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miRNA editing landscape reveals miR-34c regulated spermatogenesis through structure and target change in pig and mouse

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ABSTRACT

Spermatogenesis has a close relationship with male infertility. MicroRNAs (miRNAs) play crucial roles in their regulation of target genes during spermatogenesis. A huge dataset of high-throughput sequencing all over the world provides the basis to dig the cryptic molecular mechanism. But how to take advantage of the big data and unearth the miRNA regulation is still a challenging problem. Here we integrated transcriptome of spermatogenesis and found miRNA regulate spermatogenesis through miRNA editing. We then compared different species and found that the distributions of miRNA editing site number and editing types among different cell types during spermatogenesis are conservative. Interesting, we further found that nearly half of the editing events occurred in the seed region in both mouse and pig. Finally, we foundmiR-34c, which is edited frequently at all stages during spermatogenesis, regulates its target genes through the RNA structure changing and shows dysfunction when it is edited. Summary, we depicted the overall profile of miRNA editing during spermatogenesis in mouse and pig and reveal miR-34c may play its roles through miRNA editing.

1. Introduction

Spermatogenesis is a complex and dynamic process [1]. Spermatogonia undergo mitosis to form primary spermatocytes. Secondary spermatocytes are generated after the first division and contain duplicate haploid chromosomes. After the second meiotic division, round spermatids with half the number of chromosomes are formed. The round spermatids' morphology is elongated undergo complex and significant changes, and eventually, mature sperm is formed [2–4]. Such a complex process requires different molecules to execute their functions at different stages [5], and a considerable part of these molecules are named of microRNAs (miRNAs), which are recognized as vital regulatory factors to regulate the expression of their target genes [6].

miRNAs are endogenous small non-coding RNAs of about 20–24 nucleotides (nt) and they show important roles in regulating their target genes within different cells. The first 2–8 nt at the 5′-end of miRNAs is the key region to recognize their target and it is called seed region [7]. Each miRNA may have multiple target genes, and the same gene may be regulated by several miRNAssynergistically. It is reported that more than 30% of genes are regulated by miRNAs and they are conserved in different species [8]. Although some miRNAs are ubiquitously expressed in different cells, about 68% of miRNAs are expressed spatiotemporally [9]. Several experiments have revealed that several miRNAs are high, exclusively or preferentially expressed in the testis and in specific testicular cell types [10–12]. Furthermore, miR-34c, miR-21, miRNA-20, and miRNA-106a are preferentially expressed in mouse spermatogonial stem cells (SSCs). In SSCs, the downregulation of miR-21 increased apoptosis and reduced SSC potency [13]. miR-20 and miR-106a were shown to regulate the renewal of SSCs by targeting *STAT3* and *Ccnd1* [14]. miR-34c has been shown to play a role in maintaining stem cell status and regulating meiosis, and overexpression of miR-34c in germ cells triggers apoptosis [15–17]. In summary,

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we can conclude that miRNAs play an important role during spermatogenesis in mouse. But for miRNAs how to control the sperm cells development is still unclear.

Recently, some studies show that miRNA editing may regulate the processing of pre-miRNAs into mature miRNAs [18,19]. As a post-transcriptional regulator, RNA editing can change nucleic acids at the RNA level without affecting the corresponding DNA sequence. In addition, it is well known that miRNAs exert their regulatory functions on target genes no matter perfect or not match with their seed sequences in the 3′-untranslated regions (UTRs). So we hypothesize that miRNA editing, especially that occurs in the miRNA seed region, will affect the binding of the miRNAs to their target genes by changing the arrangement of the seed sequence, thereby changing the miRNA target. So, we want to make it clear whether RNA editing will affect the miRNA regulation and thus affects the entire spermatogenesis process.

Based on the rapid development of second-generation sequencing and the availability of large-scale small RNA sequencing data, it is possible to verify our hypothesis. High-quality bioinformatics tools for miRNA editing calling have been developed in recent years [20,21], providing us the basic experimental and analytical conditions. Here, we collected all the available small RNA sequencing data of mouse spermatogenesis to depict the miRNA editing panorama of mouse spermatogenesis. Then we integrated them with our miRNA sequencing data during pig spermatogenesis and analyzed the results of mouse and pig from a conservative perspective. Interesting, we found that the distributions of miRNA editing site number and editing types among different cell types during spermatogenesis are conservative. Furthermore, we found that nearly half of the editing events occurred in the seed region in both mouse and pig. Finally, we found miR-34c, which is edited frequently at all stages during spermatogenesis, regulates its target genes through the RNA structure changing and shows dysfunction when it is edited. Summary, we depicted the overall profile of miRNA editing during spermatogenesis in mouse and pig and reveal miR-34c may play its roles through miRNA editing.

2. Materials and methods

2.1. Identification of miRNA editing sites

All sequencing reads will be filtered as follows: (1) the quality of each read need to be at least 20 in more than three positions, (2) adaptor sequences will be removed, (3) reads with length longer (>28 bp) or shorter (<15 bp) than the typical length of a mature miRNA will be removed. The filtered reads were trimmed by 2 nt at 3′end because of the 3′end of mature miRNAs undergoes extensive modifications [22,23]. The filtered and trimmed reads were aligned using Bowtie [24] against their corresponding species, mouse (mm10) and pig (susScr11) genomes, allowing up to one mismatch, the best alignment, and no cross-mapping. We focused on the reads aligned against genomic regions of known pre-miRNAs in miRBase (release 21) [25] for mismatch calling based on the binomial test as described in the literature [20,21]. We identified miRNA editing candidates using the cutoff of the Bonferroni-corrected *P*-value of 0.05 and the mismatch base quality score ≥30. In order to eliminate the effect of change in DNA level, we filter out the points marked in dbSNP (142) from the candidates. The scripts used for miRNA editing calling were published by Alon et al., in 2012 and are available at http://www.tau.ac.il/~elieis/miR_editing/. For the sites confirmed as miRNA editing sites, we divided them into two groups: editing sites in seed region and editing sites in the non-seed region.

2.2. Functional analysis of miRNA targets

miRNA targeting relationship, before and after editing, shows significant differences when the RNA editing occurred in the miRNA seed region. Based on the sequence before and after editing, we first used miRanda [26]software to predict the target gene set of WT miRNAs and edited miRNAs.We then performed functional enrichment analysis of the genes to find biological processes that were affected by WT miRNAs and edited miRNAs. The sequence logo is generated by the WebLogo tool [27].

3. Results

3.1. Data collection and classification integration

We detected miRNA editing sites during spermatogenesis in both pig and mouse species. Up to 26 samples of mouse non-coding RNA-seq datasets were derived from the Gene Expression Omnibus (GEO) and they conclude four type cells: spermatogonia (SG), primary spermatocytes (PSC), spermatid and sperm (Table 1). Then we integrated them with our previous miRNAs sequencing dataset with the same types of cells during pig spermatogenesis [28]. We integrated samples from the same period into one item, so we can find consistent editing sites from different studies. After length and quality control on all reads and filtered out ineligible, the reads were aligned to precursor miRNA sequences in miRBasewith release 21 [25] for mismatch calling (see methods). We screened the mismatched bases in the literature (see methods) to obtain candidate miRNA editing sites and then removed the known SNPs to get the final miRNA editing sites. According to the location information of the editing sites, we divided all the miRNA editing sites into two groups, editing sites in the seed region and editing sites in the non-seed region. Then we focused on the editing sites in the seed region. Our analysis process is shown in Fig. 1A.

Table 1
Samples' information.

Reference	GEO ID	Technique	Total Sample	Phase			
				SG	PSC	Spermatid	Sperm
Inoue K et al.,2017	GSE70890	Illumina HiSeq 1500	5	1	3	1	–
Luo M et al.,2017	GSE56522	Illumina HiSeq 2000	2	1	1	–	–
Hilz S et al.,2016	GSE83264	Illumina HiSeq 2000	4	–	4	–	–
Nixon B et al.,2015	GSE70198	Illumina HiSeq 2000	6	–	–	–	6
Gan H et al.,2011	GSE24822	Illumina Genome Analyzer	3	1	1	1	–
Meunier J et al.,2012	GSE40499	Illumina Genome Analyzer IIx	2	–	1	1	–
García-López J et al.,2015	GSE59254	Illumina HiSeq 2000	2	1	–	1	–
Nordstrand LM et al.,2012	GSE37150	Illumina Genome Analyzer IIx	2	–	2	–	–
Total	26	4	12	4	6		

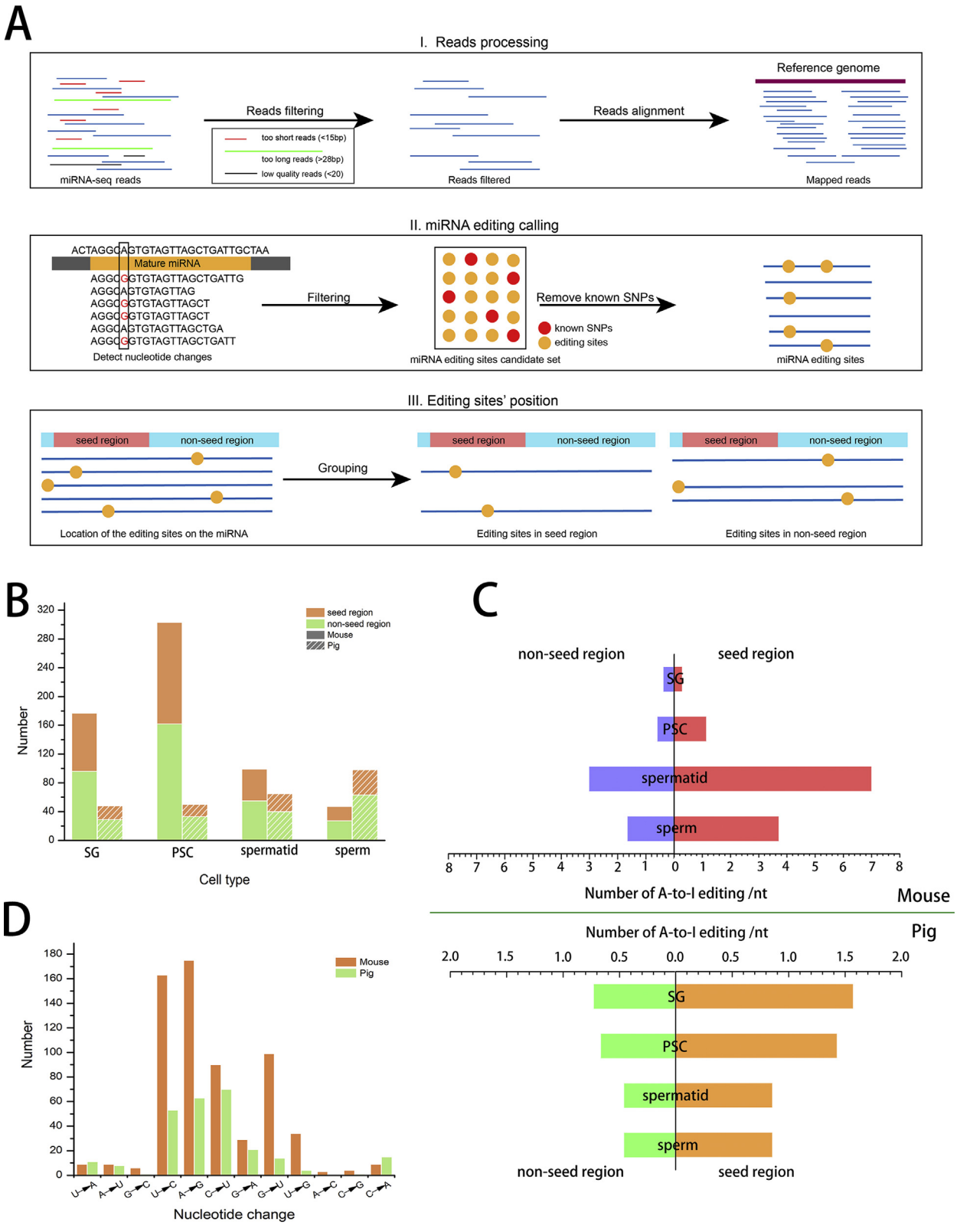


Fig. 1. Experiment process and conservative phenomenon of miRNA editing in mouse and pig. (A)The entire experiment was divided into 3 major parts. All the data were processed in the first part. The qualified reads were aligned to the reference genome. The second part is to identify the miRNA editing sites in samples and filter out the interference sites. The third part is to group all editing sites.(B) The number of editing sites in the four periods of mouse and pig and their distribution in the seed region and non-seed region. (C) The number of 12 kinds of nucleotide changes. (D) A-to-I editing distribution density in seed region and non-seed region of the two animals.

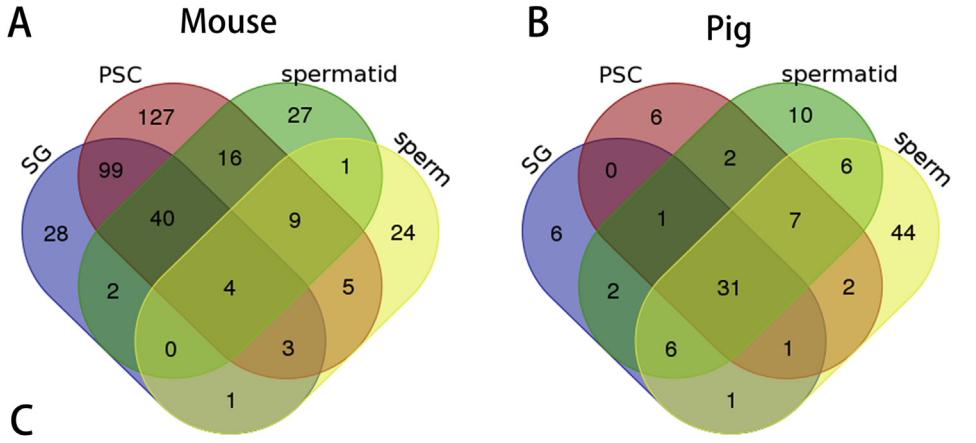
3.2. Conservative analysis of miRNA editing in mouse and pig

630 and 261 editing sites were identified from four types of male germ cells in mouse and pig, respectively. Although the number of miRNA editing sites differs between the two species, our following analysis shows that there are many consistent phenomena. All the editing sites were grouped according to the positions on the miRNAs and our results showed that no matter in mouse or in pig, nearly half of the miRNA editing occurs in the seed region (Fig. 1B).In other words, nearly half of miRNA editing events during spermatogenesis may change the regulation relationship between miRNAs and their target genes which will finally affect the expression of many genes, demonstrating the effectiveness of miRNA editing during spermatogenesis. In addition, the proportion of 12 nucleotide substitutions in mouse and pig is consistent. The number of A-to-I editing is highest among all the types of miRNA editing in both two species (Fig. 1C). Furthermore, we compared the distribution of A-to-I editing events in both mouse and pig chromosome and found that A-to-I showed high-density distribution in the seed regions of both species (Fig. 1D). In short, the distribution of miRNA editing sites number, editing types and the regional preference of A-to-I editing during spermatogenesis all show conservation between mouse and pig. Then we can boldly speculate that these conservations of miRNA editing may still exist in the spermatogenesis of other mammals.

3.3. Two mouse miRNA families frequently edited in the seed region

A comparative analysis of miRNA editing sites at different stages of the same species found that most of the editing sites in the mouse are time-specific, and so there are significantly different editing sites in different phases (Fig. 2A). While editing events in pig are stable at different stages, so most editing events occur in all four periods (Fig. 2B).

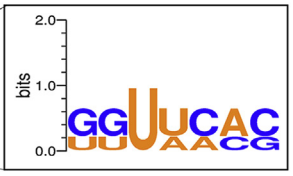
Two miRNA families in mouse present frequent editing, including miR-10 family and the miR-30 family. As shown in, (1) they occurred in all four periods. Interesting, we found they occurred in almost all nucleotides in the seed region. (2)The seed regions of each member of the two miRNA families show some sequence consistency in their editing sites. From Fig. 3, we can find that the seed region of the two miRNA show two typically features: miR-10 family, including miR-10a, miR-10 b, and miR-100 all occurred C-to-U editing in the third position without any other conservative editing types in their seed regions (Fig. 2C); on the other hand, miR-30 family, including miR-30a, miR-30 d, and miR-30e, show six conservative editing types in the seed region with the first position as non-conservative editing type (Fig. 2D). What surprised us is that among the six conservative editing types in miR-30 family, four of them are the A-to-I editing, indicating that deamination in the seed regions susceptible to be replaced by inosine (Fig. 2D).



C

miR-10 family

miRNA name	Sequence of seed region	Sequence of edited seed region
miR-10a	ACCCUGU	GGUUCAC
miR-10b	ACCCUGU	UGUACAC
miR-100	ACCCGUA	GUUUACG



D

miR-30 family

miRNA name	Sequence of seed region	Sequence of edited seed region
miR-30a	GUAAACA	AGGGGUG
miR-30d	GUAAACA	AGGGGUG
miR-30e	GUAAACA	UGGGGUG

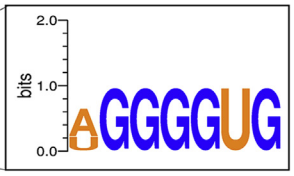


Fig. 2. Two miRNA families in time-specific miRNA editing. (A) miRNA editing in mouse has stage specificity. (B) Editing events in pig are stable at different stages. (C) Sequences of the seed regions of the 3 miRNA members of the miR-10 family before and after editing. (D) Sequences of the seed regions of the 3 miRNA members of the miR-30 family before and after editing.

3.4. Association of miR-34c miRNA editing and male diseases

In order to detect the relationship between miRNA editing and male diseases, we downloaded the list of all human disease-related miRNAs from the HMDD database [29].40 miRNAs are clearly indicated to be related to male reproductive diseases and are mainly divided into two categories: male infertility-associated miRNAs and azoospermia-associated miRNAs, both of them have 20separately. Among them, six miRNAs were detected miRNA editing in the seed region at different stages of spermatogenesis in mouse. miR-34c, miR-122 and miR-374 b are related to male infertility [30], while miR-17, miR-20a and miR-18a are related to azoospermia [31]. Here we focused on miR-34c, whose seed region has been edited in all four stages (Fig. 3A).

Top 10 miRNAs with high editing frequency and seed region edited in the four stages during mouse spermatogenesis were filtered. Then we analyzed the relationship between editing frequency and expression. Surprising, we found that miR-34c is the only miRNA that has been edited in all four stages and has high expression level (Fig. 3B). Seven nucleotides in the miR-34c seed region have been edited and there are two substitutions for each nucleotide in mouse (Fig. 3C). While six nucleotides in miR-34c seed region have been edited in pig and there is only one substitution per nucleotide (Fig. 3D). As our and other group reports, miR-34c plays an important regulatory role in meiosis, germ cell apoptosis, and stem cell differentiation [15–17]. Combining this result, we speculate that miR-34c affects the entire spermatogenesis process by miRNA editing with species specifically.

3.5. Functional effects of RNA editing in miR-34c

Wide-type (WT) miR-34c is involved in many processes of spermatogenesis: stem cell differentiation, mitosis, mitotic and cell motility (Fig. 4A). It has been reported that miR-34c affects the apoptosis of male germ stem cells by regulating its target gene *Alf1* [15]. It can also influence meiotic and the differentiation of spermatogonial stem cells through the regulation of the gene *Nanos2* [17](Fig. 4B). Interestingly, when the fourth nucleotide of the WT miR-34c seed region undergoes A-to-I editing, the edited miR-34c redirects to a new set of target genes. There is no *Alf1* and *Nanos2* in the predicted target genes set for miR-34c with miRNA editing. This means that the edited miR-34c loses its regulatory effects on the genes *Alf1* and *Nanos2*. We got 3463 targets of WT miR-34c and 833 targets of edited miR-34c based on their sequence (see methods), with 274 genes overlapping between the two sets, accounting for 7.9% of the original target genes set (Fig. 4C). In other words, the occurrence of RNA editing changed more than 90% targeting relationship of miR-34c. Functional enrichment analysis of

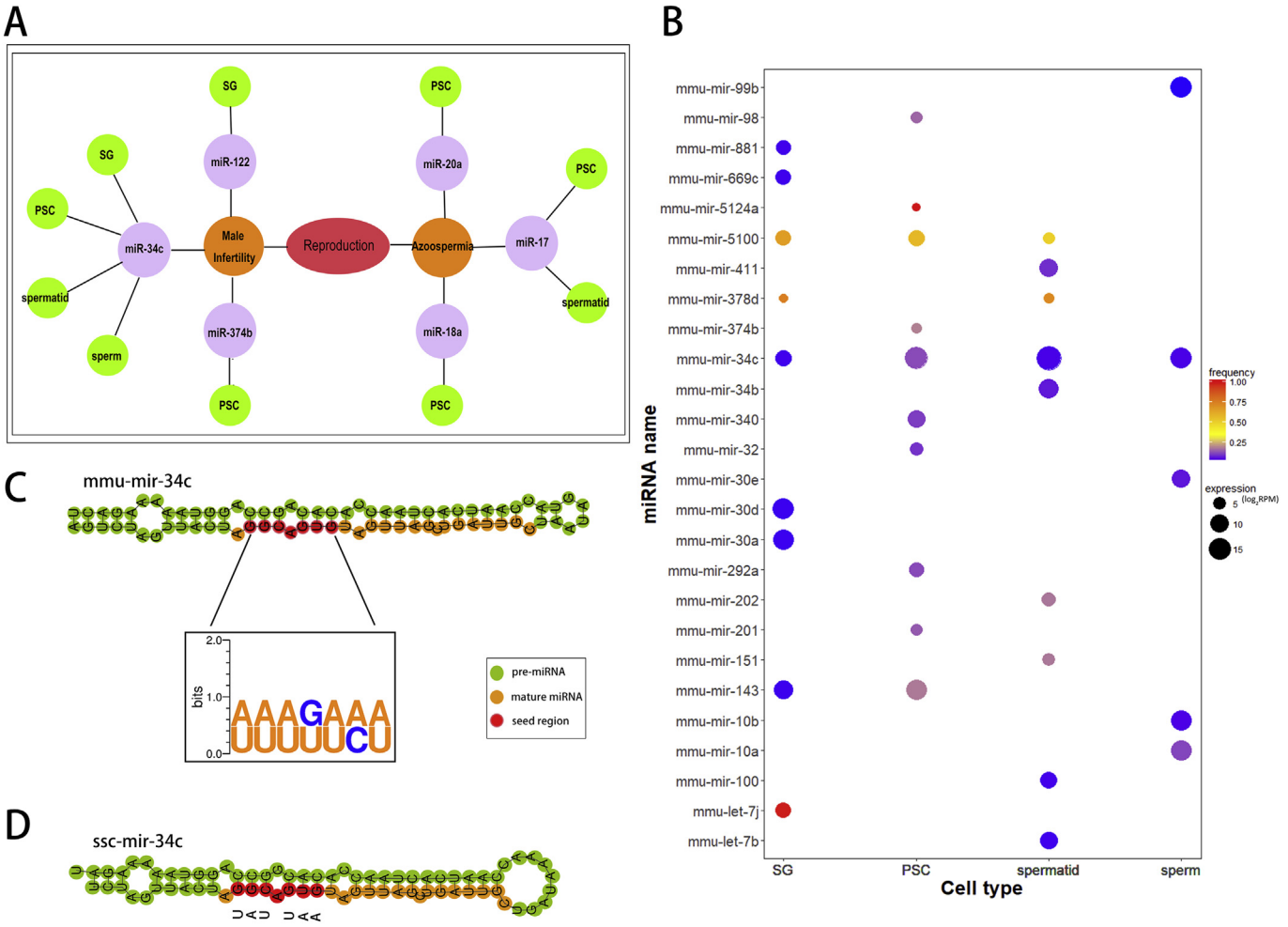


Fig. 3. Highly expressed miR-34c is frequently edited. (A) The seed regions of six miRNAs which related to male reproductive disease were detected editing events during spermatogenesis in mouse. (B) In the four stages of the mouse, some miRNAs which RNA editing occurs in the seed region and the editing frequency is the top 10. (C) The secondary structure of mouse pre-mir-34c and miRNA editing in its seed region. (D) The secondary structure of pig pre-mir-34c and miRNA editing in its seed region.

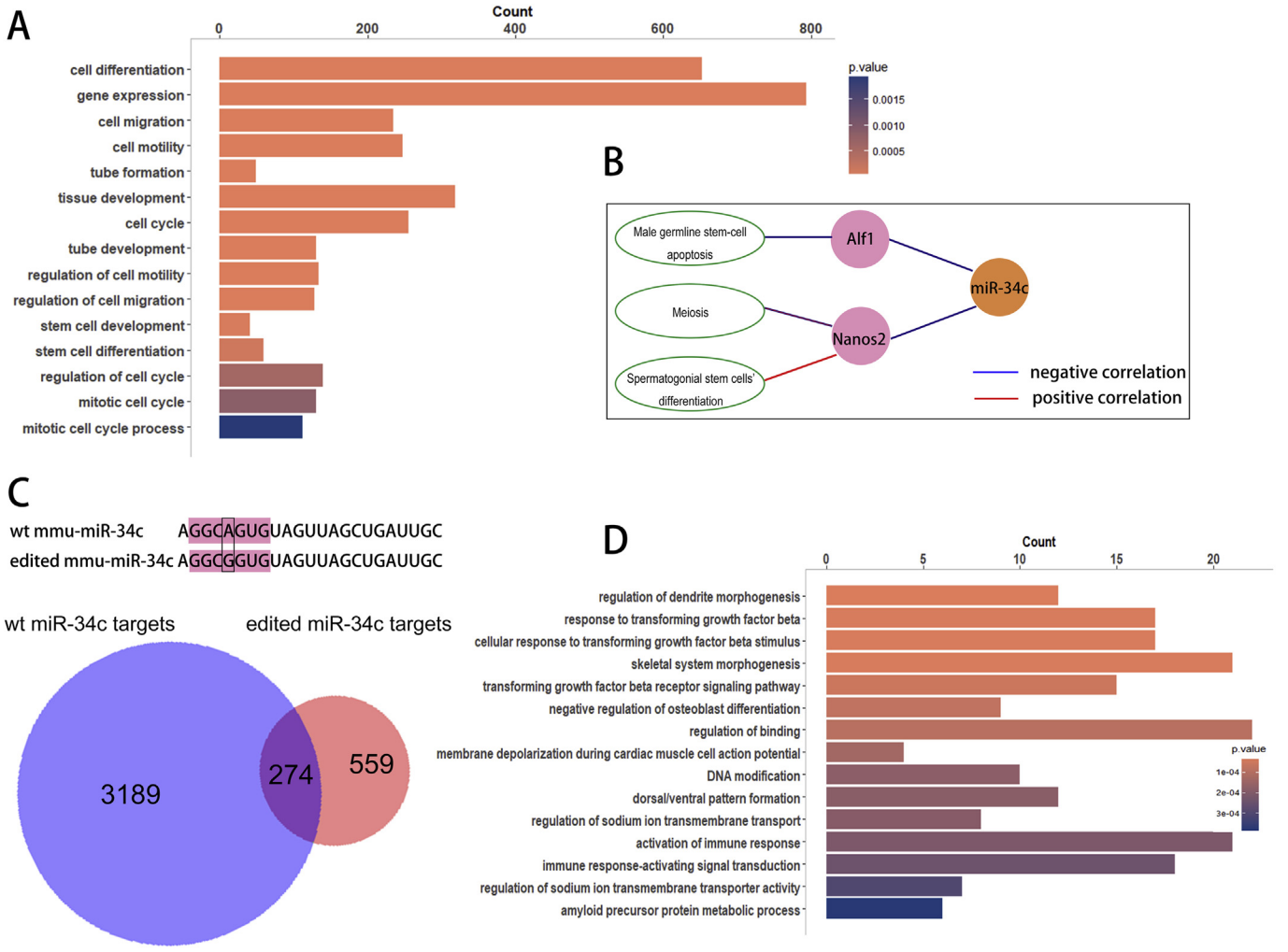


Fig. 4. Changes in function of miR-34c before and after editing. (A) Some biological functions related to WT miR-34c. (B) WT miR-34c influences meiosis, apoptosis of male germ cells and differentiation of spermatogonial stem cells through its regulatory functions on Alf1 and Nanos2. (C) Predicted target genes of WT miR-34c and edited miR-34c. (D) Some biological functions related to edited miR-34c.

the edited miR-34c target genes revealed that these genes are involved in processes such as immunization, and almost no spermatogenesis-related process is found (Fig. 4D). Edited miR-34c is likely to no longer perform its original function and cannot regulate spermatogenesis.

4. Discussion

In this study, based on data integration methods, we use big data of high-throughput sequencing to explore the molecular mechanisms that miRNAs play in spermatogenesis in mouse and pig from the perspective of miRNA editing. We identified the miRNA editing sites of spermatogonia, primary spermatocytes, spermatids, and mature sperm in the two species. The results show that mouse had more miRNA editing sites than pig. However, there are many miRNA editing phenomena that show consistency between two species, such as the distribution of editing number, editing types, and the distribution density of A-to-I editing in the seed region and non-seed region. No matter in mouse or in pig, nearly half of the miRNA editing events occur in the seed region, meaning that nearly half of editing events may affect miRNA function. The distribution density of A-to-I editing in the seed region is much higher than in the non-seed region, future demonstrating the frequency of RNA editing in the seed region and the effectiveness of RNA editing. Among the miRNAs that have been edited in seed regions,miR-34c is the only one that has been edited in all four stages and has high expression level. It is known that miR-34c plays an important role in spermatogenesis. It can regulate meiosis and its deletion mutant can lead to male infertility [32]. Our result suggests that RNA editing will change its function by altering the targets of miR-34c. Especially, when the fourth nucleotide of the miR-34c seed region is edited, the number and function of its target genes will change greatly.

Existing experiments have pointed out that miRNA is indispensable for spermatogenesis, and miR-34c has also been shown to be related to male infertility. However, the molecular mechanism of miR-34c dynamic regulation during spermatogenesis is not clear. Previous studies on miRNA editing mainly focused on cancer. Our study for the first time links male infertility and spermatogenesis with miRNA editing and analyzes the effects of miRNA editing on spermatogenesis. We focus on the miRNAs that have been edited in the seed region. This part of the miRNA may not be able to bind to its original target genes finely because of changes in the seed sequence and thus leads to dysfunction in spermatogenesis. Our study shows that many miRNAs related to male reproductive diseases have undergone RNA editing, especially miR-34c, which was detected miRNA editing in many stages. The edited miR-34c almost lost its regulatory function during spermatogenesis, which is

consistent with our hypothesis. In short, big data mining allows us to establish a comprehensive standard map of miRNA editing for the entire spermatogenesis.

Our current study was conducted on mouse and pig. miRNA editing showed some conservation in the spermatogenesis of the two species. However, whether these conservations of miRNA editing will present in other animals, especially primates is still not clear and need more study. In our analysis, some miRNAs such as miR-34c showed frequent editing during spermatogenesis. In other organizations or biological processes, which miRNAs are edited, what are the conservativeness and specificity of miRNA editing among different tissues? These problems are still worth exploring in future.

Conflicts of interest

The authors declare no competing financial interests.

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