Comprehensive analysis of circRNA expression profiles and circRNA-associated competing endogenous RNA networks in the development of mouse thymus

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Abstract
The thymus plays an irreplaceable role as a primary lymphoid organ. However, the complicate processes of its development and involution are incompletely understood. Accumulating evidence indicates that non-coding RNAs play key roles in the regulation of biological development. At present, the studies of the circRNA profiles and of circRNA-associated competing endogenous RNAs (ceRNAs) in the thymus are still scarce. Here, deep-RNA sequencing was used to study the biological mechanisms underlying the development process (from 2-week-old to 6-week-old) and the recession process (from 6-week-old to 3-month-old) of the mouse thymus. It was found that 196 circRNAs, 233 miRNAs and 3807 mRNAs were significantly dysregulated. The circRNA-associated ceRNA networks were constructed in the mouse thymus, which were mainly involved in early embryonic development and the proliferation and division of T cells. Taken together, these results elucidated the regulatory roles of ceRNAs in the development and involution processes of the mouse thymus.

KEYWORDS
cRNA network, circRNA, thymus development

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1 | INTRODUCTION

The thymus is defined as a primary lymphoid organ with its inimitable role in T cell maturation, education and selection. It is the first formed lymphoid organ, but may also the most rapidly ageing tissue in the body. In response to the demand for large numbers of mature T cells, the thymus grows fast in the early stages of postnatal life and reaches a plateau at 4-6 weeks of age in the mouse. Afterwards, it starts the ageing process, of which the typical phenotype is thymic involution. The significantly decreased size and structural complexity of the thymus with age were manifested by the loss of thymocytes and thymic epithelial cells and the increase in the content of adipocytes. Actually, the processes about the rapid development and gradual involution of the thymus are still unclear.

MicroRNAs (miRNAs) are single-stranded (average size 22 nt) non-coding RNAs that regulate gene expression at the post-transcriptional level. Through their broad effects on gene expression, miRNAs participate in the regulation of many cellular processes such as organismal development, cellular differentiation and apoptosis, as well as mitochondrial metabolism. MiRNAs also take part in many aspects of T cell immunity such as T cell maturation, differentiation, activation and ageing. For example, some miRNAs including miR-155, miR-181c, miR-9 and miR-31 can regulate T cell activation by modulating the IL-2 signalling pathway. Moreover, miR-181a-5p and miR-195a-5p expressed in thymic epithelial cells are involved in thymus involution, which directly target transforming growth factor β receptor 1 (Tgfbr1) and Smad family member 7 (Smad7) respectively.

Circular RNAs (circRNAs) are a class of single-stranded closed RNA molecules without 3′-poly (A) and 5′-cap structures, which have been widely found in plants, animals and human beings. The cell-type-specific, tissue-specific or developmental stage-specific expression profiles of circRNAs suggest their regulatory functions in biological processes. Numerous studies have reported that circRNAs are closely related to the tumorigenesis, neurodegenerative diseases, cardiovascular diseases and immune diseases. Some circRNAs might be involved in post-transcriptional regulation by functioning as ‘sponges’ of miRNAs. Therefore, the circRNA-miRNA-mRNA networks may influence multiple biological pathways. At present, integrative analysis of circRNA-associated competing endogenous RNAs (ceRNA) networks in the development and involution of the thymus are still scarce.

To gain further insight into the molecular events associated with thymic development and involution, RNA-seq data were systematically analysed to identify aberrantly expressed circRNAs, miRNAs and mRNAs among mice thymuses at 2, 6 weeks and 3 months, respectively. In addition, the circRNA-miRNA-mRNA networks were constructed. This is the first comprehensive high-throughput sequencing analysis of circRNA, miRNA and mRNA expression profiles in the thymus, which deepen our understanding of thymic development and involution.

2 | MATERIALS AND METHODS

2.1 | Animal tissues preparation

Male C57BL/6 mice were purchased from Beijing HFK Bioscience Co. Ltd and maintained in a specific-pathogen-free environment. The mice were provided free access to standard diet until they met age requirements (2 weeks, 6 weeks and 3 months). Two biological replicates at each time point were used for sequencing. All the animal study protocols were approved by the Committee for the Ethics of Animal Experiments, Shenzhen Peking University-The Hong Kong University of Science and Technology Medical Center (SPHMC; protocol number 2011-004). The thymuses harvested from each group were placed in cryopreservation tubes and immediately immersed in liquid nitrogen (−196°C) for preservation.

2.2 | RNA extraction

Total RNA from each thymus sample was isolated with the TRizol Reagent kit (Invitrogen) and RNA degradation and contamination were monitored on 1% agarose gels. NanoPhotometer spectrophotometer (IMPLEN) was used to check the RNA purity. RNA concentration was measured with the Qubit RNA Assay Kit in Qubit 2.0 Flurometer (Life Technologies), and RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies). All the RNA samples had an RNA Quality Index ≥8.

2.3 | RNA-sequencing and miRNA-sequencing

Details of the RNA-seq and miRNA-seq methods are described in Table S15.

2.4 | Differential expression analysis

The differentially expressed circRNAs (DEcircRNAs), mRNAs (DEmRNAs) and miRNAs (DEmiRNAs) were identified using DESeq Software Packages (http://bioconductor.org/packages/release/bioc/html/DESeq.html). P value and log_{2} FC were used to screen differential transcripts with P < .05 and |log_{2} FC| > 1.

2.5 | Integrated analysis of circRNAs-miRNAs-mRNAs

CircRNAs were blasted against the circBase for annotation. Those cannot be annotated were defined as novel circRNAs. For circRNAs that have been annotated in circBase, the target relationship with miRNAs can be predicted by StarBase (v2.0). For novel circRNAs, three softwares Mireap, Miranda (v3.3a) and TargetScan (v7.0) were used to predict targets. For the prediction of mRNAs interacting
with circRNAs and miRNAs, miRTarBase (v6.1) was used to predict mRNAs targeted by miRNAs sponge. The triple network was finally built based on the ceRNA theory and the resulting correlation of circRNAs-miRNAs-mRNAs can be visualized by Cytoscape 3.01.

2.6 | KEGG enrichment analysis of differentially expressed genes

To further understand the underlying biological mechanisms and pathways of ceRNA-related genes, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was implemented using the clusterProfiler R package.

2.7 | Data access

All raw and processed sequencing data have been submitted to the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE139653.

3 | RESULTS

3.1 | Changes in thymus tissues

The age-related changes were observed in the thymuses derived from the 2-week-old, 6-week-old and 3-month-old male mice (Figure S1). Consistent with the literature, the mouse thymus grows rapidly after birth, reaches the maximal size by sexual maturity and then gradually involutes.\(^7\) The volume of the thymus increases significantly in the 6-week-old mouse compared with that in the 2-week-old mouse. As expected, the thymus shows remarkable atrophy in 3-month-old mouse compared with that in 6-week-old mouse.

3.2 | Identification of differentially expressed genes

A total of 94 differentially expressed circRNAs (DEcircRNAs, 45 up-regulated and 49 down-regulated), 154 differentially expressed miRNAs (DEmiRNAs, 38 up-regulated and 116 down-regulated)
and 1836 differentially expressed mRNAs (DEmRNAs, 1018 up-regulated and 818 down-regulated) were identified in the 6-week-old mice relative to the 2-week-old mice (Figure 1A,D,G). In the 3-month-old mice, 102 DEcircRNAs (54 up-regulated and 48 down-regulated), 79 DEmiRNAs (47 up-regulated and 32 down-regulated) and 1971 DEmRNAs (1005 up-regulated and 966 down-regulated) were observed relative to the 6-week-old mice (Figure 1B,E,H). Cluster analyses and heat-maps about the expression of these circRNAs, miRNAs and mRNAs were conducted respectively (Figure 1C,F,I). Detailed information is listed in Table S1-S6.

3.3 | Construction of the ceRNA network

According to the ceRNA hypothesis, competing endogenous RNAs (ceRNAs) members can regulate each other through competing for the same miRNA response elements (MREs). RNA transcripts communicate with the ceRNA language. The ceRNA networks in the mouse thymus were constructed based on our RNA-seq data. As the Venn diagram shown in the Figure 2, we divided the differentially expressed transcripts (circRNAs, miRNAs and mRNAs) into two groups: (a) 2W/6W (+) 6W/3M (−): differential expression in 2W vs 6W group but not in 6W vs 3M group. We consider these transcripts in the group as the factors that play a vital role in the rapid development of the thymus. (b) 2W/6W (−) 6W/3M (+): no differential expression in 2W vs 6W group, but differential expression in 6W vs 3M group. The differential transcripts in this group are thought to be factors that play an important role in the early decline of the thymus.

A total of 40 circRNAs, 136 miRNAs and 523 mRNAs were significantly dysregulated in the 2W/6W (+) 6W/3M (−) group, which were selected to construct ceRNA networks (Tables S7 and S8). The ceRNA networks included both positive and negative links (Figure 3A,B). Figure 3A shows the decreased circRNAs, increased miRNAs and decreased mRNAs and Figure 3B shows the increased circRNAs, decreased miRNAs and increased mRNAs. In the 2W/6W (−) 6W/3M (+) group, 45 DEcircRNAs, 65 DEmiRNAs and 407 DEmRNAs were used to construct the ceRNA networks (Table S9 and S10). As mentioned above, the ceRNA networks also include two types of links in this group (Figure 4A,B). Figure 4A shows the decreased circRNAs, increased miRNAs and decreased mRNAs and Figure 4B shows the increased circRNAs, decreased miRNAs and increased mRNAs. Information about the differentially expressed transcripts used to construct the networks can be found in Tables S7-S10. And the top15 DEcircRNAs between groups was shown in Tables 1 and 2.

3.4 | Functional enrichment analysis

Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis was used to determine the underlying biological mechanisms and pathways in the mouse thymus. Bubble charts were used to represent the top 20 pathways in 2W/6W (+) 6W/3M (−) group (Figure 5A,B) and 2W/6W (−) 6W/3M (+) group (Figure 5C,D), respectively. Each group included both positive and negative regulation. In the 2W/6W (+) 6W/3M (−) group, ceRNA gene-related KEGG pathways were involved in pyruvate metabolism, lysine degradation, PI3K-Akt signalling pathway, Ras signalling pathway, cytokine-cytokine receptor interaction, insulin signalling pathway and so on (Tables S11 and S12). The ceRNA genes in the 2W/6W (−) 6W/3M (+) group were associated with biological processes including fatty acid biosynthesis and metabolism, regulation of lipolysis in adipocyte, pyruvate metabolism, insulin secretion, T cell receptor signalling pathway, regulation of autophagy, etc (Table S13 and S14).
The thymus is a vital immune organ in humans and other mammals which is the primary site of T cell development. The thymus provides a specialized environment to facilitate T cell maturation and generate an extremely diverse T cell repertoire. It also establishes its self-tolerance to prevent autoimmune diseases. The thymus begins the involution process and apparently exhibits symptoms of age-related changes at an accelerated rate relative to other organs. Previous studies have found that some miRNAs play an important role in thymic development and involution. However, the role of circRNA-associated ceRNA networks in these processes is unknown. We designed this experiment to further investigate the molecular mechanisms underlying these complex processes of the post-natal rapid development and subsequent gradual decline in the thymus.

Consistent with the literature, the mouse thymus undergoes rapid growth after birth. After reaching its maximum size at 6 weeks, the mouse thymus begins to decline. To identify the key molecules involved in these processes and understand their possible mechanisms, RNA-seq was performed to compare the expression of circRNA, miRNA and mRNA in the thymus of 2-week-old, 6-week-old and 3-month-old mice.

By systematically analysing the sequencing data, we identified DEcircRNAs, DEmiRNAs and DEMRNAs in the 2W vs 6W group and the 6W vs 3M group (fold change ≥2.0 and \( P < .05 \)), and mainly constructed the ceRNA networks of the 2W/6W (+) and 6W/3M (−) group. These circRNA-associated ceRNA networks are potentially involved in the development and early decline of thymus. For instance, mmu_circ_0001023, novel_circ_003800 were identified as ceRNAs of mmu-miR-434-5p, which targets lds. The expression of lds was higher in 6-week-old mice than that in 2-week-old mice.
mice. *Id5* are necessary for skeletal myogenesis,\(^{31}\) cardiac development\(^{32}\) and also play key roles in the differentiation and proliferation of T cells.\(^{33,34}\) Moreover, mmu_circ_0000909 was found to be a ceRNA of mmu-miR-92a-1-5p, which targets *KMT2D*. *KMT2D* is a major enhancer of H3K4me1/2 methyltransferase which is essential for early embryonic development in mice.\(^{35}\) There is evidence that *KMT2D* is required for cell-type-specific gene expression and cell differentiation in model systems for adipogenesis and myogenesis.\(^{36}\) Its mutation is closely related to the occurrence of T and B cell-associated lymphoma.\(^{37,38}\) In the 2W/6W (−) 6W/3M (+) group, mmu_circ_0000459, novel_circ_000862, novel_circ_001902 and novel_circ_002116 were identified as ceRNAs of mmu-miR-125a-5p, which targets *Erap1*. *Erap1* is an M1 zinc metalloprotease family member which has a significant impact on peptide processing function and the repertoire of peptides presented. In addition, the deregulation of *ERAP1* expression in mice results in increased production of proinflammatory cytokines.\(^{39}\) The occurrence of inflammation is closely related to thymic ageing.\(^{4,41}\) The down-regulation of *Erap1* in 3-month-old mice may be associated with the early thymic decline. Besides, mmu_circ_0001431, novel_circ_004960, novel_circ_005000 and novel_circ_005893 were identified as ceRNAs of mmu-miR-135a-5p, which targets *Celf1*. *Celf1*, a member of the *Celf* family, belongs to RNA-binding proteins. Previous research has indicated that *Celf1* may affect the embryonic development process\(^{42,43}\) and involve in the proliferation and division of T cells.\(^{44}\) Other links in these ceRNA networks are listed in Tables S7-S10.

In order to clarify ceRNA functions in mouse thymus, KEGG pathway enrichment analysis was used to determine the underlying biological pathways. The metabolism pathways such as pyruvate metabolism, fatty acid metabolism were significantly enriched pathways in both groups. Metabolism interconnects with...
signalling events to influence cell cycle, differentiation, cell death and immunological function.\textsuperscript{45} Yang et al\textsuperscript{46} reported that mTORC1 coordinated multiple metabolic programmes in T cells including glycolysis, lipid synthesis and oxidative phosphorylation to affect lymphocyte activation and fate decisions in adaptive immunity. In addition, Ras signalling pathway was found to be enriched in the 2W/6W (+) 6W/3M (−) group. Some evidence indicates that the activation of the Ras signalling pathway is critical for T cell development in the thymus.\textsuperscript{47-49} In the 2W/6W (−) 6W/3M (+) group, the expression of DEmRNAs related to the Insulin secretion pathway was significantly down-regulated. Insulin is a key hormone which controls the metabolism of carbohydrates, proteins and lipids. Previous studies indicated that insulin receptor signalling controlled T cell proliferation, cytokine production and regulated adaptive immunity.\textsuperscript{50,51} It suggests that insulin signalling pathway may modulate the thymus ageing through controlling the development of the thymocytes.

In summary, we demonstrated the circRNA-associated ceRNA profiles of the mouse thymus at different time points by using deep-RNA-seq analysis for the first time. These results shed light on the possible contribution of the ceRNAs to the development and involution of the thymus.

### TABLE 1
Top 15 DEcircRNAs in the thymus between 2-wk-old and 6-wk-old mouse

| CircRNA ID       | T-2W_RPM | T-6W_RPM | log2(FC) | P value |
|------------------|----------|----------|----------|---------|
| novel_circ_002600 | 6282.377 | 2524.365 | -1.31539 | 5.80E-08 |
| mmu_circ_0000130 | 1739.733 | 411.06649| -2.08142 | 9.51E-05 |
| novel_circ_002602 | 3203.865 | 1161.256 | -1.46413 | 5.00E-08 |
| novel_circ_002600 | 2524.365 | 5384.594 | -1.09292 | 5.23E-07 |
| novel_circ_002601 | 1161.256 | 4700.284 | 2.017062 | 3.25E-14 |
| mmu_circ_0001100 | 22.81646 | 944.118  | 5.37082  | 4.86E-07 |
| novel_circ_002600 | 2524.365 | 5384.594 | 1.092917 | 5.23E-07 |
| novel_circ_002600 | 22.81646 | 944.118  | 5.37082  | 4.86E-07 |
| novel_circ_002600 | 22.81646 | 944.118  | 5.37082  | 4.86E-07 |

Abbreviation: FC, fold change; RPM, reads per million mapped reads.

### TABLE 2
Top 15 DEcircRNAs in the thymus between 6-wk-old and 3-mo-old mouse

| CircRNA ID       | T-6W_RPM | T-3M_RPM | log2(FC) | P value |
|------------------|----------|----------|----------|---------|
| novel_circ_002601 | 1161.256 | 4700.284 | 2.017062 | 3.25E-14 |
| novel_circ_002602 | 1047.173 | 4015.974 | 1.939249 | 2.34E-09 |
| mmu_circ_0001100 | 22.81646 | 944.118  | 5.37082  | 4.86E-07 |
| novel_circ_002600 | 2524.365 | 5384.594 | 1.092917 | 5.23E-07 |
| novel_circ_002600 | 22.81646 | 944.118  | 5.37082  | 4.86E-07 |
| novel_circ_002600 | 22.81646 | 944.118  | 5.37082  | 4.86E-07 |
| novel_circ_002600 | 22.81646 | 944.118  | 5.37082  | 4.86E-07 |

Abbreviations: FC, fold change; RPM, reads per million mapped reads.
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FIGURE 5  KEGG pathways analysis in the thymus. KEGG pathway enrichment analysis of up and down-regulated mRNAs with top twenty Enrichment score. (A) 2W/6W (+) 6W/3M (−) group: up-regulated mRNAs. (B) 2W/6W (+) 6W/3M (−) group: down-regulated mRNAs. (C) 2W/6W (-) 6W/3M (+) group: up-regulated mRNAs. (D) 2W/6W (-) 6W/3M (+) group: down-regulated mRNAs. The Y-axis label represents the pathway and the X-axis label represents the rich factor. The colour and size of the bubble represent enrichment significance and the amount of differentially expressed genes enriched in the pathway, respectively.
CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

AUTHORS CONTRIBUTIONS
WZ, JW and YZ designed the study. WL, NM, BY, YZ, YY, QL and T.YW. participated in the animal experiments including tissue collection and RNA/protein extraction. WL, NM, XFC., JW and WZ analysed the data and wrote the paper. The final manuscript has been seen and approved by all authors.

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**SUPPORTING INFORMATION**
Additional supporting information may be found online in the Supporting Information section.

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