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TGF-β1 resulting in differential microRNA expression in bovine granulosa cells

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28

Abstract

To explore the expression profile of the cellular miRNAs in bovine ovarian granulosa cells responding to transforming growth factor-β1 (TGF-β1), the effect of TGF-β1 on cell proliferation was firstly investigated by CCK-8 method and the results showed that there was a significant inhibitory effect on bovine granulosa cell proliferation treated with 5/10 ng/mL human recombinant TGF-β1 for 24 h compared to the control ($P < 0.05$). Then, we performed high-throughput sequencing of two small RNA libraries prepared from cultured bovine granulosa cells stimulated with or without 10 ng/mL human recombinant TGF-β1. A total of 13,257,248 and 138,726,391 clean reads per library were obtained from TGF-β1 and control groups, respectively. There were 498 and 499 bovine-specific exist miRNAs (exist miRNAs), 627 and 570 conserved known miRNAs (known miRNAs), and 593 and 585 predicted novel miRNAs in TGF-β1 and control groups, respectively. A total of 78 miRNAs with significant differential expression, including 39 up-regulated miRNAs and 39 down-regulated miRNAs were identified in the TGF-β1 group compared with the control. Real-time quantitative PCR analyses of bta-miR-106a and bta-miR-1434-5p showed that their up-expressions were interrupted by SB431542, an inhibitor that blocks TGFβ1/Smad signaling, which supported the sequencing data. GO analysis showed involvement of the predicted genes of the differentially expressed miRNAs in a broad spectrum of cell biological processes, cell components, and molecular functions. KEGG pathway analysis of the predicted miRNA targets further indicated that these differentially expressed miRNAs are involved in various signaling pathways, such as Wnt, MAPK, and TGF-β signaling, which might be involved in follicular development. These results provide valuable information on the composition, expression, and function of miRNAs in bovine granulosa cells responding to

TGF-β1, and will aid in understanding the molecular mechanisms of TGF-β1 in granulosa cells.

Keywords: microRNA; TGF-β1; bovine granulosa cells; high-throughput sequencing

1. Introduction

The transforming growth factor-beta (TGF-β) superfamily, which consists of more than 35 structurally related members, has been further classified into several subfamilies including the prototypic TGF-β subfamily (TGF-β1, TGF-β2, and TGF-β3), bone morphogenetic protein subfamily (20 members), growth and differentiation factor subfamily (at least nine members), and activin/inhibin subfamily (including activin A, AB, and B, and inhibin A and B)(Kaivo-oja et al., 2006). These molecules have wide-ranging influences on many tissue and organ systems. As an important member of transforming growth factor-beta (TGF-β) superfamily, TGF-β1 was firstly identified in human platelets as a protein with a molecular mass of 25 kilodaltons (Drummond, 2005; Miller and Hill, 2016). It plays an important role of regulation follicular development. In vitro, TGF-β1 stimulates the growth of pre-antral follicles dissected from adult mice ovaries, but does not affect the size of pre-antral follicles dissected from immature mice ovaries(Liu et al., 1999). The bovine follicular fluid concentration of TGF-β1 is negatively correlated with estradiol and follicle size at the early stage of development of the first-wave cohort of bovine ovarian follicles(Ouellette et al., 2005). TGF-β1 in the presence of follicle-stimulating hormone (FSH) increases E(2) secretion and mRNA expression of E(2)-related enzymes, but inhibits E(2) secretion and decreases mRNA expression of the FSH receptor in bovine granulosa cells(Zheng et al., 2008). TGF-α and TGF-β1 inhibit buffalo follicular survival and induce oocyte apoptosis (Sharma et al., 2010).

MicroRNAs (miRNAs) are single-stranded, small non-coding RNAs (typically around 22 nt) that negatively regulate the expression of target genes in a post-transcriptional manner by interacting with the 3' untranslated regions of their target mRNAs to degrade them(Yates et al., 2013). Increasing evidence indicates that the influence of miRNA on ovarian functions primarily occurs through their actions on ovarian somatic cells such as granulosa cells(Fiedler et al., 2008; Carletti et al., 2010; Donadeu et al., 2012; Miles et al., 2012). For example, miR-224 can promote the secretion of 17β-estradiol in mouse pre-antral follicular granulosa cells by promoting the expression of the cytochrome P450, polypeptide 1 and CYP19A1(Yao et al., 2010a). The expression of miRNAs is also regulated by hormones, ovarian granulosa cells after 12 hours of FSH treatment, 17 miRNAs were up-regulate, 14 miRNAs down-regulate, while the levels of progesterone increased(Yao et al., 2010b). However, little is known about the role of cytokines, such as TGF-β, in the functional involvement of miRNAs in follicular development.

In this study, the effect of TGF-β1 on bovine granulosa cell proliferation was investigated, and high-throughput RNA sequencing was performed to investigate whether TGF-β1 induces differential microRNA expression in bovine granulosa cells. The comprehensive miRNA expression data will provide further information on the function of TGF-β1 as well as molecular evidence to elucidate the regulatory mechanisms of bovine granulosa cells.

2. Materials and methods

2.1. Isolation and culture of bovine granulosa cells

Cow and heifer ovaries were obtained from a local slaughterhouse just after the animals had been slaughtered. After transporting to the laboratory between 25°C and 35°C, the ovaries were

washed three times with pre-warmed phosphate buffer saline (pH 7.2–7.4) containing 1% penicillin-streptomycin(Gibco Co.). Healthy follicles and atresia follicles were distinguished as previously described(Kaivo-oja et al., 2006). In our study, granulosa cells were mainly collected from healthy follicles (4–8 mm) by aspiration using an 18 G needle and plastic 10-mL syringe, and washed in Dulbecco's modified Eagle's medium (DMEM) (Sigma Co.). Then, the granulosa cell suspension was centrifuged, re-suspended, and seeded at a density of 1×10^6 cells per 60-mm culture plate in 3 mL DMEM containing 10% fetal bovine serum(Gibco Co.), 1% penicillin-streptomycin, and 1% glutamine(Gibco Co.). The granulosa cells were cultured at 37°C in a 5% CO_2 atmosphere. To the end of all experiments in this paper, the cell passage was not more than three generations.

2.2. CCK-8 analysis of bovine granulosa cell proliferation

After $1.0x10^4$ cells/well inoculated in 96-well plate, 100 μL of culture medium was added to each well. When the cells reached 70%, the wells began to treat with low serum(5% fetal bovine serum) for 12 h to cell synchronize, then were replaced by each treatment medium, in which other components were same to 2.2. DMEM medium but had different concentrations (0, 2, 5, 10, 50 ng/mL) of human recombinant TGF-β1 protein (Novoprotein Co., China). Gene cloning and sequencing showed that TGF-β in mice, cattle, pigs, monkeys and chickens had a high degree of homology with human(Chen et al., 2011), so human recombinant TGF-β1 was used in this study. When the plates were incubated for 24 h and 48 h, the CCK-8 solution (Beyotime, China) was separately added to each well and incubated for 2.5 hours. Then, the optical density （OD） in each well at 450 nm was measured using the SpectraMax M5 multifunctional microplate reader

(Molecular Devices, USA). The OD value of each well for cells was the OD after subtracting the control blank well, which had no cells but had the same culture medium. The experiment has three biological replicates for each treatment.

2.3. Library construction and sequencing

According to the above experiment result of TGF-β1 effect on bovine granulosa cell proliferation, the concentration of 10 ng/mL human recombinant TGF-β1 protein was used to treat the bovine granulosa cells. At the beginning, the density of 1×10^6 cells per 60-mm culture plate was incubated in 3 mL culture medium which was same to 2.2. When cells reached 70%, the methods of cell synchronization and TGF-β1 treatment were taken to each plate. After treatment for 24 h, TGF-β1 treatment (10 ng/mL) and control group (0 ng/mL) of three biological replicates were harvested for cell RNA extraction.

Each group total RNA was extracted from the cultured granulosa cells collected from three plates together using a miRNeasy Mini Kit (Cat#217004, Qiagen, GmBH, Germany) according to the manufacturer's protocol. For quality control, the integrity of the total RNA was measured on a 2100 Bio-analyzer system (Agilent). RNA fragments of 16–35 nt were purified from a polyacrylamide gel and ligated with 5′ and 3′ adaptors using T4 RNA ligase. Reverse transcription followed by PCR was used to create cDNA constructs based on the small RNAs ligated with 3′ and 5′ adapters. Subsequently, the amplified cDNA constructs were purified from an agarose gel to prepare for sequencing analysis using a Genome Analyzer (Illumina, CA, USA) according to the manufacturer's instructions.

2.4. Filtering of clean tags

Reads obtained from the sequencing machine included other reads containing adapters or low quality bases, which would affect the following assembly and analysis. Thus, to obtain clean tags, raw reads were further filtered according to the following rules: 1) removal of low quality reads containing more than one low quality (Q-value: ≤20) base or containing unknown nucleotides (N); 2) removal of reads without 3′ adapters; 3) removal of reads containing 5′ adapters; 4) removal of reads containing 3′ and 5′ adapters but no small RNA fragments between them; 5) removal of reads containing polyA in a small RNA fragment; 6) removal of reads shorter than 18 nt (not including adapters).

2.5. Alignment and identification of small RNAs

2.5.1. Alignment of small RNAs

All clean tags were aligned with small RNAs in the GeneBank database (Release 209.0) to identify and remove rRNA, scRNA, snoRNA, snRNA, and tRNA. In addition, all clean tags were aligned with small RNAs in the Rfam database (version 11) to identify and remove rRNA, scRNA, sonRNA, snRNA, and tRNA. All clean tags were also aligned with a reference genome. Tags mapped to exons or introns might be fragments from mRNA degradation. Therefore, these tags were removed. Tags mapped to repeat sequences were also removed.

2.5.2. Identification and validation of miRNAs

All clean tags were then searched against the miRBase database (Release 21) to identify bovine-specific exist miRNAs (exist miRNAs) and conserved known miRNAs of other animal

154 species (known miRNAs). All unannotated tags were aligned with the reference genome.
155 According to their genome positions and hairpin structures predicted by Mireap_v0.2 software,
156 novel miRNA candidates were identified.

157 2.5.3. Small RNA annotation summary

158 After tags were annotated as described above, the annotation results were determined in this
159 priority order: rRNA etc > exist miRNA > exist miRNA edit > known miRNA > repeat > exon >
160 novel miRNA > intron. Tags that could not be annotated as any of the above molecules were
161 recorded as unann.

162 2.6. Analysis of differentially expressed miRNAs

163 Total miRNA consists of exist, known, and novel miRNAs. Based on their expression in each
164 sample, the miRNA expression level was calculated and normalized to transcripts per million
165 (TPM). The formula is as follows: TPM=actual miRNA counts/total counts of clean tags × 10^6. To
166 identify differentially expressed miRNA, miRNAs with a fold change ($\log_2$) of ≥1 or -1 and
167 *P*-value of <0.05 in a comparison were considered as significant differentially expressed miRNAs.

168 2.7. Target gene prediction and functional enrichment 169 analysis

170 2.7.1. Target gene prediction

171 Based on the sequences of exist, known, and novel miRNAs, candidate target genes were
172 predicted by three software packages: RNAhybrid (v2.1.2) + svm-light (v6.01), miRanda (v3.3a),

and TargetScan (v7.0). The intersection of the three results were more credible to choose predicted miRNA target genes.

2.7.2. Gene ontology (GO) enrichment analysis

The predicted miRNA target genes were then subjected to the Gene Ontology (GO) enrichment analysis(Ashburner et al., 2000). The target genes were mapped to GO terms in the Gene Ontology database (http://www.geneontology.org/), and significantly enriched GO terms were defined by the hypergeometric test. The calculated *P*-value underwent false discovery rate (FDR) correction with an FDR of ≤1 as the threshold.

2.7.3. Pathway enrichment analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg) pathway enrichment analysis was performed to further understand gene biological functions. The calculated *P*-value underwent FDR correction with an FDR of ≤1 as the threshold. Pathways meeting this condition were defined as significantly enriched pathways.

2.8. Confirmation of miRNA expression by quantitative RT-PCR

After cell synchronization with low serum(5% fetal bovine serum) for 12 h, bovine granulosa cells were treated with 10μM SB431542(+) or without SB431542(-) for 30 min, then were treated with 10ng/ mL(+) or 0ng/ mL(-) of human recombinant TGF-β1 protein for 24 h. SB431542, which was an inhibitor that blocks phosphorylation of Smad2/3 that are TGFβ1/Smad signaling downstream molecules, was selected to prove whether the effect what we saw in transcription of

193 miRNAs was due to the TGF-β1 protein treatment. Each treatment has three replicates, and other
194 methods and conditions were same to 2.3.

195 Total RNA was separately isolated from bovine granulosa cells in each plate using TRIzol
196 reagent. DNA contamination was removed by incubation with RNase-free DNase I (NEB, USA)
197 for 45 min at 37 °C. Approximately 2 μg total RNA and 2 μg Poly(T) adaptor (TIANGEN, China)
198 was reverse transcribed in a 20 μL reaction volume using a One Step miRNA cDNA Synthesis Kit
199 (TIANGEN, China). The tailing and reverse transcription reactions were incubated at 37°C for 60
200 min and 95°C for 5 min, respectively. The expression levels of the miRNAs were validated using a
201 Real-time PCR System (Bio-Rad, USA) and SYBR Premix Ex Taq kit (Takara, Dalian, China).
202 The miRNA PCR system was in a 20-μL PCR reaction containing SYBR Premix Ex Taq II 10 μL,
203 miRNA-specific forward primer（10 μmol·L^{-1}, Invitrogen Biotechnology Co. Ltd ,China) 0.8 μL
204 and universal reverse primer（10 μmol·L^{-1}, TIANGEN, China）0.8 μL, 1 μL miRNA cDNA product,
205 ddH_2O 7.4 μL. Temperature cycles were as follows: 95°C for 30 s ; followed by 40 PCR cycles of
206 95 ℃ for 5 s, 60°C for 30 s, 72 ℃ for 5 s, and plate-reading. This was followed by an extension
207 of 5 min at 72 ℃, plate-reading every other 0.2 ℃ from 55 to 95 ℃ for drawing melting curves.
208 The product was then gel-extracted and cloning sequenced by Invitrogen Biotechnology Co.
209 Ltd ,China. The reactions were repeated three times for every sample running on a 96-well PCR
210 plate. The intra- and inter-assay coefficients of variation (CV) of Ct values of the optimal assay
211 were less than 4%. miRNA quantifications were performed by the $2^{-\Delta\Delta Ct}$ method and results for
212 each miRNA were presented as ratios relative to the U6 snRNA. The results were represented as
213 the mean ± standard deviation (S.D.). All primers for qRT-PCR were listed in Table S1.

3. Results

3.1. Effect of TGF-β1 on bovine granulosa cell proliferation

The cell proliferation in bovine ovarian granulosa cells treated with 0, 2, 5, 10, 50 ng/ mL TGF-β1 for 24 h and 48 h indicated that there was a significant inhibitory effect on cell proliferation of 5 ng/mL TGF-β1 treatment group compared with the control group (0 ng/mL) ($P > 0.05$), and showed a dose-dependent, but the difference was not significant after 10 ng/mL ($P < 0.05$) at 24 h, and the difference at 48h in each group was similar to that of 24 h (Fig.1). Therefore, 10ng/mL for 24 h was selected as the TGF-β1 treatment conditions in the following experiments.

3.2. Overview of high-throughput sequencing data

To investigate the small RNA expression profiles of granulosa cells, two cDNA libraries of small RNAs were prepared from the pool of the three culture plates of bovine granulosa cells treated with or without TGF-β1.

In total, 15,517,878 and 14,470,367 raw reads were obtained from TGF-β1 and control libraries, respectively. After elimination of low quality reads, adaptors and contaminating sequences, 13,872,639 and 13,257,248 clean reads with lengths of 16–35 nt remained in the TGF-β1 and control groups, respectively (Table 1). The overall size distributions of the sequenced reads from the two libraries were very similar with the 22 nt class being the most abundant (Fig.2).

3.3. Alignment and identification of small RNAs

3.3.1. Alignment and annotation of small RNAs

All clean tags were aligned with small RNAs in the GeneBank database (Release 209.0) to identify and remove rRNA, scRNA, snoRNA, snRNA, and tRNA, accounting for 420,615 and 473,148 unique sequence reads in small RNA libraries of control and TGF-β1, respectively (Fig.3 A, B).

In addition, all clean tags were aligned with small RNAs in the Rfam database (version 11) to identify and remove rRNA, scRNA, sonRNA, snRNA, and tRNA, accounting for 39,005 and 439,468 unique sequence reads in small RNA libraries of control and TGF-β1, respectively, (Fig.3 C, D).

Tags mapped to exons or introns might be fragments from mRNA degradation and were removed (Fig.3 E, F).

All clean tags were also aligned with the reference genome (Fig.4 A, B). Of the clean reads, 145,459 and 125,690 unique sequence reads in the small RNA libraries of control and TGF-β1, respectively, were matched to the bovine genome.

3.3.2. Identification of small RNAs

After tags were annotated, the small RNA annotation summary showed that 5855, 5897, and 32608, and 32058, 1529, and 1513 unique sequence reads from small RNA libraries of control and TGF-β1 were exist, known, or novel miRNAs, respectively (Table 2). Among these reads, all small RNA sequences were searched against the bovine miRNA database to identify exist, known, and novel miRNAs in TGF-β1 and control groups as described in the Materials and methods. A

252 total of 498 and 499 exist bovine miRNAs were identified in TGF-β1 and control groups,
253 respectively, including 461 miRNAs found in both groups (Fig.5). A total of 627 and 570 known
254 miRNAs were identified in TGF-β1 and control groups, respectively, including 453 miRNAs
255 found in both groups (Fig.5). A total of 593 and 585 novel bovine miRNAs were identified in
256 TGF-β1 and control groups, respectively, including 546 miRNAs found in both groups (Fig.5). We
257 also found a nucleotide bias in both the first and each position of exist, known, and novel miRNAs
258 in the annotation results (Fig.6 A–L).

259 3.4. Analysis of differentially expressed miRNAs

260 Differentially expressed miRNAs might play important roles in biological processes resulting
261 from TGF-β1 stimulation of bovine granulosa cells. Differentially expressed miRNAs between the
262 two libraries were identified by comparing the normalized expression data of the 1719 and 1653
263 miRNAs in TGF-β1 and control groups. In total, 78 differentially expressed miRNAs (fold change
264 ($\log_2$): $\geq \pm 1$; $P < 0.05$) were identified between TGF-β1 and control groups (Table 3). Among
265 them, 9 and 11 exist miRNAs, 15 and 11 known miRNAs, 15 and 17 novel miRNAs were
266 up-regulated and down-regulated in TGF-β1 and control groups, respectively (Fig.5).

267 3.5. TGF-β1 affects the miRNA expression by Smad
268 signalling

269 Bta-miR-106a and bta-miR-1434-5p, which were two significantly up-expressed miRNAs by
270 high throughput analysis, were randomly chosen to prove the TGF-β1 effect by qRT-PCR. The
271 results showed that the up-expression of bta-miR-106a and bta-miR-1434-5p were interrupted by
272 treatment of SB431542 to the bovine granular cell (Fig.7), in which SB4342 was an inhibitor that

blocked phosphorylation of Smad2/3 that were TGFβ1/Smad signaling downstream molecules.

3.6. Target prediction for significant differentially expressed miRNAs and functional analysis

To better understand the specific functions of significant differentially expressed miRNAs, the predicted miRNA targets were annotated by GO enrichment and KEGG pathway analysis. Specific GO of the bovine target genes was involved mainly in biological processes (e.g., metabolic process, localization, transport, cell cycle, nuclear transport, cell division, lipid metabolic process, mitotic cell cycle, nuclear division, mitotic nuclear division, cell differentiation, cellular developmental process, and carbohydrate metabolic process) (Table S2), cell components (e.g., cytoplasm, intracellular part, intracellular membrane-bounded organelle, intracellular membrane-bounded organelle, organelle, intracellular organelle, endomembrane system, and cytoplasmic part) (Table S3), and molecular function (e.g., catalytic activity, binding, transferase activity, transferase activity, kinase activity, and oxidoreductase activity) (Table S4).

Importantly, with the exception of the above mentioned biological functions, some second GO term clusters of the targets of the significant differentially expressed miRNAs were associated with follicle development, i.e., follicular dendritic cell differentiation, ovarian cumulus expansion, and pre-antral ovarian follicle growth, which might be associated with the physiology of bovine granulosa cells in response to TGF-β1.

Following GO analysis, KEGG was then used for pathway enrichment analysis of predicted target genes of the significant differentially expressed miRNAs. The results showed that total of 238 KEGG pathways and 157 significant difference expression target genes were predicted (Fig.8

294 A-C and table S5), suggesting that many pathways were associated with some miRNAs in the
295 bovine granulosa cell response to TGF-β1.

296 **4. Discussion**

297 In recent years, high-throughput sequencing has become an effective method for analyzing
298 miRNA expression profiles of animals. Based on the high throughput sequencing technology, the
299 sRNA digital analysis model has the advantages of high flux, less demand for samples, high
300 precision and simple operation, which can quickly and comprehensively analyze miRNA
301 differential expression between samples. TGF-β1 plays roles in follicular and granulosa cell
302 functions(Liu et al., 1999; Ouellette et al., 2005; Zheng et al., 2008; Sharma et al., 2010), and this
303 study also indicated there was an inhibitory effect on bovine granulosa cell proliferation. Although
304 many genes and miRNAs involved in follicular or granulosa cell functions in ovaries have been
305 identified(Tripurani et al., 2010; Hossain et al., 2012; Miles et al., 2012), little research has been
306 conducted on TGF-β1-responsive miRNAs in TGF-β1 at a genome-wide scale. In the present
307 study, we used a high-throughput sequencing approach to sequence two small RNA libraries
308 prepared from cultured granulosa cells treated with or without TGF-β1.

309 In this study, the major peak for bovine granulosa cells was at 22 nt, which similar to the
310 small RNA length distribution of bovine cumulus-oocyte complexes(Miles et al., 2012) ,typical
311 length of animal miRNAs and granulosa cells of subordinate and dominant follicles
312 (Salilew-Wondim et al., 2014). As similar as previous study has shown that the small RNA length
313 distribution pattern of the bovine ovary exhibits a major peak at 21 nt (Hossain et al., 2009),
314 increasing the accuracy of the identification results.

315 Previous reports have revealed the expression patterns of miRNAs in the bovine ovary and

316 granulosa cells (Hossain et al., 2009; Salilew-Wondim et al., 2014; Gebremedhn et al., 2015). Our
317 results showed 498 and 499 specific bovine mature exist miRNAs in TGF-β1 and control groups,
318 respectively, including 461 miRNAs in both groups. Of these miRNAs, 315 and 323 miRNAs
319 have been respectively detected in bovine granulosa cells of pre-ovulatory dominant and
320 subordinate follicles during the late follicular phase of the estrous cycle(Gebremedhn et al., 2015)
321 and 291–318 known miRNAs were detected in granulosa cells of subordinate and dominant
322 follicle during the early luteal phase of the bovine estrous cycle using miRNA deep
323 sequencing(Salilew-Wondim et al., 2014). This difference might be due to the TGF-β1 stimulation
324 of granulosa cells. In addition, we found that 9 and 11 specific bovine exist miRNAs were
325 up-regulated and down-regulated in TGF-β1 and control groups. Some members among these
326 miRNAs have various roles and targets, especially in the ovary. For example, miR-106a might
327 play a role in the development of bovine oocytes(Miles et al., 2012). MiR-126 targets talin2 in the
328 bovine corpus luteum, suggesting its putative involvement in development of the bovine corpus
329 luteum during the estrous cycle(Dai et al., 2014). MiR-212 is involved in the regulation of cell
330 survival, steroidogenesis, and differentiation during follicle selection and ovulation in the
331 mon-ovular equine ovary(Schauer et al., 2013).MiR-212 is also highly upregulated following
332 luteinizing hormone (LH)/human chorionic gonadotropin induction in periovulatory mouse
333 granulosa cells(Fiedler et al., 2008), but exhibits down-regulation caused by FSH in human MII
334 oocytes (Xu et al., 2011). MiR-129-5p inhibits ovarian cancer cell proliferation and survival via
335 direct suppression of transcriptional co-activators YAP and TAZ(Tan et al., 2015). Our study
336 showed that TGF-β1 stimulation down-regulated and up-regulated some miRNAs in bovine
337 granulosa cells, suggesting their possible roles in TGF-β1-mediated functions. The expression of

two random miRNAs in TGF-β1 and control groups were confirmed by qRT-PCR and were consistent with the results of high-throughput sequencing, increasing the accuracy of the identification results. And the present study also showed that the up-expressions of two random miRNAs, bta-miR-106a and bta-miR-1434-5p, were interrupted by SB431542, an inhibitor that blocks phosphorylation of Smad2/3 that are TGFβ1/Smad signaling downstream molecules, suggesting that the granular cell miRNA different expression resulting from TGF-β1 was at least partly through the classic TGFβ1/Smad signal pathway and proving that what we saw was due to the treatment of the cells with the TGF-β1. The mechanism in mouse study has showed that TGF-β1 treatment in granulosa cells caused up-expression of miR-224, and miR-224 regulated the expression of Smad4 gene which was one of genes involved in TGF-beta signaling pathway(Yao et al., 2010a). However, the mechanism of action of TGF-β1 on the expression of miRNAs in cattle is not yet clear, which need to be further studied.

A total of 157 target genes for differentially expressed miRNAs were predicted by GO analysis, and further KEGG pathway analysis demonstrated that differentially expressed miRNAs were involved in various signaling pathways, including metabolic pathways, Wnt signaling, MAPK signaling, TGF-β signaling path, etc. Among the KEGG pathways, hybrid of Wingless and Int(WNT), mitogen-activated protein kinase (MAPK), and TGF-β signaling pathways were associated with follicle or granulosa cell functions. For example, WNT signaling plays an important role in regulation of ovarian follicle maturation and steroid production(Hernandez Gifford, 2015), and follicle development(Li et al., 2014). Canonical WNT signaling inhibits FSH-mediated steroidogenesis in primary cultures of rat granulosa cells(Stapp et al., 2014). MAPK3/1 in ovarian granulosa cells are essential for female fertility(Fan et al., 2009). The

MAPK3/1-dependent pathway mediates the surge of pre-ovulatory LH that induces the differential expression of transcripts encoding key steroidogenic enzymes essential for E(2) and P(4) synthesis in mural and cumulus granulosa cells(Su et al., 2006). The intra-ovarian TGF-β signaling pathway is a critical regulator of folliculogenesis and ovarian functions. Disorder of ligands or receptors may influence ovarian functions, leading to several reproductive pathologies or infertility(Kaivo-oja et al., 2006; Knight and Glister, 2006; Kristensen et al., 2014; Persani et al., 2014; Chang et al., 2016) .

In summary, we constructed two miRNA libraries from TGF-β1-stimulated and unstimulated bovine granulosa cells. To identify and characterize TGF-β1-responsive miRNAs in bovine granulosa cells, miRNAs were sequenced at a genome-wide scale using the Solexa technique. Seventy-eight miRNAs were expressed differentially, of which two miRNAs were validated by qRT-PCR, suggesting expression characteristics in response to TGF-β1 treatment of bovine granulosa cells. Target genes were predicted for differentially expressed miRNAs, and the functional annotations of these target genes were analyzed. The results showed involvement of the predicted genes in a broad spectrum of cell biological processes, cell components, and molecular functions. The KEGG pathway analysis of the predicted miRNA targets further indicated that these differentially expressed miRNAs are involved in various signaling pathways, such as Wnt, MAPK, TGF-β signaling, which might be involved in follicular development. Our study supports and further extends the knowledge of the possible regulatory roles of miRNAs and their targeted pathways in molecular and cellular mechanisms of bovine granulosa cells in response to TGF-β signaling.

Conflict of interest

None of the authors have any conflict of interest to declare.

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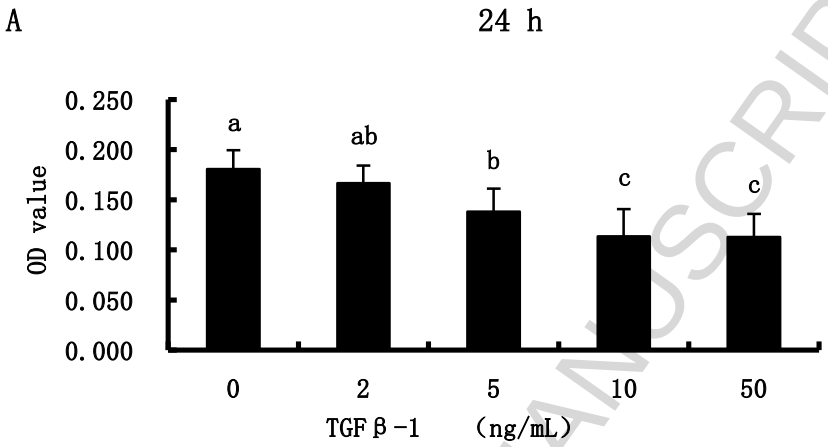
Figure captions

Fig.1. Effect of TGF-β1 on bovine granulosa cell proliferation.

A. The optical density （OD）at 450 nm of bovine granulosa cell with different concentrations(0, 2, 5, 10, 50 ng/mL) of human recombinant TGF-β1 protein treatment for 24h

B. The optical density （OD）at 450 nm of bovine granulosa cell with different concentrations(0, 2, 5, 10, 50 ng/mL) of human recombinant TGF-β1 protein treatment for 48h

Bars show the mean ± SD of three independent experiments and different lowercase letters above bars show significant differences ($P < 0.05$).



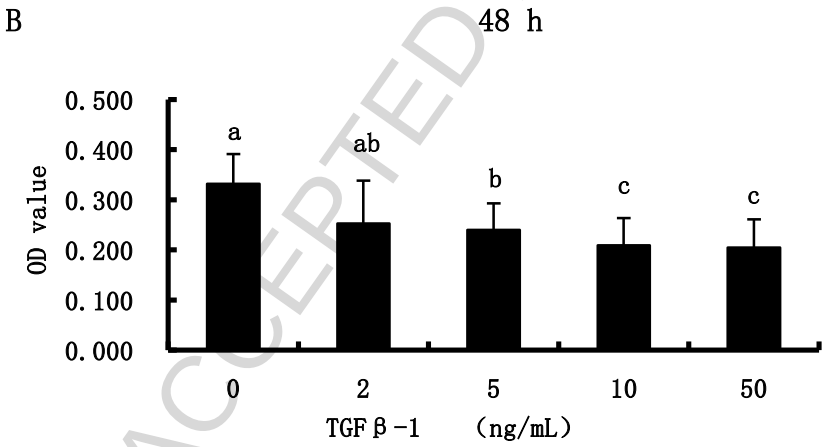


Fig.2. Length distribution of small RNAs in control and TGF-β1-treated bovine granulosa cells.

Vertical axis represents the frequency of small RNAs with different sizes. Horizontal axis shows small RNAs with different sizes.

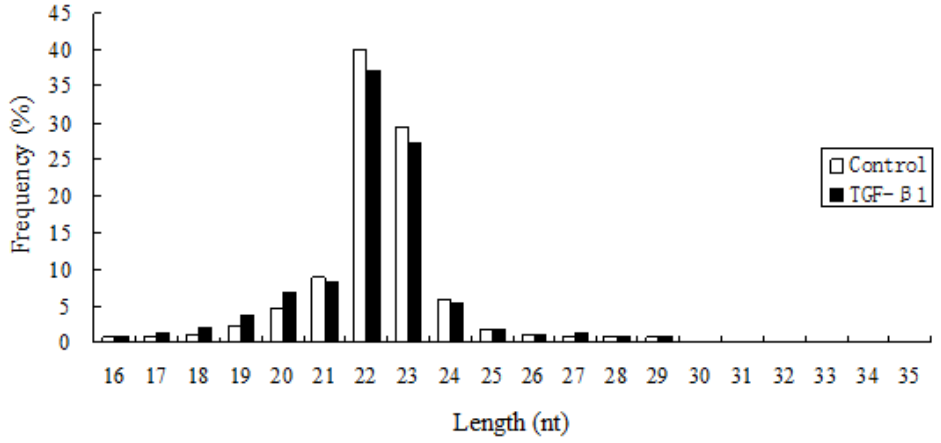


Fig. 3. Alignment to Genebank, Rfam, and exon and intron.

A. Alignment (unique and total unknown sRNAs) to Genebank for control cells.
B. Alignment (unique and total unknown sRNAs) to Genebank for TGF-β1-treated cells.
C. Alignment (unique and total unknown sRNAs) to Rfam for control cells.
D. Alignment (unique and total unknown sRNAs) to Rfam for TGF-β1-treated cells.
E. Exon and intron alignment (unique and total unknown sRNAs) to Rfam for control cells.
F. Exon and intron alignment (unique and total unknown sRNAs) for TGF-β1-treated cells.

Fig.4. Alignment results of small RNA sequences in Genome.

A. Distribution of miRNAs of control cells in Genome.
B. Distribution of miRNAs of TGF-β1-treated cells in Genome.
Blue columns indicate small RNAs mapped to the sense strand. Red columns indicate small RNAs mapped to the anti-sense strand.

Fig.5. MiRNAs of exist, known and novel were up-regulated and down-regulated in TGF-β1 and control groups.

Fig.6. Frequency of four nucleotides in the first position and at each position of small RNA.

A .Frequency of four nucleotides in the first position of exist miRNAs in control cells
B .Frequency of four nucleotides in the first position of exist miRNAs in control cells

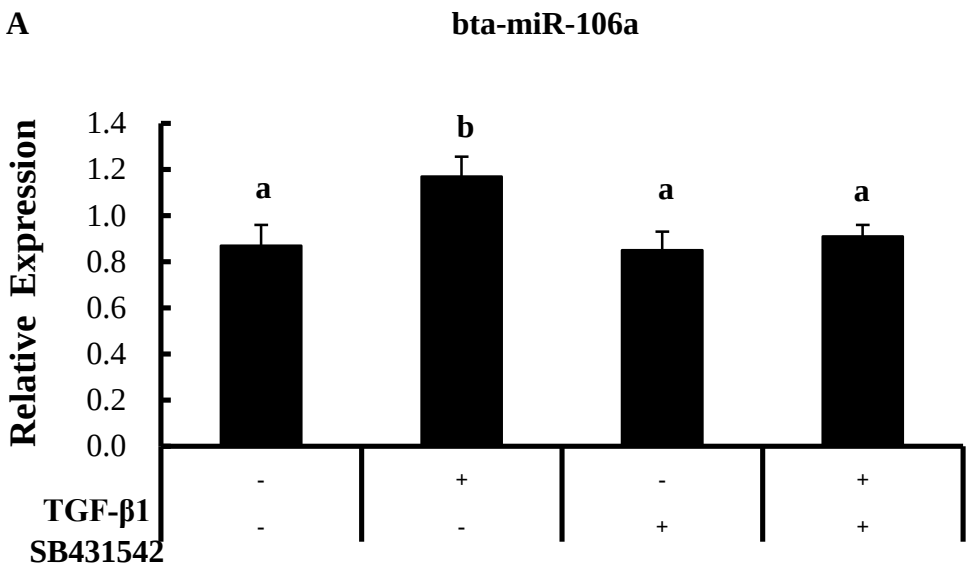
C .Frequency of four nucleotides in the first position of exist miRNAs in TGF-β1-treated cells
D .Frequency of four nucleotides in the first position of exist miRNAs in TGF-β1-treated cells
E .Frequency of four nucleotides in the first position of known miRNAs in control cells
F. Frequency of four nucleotides in the first position of known miRNAs RNA in control cells
G. Frequency of four nucleotides in the first position of known miRNAs in TGF-β1-treated cells
H. Frequency of four nucleotides in the first position of known miRNAs in TGF-β1-treated cells
I .Frequency of four nucleotides in the first position of novel miRNAs in control cells
J. Frequency of four nucleotides in the first position of novel miRNAs in control cells
K. Frequency of four nucleotides in the first position of novel miRNAs in TGF-β1-treated cells
L. Frequency of four nucleotides in the first position of novel miRNAs in TGF-β1-treated cells

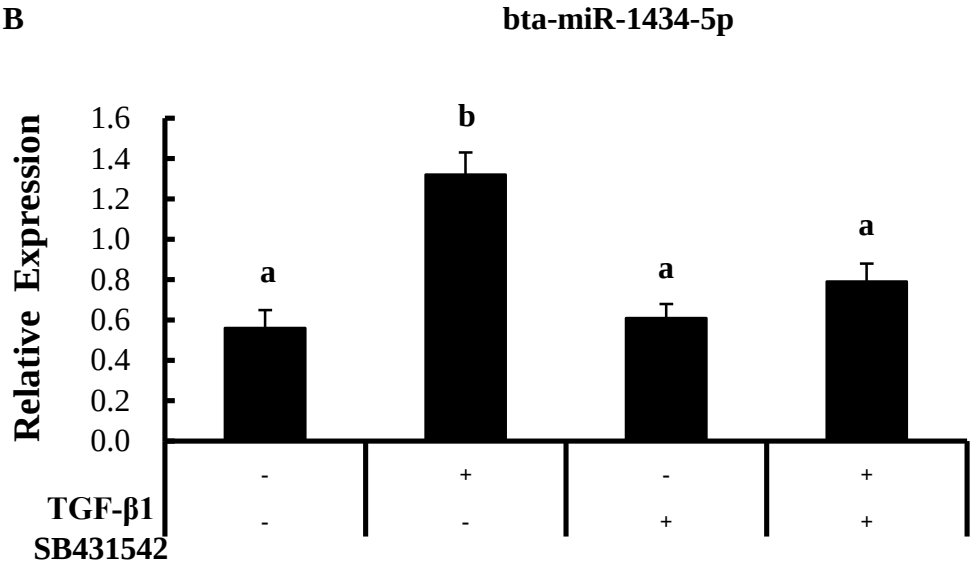
Fig.7. Effect of TGF-β1 on the expression level of bta-miR-106a and bta-miR-1434-5p in bovine granulosa cells measured by qRT-PCR.

A. The effect of TGF-β1 on the bta-miR-106a expression level.

B. The effect of TGF-β1 on the bta-miR-1434-5p expression level.

After cell synchronization with low serum(5% fetal bovine serum) for 12h , bovine granulosa cells were treated with 10 μ M SB431542(+) or without SB431542(-) for 30 min, then were treated with 10ng/ mL(+) or 0ng/ mL(-) of human recombinant TGF-β1 protein treatment for 24h and total RNA was separately extracted for qRT-PCR. Data were normalized to U6 snRNA levels and shown as mean ± SD of three independent experiments. Different lowercase letters above bars show significant differences ($P < 0.05$).





455

456 **Fig.8. Members of WNT, MAPK and TGF-β pathways predicted to be targeted by differentially**
457 **expressed miRNAs in TGF-β and control bovine granulosa cells.**

458 A. WNT pathway; B. MAPK pathway; C. TGF-β pathway
459 Red box indicates differentially expressed miRNA target genes.

460

461 **Tables**

462 **Table 1 Raw data of small RNAs in control and TGF-β1-treated bovine granulosa cells.**

	control		TGF-β1	
type	count	percent(%)	count	percent(%)
Total reads	14470367	100%	15517878	100%
High quality	14116273	97.55%	15126489	97.48%
3'adapter null	17607	0.12%	22376	0.15%
Insert null	243362	1.72%	457858	3.03%
5'adapter contaminants	18468	0.13%	30115	0.20%
Smaller than 18nt	578251	4.10%	741036	4.90%
Poly A	1337	0.01%	2465	0.02%
Clean reads	13257248	93.91%	13872639	91.71%

463

464 **Table 2 Small RNA annotation summary of control and TGF-β1-treated cells.**

	control				TGF–β			
	Tag number (unique)	percent (%)	Tag abundance (total)	percent (%)	Tag number (unique)	percent (%)	Tag abundance (total)	percent (%)
total	460974	100%	13257248	100%	515148	100%	13872639	100%
Exist-miRNA	5855	1.27%	7945668	59.93%	5897	1.14%	7800226	56.23 %
Exist-mirna-edit	16155	3.50%	722421	5.45%	15913	3.09%	699387	5.04%
Exon-antisense	1051	0.23%	8788	0.07%	1373	0.27%	9742	0.07%
Exon-sense	4530	0.98%	7681	0.06%	5958	1.16%	9884	0.07%
Intron-antisense	2	0.00%	107	0.00%	6	0.00%	93	0.00%
Intron-sense	2660	0.58%	4969	0.04%	3512	0.68%	6707	0.05%
Known-miRNA	32608	7.07%	299675	2.26%	32058	6.22%	278272	2.01%
Novel-miRNA	1529	0.33%	9210	0.07%	1513	0.29%	9802	0.07%
rRNA	43769	9.49%	610010	4.60%	46117	8.95%	781913	5.64%
scRNA	90	0.02%	155	0.00%	92	0.02%	196	0.00%
snRNA	4161	0.90%	83886	0.63%	4532	0.88%	132394	0.95%
snoRNA	7151	1.55%	89383	0.67%	7723	1.50%	135792	0.98%
tRNA	37432	8.12%	750891	5.66%	39862	7.74%	843563	6.08%
unann	303981	65.94%	2724404	20.55%	350592	68.06%	3164668	22.81 %

465

466

467 **Table 3 Different exist, known, and novel miRNAs in control and TGF-β1-treated bovine**
468 **granulose cells.**

miR-name		Sequence(5'-3')	Length	TPM		fold-change(log_2 TGF-b1/Control)	*P*-value
				Control	TGF-β1		
Exit	bta-miR-106a	AAAAGTGCTTACAGTGCAGGTA	22	2.34	6.15	1.39	0
	bta-miR-126-5p	CATTATTACTTTTGGTACGCG	21	0.11	1.02	3.2	0.01
	bta-miR-1277	TACGTAGATATATATGTATTTT	22	3.12	1.25	-1.32	0.01
	bta-miR-129	CTTTTTGCGGTCTGGGCTTGCT	22	46.56	17.98	-1.37	0
	bta-miR-129-3p	AAGCCCTTACCCCAAAAAGCAT	22	8.8	4.32	-1.03	0
	bta-miR-129-5p	CTTTTTGCGGTCTGGGCTTGCT	22	46.56	17.98	-1.37	0
	bta-miR-141	TAACACTGTCTGGTAAAGATGG	22	15.26	4.78	-1.67	0
	bta-miR-1434-5p	GTACATGATGACTAAAATTTCT	22	0.45	2.16	2.28	0
	bta-miR-200c	TAATACTGCCGGGTAATGATGGA	23	11.03	3.87	-1.51	0
	bta-miR-212	ACCTTGGCTCTAGACTGCTTACT	23	2.34	6.6	1.5	0
	bta-miR-2285l	AAAACCCGCATGAACTTTTTGGC	23	1.67	0.57	-1.55	0.03
	bta-miR-2316	ACTCCGGCCTGGACTGCGGCGGG	23	0.22	1.25	2.49	0.01
	bta-miR-2453	TCCTCAGGGCAGGAAGTGCGCAG	23	2.78	1.37	-1.03	0.04
	bta-miR-2454-3p	TCTCCTCTGGCCGCTCTCCT	20	1	0.01	-6.65	0
	bta-miR-2478	GTATCCCACTTCTGACACCA	20	7.8	16.16	1.05	0
	bta-miR-29d-5p	TGACCGATTTCTCCTGGTGTT	21	2.78	0.8	-1.81	0
	bta-miR-339b	TCCCTGTCCTCCAGGAGCTC	20	48.68	143.04	1.56	0
	bta-miR-33b	GTGCATTGCTGTTGCATTGC	20	7.35	15.82	1.11	0
	bta-miR-6121-3p	CCGGATGATGGACACTGAGG	20	14.37	30.61	1.09	0
	bta-miR-95	TCAACGGGTATTTATTGAGCA	22	1.89	0.68	-1.47	0.03
Known	mir-129	CTTTTTGCGGTCTGGGTTTGC	21	4.9	2.05	-1.26	0
	mir-132	TAACAGTCTACAGCCATGGTCGTA	24	4.46	9.9	1.15	0
	mir-1338	AGGACTGTCCGACCTGAGAATG	22	1.11	0.11	-3.29	0.01
	mir-1384	GCAATTTTTGGAAAAAAG	18	5.57	11.38	1.03	0
	mir-1386	CTCCTGGCTGGCTCGCCA	18	124.32	258.2	1.05	0
	mir-141	TAACACTGTCTGGTAAAGATGGCT	24	3.45	0.91	-1.92	0
	mir-1599	AGGGGGGAAAAAAAAAAA	18	6.68	16.27	1.28	0
	mir-1777	CTGGGGGCGGTGGGGGGCGGG	21	1.67	3.41	1.03	0.02
	mir-190	TGATATGTTTGATATATTAGGTTA	24	2.9	1.25	-1.21	0.02
	mir-200	TAATACTGCCTGGTAATGATGAC	23	7.24	1.93	-1.9	0
	mir-222	AGCTACATCTGGCTACTGGGTCTT	24	231.37	96.73	-1.26	0.01
	mir-2299	TCCGGGGAATGGATCCAGCGTGT	23	4.46	2.16	-1.04	0.01
	mir-2313	CCAGTTCTACGCTGCATGCCT	21	3.9	1.82	-1.1	0.01
	mir-2336	CTAACCGTAACTTTGAAGTGCTA	23	1.34	0.34	-1.97	0.02
	mir-2419	ATCGCATCAACACTCGTCCATT	22	1.34	2.84	1.09	0.03
	mir-2981	CGAGGCGGGCGGGCGGGG	18	1.34	2.84	1.09	0.03

	mir-3120	CACAGCAAGTGTAGACAGGCAA	22	0.22	1.14	2.35	0.02
	mir-335	TCAAGAGCAATAGCGAAAAATGA	23	0.33	1.48	2.15	0.01
	mir-352	AGAGTAGTAGGTTGCATAGTT	21	2.01	0.68	-1.55	0.02
	mir-4286	ACCCCACTCTCGGTACCA	18	2.78	5.58	1	0
	mir-4429	AAAAGCTGGGTTGAGAGGCGA	21	1	2.96	1.56	0
	mir-6121	GCCGGATGATGGACACTGAGA	21	0.33	2.16	2.69	0
	mir-6516	TCATGTATGATACTGCAAACAGAA	24	5.24	2.5	-1.06	0
	mir-6747	CTCCCTTCCTCTCCACCA	18	2.56	5.46	1.09	0
	mir-7550	TTCCGGCTCGAAGGACCA	18	9.58	34.71	1.86	0
	mir-7977	TTCCCGGCCAATGCACCA	18	19.49	43.01	1.14	0
Novel	novel-m0009-3p	AGCCGGCTCTTGGGCTGTCCGCCT	24	1.89	0.34	-2.47	0
	novel-m0089-5p	AATCTAAGCTCCTATTTTTGGA	22	0.45	2.05	2.2	0
	novel-m0100-3p	GCGGGCTTCCCTGGTGGCTCAGCT	24	1.89	0.01	-7.57	0
	novel-m0130-3p	GGCCAGGGGCGTGTCGGGCTCT	22	0.56	1.82	1.71	0.01
	novel-m0141-5p	CGGGGAGGATGGAGCCTGGCT	21	2.67	0.91	-1.55	0.01
	novel-m0162-3p	AGGGAGTCCCTGGTAGTTCAGT	22	2.34	0.46	-2.36	0
	novel-m0175-3p	TAAAAAGCGTAAGATTTCCCT	21	0.67	1.82	1.45	0.03
	novel-m0191-3p	CAGGTCCCTGCCGGGCGGAGA	21	0.22	1.14	2.35	0.02
	novel-m0199-3p	CATCTGGTCCAGTGTGCCCGC	21	1.11	0.23	-2.29	0.02
	novel-m0200-5p	CATCTGGTCCAGTGTGCCCGC	21	1.11	0.23	-2.29	0.02
	novel-m0201-5p	CATCTGGTCCAGTGTGCCCGC	21	1.11	0.23	-2.29	0.02
	novel-m0220-5p	TGATGTGATGTGATCTGATC	20	0.11	1.02	3.2	0.01
	novel-m0227-5p	TAGGCCAAAAAGTTAACTTGGG	22	0.33	1.25	1.91	0.03
	novel-m0237-5p	ATGCCTTCCCCAGCCTCCGAGC	22	2.34	0.34	-2.78	0
	novel-m0247-5p	CAGGAGCGGTTTGCTGCCAGC	21	0.56	2.05	1.88	0.01
	novel-m0250-3p	TATCAGTTGTGTCTGACTCTTT	22	0.56	2.28	2.03	0
	novel-m0266-3p	TGAGCTCAGACATCCTGCCCCT	22	1.23	0.34	-1.84	0.04
	novel-m0275-3p	TGCCGGGGACCCGGAGCCCCAGG	23	0.01	1.14	6.83	0
	novel-m0313-3p	TATCAGTTGTGTCTGACTCTTT	22	0.56	2.28	2.03	0
	novel-m0318-3p	GCGACTTCTGGGGAGCCTGGTGT	23	2.01	0.8	-1.33	0.03
	novel-m0361-5p	TCTCTTATTCCATTAACTTTAG	22	0.01	1.02	6.68	0
	novel-m0363-5p	GAGGGGGCGGGGCGGGGTCC	20	1.23	0.34	-1.84	0.04
	novel-m0378-3p	CTGCCGAGGCTGTGGCTGGA	20	0.33	1.25	1.91	0.03
	novel-m0384-3p	AAGGGACCTGAATGAACTTTTT	22	0.67	1.82	1.45	0.03
	novel-m0392-3p	CCCCCACTTGCATGACCCTGAGA	23	1.23	0.23	-2.43	0.01
	novel-m0395-5p	CACCCTTTCCAGTGCCCTTTTAAG	24	1.45	0.46	-1.67	0.03
	novel-m0397-3p	GCGGGCTTCCCTGGTGGCTCAGCT	24	2.23	0.01	-7.8	0
	novel-m0401-3p	TCCGAGCGCCGACGCAGCCCAGC	23	2.9	0.91	-1.67	0
	novel-m0412-3p	CTCACAAGCAGCTAAGCCCTGC	22	0.67	2.16	1.69	0.01
	novel-m0431-5p	TGGACCACCAGAGAAGTCCACTGG	24	1.11	0.11	-3.29	0.01
	novel-m0439-5p	AGGAGTCATTCACTCTCAGGA	21	0.22	1.48	2.73	0

	novel-m0463-3p	TGATTGGCATTTCTTAGAGTGGA	23	3.45	1.59	-1.12	0.01

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574 **Highlights**

575 We obtained miRNA profiles of bovine granulosa cells with/without TGF-β1 stimulus.

576 TGF-β1 affects the miRNA expression by Smad signaling.

577 The miRNAs were predicted to be involved in follicular development by GO and KEGG.

578

579 Abbreviations

580 TGF-β1,transforming growth factor-β1; OD, optical density; miRNA, microRNA; GO, Gene
581 ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; qRT-PCR, quantitative reverse
582 transcription -polymerase chain reaction.

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587 **Fig. 3**

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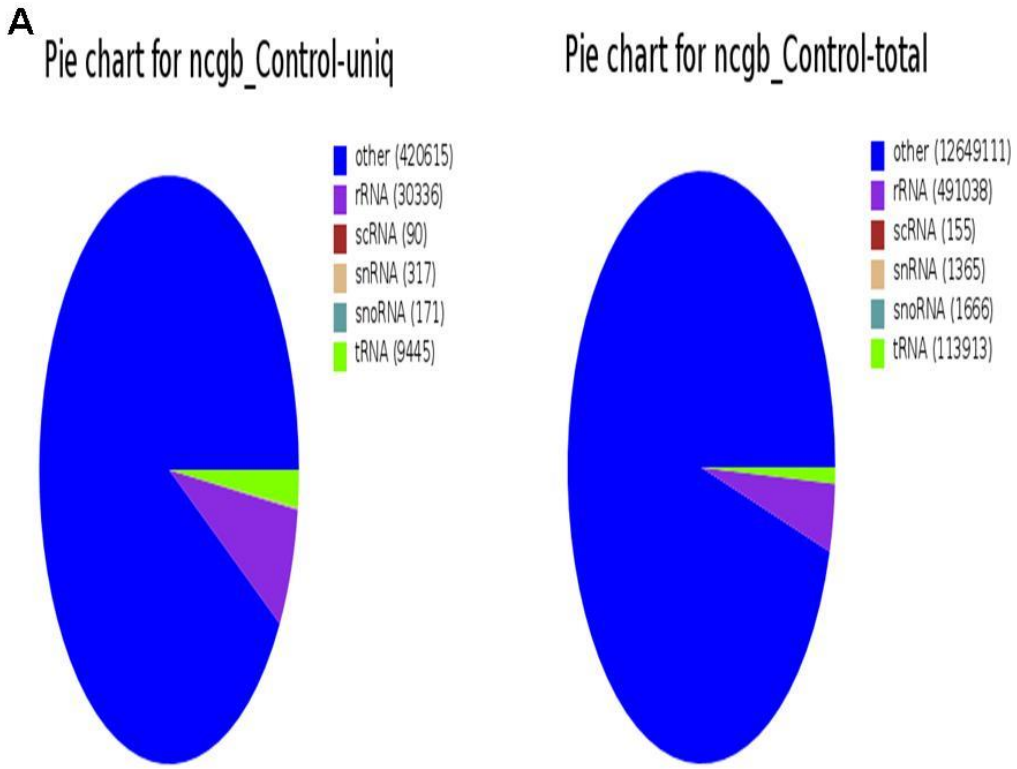

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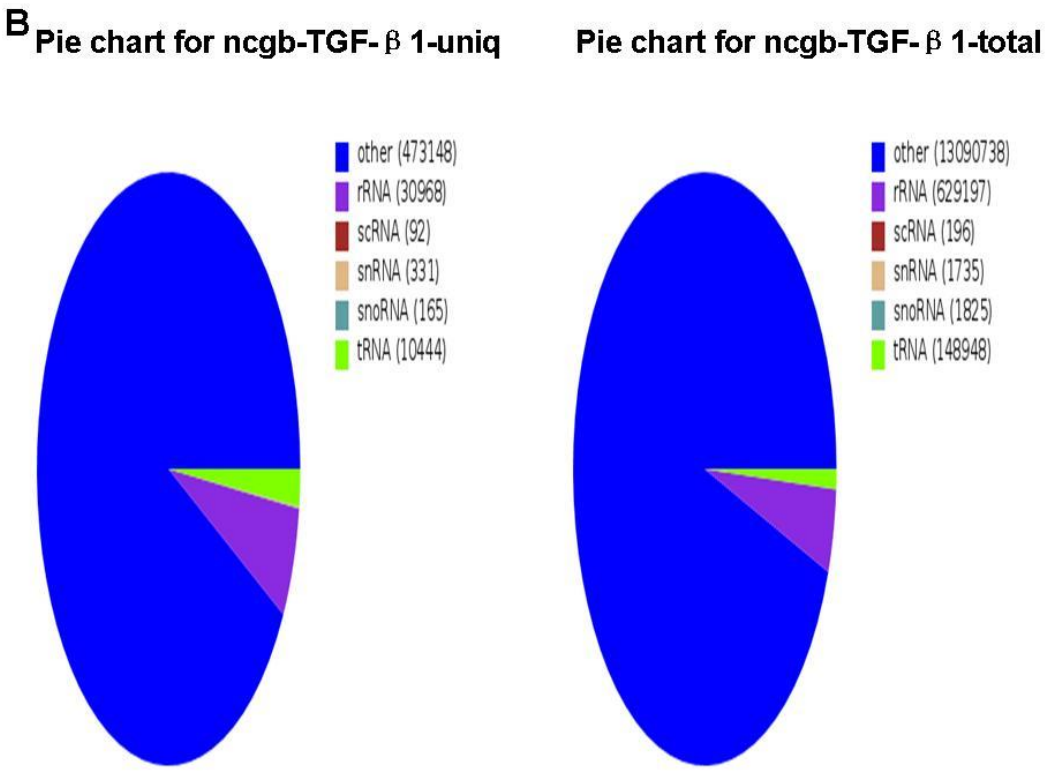

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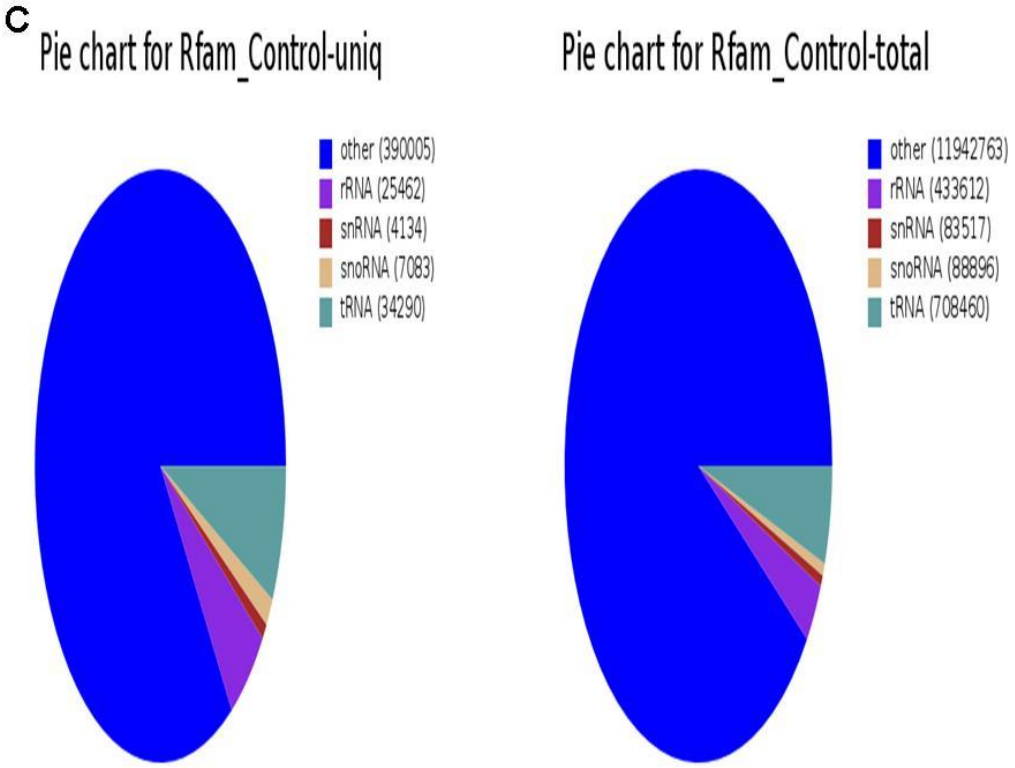

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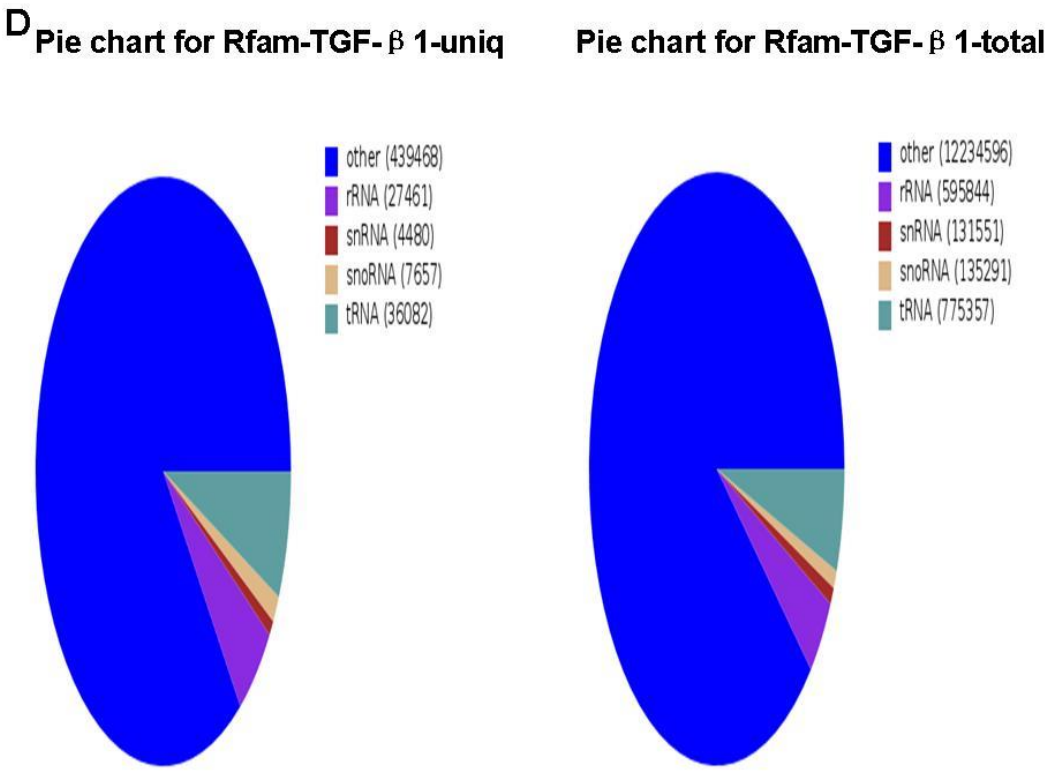

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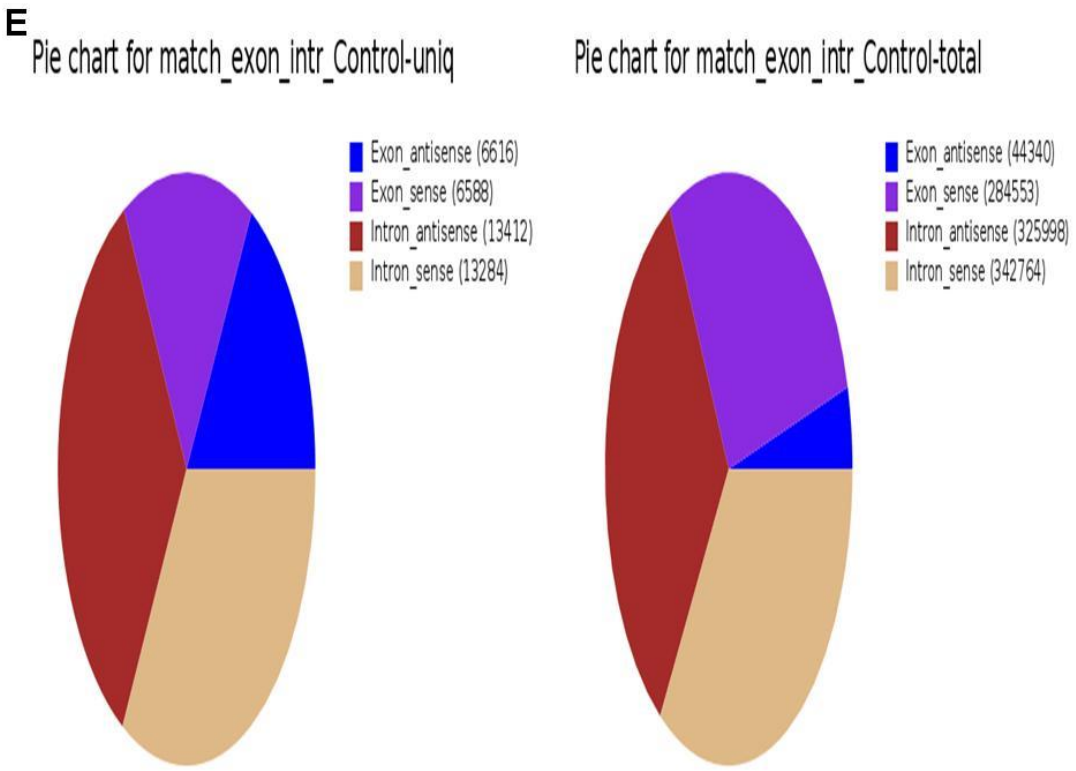

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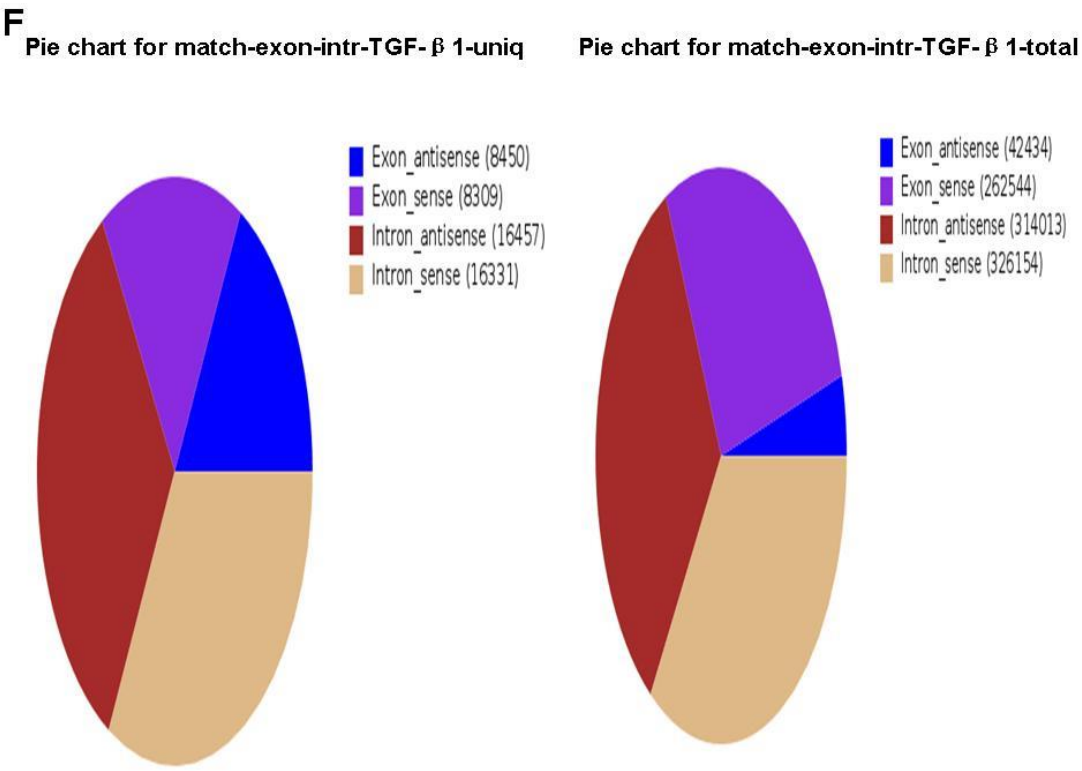

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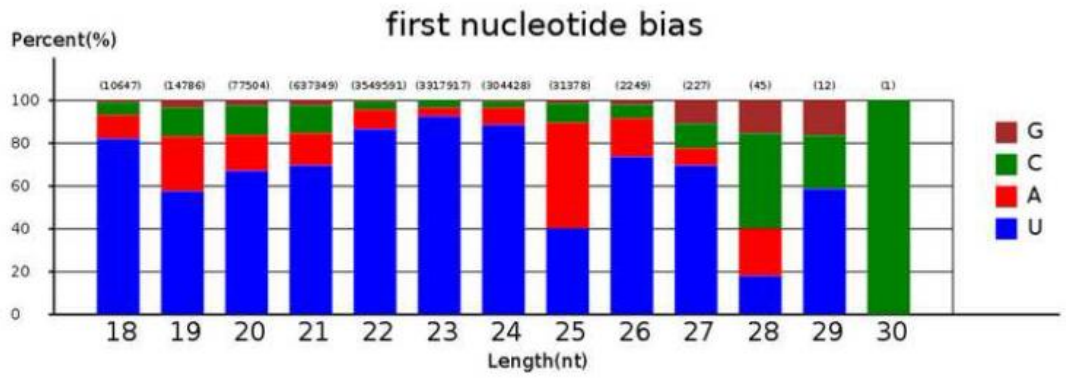
601 **Fig. 4**



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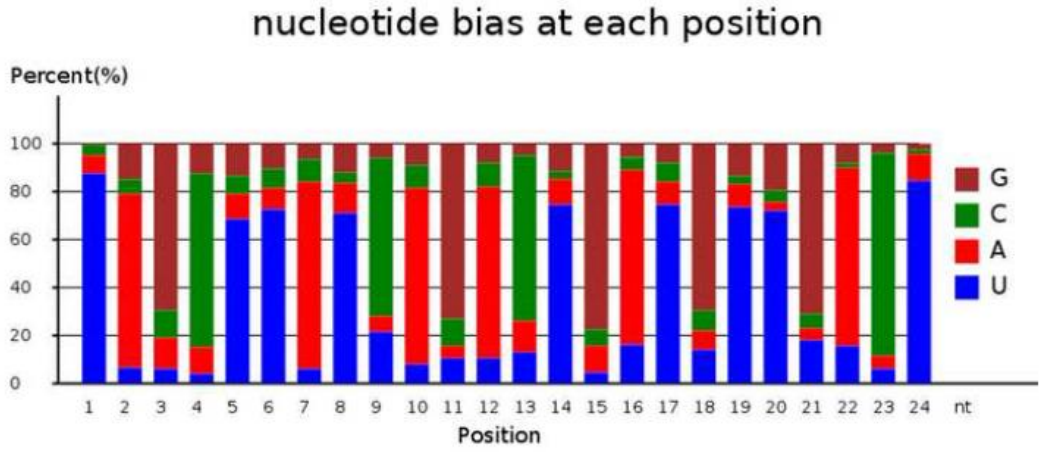

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606 **Fig. 5**

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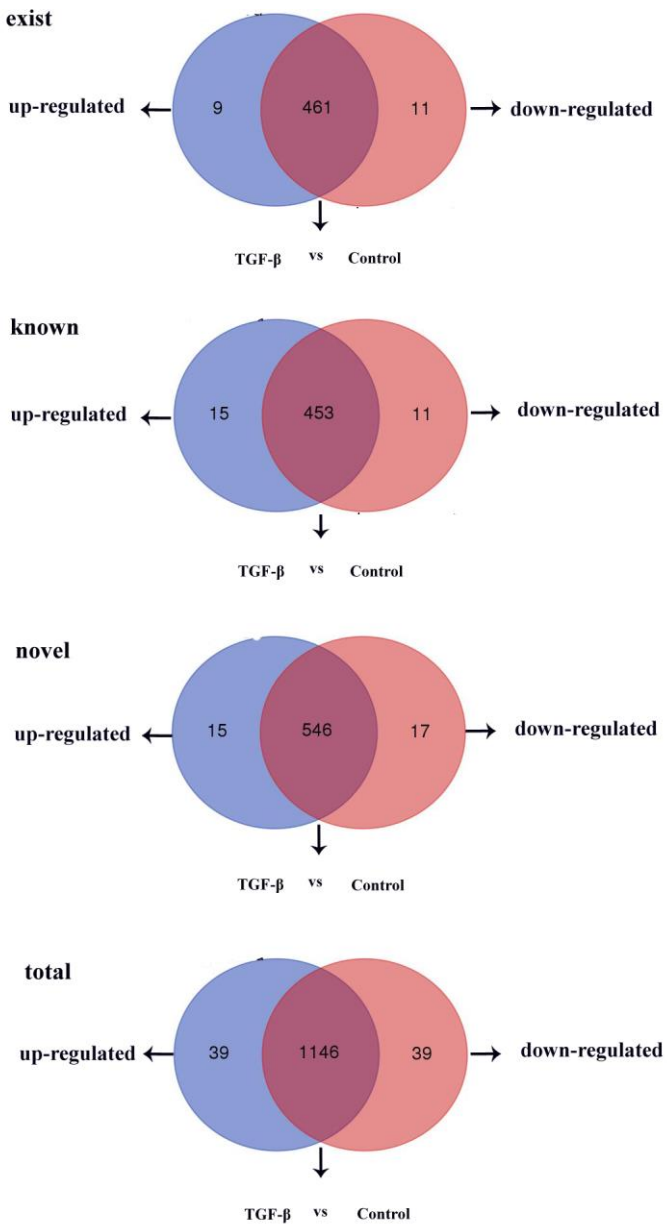

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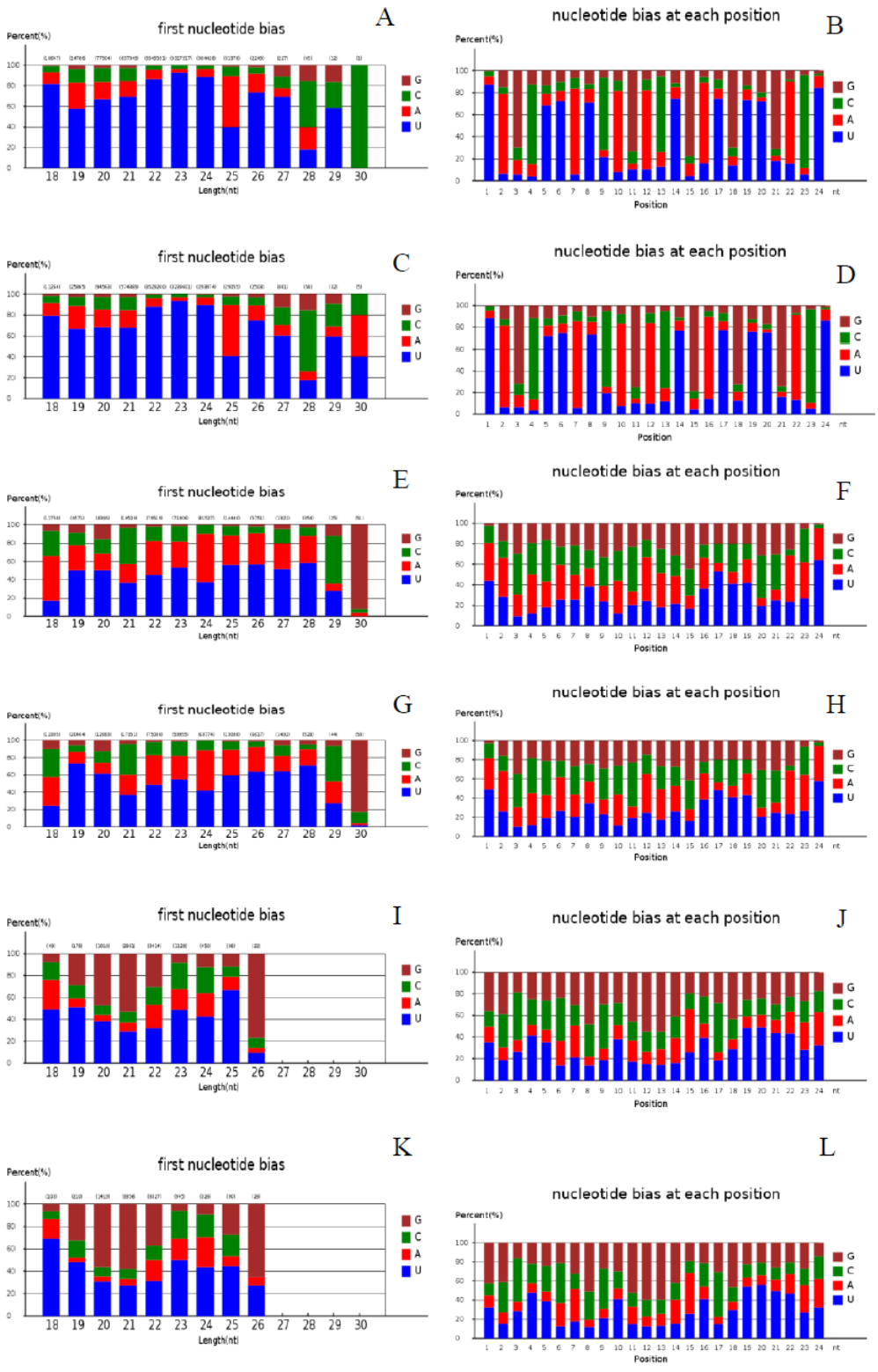
610 **Fig. 6**



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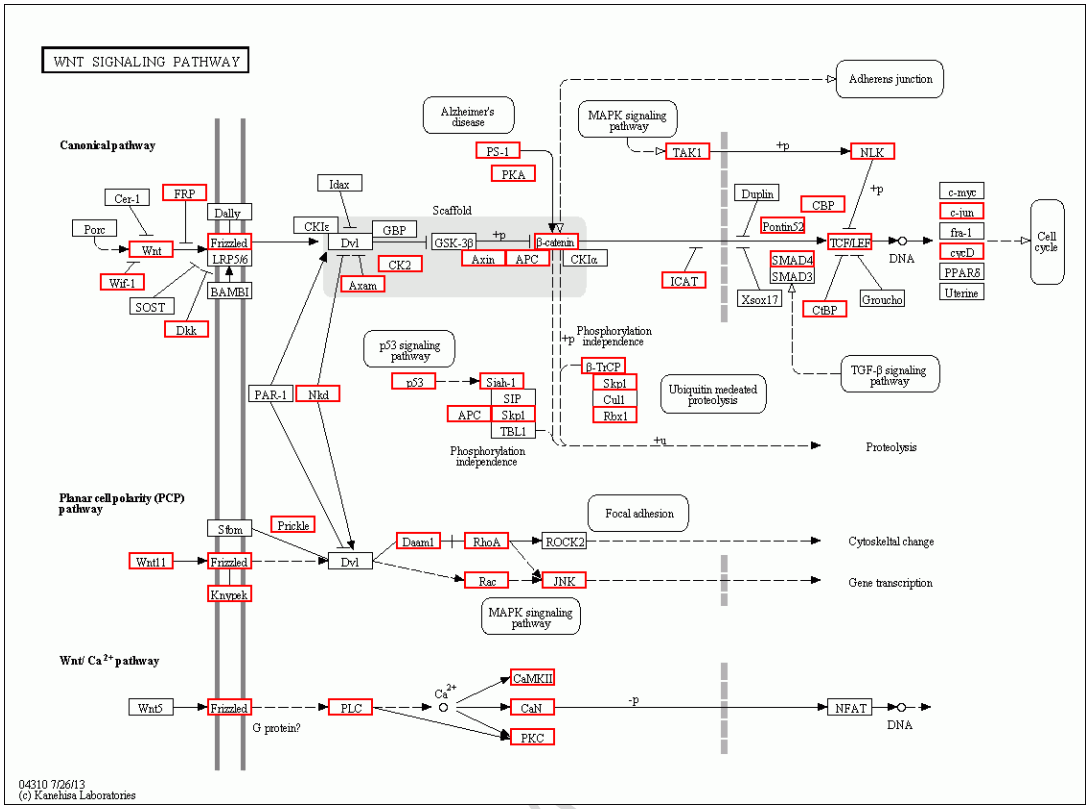
613 **Fig. 8**



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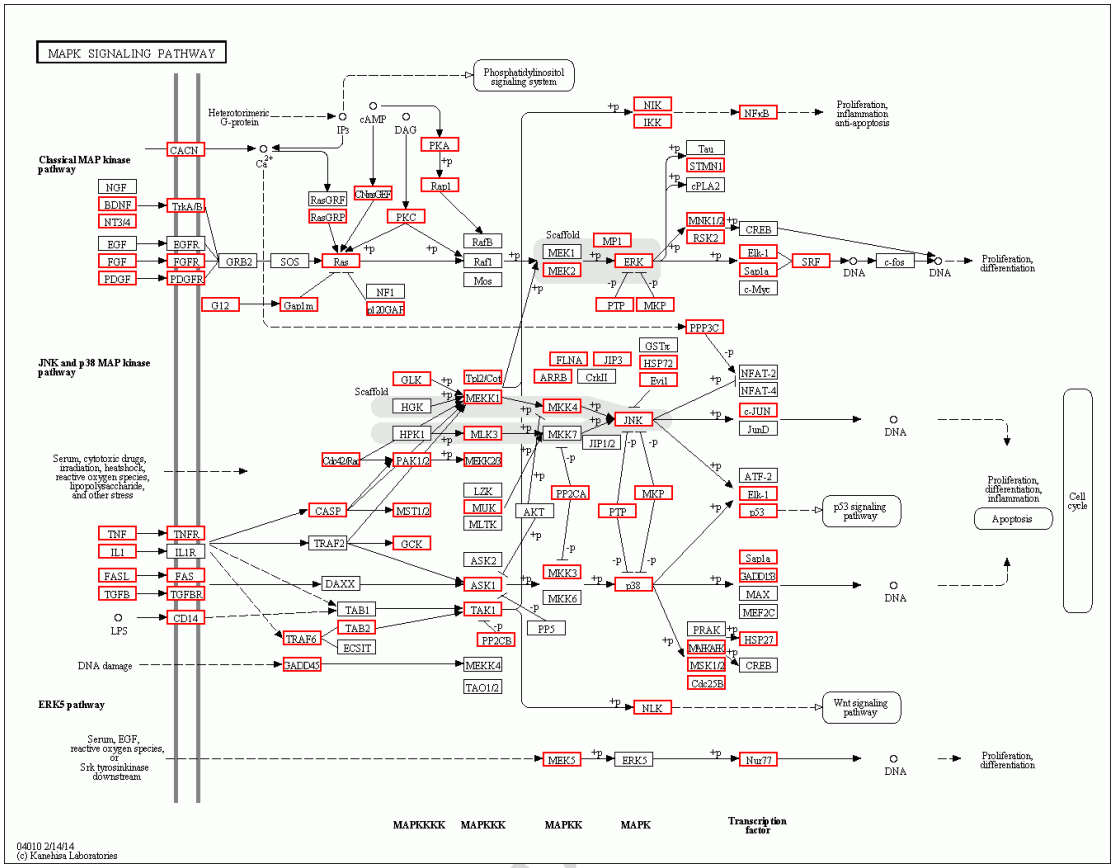

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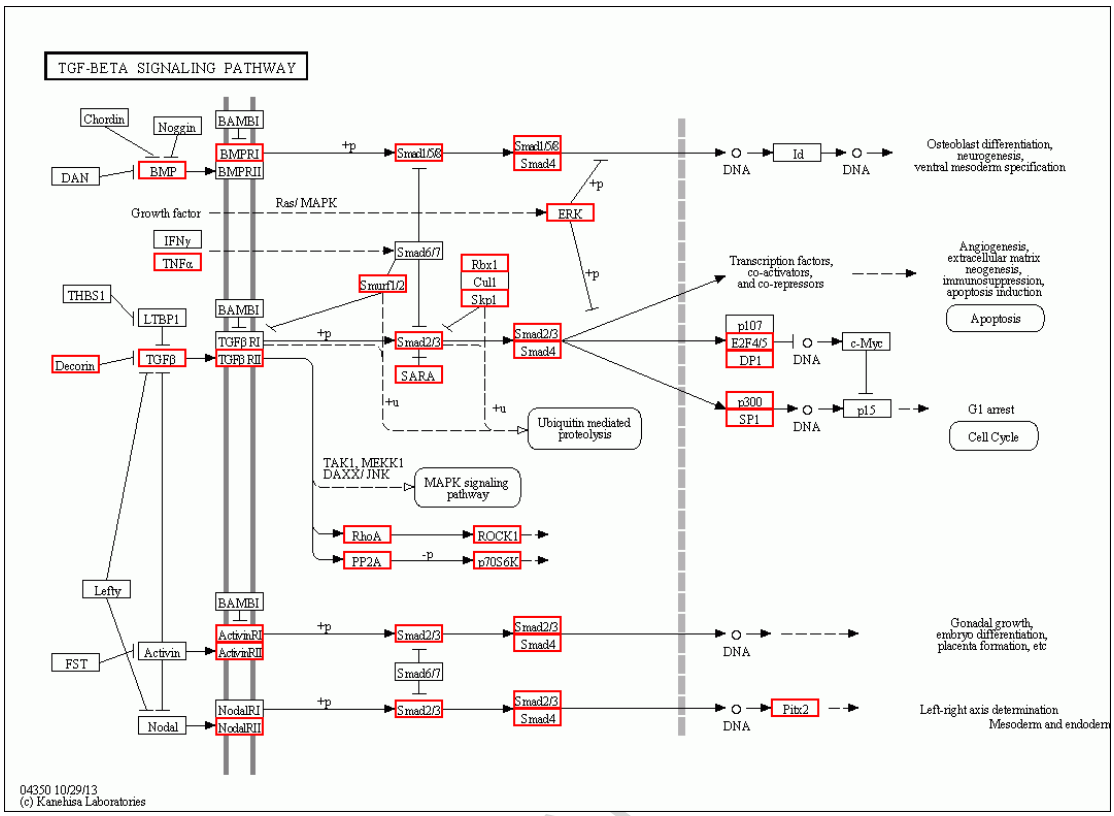

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