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# Identification, characterization and functional analysis of gonadal long noncoding RNAs in a protogynous hermaphroditic teleost fish, the ricefield eel (*Monopterus albus*)

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## Abstract

**Background:** An increasing number of long noncoding RNAs (lncRNAs) have been found to play important roles in sex differentiation and gonad development by regulating gene expression at the epigenetic, transcriptional and posttranscriptional levels. The ricefield eel, *Monopterus albus*, is a protogynous hermaphroditic fish that undergoes a sequential sex change from female to male. However, the roles of lncRNA in the sex change is unclear.

**Results:** Herein, we performed RNA sequencing to analyse lncRNA expression patterns in five different stages of *M. albus* development to investigate the roles of lncRNAs in the sex change process. A total of 12,746 lncRNAs (1503 known lncRNAs and 11,243 new lncRNAs) and 2901 differentially expressed lncRNAs (DE-lncRNAs) were identified in the gonads. The target genes of the DE-lncRNAs included *foxo1*, *foxm1*, *smad3*, *foxr1*, *camk4*, *ar* and *tgfb3*, which were mainly enriched in signalling pathways related to gonadal development, such as the insulin signalling pathway, MAPK signalling pathway, and calcium signalling pathway. We selected 5 highly expressed DE-lncRNAs (*LOC109952131*, *LOC109953466*, *LOC109954337*, *LOC109954360* and *LOC109958454*) for full length amplification and expression pattern verification. They were all expressed at higher levels in ovaries and intersex gonads than in testes, and exhibited specific time-dependent expression in ovarian tissue incubated with follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG). The results of quantitative real-time PCR (qRT-PCR) analysis and a dual-luciferase assay showed that *znf207*, as the gene targeted by *LOC109958454*, was expressed in multiple tissues and gonadal developmental stages of *M. albus*, and its expression was also inhibited by the hormones FSH and hCG.

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**Conclusions:** These results provide new insights into the role of lncRNAs in gonad development, especially regarding natural sex changes in fish, which will be useful for enhancing our understanding of sequential hermaphroditism and sex changes in the ricefield eel (*M. albus*) and other teleosts.

**Keywords:** RNA-seq, *Monopterus albus*, Gonadal development, lncRNA

## Background

Long noncoding RNAs (lncRNAs) are transcript with a total length of more than 200 nucleotides that are little or no significant protein coding capacity [1, 2]. Compared with mRNAs, lncRNAs are more abundant in the nucleus [3–5]. They can exert their biological functions by acting on protein-coding genes in *cis* and *trans* ways [6–9]. Many studies have shown that lncRNAs are involved in several biological processes, including germ cell development [10], meiosis [11], gamete formation [12, 13] and sex differentiation [14].

lncRNAs have been found to play important roles in the reproductive development of organisms. The lncRNAs *TCONS\_00025195* and *TCONS\_00025196* may be involved in regulating the expression of SOX9 in human testes [15]. Most of the highly expressed lncRNAs in the testes of adult mice were found to be testis specific and were expressed at the beginning of and after spermatogenesis [16]. A total of 24,601 lncRNAs were identified in ovaries of *Columba livia*, and 148 lncRNAs were significantly differentially expressed between high and low egg production performance groups, and the target gene *FOXP2* of the lncRNA *MSTRG.7894.4* is involved in the regulation of an oestrogen receptor [17]. We identified 5317 lncRNAs and 297 differentially expressed lncRNAs (DE-lncRNAs) in *Oryzias latipes* ovaries and testes, of which lncRNA *MSTRG.14827.1* may play a key role in gametogenesis [18]. In total, 28,500 lncRNAs were identified in the ovaries and testes of *Pelodiscus sinensis*, and the target genes for the 10,495 DE-lncRNAs included several genes involved in gonadal development [19]. In addition, 962 DE-lncRNAs related to sex differentiation were found to be involved in the sex reversal induced by mifepristone (RU486) in XX *Oreochromis niloticus* gonads [20].

The ricefield eel, *Monopterus albus*, is increasingly recognized as a new model species for the study of sequential hermaphroditism and sex change [21–23]. *M. albus* is a hermaphroditic fish with reproductive traits that natural sex reversal from female to male through an intersex stage during its life cycle [22, 24]. To comprehensively reveal the molecular mechanisms of the sex change process and the potential role of lncRNAs in *M. albus*, RNA sequencing (RNA-seq) of gonadal tissues representing five sexual stages (ovary stage (OV), early intersexual stage (IE), middle intersexual stage (IM), late intersexual

stage (IL) and testis stage (TE)) was performed in the present study. The functional lncRNAs, target genes and their correlations associated with both sex determination and gonad development were explored. This study provides novel insights into lncRNA function and the mechanisms of sequential hermaphroditism in fish and other vertebrates.

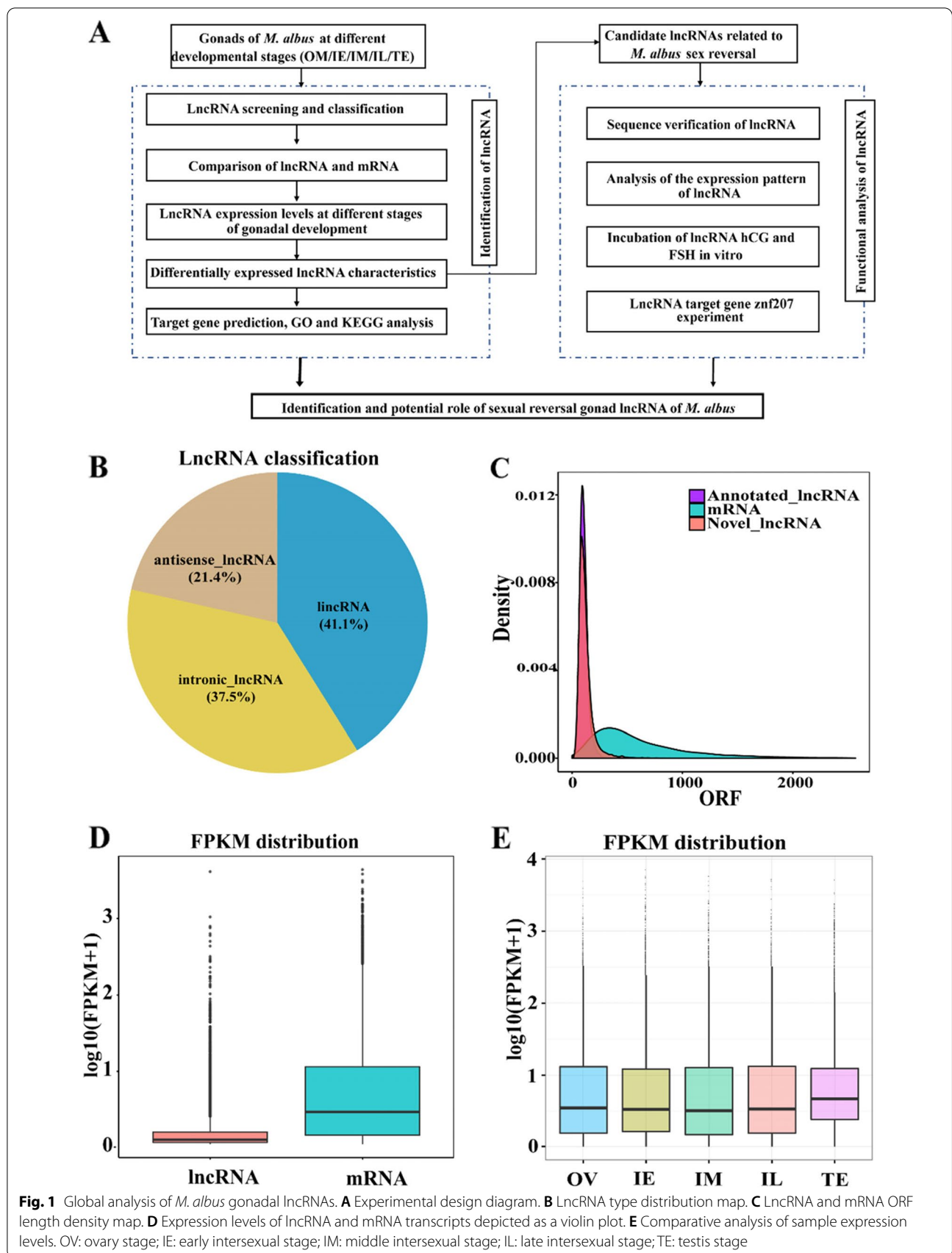
## Results

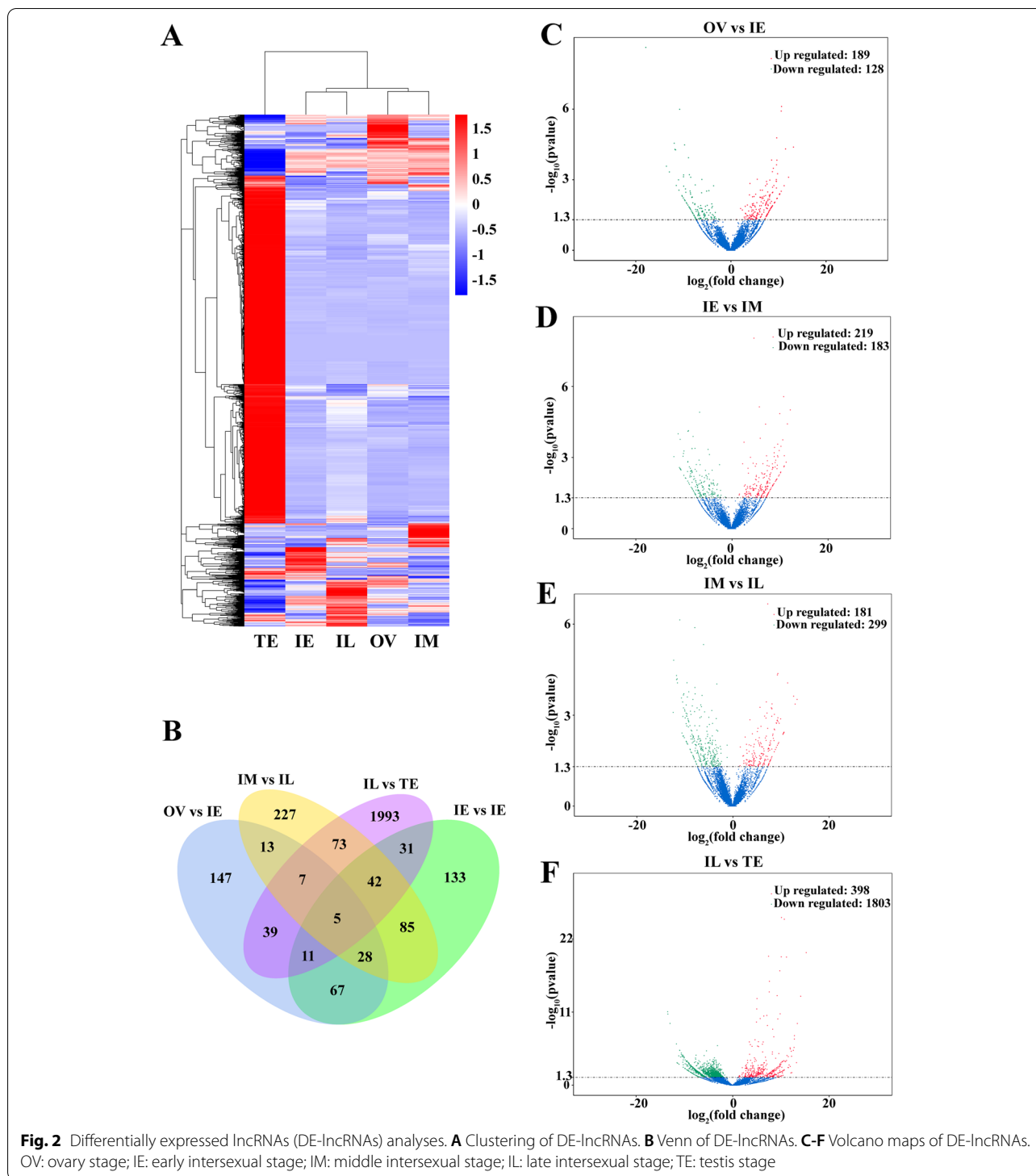
### Identification and characterization of gonadal lncRNAs

To study the potential role of lncRNAs in fish gonads, we used *M. albus* gonads at five developmental stages (OV, IE, IM, IL, TE) for RNA-seq and cDNA libraries. At least 101,886,556 clean reads were obtained. The Q20 percentage > 97.51%, Q30 percentage > 93.68%, GC content between 47.63–48.71% and percentage of error < 0.01% (Additional file 1: Table S1). These values indicated that the RNA-seq data were reliable.

In this study, a total of 1503 known lncRNAs and 11,243 new lncRNAs (Additional file 2: Fig. S1) were identified in libraries constructed from *M. albus* gonads. The whole experimental process is shown in Fig. 1A. After each step of screening, the final proportion of different types of lncRNAs (Fig. 1B), including lincRNAs (41.1%), antisense lncRNAs (21.4%), and intronic lncRNAs (37.5%), was determined. The ORF length and average FPKM of lncRNAs were smaller than those of mRNAs (Fig. 1C–D). By comparing the box plots of the quantitative results for different samples (Fig. 1E), the overall expression level in the male stage among different samples was found to be higher than that in the female/intersex stages ( $P < 0.05$ ).

The DE-lncRNAs ( $P < 0.05$ ) were counted according to the gonadal development of *M. albus* (Fig. 2, Additional file 3: Dataset S1). The aggregation of DE-lncRNAs at different gonadal stages indicated that ovarian and intersex expression clustered into one branch, and testis into one branch (Fig. 2A). Venn diagram analysis showed that there was a total of 2901 DE-lncRNAs, and 5 lncRNAs were differentially expressed throughout gonadal development (Fig. 2B). In OV vs. IE, 189 lncRNAs were up regulated and 128 lncRNAs were down regulated (Fig. 2C); in IE vs. IM, 219 lncRNAs were up regulated and 183 lncRNAs were down regulated (Fig. 2D); in IM vs. IL, 181 lncRNAs were up regulated and 299 lncRNAs were down regulated (Fig. 2E); and in IL vs. TE, 398 lncRNAs were up regulated and 1803 lncRNAs were down





regulated (Fig. 2F). The results showed that the number of DE-lncRNAs gradually increased from the female to intersex to male stages, especially the number of DE-lncRNAs from IL to TE.

**GO and KEGG analyses of lncRNA target genes**

In this study, we predicted the *trans*-target genes of lncRNAs, predicted the potential function of lncRNAs, and performed Gene Ontology (GO) cluster analysis (Table 1, Additional file 4: Fig. S2, Additional file 5: Dataset S2,

Additional file 6: Dataset S3) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis (Table 2, Additional file 7: Fig. S3, Additional file 8: Dataset S4, Additional file 6: Dataset S3). The significantly enriched ( $q$ -value<0.05) GO terms were involved in regulation of cellular processes, regulation of biological processes and cellular components, and some *trans*-target genes of the lncRNAs were related to gonadal development, such as *foxo1*, *foxm1*, *smad3*, *foxr1*, *camk4* and *ar*. Genes that were significantly enriched in several KEGG pathways ( $P$ <0.05) were identified as being involved in gonadal development, including *XLOC\_059262* and its target *foxo1* in the insulin signalling pathway, *XLOC\_091218* and its target *tgfb3* in the MAPK signalling pathway, and *XLOC\_169543* and its target *camk4* in the calcium signalling pathway. Significantly, *XLOC\_181771* and its target *smad3* and *XLOC\_091218*

**Table 2** DE-lncRNAs and their targets among the significantly enriched KEGG pathways

Comparison	lncRNA (number)	Trans-targets	KEGG
OV vs. IE/ IE vs. IM/ IM vs. IL/ IL vs. TE	<i>XLOC_059262</i> (45)	<i>foxo1</i>	Insulin signaling pathway
	<i>XLOC_091218</i> (40)	<i>tgfb3</i>	MAPK signaling pathway
	<i>XLOC_169543</i> (38)	<i>camk4</i>	Calcium signaling pathway
OV vs. IE/ IL vs. TE	<i>XLOC_181771</i> (35)	<i>smad3</i>	Endocytosis
	<i>XLOC_091218</i> (40)	<i>tgfb3</i>	Endocytosis

Note: lncRNA (number): the listed lncRNA Gene IDs are those with the smallest  $P$ -values, and numbers in parentheses are the numbers of lncRNAs; see Schedule Data 4 for details. *OV* Ovary stage, *IE* Early intersexual stage, *IM* Middle intersexual stage, *IL* Late intersexual stage, *TE* Testis stage

**Table 1** DE-lncRNAs and their targets among the significantly enriched GO terms

Comparison	DE-lncRNA (number)	Trans-targets	GO terms	Term type
OV vs. IE	<i>XLOC_059262</i> (45)	<i>foxo1</i>	Regulation of biological process	BP
	<i>XLOC_138801</i> (29)	<i>foxm1</i>	Regulation of cellular process	BP
IE vs. IM	<i>XLOC_181771</i> (35)	<i>smad3</i>	Regulation of cellular process	BP
	<i>XLOC_138801</i> (29)	<i>foxm1</i>	Regulation of biological process	BP
			Biological regulation	BP
	<i>XLOC_064006</i> (2)	<i>foxr1</i>	Regulation of biological process	BP
			Biological regulation	BP
IM vs. IL	<i>XLOC_059262</i> (45)	<i>foxo1</i>	Regulation of biological process	BP
	<i>XLOC_181771</i> (35)	<i>smad3</i>	Regulation of biological process	BP
	<i>XLOC_138801</i> (29)	<i>foxm1</i>	Regulation of cellular process	BP
			Biological regulation	BP
	<i>XLOC_169543</i> (38)	<i>camk4</i>	Cellular process	BP
IL vs. TE	<i>XLOC_059262</i> (45)	<i>foxo1</i>	Transmembrane signaling	MF
			Receptor activity	MF
	<i>XLOC_181771</i> (35)	<i>smad3</i>	Regulation of cellular process	BP
			Biological regulation	BP
	<i>XLOC_138801</i> (29)	<i>foxm1</i>	Biological regulation	BP
	<i>XLOC_169543</i> (38)	<i>camk4</i>	Membrane	CC
	109,964,303 (3)	<i>ar</i>	Signaling receptor activity	MF
			Signal transducer activity	MF
			Receptor activity	MF
			Molecular transducer activity	MF
			Signal transduction	BP
			Single organism signaling	BP
			Signaling	BP
			Cell communication	BP
			Regulation of biological process	BP
			Cellular response to stimulus	BP
			Response to stimulus	BP

Note: lncRNA (number): the listed lncRNA Gene IDs are those with the smallest  $P$ -values, and numbers in parentheses are the numbers of lncRNAs; see Schedule Data 4 for details. *OV* Ovary stage, *IE* Early intersexual stage, *IM* Middle intersexual stage, *IL* Late intersexual stage, *TE* Testis stage. *BP* Biological process, *CC* Cellular component, *MF* Molecular function

and its target *tgfb3* were enriched in endocytosis pathways.

#### Validation of DE-lncRNAs

Through PCR technology, we successfully amplified 5 highly expressed DE-lncRNAs, namely, *LOC109952131* (450 bp), *LOC109953466* (320 bp), *LOC109954337* (239 bp), *LOC109954360* (424 bp) and *LOC109958454* (454 bp). In addition, we showed the location of the lncRNAs on the *M. albus* chromosome and their exon number (Fig. 3A).

To verify the authenticity of the RNA-seq data, we conducted qRT-PCR analysis to determine the expression levels of 5 randomly selected DE-lncRNAs in the gonads at the 5 developmental stages. The lncRNA expression trend obtained by qRT-PCR was consistent with the RNA-seq results, confirming that the RNA-seq data were reliable (Fig. 3 B-F). Five DE-lncRNAs were expressed at significantly lower levels in the male stage than in the female and intersex stages ( $P < 0.05$ ). The significant difference in expression between the male and female/intersex stages further indicated that lncRNAs may regulate the gonadal development of *M. albus*.

#### Expression of five DE-lncRNAs in the ovary after incubation with FSH and hCG

Incubation with FSH increased lncRNA expression in a time-dependent manner, and the overall trend was a decrease followed by an increase (Fig. 4E, G and I). Except for *LOC109952131* (Fig. 4A) and *LOC109953466* (Fig. 4C) incubated at 1 ng/mL and 5 ng/mL, all the samples showed the highest expression level at 1 h of incubation. Incubation with hCG increased lncRNA expression in a time-dependent manner, and the overall trend was an increase followed by a decrease (Fig. 4B, D and H). Except for *LOC109954337* (Fig. 4F) incubated at 10.0 IU/mL and *LOC109954454* (Fig. 4J) incubated at 10.0 IU/mL, all the samples showed the highest expression levels at 2 h of incubation.

#### Identification of the *LOC109958454* target gene *znf207*

As shown in Fig. 5A, *LOC109958454* is 11.108 kb away from its colocalized mRNA *znf207*. The results of tissue distribution experiments showed that *znf207* was widely distributed in gonadal tissues (ovary and testis) and non-gonadal tissues (eyes, pituitary gland, heart, etc.) of *M. albus* (Fig. 5B). In contrast to *LOC109958454*, the expression of *znf207* continued to increase, and the expression in the male stage was higher than that in the female and intersex stages during the gonadal development process (Fig. 5C). The biological relationship of lncRNAs is often related to their subcellular location. The results showed that *LOC109958454* and *znf207* were specifically

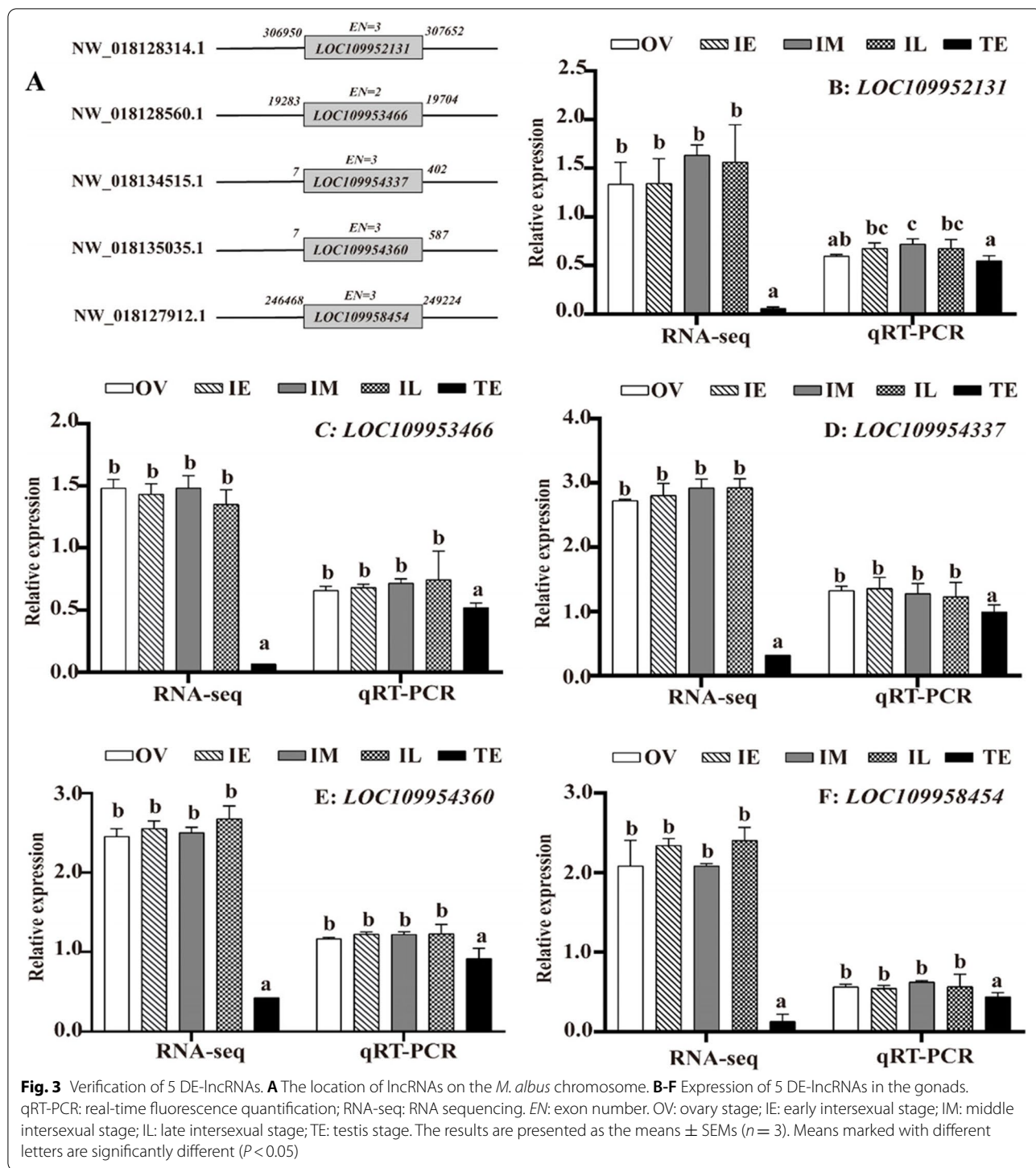
expressed in the nucleus (Fig. 5D). The results of the dual-luciferase assay showed that *LOC109958454* could inhibit the expression of *znf207*, and this inhibition disappeared or was weakened when the binding site was mutated (Fig. 5E). The expression of *znf207* at each time point after incubation with FSH and hCG divided by that in the control group (0 h) was mostly less than 1. FSH and hCG inhibited the expression of *znf207* (Fig. 5F-G). In short, *znf207* is the target gene of *LOC109958454*, and its expression level is affected by hormones.

## Discussion

### Function of lncRNAs in gonadal development

Transcriptome sequencing of Meishan pig and Yorkshire ovarian tissues revealed 3827 lncRNAs and 192 DE-lncRNAs [25]. In Duroc pigs ovaries on days 0, 2, and 4 of follicular development, 140 lncRNAs were found to be differentially expressed in pairwise comparisons [26]. There were 115 DE-lncRNAs between the goat ovarian follicular phase and luteal phase [27]. A total of 1118 DE-lncRNAs were found based on the comparison between the testes of 3 month old and 9 month old sheep [28]. In our research, the number of DE-lncRNAs corresponding to OV vs. IE, IE vs. IM, IM vs. IL and IL vs. TE was 317, 402, 408, and 2201, respectively. Analysis of the results of lncRNA identification in different species and the differential expression at different developmental stages suggested that lncRNAs also play roles in gonadal development in *M. albus*.

lncRNAs have a wide range of functions in animal reproduction and development, including in germ cell growth, meiosis, gametogenesis, sex hormone response, and sex determination [29–31]. A recent study provided the first evidence that the lncRNA *H19* is associated with polycystic ovary syndrome (PCOS) in women and that increased levels of lncRNA *H19* are a risk factor for PCOS [32]. The expression of lncRNAs (such as *XR\_001917388.1*) in goat ovaries during the follicular phase is higher than that in the luteal phase and may regulate oogenesis and oocyte maturation [27]. The lncRNA *TCONS\_00030774* is located approximately 500 bp downstream of the estrogen receptor 1 (*ESR1*), which indicates that it plays a role in porcine ovarian development [25]. The lncRNAs *1700108J01Rik* and *1700101O22Rik*, both of which are mouse testis specific lncRNAs, are specifically expressed in the testicular germ cells of premeiosis and round spermatids, which coincides with the reactivation of transcription during spermatogenesis [16]. The lncRNA *NONMMUT074098.2* (*Lnc10*) can cause abnormal spermatogenesis by promoting germ cell apoptosis. After *Lnc10* was knocked down, the morphology of the testis changes, and the average quality decreases [33]. In addition, lncRNAs can regulate



(See figure on next page.)

**Fig. 4** In vitro regulatory effect of hCG and FSH on the expression of DE-lncRNAs in the ovaries of *M. albus*. **A, B** LOC109952131 expression patterns in ovaries after incubation with FSH and hCG. **C, D** LOC109953466 expression pattern in ovaries after incubation with FSH and hCG. **E, F** LOC109954337 expression pattern in ovaries after incubation with FSH and hCG. **G, H** LOC109954360 expression pattern in ovaries after incubation with FSH and hCG. **I, J** LOC109958454 expression pattern in ovaries after incubation with FSH and hCG. The results are presented as the means  $\pm$  SEMs ( $n = 3$ ). Means marked with different letters are significantly different ( $P < 0.05$ )

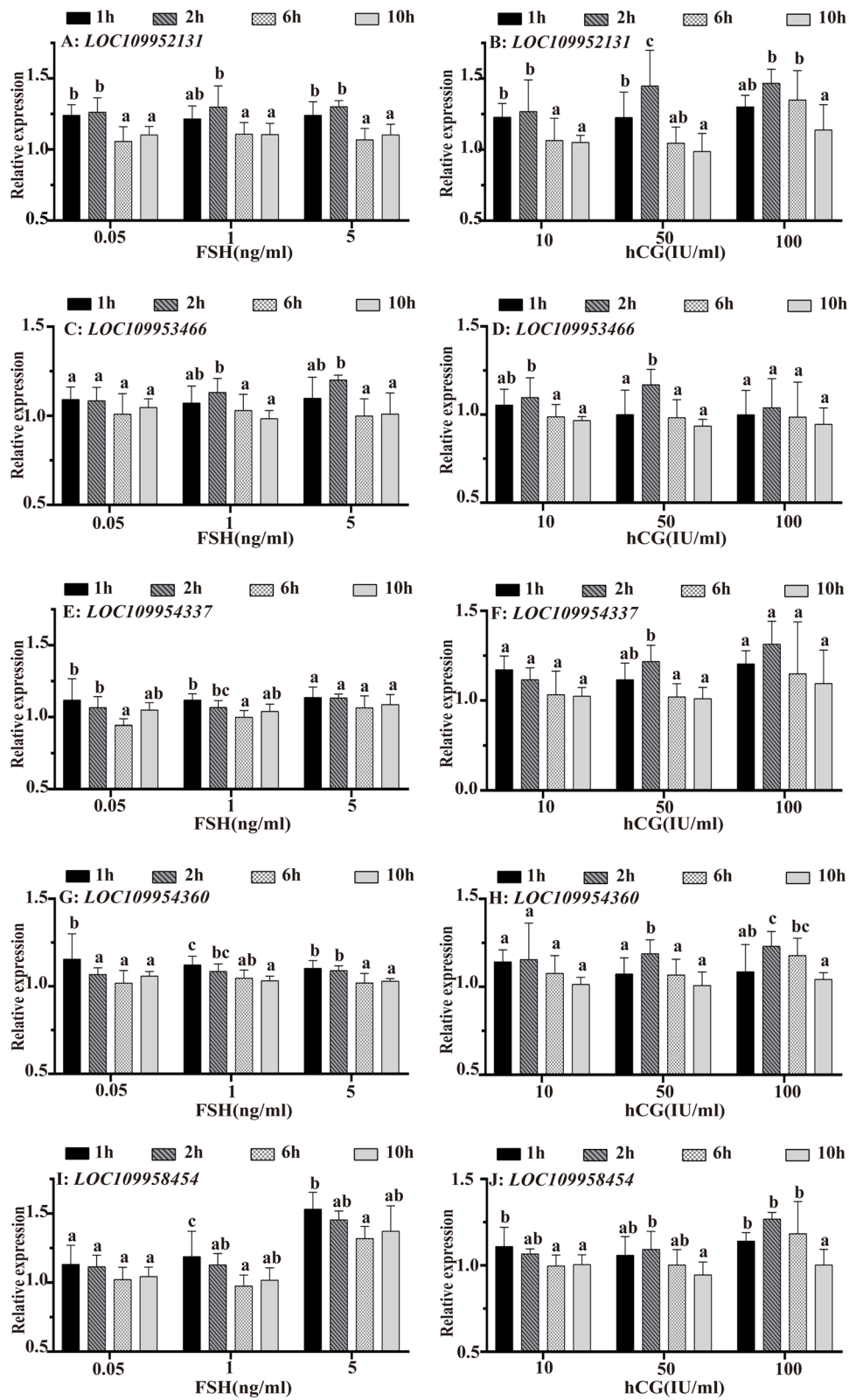
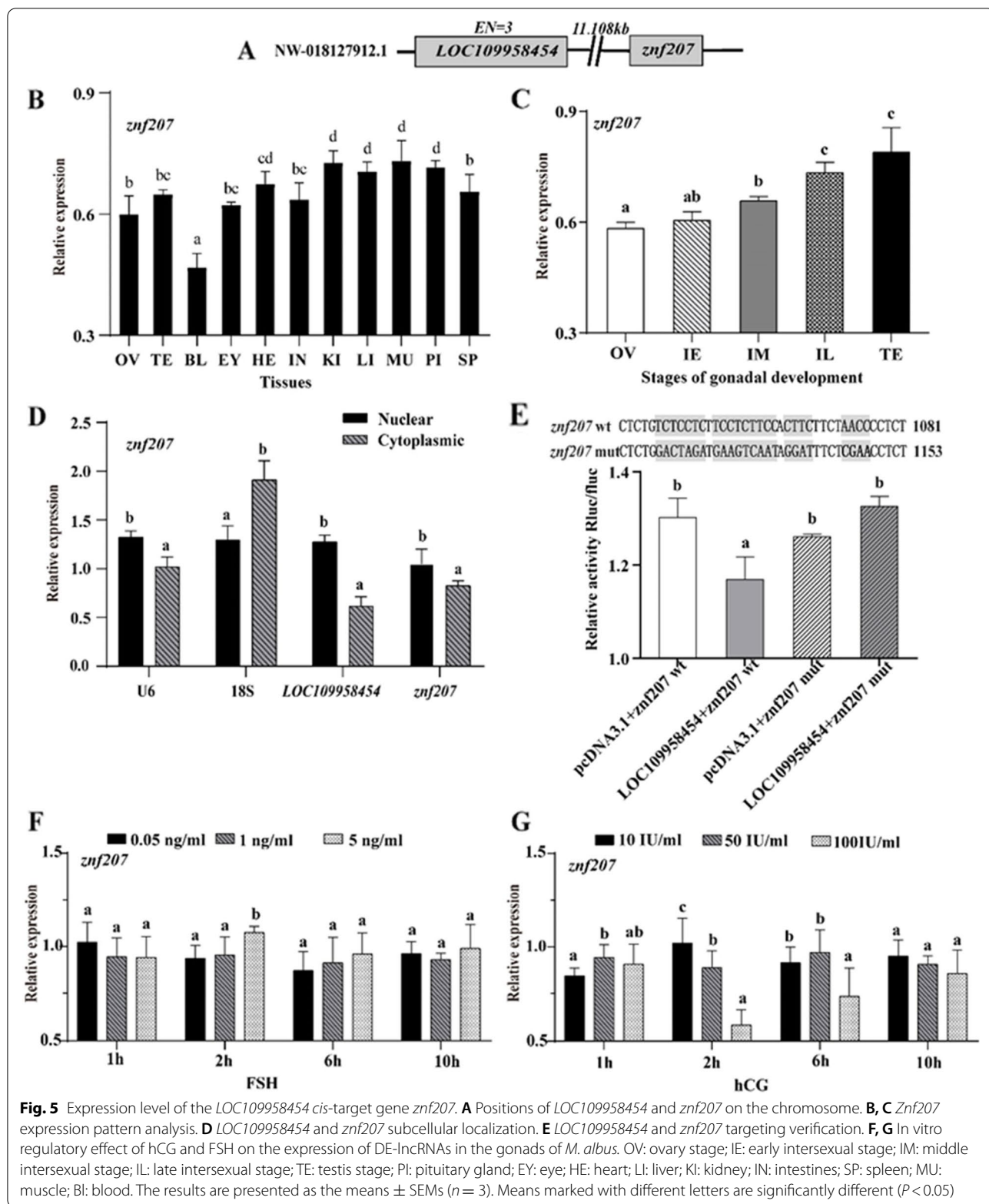


Fig. 4 (See legend on previous page.)





the gonadal development of *Mauremys mutica* [14], *P. sinensis* [34] and *Scylla paramamosain* [35] and affect sex differentiation. In our study, the *cis*-target genes of the lncRNAs included a large number of genes involved in gonadal development, including *igf1* [36], *sox9* [37], *gdf9* [38], and *smad2* [24] (Additional file 9: Dataset S5). Therefore, lncRNAs may play roles in the gonadal development process in *M. albus*, and the specific mechanism needs to be further studied.

#### DE-lncRNA target genes and their GO and KEGG analyses

A total of 12,746 lncRNAs were identified from the gonads of *M. albus* by RNA-seq, and 2901 of the lncRNAs were differentially expressed. In this study, some of the predicted *trans*-target genes were related to gonadal development, such as *foxo1*, *foxm1*, *smad3*, *foxr1*, *camk4*, *ar* and *tgfb3* (Table 1 and Table 2). The *XLOC\_059262* target gene *foxo1*, *XLOC\_138801* target gene *foxm1* and *XLOC\_064006* target gene *foxr1* are all fox genes, which play important roles in the sex determination and gonadal development of bony fish [39]. The target gene of *XLOC\_181771*, *smad3*, is involved in endocytosis and is also a member of the TGF- $\beta$  family. That the gene *smad3* may be the main signal transduction molecule that, via activin, stimulates the expression of FSH $\beta$  in goldfish [40]. The target gene *camk4* of *XLOC\_169543* in the calcium signalling pathway is expressed at high levels. In mice lacking *camk4*, spermatogenesis and basic nucleoprotein exchange are impaired, leading to male sterility [41]. Similarly, female fertility in *camk4* deficient muscle groups is significantly reduced due to follicular development and ovulation damage [42]. *ar* is an important receptor in the supporting cells of the testis. It plays an important role in maintaining the number of spermatogonia and the integrity of the blood testis barrier, in the completion of meiosis, in the adhesion of sperm cells and in the fertilization of sperm and other spermatogenesis processes [43, 44]. In this study, *ar* was enriched in only IL vs. TE, indicating that it plays an important role in the development of the testis during the sex reversal process of *M. albus*. Mutation of the *XLOC\_003298* target gene *tgfb3* may be related to male sterility [45]. In addition, *tgfb3* has a key role related to meiotic arrest and the development of oocytes [46, 47].

The predicted target genes of the DE-lncRNAs were analysed by GO and KEGG cluster analyses. In our research, the insulin signalling pathway, MAPK signalling pathway, and calcium signalling pathway were found to be enriched. The lncRNA GAS5 may regulate the expression of PARP1 by recruiting the transcription factor E2F4 to its promoter, affecting the activity of the MAPK signalling pathway, promoting apoptosis and causing G0/G1

arrest in ovarian cancer cells [48]. The results in zebrafish (*Danio rerio*) show that Corticotropin-releasing hormone (CRH) and its receptor inhibit estrogen production and synthesis, and the inhibition of CRH $\alpha$  is partly mediated through the p38 MAPK signalling pathway [49]. Injection of IRS (an insulin signaling pathway component) dsRNA into adult female oriental fruit fly significantly reduced IRS transcript levels, thereby inhibiting ovarian development, and the average size of the ovaries was reduced by 33% compared to controls [50]. A calcium signaling pathway, initiated by internal stores, is required for acute LH-induced steroidogenesis [51]. And calcium is also an important component of the signal transduction pathway, which is an important regulatory component of meiosis during oogenesis in mouse [52]. In our previous study, insulin, MAPK and calcium signalling pathway were involved in the process of *M. albus* cell apoptosis [53]. In this study, these pathways were also enriched in the whole process of sexual reversal of *M. albus*, which suggested that it may be involved in the whole process of gonadal development, especially in the process of ovarian development and apoptosis. Receptor-mediated endocytosis uptake of multiple ligands, such as hormones, growth factors, and transport molecules, is required for gonadal development [54]. In teleost fish, vitellogenin (Vtg) is released into the blood circulation through Vtg receptor-mediated endocytosis, and combines with oocytes to form yolk granules, thereby participating in fish ovary growth [55]. In our previous report, endocytosis was associated with oocyte apoptosis during the female-to-male sex transition of *M. albus* [53]. In this study, endocytosis was enriched during the gonad development from OV to IE and from IL to TE, indicating that endocytosis may be involved in the process of oocyte development and sexual reversal of *M. albus*. The specific mechanism remains to be further explored.

#### Effect of FSH and hCG on lncRNAs

FSH and hCG levels can affect gonadal development [56, 57]. Studies have found that FSH can promote the growth of *M. albus* oocytes, playing an important role in the transition from primary follicle growth to secondary follicle growth, which is an important step in oocyte maturation [58]. hCG promotes gonad maturation in *Trachinotus blochii* [59] and *Anguilla japonica* [60]. Interestingly, the expression levels of lncRNAs can affect the levels of hormones (such as FSH) and are regulated by hormones (such as hCG). For example, after interfering with the expression of the lncRNA *TCONS\_00066406*, the expression level of FSH is reduced, affecting female reproduction [61, 62]. Moreover, the lncRNA *HAS2-as1* is an hCG target that promotes the expression of HAS2 and may play a role in regulating cumulus

expansion and migration [63]. In our previous study, FSH and hCG were used to promote the expression of the lncRNA *LOC109960696* *trans*-target gene *Smad2* in *M. albus* ovarian tissue in a time- and dose-dependent manner, respectively [24]. We successfully amplified 5 highly expressed DE-lncRNAs (*LOC109952131*, *LOC109953466*, *LOC109954337*, *LOC109954360* and *LOC109958454*). The results showed that the 5 lncRNAs were expressed at higher levels in the ovary and intersexual stage than in the testis. Incubation with FSH and hCG increased the expression of the 5 lncRNAs in a time-dependent manner. In summary, it was shown that the expression of lncRNAs is regulated by the levels of hormones, which in turn affects gonadal development in organisms.

#### Potential effect of the *LOC109958454 cis*-target gene *znf207* on gonadal development

Many scholars have also explored the interactions between target genes and lncRNAs. In *C. livia*, RNA-seq analysis showed that *FOXK2* was a target gene of the lncRNA *MSTRG.7894.4* involved in the regulation of the oestrogen receptor, and qRT-PCR experiments showed that the mRNA expression level of the *ER $\alpha$*  gene in the high egg production performance group was significantly higher than that in the low egg production performance group [17, 64]. In *S. paramamosain*, the expression level and complementary sequence of lncRNA-*ncr2* were found to be higher in the ovary than in the testis, and the expression levels of its target genes *Sp-jcb2* and *Sp-jcb3* in the ovary were significantly lower than those in the testis [35]. In *Fenneropenaeus merguensis*, the expression of *lncPV13* was inhibited and the expression of vitellogenin (Vg) was significantly decreased in the ovary on day 7 after injection of lncPV13-specific double-stranded RNA (*dslncPV13*). In contrast, the expression of gonad-inhibiting hormone (GIH) was significantly increased in *lncPV13*-knockout shrimp [65]. We found that the *cis*-target gene *znf207* was located 11.108 kb downstream of *LOC109958454*. This gene was widely expressed in various tissues of *M. albus*, and its expression in the testis was higher than that in the intersex stages and ovaries.

*Znf207* belongs to the zinc finger protein family, members of which are involved in a variety of biological processes, including chromatin remodelling, transcription activation/inhibition, DNA repair, apoptosis regulation, protein folding and assembly, stress response, and cell proliferation and differentiation [66, 67]. Nuclear lncRNAs are involved in a variety of biological processes, including chromatin organization, transcription and posttranscriptional gene expression, and serve as structural scaffolds for the nuclear domain [68]. Depletion of *znf207* leads to an increase in the formation of

RNA-DNA hybrids (R-loops), which leads to DNA damage and p53 activation in cells, which in turn leads to cell apoptosis or senescence [69]. Loss of *znf207* gene expression is beneficial for the treatment of *tp53* mutant ovarian cancer [70]. In our study, *LOC109958454* and *znf207* were found to be expressed mainly in the nucleus and to have a targeting relationship; *LOC109958454* inhibits the expression of *znf207*. In addition, FSH and hCG inhibited the expression of *znf207*. The lncRNA and hormones could regulate the expression of *znf207*; the specific mechanism remains to be further explored.

#### Conclusion

To the best of our knowledge, our study is the first systematic report of lncRNA sequencing for the five stages of gonadal development during sexual reversal of *M. albus*, and many candidate lncRNAs related to gonadal development were discovered. We showed that 5 lncRNAs had higher levels in the female and intersex stages and that their expression levels were affected by the hormones FSH and hCG. And *LOC109958454* and *znf207* were expressed mainly in the nucleus rather than the cytoplasm, verifying their targeting relationship, and found that the expression of *znf207* was suppressed by hormones. The above experiments showed that many lncRNAs are involved in the gonadal development of *M. albus* and may function through target genes. This study provides a new theoretical basis for the study of gonadal development in teleost fish and other animals.

#### Methods

##### Sample collection and preparation

Wild *M. albus* ( $n = 300$ ) were purchased from a local market in Chengdu, Sichuan. These fish were kept under the natural temperature and photoperiod in the laboratory. All procedures and investigations used for animal research were subject to approval and performed in accordance with the guidelines of the ethics committee (Approval No.20190031).

Fish were anaesthetized with 0.02% tricaine buffer (80  $\mu\text{g/L}$ ) (Sigma, LA, USA) for 10 min after a 24 h fast, and the tissues, including half of the gonads, pituitary gland, eye, heart, kidney, intestines, spleen, muscle and blood, were collected and immediately stored in liquid nitrogen at  $-80^{\circ}\text{C}$ . The other half of the fresh gonads were immediately fixed in Bouin's solution for 24 h and then stored in 75% ethanol until paraffin embedding.

Sections were serially cut at a thickness of 5  $\mu\text{m}$  using a slicer (Leica, Nussloch, Germany) and stained with haematoxylin/eosin [24]. Through gonadal histological sectioning, gonadal samples from different stages of the *M. albus* sex reversal process were verified. With reference to the previous reports [24, 45, 71], according to the

proportion of male and female germ cell development, the transition from female to male sex was classified into five phases: ovary in mature stage (OV) (Fig. 6A), early intersexual stage gonad (IE) (Fig. 6B), middle intersexual stage gonad (IM) (Fig. 6C), late intersexual stage gonad (IL) (Fig. 6D), and testis (TE) (Fig. 6E).

### Sequencing method and quality control

We used *M. albus* gonad samples for RNA-seq at the 5 developmental stages, with 3 biological replicates in each stage, and for identification and analysis of lncRNAs. Raw data in FASTQ format were first processed through in-house Perl scripts. In this step, clean data were obtained by removing reads containing adapters, reads containing poly-N sequences and low-quality reads from the raw data. At the same time, the Q20, Q30 and GC contents of the clean data were calculated. All subsequent analyses were based on clean data of high quality.

### LncRNA identification

Cuffmerge was used to merge the transcripts spliced from each sample, and the transcripts with uncertain chain directions were removed to obtain the complete transcriptome information from this sequencing data [72]. Finally, five basic principles were used to filter the data: (1) transcripts with exon number  $\geq 2$ ; (2) transcripts with transcript length  $> 200$  bp; (3) screening of

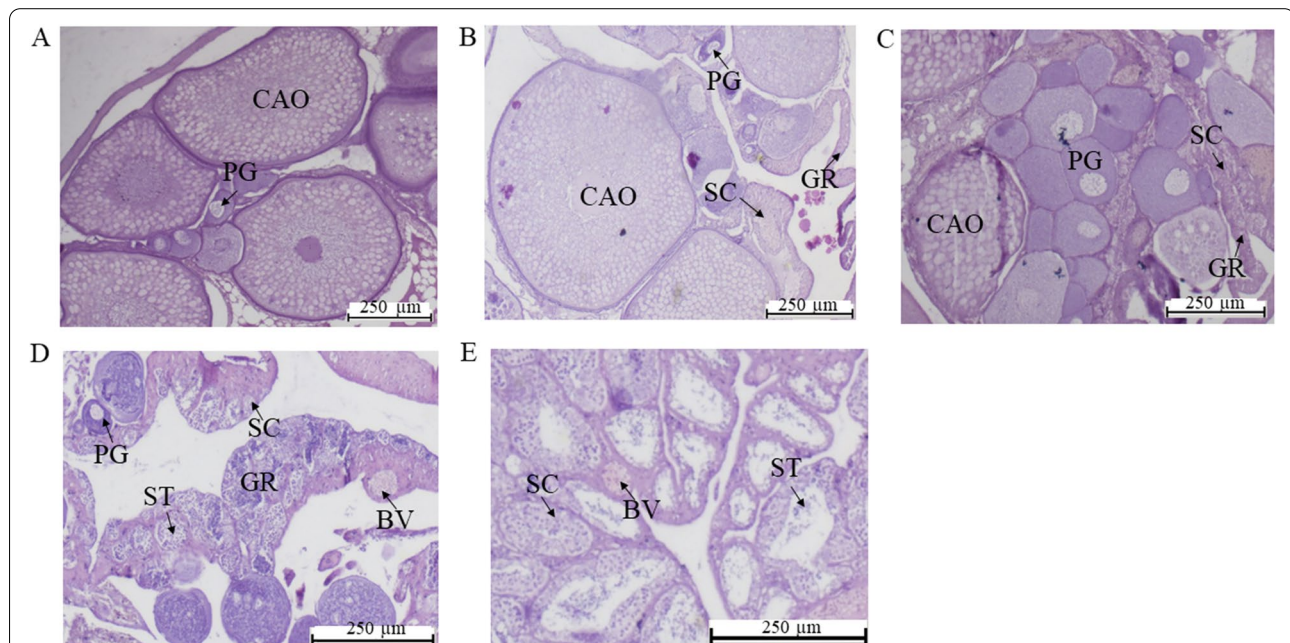
transcripts with known annotations; (4) calculation of the expression of each transcript by Cuffquant, selecting transcripts with fragments per kilobase of transcript per million mapped reads (FPKM) value  $\geq 0.5$ ; and (5) use of Coding Potential Calculator (CPC) [73] to filter transcripts with coding potential.

### Differential expression of lncRNAs

After screening, String Tie-eB [74] software was used to quantitatively analyse the transcripts, including lncRNAs, and obtain the FPKM values of the transcripts of each sample. With regard to statistical significance, lncRNAs were analysed as a whole. The DE-lncRNAs were statistically analysed using the edgeR program with default parameters:  $p$ -value  $< 0.05$  and  $|\log_2(\text{fold change})| > 1$ .

### Target gene prediction, GO and KEGG enrichment analyses

For position-related target gene analysis, the *cis*-target genes were predicted based on the positional relationship between lncRNAs and mRNAs, and the screening range was within 100 kb. Target gene prediction was based on the expression correlation between lncRNAs and mRNAs, and the screening condition was Pearson's correlation coefficient  $> 0.95$ . It should be noted that the mRNA data came from our laboratory, and RNA-seq was performed using the same batch of samples as those used for lncRNA analysis. GO enrichment analysis of DE-lncRNA target genes was performed by the Goseq R



**Fig. 6** Results of identification of the gonad development stages of *M. albus*. **A:** Ovary stage (OV), **B:** early intersexual gonad (IE), **C:** middle intersexual gonad (IM), **D:** late intersexual gonad (IL), **E:** testis stage (TE). CAO: cortical alveolar oocyte, PG: primary growth stage, GR: gonadal ridge, SC: spermatocyte, ST: spermatid, BV: blood vessel

software package [75], and GO entries with  $q$ -value  $< 0.05$  were considered to be significantly enriched. KEGG is a high-throughput experimental database generated from a large-scale molecular data set by using a bioinformatics database resource for biological systems. A pathway enriched with  $P < 0.05$  was defined as being significantly enriched by the associated differentially expressed gene. We used KOBAS software [76] to detect the enrichment of KEGG pathways by lncRNA target genes.

#### Cloning of 5 full length DE-lncRNAs

Using gonadal cDNAs as templates and the primer sequences shown in Additional file 10: Table S2, the initial fragments of the full length DE-lncRNAs were amplified by nested PCR. The final volume of the PCR mixture was 20  $\mu$ L, which contained 2  $\mu$ L of gonadal cDNA, 1  $\mu$ L of each of the upstream and downstream primers (10  $\mu$ M), 6  $\mu$ L of water, and 10  $\mu$ L of 2  $\times$  PCR Mix (Vazyme, Nanjing, China). The PCR program included 95  $^{\circ}$ C for 3 min, 36 cycles (95  $^{\circ}$ C for 0.5 min, TMC for 0.5 min, 72  $^{\circ}$ C for 1.5 min), at 72  $^{\circ}$ C for 10 min and storage at 4  $^{\circ}$ C until use. The gel recovery product was cloned into the pMD-19 T vector (Vazyme, Nanjing, China) and sequenced using universal primers.

#### Expression patterns of the DE-lncRNAs determined by qRT-PCR

cDNA was obtained from gonadal and other tissues (pituitary gland, eye, heart, kidney, intestines, spleen, muscle, blood) at the five developmental stages. qRT-PCR analysis (by the double internal reference method) was used to determine the expression levels of lncRNAs in the gonads at the different developmental stages. The genes *ef1a* and *rpl17* (the GenBank accession numbers are kc011266 and kc011267, respectively) were identified as the most stable genes in *M. albus* at different stages of gonadal development and in different tissues [77] and were therefore used as internal controls. The primer sequences are shown in Additional file 11: Table S3. The CFX system (Bio-Rad, Chicago, USA) was used for qRT-PCR. The PCR mixture contained 1  $\mu$ L of cDNA template, 0.4  $\mu$ M upstream and downstream primers, and 10  $\mu$ L of 2  $\times$  SYBR Green MasterMix (TaKaRa Bio, Dalian, China). PCR was carried out at 95  $^{\circ}$ C for 2 min, followed by 40 cycles of 95  $^{\circ}$ C for 0.5 s and 57–61  $^{\circ}$ C for 0.5 min and then by signal collection. The melting curve was generated as follows: 95  $^{\circ}$ C for 5 s, followed by heating from 65  $^{\circ}$ C–95  $^{\circ}$ C, increasing the temperature by 5  $^{\circ}$ C every 5 s, and then by signal collection. The amplification curve showed the expression levels and melting curve analysis confirmed the specificity of qRT-PCR amplification; CFX Manager software was used to analyse gene expression. Calculation of the copy numbers of the lncRNA and internal reference genes in CFX

Manager was based on DNA fragments, ranging from  $10^2$  to  $10^9$  copies [38]. The transcriptional expression level of the DE-lncRNA was calculated as follows:

$$C_{\ln \text{ lncRNA}} / \sqrt{C_{ef1a} * C_{rpl17}}$$

The expression levels of the lncRNA target genes were determined by the same method.

#### Expression patterns of 5 lncRNAs and *znf207* after incubating ovaries with hCG and FSH in vitro

The expression patterns of 5 DE-lncRNAs (*LOC109952131*, *LOC109953466*, *LOC109954337*, *LOC109954360* and *LOC109958454*) and the target gene *znf207* of *LOC109958454* were analysed after incubating ovaries with human chorionic gonadotropin (hCG) and follicle-stimulating hormone (FSH) in vitro. M199 medium (Sigma, Shanghai, China) was applied on ice, and *M. albus* ovaries were washed and dissected. Ovarian tissue (50–100 mg) was placed in a 24-well petri dish, and 1 mL of L15 containing penicillin (0.1  $\mu$ g/mL, Gibco, Massachusetts, USA) and streptomycin (0.1 mg/mL, Gibco, Massachusetts, USA) was added to the basal medium; tissue culture was performed at 28  $^{\circ}$ C in a humidified incubator. Stock solutions of hCG and FSH (Sigma, Shanghai, China) were prepared in saline solution at  $10^3$  times higher than the final concentration. After pre-incubation in normal saline for 2 h, FSH (0.05, 1.0, or 5.0 ng/ml), hCG (10, 50, or 100 IU/ml) or normal saline (control group) were incubated for 1, 2, 4, or 10 h with 3 replicates per treatment. After incubation, total RNA from ovarian tissue was extracted with TRIzol reagent (Invitrogen, Chicago, USA), cDNA was synthesized by reverse transcription, and qRT-PCR was performed to analyse the expression of lncRNAs. The results of the experimental group were compared to those of the control group as the final mapping data. Please refer to section 2.7 for the method.

#### Subcellular localization analysis of *LOC109958454* and *znf207*

The ovarian tissue samples of *M. albus* were cleaned and dissected and then placed in cold Leibovitz's L-15 medium (Gibco, Massachusetts, USA). Tweezers were used to continuously peel off the oocytes and connective tissue. After mixing the oocytes, the samples were divided into 5 groups, taking care to not break the cells during this process. Resuspend the cell pellet in precooled PBS (Gibco, Massachusetts, USA), and then, prechilled HLB (Foregene, Chengdu, China) was added at least 5 times the volume of the cell pellet, followed by the addition of 100 U of RNase inhibitor (G-Clone, Beijing, China). The mixture

was vortexed for 10 s and centrifuged at 1000×g and 4°C for 5 min. The supernatant (cytoplasm) was carefully aspirated into a new 1.5 mL EP tube, and the pellet (nucleus) was washed with 1 mL of HLB. An RNAPrep Pure Micro Kit (Tiangen, Beijing, China) was used to extract cytoplasmic and nuclear RNA, which was then reverse transcribed to cDNA; *U6* and *18S* were used as internal reference genes to perform qRT-PCR experiments on the lncRNAs. Please refer to section 2.7 for the method.

### Plasmid construction and dual-luciferase assay

The full length *LOC109958454*, *znf207* and *znf207 mut* cDNAs were amplified from *M. albus* and cloned into the PSE5390 vector (Sangon Biotech, Shanghai, China) to obtain PSE5390-*LOC109958454*, PSE5390-*znf207* and PSE5390-*znf207 mut*, respectively. Then, 293 T cells (Sangon Biotech, Shanghai, China) were inoculated into a 24 well plate at 30–50% confluence, with 3 replicate wells for each group. Cell transfection was performed using Lipo2000 (Invitrogen, Chicago, USA). Then, 200 μL of diluted 1× PLB was added to each well for cell lysis, and 20 μL of LAR II was added to detect firefly luciferase activity with a microplate reader (Tecan, Shanghai, China). After adding 20 μL of Stop&Glo Reagent, the microplate reader was used to detect Renilla luciferase activity. The Renilla luciferase activity values were divided by the firefly luciferase activity values, and the average values were plotted.

### Statistical analysis

All the data are expressed as the mean ± S.E.M. The data were subjected to single-factor analysis of variance, followed by Tukey's multiple comparison test, with SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate statistical significance.

### Abbreviations

lncRNAs: Long noncoding RNAs; DE-lncRNAs: Differentially expressed lncRNAs; FSH: Follicle stimulating hormone; hCG: Human chorionic gonadotropin; qRT-PCR: Quantitative real-time PCR; OV: Ovary stage; IE: Early intersexual stage; IM: Middle intersexual stage; IL: Late intersexual stage; TE: Testis stage; GO: GENE Ontology; BP: Biological process; CC: Cellular component; MF: Molecular function; KEGG: Kyoto Encyclopedia of Genes and Genomes; EN: Exon number; PI: Pituitary gland; EY: Eye; HE: Heart; LI: Liver; KI: Kidney; IN: Intestines; SP: Spleen; MU: Muscle; BL: Blood; PCOS: Polycystic ovary syndrome; ESR1: The estrogen receptor 1; LH: Luteinizing hormone.

### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12864-022-08679-2>.

**Additional file 1: Table S1.** Quality inspection of transcriptome data.

**Additional file 2: Fig. S1.** Identification results of new lncRNA.

**Additional file 3: Dataset S1.** DE-lncRNA annotation.

**Additional file 4: Fig. S2.** GO enrichment analysis of DE-lncRNA *trans*-target genes.

**Additional file 5: Dataset S2.** GO terms with significant differences among different developmental stages.

**Additional file 6: Dataset S3.** DE-lncRNAs and their targets in the significantly enriched GO terms and KEGG pathways.

**Additional file 7: Fig. S3.** KEGG enrichment analysis of DE-lncRNA *trans*-target genes.

**Additional file 8: Dataset S4.** KEGG pathways with significant differences among different developmental stages.

**Additional file 9: Dataset S5.** DE-lncRNAs and their *cis*-target genes.

**Additional file 10: Table S2.** Primers used for cloning.

**Additional file 11: Table S3.** Primers used for qRT-PCR and incubation.

### Acknowledgements

Not applicable.

### Authors' contributions

ZH: Conceptualization; formal analysis; investigation; writing, original and draft; supervision; and project administration. LJY: Conceptualization; methodology; investigation; resources; data statistics; and writing, original and draft. DYY: Supervision and validation. ZJM: Writing and review. FQD: Conceptualization and investigation. ZDH: Investigation. JXH: Investigation. HJC: Investigation. LZ: Investigation. YP: Resources. YYJ: Resources. QQC: Resources. KG: Resources. JXX: Resources. BLL: Resources. XBG: Validation; writing and editing. XLH: Conceptualization and investigation. SYY: Investigation and resources. MWZ: Investigation. TMY: Project administration; resources; supervision; validation; and writing, review and editing. All authors read and approved the final manuscript.

### Funding

This research was supported by the National Natural Science Foundation of China [grant numbers 31972777, 2019; 31402286, 2015].

### Availability of data and materials

The datasets generated and analysed during the current study are available in the Sequence Read Archive of National Center for Biotechnology Information database with accession number PRJNA798587 (<https://dataview.ncbi.nlm.nih.gov/object/PRJNA798587>). The datasets analysed during this study are included in this published article and its supplementary information files. Please contact Zhi He ([zhihe@sicau.edu.cn](mailto:zhihe@sicau.edu.cn)) if someone wants to request the data from this study.

### Declarations

#### Ethics approval and consent to participate

All experimental protocols involved in fishes in this study were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Sichuan Agricultural University and ARRIVE guidelines (<https://arriveguidelines.org>). All procedures and investigations were reviewed and approved by the Animal Research and Ethics Committees of Sichuan Agricultural University and performed in accordance with the guidelines of the committee (Approval No.20190031).

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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Received: 14 February 2022 Accepted: 9 June 2022  
Published online: 20 June 2022

## References

- Novikova IV, Hennelly SP, Tung CS, Sanbonmatsu KY. Rise of the RNA machines: exploring the structure of long non-coding RNAs. *J Mol Biol.* 2013;425(19):3731–46. <https://doi.org/10.1016/j.jmb.2013.02.030>.
- Ulitsky I, Bartel DP. lincRNAs: genomics, evolution, and mechanisms. *Cell.* 2013;154(1):26–46. <https://doi.org/10.1016/j.cell.2013.06.020>.
- Derrien T, Johnson R, Bussotti G, Tanzer A, Guigó R. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. *Genome Res.* 2012;22(9):1775–89. <https://doi.org/10.1101/gr.132159.111>.
- Guo CJ, Ma XK, Xing YH, Zheng CC, Xu YF, Shan L, et al. Distinct processing of lincRNAs contributes to non-conserved functions in stem cells. *Cell.* 2020;181(3):621–36. <https://doi.org/10.1016/j.cell.2020.03.006>.
- Tian B, Manley JL. Alternative polyadenylation of mRNA precursors. *Nat Rev Mol Cell Biol.* 2016;18(1):18–30. <https://doi.org/10.1038/nrm.2016.116>.
- Csörba T, Questa JI, Sun Q, Dean C. Antisense COOLAIR mediates the coordinated switching of chromatin states at FLC during vernalization. *Proc Natl Acad Sci.* 2014;111(45):16160–5. <https://doi.org/10.1073/pnas.1419030111>.
- Rosa S, Duncan S, Dean C. Mutually exclusive sense-antisense transcription at FLC facilitates environmentally induced gene repression. *Nat Commun.* 2016;7:13031. <https://doi.org/10.1038/ncomms13031>.
- Yap KL, Li S, Cabello AMM, Raguz S, Zeng L, Mujtaba S, et al. Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. *Mol Cell.* 2010;38(5):662–74. <https://doi.org/10.1016/j.molcel.2010.03.021>.
- Holdt LM, Hoffmann R, Sass K, Langenberger D, Scholz M, Krohn K, et al. Alu elements in ANRIL non-coding RNA at chromosome 9p21 modulate atherogenic cell functions through trans-regulation of gene networks. *PLoS Genet.* 2013;9(7):e1003588. <https://doi.org/10.1371/journal.pgen.1003588>.
- Song FB, Wang LM, Zhu WB, Dong ZJ. Long noncoding RNA and mRNA expression profiles following igf3 knockdown in common carp, *Cyprinus carpio*. *Sci Data.* 2019;6:190024. <https://doi.org/10.1038/sdata.2019.24>.
- van Werven FJ, Neuert G, Hendrick N, Lardenois A, Buratowski S, van Oudenaarden A, et al. Transcription of two long noncoding RNAs mediates mating-type control of gametogenesis in budding yeast. *Cell.* 2012;150(6):1170–81. <https://doi.org/10.1016/j.cell.2012.06.049>.
- Herrera L, Ottolenghi C, Garcia-Ortiz JE, Pellegrini M, Manini F, Ko MSH, et al. Mouse ovary developmental RNA and protein markers from gene expression profiling. *Dev Biol.* 2005;279(2):271–90. <https://doi.org/10.1016/j.ydbio.2004.11.029>.
- Laiho A, Kotaja N, Gyenesei A, Sironen A. Transcriptome profiling of the murine testis during the first wave of spermatogenesis. *PLoS One.* 2013;8(4):e61558. <https://doi.org/10.1371/journal.pone.0061558>.
- Zhu YY, Liu XL, Wang YK, Li W, Hong XY, Zhu XP, et al. Screening and preliminary analysis of lincRNA and mRNA related to sex regulation in yellow-throated turtle (*Mauremys mutica*). *J Fish China.* 2020;44(12):1960–75 (in chinese). <https://doi.org/10.11964/jfc.20200312187>.
- Rastetter RH, Smith CA, Wilhelm D. The role of non-coding RNAs in male sex determination and differentiation. *Reproduction.* 2015;150(3):R93–107. <https://doi.org/10.1530/REP-15-0106>.
- Song XH, Kyi-Tha-Thu C, Takizawa T, Naing BT, Takizawa T. 1700108J01Rik and 1700101O22Rik are mouse testis-specific long non-coding RNAs. *Histochem Cell Biol.* 2018;149(5):517–27. <https://doi.org/10.1007/s00418-018-1642-4>.
- Mao HG, Xu XL, Cao HY, Dong XY, Zou XT, Xu NY, et al. Comparative transcriptome profiling of mRNA and lincRNA of ovaries in high and low egg production performance in domestic pigeons (*Columba livia*). *Front Genet.* 2021;12:571325. <https://doi.org/10.3389/fgene.2021.571325>.
- Guo SY, Zhong Y, Zhang Y, Zhu YF, Guo J, Fu YS, et al. Transcriptome analysis provides insights into long noncoding RNAs in medaka gonads. *Comp Biochem Physiol part D genomics. Proteomics.* 2021;100842. <https://doi.org/10.1016/j.cbd.2021.100842>.
- Ma X, Cen SS, Wang LM, Zhang C, Wu LM, Tian X, et al. Genome-wide identification and comparison of differentially expressed profiles of miRNAs and lincRNAs with associated ceRNA networks in the gonads of Chinese soft-shelled turtle, *Pelodiscus sinensis*. *BMC Genomics.* 2020;21(1):443. <https://doi.org/10.1186/s12864-020-06826-1>.
- Cai J, Li L, Song L, Xie L, Luo F, Sun S, et al. Effects of long term antiprogesterone mifepristone (RU486) exposure on sexually dimorphic lincRNA expression and gonadal masculinization in Nile tilapia (*Oreochromis niloticus*). *Aquat Toxicol.* 2019;215:105289. <https://doi.org/10.1016/j.aquatox.2019.105289>.
- Shapiro DY. Plasticity of gonadal development and protandry in fishes. *J Exp Zool.* 1992;261(2):194–203. <https://doi.org/10.1002/jez.1402610210>.
- Cheng HH, He Y, Zhou RJ. Swamp eel (*Monopterus albus*). *Trends Genet.* 2021;37(12):1137–8. <https://doi.org/10.1016/j.tig.2021.09.007>.
- Cheng H, Guo Y, Yu Q, Zhou R. The rice field eel as a model system for vertebrate sexual development. *Cytogenet Genome Res.* 2003;101(3–4):274–7. <https://doi.org/10.1159/000074348>.
- He Z, Deng FQ, Xiong S, Cai YP, He ZD, Wang XY, et al. Expression and regulation of smad2 by gonadotropins in the protogynous hermaphroditic ricefield eel (*Monopterus albus*). *Fish Physiol Biochem.* 2020;46(3):1155–65. <https://doi.org/10.1007/s10695-020-00778-9>.
- Wang Z, Yang Y, Li S, Li K, Tang Z. Analysis and comparison of long non-coding RNAs expressed in the ovaries of Meishan and Yorkshire pigs. *Anim Genet.* 2019;50(6):660–9. <https://doi.org/10.1111/age.12849>.
- Liu Y, Li MX, Bo XW, Li T, Ma LP, Zhai TJ, et al. Systematic analysis of long non-coding RNAs and mRNAs in the ovaries of duroc pigs during different follicular stages using RNA sequencing. *Int J Mol Sci.* 2018;19(6):1722. <https://doi.org/10.3390/ijms19061722>.
- Liu Y, Qi B, Xie J, Wu XQ, Ling YH, Cao XY, et al. Filtered reproductive long non-coding RNAs by genome-wide analyses of goat ovary at different estrus periods. *BMC Genomics.* 2018;19(1):866. <https://doi.org/10.1186/s12864-018-5268-7>.
- Yang H, Wang F, Li FZ, Ren CF, Pang J, Wan YJ, et al. Comprehensive analysis of long non-coding RNA and mRNA expression patterns in sheep testicular maturation. *Biol Reprod.* 2018;99(3):650–61. <https://doi.org/10.1093/biolre/iy088>.
- Fatica A, Bozzoni I. Long non-coding RNAs: new players in cell differentiation and development. *Nat Rev Genet.* 2014;15(1):7–21. <https://doi.org/10.1038/nrg3606>.
- Taylor DH, Chu TE, Spektor R, Soloway PD. Long non-coding RNA regulation of reproduction and development. *Mol Reprod Dev.* 2015;82(12):932–56. <https://doi.org/10.1002/mrd.22581>.
- Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. *Nat Rev Genet.* 2009;10(3):155–9. <https://doi.org/10.1038/nrg2521>.
- Qin L, Huang CC, Yan XM, Wang Y, Li ZY, Wei XC. Long non-coding RNA H19 is associated with polycystic ovary syndrome in Chinese women: a preliminary study. *Endocr J.* 2019;66(7):587–95. <https://doi.org/10.1507/endocrj.EJ19-0004>.
- Li K, Zhong SS, Luo YS, Zou DF, Li MZ, Li YH, et al. A long noncoding RNA binding to QKI-5 regulates germ cell apoptosis via p38 MAPK signaling pathway. *Cell Death Dis.* 2019;10(10):699–715. <https://doi.org/10.1038/s41491-019-1941-2>.
- Zhang J, Yu P, Zhou QY, Li XL, Ding SQ, Su SP, et al. Screening and characterisation of sex differentiation-related long non-coding RNAs in Chinese soft-shell turtle (*Pelodiscus sinensis*). *Sci Rep.* 2018;8(1):8630–9. <https://doi.org/10.1038/s41598-018-26841-3>.
- Yang XL, Ikhwanuddin M, Li XC, Lin F, Wu QY, Zhang YL, et al. Comparative transcriptome analysis provides insights into differentially expressed genes and long non-coding RNAs between ovary and testis of the mud crab (*Scylla paramamosain*). *Mar Biotechnol (NY).* 2018;20(1):20–34. <https://doi.org/10.1007/s10126-017-9784-2>.
- Yuan CC, Chen KR, Zhu YF, Yuan YM, Li MY. Medaka igf1 identifies somatic cells and meiotic germ cells of both sexes. *Gene.* 2018;642:423–9. <https://doi.org/10.1016/j.gene.2017.11.037>.
- Kanai Y, Hiramatsu R, Matoba S, Kidokoro T. From SRY to SOX9: mammalian testis differentiation. *J Biochem.* 2005;1;13–9. <https://doi.org/10.1093/jb/mvi098>.
- He Z, Wu YS, Xie J, Wang TX, Zhang LH, Zhang WM. Growth differentiation factor 9 (Gdf9) was localized in the female as well as male germ cells in a protogynous hermaphroditic teleost fish, ricefield eel *Monopterus albus*.

- Gen Comp Endocrinol. 2012;178(2):355–62. <https://doi.org/10.1016/j.ygcen.2012.06.016>.
39. Yuan J, Tao WJ, Cheng YY, Huang BF, Wang DS. Genome-wide identification, phylogeny, and gonadal expression of fox genes in Nile tilapia, *Oreochromis niloticus*. *Fish Physiol Biochem*. 2014;40(4):1239–52. <https://doi.org/10.1007/s10695-014-9919-6>.
  40. Lau MT, Ge W. Cloning of smad2, smad3, smad4, and smad7 from the goldfish pituitary and evidence for their involvement in activin regulation of goldfish FSHbeta promoter activity. *Gen Comp Endocrinol*. 2005;141(1):22–38. <https://doi.org/10.1016/j.ygcen.2004.10.019>.
  41. Wu JY, Ribar TJ, Cummings DE, Burton KA, Means AR. Spermiogenesis and exchange of basic nuclear proteins are impaired in male germ cells lacking Camk4. *Nat Genet*. 2000;25(4):448–52. <https://doi.org/10.1038/78153>.
  42. Wu JY, Gonzalez-Robayna IJ, Richards JS, Means AR. Female fertility is reduced in mice lacking Ca2+/calmodulin-dependent protein kinase IV. *Endocrinology*. 2000;141(12):4777–83. <https://doi.org/10.1210/en.141.12.4777>.
  43. Zhao ZQ, Qiao L, Dai ZN, He QJ, Lan X, Huang SY, et al. LncNONO-AS regulates AR expression by mediating NONO. *Theriogenology*. 2020;145:198–206. <https://doi.org/10.1016/j.theriogenology.2019.10.025>.
  44. O'Hara L, Smith LB. Androgen receptor roles in spermatogenesis and infertility. *Best Pract Res*. 2015;29(4):595–605. <https://doi.org/10.1016/j.beem.2015.04.006>.
  45. Drożdżik M, Kaczmarek M, Malinowski D, Broś U, Kazienko A, Kurzawa R, et al. TGFβ3 (TGFB3) polymorphism is associated with male infertility. *Sci Rep*. 2015;5:17151. <https://doi.org/10.1038/srep17151>.
  46. Jackowska M, Kempisty B, Woźna M, Piotrowska H, Antosik P, Zawierucha P, et al. Differential expression of GDF9, TGFB1, TGFB2 and TGFB3 in porcine oocytes isolated from follicles of different size before and after culture in vitro. *Acta Vet Hung*. 2013;61(1):99–115. <https://doi.org/10.1556/AVet.2012.061>.
  47. Yang J, Zhang Y, Xu XT, Li J, Yuan FF, Bo SM, et al. Transforming growth factor-β is involved in maintaining oocyte meiotic arrest by promoting natriuretic peptide type C expression in mouse granulosa cells. *Cell Death Dis*. 2019;10(8):558. <https://doi.org/10.1038/s41419-019-1797-5>.
  48. Long XR, Song KQ, Hu H, Tian Q, Wang WJ, Dong Q, et al. Long non-coding RNA GAS5 inhibits DDP-resistance and tumor progression of epithelial ovarian cancer via GAS5-E2F4-PARP1-MAPK axis. *J Exp Clin Cancer Res*. 2019;38(1):345–60. <https://doi.org/10.1186/s13046-019-1329-2>.
  49. Zhou H, Chen A, Lu WQ. Corticotropin-releasing hormone reduces basal estradiol production in zebrafish follicular cells. *Mol Cell Endocrinol*. 2021;527(3):111222. <https://doi.org/10.1016/j.mce.2021.111222>.
  50. Xu KK, Yang WJ, Tian Y, Wu YB, Wang JJ. Insulin signaling pathway in the oriental fruit fly: the role of insulin receptor substrate in ovarian development. *Gen Comp Endocrinol*. 2014;216:125–33. <https://doi.org/10.1016/j.ygcen.2014.11.022>.
  51. Abdou HS, Gabrielle V, Tremblay JJ. The calcium signaling pathway regulates Leydig cell steroidogenesis through a transcriptional Cascade involving the nuclear receptor NR4A1 and the steroidogenic acute regulatory protein. *Endocrinology*. 2013;154(1):511–20. <https://doi.org/10.1210/en.2012-1767>.
  52. Lefèvre B, Nagyova E, Pesty A, Testart J. Acquisition of Meiotic Competence is Related to the functionality of the phosphoinositide/calcium signaling pathway in the mouse oocyte. *Exp Cell Res*. 1997;236(1):193–200. <https://doi.org/10.1006/excr.1997.3720>.
  53. He Z, Deng FQ, Yang DY, He ZD, Hu JX, Ma ZJ, et al. Crosstalk between sex-related genes and apoptosis signaling reveals molecular insights into sex change in a protogynous hermaphroditic teleost fish, ricefield eel *Monopterus albus*. *Aquaculture*. 2022;552:737918. <https://doi.org/10.1016/j.aquaculture.2022.737918>.
  54. Mizuta H, Mushiobira Y, Nagata J, Todo T, Hara A, Reading BJ, et al. Ovarian expression and localization of clathrin (Cltc) components in cutthroat trout, *Oncorhynchus clarki*: evidence for Cltc involvement in endocytosis of vitellogenin during oocyte growth. *Comp Biochem Physiol A Mol Integr Physiol*. 2017;212:24–34. <https://doi.org/10.1016/j.cbpa.2017.06.021>.
  55. Morini M, Lafont AG, Maugars G, Baloché S, Dufour S, Asturiano JF, et al. Identification and stable expression of vitellogenin receptor through vitellogenesis in the European eel. *animal*. 2020;1-10. <https://doi.org/10.1017/S1751731119003355>.
  56. Lubzens E, Young G, Bobe J, Cerdà J. Oogenesis in teleosts: how eggs are formed. *Gen Comp Endocrinol*. 2010;165(3):367–89. <https://doi.org/10.1016/j.ygcen.2009.05.022>.
  57. Schulz RW, França LRD, Lareyre JJ, Gac FL, Chiarini-Garcia H, Nobrega RH, et al. Spermatogenesis in fish. *Gen Comp Endocrinol*. 2010;165(3):390–411. <https://doi.org/10.1016/j.ygcen.2009.02.013>.
  58. Wu YS, He Z, Zhang LH, Jiang H, Zhang WM. Ontogeny of immunoreactive Lh and Fsh cells in relation to early ovarian differentiation and development in protogynous hermaphroditic ricefield eel *Monopterus albus*. *Biol Reprod*. 2012;86(3):93. <https://doi.org/10.1095/biolreprod.111.095646>.
  59. Putra W, Mullah A. Combination effects human chorionic gonadotropin hormone and ovaprim distribution on the time latency, percentage of fertilization, hatching and survival of silver pompano (*Trachinotus blochii*) larvae fish. *IOP Conf Ser: Earth Environ Sci*. 2019;278(1):012063. <https://doi.org/10.1088/1755-1315/278/1/012063>.
  60. Ohta H, Tanaka H. Relationship between serum levels of human chorionic gonadotropin (hCG) and 11-ketotestosterone after a single injection of hCG and induced maturity in the male Japanese eel, *Anguilla japonica*. *Aquaculture*. 1997;153(1-2):123–34. [https://doi.org/10.1016/S0044-8486\(97\)00020-3](https://doi.org/10.1016/S0044-8486(97)00020-3).
  61. Yang H, Ma JY, Wang ZB, Yao XL, Zhao J, Zhao XY, et al. Genome-wide analysis and function prediction of Long noncoding RNAs in sheep pituitary gland associated with sexual maturation. *Genes (Basel)*. 2020;11(3):320. <https://doi.org/10.3390/genes11030320>.
  62. Heidi K, Marion A, Jenni MJ, Pauliina D, Damdimopoulos AE, Juha K, et al. The Hydroxysteroid (17β) dehydrogenase family gene HSD17B12 is involved in the prostaglandin synthesis pathway, the ovarian function, and regulation of fertility. *Endocrinology*. 2016;10:3719–30. <https://doi.org/10.1210/en.2016-1252>.
  63. Yung Y, Ophir L, Yerushalmi GM, Baum M, Hourvitz A, Maman E. HAS2-AS1 is a novel LH/hCG target gene regulating HAS2 expression and enhancing cumulus cells migration. *J Ovarian Res*. 2019;12(1):21. <https://doi.org/10.1186/s13048-019-0495-3>.
  64. Liu Y, Ao X, Jia Z, Bai XY, Xu Z, Hu G, et al. FOXK2 transcription factor suppresses ERα-positive breast Cancer cell growth through Down-regulating the stability of ERα via mechanism involving BRCA1/BARD1. *Sci Rep*. 2015;5(4):8796. <https://doi.org/10.1038/srep08796>.
  65. Thepsuwan T, Rungrasamee W, Sangket U, Whankaew S, Sathapondecha P. Long non-coding RNA profile in banana shrimp, *Fenneropenaeus merguensis* and the potential role of lncPV13 in vitellogenesis. *Comp Biochem Physiol A*. 2021. <https://doi.org/10.1016/j.cbpa.2021.111045>.
  66. Cassandri M, Smirnov A, Novelli F, Pitolli C, Agostini M, Malewicz M, et al. Zinc-finger proteins in health and disease. *Cell Death Dis*. 2017;3:17071. <https://doi.org/10.1038/cddiscovery.2017.71>.
  67. Hajikhezri Z, Darweesh M, Akusjrvi G, Punga T. Role of CCCH-type zinc finger proteins in human adenovirus infections. *Viruses*. 2020;12(11):1322. <https://doi.org/10.3390/v12111322>.
  68. Sun QY, Hao QY, Prasant KV. Nuclear long noncoding RNAs: key regulators of gene expression. *Trends Genet*. 2018;34(2):142–57. <https://doi.org/10.1016/j.tig.2017.11.005>.
  69. Wan YH, Zheng XB, Chen HY, Guo YX, Jiang H, He XN, et al. Splicing function of mitotic regulators links R-loop-mediated DNA damage to tumor cell killing. *J Cell Biol*. 2015;209(2):235–46. <https://doi.org/10.1083/jcb.201409073>.
  70. Liu C, Banister CE, Buckhaults PJ. Spindle assembly checkpoint inhibition can Resensitize p53-null stem cells to Cancer chemotherapy. *Cancer Res*. 2019;79(1):2392–403. <https://doi.org/10.1158/0008-5472.CAN-18-3024>.
  71. Zhang Y, Zhang WM, Yang HY, Zhou WL, Hu CQ, Zhang LH. Two cytochrome P450 aromatase genes in the hermaphrodite ricefield eel *Monopterus albus*: mRNA expression during ovarian development and sex change. *J Endocrinol*. 2008;199(2):317–31. <https://doi.org/10.1677/JOE-08-0303>.
  72. Ghosh S, Chan CK. Analysis of RNA-seq data using topHat and cufflinks. *Methods Mol Biol*. 2016;1374:339–61. [https://doi.org/10.1007/978-1-4939-3167-5\\_18](https://doi.org/10.1007/978-1-4939-3167-5_18).
  73. Kong L, Zhang Y, Ye ZQ, Liu XQ, Zhao SQ, Wei L, et al. CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine. *Nucleic Acids Res*. 2007;35:W345–9. <https://doi.org/10.1093/nar/gkm391>.



74. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nat Protoc.* 2016;11(9):1650–67. <https://doi.org/10.1038/nprot.2016.095>.
75. Young MD, Wakefield MJ, Smyth GK, Oshlack A. Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biol.* 2010;11(2):R14. <https://doi.org/10.1186/gb-2010-11-2-r14>.
76. Mao XZ, Cai T, Olyarchuk JG, Wei LP. Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. *Bioinformatics.* 2005;21(19):3787–93. <https://doi.org/10.1093/bioinformatics/bti430>.
77. Hu Q, Guo W, Gao Y, Tang R, Li DP. Reference gene selection for real-time RT-PCR normalization in rice field eel (*Monopterus albus*) during gonad development. *Fish Physiol Biochem.* 2014;40(6):1721–30. <https://doi.org/10.1007/s10695-014-9962-3>.

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