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STRA8 induces transcriptional changes in germ cells during spermatogonial development

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Abstract

Spermatogonial development is a key process during spermatogenesis to prepare germ cells to enter meiosis. While the initial point of spermatogonial differentiation is well-characterized, the development of spermatogonia from the onset of differentiation to the point of meiotic entry has not been well defined. Further, STRA8 is highly induced at the onset of spermatogonial development but its function in spermatogonia has not been defined. To better understand how STRA8 impacts spermatogonia, we performed RNA-sequencing in both wild-type and STRA8 knockout mice at multiple timepoints during retinoic acid (RA)-stimulated spermatogonial development. As expected, in spermatogonia from wild-type mice we found that steady-state levels of many transcripts that define undifferentiated progenitor cells were decreased while transcripts that define the differentiating spermatogonia were increased as a result of the actions of RA. However, the spermatogonia from STRA8 knockout mice displayed a muted RA response such that there were more transcripts typical of undifferentiated cells and fewer transcripts typical of differentiating cells following RA action. While spermatogonia from STRA8 knockout mice can ultimately form spermatocytes that fail to complete meiosis, it appears that the defect likely begins as a result of altered messenger RNA levels during spermatogonial differentiation.

KEYWORDS
retinoic acid, spermatogenesis, spermatogonia, STRA8, testis

1 | INTRODUCTION

The production of sperm via the process of spermatogenesis is a critical component for male fertility. Self-renewing stem cells reside within the seminiferous tubules and generate progenitor cells that are poised to respond to a retinoic acid (RA) pulse. In the mouse, when an RA pulse reaches these poised undifferentiated spermatogonia (A_{undiff}), a process is set in motion whereby the spermatogonial pool expands and begins to differentiate. The A_{undiff} spermatogonia are transformed into A1 differentiating spermatogonia in a process termed the A-to-A1 transition that initiates spermatogonial differentiation. Over the course of one cycle of the seminiferous epithelium in the mouse, spermatogonia will undergo six coordinated cell divisions to ultimately form spermatocytes. While the expansion of spermatogonia via mitosis is well-defined (Busada et al., 2015; Griswold, 2016; Hermann et al., 2015; Lord et al., 2018; Niedenberger et al., 2015; Velte et al., 2019), the

molecular characterization of the progression of undifferentiated spermatogonia to become preleptotene spermatocytes is less well-characterized.

The normal progression of the cycle of the seminiferous epithelium results in cells of all stages of development being present within the testis at a given time to maintain continual fertility. Thus, the adult testis is a continuum of types of developing germ cells. The four main types of male germ cells (spermatogonia, spermatocytes, round spermatids, and elongating spermatids) can be discerned by their unique morphological traits (Russell et al., 1990). Some further distinctions can be made within these populations based on the compaction and location of chromosomes within the nucleus (spermatogonia and spermatocytes) or amounts of cytoplasm present and acrosome shape (spermatids; Chiarini-Garcia & Russell, 2001; Leblond & Clermont, 1952). However, the subpopulations of differentiating spermatogonia do not have obvious morphological differences, and can only be characterized via transmission electron microscopy (TEM) based on heterochromatin percentage and location within the nuclear envelope (Chiarini-Garcia & Russell, 2002).

To reduce the heterogeneity of germ cells within the testis, we employed a spermatogenesis synchrony protocol using WIN 18,446 and RA (Hogarth et al., 2013). WIN 18,446 blocks the ALDH1A enzymes responsible for the second step in the conversion of vitamin A to RA (Arnold et al., 2015). Animals fed this compound for several days before the initial RA pulse that occurs in the testis around 3–6 days post-partum (dpp) build up a pool of undifferentiated A spermatogonia that are poised to respond to RA. When RA is then injected into these mice, the vast majority of germ cells (excepting spermatogonial stem cells that are protected from differentiating in the presence of RA) will simultaneously differentiate, generating a testis that over time is enriched for given types of germ cells (Agrimson et al., 2016, Hogarth et al., 2013). This synchrony remains for at least several rounds of spermatogenesis following the initial RA injection (Hogarth et al., 2013). The spermatogonial subtypes (A_{undiff}, A1, A2, A3, A4, intermediate [Int], and B) and preleptotene spermatocytes are present in a time-dependent manner following synchrony and have been characterized using TEM (Agrimson et al., 2016). In the adult mouse, these progressive changes occur over one cycle of the seminiferous epithelium lasting 8.6 days, while in the first round of spermatogenesis the cycle takes around 6 days (Clermont, 1972; Hogarth et al., 2013; Kluin et al., 1982).

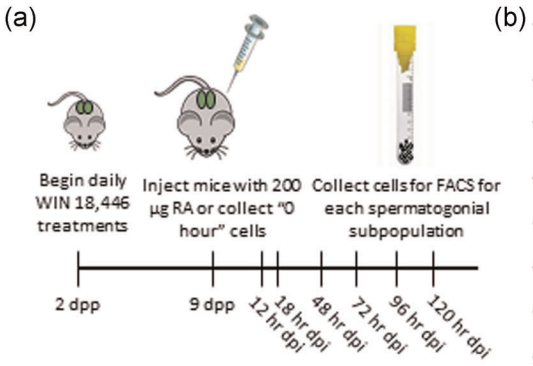
Our goal was to examine the transcriptome during spermatogonial progression from A_{undiff} cells through B spermatogonia in STRA8 knockout (STRA8 KO) mice and to compare those results with those from wild-type (WT) mice. To do this, we isolated purified spermatogonial subpopulations and used RNA sequencing (RNA-seq) to characterize transcriptome changes in the first round of germ cells to undergo spermatogonial differentiation. Previous work with STRA8 KO mice has shown that undifferentiated spermatogonia abnormally accumulate as early as 10 dpp, as shown by immunohistochemistry with LIN28 and ZBTB16, and that differentiating spermatogonia were depleted. These studies concluded that STRA8 promotes, but is not strictly required for, spermatogonial differentiation (Anderson et al., 2008; Endo et al., 2015; Koubova et al., 2006, 2014). STRA8 KO mice on a congenic background have a defect in initiating meiosis, while those on a mixed genetic background can initiate but not complete meiosis (Endo et al., 2015; Mark et al., 2008). It has also been shown that STRA8 acts as a broad transcriptional activator primarily in areas of already accessible chromatin in preleptotene spermatocytes at the time of meiotic entry (Kojima et al., 2019). Our study shows that the alterations in STRA8 KO spermatogonial transcriptomes before the formation of preleptotene spermatocytes results in abnormalities before meiosis.

2 | RESULTS

2.1 | Transcriptional changes during spermatogonial development

To address how STRA8 impacts transcriptional changes throughout spermatogonial development, we first had to define the gene expression patterns seen in WT mice in known spermatogonial subpopulations. We utilized a PIWIL2-eGFP-derived mouse line in which germ cells in the prenatal stage through early spermatids express enhanced green fluorescent protein (eGFP; Aravin et al., 2008). We synchronized spermatogonial development in these animals using a previously described WIN 18,446/RA method to generate testes filled with predominantly a single defined spermatogonial subtype and used fluorescence-activated cell sorting (FACS) to isolate the enriched germ cell type away from associated somatic cells (Agrimson et al., 2016; Arnold et al., 2015; Hogarth et al., 2013; Figure 1a,b, FACS purity graph shown in Figure S1). The timing we used corresponded to that previously used to obtain the spermatogonial subtypes shown in Figure 1b so the spermatogonial subpopulations should roughly correspond to those as designated. We extracted RNA from each sample and used RNA-seq to analyze the steady-state messenger RNA (mRNA) levels across spermatogonial development. High replicability was seen between the two biological replicates in both WT and STRA8 KO mice at each timepoint, shown by close clustering in the principal component analysis (PCA) analysis (Figure 1c).

Our sequencing results revealed that 17,523 genes were expressed (fragments per kilobase of transcript per million mapped reads [FPKM] ≥ 1) in at least one of the times corresponding roughly to the spermatogonial subtypes in the WT animals. Of these, 13,605 (77.6%) were expressed in all assayed spermatogonial subpopulations. The number of genes expressed in the STRA8 KO cells was very similar, with 17,376 genes expressed in at least one spermatogonial subtype and 13,826 (79.6%) expressed in all spermatogonial subtypes. While the overall number of expressed genes was similar between the WT and KO cells, there were many fewer changes in the steady-state mRNA levels by the end of spermatogonial development in the KO (Figure 2a). We then determined whether genes that were differentially regulated in the KO were also differentially regulated in the WT. For 12–96 h post-RA, (A1/A2 through Int spermatogonia), differentially expressed genes shared by the WT and



(b)

Time after RA injection	Cell Types Present
0 hours	Undifferentiated A spermatogonia
12 hours	Undifferentiated A spermatogonia, RA-induced genes activated
18 hours	A1/A2 spermatogonia
48 hours	A3 spermatogonia
72 hours	A4 spermatogonia
96 hours	Intermediate spermatogonia
120 hours	B spermatogonia

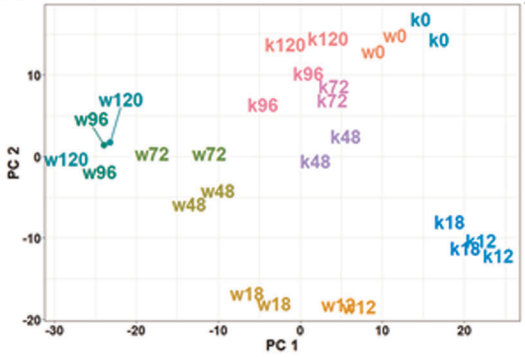


FIGURE 1 (a) Experiment schematic. PIWIL2-eGFP × STRA8 wild-type (WT) or knockout (KO) mice were treated with WIN 18,446 daily for 7 days starting at 2 days post-partum (dpp). Mice received a retinoic acid (RA) injection on Day 8 of treatment (9 dpp). Cells were collected via fluorescence-activated cell sorting (FACS) at the timepoints listed in (b) to collect given spermatogonial subtypes. (b) Cell types present at given times after WIN 18,446/RA synchrony. Original data from Agrimson et al. (2016). (c) Principal component analysis (PCA) of 0-, 12-, 18-, 48-, 72-, 96-, and 120-h post-RA WT and STRA8 KO samples generated using the top 200 most variable genes. Two biological replicates at each timepoint were sequenced and nearby clustering shows high replicate similarity. k0, KO 0-h post-RA; k12, KO 12-h post-RA; k18, KO 18-h post-RA; k48, KO 48-h post-RA; k72, KO 72-h post-RA; k96, KO 96-h post-RA; k120, KO 120-h post-RA; w0, WT 0-h post-RA (A_{undiff}); w12, WT 12-h post-RA (RA-induced genes activated); w18, WT 18-h post-RA (A1/A2 spermatogonia); w48, WT 48-h post-RA (A3 spermatogonia); w72, WT 72-h post-RA (A4 spermatogonia); w96, WT 96-h post-RA (Int spermatogonia); w120, WT 120-h post-RA (B spermatogonia)

KO made up 12%–37% of the total differentially expressed genes (Figure 2b,c). There were only about 1% shared downregulated genes in the 120-h KO, reflecting the large difference in the number of differentially expressed genes between the WT and KO for this timepoint.

In addition to those genes which were changed in either the WT or KO from their A_{undiff} transcript levels, we also assessed which transcripts were differentially expressed between the WT and KO cells at each timepoint (Figure 3). Interestingly, we found more genes at all timepoints except for 0 h which were enriched in STRA8 KO cells compared with WT cells. Overall, these data suggest that in the absence of STRA8 there are a substantial number of genes whose expression profiles change by the end of spermatogonial development.

2.2 | Transcripts characteristic of undifferentiated spermatogonia stay elevated in STRA8 KO cells

It has previously been shown via immunohistochemistry that STRA8 KO spermatogonia retain more proteins associated with undifferentiated spermatogonia (Endo et al., 2015). We evaluated how the initial actions of RA in our synchrony system could explain these transcriptome changes. We analyzed a variety of transcripts that are indicative of the A_{undiff} population, including neurogenin 3 (*Neurog3*), SRY-box transcription factor 3 (*Sox3*), zinc finger and BTB domain containing 16 (*Zbtb16*), lin-28 homolog A (*Lin28a*), and nanos C2HC-type zinc finger 2 (*Nanos2*; Buaas et al., 2004; Costoya et al., 2004; Raverot et al., 2005; Sada et al., 2009; Suzuki et al., 2007; Yoshida et al., 2004; Zheng et al., 2009; Figure 4a). These genes had detectable transcript levels throughout spermatogonial development, and transcript levels were notably lower by 12 or 18 h post-RA. While this decrease in transcripts immediately following RA action was mimicked in the STRA8 KO cells, mRNA levels of these genes were increased at later stages during development compared with the STRA8 WT cells. At 120 h post-RA, all of these transcripts were about 50% more abundant in the KO cells than in the WT. Some markers of undifferentiated spermatogonia including POU class 5 homeobox 1 (*Pou5f1*, formerly *Oct3/4*) did not show such an immediate reduction in transcript levels at the onset of spermatogonial differentiation, but still retained over 50% more transcripts by the end of spermatogonial development (Nichols et al., 1998; Pesce et al., 1998).

(a)

Time post-RA (hours)	# WT Transcripts Increased Relative to A_{undiff}	# WT Transcripts Decreased Relative to A_{undiff}	# KO Transcripts Increased Relative to A_{undiff}	# KO Transcripts Decreased Relative to A_{undiff}
12	417	597	390	414
18	347	800	308	482
48	457	611	167	389
72	313	474	174	163
96	1037	1219	212	163
120	1236	1355	196	25

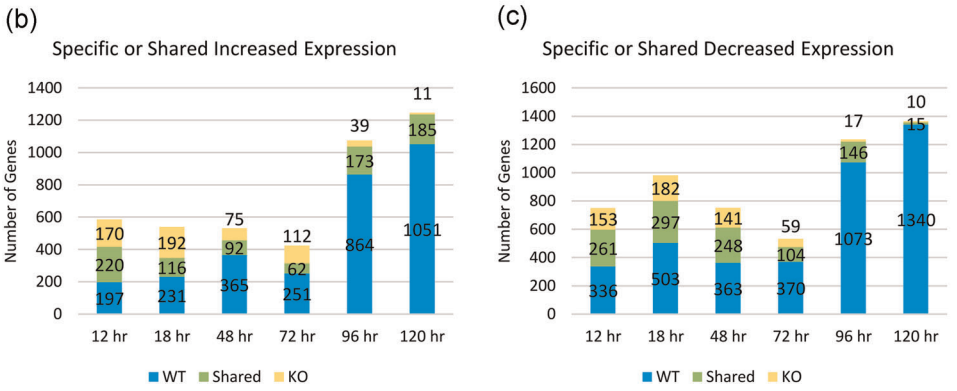


FIGURE 2 (a) Numbers of genes at each timepoint for wild-type (WT) and STRA8 knockout (KO) cells during spermatogonial development whose steady-state levels increased or decreased relative to the corresponding A_{undiff} population. (b) Increased and (c) decreased expression specifically in the WT or STRA8 KO or in both the WT and KO (shared) during spermatogonial development, relative to 0-h post-RA (A_{undiff}). Blue bars show genes with increased or decreased expression specifically in the WT cells, yellow bars show genes with increased or decreased expression specifically in the STRA8 KO cells, and green bars show genes with increased or decreased expression in both the WT and STRA8 KO cells. Differentially expressed genes determined by $\log_2$ fold-change ≥1 or ≤−1, false discovery rate ≤ 0.05

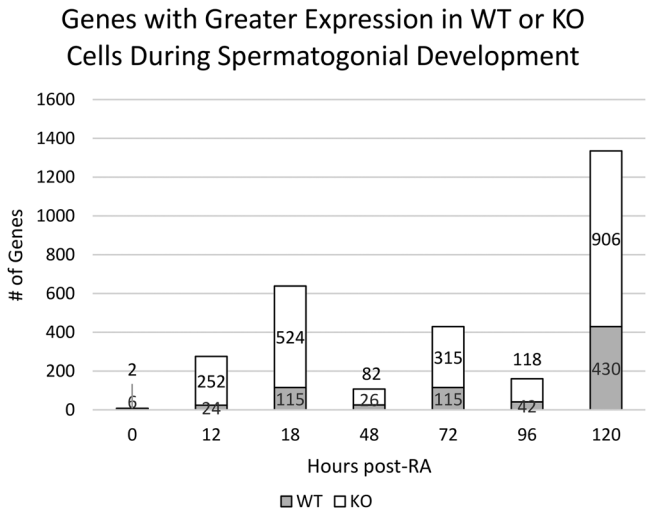


FIGURE 3 Differentially expressed genes between corresponding wild-type (WT) and (KO) cells at 0- to 120-h post-retinoic acid (RA). Differentially expressed genes determined by R DESeq2 package (Love et al., 2014) capturing genes that had a $\log_2$ fold-change ≥1 and false discovery rate ≤ 0.05. Gray bars represent genes that were more highly expressed in the WT cells, white bars represent genes that were more highly expressed in KO cells

As our synchrony system is unique, we performed immunostaining for ZBTB16 at 96 h post-RA to determine if the increase in transcript levels in the STRA8 KO cells compared with the WT was reflected at the protein level. We found there were significantly more cells with detectable ZBTB16 staining in the STRA8 KO compared with the WT cells (Figure 4b). These data indicate that at least some of the retained transcripts characterizing undifferentiated spermatogonia result in increased protein levels.

2.3 | Some transcripts characteristic of differentiating spermatogonia are decreased in STRA8 KO spermatogonia

Previous work has shown that in addition to an increase in the A_{undiff} population in STRA8 KO testes on a congenic background, there is also a decrease in the number of B spermatogonia in these mice (Endo et al., 2015). While we saw increased expression of transcripts characteristic of A_{undiff} cells in the STRA8 KO, we found that many transcripts characteristic of differentiating spermatogonia were not significantly changed following RA stimulation. KIT (KIT proto-oncogene, receptor tyrosine kinase) has been used as a common

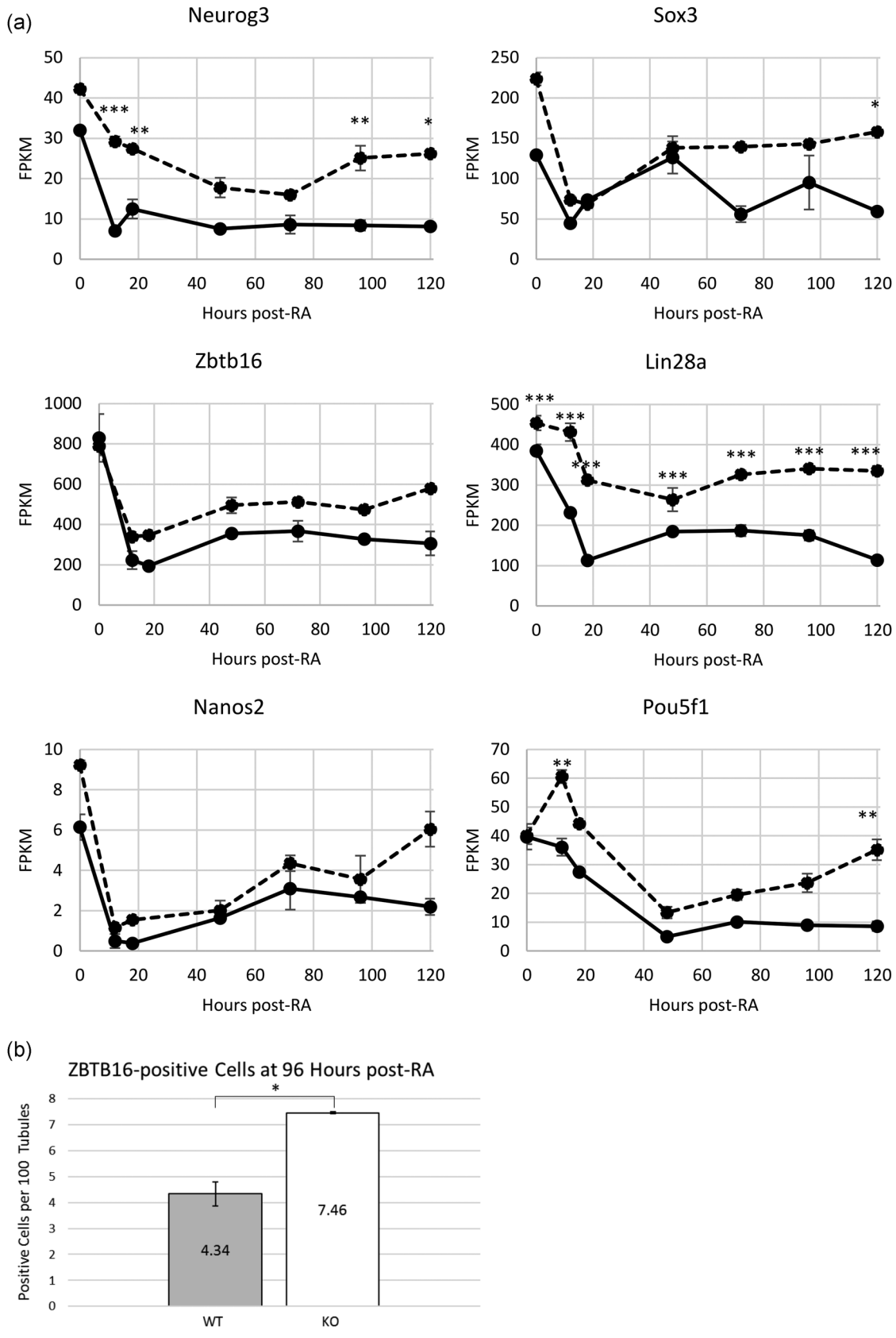


FIGURE 4 (See caption on next page)

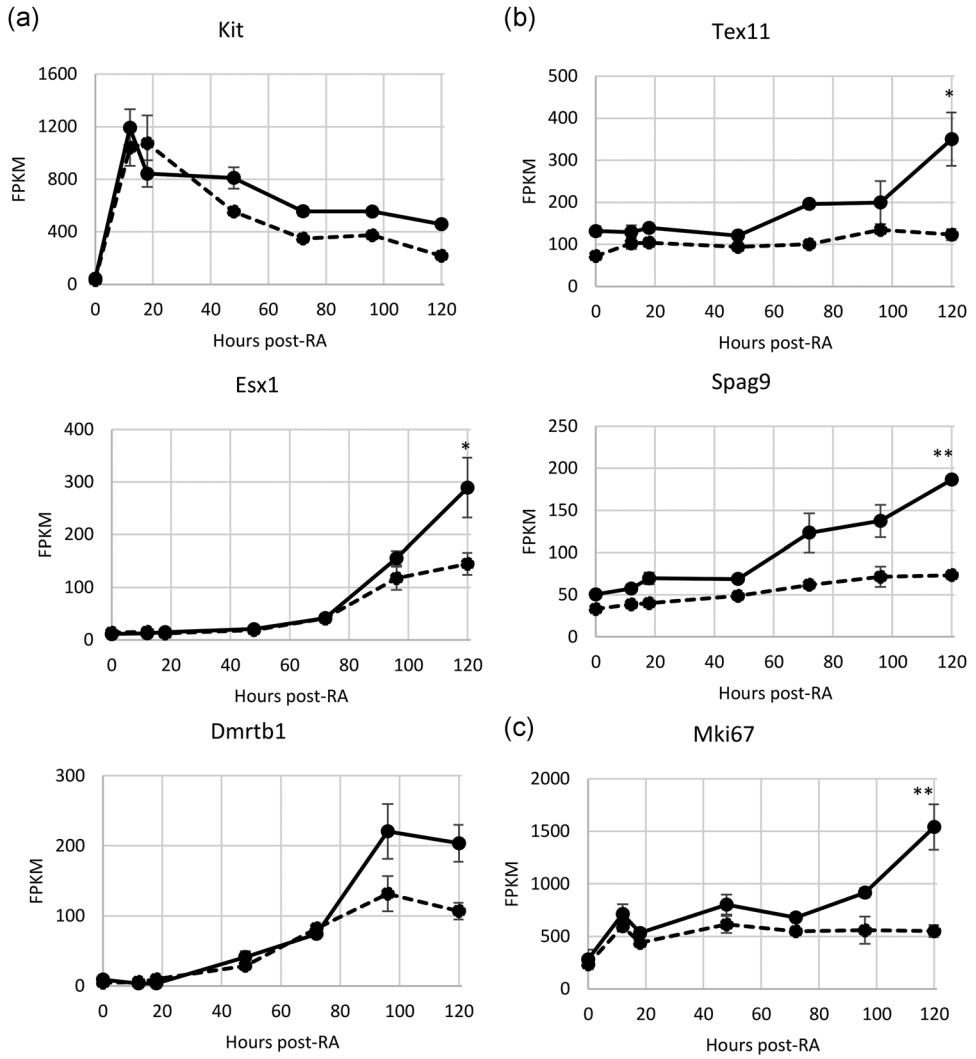


FIGURE 5 (a) Transcripts characteristic of differentiating spermatogonia in wild-type (WT) and STRA8 knockout (KO) cells (*Kit*, *Esx1*, and *Dmrtb1*). (b) Genes that have an increase in expression later during spermatogonial development (48–120 h post-retinoic acid [RA]; *Tex11* and *Spag9*). While there is an increase in WT expression, this increase is absent or muted in the STRA8 KO cells. (c) *Mki67* transcript expression throughout spermatogonial development. Solid lines represent WT data, dashed lines represent STRA8 KO data. Data represent the average of two biological replicates, error bars represent ± *SEM*. Asterisks represent significance in a one-way analysis of variance followed by a Tukey's multiple comparison honestly significant difference test, $^{*}p \le .05$, $^{**}p \le .01$, $^{***}p \le .001$

marker of differentiating spermatogonia, as the protein product is present in all subtypes of differentiating spermatogonia (Schrans-Stassen et al., 1999; Yoshinaga et al., 1991). *Kit* expression is necessary for DNA synthesis in differentiating spermatogonia and is thought to be epigenetically controlled via DNA methylation at spermatogonial differentiation (Rossi et al., 2000; Shirakawa et al., 2013). Consistent with previous data, we found that *Kit* mRNA expression was detectable but relatively low in undifferentiated A spermatogonia (Figure 5a; Niedenberger et al., 2015; Schrans-Stassen et al., 1999; Q.-E. Yang et al., 2013). When RA was administered, *Kit* levels showed one of the greatest increases, and mRNA levels remained elevated throughout spermatogonial development, going from an average of 50 FPKM to over 1000 after RA was injected, and maintaining high expression levels over 400 FPKM

FIGURE 4 (a) Pluripotency transcripts characteristic of undifferentiated spermatogonia in wild-type (WT) and STRA8 knockout (KO) cells. Solid lines represent WT data, dashed lines represent STRA8 KO data. Data represent the average of two biological replicates, error bars represent ± *SEM*. Asterisks represent significance in a one-way analysis of variance followed by a Tukey's multiple comparison honestly significant difference test, $^{*}p \le .05$, $^{**}p \le .01$, $^{***}p \le .001$. (b) ZBTB16-positive cells at 96 h post-retinoic acid (RA). Values represent the number of positive cells per 100 tubules, data averaged from three replicates. Asterisks show a significant difference in a paired Student's *t* test, two-tailed $p < .05$. Gray bars show WT values, white bars show STRA8 KO values. Error bars represent ± *SEM*

for the duration of spermatogonial development. While STRA8 KO cells did not show significantly lower levels of *Kit* expression throughout spermatogonial development, there were fewer KIT-positive spermatogonia at 96 h post-RA (roughly corresponding to Int spermatogonia) in the STRA8 KO compared with WT testes (Figure S2). We found similar trends with other common markers of differentiating spermatogonia, including DMRT-like family B with proline-rich C-terminal 1 (*Dmrtb1*, *Dmrt6*) and ESX homeobox 1 (*Esx1*; Figure 5a; Yeh et al., 2005; Zhang et al., 2014), both showed a reduced number of transcripts in the KO cells by the end of spermatogonial development (Yeh et al., 2005; Zhang et al., 2014). There were generally at least 50% more transcripts in the WT compared with the KO by 120 h post-RA for these transcripts associated with differentiating spermatogonia.

2.4 | Transcripts with greater levels later in spermatogonial development are comparatively reduced in STRA8 KO cells

Although some transcripts that reportedly define differentiating spermatogonia did not show large differences in WT and STRA8 KO cells, we did find a notable group of genes that showed a muted increase in transcript levels in the KO relative to the WT. These transcripts did not show an immediate change in response to RA, but rather increased at later times during the course of spermatogonial development and roughly corresponded to the A3, A4, Int, or B spermatogonial populations. This group of transcripts includes sperm-associated antigen 9 (*Spag9*) and testis expressed 11 (*Tex11*; Figure 5b). TEX11 shows a meiotic defect when knocked out due to altered recombination rates (F. Yang et al., 2015). SPAG9 has been noted to be in spermatids and is important for zona pellucida binding (Jagadish et al., 2005; Shankar et al., 1998). There was an increase in transcripts for both of these genes noted in the WT cells that were muted in the corresponding KO cell populations. The lack of an equal response in STRA8 KO cells at the same timepoint shows that the steady-state levels of some transcripts are lower due to the lack of STRA8. While the role of these genes has not been noted to occur until meiosis or post-meiosis, the decreased numbers of transcripts seen in STRA8 KO spermatogonia may lead to a later meiotic defect.

In addition to genes that increase later in spermatogonial development with a known role in the testis, we also evaluated the expression of *Mki67*. This marker of proliferation has been widely used to examine cancer cells as well as an indicator of an active cell cycle in multiple cell types, including spermatogonia (Schlüter et al., 1993; Sobecki et al., 2016; Steger et al., 1998). We noted that WT cells showed an increase in *Mki67* transcripts starting by 12 h post-RA injection and transcript levels continued to rise throughout spermatogonial development (Figure 5c). In the KO, transcript levels increased following RA injection similarly to WT cells, but the increase seen in the WT by 96 and 120 h post-RA was absent in the KO. The lower levels of this proliferation marker, especially by the end of spermatogonial development, is additional evidence that fewer differentiated cells properly complete spermatogonial development in STRA8 KO mice.

2.5 | Expression of meiotic genes in STRA8 KO cells

Due to the role of STRA8 in meiotic entry and progression, we examined how the expression of meiosis-associated genes changed during spermatogonial development. STRA8 KO mice on a congenic background did not have germ cell development past the stage of preleptotene spermatocytes (Endo et al., 2015). Mice on a mixed genetic background showed about 80% of tubules with preleptotene or leptotene spermatocytes, but very few cells made it past this developmental stage (Mark et al., 2008). Previous whole testis transcriptome data has shown that many meiotic transcripts are already present before spermatogonial differentiation, suggesting that although A_{undiff} do not immediately enter meiosis when RA is present, these cells have at some level already committed to that path (Evans et al., 2014). We utilized the Gene Ontology database (GO Ontology database DOI: 10.5281/zenodo.3727280; released February 23, 2020) and found terms that were annotated for meiosis and excluded for mitosis. We found 129 meiosis-related genes that were expressed in at least one WT spermatogonial subtype, and 125 that were already over 1 FPKM in the A_{undiff} population. In addition to the static values of gene expression for the different cell populations, we also examined how levels of meiotic genes changed over time. We observed the greatest number of meiosis-associated transcripts in B spermatogonia, just before their transformation into preleptotene spermatocytes. Thus, while meiotic transcripts are already seen before spermatogonial differentiation, they reached an elevated level just before the formation of preleptotene spermatocytes.

In the STRA8 KO, 127 meiosis-related genes were expressed at ≥1 FPKM at any timepoint, and 119 of these were expressed at ≥1 FPKM at all timepoints from A_{undiff} through B spermatogonia. In accordance with the fewer differentially expressed genes overall in the STRA8 KO compared with WT spermatogonial subpopulations, we observed fewer differentially expressed meiotic genes at each timepoint in the STRA8 KO compared with the WT (Figure 6). The differences in the levels of meiotic transcripts seen between the WT and KO cells, in addition to other transcriptome differences during spermatogonial development, may explain why the STRA8 KO cells are not prepared to properly complete meiosis.

3 | DISCUSSION

RA is absolutely required and is a trigger for the transition of A_{undiff} spermatogonia into the differentiation pathway, and STRA8 is a major marker of this signal. However, spermatogonia can differentiate in the absence of STRA8, and on a mixed genetic background most spermatocytes enter meiosis and proceed to leptonema, while some even progress to the pachynema stage (Anderson et al., 2008; Baltus et al., 2006; Mark et al., 2008). After one cycle of the

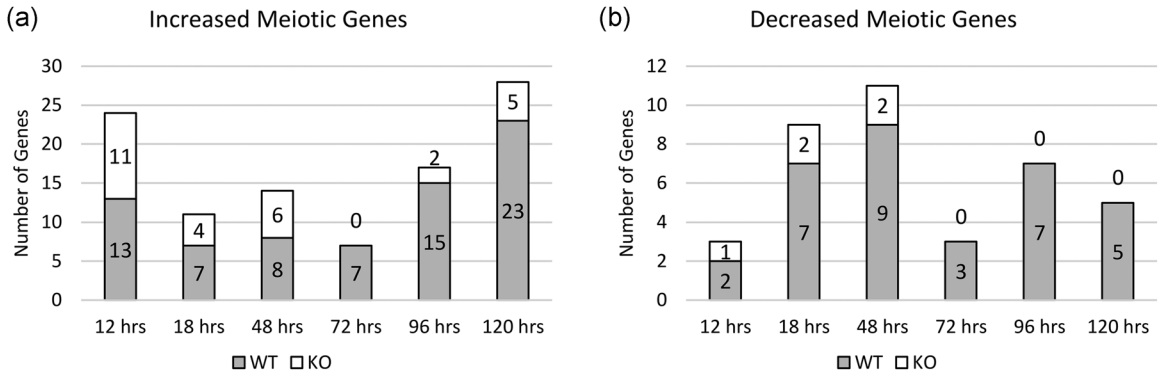


FIGURE 6 Numbers of (a) increased and (b) decreased meiotic transcripts in wild-type (WT) and STRA8 knockout (KO) cells during spermatogonial development. Fewer differentially expressed meiotic genes were observed in STRA8 KO cells at all spermatogonial subpopulations. Differentially expressed genes determined by $\log_2$ fold-change ≥ 1 and false discovery rate ≤ 0.05. Gray bars show differentially expressed genes compared with A_{undiff} in WT cells, white bars show differentially expressed genes compared with A_{undiff} in KO cells

seminiferous epithelium (8.6 days in the adult mouse), the spermatogonia have transitioned into preleptotene spermatocytes where they again are presented with an endogenous RA pulse and express STRA8 (Hogarth et al., 2013). It has been shown that the expression of STRA8 and initiation of meiosis does not depend on this second pulse of endogenous RA, or those cells at least respond to a much lower concentration of RA (Teletin et al., 2019). Thus, the RA pulse primarily regulates the onset of spermatogonial differentiation.

While the role of STRA8 in meiotic cells has been somewhat defined, we wanted to understand how a lack of STRA8 impacts spermatogonial development. We found that well before the formation of preleptotene spermatocytes, spermatogonial transcriptomes already show many alterations when STRA8 is ablated. Notably, we saw an accumulation of transcripts that have been used to define undifferentiated spermatogonia at later points following the A-to-A1 transition in STRA8 KO cells. These included pluripotency genes *Neurog3*, *Sox3*, *Lin28a*, *Zbtb16*, *Pou5f1*, and *Nanos2*. Additionally, we found transcripts including *Spag9* and *Tex11* with notably higher abundance at 48–120 h post-RA in WT but not STRA8 KO cells. Thus, transcripts that define each population of spermatogonia showed differences in their steady-state mRNA levels at several points during spermatogenesis. Together, these transcriptome abnormalities likely contribute to the failure of STRA8 KO cells to complete meiosis, as seen previously in mixed genetic background mice (Mark et al., 2008). Many of the meiosis-associated transcripts in STRA8 KO spermatogonia also did not show changes similar to those observed in the WT, giving further evidence why these cells may be unable to properly complete meiosis. Additionally, we corroborated previous results via immunohistochemistry indicating an increase in undifferentiated spermatogonia and a decrease in differentiating spermatogonia in STRA8 KO mice at 96 h post-RA, roughly corresponding to the Int spermatogonia stage (Agrimson et al., 2016; Endo et al., 2015). Together with the lower levels of proliferation marker *Mki67*, these data indicate that fewer cells make it to preleptotene spermatocytes, and those that do have abnormal transcriptomes before reaching the preleptotene spermatocyte stage.

The changes between the WT and STRA8 KO cells throughout development are shown well in PCA analysis (Figure 1c). Both the WT and STRA8 KO cells cluster closely together before RA injection at the 0-h timepoint, reflecting the A_{undiff} populations. After RA is injected, there are many differentially expressed genes between the 12 and 18 h post-RA cells relative to 0 h, resulting in clustering further away from the A_{undiff} populations. While the WT spermatogonia continue in a trajectory further away from the undifferentiated cells, by 120 h post-RA STRA8 KO cells are more closely clustered with the A_{undiff} cells. This further highlights how the retention of transcripts associated with undifferentiated spermatogonia in the STRA8 KO, including those indicating pluripotency, dramatically changes the transcriptomes of spermatogonia. By the B spermatogonial stage, the STRA8 KO spermatogonia are similar to the undifferentiated population such that it seems likely they are unable to properly respond to the second RA pulse they would encounter to form normal preleptotene spermatocytes that are able to progress through and complete meiosis, as their gene expression indicates that they would instead respond more similarly to re-entering spermatogonial differentiation. The direct action of STRA8 on certain genes in preleptotene spermatocytes has been previously demonstrated in mice with a congenic background (Kojima et al., 2019). While our data cannot determine whether the effects of STRA8 on spermatogonial transcriptomes are direct or indirect, our goal was to simply show the overall effect of the lack of STRA8 during spermatogonial development. We assert that the meiotic defects seen in STRA8 KO mice likely have their roots in abnormal transcriptomes that occur during spermatogonial development.

Our use of spermatogenesis synchrony enabled us to look at the transcriptomes across spermatogonial development in WT and STRA8 KO cells. In the WT cells, we used STRA8 as a proxy to determine whether normal differentiation had occurred via the actions of RA, with good evidence that our synchrony protocol robustly blocked RA action and thus halted germ cell developmental progression for a prolonged time (Beedle et al., 2019). It is key to remember that these mice were treated with exogenous doses of RA to stimulate synchronous spermatogenesis. RA acts at the local level on RA receptors and the

concentration of RA acting on those receptors depends on transport and degradation processes. We do not know whether the exogenous dose of RA mimics the endogenous levels, but we do not see cell death that could arise from high levels of RA (Hogarth et al., 2013). Spermatogonial development was also only assayed for the first round of cells to undergo spermatogenesis, and such values or trends may differ in the second and subsequent rounds when RA is potentially provided by sources other than Sertoli cells (Endo et al., 2017; Raverdeau et al., 2012; Sugimoto et al., 2012) However, our study of the first round of spermatogenesis allows us to isolate highly purified cell populations corresponding to those previously determined by Agrimson et al. (2016), and examine differentiating subpopulations throughout the course of spermatogonial development. These results, particularly for the WT, can also be compared with recent single-cell testis transcriptomes that have been generated. We find that for genes expressed in differentiating spermatogonia, our data confirms what has been seen in single-cell RNA-seq experiments as cells progress through pseudotime. Green et al. (2018) describe three distinct differentiating spermatogonial clusters presumably corresponding to A1–A4 spermatogonia, Int-B spermatogonia, and B spermatogonia and preleptotene spermatocytes in adult mice. Our A1–A4 data showed higher transcript levels in those populations than in Int and B spermatogonia for genes including TEA domain transcription factor 2 (*Tead2*) and PHD finger protein 13 (*Phf13*). *Esx1* was described via single-cell sequencing as an Int-B marker, which we identified as a gene with a large increase in transcript levels starting at the Int stage. Additionally, Chen et al. (2018) used the synchronization system followed by single-cell RNA-seq to examine gene expression throughout the course of spermatogenesis. Although they only examined A1, Int, and B spermatogonia, the trends in our data are very similar to their results. Our purified cell populations also allow us to not only cluster gene expression profiles, but to assign these profiles to a known spermatogonial subtype and to obtain a significantly greater depth of sequencing.

Overall, our data set shows the transcriptomes of the differentiating spermatogonial subtypes across the first wave of spermatogonial development in both WT and STRA8 KO cells. We show differences in the STRA8 KO compared with WT cells during spermatogonial development, defining how the lack of STRA8 even before meiosis prevents normal germ cell development leading to cells that are unable to complete meiosis. These data help to uncover the effects STRA8 causes during spermatogonial development which has previously been unexplored. While STRA8 has previously been thought to aid in but overall be dispensable for spermatogonial development, we argue that the transcriptional changes which occur via the initial actions of RA triggering STRA8 activation are indeed necessary for proper spermatogonial development to ultimately lead to cells that can complete spermatogenesis.

4 | MATERIALS AND METHODS

4.1 | Animals

All animal experiments were approved by the Washington State University Animal Care and Use committees and were conducted in accordance with the Principles for the Care and Use of Research Animals of the National Institutes of Health. Tg(Piwil2/EGFP) 1GHan/J mice (denoted here as PIWIL2-eGFP) were obtained from the Jackson Laboratories (stock no. 012276; Aravin et al., 2008). These mice express GFP under control of the piwi like RNA-mediated gene silencing 2 (*Piwil2*) promoter and expression is seen prenatally through early spermatids and were produced on a C57BL/6 × SJL background (Lee et al., 2006). PIWL2-eGFP mice were crossed with B6.Cg-*Stra8*^{tm1Dcp}/J mice (C57BL/6 × 129S4/SvJae background) that contain a targeted deletion exons 2–7 of the *Stra8* gene resulting in nondetectable levels of STRA8 protein (stock no. 023805; Jackson Laboratories, gift from David Page; Baltus et al., 2006). PIWIL2-eGFP mice were crossed with this line to generate PIWIL2-eGFP, STRA8 WT or PIWIL2-eGFP, STRA8 KO mice used for the WT and STRA8 KO data, respectively. Animals were housed in a humidity- and temperature-controlled environment with food and water provided ad libitum. At the time of tissue collection, mice were euthanized via carbon dioxide asphyxiation followed by either decapitation (<21 dpp) or cervical dislocation (21 dpp and older).

4.1.1 | Mouse genotyping

Tail clips were taken from neonatal mice to identify genotypes before euthanasia. These tail clips were lysed in 75 µl of 25-mM NaOH, 200-µM EDTA (pH 12) in a 95°C heat block for 1 h, with manual dissociation at 30 min. 75-µl neutralization buffer (40 mM Tris-HCl, pH 5) was then added before DNA was used for genotyping reactions. PIWIL2-eGFP mice were assayed for the presence of the transgene using primers specific to eGFP (forward: 5′-CTGACTC CTGATGAAGTGTTATAGCC-3′, reverse: 5′-TCCTTGAAGAAGATGG TGCGCTCCT-3′). Presence of a band at ~500 bp signified the presence of the transgene. STRA8 presence was detected using primer 1: 5′-AGCTGCAGAAGCTTGAGCCT-3′; primer 2: 5′-AGGTCAGGCT GCTAGGATGC-3′; and primer 3: 5′-TCCGATAGCTTGGCTGCAG GTC-3′. Wild-type *Stra8* results in a band at ~280 bp, and the *Stra8* KO produces a band at ~180 bp.

4.1.2 | WIN 18,446/RA treatments

Spermatogenesis synchronization was performed as previously described (Hogarth et al., 2011). Briefly, mice were treated with 100 mg/kg body weight WIN 18,446 suspended in 1% gum tragacanth every 24 h for 7 days starting at 2 dpp. At 9 dpp, mice were injected intraperitoneally with 200-µg RA diluted in 10-µl dimethyl sulfoxide. Mice were then euthanized and testes collected at 12, 18, 48, 72, 96, or 120 h following the RA injection to roughly correspond with cell populations following synchrony as described by Agrimson et al. (2016). For "0 h" collections, mice received 7 days of WIN 18,446 treatment and then were euthanized without receiving an RA injection.

4.1.3 | Cell sorting

Testes to be used for cell sorting were dissected and the tunica removed. Tubules were placed in 1× Hank's Balanced Salt Solution (HBSS; 14175145; Thermo Fisher Scientific) until they are transferred to a Petri dish with 5-ml trypsin/EDTA (25200056; Thermo Fisher Scientific) and 0.5-ml DNase (7 mg/ml in HBSS, 9003-98-9; Sigma-Aldrich). Tubules were manually dissociated via pipetting and then incubated for 5 min at 37°C. One milliliter DNase was then added and tubules were dissociated again. The cells were incubated for another 5 min at 37°C, and these steps were repeated twice more. Fetal bovine serum (850 µl) was then added and the suspension was strained through a 30-µm filter. The Petri dish was rinsed with 4-ml HBSS to remove any remaining cells that were then also filtered, followed by a final 1-ml HBSS wash through the filter. Cell number in the suspension was estimated using a hemocytometer. Cells were spun down at 600*g* for 7 min at 4°C, then resuspended in 12.5% DNase solution diluted in DPBS (14190235; Thermo Fisher Scientific) at 4 million cells/ml. FACS was performed with an SH800 machine (Sony Biotechnology) to distinguish eGFP-positive and -negative populations. eGFP-positive cells were collected into a tube containing 0.5-ml dPBS. Post sort analysis revealed over 95% of cells collected were GFP-positive (representative figure shown in Figure S1). Cells were then spun down at 600*g* for 7 min at 4°C, then put into 0.5-ml TRIzol (Invitrogen), pipetted up and down, and stored at −20°C until RNA was extracted.

4.1.4 | RNA sample preparation

For each sample, cells from three to eight mice were combined during RNA preparation. To prepare cells for RNA-seq, the TRIzol RNA isolation procedure was followed according to the manufacturer's instructions. Briefly, frozen samples were thawed and incubated for an additional 5 min at room temperature. 0.2-ml chloroform per 1 ml TRIzol was added and tubes were briefly mixed by vortex. Samples were then incubated at room temperature for 2–3 min and then spun at 12,000*g* for 15 min at 4°C. The aqueous phase was transferred to a clean tube and isopropanol was added to precipitate the RNA (0.5 ml per 1 ml TRIzol used). Tubes were turned gently to mix the solution then incubated 10 min at room temperature. Samples were then spun at 12,000*g* for 10 min at 4°C. Supernatant was removed and the RNA pellet was washed with 1-ml 75% ethanol. The pellet was centrifuged at 7500*g* for 5 min at 4°C, then residual ethanol was removed via gentle pipetting and evaporation. RNA pellets were resuspended in 20-µl warm nuclease-free water and stored at −80°C until further use. To enrich for mRNA, an NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490S; New England Biolabs) was used according to the manufacturer's protocol.

4.1.5 | RNA-sequencing and data analysis

Ten nanograms of mRNA were used to make barcoded libraries using the Ion Total RNA-seq Kit V2 (Life Technologies). The resulting libraries were quantified and pooled to 75 pM before loading onto Ion P1 semiconductor chips using an Ion Chef and sequenced on an Ion Proton sequencer. Reads were mapped to the *Mus musculus* genome v10 and expression values were calculated using a Bowtie/TopHat/Cufflinks pipeline incorporated into the Ion Torrent Suite v.5.0.5 (Langmead et al., 2009; Trapnell et al., 2012). PCA analysis was conducted using the R package FactoMineR (Lê et al., 2008) and differential gene expression was determined using the R DESeq2 package (Love et al., 2014). Differentially expressed genes were defined as those that had a $\log_2$ fold change ≥ 1 and a false discovery rate ≤ 0.05. Statistical significance of expression differences for transcripts displayed in Figures 4 and 5 was evaluated using a one-way analysis of variance followed by a Tukey's multiple comparison HSD test. Adjusted $p < .05$ were considered significant.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Project conceptualization: Michael D. Griswold and Rachel L. Gewiss. *Data acquisition*: Rachel L. Gewiss. *Manuscript writing and editing*: Rachel L. Gewiss, Eric A. Shelden, and Michael D. Griswold. *Analysis*: Rachel L. Gewiss, Eric A. Shelden, and Michael D. Griswold. *Figures*: Rachel L. Gewiss and Eric A. Shelden. *Funding acquisition*: Michael D. Griswold.

DATA AVAILABILITY STATEMENT

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE155307 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155307).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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