Tsuda H, et al. Germ cell specific protein VASA is over-expressed in epithelial ovarian cancer and disrupts DNA damage-induced G2 checkpoint. Gynecologic Oncology. 2008;111(2):312-9.
56. Öner Ç, Turgut Coşan D, Çolak E. Estrogen and Androgen Hormone Levels Modulate the Expression of PIWI Interacting RNA in Prostate and Breast Cancer. PLOS One. 2016;11(7):e0159044.
57. Adamo P, Ladomery M. The oncogene ERG: a key factor in prostate cancer. Oncogene. 2016;35(4):403-14.
58. Mitchell S, Abel P, Ware M, Stamp G, Lalani EN. Phenotypic and genotypic characterization of commonly used human prostatic cell lines. BJU International. 2000;85(7):932-44.
59. Kim S-H, Park E-R, Cho E, Jung W-H, Jeon J-Y, Joo H-Y, et al. Mael is essential for cancer cell survival and tumorigenesis through protection of genetic integrity. Oncotarget. 2017;8(3):5026-37.
60. Simon B, Kirkpatrick JP, Eckhardt S, Reuter M, Rocha EA, Andrade-Navarro MA, et al. Recognition of 2′-O-methylated 3′-end of piRNA by the PAZ domain of a Piwi protein. Structure. 2011;19(2):172-80.

Supplementary Figures

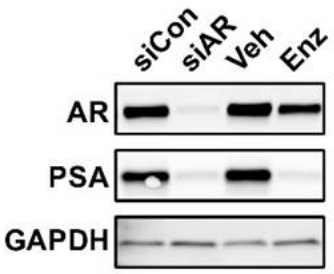

Fig. S1 Western blots showing AR and PSA levels after knockdown with siAR and enzalutamide (ENZ) treatment in LNCaP cells (unpublished, Butler Lab). siControl (siCon) and DMSO (Veh) were controls for the knockdown and treatment respectively.

Supplementary Tables

Table S1. Histopathological features of prostate tissues used in the Fluidigm qRT-PCR. Age and PSA levels were recorded when the patient underwent radical prostatectomy.

Benign prostate tissues (n=9)		
Age	Median (range)	71 (64–82)
PSA (ng/mL)	Median (range)	5.2 (2–170)
Prostate adenocarcinoma, early stage (n=10)		
Age	Median (range)	62 (51–75)
PSA (ng/mL)	Median (range)	8.8 (2.2–26)
Gleason score	Primary Secondary	3 4
Prostate adenocarcinoma, late stage (n=18)		
Age	Median (range)	64 (56–74)
PSA (ng/mL)	Median (range)	7.2 (2.2–14.5)
Gleason score	Primary Secondary	4, 5 3 – 5

Table S2. Taqman assays used in the Fluidigm qRT-PCR

Gene	Assay ID
PIWIL1	Hs01041737_m1
PIWIL2	Hs01032720_m1
PIWIL3	Hs00908825_m1
PIWIL4	Hs00381509_m1
DDX4	Hs00987125_m1
HENMT1	Hs00989130_m1

HPRT1	Hs02800695_m1
MAEL	Hs00262601_m1
PLD6	Hs00381651_m1
TDRD1	Hs00229805_m1
TDRD9	Hs00403678_m1

CHAPTER 4: GENERAL DISCUSSION

4.1 Conclusions

There is an increasing interest in the role of piRNA pathway genes in cancer and a growing body of work supports that this pathway plays a role in fundamental aspects of cancer development and progression (1, 2). However, differences in expression and function in various cancers suggest that piRNA pathway genes may have cancer-specific expression profiles and functions (3). Indeed, this was observed in our investigation looking into the expression profiles of a set of 10 piRNA pathway genes (*PIWIL1-4*, *DDX4*, *HENMT1*, *MAEL*, *PLD6*, *TDRD1* and *TDRD9*) in two understudied cancers in this area, ovarian (OC) and prostate cancer (PCa). Comparing our results in high grade serous OC (HGSOC) (chapter 2) and PCa (chapter 3), we see different expression patterns in a majority of genes examined (*PIWIL1*, *PIWIL2*, *DDX4*, *MAEL*, *PLD6*, *TDRD1* and *TDRD9*) between benign and cancer samples. More detailed investigation comparing the expression of these genes in two different OC diseases (low and high grade OC), demonstrated again different expression levels of piRNA pathway genes (*PIWIL1*, *PIWIL3*, *PLD6* and *TDRD1*), suggesting that the pathway genes may even have cancer subtype-specific expression.

To the best of our knowledge, HGSOC and PCa patient survival analysis based on different expression levels of piRNA pathway genes have not been performed. Our comprehensive analysis of high and low expression of piRNA pathway genes in progression free, post-progression free and overall survival of HGSOC patients demonstrated potential clinical relevance of *PIWIL1*, *DDX4*, *MAEL* and *TDRD1* in early stage HGSOC,

and *PIWIL1*, *PIWIL2*, *PIWIL4*, *MAEL*, *PLD6* and *TDRD9* in late stage HGSOC. In PCa, we showed that altered expression of a gene signature consisting the 10 piRNA pathway genes and individual genes *PIWIL4*, *DDX4* and *TDRD1* were significantly associated with poorer disease-free survival. We concur with studies in other cancers proposing the piRNA pathway genes as potential biomarkers and therapeutic targets for HGSOC and PCa (4-7). To utilise components of the piRNA pathway as biomarkers, it would be more beneficial for patients if less invasive procedures such as blood tests were used in place of a biopsy. Preliminary work has demonstrated this possibility where significantly lower levels of piRNAs (piR-651 and piR-823) were detected in the peripheral blood of gastric cancer patients as compared to controls (8).

The development of chemoresistance and patients relapsing are concerning issues in cancer as they are currently largely incurable, contributing to cancer related deaths. Therefore, a better understanding of the pathways underpinning chemoresistance is vital. Through our pilot study with a small sample size in HGSOC, we showed genes from the piRNA pathway (*PIWIL3*, *PIWIL4*, *MAEL*, *PLD6*, *TDRD1* and *TDRD9*) potentially playing roles in the progression of chemoresistance. Analysing the tumours of PCa patients with and without relapse showed trends in expression of piRNA pathway genes *DDX4*, *HENMT1* and *PLD6*. While *PLD6* was the only gene altered in both cancers in this context, its role in cancer has not been studied. This hints at these genes also potentially having cancer-specific expression in the development of resistance or patient relapse.

OC and PCa cells are hormone sensitive where previous studies have shown follicle stimulating hormone (FSH) and luteinising hormone (LH) promoting OC proliferation and migration while androgen is known to regulate the proliferation and apoptosis of PCa cells (9-13). As piRNA pathway genes have been shown to play a role in cell proliferation and migration in other cancers, we investigated their expression in relation to FSH, LH and androgen treatment (14). We observed increased *PIWIL2* expression in HGSOC cell line, OV-90 after high dose FSH treatment, indicating a possible modulation of *PIWIL2* by FSH. Moreover, treatment of OV-90 cells with both FSH and LH did not induce any change in *PIWIL2* expression. This may be due to antagonistic effects of FSH and LH as seen in a past study (9). Transcriptomic studies on PCa cell line, LNCaP after enzalutamide treatment (decreased androgen receptor (AR) activity) and AR knockdown showed trends in the expression of piRNA pathway genes *HENMT1*, *PLD6* and *TDRD1*. While these trends were not significant, they showed possible effects of androgen on piRNA pathway gene expression. In addition to FSH, LH and androgen, other hormones including estradiol-17β, 17α-methyltestosterone and fadrozole have been reported to affect expression of the piRNA pathway in fishes and frogs (15, 16). Altogether, hormones may affect expression of the piRNA pathway genes and potentially cause an effect in hormone-sensitive cancers.

As the effects on motility and invasion of cells from manipulation of *PIWIL1*, *PIWIL2* and *MAEL* expression have been studied in more detail in other cancers, we investigated this aspect by overexpressing *PIWIL1*,

PIWIL2 and *MAEL* in HGSOC (OVCAR-3 and OV-90) and castrate resistant prostate cancer (CRPC) (22RV1) cell lines. In contrast to other cancers showing cancer promoting effects, we demonstrated that overexpression resulted in decreased motility and invasion *in vitro* (HGSOC and CRPC) and *in vivo* (HGSOC) (17-21). These contrasting results may be due to HGSOC and CRPC having unique gene mutations that set them apart from other cancers. This includes HGSOC having high rates (~100%) of *TP53* mutations and CRPC having the prevalent *TMPRSS2:ERG* fusion where both mutations have been linked to transposable elements (TEs) and the piRNA pathway (22-25). The similar outcome of overexpressing *PIWIL1*, *PIWIL2* and *MAEL* in HGSOC and CRPC may be attributed by them having mutations in a common set of genes such as *BRCA1* and *BRCA2* which predisposes women and men to HGSOC and PCa respectively (26, 27). A recent study has shown that *BRCA1* is a target gene of seven piRNAs in neuroblastoma cells (28). Therefore, the relationship between *BRCA1* and *BRCA2* mutation and piRNA pathway expression needs further investigation.

Further, we explored the effects of overexpressing *PIWIL1* (*P1Δ17*) and *PIWIL2* (*PL2L60*) mutants on the motility and invasion of OVCAR-3, OV-90 and 22RV1 cells. *P1Δ17* was proposed to have a truncated PIWI domain which may impair its endonuclease activity while *PL2L60* with truncated PAZ domain was proposed to have impaired piRNA binding (29-31). In contrast to the empty vector control and *PIWIL1* overexpression, *P1Δ17* overexpression promoted motility in OVCAR-3 cells. However, the effect of

overexpressing *P1Δ17* in OV-90 and 22RV1 cells was similar to *PIWIL1*. While OVCAR-3, OV-90 and 22RV1 cells overexpressing *PL2L60* had decreased motility and invasion compared to the empty vector control, cells overexpressing *PL2L60* had increased motility (22RV1) and invasion (OV-90) compared to overexpressing wildtype *PIWIL2*. We postulate that the differences in motility and invasion induced by the mutants may be attributed to their truncated domains likely affecting their endonuclease activity and piRNA binding.

4.2 Future Directions

The piRNA pathway has become an important aspect of understanding cancer development and progression. The increasing number of genes in this pathway and their associated regulatory networks opened up new avenues of exploration into better understanding cancer development and progression. With changes consistently observed across the 10 piRNA pathway genes in normal, benign, low grade OC, HGSOC and PCa tissues, expanding expression and patient survival analysis in independent patient cohorts would be required for validation. This can better inform their potential as novel biomarkers assisting with patient diagnosis or prognosis. The effects of chemoresistance, cancer treatment and hormones on the piRNA pathway are largely unexplored but are important. To address the trends in piRNA pathway gene expression observed in chemoresistant HGSOC (*PIWIL3*, *PIWIL4*, *DDX4*, *MAEL*, *PLD6*, *TDRD1* and *TDRD9*) and relapsed PCa (*DDX4*, *HENMT1* and *PLD6*) patients, a larger patient cohort is needed to validate these expression changes. Downstream work can

then investigate if chemoresistant HGSOC and enzalutamide resistant PCa cell lines can regain sensitivity to treatment after overexpression or knockdown of piRNA pathway genes which would link the piRNA pathway genes more directly to the mechanisms of chemoresistance.

This study also investigated potential effects of hormone treatment on piRNA pathway genes. Here, we observed increased *PIWIL2* expression after FSH treatment. Further work could determine dose-response in OV-90, additional HGSOC cell lines and primary HGSOC cells. piRNA pathway genes interact with other genes outside this pathway including established players in cancer development (18, 21, 32, 33). Investigation into components of downstream signalling pathways of FSH such as PI3K/AKT or AKT/mammalian target of rapamycin (mTOR) potentially binding the *PIWIL2* promoter for direct expression regulation can be analysed using luciferase assays (34). Our transcriptomic analysis of enzalutamide treated and AR knocked down LNCaP cells was only able to detect the expression of three piRNA pathway genes. For detection and quantitative expression of the selected 10 piRNA pathway genes, qRT-PCRs can be carried out to provide a more comprehensive picture of how these genes may be affected.

It becomes increasingly clear that the piRNA pathway acts in complex ways and the dissimilar effects of overexpressing piRNA pathway genes on the motility and invasion of different cancers may be attributed by the interaction of piRNAs and the pathway genes. There have been studies of certain piRNA species (piR-651 and piR-823) having tumour suppressive and oncogenic properties in gastric cancer and multiple myeloma (35-37).

Moreover, a recent study demonstrated that piRNA-DQ593109 and PIWIL1 can form a complex to execute functions beyond TE repression including regulating the permeability of blood-tumour barrier (38). To explore this, RNA immunoprecipitation and RNA sequencing will be needed to investigate if in different cancers, different piRNA species are interacting with the pathway genes. Preliminary work by Wylie, Jones (39) in *Drosophila* proposed that TP53 may collaborate with the piRNA pathway to repress TEs. As TP53 can regulate a plethora of pathways including DNA damage response, apoptosis and cell growth, future work in cancer cells can inform their differential effects in HGSOC which contains a high rate of *TP53* mutations (22, 40). In PCa and CRPC, the *TMPRSS2:ERG* fusion is prevalent (25, 41). Its formation has been linked to *PIWIL1* and *PIWIL2* while ERG has been proposed to regulate *TDRD1* expression (23, 42). Therefore, subsequent studies performed can compare the motility and invasion of PCa and CRPC cells overexpressing piRNA pathway genes in the presence and absence of this fusion.

Further research into the relationship of *BRCA* genes and the piRNA pathway can provide additional insights into their roles in cancers of the reproductive system. As BRCAs are key in the DNA damage repair mechanism, cancer cells can be irradiated to induce DNA damage (43). Immunofluorescence staining can then be performed to identify any co-localization of BRCA and piRNA pathway proteins. To address the potential impairment of piRNA binding and endonuclease activity in P1Δ17 and PL2L60, RNA immunoprecipitation can be carried out in parallel in cell

systems containing only wildtype gene or only mutant gene to compare the abundance and lengths of piRNAs pulled down.

The enigmatic piRNA pathway has emerged as an important player in cancer development and progression. Here, we showed their differential expression profiles in OC and PCa, association with prognosis, chemoresistance, hormone treatment and effects on cancer progression through motility and invasion assays. Furthermore, our work demonstrated the novelty of the piRNA pathway having cancer-specific effects. More work is needed to connect the piRNA pathway landscape in cancer and their interactions with other pathways to refine the picture of the complex roles of this pathway in different cancers.

References

1. Liu Y, Dou M, Song X, Dong Y, Liu S, Liu H, et al. The emerging role of the piRNA/piwi complex in cancer. Molecular Cancer. 2019;18(1):123.
2. Hanahan D, Weinberg Robert A. Hallmarks of Cancer: The Next Generation. Cell. 2011;144(5):646-74.
3. Lee E, Jacob R, Grützner F. PIWI-Interacting RNAs (piRNAs) and Cancer. Cancer and Noncoding RNAs: Elsevier; 2018. p. 131-50.
4. Xiao L, Lanz RB, Frolov A, Castro PD, Zhang Z, Dong B, et al. The germ cell gene TDRD1 as an ERG target gene and a novel prostate cancer biomarker. The Prostate. 2016;76(14):1271-84.
5. He G, Chen L, Ye Y, Xiao Y, Hua K, Jarjoura D, et al. Piwil2 expressed in various stages of cervical neoplasia is a potential complementary marker for p16INK4a. American Journal of Translational Research. 2010;2(2):156.
6. Kim KH, Kang Y-J, Jo J-O, Ock MS, Moon SH, Suh DS, et al. DDX4 (DEAD box polypeptide 4) colocalizes with cancer stem cell marker CD133 in ovarian cancers. Biochemical and Biophysical Research Communications. 2014;447(2):315-22.
7. Krishnan P, Ghosh S, Graham K, Mackey J, Kovalchuk O, Damaraju S. Piwi-interacting RNAs and PIWI genes as novel prognostic markers for breast cancer. Oncotarget. 2016.
8. Cui L, Lou Y, Zhang X, Zhou H, Deng H, Song H, et al. Detection of circulating tumor cells in peripheral blood from patients with gastric cancer using piRNAs as markers. Clinical Biochemistry. 2011;44(13):1050-7.

9. Zheng W, Lu JJ, Luo F, Zheng Y, Feng Y-j, Felix JC, et al. Ovarian Epithelial Tumor Growth Promotion by Follicle-Stimulating Hormone and Inhibition of the Effect by Luteinizing Hormone. Gynecologic Oncology. 2000;76(1):80-8.
10. Feng D, Zhao T, Yan K, Liang H, Liang J, Zhou Y, et al. Gonadotropins promote human ovarian cancer cell migration and invasion via a cyclooxygenase 2-dependent pathway. Oncology Reports. 2017;38(2):1091.
11. Mertens-Walker I, Bolitho C, Baxter RC, Marsh DJ. Gonadotropin-induced ovarian cancer cell migration and proliferation require extracellular signal-regulated kinase 1/2 activation regulated by calcium and protein kinase Cδ. Endocrine-Related Cancer. 2010;17(2):335.
12. Choi J-H, Choi K-C, Auersperg N, Leung PC. Gonadotropins activate proteolysis and increase invasion through protein kinase A and phosphatidylinositol 3-kinase pathways in human epithelial ovarian cancer cells. Cancer Research. 2006;66(7):3912-20.
13. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nature Reviews Cancer. 2001;1(1):34-45.
14. Tan Y, Liu L, Liao M, Zhang C, Hu S, Zou M, et al. Emerging roles for PIWI proteins in cancer. Acta Biochimica et Biophysica Sinica. 2015;47(5):315-24.
15. Zhang D, Duarte-Guterman P, Langlois VS, Trudeau VL. Temporal expression and steroidal regulation of piRNA pathway genes (mael, piwi, vasa) during Silurana (Xenopus) tropicalis embryogenesis and early larval development. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 2010;152(2):202-6.
16. Wang H, Wang B, Liu J, Li A, Zhu H, Wang X, et al. Piwil1 gene is regulated by hypothalamic-pituitary-gonadal axis in turbot (Scophthalmus maximus): A different effect in ovaries and testes. Gene. 2018;658:86-95.
17. Wang D-W, Wang Z-H, Wang L-L, Song Y, Zhang G-Z. Overexpression of hiwi promotes growth of human breast cancer cells. Asian Pacific Journal of Cancer Prevention. 2013;15(18):7553-8.
18. Lee JH, Jung C, Javadian-Elyaderani P, Schweyer S, Schütte D, Shoukier M, et al. Pathways of Proliferation and Antiapoptosis Driven in Breast Cancer Stem Cells by Stem Cell Protein Piwil2. Cancer Research. 2010;70(11):4569-79.
19. Xie Y, Yang Y, Ji D, Zhang D, Yao X, Zhang X. Hiwi downregulation, mediated by shRNA, reduces the proliferation and migration of human hepatocellular carcinoma cells. Molecular Medicine Reports. 2015;11:1455+.
20. Qu X, Liu J, Zhong X, Li X, Zhang Q. PIWIL2 promotes progression of non-small cell lung cancer by inducing CDK2 and Cyclin A expression. Journal of Translational Medicine. 2015;13(1):1.
21. Liu L, Dai Y, Chen J, Zeng T, Li Y, Chen L, et al. Maelstrom promotes hepatocellular carcinoma metastasis by inducing epithelial-mesenchymal transition by way of Akt/GSK-3β/Snail signaling. Hepatology. 2014;59(2):531-43.
22. Vang R, Levine DA, Soslow RA, Zaloudek C, Shih I-M, Kurman RJ. Molecular alterations of TP53 are a defining feature of ovarian high-grade serous carcinoma: a rereview of cases lacking TP53 mutations in The

Cancer Genome Atlas Ovarian Study. International Journal of Gynecological Pathology. 2016;35(1):48.
23. Lin C, Yang L, Tanasa B, Hutt K, Ju B-g, Ohgi KA, et al. Nuclear receptor-induced chromosomal proximity and DNA breaks underlie specific translocations in cancer. Cell. 2009;139(6):1069-83.
24. Tiwari B, Jones AE, Abrams JM. Transposons, p53 and genome security. Trends in Genetics. 2018;34(11):846-55.
25. Holcomb IN, Young JM, Coleman IM, Salari K, Grove DI, Hsu L, et al. Comparative analyses of chromosome alterations in soft-tissue metastases within and across patients with castration-resistant prostate cancer. Cancer Research. 2009;69(19):7793-802.
26. Cunningham JM, Cicek M, Larson N, Davila J, Wang C, Larson M, et al. Clinical characteristics of ovarian cancer classified by BRCA1, BRCA2, and RAD51C status. Scientific Reports. 2014;4.
27. Gallagher DJ, Gaudet MM, Pal P, Kirchhoff T, Balistreri L, Vora K, et al. Germline BRCA mutations denote a clinicopathologic subset of prostate cancer. Clinical Cancer Research. 2010;16.
28. Roy J, Mallick B. Investigating piwi‐interacting RNA regulome in human neuroblastoma. Genes, Chromosomes and Cancer. 2018;57(7):339-49.
29. Ye Y, Yin D-T, Chen L, Zhou Q, Shen R, He G, et al. Identification of Piwil2-Like (PL2L) Proteins that Promote Tumorigenesis. PLOS One. 2010;5(10):e13406.
30. Lim SL, Ricciardelli C, Oehler MK, De Arao Tan IMD, Russell D, Grützner F. Overexpression of piRNA Pathway Genes in Epithelial Ovarian Cancer. PLOS One. 2014;9(6):e99687.
31. Bamezai S, Rawat VPS, Buske C. Concise Review: The Piwi-piRNA Axis: Pivotal Beyond Transposon Silencing. Stem Cells. 2012;30(12):2603-11.
32. Li D, Sun X, Yan D, Huang J, Luo Q, Tang H, et al. Piwil2 modulates the proliferation and metastasis of colon cancer via regulation of matrix metallopeptidase 9 transcriptional activity. Experimental Biology and Medicine. 2012;237(10):1231-40.
33. Li C, Zhou X, Chen J, Lu Y, Sun Q, Tao D, et al. PIWIL1 destabilizes microtubule by suppressing phosphorylation at Ser16 and RLIM-mediated degradation of stathmin1. Oncotarget. 2015;6(29):27794-804.
34. Casarini L, Crépieux P. Molecular Mechanisms of Action of FSH. Front Endocrinol (Lausanne). 2019;10:305-.
35. Cheng J, Deng H, Xiao B, Zhou H, Zhou F, Shen Z, et al. piR-823, a novel non-coding small RNA, demonstrates in vitro and in vivo tumor suppressive activity in human gastric cancer cells. Cancer Letters. 2012;315(1):12-7.
36. Yan H, Wu Q, Sun C, Ai L, Deng J, Zhang L, et al. piRNA-823 contributes to tumorigenesis by regulating de novo DNA methylation and angiogenesis in multiple myeloma. Leukemia. 2015;29(1):196-206.
37. Cheng J, Guo J-M, Xiao B-X, Miao Y, Jiang Z, Zhou H, et al. piRNA, the new non-coding RNA, is aberrantly expressed in human cancer cells. Clinica Chimica Acta. 2011;412(17–18):1621-5.

38. Shen S, Yu H, Liu X, Liu Y, Zheng J, Wang P, et al. PIWIL1/piRNA-DQ593109 Regulates the Permeability of the Blood-Tumor Barrier via the MEG3/miR-330-5p/RUNX3 Axis. Molecular Therapy - Nucleic Acids. 2018;10:412-25.
39. Wylie A, Jones AE, D'Brot A, Lu W-J, Kurtz P, Moran JV, et al. p53 genes function to restrain mobile elements. Genes & Development. 2016;30(1):64-77.
40. Brady CA, Attardi LD. p53 at a glance. Journal of Cell Science. 2010;123(15):2527-32.
41. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun X-W, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science. 2005;310(5748):644-8.
42. Boormans JL, Korsten H, Ziel‐van der Made AJ, van Leenders GJ, de Vos CV, Jenster G, et al. Identification of TDRD1 as a direct target gene of ERG in primary prostate cancer. International Journal of Cancer. 2013;133(2):335-45.
43. Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nature Reviews Cancer. 2012;12(1):68-78.

BIBLIOGRAPHY

Adamo, P., and M. Ladomery, 2016 The oncogene ERG: a key factor in prostate cancer. Oncogene 35**:** 403-414.

Aravin, A., D. Gaidatzis, S. Pfeffer, M. Lagos-Quintana, P. Landgraf *et al.*, 2006 A novel class of small RNAs bind to MILI protein in mouse testes. Nature 442**:** 203-207.

Aravin, A. A., M. Lagos-Quintana, A. Yalcin, M. Zavolan, D. Marks *et al.*, 2003 The Small RNA Profile during Drosophila melanogaster Development. Developmental Cell 5**:** 337-350.

Aravin, A. A., N. M. Naumova, A. V. Tulin, V. V. Vagin, Y. M. Rozovsky *et al.*, 2001 Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the D. melanogaster germline. Current Biology 11**:** 1017-1027.

Aravin, A. A., R. Sachidanandam, D. Bourc'his, C. Schaefer, D. Pezic *et al.*, 2008 A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. Molecular Cell 31**:** 785-799.

Aravin, A. A., R. Sachidanandam, A. Girard, K. Fejes-Toth and G. J. Hannon, 2007 Developmentally Regulated piRNA Clusters Implicate MILI in Transposon Control. Science 316**:** 744-747.

Australia, H., 2017 Ovarian cancer symptoms.

Babatunde, K. A., A. Najafi, P. Salehipour, M. H. Modarressi and M. B. Mobasheri, 2017 Cancer/Testis genes in relation to sperm biology and function. Iranian Journal of Basic Medical Sciences 20**:** 967-974.

Bamezai, S., V. P. S. Rawat and C. Buske, 2012 Concise Review: The Piwi-piRNA Axis: Pivotal Beyond Transposon Silencing. Stem Cells 30**:** 2603-2611.

Birkbak, N. J., A. C. Eklund, Q. Li, S. E. McClelland, D. Endesfelder *et al.*, 2011 Paradoxical relationship between chromosomal instability and survival outcome in cancer. Cancer Research 71**:** 3447-3452.

Boormans, J. L., H. Korsten, A. J. Ziel‐van der Made, G. J. van Leenders, C. V. de Vos *et al.*, 2013 Identification of TDRD1 as a direct target gene of ERG in primary prostate cancer. International Journal of Cancer 133**:** 335-345.

Bostwick, D. G., and L. Cheng, 2012 Precursors of prostate cancer. Histopathology 60**:** 4-27.

Bourgeois, D. L., K. A. Kabarowski, V. L. Porubsky and P. K. Kreeger, 2015 High-grade serous ovarian cancer cell lines exhibit heterogeneous responses to growth factor stimulation. Cancer Cell International 15**:** 112-112.

Boussios, S., M. Moschetta, G. Zarkavelis, A. Papadaki, A. Kefas *et al.*, 2017 Ovarian sex-cord stromal tumours and small cell tumours: Pathological, genetic and management aspects. Critical Reviews in Oncology/Hematology 120**:** 43-51.

Brady, C. A., and L. D. Attardi, 2010 p53 at a glance. Journal of Cell Science 123**:** 2527-2532.

Brase, J. C., M. Johannes, H. Mannsperger, M. Fälth, J. Metzger *et al.*, 2011 TMPRSS2-ERG-specific transcriptional modulation is associated with

prostate cancer biomarkers and TGF-β signaling. BMC Cancer 11: 1-8.
Bray, F., J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre *et al.*, 2018 Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians 68: 394-424.
Brennecke, J., A. A. Aravin, A. Stark, M. Dus, M. Kellis *et al.*, 2007 Discrete Small RNA-Generating Loci as Master Regulators of Transposon Activity in Drosophila. Cell 128: 1089-1103.
Cai, C., H. Wang, Y. Xu, S. Chen and S. P. Balk, 2009 Reactivation of Androgen Receptor–Regulated TMPRSS2: ERG Gene Expression in Castration-Resistant Prostate Cancer. Cancer Research 69: 6027-6032.
Casarini, L., and P. Crépieux, 2019 Molecular Mechanisms of Action of FSH. Frontiers in Endocrinology 10: 305-305.
Cavanagh, H., and K. M. A. Rogers, 2015 The role of BRCA1 and BRCA2 mutations in prostate, pancreatic and stomach cancers. Hereditary Cancer in Clinical Practice 13: 16.
Cerami, E., J. Gao, U. Dogrusoz, B. E. Gross, S. O. Sumer *et al.*, 2012 The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data, pp. AACR.
Chen, C., J. Liu and G. Xu, 2013 Overexpression of PIWI proteins in human stage III epithelial ovarian cancer with lymph node metastasis. Cancer Biomarkers 13: 315-321.
Chen, C., T. J. Nott, J. Jin and T. Pawson, 2011 Deciphering arginine methylation: Tudor tells the tale. Nature Reviews Molecular Cell Biology 12: 629-642.
Chen, Z., Q. Che, X. He, F. Wang, H. Wang *et al.*, 2015 Stem cell protein Piwil1 endowed endometrial cancer cells with stem-like properties via inducing epithelial-mesenchymal transition. BMC Cancer 15: 811.
Cheng, J., H. Deng, B. Xiao, H. Zhou, F. Zhou *et al.*, 2012 piR-823, a novel non-coding small RNA, demonstrates in vitro and in vivo tumor suppressive activity in human gastric cancer cells. Cancer Letters 315: 12-17.
Cheng, J., J.-M. Guo, B.-X. Xiao, Y. Miao, Z. Jiang *et al.*, 2011 piRNA, the new non-coding RNA, is aberrantly expressed in human cancer cells. Clinica Chimica Acta 412: 1621-1625.
Chirn, G.-w., R. Rahman, Y. A. Sytnikova, J. A. Matts, M. Zeng *et al.*, 2015 Conserved piRNA Expression from a Distinct Set of piRNA Cluster Loci in Eutherian Mammals. PLOS Genetics 11: e1005652.
Choi, J.-H., K.-C. Choi, N. Auersperg and P. C. Leung, 2006 Gonadotropins activate proteolysis and increase invasion through protein kinase A and phosphatidylinositol 3-kinase pathways in human epithelial ovarian cancer cells. Cancer Research 66: 3912-3920.
Chuma, S., M. Hosokawa, K. Kitamura, S. Kasai, M. Fujioka *et al.*, 2006 Tdrd1/Mtr-1, a tudor-related gene, is essential for male germ-cell differentiation and nuage/germinal granule formation in mice. Proceedings of the National Academy of Sciences 103: 15894-15899.

Cora, E., R. R. Pandey, J. Xiol, J. Taylor, R. Sachidanandam *et al.*, 2014 The MID-PIWI module of Piwi proteins specifies nucleotide- and strand-biases of piRNAs. RNA 20**:** 773-781.
Cui, L., Y. Lou, X. Zhang, H. Zhou, H. Deng *et al.*, 2011 Detection of circulating tumor cells in peripheral blood from patients with gastric cancer using piRNAs as markers. Clinical Biochemistry 44**:** 1050-1057.
Cunningham, J. M., M. Cicek, N. Larson, J. Davila, C. Wang *et al.*, 2014 Clinical characteristics of ovarian cancer classified by BRCA1, BRCA2, and RAD51C status. Scientific Reports 4.
Czech, B., and G. J. Hannon, 2016 One Loop to Rule Them All: The Ping-Pong Cycle and piRNA-Guided Silencing. Trends in Biochemical Sciences.
Czech, B., M. Munafò, F. Ciabrelli, E. L. Eastwood, M. H. Fabry *et al.*, 2018 piRNA-guided genome defense: from biogenesis to silencing. Annual Review of Genetics 52**:** 131-157.
De Angelis, G., H. G. Rittenhouse, S. D. Mikolajczyk, L. Blair Shamel and A. Semjonow, 2007 Twenty Years of PSA: From Prostate Antigen to Tumor Marker. Reviews in Urology 9**:** 113-123.
Dehm, S. M., L. J. Schmidt, H. V. Heemers, R. L. Vessella and D. J. Tindall, 2008 Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. Cancer Research 68**:** 5469-5477.
Ding, Y., H. Wu, C. Warden, L. Steele, X. Liu *et al.*, 2016 Gene expression differences in prostate cancers between young and old men. PLOS Genetics 12**:** e1006477.
Esteller, M., 2011 Non-coding RNAs in human disease. Nature Reviews Genetics 12**:** 861-874.
Feldman, B. J., and D. Feldman, 2001 The development of androgen-independent prostate cancer. Nature Reviews Cancer 1**:** 34-45.
Feng, D., T. Zhao, K. Yan, H. Liang, J. Liang *et al.*, 2017 Gonadotropins promote human ovarian cancer cell migration and invasion via a cyclooxygenase 2-dependent pathway. Oncology Reports 38**:** 1091.
Findley, S. D., M. Tamanaha, N. J. Clegg and H. Ruohola-Baker, 2003 Maelstrom, a Drosophila spindle-class gene, encodes a protein that colocalizes with Vasa and RDE1/AGO1 homolog, Aubergine, in nuage. Development 130**:** 859-871.
Fite, K., 2017 Dysregulation of Phospholipase D (PLD) isoforms increases breast cancer cell invasion, pp. Wright State University.
Flaum, N., E. J. Crosbie, R. J. Edmondson, M. J. Smith and D. G. Evans, 2020 Epithelial ovarian cancer risk: A review of the current genetic landscape. Clinical Genetics 97**:** 54-63.
Furusato, B., C.-L. Gao, L. Ravindranath, Y. Chen, J. Cullen *et al.*, 2008 Mapping of TMPRSS2–ERG fusions in the context of multi-focal prostate cancer. Modern Pathology 21**:** 67-75.
Gainetdinov, I. V., S. A. Kondratieva, Y. V. Skvortsova, M. V. Zinovyeva, E. A. Stukacheva *et al.*, 2016 Distinguishing epigenetic features of preneoplastic testis tissues adjacent to seminomas and nonseminomas. Oncotarget 7**:** 22439.

Gallagher, D. J., M. M. Gaudet, P. Pal, T. Kirchhoff, L. Balistreri *et al.*, 2010 Germline BRCA mutations denote a clinicopathologic subset of prostate cancer. Clinical Cancer Research 16.
Gambichler, T., C. Kohsik, A.-K. Höh, K. Lang, H. U. Käfferlein *et al.*, 2017 Expression of PIWIL3 in primary and metastatic melanoma. Journal of Cancer Research and Clinical Oncology 143**:** 433-437.
Gao, J., B. A. Aksoy, U. Dogrusoz, G. Dresdner, B. Gross *et al.*, 2013 Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Science Signaling 6**:** pl1-pl1.
Genzor, P., S. C. Cordts, N. V. Bokil and A. D. Haase, 2019 Aberrant expression of select piRNA-pathway genes does not reactivate piRNA silencing in cancer cells. Proceedings of the National Academy of Sciences 116**:** 11111-11112.
Gera, S., S. Kumar S, S. N. Swamy, R. Bhagat, A. Vadaparty *et al.*, 2018 Follicle-Stimulating Hormone Is an Autocrine Regulator of the Ovarian Cancer Metastatic Niche Through Notch Signaling. Journal of the Endocrine Society 3**:** 340-357.
Gharwan, H., K. P. Bunch and C. M. Annunziata, 2015 The role of reproductive hormones in epithelial ovarian carcinogenesis. Endocrine-Related Cancer 22**:** R339-R363.
Ghildiyal, M., and P. D. Zamore, 2009 Small silencing RNAs: an expanding universe. Nature Reviews Genetics 10**:** 94-108.
Goldstein, A. S., J. Huang, C. Guo, I. P. Garraway and O. N. Witte, 2010 Identification of a Cell of Origin for Human Prostate Cancer. Science 329**:** 568.
Gomez-Cambronero, J., 2014 Phospholipase D in cell signaling: from a myriad of cell functions to cancer growth and metastasis. Journal of Biological Chemistry 289**:** 22557-22566.
Grasso, C. S., Y.-M. Wu, D. R. Robinson, X. Cao, S. M. Dhanasekaran *et al.*, 2012 The mutational landscape of lethal castration-resistant prostate cancer. Nature 487**:** 239-243.
Guijo, M., M. Ceballos-Chávez, E. Gómez-Marín, L. Basurto-Cayuela and J. C. Reyes, 2017 Expression of TDRD9 in a subset of lung carcinomas by CpG island hypomethylation protects from DNA damage. Oncotarget 9**:** 9618-9631.
Gunawardane, L. S., K. Saito, K. M. Nishida, K. Miyoshi, Y. Kawamura *et al.*, 2007 A Slicer-Mediated Mechanism for Repeat-Associated siRNA 5' End Formation in Drosophila. Science 315**:** 1587-1590.
Guo, Z., X. Yang, F. Sun, R. Jiang, D. E. Linn *et al.*, 2009 A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion–resistant growth. Cancer Research 69**:** 2305-2313.
Gupta, S., K. Iljin, H. Sara, J. P. Mpindi, T. Mirtti *et al.*, 2010 FZD4 as a mediator of ERG oncogene–induced WNT signaling and epithelial-to-mesenchymal transition in human prostate cancer cells. Cancer Research 70**:** 6735-6745.
Győrffy, B., A. Lánczky and Z. Szállási, 2012 Implementing an online tool for genome-wide validation of survival-associated biomarkers in ovarian-

cancer using microarray data from 1287 patients. Endocrine-Related Cancer 19**:** 197-208.
Hafeez, B. B., V. M. Adhami, M. Asim, I. A. Siddiqui, K. M. Bhat *et al.*, 2009 Targeted knockdown of Notch1 inhibits invasion of human prostate cancer cells concomitant with inhibition of matrix metalloproteinase-9 and urokinase plasminogen activator. Clinical Cancer Research 15**:** 452-459.
Han, Y. N., Y. Li, S. Q. Xia, Y. Y. Zhang, J. H. Zheng *et al.*, 2017 PIWI Proteins and PIWI-Interacting RNA: Emerging Roles in Cancer. Cellular Physiology and Biochemistry 44**:** 1-20.
Hanahan, D., and Robert A. Weinberg, 2011 Hallmarks of Cancer: The Next Generation. Cell 144**:** 646-674.
Harris, W. P., E. A. Mostaghel, P. S. Nelson and B. Montgomery, 2009 Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion. Nature Clinical Practice Urology 6**:** 76-85.
Hashimoto, H., T. Sudo, Y. Mikami, M. Otani, M. Takano *et al.*, 2008 Germ cell specific protein VASA is over-expressed in epithelial ovarian cancer and disrupts DNA damage-induced G2 checkpoint. Gynecologic Oncology 111**:** 312-319.
He, G., L. Chen, Y. Ye, Y. Xiao, K. Hua *et al.*, 2010 Piwil2 expressed in various stages of cervical neoplasia is a potential complementary marker for p16INK4a. American Journal of Translational Research 2**:** 156.
Hempfling, A.-L., S. L. Lim, D. Adelson, J. Evans, A. E. O'Connor *et al.*, 2017 Expression patterns of HENMT1 and PIWIL1 in human testis-Implications for transposon expression. Reproduction**:** REP-16-0586.
Hirakata, S., and M. C. Siomi, 2016 piRNA biogenesis in the germline: From transcription of piRNA genomic sources to piRNA maturation. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms 1859**:** 82-92.
Holcomb, I. N., J. M. Young, I. M. Coleman, K. Salari, D. I. Grove *et al.*, 2009 Comparative analyses of chromosome alterations in soft-tissue metastases within and across patients with castration-resistant prostate cancer. Cancer Research 69**:** 7793-7802.
Holzbeierlein, J., P. Lal, E. LaTulippe, A. Smith, J. Satagopan *et al.*, 2004 Gene expression analysis of human prostate carcinoma during hormonal therapy identifies androgen-responsive genes and mechanisms of therapy resistance. The American Journal of Pathology 164**:** 217-227.
Horwich, M. D., C. Li, C. Matranga, V. Vagin, G. Farley *et al.*, 2007 The Drosophila RNA Methyltransferase, DmHen1, Modifies Germline piRNAs and Single-Stranded siRNAs in RISC. Current Biology 17**:** 1265-1272.
Hosokawa, M., M. Shoji, K. Kitamura, T. Tanaka, T. Noce *et al.*, 2007 Tudor-related proteins TDRD1/MTR-1, TDRD6 and TDRD7/TRAP: domain composition, intracellular localization, and function in male germ cells in mice. Developmental biology 301**:** 38-52.

Iliev, R., M. Stanik, M. Fedorko, A. Poprach, P. Vychytilova-Faltejskova *et al.*, 2016 Decreased expression levels of PIWIL1, PIWIL2, and PIWIL4 are associated with worse survival in renal cell carcinoma patients. OncoTargets and Therapy 9**:** 217.

Iorio, M. V., R. Visone, G. Di Leva, V. Donati, F. Petrocca *et al.*, 2007 MicroRNA signatures in human ovarian cancer. Cancer Research 67**:** 8699-8707.

Ivarsson, K., K. Sundfeldt, M. Brännström, P. r. Hellberg and P. O. Janson, 2001 Diverse effects of FSH and LH on proliferation of human ovarian surface epithelial cells. Human Reproduction 16**:** 18-23.

Iwasaki, Y. W., M. C. Siomi and H. Siomi, 2015 PIWI-Interacting RNA: Its Biogenesis and Functions. Annual Review of Biochemistry 84**:** 405-433.

Jiang, L., W.-J. Wang, Z.-W. Li and X.-Z. Wang, 2017 Downregulation of Piwil3 suppresses cell proliferation, migration and invasion in gastric cancer. Cancer Biomarkers 20**:** 499-509.

Jiang, S., L. Zhao, Y. Lu, M. Wang, Y. Chen *et al.*, 2014 Piwil2 inhibits keratin 8 degradation through promoting p38-induced phosphorylation to resist Fas-mediated apoptosis. Molecular and Cellular Biology 34**:** 3928-3938.

Kacprzyk, L. A., M. Laible, T. Andrasiuk, J. C. Brase, S. T. Börno *et al.*, 2013 ERG Induces Epigenetic Activation of Tudor Domain-Containing Protein 1 (TDRD1) in ERG Rearrangement-Positive Prostate Cancer. PLOS One 8**:** e59976.

Karantanos, T., P. G. Corn and T. C. Thompson, 2013 Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate-resistance and novel therapeutic approaches. Oncogene 32**:** 5501.

Kazazian, H. H., 2004 Mobile Elements: Drivers of Genome Evolution. Science 303**:** 1626-1632.

Kim, K. H., Y.-J. Kang, J.-O. Jo, M. S. Ock, S. H. Moon *et al.*, 2014 DDX4 (DEAD box polypeptide 4) colocalizes with cancer stem cell marker CD133 in ovarian cancers. Biochemical and Biophysical Research Communications 447**:** 315-322.

Kim, S., Y. Han, S. I. Kim, H.-S. Kim, S. J. Kim *et al.*, 2018 Tumor evolution and chemoresistance in ovarian cancer. NPJ Precision Oncology 2**:** 1-9.

Kim, S.-H., E.-R. Park, E. Cho, W.-H. Jung, J.-Y. Jeon *et al.*, 2017 Mael is essential for cancer cell survival and tumorigenesis through protection of genetic integrity. Oncotarget 8**:** 5026-5037.

Krishnan, P., S. Ghosh, K. Graham, J. Mackey, O. Kovalchuk *et al.*, 2016 Piwi-interacting RNAs and PIWI genes as novel prognostic markers for breast cancer. Oncotarget.

Kron, K. J., A. Murison, S. Zhou, V. Huang, T. N. Yamaguchi *et al.*, 2017 TMPRSS2–ERG fusion co-opts master transcription factors and activates NOTCH signaling in primary prostate cancer. Nature Genetics 49**:** 1336.

Kumar, V., and P. Majumder, 1995 Prostate gland: structure, functions and regulation. International Urology and Nephrology 27**:** 231-243.

Kuramochi-Miyagawa, S., T. Watanabe, K. Gotoh, K. Takamatsu, S. Chuma *et al.*, 2010 MVH in piRNA processing and gene silencing of retrotransposons. Genes & Development 24**:** 887-892.
Kuramochi-Miyagawa, S., T. Watanabe, K. Gotoh, Y. Totoki, A. Toyoda *et al.*, 2008 DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. Genes & Development 22**:** 908-917.
Kurman, R. J., and I.-M. Shih, 2016 The Dualistic Model of Ovarian Carcinogenesis: Revisited, Revised, and Expanded. The American Journal of Pathology 186**:** 733-747.
Kurman, R. J., K. Visvanathan, R. Roden, T. C. Wu and I.-M. Shih, 2008 Early Detection and Treatment of Ovarian Cancer: Shifting from Early Stage to Minimal Volume of Disease Based on a New Model of Carcinogenesis. American Journal of Obstetrics and Gynecology 198**:** 351-356.
Law, P. T.-Y., H. Qin, A. K.-K. Ching, K. P. Lai, N. N. Co *et al.*, 2013 Deep sequencing of small RNA transcriptome reveals novel non-coding RNAs in hepatocellular carcinoma. Journal of Hepatology 58**:** 1165-1173.
Lee, E., 2020 (University of Adelaide, Adelaide, South Australia, Australia). Personal communication.
Lee, E., R. Jacob and F. Grützner, 2018 PIWI-Interacting RNAs (piRNAs) and Cancer, pp. 131-150 in *Cancer and Noncoding RNAs*. Elsevier.
Lee, J. H., C. Jung, P. Javadian-Elyaderani, S. Schweyer, D. Schütte *et al.*, 2010 Pathways of Proliferation and Antiapoptosis Driven in Breast Cancer Stem Cells by Stem Cell Protein Piwil2. Cancer Research 70**:** 4569-4579.
Lheureux, S., M. Braunstein and A. M. Oza, 2019 Epithelial ovarian cancer: evolution of management in the era of precision medicine. CA: A Cancer Journal for Clinicians 69**:** 280-304.
Li, C., X. Zhou, J. Chen, Y. Lu, Q. Sun *et al.*, 2015 PIWIL1 destabilizes microtubule by suppressing phosphorylation at Ser16 and RLIM-mediated degradation of stathmin1. Oncotarget 6**:** 27794-27804.
Li, D., X. Sun, D. Yan, J. Huang, Q. Luo *et al.*, 2012a Piwil2 modulates the proliferation and metastasis of colon cancer via regulation of matrix metallopeptidase 9 transcriptional activity. Experimental Biology and Medicine 237**:** 1231-1240.
Li, J., O. Fadare, L. Xiang, B. Kong and W. Zheng, 2012b Ovarian serous carcinoma: recent concepts on its origin and carcinogenesis. Journal of Hematology & Oncology 5**:** 8.
Li, J., L. Xu, Z. Bao, P. Xu, H. Chang *et al.*, 2017 High expression of PIWIL2 promotes tumor cell proliferation, migration and predicts a poor prognosis in glioma. Oncology Reports 38**:** 183-192.
Li, W., J. Martinez-Useros, N. Garcia-Carbonero, M. J. Fernandez-Aceñero, A. Orta *et al.*, 2020 The Clinical Significance of PIWIL3 and PIWIL4 Expression in Pancreatic Cancer. Journal of Clinical Medicine 9**:** 1252.
Li, Y., M. Alsagabi, D. Fan, G. S. Bova, A. H. Tewfik *et al.*, 2011 Intragenic rearrangement and altered RNA splicing of the androgen receptor in a

cell-based model of prostate cancer progression. Cancer Research 71**:** 2108-2117.
Li, Y., S. C. Chan, L. J. Brand, T. H. Hwang, K. A. Silverstein *et al.*, 2013 Androgen receptor splice variants mediate enzalutamide resistance in castration-resistant prostate cancer cell lines. Cancer Research 73**:** 483-489.
Liang, D., Z. Fang, M. Dong, C. Liang, C. Xing *et al.*, 2012 Effect of RNA interference-related HiWi gene expression on the proliferation and apoptosis of lung cancer stem cells. Oncology Letters 4**:** 146-150.
Lim, S. L., Z. P. Qu, R. D. Kortschak, D. M. Lawrence, J. Geoghegan *et al.*, 2015 HENMT1 and piRNA Stability Are Required for Adult Male Germ Cell Transposon Repression and to Define the Spermatogenic Program in the Mouse. PLOS Genetics 11**:** e1005620.
Lim, S. L., C. Ricciardelli, M. K. Oehler, I. M. D. De Arao Tan, D. Russell *et al.*, 2014 Overexpression of piRNA Pathway Genes in Epithelial Ovarian Cancer. PLOS One 9**:** e99687.
Lim, S. L., E. Tsend-Ayush, R. D. Kortschak, R. Jacob, C. Ricciardelli *et al.*, 2013 Conservation and Expression of PIWI-Interacting RNA Pathway Genes in Male and Female Adult Gonad of Amniotes. Biology of Reproduction 89**:** 136, 131-113.
Lin, C., L. Yang, B. Tanasa, K. Hutt, B.-g. Ju *et al.*, 2009 Nuclear receptor-induced chromosomal proximity and DNA breaks underlie specific translocations in cancer. Cell 139**:** 1069-1083.
Linder, P., 2006 Dead-box proteins: a family affair—active and passive players in RNP-remodeling. Nucleic Acids Research 34**:** 4168-4180.
Linder, S., H. G. van der Poel, A. M. Bergman, W. Zwart and S. Prekovic, 2019 Enzalutamide therapy for advanced prostate cancer: efficacy, resistance and beyond. Endocrine-Related Cancer 26**:** R31-R52.
Lisio, M.-A., L. Fu, A. Goyeneche, Z.-h. Gao and C. Telleria, 2019 High-Grade Serous Ovarian Cancer: Basic Sciences, Clinical and Therapeutic Standpoints. International Journal of Molecular Sciences 20**:** 952.
Liu, J. J., R. Shen, L. Chen, Y. Ye, G. He *et al.*, 2010 Piwil2 is expressed in various stages of breast cancers and has the potential to be used as a novel biomarker. International Journal of Clinical and Experimental Pathology 3**:** 328-337.
Liu, L., Y. Dai, J. Chen, T. Zeng, Y. Li *et al.*, 2014 Maelstrom promotes hepatocellular carcinoma metastasis by inducing epithelial-mesenchymal transition by way of Akt/GSK-3β/Snail signaling. Hepatology 59**:** 531-543.
Liu, X., Y. Sun, J. Guo, H. Ma, J. Li *et al.*, 2006 Expression of hiwi gene in human gastric cancer was associated with proliferation of cancer cells. International Journal of Cancer 118**:** 1922-1929.
Liu, X., J. Zheng, Y. Xue, H. Yu, W. Gong *et al.*, 2018 PIWIL3/OIP5-AS1/miR-367-3p/CEBPA feedback loop regulates the biological behavior of glioma cells. Theranostics 8**:** 1084-1105.
Liu, Y., M. Dou, X. Song, Y. Dong, S. Liu *et al.*, 2019 The emerging role of the piRNA/piwi complex in cancer. Molecular Cancer 18**:** 123.

Lokman, N. A., A. S. F. Elder, C. Ricciardelli and M. K. Oehler, 2012 Chick Chorioallantoic Membrane (CAM) Assay as an In Vivo Model to Study the Effect of Newly Identified Molecules on Ovarian Cancer Invasion and Metastasis. International Journal of Molecular Sciences 13**:** 9959-9970.

Lokman, N. A., Z. K. Price, E. K. Hawkins, A. M. Macpherson, M. K. Oehler *et al.*, 2019 4-Methylumbelliferone Inhibits Cancer Stem Cell Activation and Overcomes Chemoresistance in Ovarian Cancer. Cancers 11**:** 1187.

Lomas, D. J., and H. U. Ahmed, 2020 All change in the prostate cancer diagnostic pathway. Nature Reviews Clinical Oncology**:** 1-10.

Lu, Y., K. Zhang, C. Li, Y. Yao, D. Tao *et al.*, 2012 Piwil2 Suppresses P53 by Inducing Phosphorylation of Signal Transducer and Activator of Transcription 3 in Tumor Cells. PLOS One 7**:** e30999.

Malone, C. D., J. Brennecke, M. Dus, A. Stark, W. R. McCombie *et al.*, 2009 Specialized piRNA Pathways Act in Germline and Somatic Tissues of the Drosophila Ovary. Cell 137**:** 522-535.

Mani, R.-S., S. A. Tomlins, K. Callahan, A. Ghosh, M. K. Nyati *et al.*, 2009 Induced chromosomal proximity and gene fusions in prostate cancer. Science 326**:** 1230-1230.

Mani, S. R., and C. E. Juliano, 2013 Untangling the web: The diverse functions of the PIWI/piRNA pathway. Molecular Reproduction and Development 80**:** 632-664.

Martinez, V. D., E. A. Vucic, K. L. Thu, R. Hubaux, K. S. Enfield *et al.*, 2015 Unique somatic and malignant expression patterns implicate PIWI-interacting RNAs in cancer-type specific biology. Scientific Reports 5.

Mathioudakis, N., A. Palencia, J. Kadlec, A. Round, K. Tripsianes *et al.*, 2012 The multiple Tudor domain-containing protein TDRD1 is a molecular scaffold for mouse Piwi proteins and piRNA biogenesis factors. RNA 18**:** 2056-2072.

Matulonis, U. A., A. K. Sood, L. Fallowfield, B. E. Howitt, J. Sehouli *et al.*, 2016 Ovarian cancer. Nature Reviews Disease Primers 2**:** 1-22.

Mertens-Walker, I., R. C. Baxter and D. J. Marsh, 2012 Gonadotropin signalling in epithelial ovarian cancer. Cancer Letters 324**:** 152-159.

Mertens-Walker, I., C. Bolitho, R. C. Baxter and D. J. Marsh, 2010 Gonadotropin-induced ovarian cancer cell migration and proliferation require extracellular signal-regulated kinase 1/2 activation regulated by calcium and protein kinase Cδ. Endocrine-Related Cancer 17**:** 335.

Miller, D. C., K. S. Hafez, A. Stewart, J. E. Montie and J. T. Wei, 2003 Prostate carcinoma presentation, diagnosis, and staging. Cancer 98**:** 1169-1178.

Mitchell, S., P. Abel, M. Ware, G. Stamp and E. N. Lalani, 2000 Phenotypic and genotypic characterization of commonly used human prostatic cell lines. BJU International 85**:** 932-944.

Mohn, F., D. Handler and J. Brennecke, 2015 piRNA-guided slicing specifies transcripts for Zucchini-dependent, phased piRNA biogenesis. Science 348**:** 812-817.

Ng, K. W., C. Anderson, E. A. Marshall, B. C. Minatel, K. S. S. Enfield *et al.*, 2016 Piwi-interacting RNAs in cancer: emerging functions and clinical utility. Molecular Cancer 15**:** 5.
Olivier, M., M. Hollstein and P. Hainaut, 2010 TP53 mutations in human cancers: origins, consequences, and clinical use. Cold Spring Harbor Perspectives in Biology 2**:** a001008.
Olson, S. H., L. Mignone, C. Nakraseive, T. Caputo, R. Barakat *et al.*, 2001 Symptoms of ovarian cancer. Obstetrics & Gynecology 98**:** 212-217.
Öner, Ç., D. Turgut Coşan and E. Çolak, 2016 Estrogen and Androgen Hormone Levels Modulate the Expression of PIWI Interacting RNA in Prostate and Breast Cancer. PLOS One 11**:** e0159044.
Ozata, D. M., I. Gainetdinov, A. Zoch, D. O'Carroll and P. D. Zamore, 2019 PIWI-interacting RNAs: small RNAs with big functions. Nature Reviews Genetics 20**:** 89-108.
Palma Flores, C., R. García-Vázquez, D. Gallardo Rincón, E. Ruiz-García, H. Astudillo de la Vega *et al.*, 2017 MicroRNAs driving invasion and metastasis in ovarian cancer: Opportunities for translational medicine. International Journal of Oncology 50**:** 1461-1476.
Pan, Y., M. Hu, H. Liang, J.-j. Wang and L.-j. Tang, 2012 The expression of the PIWI family members miwi and mili in mice testis is negatively affected by estrogen. Cell and Tissue Research 350**:** 177-181.
Park, J. W., J. K. Lee, J. W. Phillips, P. Huang, D. Cheng *et al.*, 2016 Prostate epithelial cell of origin determines cancer differentiation state in an organoid transformation assay. Proceedings of the National Academy of Sciences 113**:** 4482-4487.
Perner, S., J.-M. Mosquera, F. Demichelis, M. D. Hofer, P. L. Paris *et al.*, 2007 TMPRSS2-ERG fusion prostate cancer: an early molecular event associated with invasion. The American Journal of Surgical Pathology 31**:** 882-888.
Pharoah, P. D., and B. A. Ponder, 2002 The genetics of ovarian cancer. Best Practice & Research Clinical Obstetrics & Gynaecology 16**:** 449-468.
Prat, J., 2012 Ovarian carcinomas: five distinct diseases with different origins, genetic alterations, and clinicopathological features. Virchows Archiv 460**:** 237-249.
Prat, J., and F. C. o. G. Oncology, 2014 Staging classification for cancer of the ovary, fallopian tube, and peritoneum. International Journal of Gynecology & Obstetrics 124**:** 1-5.
Qu, X., J. Liu, X. Zhong, X. Li and Q. Zhang, 2015 PIWIL2 promotes progression of non-small cell lung cancer by inducing CDK2 and Cyclin A expression. Journal of Translational Medicine 13**:** 1.
Ramat, A., and M. Simonelig, 2020 Functions of PIWI Proteins in Gene Regulation: New Arrows Added to the piRNA Quiver. Trends in Genetics.
RCOG/BSGE, 2011 Management of suspected ovarian masses in premenopausal women.
Reuter, M., S. Chuma, T. Tanaka, T. Franz, A. Stark *et al.*, 2009 Loss of the Mili-interacting Tudor domain–containing protein-1 activates transposons and alters the Mili-associated small RNA profile. Nature Structural & Molecular Biology 16**:** 639.

Roy, J., and B. Mallick, 2018 Investigating piwi-interacting RNA regulome in human neuroblastoma. Genes, Chromosomes and Cancer 57: 339-349.
Roy, R., J. Chun and S. N. Powell, 2012 BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nature Reviews Cancer 12: 68-78.
Saito, K., Y. Sakaguchi, T. Suzuki, T. Suzuki, H. Siomi *et al.*, 2007 Pimet, the Drosophila homolog of HEN1, mediates 2′-O-methylation of Piwi-interacting RNAs at their 3′ ends. Genes & Development 21: 1603-1608.
Salomon-Perzyński, A., M. Salomon-Perzyńska, B. Michalski and V. Skrzypulec-Plinta, 2017 High-grade serous ovarian cancer: the clone wars. Archives of Gynecology and Obstetrics 295: 569-576.
Sandberg, A. A., 1980 Endocrine control and physiology of the prostate. The Prostate 1: 169-184.
Sartor, O., and J. S. de Bono, 2018 Metastatic prostate cancer. New England Journal of Medicine 378: 645-657.
Sato, K., and M. C. Siomi, 2015 Functional and structural insights into the piRNA factor Maelstrom. FEBS letters 589: 1688-1693.
Sato, K., and M. C. Siomi, 2020 The piRNA pathway in Drosophila ovarian germ and somatic cells. Proceedings of the Japan Academy, Series B 96: 32-42.
Schmitz, S. U., P. Grote and B. G. Herrmann, 2016 Mechanisms of long noncoding RNA function in development and disease. Cellular and Molecular Life Sciences 73: 2491-2509.
Schudrowitz, N., S. Takagi, G. M. Wessel and M. Yajima, 2017 Germline factor DDX 4 functions in blood-derived cancer cell phenotypes. Cancer Science 108: 1612-1619.
Shen, S., H. Yu, X. Liu, Y. Liu, J. Zheng *et al.*, 2018 PIWIL1/piRNA-DQ593109 Regulates the Permeability of the Blood-Tumor Barrier via the MEG3/miR-330-5p/RUNX3 Axis. Molecular Therapy - Nucleic Acids 10: 412-425.
Shibue, T., and R. A. Weinberg, 2017 EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. Nature Reviews Clinical Oncology 14: 611.
Shoji, M., T. Tanaka, M. Hosokawa, M. Reuter, A. Stark *et al.*, 2009 The TDRD9-MIWI2 complex is essential for piRNA-mediated retrotransposon silencing in the mouse male germline. Developmental Cell 17: 775-787.
Siddiqi, S., M. Terry and I. Matushansky, 2012 Hiwi mediated tumorigenesis is associated with DNA hypermethylation. PLOS One 7: e33711.
Siegel, R. L., K. D. Miller and A. Jemal, 2020 Cancer statistics, 2020. CA: A Cancer Journal for Clinicians 70: 7-30.
Sienski, G., D. Dönertas and J. Brennecke, 2012 Transcriptional Silencing of Transposons by Piwi and Maelstrom and Its Impact on Chromatin State and Gene Expression. Cell 151: 964-980.

Simon, B., J. P. Kirkpatrick, S. Eckhardt, M. Reuter, E. A. Rocha *et al.*, 2011 Recognition of 2′-O-methylated 3′-end of piRNA by the PAZ domain of a Piwi protein. Structure 19**:** 172-180.
Singh, G., J. Roy, P. Rout and B. Mallick, 2018 Genome-wide profiling of the PIWI-interacting RNA-mRNA regulatory networks in epithelial ovarian cancers. PLOS One 13**:** e0190485.
Siomi, M. C., T. Mannen and H. Siomi, 2010 How does the Royal Family of Tudor rule the PIWI-interacting RNA pathway? Genes & Development 24**:** 636-646.
Siomi, M. C., K. Sato, D. Pezic and A. A. Aravin, 2011 PIWI-interacting small RNAs: the vanguard of genome defence. Nature Reviews Molecular Cell Biology 12**:** 246-258.
Slack, F. J., and A. M. Chinnaiyan, 2019 The role of non-coding RNAs in oncology. Cell 179**:** 1033-1055.
Slomovitz, B., C. Gourley, M. S. Carey, A. Malpica, I.-M. Shih *et al.*, 2020 Low-grade serous ovarian cancer: State of the science. Gynecologic Oncology 156**:** 715-725.
Song, E., 2016 *The long and short non-coding RNAs in cancer biology*. Springer.
Song, K., L. Dai, X. Long, W. Wang and W. Di, 2020 Follicle-stimulating hormone promotes the proliferation of epithelial ovarian cancer cells by activating sphingosine kinase. Scientific Reports 10**:** 1-13.
Soper, S. F. C., G. W. van der Heijden, T. C. Hardiman, M. Goodheart, S. L. Martin *et al.*, 2008 Mouse Maelstrom, a component of nuage, is essential for spermatogenesis and transposon repression in meiosis. Developmental Cell 15**:** 285-297.
Syed, V., G. Ulinski, S. C. Mok and S.-M. Ho, 2002 Reproductive hormone-induced, STAT3-mediated interleukin 6 action in normal and malignant human ovarian surface epithelial cells. Journal of the National Cancer Institute 94**:** 617-629.
Tan, M., H. T. van Tol, D. Rosenkranz, E. F. Roovers, M. J. Damen *et al.*, 2020 PIWIL3 forms a complex with TDRKH in mammalian oocytes. Cells 9**:** 1356.
Tan, T. Z., H. Yang, J. Ye, J. Low, M. Choolani *et al.*, 2015a CSIOVDB: a microarray gene expression database of epithelial ovarian cancer subtype. Oncotarget 6**:** 43843.
Tan, Y., L. Liu, M. Liao, C. Zhang, S. Hu *et al.*, 2015b Emerging roles for PIWI proteins in cancer. Acta Biochimica et Biophysica Sinica 47**:** 315-324.
Telloni, S. M., 2017 Tumor Staging and Grading: A Primer, pp. 1-17 in *Molecular Profiling: Methods and Protocols*, edited by V. Espina. Springer New York, New York, NY.
Teo, M. Y., D. E. Rathkopf and P. Kantoff, 2019 Treatment of advanced prostate cancer. Annual Review of Medicine 70**:** 479-499.
Tiwari, B., A. E. Jones and J. M. Abrams, 2018 Transposons, p53 and genome security. Trends in Genetics 34**:** 846-855.
Tomlins, S. A., D. R. Rhodes, S. Perner, S. M. Dhanasekaran, R. Mehra *et al.*, 2005 Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310**:** 644-648.

Torre, L. A., B. Trabert, C. E. DeSantis, K. D. Miller, G. Samimi *et al.*, 2018 Ovarian cancer statistics, 2018. CA: A Cancer Journal for Clinicians 68**:** 284-296.

Vang, R., D. A. Levine, R. A. Soslow, C. Zaloudek, I.-M. Shih *et al.*, 2016 Molecular alterations of TP53 are a defining feature of ovarian high-grade serous carcinoma: a rereview of cases lacking TP53 mutations in The Cancer Genome Atlas Ovarian Study. International Journal of Gynecological Pathology 35**:** 48.

von Eyss, B., L. A. Jaenicke, R. M. Kortlever, N. Royla, K. E. Wiese *et al.*, 2015 A MYC-Driven Change in Mitochondrial Dynamics Limits YAP/TAZ Function in Mammary Epithelial Cells and Breast Cancer. Cancer Cell 28**:** 743-757.

Wang, D.-W., Z.-H. Wang, L.-L. Wang, Y. Song and G.-Z. Zhang, 2013 Overexpression of hiwi promotes growth of human breast cancer cells. Asian Pacific Journal of Cancer Prevention 15**:** 7553-7558.

Wang, H., B. Wang, J. Liu, A. Li, H. Zhu *et al.*, 2018 Piwil1 gene is regulated by hypothalamic-pituitary-gonadal axis in turbot (Scophthalmus maximus): A different effect in ovaries and testes. Gene 658**:** 86-95.

Wang, Q., W. Li, X. S. Liu, J. S. Carroll, O. A. Jänne *et al.*, 2007 A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. Molecular Cell 27**:** 380-392.

Wang, Q.-E., C. Han, K. Milum and A. A. Wani, 2011 Stem cell protein Piwil2 modulates chromatin modifications upon cisplatin treatment. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 708**:** 59-68.

Wang, X., X. Tong, H. Gao, X. Yan, X. Xu *et al.*, 2014 Silencing HIWI suppresses the growth, invasion and migration of glioma cells. International Journal of Oncology 45**:** 2385+.

Wang, Y., Y. Jiang, N. Ma, B. Sang, X. Hu *et al.*, 2015 Overexpression of Hiwi Inhibits the Growth and Migration of Chronic Myeloid Leukemia Cells. Cell Biochemistry and Biophysics 73**:** 117-124.

Watson, P. A., V. K. Arora and C. L. Sawyers, 2015 Emerging mechanisms of resistance to androgen receptor inhibitors in prostate cancer. Nature Reviews Cancer 15**:** 701.

Webb, P. M., and S. J. Jordan, 2017 Epidemiology of epithelial ovarian cancer. Best Practice & Research Clinical Obstetrics & Gynaecology 41**:** 3-14.

Wenda, J. M., D. Homolka, Z. Yang, P. Spinelli, R. Sachidanandam *et al.*, 2017 Distinct Roles of RNA Helicases MVH and TDRD9 in PIWI Slicing-Triggered Mammalian piRNA Biogenesis and Function. Developmental Cell 41**:** 623-637.e629.

Wylie, A., A. E. Jones, A. D'Brot, W.-J. Lu, P. Kurtz *et al.*, 2016 p53 genes function to restrain mobile elements. Genes & Development 30**:** 64-77.

Xiao, L., R. B. Lanz, A. Frolov, P. D. Castro, Z. Zhang *et al.*, 2016 The germ cell gene TDRD1 as an ERG target gene and a novel prostate cancer biomarker. The Prostate 76**:** 1271-1284.

Xiao, L., Y. Wang, Y. Zhou, Y. Sun, W. Sun *et al.*, 2010 Identification of a novel human cancer/testis gene MAEL that is regulated by DNA methylation. Molecular Biology Reports 37**:** 2355-2360.
Xie, Y., Y. Yang, D. Ji, D. Zhang, X. Yao *et al.*, 2015 Hiwi downregulation, mediated by shRNA, reduces the proliferation and migration of human hepatocellular carcinoma cells. Molecular Medicine Reports 11**:** 1455+.
Xiol, J., P. Spinelli, M. A. Laussmann, D. Homolka, Z. Yang *et al.*, 2014 RNA clamping by Vasa assembles a piRNA amplifier complex on transposon transcripts. Cell 157**:** 1698-1711.
Xu, Y., S.-Y. Chen, K. N. Ross and S. P. Balk, 2006 Androgens induce prostate cancer cell proliferation through mammalian target of rapamycin activation and post-transcriptional increases in cyclin D proteins. Cancer Research 66**:** 7783-7792.
Yan, H., Q. Wu, C. Sun, L. Ai, J. Deng *et al.*, 2015 piRNA-823 contributes to tumorigenesis by regulating de novo DNA methylation and angiogenesis in multiple myeloma. Leukemia 29**:** 196-206.
Yang, L., L. Bi, Q. Liu, M. Zhao, B. Cao *et al.*, 2015a Hiwi Promotes the Proliferation of Colorectal Cancer Cells via Upregulating Global DNA Methylation. Disease Markers 2015**:** 10.
Yang, Y., X. Zhang, D. Song and J. Wei, 2015b Piwil2 modulates the invasion and metastasis of prostate cancer by regulating the expression of matrix metalloproteinase-9 and epithelial-mesenchymal transitions. Oncology Letters 10**:** 1735-1740.
Ye, Y., D.-T. Yin, L. Chen, Q. Zhou, R. Shen *et al.*, 2010 Identification of Piwil2-Like (PL2L) Proteins that Promote Tumorigenesis. PLOS One 5**:** e13406.
Yuan, L., Y. Xiao, Q. Zhou, D. Yuan, B. Wu *et al.*, 2014 Proteomic analysis reveals that MAEL, a component of nuage, interacts with stress granule proteins in cancer cells. Oncology Reports 31**:** 342-350.
Zamudio, N., and D. Bourc'his, 2010 Transposable elements in the mammalian germline: a comfortable niche or a deadly trap? Heredity 105**:** 92.
Zeng, G., D. Zhang, X. Liu, Q. Kang, Y. Fu *et al.*, 2017 Co-expression of Piwil2/Piwil4 in nucleus indicates poor prognosis of hepatocellular carcinoma. Oncotarget 8**:** 4607.
Zhang, D., P. Duarte-Guterman, V. S. Langlois and V. L. Trudeau, 2010 Temporal expression and steroidal regulation of piRNA pathway genes (mael, piwi, vasa) during Silurana (Xenopus) tropicalis embryogenesis and early larval development. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 152**:** 202-206.
Zhang, D., X. Wu, X. Liu, C. Cai, G. Zeng *et al.*, 2017 Piwil2-transfected human fibroblasts are cancer stem cell-like and genetically unstable. Oncotarget 8**:** 12259.
Zhang, H., Y. Ren, H. Xu, D. Pang, C. Duan *et al.*, 2013a The expression of stem cell protein Piwil2 and piR-932 in breast cancer. Surgical Oncology 22**:** 217-223.

Zhang, Y., L. Cao, D. Nguyen and H. Lu, 2016 TP53 mutations in epithelial ovarian cancer. Translational Cancer Research 5**:** 650-663.
Zhang, Z., Y. Zhu, Y. Lai, X. Wu, Z. Feng *et al.*, 2013b Follicle-stimulating hormone inhibits apoptosis in ovarian cancer cells by regulating the OCT4 stem cell signaling pathway. International Journal of Oncology 43**:** 1194-1204.
Zheng, W., J. J. Lu, F. Luo, Y. Zheng, Y.-j. Feng *et al.*, 2000 Ovarian Epithelial Tumor Growth Promotion by Follicle-Stimulating Hormone and Inhibition of the Effect by Luteinizing Hormone. Gynecologic Oncology 76**:** 80-88.
Zhou, Y., H. Zhong, S. Liu, F. Yu, J. Hu *et al.*, 2014 Elevated expression of Piwi and piRNAs in ovaries of triploid crucian carp. Molecular and Cellular Endocrinology 383**:** 1-9.