Identification and characterization of miRNAs and lncRNAs of coho salmon (*Oncorhynchus kisutch*) in normal immune organs.

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Abstract

MicroRNAs (miRNAs) and long noncoding RNAs (lncRNAs) are two relevant non-coding RNAs (ncRNAs) class. *Oncorhynchus kisutch* (coho salmon) is an important aquaculture pacific salmon species without report of miRNAs and a very limited register of lncRNAs. To gain knowledge about the interaction and discovery of miRNAs and lncRNAs in coho salmon we used high-throughput sequencing technology to sequence small and transcriptome libraries from three immune organs. A total of 163 mature miRNAs and 4,975 lncRNAs were discovered. The profiles of expression of both ncRNAs indicated that liver and head-kidney share relatively similar expression patterns. We identified 814 and 181 putative target sequences for 1,048 lncRNAs and 47 miRNAs, respectively. The results obtained provide new information and enlarge our understanding of the diversities of ncRNAs in coho salmon.

Keywords: Long non-coding RNA, microRNA, gene expression, target gene, *Oncorhynchus kisutch*, high-throughput sequencing.

1. Introduction

Current genome-wide transcriptome studies have detailed the complexity of eukaryotic transcriptomes and shown that a significant fraction of the genome gives rise to ncRNAs (Shabalina et al., 2004; Werey et al., 2011; Yamamura et al., 2018). ncRNA types present similar post-transcriptional modifications as protein-coding genes, such as splicing, but with the absence of an open reading frame (Nachtergaele and He, 2017). These ncRNAs molecules comprise ribosomal RNAs, transfer RNAs, small RNAs (e.g., miRNAs) and lncRNAs. Over the last years special consideration has been given to the class of miRNAs and lncRNAs in a wide taxonomic level. These studies have determined the key role of these molecules in the regulation of important cellular processes such as dosage compensation, imprinting, regulation of chromatin states cell fate determination, and gene silencing (Palazo et al., 2015; Wang et al., 2018). Moreover, deregulation of miRNAs and lncRNAs expression is associated with various types of diseases including cancers, immune disorders, and infections (Jiang et al., 2009; Joaquina Delás and Hannon, 2017). MiRNAs are single-stranded ncRNAs molecules of approximately 22 nucleotides (nt) in length, that regulate gene expression through translational repression and/or transcript cleavage in healthy and disease conditions of several organisms (Huang et al., 2011). In animals, this post transcriptional regulation is mainly associated with the imperfect base paring to target mRNAs in the 3' untranslated regions and recruit the RNA-induced silencing complex with subsequent down-regulation of the corresponding target genes (Feyder and Gof, 2016). In this way, miRNAs can regulate several thousands of mRNAs targets, which may include up to 30% of all protein-coding genes. LncRNAs are a relatively new class of ncRNAs with a length ranging from 200 nt to 100 kilobases (Kung et al., 2013). LncRNAs can regulate gene expression by mechanisms such as binding to the promoter DNA of target genes, inhibiting RNA polymerase activity, or by degradation of target mRNAs (Marchese et al., 2016). Moreover, several studies have showed that lncRNAs can act as target of miRNAs, suppressing the interaction between miRNAs and coding genes, or encoding certain miRNAs as precursors (Salmen et al., 2011; Jeggari et al., 2012). Similarly, studies have shown that miRNAs can reduce the stability of lncRNAs (Yoon et al., 2012, Liu et al., 2013) and that lncRNAs can compete with miRNAs through interactions with protein-coding genes (Faghihi et al., 2010; Franklin et al., 2013). In comparison with the huge very large number of studies focused on the development and evaluation of miRNAs and lncRNAs activities in plant and animal models (Li et al., 2015; Gebert and MacRae, 2019), miRNA and lncRNA studies in aquaculture fish are relatively limited (Rasal et al., 2016; Wang et al., 2017). However, in recent years salmonids have drawn much interest in the develop and characterization of miRNAs associated c viral and bacterial diseases (Rasal et al., 2016; Andreassen et al., 2017; Valenzuela-Miranda et al., 2017; Cao et al 2018; Woldemariam et al., 2019). Coho salmon is one of the six pacific salmon species inhabiting North America and Asia, and, it is an important anadromous aquaculture resource species and a valuable organism for ecological studies (Gustafson, 2007). In the last years coho salmon has been the subject of several studies, focused mainly on its natural resistance to some diseases compared to other salmonids (e,g, *Salmo salar*, Atlantic salmon) (Fast et al., 2002; Braden et al., 2012). Therefore, considering the key role of miRNAs and lncRNAs as internal gene regulators, the aim of this work was to identify and characterize microRNAs and lncRNAs from normal conditions of immune organs such as liver, spleen and head-kidney in coho salmon in addition to the evaluation of its expression levels using high-throughput sequencing technology.

2. Results and discussion

2.1. *de novo* assembly and identification of lncRNAs

To gain knowledge about lncRNAs in key immunological organs in coho salmon, total RNAs from kidney, liver and spleen were used to develop 6 pooled paired-end cDNA libraries and sequenced them on the Illumina HiSeq 2000 platform. The run generated 660,629,536 raw reads. Following the processing trimming steps, the sequence sets were reduced to 656,933,313 reads, with a minimum length of 50 bp. Through the assembly method used, we obtained 261,110 individual assembled transcripts with an N50 and an average length of 1,014 and 653.36 bp, respectively. The 261,110 individual assembled transcripts were aligned against UniProt, Swiss-Prot (E-value cut-off of $1xE^{-4}$) and non-redundant databases (NCBI nr and nt) using a BLASTX local version (E-value cut-off of $1xE^{-6}$). A total of 60.32% individual assembled transcripts were annotated, with the higher level of homology to Atlantic salmon. Because there is no clear method to discovery lncRNAs, we develop a rigorous pipeline to remove transcript with coding signal. Following our pipeline, 4,975 lncRNA transcripts were obtained including 3,910 intergenic (78.59%), 404 anti-sense (8.12%) and 661 intronic (13.28%), with a size range from 200 to 3,347 nt and an average of 449.7. A similar number of expressed lncRNA genes (3,768-5,636) and average size have been obtained for healthy adult coho salmon (skin and head -kidney tissues) and Atlantic salmon (gill, head-kidney, and liver tissues) species (Boltaña et al., 2016; Valenzuela-Muñoz et al., 2018). Of the total of lncRNA transcripts detected, almost 80% were categorized as long intergenic noncoding RNAs (lincRNAs), which is coherent with those reported in a wide range of vertebrates (Amaral et al., 2011) (Supplementary Table S1). From the variety of lncRNA classes, lincRNAs share the largest number of characteristics with mRNAs (Guttman et al., 2009), including their physical localization (Engreitz et al., 2016), which maximizes its potential effect in the gene regulation process. In addition to the total lncRNAs identified, only 1,805 (36%) were assigned to different chromosome positions or unlocalised/ unplaced scaffolds of coho salmon, without a clear enrichment of some chromosome in particular (Fig. 1A).

2.2. Conservation and classification of predicted lncRNAs

To evaluate putative salmonid orthologs of the predicted lncRNAs, we used CRB-Blast to compare with available lncRNAs of Atlantic salmon and *Oncorhynchus mykiss*, rainbow trout. The analysis showed that of total of 4,975 lncRNAs predicted, 2,911 were conserved. Among them, 918 (33.70%) predicted lncRNA transcripts have corresponding orthologs in Atlantic salmon, 1,382 (66.30 %) in rainbow trout, and 600 (20.61%) coho salmon lncRNA transcripts have common orthologs in Atlantic salmon and rainbow trout. Therefore, we have discovered 2,064 lncRNAs new to salmonids. The INFERNAL classification of coho salmon lncRNAs, which incorporates a covariance model based on a combination of sequence consensus and RNA secondary structure, showed a lower number of ncRNA annotated families. Only ten conserved coho salmon lncRNA families were detected, including one long interspersed nuclear element (UnaL2), one ribonucleoprotein (RNase_MRP), one selenocysteine insertion sequence (SECIS_1), one small nucleolar RNA (U3), one transfer RNA (tRNA), and five microRNAs conserved families. Only a maximum of 2 elements (lncRNA) were detected in these families (Supplementary Table S2).

2.3. Small RNA sequence analysis

To identify small RNAs associated toc- key immunological organs in coho salmon, total RNAs from kidney, liver and spleen were used to develop 6 small RNA libraries and high throughput sequencing. An average of 74,876,717, 49,093,981 and 44,548,613 raw reads were collected from kidney, liver and spleen libraries, respectively. After quality and adaptor trimming, 17,842,652, 15,346,168 and 10,084,609 clean reads of 16-30 nt were available from the kidney, liver and spleen organs/libraries,

respectively (Supplementary Table S3). The analysis of length distribution showed that most reads ranged from 21 nt to 24 nt, with 22 nt and 23 nt as the two main size groups. This is congruent with the size distribution reported for different fishes (Herkenhoff et al., 2018). The length distribution found was similar for the three organs analyzed (Fig. 2).

2.4. Identification of known and novel miRNAs

Combining data from all organs/libraries, a total of 146 known miRNAs were identified, belonging to 43 miRNA families (Supplementary Table S4). The number of these miRNAs varied with respect to the families, with LET-7 as the family with most members (19), followed by the families oki-miR-30 (7 members) and oki-miR-15, oki-miR-146, oki-miR-12, oki-miR-10, oki-miR-26, and oki-miR-1338 (all with 5 members) and several families (76%) with only 1 member. Since its first description (Lee et al., 1993), the LET-7 miRNA family has been reported in a wide range of tissues and animals and with a great diversity of family members (Zhao et al., 2017). For teleost, the LET-7 family has been associated mainly with the regulation of growth. (Zhao et al., 2017). Interesting, the miR-30 family has been reported as one of the miRNA families most abundant in Atlantic salmon infected with *Piscirickettsia salmonis* (Valenzuela-Miranda et al., 2017). This is consistent with the diversity of roles described for this family, participating in both, development of tissues and organs and diseases (Mao et al., 2018). In contrast, some few conserved miRNAs reported in other studies (e.g., miR-122) (Bizuayehu and Babiak, 2014) were not identified. Although currently there are several miRNA bioinformatics tools for miRNA sequence analysis (Liu et al., 2014), miRDeep is one of the most well-known and popularly used miRNA-seq tool. A useful aspect of the miRDeep tool, is it score parameter, which is a likelihood that a determined miRNA be true, incorporating in its calculation elements such as the presence of a short 3' overhang on the mature sequence and lengths and relative frequencies of reads that align to precursors, among others. In this way, is recommended select the lowest miRDeep score cutoff that yielded the highest signal-to-noise ratio (Friedländer et al., 2008). In the present study this cutoff was 4. Although the application of this relatively stringent score cutoff result in the exclusion of some few known miRNAs reported in another studies, our aim was avoid the overestimating the miRNAs. Therefore, the miRNAs detected in this study are robust and represent the minimum of expected miRNAs loci expressed in normal coho individuals. In addition, of the total miRNAs identified, 138 (94%) were assigned to different chromosome positions of coho salmon, with the higher frequency of miRNAs mapped in the LG2, LG6, LG26 and LG28 chromosomes (Fig. 1B). A total of 20 new miRNAs were identified, with a lower number of miRNAs (1 element) shared among the 3 organs (Supplementary Table S5).

2.5. Annotation of lncRNA and miRNA targets

To evaluate the potential functionality of the obtained lncRNAs, candidate target genes of these lncRNAs were predicted by their neighborhood with protein-coding genes. A total of 814 targets were determined for 1,048 lncRNAs. GO analysis of the target protein-coding genes showed that regulation of biological processes, biological regulation, metabolic processes, and cellular processes were significantly enriched categories of biological process. In the case of the cellular components category, the top enriched terms were cell, cell component, and organelle, and for the molecular function category the enriched terms were localization and catalytic activity (Fig. 3). The pathway analysis showed that 94 pathways were significantly enriched, with pathways in cancer, endocytosis, PI3K-Akt signaling, regulation of acting cytoskeleton, and HILV infection, as the most abundant (Fig. 4). In the case of miRNAs, target protein-coding genes were predicted by the miRanda and PITA algorithms. Both programs showed that 37 conserved miRNA families and 10 new miRNAs targeted 181 protein-coding genes (Supplementary Table S6). The pathway enrichment analysis of these target mRNAs genes determined that 12 pathways were significantly enriched, with the cytokine-cytokine receptor

interaction and endocytosis pathways as the most abundant (Fig. 5). In the case of GO, enriched terms of cell, cell part and organelle part were the most abundant in the cellular component category. In the molecular function category, the most abundantly enriched terms were the multicellular organismal process, the development process, and binding. In the biological process category, the most abundant terms were the cellular process, the biological regulation and regulation of biological process (Fig. 6). The overlap of similar GO biological process categories among both ncRNAs class would imply that both lncRNAs and miRNAs would be acting, under normal conditions, fundamentally in similar cell functions.

2.6. Interactions among lncRNAs and miRNAs

Of the total of lncRNAs predicted, six lncRNAs (0.12%) were precursors of sixteen mirRNAs from three conserved families (LET-7, oki-miR-16 and oki-miR-29). Moreover, the results of miRanda and PITA showed that 1,339 lncRNAs (27.00%) will be putative targets of 148 miRNAs (distributed in 41 families of conserved miRNAs and 20 new miRNAs) (Supplementary Table S7). Although experimental validation (e.g., crosslinking immunoprecipitation-sequencing) is fundamental to establish miRNAs-lncRNAs interactions, our results will support the hypothesis of lncRNA sponge or competing endogenous RNA (Salmena et al., 2011) in coho salmon, where lncRNAs can serve as targets to miRNAs, minimizing their regulatory role on mRNAs.

2.7. Differentially expressed lncRNAs and miRNAs

The differentially expressed (DE) lncRNAs and miRNAs genes were determined with DESeq2. In a global level, without considering tissue origin, a total of 807 lncRNAs genes were identified as DE, with 540 (67%) and 267 (33%) lncRNAs genes down-regulated and up-regulated, respectively. In the case of conserved and new miRNAs, a total of 140 genes were identified as DE with 90 (64.29%) and 50 (35.71%) down-regulated and up-regulated, respectively. At an individual level, within tissues, the expression level of the different annotated lncRNAs and miRNAs varied over a wide range, with read number from only a few to hundreds of thousands. There were 14 lncRNAs with relatively higher expression level, with over 200 reads across all the three libraries. A heatmap clustering of these 14 lncRNAs showed that liver and head-kidney were first clustered together, suggesting that both tissues share relatively similar expression patterns (Fig. 7A). In the case of miRNAs, there were 28 miRNAs with relatively higher expression level (belonging to 11 families), with over 1,000 reads across the three libraries. A heatmap clustering of these 28 miRNAs showed a pattern similar to that of lncRNAs, where liver and head-kidney were first clustered together (Fig. 7B). In several animals, the expression of both classes of ncRNAs have been demonstrated as marked by an elevated tissue/organ and biological stage specificity (Ludwig et al., 2016; Kern et al., 2018). Clearly, lncRNAs and miRNAs of coho salmon are following the same pattern. In addition, several of these 11 miRNAs families have been reported in previous studies of salmonids infected with pathogens (e.g., miR-30, miR-21, miR-10 and miR-146) (Andreassen, et al., 2017; Valenzuela-Miranda et al., 2017) however, in a specific gene member level, there are not a clear agreement. For example, Andreassen, et al. (2017) have showed that ssa-miR-146b-5p, ssa-miR-146a-3p and ssa-miR-146a-3-3p are over expressed in Atlantic salmon individuals infected with salmonid alphavirus (here was oki-miR-146a-1-5p). Likewise, Valenzuela-Miranda et al., (2017) identified an over regulation of miR-21a-1 in Atlantic salmon infected with *Piscirickettsia salmonis* (here was oki-miR-21-1-5p). Although further studies are necessary, the present results will suggest that salmonids, under normal and extreme conditions (e.g., infected), may show similar regulated miRNA families but not the same specific regulated miRNA families gene members.

In other hand, using the criterion from SPM and TPM/FPKM defined, the spleen expressed the highest number of selectively-tissue lncRNAs genes (16), followed by head-kidney (8) and liver (6) (Supplementary Table S8). The same criterion indicated oki-miR-21 as the only gene with selective expression in kidney, oki-miR-99, oki-miR-22 and oki-miR-100 with selective expression in liver, and oki-miR-2188 and oki-miR-30 with selective expression in spleen.

In conclusion, this study represents the first comprehensive bioinformatics analysis of lncRNAs and miRNAs expressed in immune organs of healthy coho salmon organisms. We have identified and characterized 4,975 lncRNAs (2,064 new to salmonids) and 163 mature miRNAs (20 new to salmonids). Salmonid RNA-sequencing studies have focused fundamentally on addressing specific extreme biological questions, such as disease challenge and nutrition experiments. This has generated a gap in knowledge and characterization of coho salmon transcriptome under normal conditions. Therefore, the results presented here represent background data for future studies to compare normal lncRNAs and/or miRNAs expression profiles of immune organs.

3. Materials and methods

3.1. Tissue collection, RNA isolation and Sequencing

All the animal experiments in this study were approved and are in agreement with the Institutional Ethics Committee of the Universidad de Santiago guidelines. Individual fish had not been vaccinated nor exposed to fish diseases and were deemed pathogen free by the regional fish health authorities. Six healthy smolt individuals (unselected, mixed-sex; mean mass ± SEM: 105.18 ± 1.6 g; mean length ± SEM: 22.0 ± 0.13 cm) were obtained from the private company Marine Farm, Chile. Samples from head-kidney, liver, and spleen were collected from each individual and stored in RNA later at -80°C. We have focused on these organs, because kidney and spleen are relevant lymphoid organs (Zapata et al., 2006) and liver has been shown to produce key immune proteins in teleost (Wu et al., 2016). Total RNA was isolated individually, using the miRNeasy Mini Kit (Qiagen, Holland) following the manufacturer's instructions. The RNA concentration, purity and integrity were determined by spectrophotometry (BioPhotometer, Eppendorf) and with the Agilent Bioanalyser 2100, respectively. For each organ, two equal total RNA pools were constructed (pool 1, individuals 1-3 and pool 2, individuals 4-6). The six pools were sent to the Génome Québec Innovation Centre, Canada, for small RNA (TruSeq Small RNA Sample protocol) and paired-end cDNA libraries (mRNAs) construction and sequencing on the Illumina HiSeq 2000 platform; each pool was sequenced two times. The raw sequence has been deposited in the Sequence Read Archive (SRA), accession number National Center for Biotechnology Information (NCBI) **SRP# 058682**.

3.2. Transcriptome (mRNA) Sequence Analysis and Identification of candidate lncRNAs

The pre-processing of the obtained reads was performed to remove short reads, low quality regions and adapter contamination using Cutadapt (v.1.8) (Martin, 2011). Duplicate reads were removed using Prinseq standalone version (Schmieder and Edwards, 2011). The resulting reads were aligned to the reference genome Okis_V1 (GenBank assembly accession: GCA_002021735.1) with HISAT2 (Kim et al., 2015) and the mapped reads were sorted by coordinates using Picard tools (http://broadinstitute.github.io/picard/R). The software Stringtie (Pertea et al., 2015) was used to assemble the transcripts for each sample independently, and then the merge option of Stringtie was used to create a reference transcriptome for this study. This reference transcriptome was compared against the public annotation of coho salmon genes (GenBank assembly accession: GCA_002021735.1), using the gffcompare software (https://github.com/gpertea/gffcompare) to classify our transcripts in the different classes defined by the position where they were mapped in the genome.

Transcripts with class code u (unknown intergenic), x (genic antisense) and i (intronic) were considered as possible lncRNA transcripts, and they were evaluated and filtered, to remove protein-coding genes, with the following criteria; (i) transcripts blasted against the non-redundant BLASTx protein database with an E-value cut-off of < 0.00001 were eliminated, ii) transcripts without match were used to search complete ORFs (from start to stop codons) using the getorf Emboss tool (http://embossgui.sourceforge.net/demo/manual/getorf.html) and transcripts with ORFs > 200 bp were discarded, iii) the remaining sequences were processed with the CPC v. 2.0 (Kang et al., 2017) and CPAT v. 1.2.4 softwares (Wang et al., 2013) to discard sequences with coding potential and iv) the potential lncRNAs were blasted against the 3'UTR sequences of the genome reference Okis_V1 (GenBank assembly accession: GCA_002021735.1) and transcript with BLASTn E-value cut-off of $<1xE^{-10}$ were also discarded.

3.3. Classification and conservation of lncRNAs

Putative orthology of the predicted lncRNAs transcripts, relative to public available lncRNAs salmonid species (Al-Tobasei et al., 2016; Paneru et al., 2016; Valenzuela-Muñoz et al., 2016), were performed with the strategy of conditional reciprocal best BLAST, using the CRB-Blast program (Aubry et al., 2014). LncRNAs with a relatively stringent E-value threshold > $1x10^{-10}$ were considered as lncRNA orthologues. Similarly, the predicted lncRNAs were classified into ncRNA families using INFERNAL V1.1 (with default parameters) (Nawrocki et al., 2013).

3.4. Small RNA Sequence Analysis

Initial raw small RNA reads were analyzed with FastQC (v.0.11.5) (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) and Prinseq (Schmieder and Edwards, 2011), to remove poor quality reads (ambiguous N and average quality score < 20), adaptor sequences, reads containing poly(A) stretches or other low complexity features and reads shorter than 16 nucleotides (nt) or longer that 30 nt. The clean reads were aligned by Bowtie2 v. 2.3.0 (Langmead and Salzberg, 2012) against Rfam 12.2 (Nawrocki et al., 2014), GtRNAdb (Chan and Lowe, 2016), SILVA 128.0 (Quast et al., 2013) and Repbase V. 22.07 (Jurka et al., 2005) database to eliminate different non-coding sRNA (e,g,, rRNA, tRNA, snRNA and snoRNA) and repetitive sequence elements.

3.5. Identification of conserved and prediction of novel miRNAs

To identify conserved miRNAs, the unique reads represented by at least 10 reads from each of the libraries, remaining from the clean step, were analyzed with miRDeep2 v. 2.0.0.8 (Friedländer et al., 2012) (default parameters) using an in-house database of mature and pre-microRNAs vertebrate from the miRBase 21.0 (http://www.mirbase.org) and from the genome reference Okis_V1 (GenBank assembly accession: GCA_002021735.1). To prevent false positive detection of miRNA precursors, a miRDeep2 score of 4 was used as a cut-off. Reads without mismatch or related (no more than one mismatch) were assigned as conserved coho salmon miRNAs. Unannotated unique reads, represented by at least 10 reads, were used to identify potential novel miRNAs with the following criteria: i) a score from miRDeep2 v. 2.0.0.8 higher than 4; ii) a signal-to-noise ratio higher that 15; and iii) length of the precursor greater than 60 nt. The new miRNAs were annotated as oki-novelx-5p/3p (x; e.g, oki-novel1-5p).

3.6. Prediction and annotation of lncRNA and miRNA targets

To predict potential lncRNA protein-coding targets, we used the option of cis-acting elements in the available Okis_V1 coho salmon genome version. In this way, we select closet protein-coding genes to lncRNAs 2.0 kb up and downstream of them, using the Bedtools software (Aaron and Quinlan, 2010). In the case of miRNAs, potential binding sites of new and conserved miRNAs were predicted using the

3′ UTRs available in the coho salmon Okis_V1 reference genome. To reduce false positive protein-coding predictions we used two target prediction algorithms, miRanda (< E^{-15} kcal/mol) (Enright, 2003) and PITA (< E^{-15} kcal/mol and score >150) (Kertesz et al., 2007) and only target site predicts by both algorithms were considered. In order to recognize functional roles of the protein-coding genes list obtained, an enrichment analysis was performed on GO terms and KEGG pathways with the software's DAVID (Huang et al., 2009) and KOBAS 3.0 (Chen et al., 2011). GO terms and KEGG pathways were considered significant with a Benjamini and Hochberg correction of $P < 0.05$.

3.7. Interactions among lncRNAs and miRNAs

To evaluate the potential role of lncRNAs as precursors of miRNAs, pre-miRNAs sequences obtained in this study were aligned to the identified lncRNAs. The cases of perfect alignment (with no gap), were considered as lncRNA harboring the respective miRNA. To evaluate if the lncRNAs obtained can act as target of the miRNAs identified, the target prediction algorithm of the miRanda (< E^{-15} kcal/mol) and PITA (< E^{-15} kcal/mol and score >150) packages were used. Only target site predicts by both algorithms were considered.

3.8. Differential expression of identified miRNAs and lncRNAs.

To compare the expression in a pairwise way among kidney, liver and spleen organs libraries, read counts for every identified miRNAs and lncRNAs were normalized into tags per million (TPM) and fragments per kilobase of exon per million mapped (FPKM), respectively. Moreover, to categorize the expression pattern of each miRNA/lncRNA a heatmap analysis was performed, transforming the TPM/FPKM data to log 2 scales. DESeq2 (Love et al., 2014) was used for differential gene expression, the transcript with absolute fold change higher that 1.0 and p-adjusted values less than 0.05 were considered as significantly differentially expressed. If the TPM/FPKM of a given miRNA/lncRNA were less than 1 in two organ libraries, these miRNA/lncRNA were not considered for further analysis of differential expression. To determine gene expression tissue-specificity, the algorithm developed by Xiao et al., (2010) was employed, with a criterion of FPKM/TPM values ≥ 1 and specificity measure (SPM) > 0.9.

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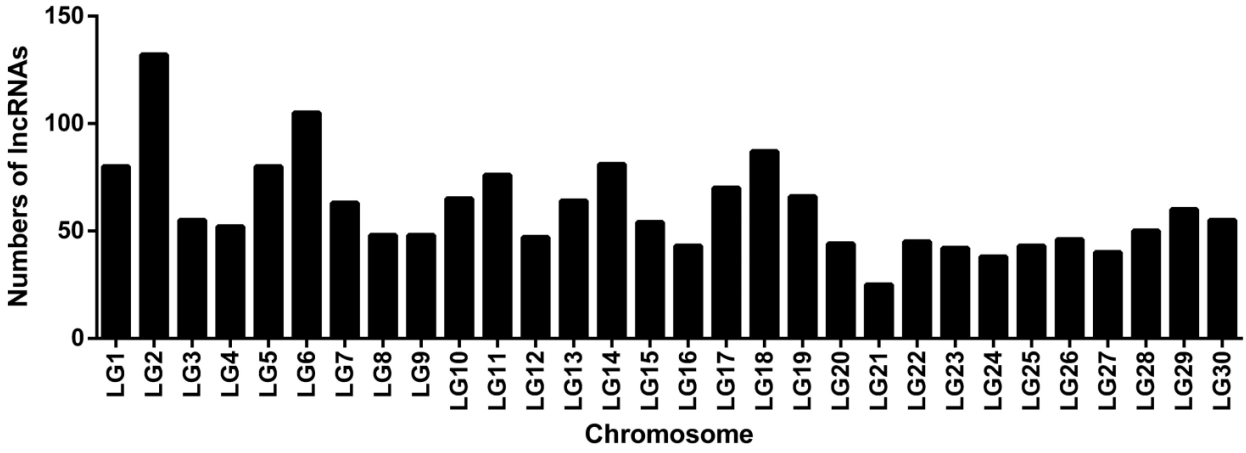

Numbers of lncRNAs
150
100
50
0
LG1
LG2
LG3
LG4
LG5
LG6
LG7
LG8
LG9
LG10
LG11
LG12
LG13
LG14
LG15
LG16
LG17
LG18
LG19
LG20
LG21
LG22
LG23
LG24
LG25
LG26
LG27
LG28
LG29
LG30
Chromosome

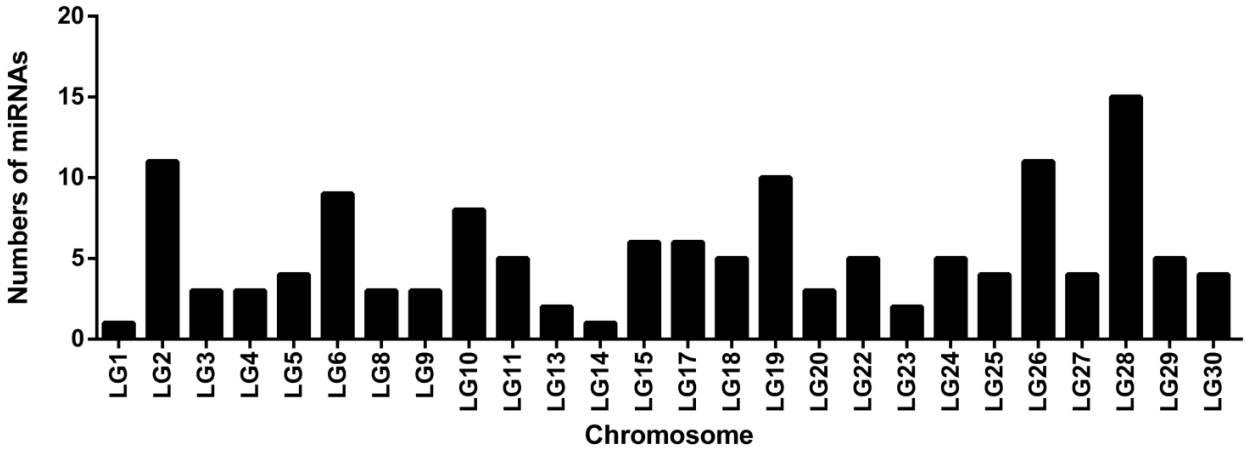


Figure 1. Distribution of the numbers of lncRNAs (A) and conserved miRNAs (B) identified on coho salmon chromosomes.

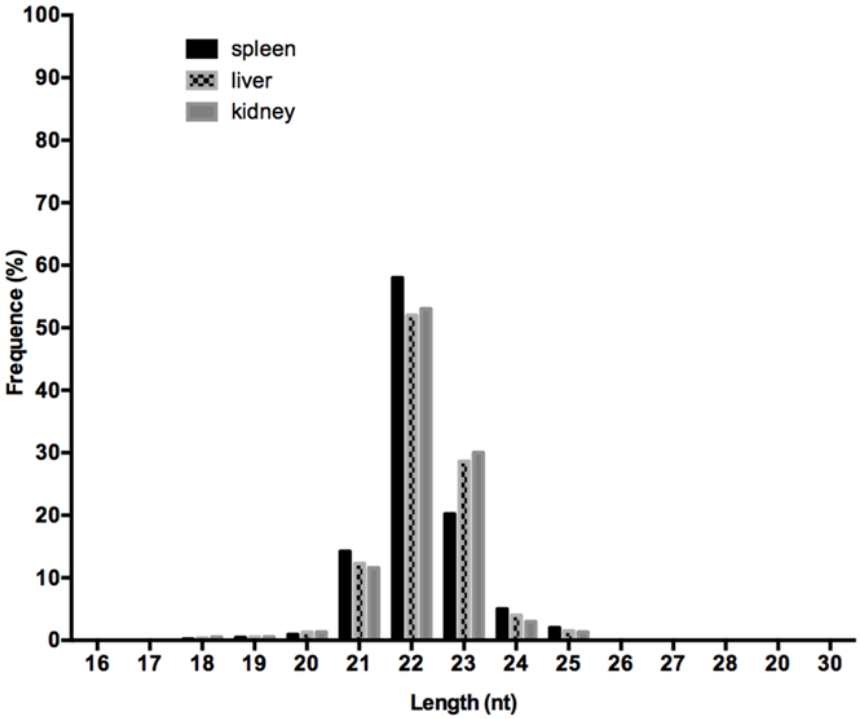
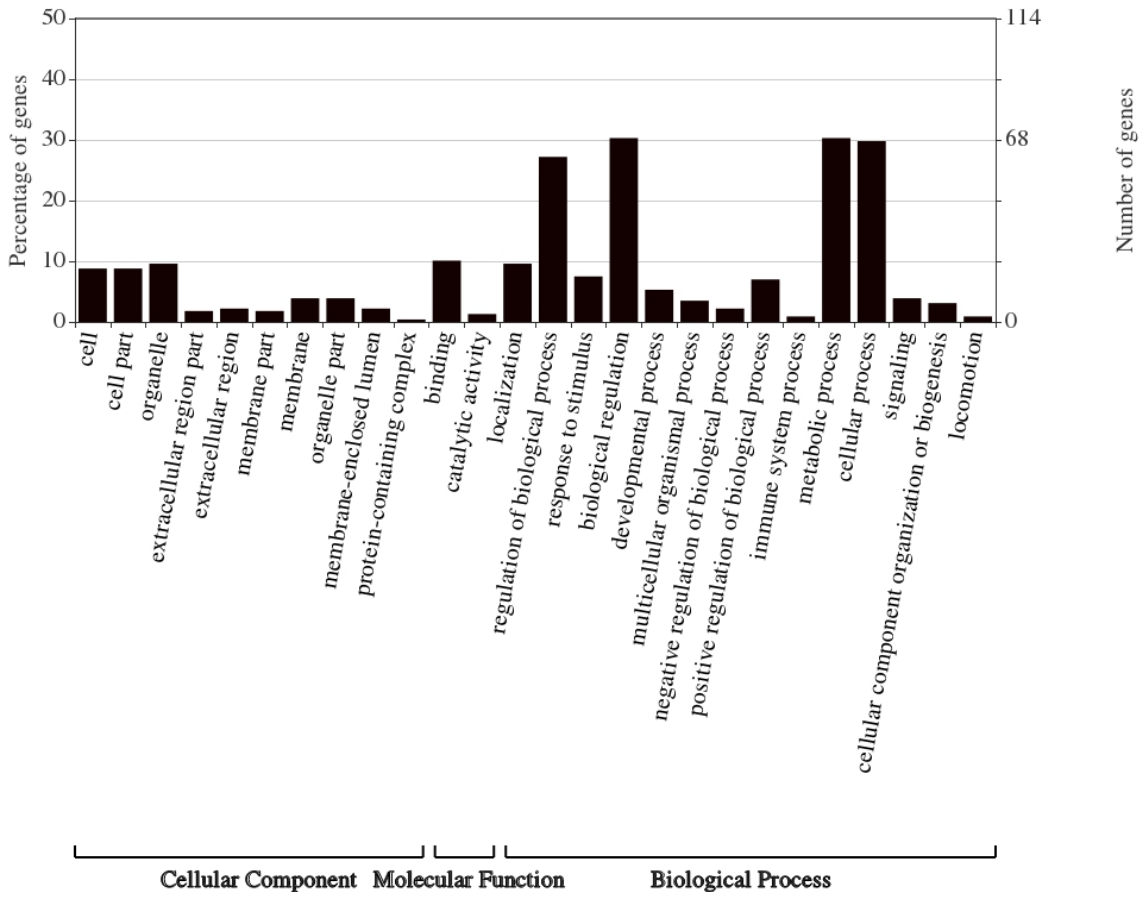


Figure 2. Frequency distribution (%) of miRNAs sizes.



548

549 Figure 3. Distribution of enriched GO terms of candidate lncRNAs protein-coding targets in coho
550 salmon.

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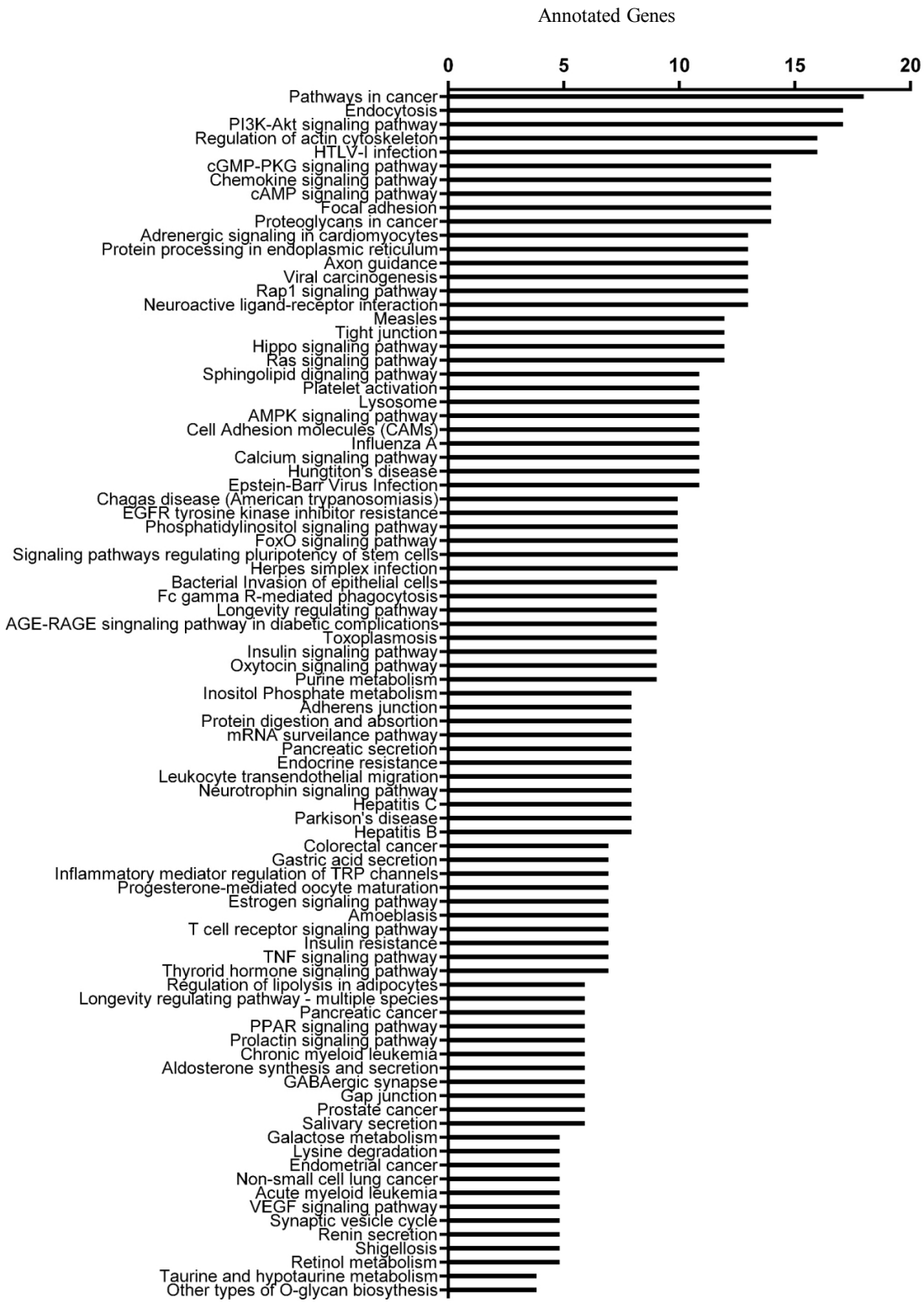

Figure 4. Distribution of enriched KEGG pathways of candidate lncRNAs protein-coding targets.

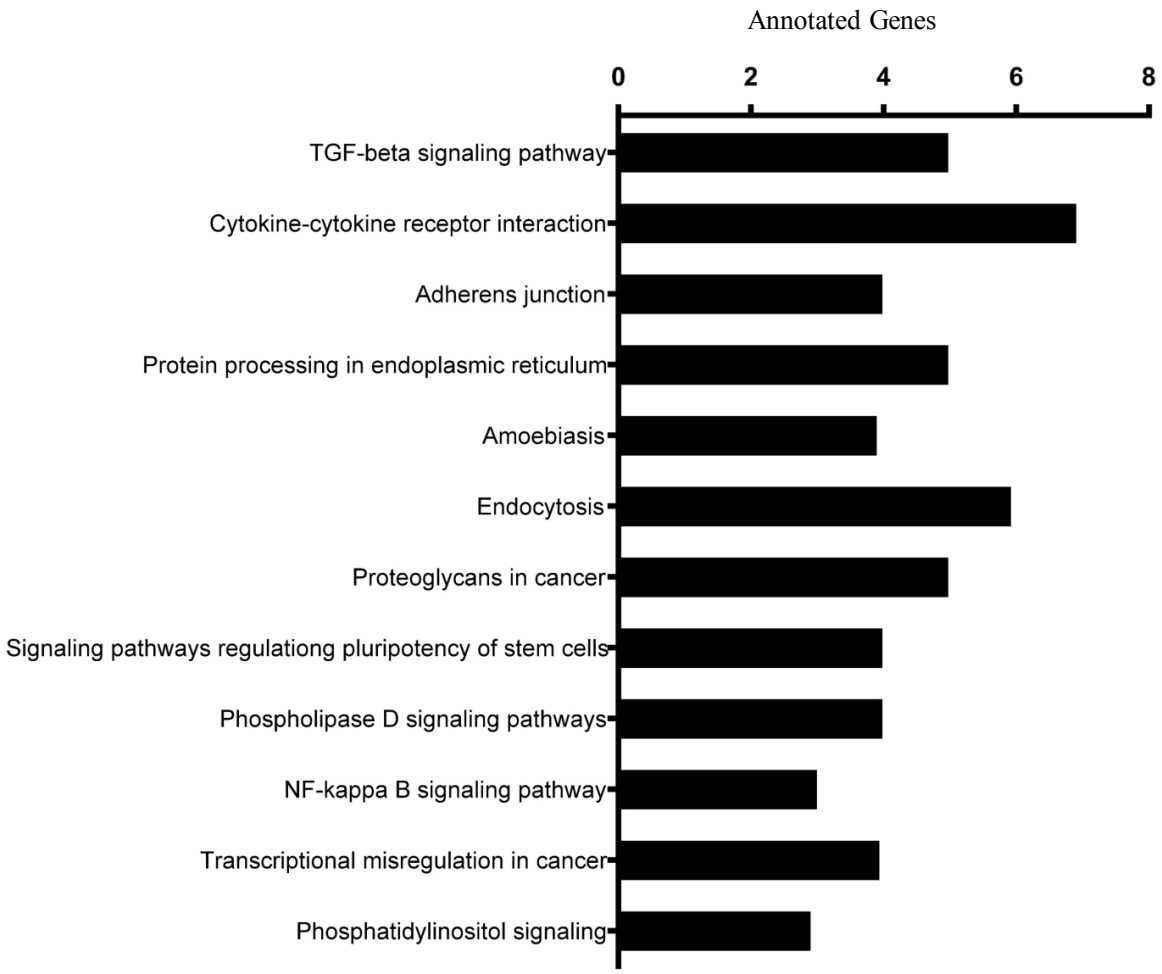

Figure 5. Distribution of enriched KEGG pathways of candidate miRNAs protein-coding targets

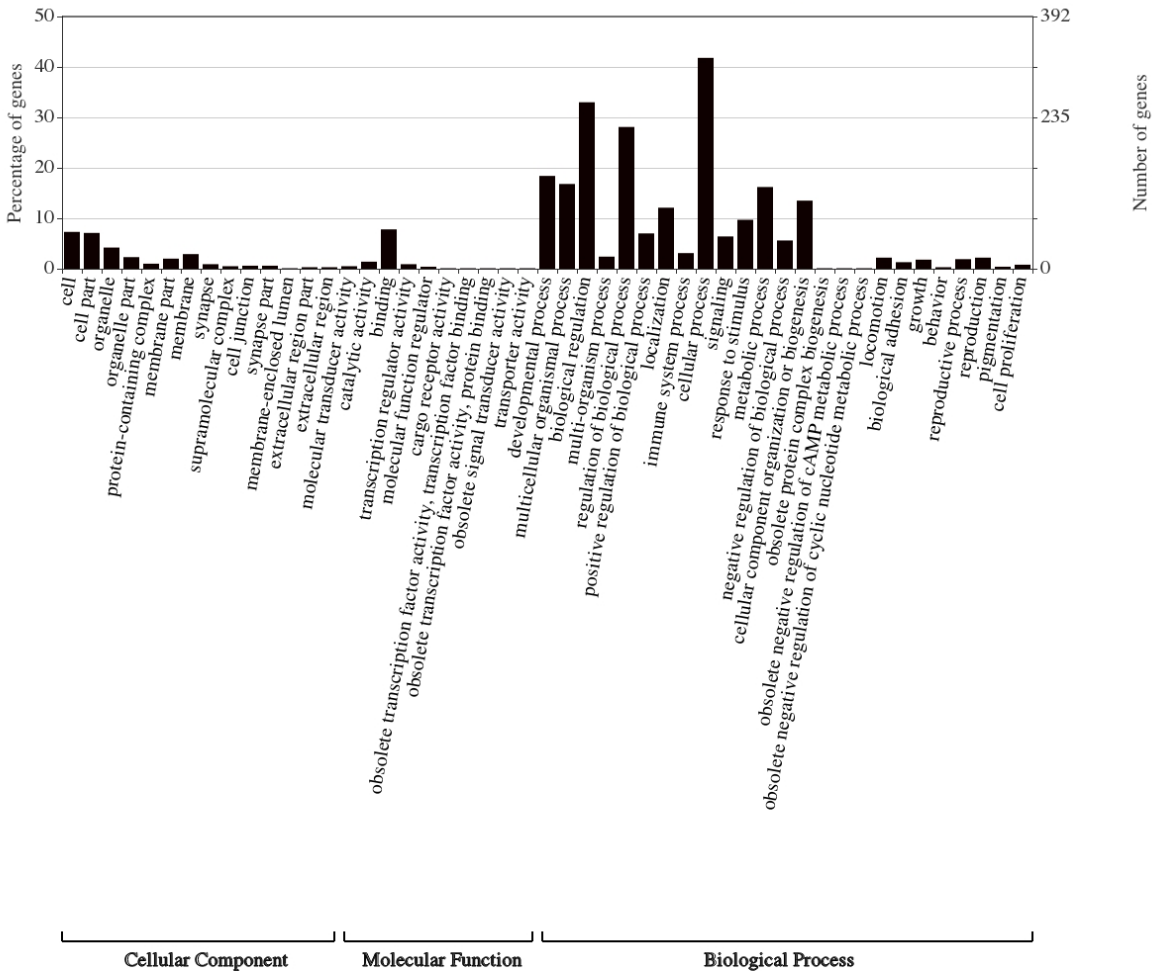

Figure 6. Distribution of enriched GO terms of candidate miRNAs protein-coding targets.

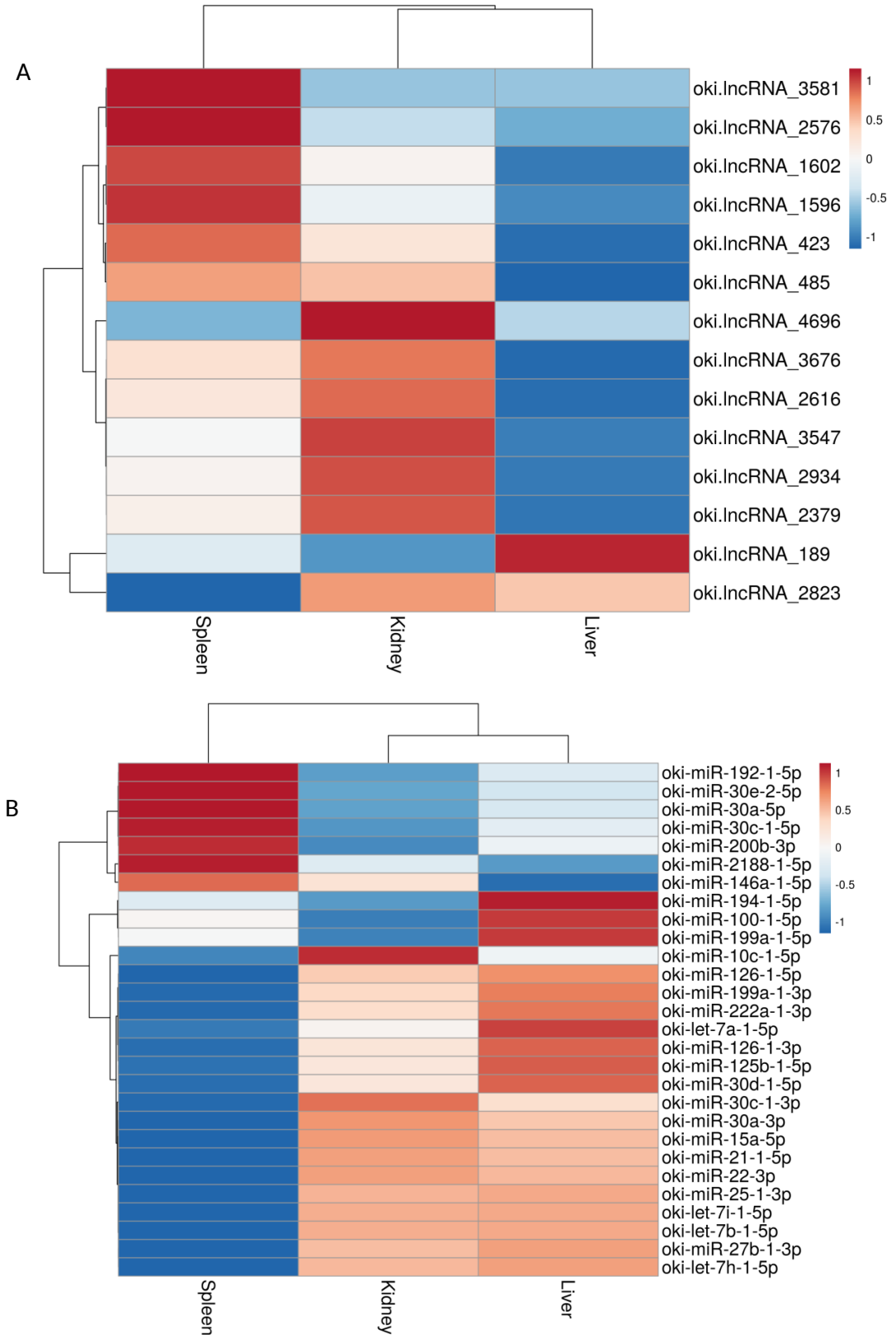

Figure 7. Heatmap clustering of top lncRNAs (A) and miRNAs (B) expressed by tissue.

Highlights

- This is first report of miRNAs in coho salmon.
- 4,975 lncRNAs and 163 mature miRNAs were identified in three normal immune organs of coho salmon.
- Both ncRNAs showed that liver and head-kidney organs share relatively similar expression patterns.
- Our results showed that 1,339 lncRNAs will be potentially the target of 148 miRNAs and only six are precursors of 16 miRNAs. Therefore, our results will support the hypothesis of lncRNA sponge or competing endogenous RNA in coho salmon.

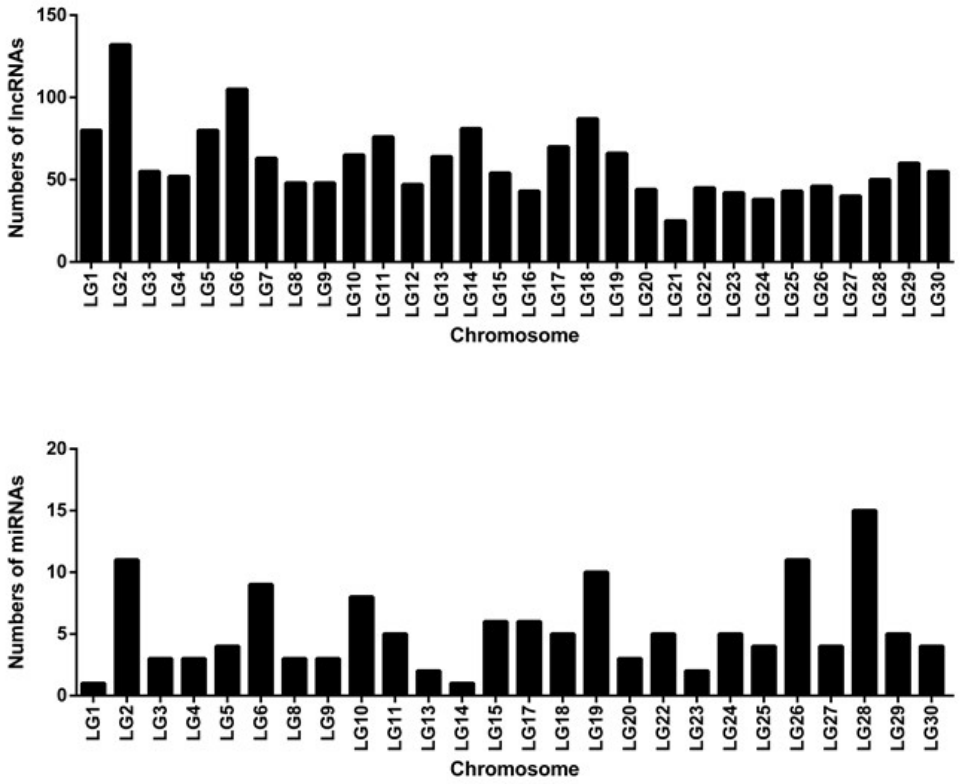
Numbers of lncRNAs
150
100
50
0
LG1 LG2 LG3 LG4 LG5 LG6 LG7 LG8 LG9 LG10 LG11 LG12 LG13 LG14 LG15 LG16 LG17 LG18 LG19 LG20 LG21 LG22 LG23 LG24 LG25 LG26 LG27 LG28 LG29 LG30
Chromosome
Numbers of miRNAs
20
15
10
5
0
LG1 LG2 LG3 LG4 LG5 LG6 LG8 LG9 LG10 LG11 LG13 LG14 LG15 LG17 LG18 LG19 LG20 LG22 LG23 LG24 LG25 LG26 LG27 LG28 LG29 LG30
Chromosome

Figure 1

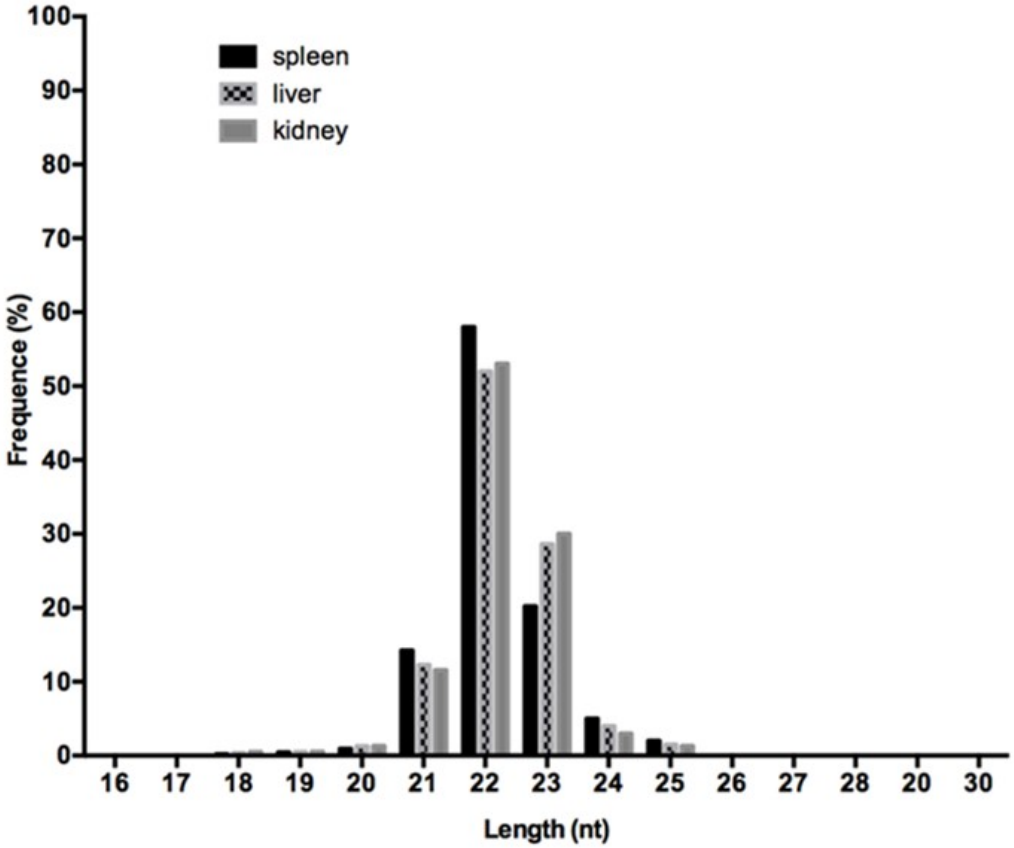
spleen
liver
kidney
Frequence (%)
100
90
80
70
60
50
40
30
20
10
0
16
17
18
19
20
21
22
23
24
25
26
27
28
20
30
Length (nt)

Figure 2

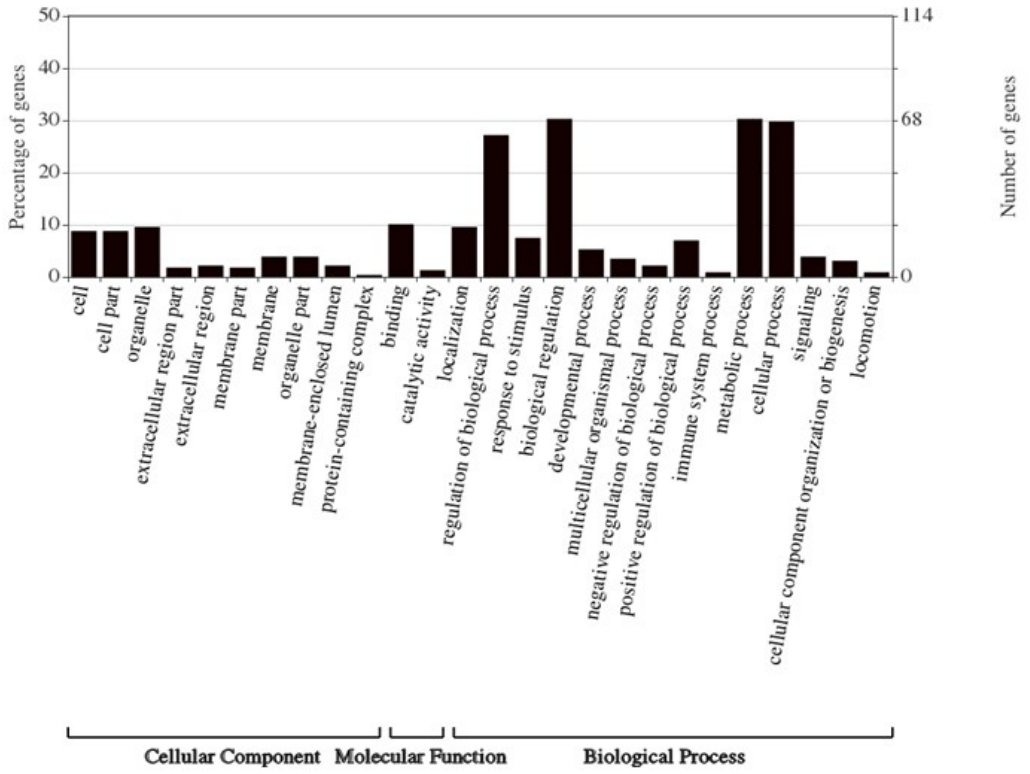
Percentage of genes
Number of genes
50
40
30
20
10
0
114
68
0
cell
cell part
organelle
extracellular region part
extracellular region
membrane part
membrane
organelle part
membrane-enclosed lumen
protein-containing complex
binding
catalytic activity
localization
regulation of biological process
response to stimulus
biological regulation
developmental process
multicellular organismal process
negative regulation of biological process
positive regulation of biological process
immune system process
metabolic process
cellular process
signaling
cellular component organization or biogenesis
locomotion
Cellular Component
Molecular Function
Biological Process

Figure 3

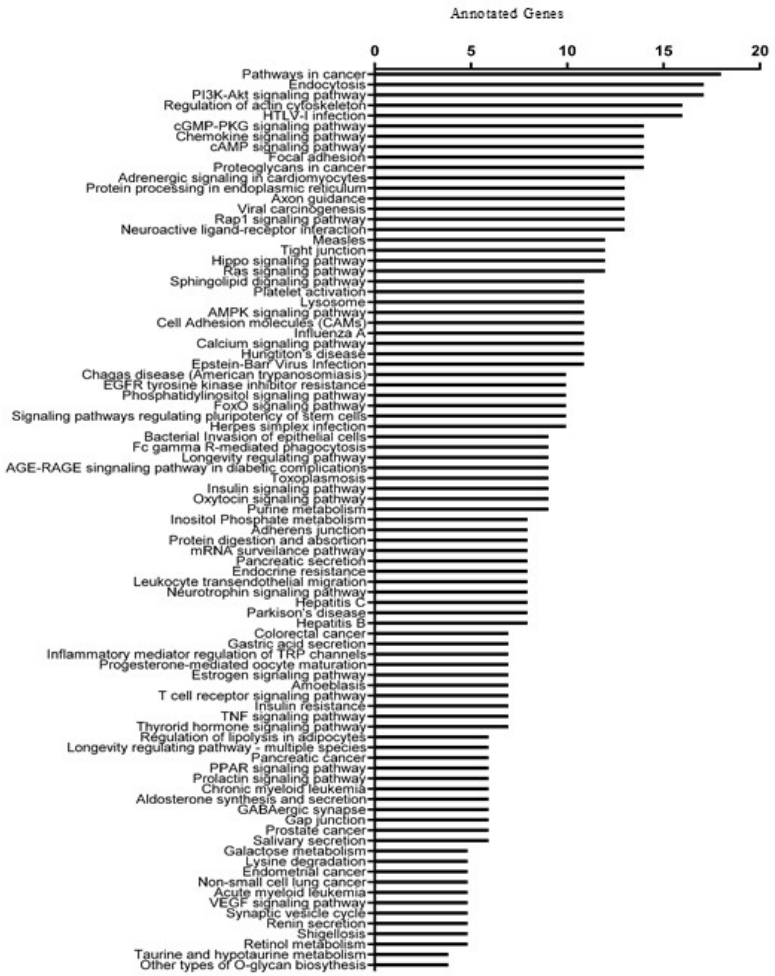
Annotated Genes
0
5
10
15
20
Pathways in cancer
Endocytosis
PI3K-Akt signaling pathway
Regulation of actin cytoskeleton
HTLV-I infection
cGMP-PKG signaling pathway
Chemokine signaling pathway
cAMP signaling pathway
Focal adhesion
Proteoglycans in cancer
Adrenergic signaling in cardiomyocytes
Protein processing in endoplasmic reticulum
Axon guidance
Viral carcinogenesis
Rap1 signaling pathway
Neuroactive ligand-receptor interaction
Measles
Tight junction
Hippo signaling pathway
Ras signaling pathway
Sphingolipid signaling pathway
Platelet activation
Lysosome
AMPK signaling pathway
Cell Adhesion molecules (CAMs)
Influenza A
Calcium signaling pathway
Huriglition's disease
Epstein-Barr Virus Infection
Chagas disease (American trypanosomiasis)
EGFR tyrosine kinase inhibitor resistance
Phosphatidylinositol signaling pathway
FoxO signaling pathway
Signaling pathways regulating pluripotency of stem cells
Herpes simplex infection
Bacterial Invasion of epithelial cells
Fc gamma R-mediated phagocytosis
Longevity regulating pathway
AGE-RAGE singnaling pathway in diabetic complications
Toxoplasmosis
Insulin signaling pathway
Oxytocin signaling pathway
Purine metabolism
Inositol Phosphate metabolism
Adherens junction
Protein digestion and absortion
mRNA surveilance pathway
Pancreatic secretion
Endocrine resistance
Leukocyte transendothelial migration
Neurotrophin signaling pathway
Hepatitis C
Parkison's disease
Hepatitis B
Colorectal cancer
Gastric acid secretion
Inflammatory mediator regulation of TRP channels
Progesterone-mediated oocyte maturation
Estrogen signaling pathway
Amoeblasis
T cell receptor signaling pathway
Insulin resistance
TNF signaling pathway
Thyrorid hormone signaling pathway
Regulation of lipolysis in adipocytes
Longevity regulating pathway - multiple species
Pancreatic cancer
PPAR signaling pathway
Prolactin signaling pathway
Chronic myeloid leukemia
Aldosterone synthesis and secretion
GABAergic synapse
Gap junction
Prostate cancer
Salivary secretion
Galactose metabolism
Lysine degradation
Endometrial cancer
Non-small cell lung cancer
Acute myeloid leukemia
VEGF signaling pathway
Synaptic vesicle cycle
Renin secretion
Shigellosis
Retinol metabolism
Taurine and hypotaurine metabolism
Other types of O-glycan biosythesis

Figure 4

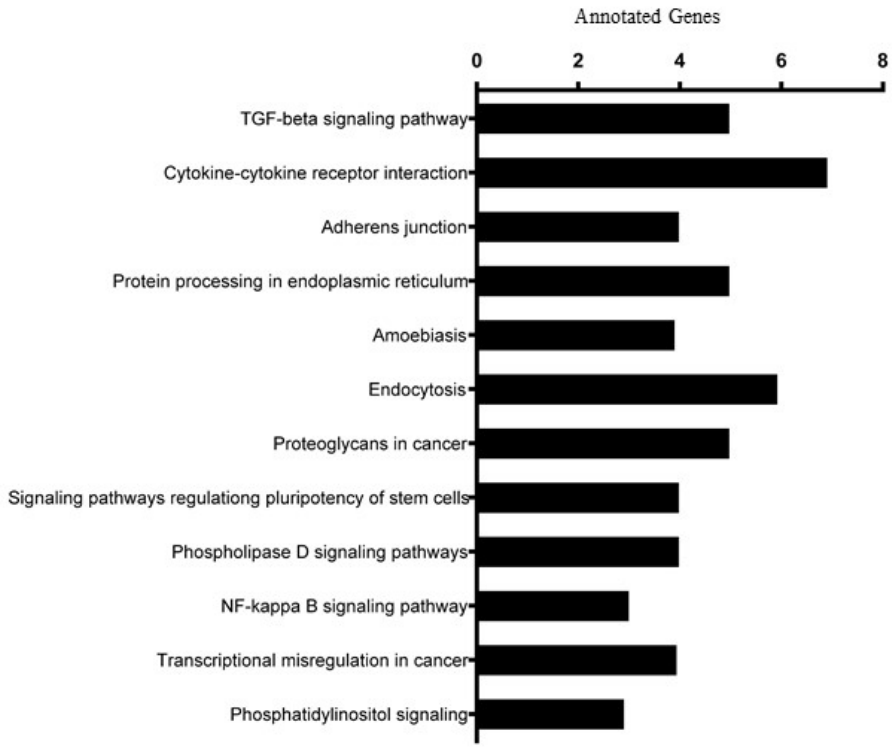
Annotated Genes
0
2
4
6
8
TGF-beta signaling pathway
Cytokine-cytokine receptor interaction
Adherens junction
Protein processing in endoplasmic reticulum
Amoebiasis
Endocytosis
Proteoglycans in cancer
Signaling pathways regulationg pluripotency of stem cells
Phospholipase D signaling pathways
NF-kappa B signaling pathway
Transcriptional misregulation in cancer
Phosphatidylinositol signaling

Figure 5

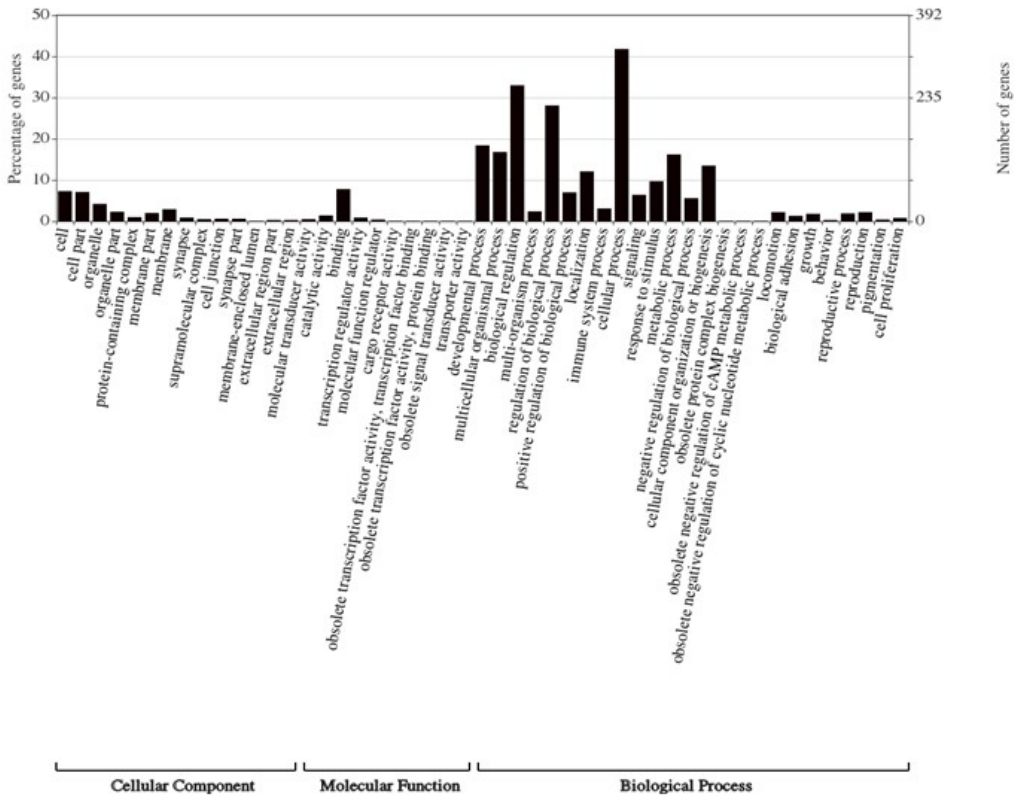
Percentage of genes
Number of genes
50
40
30
20
10
0
392
235
0
cell
cell part
organelle
protein-containing complex
membrane part
membrane
synapse
supramolecular complex
cell junction
synapse part
membrane-enclosed lumen
extracellular region part
molecular transducer activity
catalytic activity
binding
transcription regulator activity
molecular function regulator
cargo receptor activity
obsolete transcription factor activity, transcription factor binding
obsolete signal transducer activity
transporter activity
developmental process
multicellular organismal process
biological regulation
regulation of biological process
positive regulation of biological process
localization
immune system process
cellular process
signaling
response to stimulus
metabolic process
negative regulation of biological process
cellular component organization or biogenesis
obsolete protein complex biogenesis
obsolete negative regulation of cAMP metabolic process
obsolete negative regulation of cyclic nucleotide metabolic process
locomotion
biological adhesion
growth
behavior
reproductive process
reproduction
pigmentation
cell proliferation
Cellular Component
Molecular Function
Biological Process

Figure 6

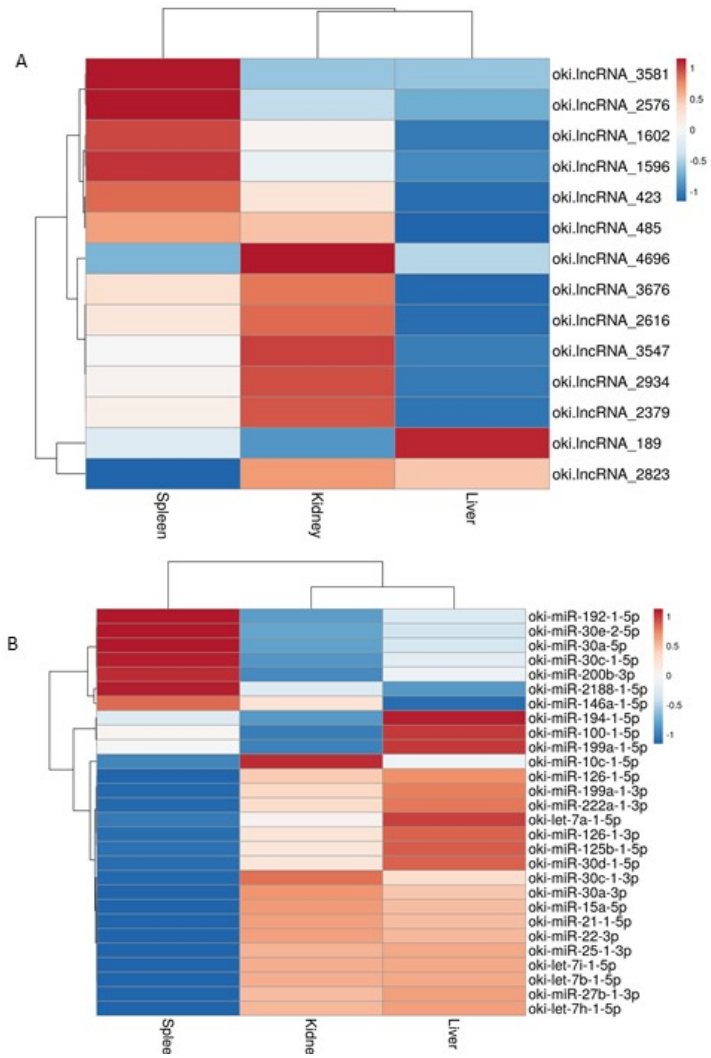
A
oki.lncRNA_3581
oki.lncRNA_2576
oki.lncRNA_1602
oki.lncRNA_1596
oki.lncRNA_423
oki.lncRNA_485
oki.lncRNA_4696
oki.lncRNA_3676
oki.lncRNA_2616
oki.lncRNA_3547
oki.lncRNA_2934
oki.lncRNA_2379
oki.lncRNA_189
oki.lncRNA_2823
Spleen
Kidney
Liver
1
0.5
0
-0.5
-1
B
oki-miR-192-1-5p
oki-miR-30e-2-5p
oki-miR-30a-5p
oki-miR-30c-1-5p
oki-miR-200b-3p
oki-miR-2188-1-5p
oki-miR-146a-1-5p
oki-miR-194-1-5p
oki-miR-100-1-5p
oki-miR-199a-1-5p
oki-miR-10c-1-5p
oki-miR-126-1-5p
oki-miR-199a-1-3p
oki-miR-222a-1-3p
oki-let-7a-1-5p
oki-miR-126-1-3p
oki-miR-125b-1-5p
oki-miR-30d-1-5p
oki-miR-30c-1-3p
oki-miR-30a-3p
oki-miR-15a-5p
oki-miR-21-1-5p
oki-miR-22-3p
oki-miR-25-1-3p
oki-let-7i-1-5p
oki-let-7b-1-5p
oki-miR-27b-1-3p
oki-let-7h-1-5p
Splee
Kidne
Liver
1
0.5
0
-0.5
-1

Figure 7