Transcriptome analysis of the epididymis from *Plag1* deficient mice suggests dysregulation of sperm maturation and extracellular matrix genes

Joanne Wong | Anastasios Damdimopoulos | Pauliina Damdimopoulou | Jemma G. Gasperoni | Stephanie C. Tran | Sylvia V.H. Grommen | Bert De Groef | Sebastian Dworkin

1Department of Physiology, Anatomy and Microbiology, School of Life Sciences, La Trobe University, Bundoora, Victoria, Australia
2Bioinformatics and Expression Analysis core facility, Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden
3Department of Clinical Science, Intervention and Technology, Karolinska Institutet, Stockholm, Sweden

Correspondence
Sebastian Dworkin, Department of Physiology, Anatomy and Microbiology, School of Life Sciences, La Trobe University, Bundoora VIC 3086, Australia. Email: s.dworkin@latrobe.edu.au

Present address
Bert De Groef, Department of Biology, KU Leuven, Leuven, Belgium

**Abstract**

**Background:** The transcription factor pleomorphic adenoma gene 1 (PLAG1) is required for male fertility. Mice deficient in PLAG1 exhibit decreased sperm motility and abnormal epididymal tubule elongation and coiling, indicating impaired sperm maturation during epididymal transit. However, the downstream transcriptomic profile of the *Plag1* knockout (KO; *Plag1*−/−) murine epididymis is currently unknown.

**Results:** In this study, the PLAG1-dependent epididymal transcriptome was characterised using RNA sequencing. Several genes important for the control of sperm maturation, motility, capacitation and the acrosome reaction were dysregulated in *Plag1*−/− mice. Surprisingly, several cell proliferation genes were upregulated, and Ki67 analysis indicated that cell proliferation is aberrantly upregulated in the cauda epididymis stroma of *Plag1*−/− mice. Gene ontology analysis showed an overall upregulation of genes encoding extracellular matrix components, and an overall downregulation of genes encoding metalloendopeptidases in the epididymides from *Plag1*−/− mice.

**Conclusion:** Together, these results suggest a defect in the epididymal extracellular matrix in *Plag1*−/− mice. These results imply that in addition to maintaining epididymal integrity directly, PLAG1 may also regulate several genes involved in the regulation of sperm maturation and capacitation. Moreover, PLAG1 may also be involved in regulating tissue homeostasis and ensuring proper structure and maintenance of the extracellular matrix in the epididymis.

1 | **INTRODUCTION**

Pleomorphic adenoma gene 1 (PLAG1) is a highly-conserved zinc-finger transcription factor required for growth,1 male fertility,2 normal sperm production and motility,3 and for normal epididymis development and morphology.2 *Plag1* knockout (KO; *Plag1*−/−) mice exhibit growth retardation during both embryonic and postnatal development, resulting in a ~30% decreased size in adulthood.1 *Plag1*−/− mice also present with substantial fertility defects. Conception rates for male *Plag1*−/− mice are reduced when paired with *Plag1*+/+ females,1 while we have previously shown in a 5 month breeding analysis that *Plag1*−/− males are completely
infertile, despite normal plugging behavior. The number of motile spermatozoa present in the semen from the cauda of KO mice was reduced by 49% compared to their Plag1+/+ counterparts and progressive motility was reduced by 80%. As spermatozoa gain motility during epididymal transit, these results indicate that sperm maturation is substantially impaired in Plag1−/− mice, however whether these effects are due to cell-autonomous defects in sperm, or nonautonomous defects due to epididymal dysfunction, is currently unknown.

The epididymis is divided into 10 transcriptionally unique segments in mice. During epididymal sperm maturation, spermatozoa undergo a precise sequence of biochemical and structural modifications, which is facilitated by the tightly regulated transcriptomes of these segments. The epididymides of Plag1−/− mice display reduced relative weight, decreased tubule coiling and elongation, and the loss of morphology of the typical bulbous shape of the cauda. It is therefore possible that the fertility defects in Plag1−/− mice are due to loss of epididymal segment identity resulting in altered gene transcription in the epididymis, and ultimately leading to impaired sperm maturation as spermatozoa transit though the duct. Disruption to gene transcription in the epididymis is known to result in dysfunctional, immature or immotile sperm in various rodent models, and has also been implicated in azoospermic human male infertility.

We hypothesised that the underlying cause of the sperm motility defects observed in Plag1−/− mice is due to aberrant transcriptional regulation in the Plag1−/− epididymis. To that end, we sought to characterise the transcriptome of the Plag1−/− epididymis in order to identify genes and pathways that are dysregulated following deletion of Plag1.

2 | RESULTS

2.1 Transcriptome analysis of Plag1−/− and Plag1+/+ mice mouse epididymis

RNA-Seq was used to analyse and compare the transcriptomes of epididymides from 7-week-old Plag1−/− and Plag1+/+ mice. Although the epididymis is often divided into 10, transcriptionally distinct segments in mice, the shortened, dysmorphic and aberrantly coiled epididymis in Plag1−/− mice made it unfeasible to accurately dissect and sequence “match” transcriptionally-analogous segments or regions from Plag1−/− and Plag1+/+ epididymides. Rather, we reasoned that analysis of the transcriptome from whole Plag1−/− epididymides would allow us to accurately determine the overall molecular profile of the Plag1−/− epididymis. We found that 1728 genes were differentially expressed in the epididymis of Plag1−/− compared to Plag1+/+ mice; 1102 genes were upregulated and 626 genes were downregulated (Figure 1). The top-10 up- and downregulated genes in Plag1−/− compared to Plag1+/+ are listed in Supporting Table 2. Additionally, we performed RNA-Seq comparing the transcriptome in epididymides from Plag1+/+ with that of Plag1+/− mice, but did not find any differentially regulated genes (Figure 1). For standard validation of the RNA-Seq, Mepla, Spink14 and Timp2 were arbitrarily chosen from the list of dysregulated genes for confirmation of differential gene expression by quantitative polymerase chain reaction (qPCR), using samples from animals separate to those that were used in the RNA-Seq. Validation of Defb9 expression, which was not differentially expressed in the epididymis from Plag1−/− mice in the RNA-Seq dataset, was also included. We validated the differential expression of Mepla, Spink14 and Timp2 in the epididymides of Plag1−/− mice; fold changes in mRNA as determined by qPCR are shown alongside fold changes as determined by RNA-Seq and mRNA expression levels determined by qPCR, confirming the differential expression of Mepla, Spink14 and Timp2 (t-test, P < .01) are shown (Figure 1). RNA-Seq indicated that Defb9 was not differentially expressed in the epididymides of Plag1−/− mice, and this was confirmed by qPCR (P = .121; Figure 1). Together, these data validate our RNA-Seq analysis as a robust methodology to determine the PLAG1-dependent transcriptome within the murine epididymis.

DAVID functional annotation analysis was performed to identify genes associated with sperm maturation that are dysregulated in the epididymis of Plag1−/− mice. This analysis revealed that a number of genes involved in sperm maturation, motility, capacitation, the acrosome reaction and the prevention of premature sperm capacitation were dysregulated in Plag1−/− mice (Tables 1,2). Surprisingly, despite the fact that the epididymis of Plag1−/− mice is underdeveloped, several cell proliferation genes were significantly upregulated in the epididymis from Plag1−/− mice. Indeed, GO analysis revealed that the GO term “Regulation of cell proliferation” was overrepresented; 18 genes significantly upregulated in the epididymis from Plag1−/− mice are categorised under this term (Table 3). Next, we performed GO analysis to identify pathways and processes the were affected in the epididymis of Plag1−/− mice, which showed an overall down-regulation of genes involved in cell migration, cell motility, metalloendopeptidase activity and metallopeptidase activity in Plag1−/− mice. Additionally, GO analysis also showed an overall upregulation
of genes associated with cell adhesion, molecular binding, and extracellular matrix (ECM) components in KOs (Figure 2). Taken together, these data indicate a defective maintenance and integrity of the epididymis in Plag1−/− mice. Enriched “biological process”, “molecular function” and “cellular component” GO terms and dysregulated genes in the epididymis of Plag1−/− mice categorised under the GO terms “Metalloendopeptidase activity” and “Extracellular matrix component” and are listed in supporting tables (supporting Tables 3-7).

2.2 | Cell proliferation in the epididymis from Plag1−/− mice

Ki67 staining and quantification was used to determine if the overall upregulation of cell proliferation genes
### TABLE 1  
Selection of significantly upregulated genes with functions related to sperm maturation in the epididymis of *Plag1* knockout mice compared to wild-type mice

| Gene symbol | Gene name | FDR   | LFC   | TPM | Known functions                                                                 | References |
|-------------|-----------|-------|-------|-----|--------------------------------------------------------------------------------|------------|
| Acrv1       | Acrosomal vesicle protein 1 | 0.027 | 1.10  | 1.43 | Involved in sperm-oolemma binding in humans                                      | 11,12      |
|             |           |       |       |     | Segment-specific expression in the mouse epididymis                             |            |
| Adam2       | A disintegrin and metallopeptidase domain 2 | 0.040 | 1.60  | 0.65 | Encodes subunit of the sperm membrane glycoprotein fertilin                      | 13-15      |
|             |           |       |       |     | Deletion results in male infertility, defective sperm migration from uterus to oviduct, and defective sperm aggregation and zona pellucida binding in mice |            |
|             |           |       |       |     | Required for ADAM3 function                                                      |            |
| Adam3       | A disintegrin and metallopeptidase domain 3 (cyritestin) | 0.031 | 1.60  | 1.91 | Deletion results in male infertility, defective sperm migration from uterus to oviduct, and defective sperm aggregation and zona pellucida binding in mice | 15-18      |
|             |           |       |       |     | Plays a role in regulating murine, human and bovine sperm motility, capacitation and acrosome reaction | 19-21      |
| Akap3       | A-Kinase anchoring protein | 0.024 | 1.88  | 0.99 | Undergoes tyrosine phosphorylation during sperm capacitation in mice, which may regulate glycolysis during capacitation | 22         |
| Aldoart1    | Aldolase 1 A, retrogene 1 | 0.026 | 1.22  | 0.29 | Mutations result in primary ciliary dyskinesia and male infertility               | 23,24      |
|             |           |       |       |     | Localized to sperm flagella in human sperm and murine sperm annulus during flagellar development | 25-27      |
| Atp1a4      | ATPase Na+/K+ transporting subunit alpha 4 | 0.039 | 1.17  | 1.65 | Deletion in mice results in reduced sperm motility; sperm unable to fertilize egg; abnormal ion regulation in sperm causing bend in sperm flagellum | 23,24      |
|             |           |       |       |     | Regulates sperm capacitation in bulls                                            |            |
| Dnajb13     | DnaJ heat shock protein family member B13 | 0.012 | 0.82  | 3.86 | Mutations result in primary ciliary dyskinesia and male infertility               | 25-27      |
|             |           |       |       |     | Localized to sperm flagella in human sperm and murine sperm annulus during flagellar development |            |
| Txndc3      | Thioredoxin domain-containing protein 3/2 | 0.023 | 1.73  | 0.40 | *Txndc3/Txndc2-deficient mice exhibit age-related sperm motility loss             | 28,29      |
| Txndc2      |           | 0.049 | 0.61  | 2.46 | *Txndc3 is required for sperm flagellar axoneme; mutations implicated in human primary ciliary dyskinesia, which is associated with male infertility |            |

Abbreviations: FDR, false-discovery rate; HET, heterozygous; KO, knockout; LFC, log-fold change KO compared to WT; TPM, transcripts per kilobase million; WT, wild-type.
| Gene symbol | Gene name                                | FDR  | LFC  | TPM       | WT     | HET    | KO     | Known functions                                                                 | References |
|-------------|------------------------------------------|------|------|-----------|--------|--------|--------|---------------------------------------------------------------------------------|------------|
| Akt2        | Thymoma viral proto-oncogene 2          | 0.043| −0.20| 30.28     | 27.85  | 27.17  | • Deletion results in decreased fertilization in vitro, sperm motility and concentration, and increased apoptotic sperm | 30         |
| BspH2       | Binder of sperm protein homolog 2        | 0.013| −0.84| 2261.00   | 2607.40| 1758.40| • Murine BSPh2 protein on sperm surface is modified during sperm capacitation    | 31         |
| Pebp1       | Phosphatidylethanolamine binding protein 1| 0.0498| −0.24| 1558.94   | 1394.72| 1384.99| • Can act as a decapacitation factor or as a membrane receptor that facilitates the loss of decapacitation factors • Localized to the acrosome and flagellum of murine and human sperm, and to bovine epididymosomes • Deletion results in premature capacitation in murine sperm | 32-35      |
| Rgn         | Regucalcin                               | 0.022| −0.45| 54.00     | 51.78  | 41.76  | • Involved in sperm maturation via regulation of epididymal Ca²⁺ levels • Overexpression in rats results in decreased epididymis epithelial cell height, decreased sperm counts and motility, and increased sperm viability and frequency of morphologically normal sperm | 36         |
| Smox        | Spermine oxidase                         | 0.045| −0.44| 25.49     | 21.43  | 20.15  | • Encoded enzyme required for spermine to spermidine oxidation • High concentrations of spermine facilitate the inhibition of the acrosome reaction in bovine sperm | 37         |
| Spink10     | Serine peptidase inhibitor 2C Kazal type 10| 0.028| −0.42| 398.56    | 367.64 | 314.47 | • Encodes a Kazal family protease inhibitor, which are crucial in regulating sperm membrane protein modifications • Imbalance between proteases and protease inhibitors results in azoospermia, low fertility and impaired sperm functions | 38,39      |
| Spinkl      | Serine protease inhibitor 2C Kazal type-like | 0.012| −0.74| 248.93    | 205.54 | 167.47 | • Acts as a decapacitation factor, suppressing premature murine sperm capacitation in vitro • Can bind to murine sperm and increase motility | 40,41      |

Abbreviations: FDR, false-discovery rate; HET, heterozygous; KO, knockout; LFC, log-fold change KO compared to WT; TPM, transcripts per kilobase million; WT, wild-type.
TABLE 3  Upregulated genes categorised under the GO term ‘Regulation of cell proliferation (GO: 0042127) in the epididymis from Plag1 knockout vs wild-type mice

| Gene symbol | Gene name                                      | FDR      | LFC       | TPM WT  | TPM HET | TPM KO |
|-------------|------------------------------------------------|----------|-----------|---------|---------|--------|
| *Sparc*     | Secreted acidic cysteine rich glycoprotein    | 1.40E–3  | 0.72      | 431.65  | 488.10  | 753.94 |
| *EdnrB*     | Endothelin receptor type B                    | 1.40E–3  | 0.90      | 4.65    | 5.05    | 8.64   |
| *Lgals9*    | Lectin, galactose binding, soluble 9          | 1.74E–3  | 0.71      | 11.00   | 12.75   | 19.00  |
| *Trf*       | Transferrin                                   | 1.61E–3  | 1.13      | 10.78   | 12.10   | 22.71  |
| *Scube2*    | Signal peptide, cub domain, EGF-like 2        | 1.85E–3  | 1.82      | 0.44    | 0.58    | 1.59   |
| *Hoxa5*     | Homeobox A5                                   | 1.25E–3  | 0.70      | 6.44    | 6.67    | 10.56  |
| *Aldh1a2*   | Aldehyde dehydrogenase family 1, subfamily A2 | 9.78E–4  | 0.87      | 15.64   | 17.51   | 29.55  |
| *Gas1*      | Growth arrest specific 1                      | 1.99E–3  | 0.56      | 18.25   | 19.26   | 28.71  |
| *Efemp1*    | Epidermal growth factor-containing fibulin-like extracellular matrix protein 1 | 1.33E–3  | 0.82      | 31.22   | 36.25   | 56.05  |
| *Tgfbr2*    | Transforming growth factor-beta receptor 2    | 1.33E–3  | 0.63      | 9.58    | 10.33   | 15.68  |
| *Timp2*     | Tissue inhibitor of metalloproteinase 2       | 1.23E–3  | 0.72      | 59.02   | 66.84   | 102.61 |
| *Cxcl12*    | Chemokine (c-x-c motif) ligand 12             | 1.40E–3  | 0.42      | 18.06   | 17.39   | 24.07  |
| *Cx3cr1*    | Chemokine (c-x3-c) receptor 1                | 1.40E–3  | 0.71      | 4.68    | 5.55    | 7.52   |
| *Ptn*       | Pleiotrophin                                  | 1.96E–3  | 0.93      | 8.14    | 8.96    | 15.02  |
| *Igf1*      | Insulin-like growth factor 1                  | 9.78E–3  | 1.23      | 6.25    | 7.58    | 14.79  |
| *Htra1*     | Htra serine peptidase 1                      | 1.40E–3  | 0.52      | 38.28   | 39.28   | 56.25  |
| *Cdh5*      | Cadherin 5                                    | 1.78E–3  | 0.79      | 7.04    | 7.70    | 13.01  |
| *Wt1*       | Wilms tumor 1 homolog                         | 2.01E–3  | 1.82      | 0.86    | 1.29    | 3.18   |

Abbreviations: FDR, false-discovery rate; HET, heterozygous; KO, knockout; LFC, log-fold change KO compared to WT; TPM, transcripts per kilobase million; WT, wild-type.

FIGURE 2  Top-5 overrepresented GO terms determined by GOrilla analysis, within the dataset of significantly dysregulated genes in epididymides from Plag1 knockout mice compared to wild-type mice. A, Overrepresented GO terms within significantly upregulated genes. B, Overrepresented GO terms within significantly downregulated genes. There was no cellular component GO terms in the list of downregulated genes.
Table 3 correlates with increased cellular proliferation, in the epididymis of Plag1−/− mice. The percentage of Ki67+ cells was significantly higher in the stroma of the cauda epididymis from Plag1−/− compared to Plag1+/+ mice ($t$-test, $P = .0007$), but not in the epithelium ($P = .9889$) (Figure 3). There was no difference in the percentage of Ki67+ cells in the epithelium or stroma in the caput and corpus between genotypes ($P > .05$). There was a significantly higher percentage of stromal cells (relative to the total number of cells) in the caput ($t$ test, $P = .0033$), corpus ($P = .0028$) and cauda ($P = .0080$) of Plag1−/− mice compared to Plag1+/+ (Figure 3). These data indicate that the stroma, albeit not the epididymal epithelium of Plag1−/− mice, is hyperproliferative relative to WT controls.

2.3 PLAG1 binding sites are not enriched in dysregulated genes

To determine if PLAG1 binding sites were enriched in the target genes determined by the RNA-Seq analysis, genes up- or downregulated in the epididymis from Plag1−/− mice were analyzed for significant enrichment of PLAG1 binding sites using the PLAG1 motif from the JASPAR database and the binding sites defined by Madissoon et al.42 This motif comprises two G-rich regions (a GRGGC Core and a G-Cluster) separated by 7 nonconserved nucleotides, and broadly takes the form "GA/GGGCNNNNNNA/GGGG/T", where N is a random nucleotide and the underlined nucleotides are largely invariant. Of all annotated murine genes, 16 163

![FIGURE 3](image-url) Expression of Ki67 in the epididymis of 7-week-old Plag1 knockout (KO) and wild-type (WT) mice. A, Immunofluorescence staining of Ki67 (red) and DAPI (nuclei, blue) in the epididymis of WTs and KOs. Arrowheads indicate epithelial Ki67 expression; arrows point to stromal Ki67 expression. Scale bars represent 50 μm. B, Quantification of Ki67-positive cells relative to total cell number in the epithelium and stroma of the epididymis of Plag1 KO compared to WT mice (n = 5 per genotype) and quantification of the number of stromal cells relative to total cell number in the epididymis (far right graph) of Plag1 KO compared to WT mice (n = 5 per genotype). Values shown are means ± SEM. **$P < .01$; ***$P < .001$ ($t$ test)
were found to have at least one PLAG1 binding site in their promoters, whereas 7390 did not have any PLAG1 binding sites, suggesting that the presence of a putative PLAG1 site is extremely commonplace, and that enrichment analysis may present more accurate indication of PLAG1 binding activity. The results of the Homer analysis, however, indicated that among the genes dysregulated in the epididymis from Plag1−/− mice, there was no significant enrichment of PLAG1 binding sites. Furthermore, the motif scanning did not reveal significant enrichment of binding sites of any other transcription factors, suggesting that the PLAG1-dependent transcriptome in the epididymis does not comprise an over-represented hierarchical pathway of any known transcription factor families.

3 | DISCUSSION

The cause of male infertility is unidentified in 30% to 45% of clinical cases, exemplifying the lack of knowledge pertaining to the underlying molecular and genetic mechanisms of sperm dysfunction. Several studies using transgenic mice have identified gene candidates involved in poor or abnormal sperm function, including Plag1, required for sperm motility, normal epididymal morphology and male fertility. However, the underlying cause of these defects is unknown and the genes that are controlled by PLAG1 in the epididymis have not yet been characterized. As PLAG1 is expressed in brain and pituitary (as well as elsewhere), we cannot rule out hormonal, metabolic, paracrine signaling or other, nonepididymal contributions to both male infertility and epididymal morphology defects we describe within our study. Nonetheless the abundant widespread expression of PLAG1 in the epithelium and stroma of the adult mouse epididymis points to an important role in male fertility. This study aimed to characterise the epididymal target genes of PLAG1, and to identify processes and functions that may be affected in the epididymis when Plag1 is deficient.

RNA-Seq results showed that there are no significantly differentially expressed genes in the epididymis from Plag1+/− mice compared to Plag1+/+, indicating that one copy of the Plag1 gene is sufficient for normal gene expression. This is in agreement with previous findings that Plag1+/− males have normal fertility, as numbers of litters born and litter size was not affected in the mice. However, Plag1+/− males do exhibit decreased epididymal tubule elongation and coiling in the caput and corpus epididymis, suggesting that although gene expression does not appear to be significantly dysregulated, the morphology of the epididymis in these mice is altered. RNA-Seq revealed a number of dysregulated genes in the Plag1−/− epididymis compared to Plag1+/+, that have known sperm maturation and storage-related functions (see refs. in Tables 1,2) and common functions are seen among these PLAG1 target genes. As spermatozoa were removed from the tissue samples, these genes identified by the RNA-Seq experiment are exclusively epididymal genes. Several genes, such as Txndc3, Txndc2, Dnajb13, Pebp1, and Rgn, are known to play a role in sperm motility or sperm flagellar development, or have been localized to the developing sperm flagellum, and several KO models of these genes exhibit defective sperm motility (see refs. in Tables 1,2). Txndc3 and Dnajb13 have been implicated in primary ciliary dyskinesia (PCD), a disease in humans resulting from dysfunction of motile cilia in which the majority of male sufferers are infertile. The role of PLAG1 in PCD has not yet been studied, but may be a worthy genetic candidate for investigation, as the emerging diagnostic approach for this disease is genetic testing.

Several dysregulated genes in the Plag1−/− epididymis play a role in regulating the acrosome reaction or sperm capacitation (see refs. in Tables 1,2). Interestingly, this includes the downregulation of two genes that encode decapacitation factors (Pebp1, Spinkl), which bind to the sperm head to prevent premature sperm capacitation (see refs. in Table 2). It is crucial for capacitation to be preserved for when the spermatozoon reaches the ovum; premature capacitation results in redundant spermatozoa that are incapable of fertilization. In addition, previous research has shown that the most severe morphological abnormalities in the epididymis of Plag1−/− mice are observed in the cauda region (lack of bulbous shape, reduced tubule elongation and coiling) and that in Plag1+/+ mice, PLAG1 is potentially secreted into the lumen in this region. Altogether, these findings may be indicative of dysfunction of the cauda epididymidis where mature spermatozoa are stored, resulting in premature capacitation and therefore infertility, and future studies should be targeted to examine differential gene expression specifically within this region.

The maintenance of epididymal integrity through the regulation of ion transport also appears to be affected; Atp1a4 and Rgn are dysregulated genes that are related to ion balance within the microenvironment of the epididymal lumen. Atp1a4 (upregulated in the Plag1−/− epididymis) encodes the α4 subunit of the Na,K-ATPase and is required for sperm fertility due to its role in maintaining sperm ion balance, which is crucial for sperm morphology and motility, and fertilization capacity. The deletion of this gene in mice results in infertility in males, reduced sperm motility and abnormal sperm tail morphology, and spermatozoa are unable to fertilize eggs.
in vitro.23 Rgn (downregulated in Plag1/−/−) encodes a Ca\(^{2+}\)-binding protein important for regulating Ca\(^{2+}\) balance, and overexpression of this gene results in increased luminal Ca\(^{2+}\) and decreased sperm motility and sperm counts.36 Dysregulation of these genes may disrupt the movement of ions, sperm ion balance or the epididymal Ca\(^{2+}\) gradient and contribute to the decreased sperm motility and infertility observed in Plag1/−/− mice, or the dysregulation of these genes in the epididymides from Plag1/−/− mice may be a compensatory mechanism. Interestingly, the expression of several genes such as Txndc3 and Dnajb13 have not been reported in the epididymis previously; Txndc3 expression was thought to be testis-specific and Dnajb13 expression has been detected on developing spermatids. However, as spermatozoa were rinsed from the epididymis tissues, it is unlikely that the expression detected was not epididymal expression.

GO analysis revealed several cell proliferation GO terms enriched in the dataset of significantly upregulated genes in the epididymis from Plag1/−/− mice, which is surprising given that the epididymides in these mice are reduced in weight, the tubule is less developed,2 and Plag1 is known to upregulate cell proliferation genes during early development.49 Ki67 quantification showed that cell proliferation was significantly increased in the cauda stroma of the epididymis from Plag1/−/− mice, but not in the caput or corpus, or in the epithelium of any region. The proportion of stromal cells, out of total cells, was significantly increased in all regions of the epididymis from Plag1/−/− mice. These results indicate that cell proliferation is upregulated specifically in the cauda region, which correlates with the morphological defects being most severe in this region in Plag1/−/− mice and may correlate with the dysregulation of ECM components shown by the RNA-Seq data, as the ECM regulates several cell behaviors including cell proliferation.50,51 Although the GO term “positive regulation of epithelial cell proliferation” was also enriched in the upregulated genes in the epididymis from Plag1/−/− mice, the results showed that cell proliferation was normal in the epithelium. The upregulation of these genes may be a compensatory mechanism for the lack of tubule of elongation, which is a defect that persists into adulthood in Plag1/−/− mice.2

GO analysis also revealed that within the list of downregulated genes in the epididymis from Plag1/−/− mice, there was significant enrichment of genes associated with the GO terms cell migration, cell motility, movement of cell or subcellular component and metalloendopeptidase/metallopeptidase activity. The Mmp1b, Mep1a, Mme and Pappa genes encoding metalloproteinases or matrix metalloproteinases (MMPs) were downregulated in the Plag1/−/− epididymis, and are important in degrading proteins in the ECM, thereby playing a crucial role in tissue development, remodeling and repair.52 MMPs are also involved in cell signaling, cell migration and the activation of growth factors,53,54 and are partly regulated by tissue inhibitors of metalloproteinases (TIMPs).52 The balance between MMPs and TIMPs is vital for ECM remodeling and maintenance,52 and it has been shown that MMPs and TIMP2 are present in the epididymal fluid in the boar, ram and stallion, suggesting a role in epididymal sperm maturation.55 Notably, Temp2, which encodes a TIMP protein, was upregulated in the Plag1/−/− epididymis. These results suggest that MMP activity may be decreased by both the downregulation of MMP genes and the upregulation of an MMP inhibitor. GO analysis also showed that among upregulated genes in the epididymides from Plag1/−/− mice, there was significant enrichment of genes mapped to the GO terms cell adhesion, glycosaminoglycan binding, ECM/region and proteinaceous ECM. Notably, a large number of collagen genes was upregulated in Plag1/−/− epididymides (Col6a3, Col12a1, Col14a6, Col15a1, Col6a1, Col23a1, Col1a2, Col6a5, Col14a3, Col4a5, Col11a1, Col3a1, Col6a2, Col5a2, Col6a6, Pcolce, Col1a1, Col9a1, Col5a3, Col5a1, Col4a1, Col14a1, Cogalt2 and Col4a4), and upregulated proteinaceous ECM genes included Spon1, Spon2, Lama2, Lamb1 and Ecm2. Overdeposition of ECM components can result in ECM stiffness or fibrosis in other tissues, leading to diseases such as cancer, cardiovascular disease,56 pulmonary fibrosis,57 and kidney sclerosis.58 ECM stiffness, however, has not yet been investigated in the epididymis. While ECM-related genes are known to be expressed in the Wolffian duct during embryonic development59 and ECM remodeling is recognized as a crucial aspect of epididymis morphogenesis,60 the role of ECM genes in the function of the adult epididymis is not well studied and the implication of ECM defects on sperm maturation are not known. As the ECM acts as a harbor for growth factors,61 it is reasonable to speculate that defective ECM structure would have an impact on the release of growth factors or other proteins that are important for sperm maturation or epididymal tissue homeostasis. Taken together, the downregulation of cell motility and MMP genes, the upregulation of ECM component genes and Temp2, and the increased cell proliferation in the cauda epididymis stroma of Plag1/−/− mice suggest a defect in the maintenance and structure of the ECM, or perhaps disruption of normal tissue homeostasis in the epididymis of Plag1/−/− mice.

PLAG1 binding site analysis on dysregulated genes in the epididymides from Plag1/−/− mice did not show significant enrichment of PLAG1 binding sites. This could indicate either that PLAG1 control of these particular genes may be upstream or indirect, that PLAG1 may act
in conjunction with other transcription factors, or perhaps that epididymal defects occurred earlier in development that we have analyzed during the present study. Moreover, motif scanning did not reveal enrichment of any other transcription factor motifs, providing further indication that the regulation of these target genes may require other transcription factors to act in concert. Some of the effects of PLAG1 deficiency on gene expression may be due to androgen imbalance; Hsd17b3 and Sultle1 (genes involved in the testosterone biosynthesis pathway) were downregulated in the testis from Plag1<sup>−/−</sup> mice and the seminal vesicles from Plag1<sup>−/−</sup> mice were disproportionately reduced in size, indicating decreased testosterone levels. Androgenic control of many epididymal genes has been established, however, some genes involved in sperm maturation are not controlled (or not entirely controlled) by androgens, suggesting that at the molecular level sperm maturation regulation is more complex than a requirement for normal androgen action. For example, Acrv1 mRNA expression in the caput mouse epididymis spikes 8 hours after gonadectomy, but normalises to control levels 1 day post gonadectomy, indicating that Acrv1 is not exclusively regulated by androgens.11

In conclusion, we investigated the genetic pathways that are regulated by PLAG1 in the mouse epididymis and identified dysregulated genes in Plag<sup>1−/−</sup> mice that may be involved in the facilitation of proper sperm maturation, regulating the acrosome reaction and regulating sperm capacitation. Further, we show that there is an overall dysregulation of genes involved in the maintenance and structure of the ECM in Plagi<sup>1−/−</sup> mice, and that cell proliferation is significantly increased in the cauda epididymis from Plag<sup>1−/−</sup> mice. However, since there was no significant enrichment of PLAG1 binding sites in the differentially expressed genes, PLAG1 control of these genes may be indirect and further investigation is required to further elucidate the precise direct targets that PLAG1 binds to and activate in order to influence proper sperm maturation. While the data presented in this study is suggestive of possible premature sperm capacitation and imbalanced ECM deposition and degradation in the epididymis from Plag<sup>1−/−</sup> mice, further experimental validation is required. Future studies analyzing the functional capabilities of mature spermatozoa from Plag<sup>1−/−</sup> mice such as the acrosome reaction, or characterization of the ECM structure in epididymides from Plag<sup>1−/−</sup> mice may be insightful. Moreover, as global defects in Plag<sup>1−/−</sup> mice may also contribute to generalized defects in tissue growth and development, targeted, tissue specific deletion of Plag1 may ultimately be necessary to determine epididymis-specific function. Although PLAG1 mutations have not yet been investigated in the context of fertility in human males; as the underlying causes of clinical male infertility are largely unidentified, we suggest Plag<sup>1</sup> as a worthy candidate for investigation.

4 | EXPERIMENTAL PROCEDURES
4.1.1. | Animals

All animal procedures undertaken in this study were approved by the Animal Ethics Committee of La Trobe University (AEC17-16) and the La Trobe Institutional Biosafety Committee (GMSC17-15). All animal care and experimental procedures were conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8 edition, 2013) of the National Health and Medical Research Council. Plag<sup>1</sup> founder mice were a kind gift from Prof. Wim Van de Ven (Laboratory for Molecular Oncology, Center for Human Genetics, Catholic University of Leuven, Belgium); the generation of the Plag<sup>1−/−</sup> mouse line has been reported previously.1 Genotypes were determined by PCR with genomic DNA isolated from ear clips as previously described.2 RNA sequencing and data analysis

Whole epididymides were used for RNA sequencing (RNA-Seq) in order to characterise the overall transcriptome of the Plag<sup>1−/−</sup> mouse epididymis. Epididymides were harvested from 7-week-old Plag<sup>1−/−</sup>, heterozygous (HET; Plag1<sup>+/−</sup>) and wild-type (WT; Plag1<sup>+/+</sup>) mice (n = 5 per genotype) euthanized by CO₂ asphyxiation, and teased apart in cold phosphate-buffered saline (PBS). The tissues were rinsed in two changes of sterile PBS to remove spermatozoa. The samples were then homogenized using a mortar and pestle in TRIzol Reagent (Invitrogen, Carlsbad, California), according to manufacturer instructions. Total RNA was treated with DNase using the TURBO DNA-free kit (Invitrogen) and subsequently purified using the Zymo RNA Clean & Concentrator-25 kit (Zymo Research Corporation, Irvine, California) following the manufacturer's instructions. RNA concentration was then determined using a Nanodrop (Thermo Fisher, Waltham, Massachusetts). RNA-Seq was carried out at the La Trobe Genomics Platform (La Trobe University, Melbourne, Victoria, Australia). Fifteen libraries were constructed using the Illumina TruSeq Stranded mRNA kit (Illumina, San Diego, California) as per manufacturer instructions and the samples were sequenced by 150-bp, paired-end sequencing using the Illumina NextSeq 500 (Illumina). The raw RNA-Seq data were quality-assessed using fastQC (http://www.bioinformatics. babraham.ac.uk/projects/fastqc/). Adapter and low-quality
fragments were removed using trimmimatic v0.32<sup>64</sup> and the clean reads were then mapped to the *Mus musculus* genome (assembly GRCm38.p6) by HISAT2 v2.0.5<sup>65</sup>. Alignment quality control was performed using RSeQC v2.6.4<sup>66</sup> and expression of each gene was determined by the featureCounts function in subread v1.4.6p5<sup>67</sup>. Genes with low count values were excluded from analysis; genes with valid expression were defined as genes with log counts per million >0.3 in at least three different replicates. In total, 20 505 genes were identified as meeting this criterion. Differential gene expression was then analyzed using edgeR v3.18.1<sup>68</sup> with false-discovery rate < 0.05 set as the cut-off. DAVID<sup>69,70</sup> was used for functional annotation analysis to identify genes associated with sperm-related gene ontology (GO) terms. The GOrilla analysis tool<sup>71</sup> was used to categorize differentially expressed genes into pathways and processes according to functional GO annotations. This allows enriched GO terms and common processes and pathways affected in the data sets to be identified. The RNA-Seq raw data have been deposited to NCBI’s Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE140576.

### 4.1 Quantitative PCR validation of RNA-Seq results

For quantitative PCR (qPCR) validation of the RNA-Seq results, epididymides were collected from different animals to those used for RNA-Seq (n = 5 for each of WT and KO). RNA was isolated as described above and cDNA was reverse-transcribed using MultiScribe reverse transcriptase (Life Technologies, Carlsbad, California), 10× M-MuLV buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, pH 8.3; New England BioLabs, Ipswich, Massachusetts), 10 mM dNTP mix (Bioline, London, UK), oligo(dT)<sub>18</sub> primer (Bioline) and 40 U/μl RiboSafe RNase Inhibitor (Bioline). Reactions were incubated for 10 minutes at 25°C, 2 hours at 42°C and then 5 minutes at 85°C in a thermocycler. qPCR was used to validate differential expression of the genes *Defb9*, *Mep1a*, *Spink14* and *Timp2* (primer sequences listed in supporting Table 1). Each qPCR amplification reaction mix contained 5 μL of cDNA, 300 nM of forward and reverse primer and 5 μL Fast SYBR Green Master Mix (Life Technologies). Reactions underwent the following qPCR protocol using the Biorad CFX96 Real Time System (Biorad, Hercules, California): 20 seconds at 95°C, 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C. All samples were measured in triplicate. *Actb* was used as the reference gene, as expression levels of this gene was not different in the epididymides of *Plag1<sup>−/−</sup>* compared to *Plag1<sup>+/+</sup>* mice according to the RNA-Seq data. qPCR data were processed and fold changes presented using LinRegPCR v.2017.0 software<sup>72</sup> as previously described. Unpaired t-tests were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, California) to compare average normalised mRNA expression levels in the epididymides of *Plag1<sup>−/−</sup>* compared to *Plag1<sup>+/+</sup>* mice, as determined by qPCR.

### 4.2 Ki67 immunofluorescence

Epididymides were collected from adult (7-week-old) mice (n = 5 for each of WT and KO), fixed for 2 hours in 4% (w/v) paraformaldehyde then cryoprotected for 2 hours in 10% (w/v) sucrose followed by 30% sucrose until the tissues sank. Tissues were stored at −80°C until sectioned into 5 μm cryosections, and subsequently stored at −80°C. Thawed sections were first treated with boiling 1 M trisodium citrate dihydrate (VWR, Radnor, Pennsylvania) for 40 minutes for antigen retrieval. Sections were then washed in phosphate-buffered saline (PBS), incubated for 2 hours in PBS containing 4% (w/v) bovine serum albumin (BSA), 0.1% (v/v) Triton X-100, 0.1 M lysine and 5% (v/v) normal goat serum (Antibodies Australia, Melbourne, Australia) (BSA-PBS-TX) at room temperature, then washed in PBS, before overnight incubation at 4°C with anti-Ki67 antibody (ab15580; Abcam, Cambridge, United Kingdom) diluted 1:500 in BSA-PBS-TX. Control slides were incubated in BSA-PBS-TX with the primary antibody omitted. Next, sections were washed in PBS, incubated for 2 hours at 4°C with goat anti-rabbit IgG Alexa Fluor 555 (A27039; Thermo Fisher, Waltham, Massachusetts) secondary antibody diluted 1:500 in BSA-PBS-TX. Sections were then washed in PBS with 0.1% Triton X-100 before application of DAPI diluted in PBS according to manufacturer instructions (Thermo Fisher). The sections were washed in PBS and mounted using aqueous mounting medium.

### 4.3 Microscopy and image analysis

Sections were imaged using an Olympus BX41 microscope with an Olympus DP25 camera (Olympus Scientific Solutions Americas Inc., Waltham, Massachusetts). The 3-6 images were taken (20× magnification) of each region (caput, corpus and cauda) for analysis using Image J software (https://imagej.nih.gov/ij/). Percentages of Ki67<sup>+</sup> cells in the epithelium and stroma, and the percentage of stromal cells (identified by cell size, shape and location) out of total cells, were determined using the “analyze
4.4 | PLAG1 binding motif scanning

The TxDB.Musculus.UCSC.mm10.knownGene R library was used to obtain all the annotated transcriptional start sites (TSS) in mouse (mm10 genome version). The JASPAR database PLAG1 motif (JASPAR MA0163.1) and the binding sites defined by Madissoon et al were used for the analysis. Two different promoter sizes were analyzed: the genomic regions spanning 300 bp upstream to 50 bp downstream of the TSS, and between 2 kb upstream to 100 bp downstream of the TSS. First we scanned selected target genes for PLAG1 sites using the Homer suite of sequence analysis tools and performed \( \chi^2 \)-square test analysis to study differences in the occurrence of the sites compared to all other promoters. Then we extended the analyses to all differentially regulated genes and subjected the promoter regions to de novo motif discovery and general motif scanning using the findMotifs.pl program in Homer. The output of these analyses is a list of significantly enriched motifs in the promoters of the differentially regulated genes over all promoters in the genome, and their similarity to known motifs.

ACKNOWLEDGEMENTS

This research was supported by internal funding from the Research Focus Area “Securing Food and Water” of La Trobe University, from the Department of Physiology, Anatomy and Microbiology, and from the School of Life Sciences, La Trobe University. JW is supported by an Australian Government Research Training Program Scholarship. The authors acknowledge the staff of the La Trobe Animal Research and Teaching Facility for animal care.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Joanne Wong: Conceptualization; data curation; formal analysis; investigation; methodology; writing-original draft; writing-review and editing. Anastasios Damdimopoulou: Data curation; formal analysis; investigation; methodology. Pauliina Damdimopoulou: Data curation; formal analysis; investigation; methodology. Jemma Gasperoni: Data curation; investigation; methodology. Stephanie Tran: Data curation; investigation; methodology. Sylvia Grommen: Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; supervision. Bert de Groef: Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; writing-review and editing. Sebastian Dworkin: Formal analysis; funding acquisition; project administration; supervision; writing-review and editing.

ORCID

Sebastian Dworkin @ https://orcid.org/0000-0001-5828-9992

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**How to cite this article:** Wong J, Damdimopoulos A, Damdimopoulos P, et al. Transcriptionome analysis of the epididymis from *Plag1* deficient mice suggests dysregulation of sperm maturation and extracellular matrix genes. *Developmental Dynamics.* 2020;249:1500–1513. [https://doi.org/10.1002/dvdy.254](https://doi.org/10.1002/dvdy.254)