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Transcriptomic analysis reveals key regulators of mammogenesis and the pregnancy-lactation cycle

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An organ unique to mammals, the mammary gland develops 90% of its mass after birth and experiences the pregnancy-lactation-involution cycle (PL cycle) during reproduction. To understand mammogenesis at the transcriptomic level and using a ribo-minus RNA-seq protocol, we acquired greater than 50 million reads each for the mouse mammary gland during pregnancy (day 12 of pregnancy), lactation (day 14 of lactation), and involution (day 7 of involution). The pregnancy-, lactation- and involution-related sequencing reads were assembled into 17344, 10160, and 13739 protein-coding transcripts and 1803, 828, and 1288 non-coding RNAs (ncRNAs), respectively. Differentially expressed genes (DEGs) were defined in the three samples, which comprised 4843 DEGs (749 up-regulated and 4094 down-regulated) from pregnancy to lactation and 4926 DEGs (4706 up-regulated and 220 down-regulated) from lactation to involution. Besides the obvious and substantive up- and down-regulation of the DEGs, we observe that lysosomal enzymes were highly expressed and that their expression coincided with milk secretion. Further analysis of transcription factors such as *Trps1*, *Gtf2i*, *Tcf7l2*, *Nupr1*, *Vdr*, *Rb1*, and *Aebp1*, and ncRNAs such as *mir-125b*, *Let7*, *mir-146a*, and *mir-15* has enabled us to identify key regulators in mammary gland development and the PL cycle.

mouse mammary gland, mammogenesis, transcriptome, rmRNA-seq, miRNAs, transcription factors

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Mammals first appeared ~200 million years ago during the Jurassic Period [1]. Linnaeus, recognizing their uniqueness in that female mammals nurse their young with milk produced by the mammary glands, classified the mammary-gland-bearing animals as belonging to the class Mammalia [2]. As one of the mammal-specific organs, the mammary gland has been widely studied at the molecular level [3], with many studies being micro-array-based [4–6]. For example, Clarkson et al. [5] performed microarray studies and identified the difference in gene expression over 12 time points during mouse mammogenesis. Rudolph et al. [7] profiled expression of 1358 mouse genes that experience significant changes in pregnancy, lactation, and mammary gland involution. Despite a growing literature of studies on the mammary gland [8–12], in-depth sampled transcriptome analyses of its different developmental stages are essential for dissecting the molecular details of its biology. RNA-seq, a sequencing-based method that accurately quantifies gene expression levels across a wide dynamic range has revolutionized transcriptomics [13,14]. Compared with microar-

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ray-based methods, RNA-seq not only offers much higher sequence coverage, but also eliminates the need for preexisting sequence information [15,16].

In the present study, we used RNA-Seq technology to examine the transcriptome of the mouse mammary gland in the pregnancy-lactation-involution (PL) cycle. To the best of our knowledge, this is the first RNA-seq-based study on the PL cycle. The provision of high-quality transcriptome data has allowed us to apply the concept of housekeeping (HK) and tissue-specific (TS or mammogenesis-specific, MS) genes [15], to define the differentially expressed genes (DEGs) during the three stages of mammogenesis. In addition, we investigated protein-coding and non-coding RNAs (ncRNAs), and focused our analyses on transcription factors [17] and lysosomal enzymes that may play key roles in the lactating mammary gland as well as in involution [18–20].

1 Materials and methods

1.1 Mouse husbandry

Six-week-old clean-class Balb/c mice purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) were continuously bred in a specific pathogen free level laboratory (constant temperature, relative humidity, and illumination; one mouse per squirrel-cage) at the Institute of Zoology, Chinese Academy of Sciences. Eight-week-old virgin females were mated with males of the same age. Females were checked twice daily for the presence of a vaginal plug. The disruption of the vaginal plug and the day of birth define day 0 of pregnancy and lactation. Females used for the involution time points were forced to wean at 20 days post lactation.

1.2 RNA sample harvesting

Three time points during the adult mouse mammary gland development were chosen for analysis according to histology: day 12 of pregnancy, day 14 of lactation, and day 7 after forced weaning (after normal feeding for 20 d). Three animals were used for each time point and fast executed by cervical dislocation. Mammary glands were surgically removed and immediately frozen in liquid nitrogen. Total RNA was extracted with Trizol reagent (Invitrogen, Paisley, UK) according to the manufacturer's protocol and the integrity and purity of the RNA was examined with spectrophotometer (Nonodrop, Thermo Scientific, Wilmington, DE, USA) and non-denaturing agarose gel electrophoresis.

1.3 Sequencing and read mapping

We used the Ribo-minus Eukaryote Kit from Invitrogen (cat.10837-08, Invitrogen, Grand Island, NY, USA) to deplete ribosomal RNAs (rRNAs) from the samples according to the manufacturer's instructions. The libraries were constructed using 1 μg of the rRNA-depleted RNAs. Sequence reads were acquired from the Life Technologies' SOLiD3 platform (Life Technologies, Grand Island, NY, USA). For sequence read mapping, we downloaded the mouse reference sequence (release-NCBIM37.56) from Ensembl (http://asia.ensembl.org/index.html), which contains 31510 genes. We also compiled an exon-exon junction library for gene mapping with 49-nt flanking sequences into both the donor and accepter sequences. Low-quality reads (where the average quality value was below 8) were filtered and the remaining reads were used for additional mapping steps. We first mapped the 50-bp full-length reads to the reference sequence with up to five mismatches, and the un-mapped reads were then mapped to our junction library. Thereafter, we repeated the above two steps for the first 45, 40, 35, 30, and 25-bp truncated reads with up to 4, 4, 3, 3, and 2-bp mismatches, respectively. Ribosomal RNA-related reads were filtered out at the beginning of the read mapping. The original data set was deposited in NCBI SRA database (accession No. SRP027516).

1.4 Gene expression analysis

We measured gene expressions by read counts that are normalized with the total number of mapped reads and gene length based on the RPKM method [21]. DEGseq1.2.2 was used for approximate identification of DEGs, some of the DEGs were evaluated via their *P*-values and fold changes. GenMAPP was used to show differential gene expression in different pathways [22]. We also used DAVID [23] for annotation, visualization, and integrated discovery and functional classification; *P*-values for GO category enrichment were calculated by using the Benjamin algorithm.

1.5 Definition of HK and TS genes

Although the exact definition of HK genes and TS genes is still in disagreement, their analyses are usually very informative [15]. The purpose of defining them is to stratify the data for in-depth analysis because HK and TS genes are very different in their overall expression levels, tissue-distributions, and mutation rates [15,24]. In this study, we defined HK and TS genes according to the analysis of the RNA-seq data and based on the expression breadth from nine mouse tissues and/or cell types. Gene expression was calculated as 'reads per kilobase of exon model per million mapped reads' (RPKM). A published method was used to determine the expression value cutoff, which removes the background expression level noise [25]. Therefore, the definition herein is based on the presence of a transcript and not expression level variability among the tissues and cells.

2 Results

2.1 An overview of gene expression regulation of the mouse mammary gland in the PL cycle

We used the ribo-minus RNA-sequencing (rmRNA-seq) method to obtain transcriptional profiles because it allows expression information to be extracted from mRNAs and other non-ribosomal RNAs. For each rmRNA library, we acquired >50 million reads; this number of mapped reads usually gives rise to a satisfactory profile of the transcriptome. After filtering the low quality reads, we mapped the high-quality reads to the mouse reference genome (containing 23241 protein coding genes) and 8269 known non-protein coding RNA sequences, including miRNA, misc_RNA, mtrRNA, retrotransposons, rRNA, snoRNA, snRNA, and pseudogenes. We obtained 17439616 (37.23% out of a total of 46833536 mapped reads), 7299414 (25.28% out of a total of 28864990 mapped reads), and 8542102 (31.83% out of a total of 26837367 mapped reads) uniquely-mapped reads, along with 29393920, 21565576, and 18295265 reads with redundantly mapped loci, from the three libraries representing the PL cycle, including pregnancy, lactation, and involution, respectively. Only the uniquely-mapped reads were used for further analyses. According to various tissue-based transcriptomic studies [25], greater than six million reads are sufficient for detecting most of the expressed genes in the human mammary gland. Hence, we are confident that our sequencing depth is adequate and the RNA-seq data provide objective profiling of the transcriptomes of the mouse mammary gland at the different developmental stages that we studied. In addition, qRT-PCR experiment on selected candidates from our data at variable expression levels confirmed our RNA-seq results (Figure S1 in Supporting Information).

Using a threshold of >2 exon reads per gene, we identified 17344, 10160, and 13739 protein-coding genes that were expressed in the pregnancy, lactation, and involution libraries, respectively, together with 1803 (pregnancy), 828 (lactation), and 1288 (involution) ncRNAs. We further defined DEGs based on their pair-wise comparison. The numbers of DEGs for the two comparisons include 4094 down-regulated and 749 up-regulated genes from pregnancy to lactation and 220 down-regulated and 4706 up-regulated genes from lactation to involution (Excel file in Supporting Information). To obtain an overview of the function of the defined DEGs, we listed the top 10 DEGs from our comparisons that were significantly different between the three mammogenesis stages (Table 1). The genes expressed at each stage are distributed among most of the chromosomes with the exception of the Y chromosome (Figure 1A) and the highly expressed genes are clustered among the different chromosomes. Although the number of sequencing reads acquired for each library has reached a rather adequate overall coverage (the total length of acquired sequences divided by the estimated length), the differences do exist as we show in Figure 1B.

Upon further inspection, such as in the exonic regions of genes, we observed a few genes on individual chromosomes that had extremely high read counts during lactation. For example, the top five genes on chromosome 5 have a total read count exceeding 10000, comprising the casein gene family, which exhibits 92.38% of the total read counts in the region. High read counts also occur on chromosomes 9 and 11 (Figure 2A). For instance, the total read counts for aminoacyl-tRNA synthetase (*Lars2*) and transferrin (*Trf*) genes collectively comprise 92.53% of the grand total of chromosome 9, while the reads for whey acidic protein (*Wap*) and fatty acid synthetase (*Fasn*) genes reach 87.07% of the total reads for chromosome 11. Among these genes, casein, *Wap*, and *Trf* are major milk proteins in mice [26,27]. *Lars2*, one of the mitochondrial synthetases, also has an extremely high read count, which indicates that leucine-rich proteins are being accumulated in the milk or that an unknown mechanism requires higher activity of this enzyme. In addition, it has been shown that the turnover rate in lactating animals is several folds higher than that of virgin and pregnant animals [28,29]. Without exception, aside from the genes with unusually high-read count, the overall read counts of all other genes for all chromosomes were reduced during lactation (Figure 2B). Finally, the read count for the sequences that mapped to the intronic and intergenic regions is similar to that of the exonic regions, as can be seen after removal of the limited number of highly expressed genes.

2.2 Expression of HK and MS genes

To investigate DEGs involved in the three developmental stages, we first looked into HK genes and then the rest after removal of the HK genes (Figure 3). We identified 4094 down-regulated and 749 up-regulated DEGs from pregnancy to lactation, together with 220 down-regulated and 4706 up-regulated DEGs from lactation to involution. Among them, after the removal of HK genes, there were 2028 HK-minus DEGs down-regulated and 276 HK-minus up-regulated DEGs from pregnancy to lactation as well as 128 HK-minus down-regulated and 1878 HK-minus up-regulated DEGs from lactation to involution. Thus, lactation is the stage where 47.73% of the down-regulated genes were up-regulated. There were 1060 genes with unrestored expression levels. However, only 110 genes had a peak-shaped profile, which indicates that gene expression is suppressed during pregnancy and involution; hence, these could be lactation-specific genes. To glean possible functions of many of the significantly differentially up- and down-regulated HK-minus genes during mammary gland development, we have highlighted 10 of the mammogenesis-specific genes in Table 1.

Table 1 Top 10 DEGs between two libraries

From pregnancy to lactation(up-regulation DEGs)

Gene	Description	Pr	La	$\log_2$(FC)	*P*-value	Ensemble gene ID
Wfdc3	WAP four-disulfide core domain 3	0.70	614.78	–9.76	0	ENSMUSG00000076434
Csn1s2b	Alpha-S2-casein-like B precursor	5.40	4245.66	–9.62	0	ENSMUSG00000061388
Wap	Whey acidic protein precursor	134.62	71884.99	–9.06	0	ENSMUSG00000000381
Lao1	L-amino acid oxidase 1	2.51	977.83	–8.60	0	ENSMUSG00000024903
Lalba	Alpha-lactalbumin precursor	6.69	2130.60	–8.32	0	ENSMUSG00000022991
Cidea	Cell death activator CIDE-A	1.35	332.21	–7.95	0	ENSMUSG00000024526
Qsox1	Sulfhydryl oxidase 1 precursor	2.56	527.88	–7.69	0	ENSMUSG00000033684
Slc34a2	Sodium-phosphate transport protein 2B	0.85	128.09	–7.24	0	ENSMUSG00000029188
Cel	Bile salt-activated lipase precursor	1.258	169.70	–7.08	0	ENSMUSG00000026818
Olah	S-acyl fatty acid synthase thioesteras	1.06	92.23	–6.44	1.69×10^{-180}	ENSMUSG00000026645

From pregnancy to lactation (down-regulation DEGs)

Gene	Description	Pr	La	$\log_2$(FC)	*P*-value	Ensemble gene ID
Unknown	Putative uncharacterized protein	71.45	0.095	9.56	5.31×10^{-205}	ENSMUSG00000067879
Prune2	Protein prune homolog 2	332.18	2.181	7.25	0	ENSMUSG00000054366
Kit	Proto-oncogene tyrosine-protein kinase Kit	6.40	0.056	6.85	1.14×10^{-42}	ENSMUSG00000005672
Pde8b	Phosphodiesterase 8B	4.85	0.063	6.28	5.85×10^{-30}	ENSMUSG00000021684
Unknown	N/A	173.34	2.25	6.27	5.48×10^{-114}	ENSMUSG00000075015
Ush1c	Harmonin	12.57	0.18	6.10	8.06×10^{-52}	ENSMUSG00000030838
Gli3	Zinc finger protein	2.33	0.04	6.10	9.16×10^{-27}	ENSMUSG00000021318
Arhgap29	Rho GTPase-activating protein 29	3.08	0.06	5.78	9.18×10^{-22}	ENSMUSG00000039831
Unknown	Putative uncharacterized protein	8.93	0.19	5.58	4.13×10^{-19}	ENSMUSG00000046764
Kank4	Motif and ankyrin repeat domain	2.81	0.06	5.56	6.24×10^{-19}	ENSMUSG00000035407

From lactation to involution (up-regulation DEGs)

Gene	Description	La	In	$\log_2$(FC)	*P*-value	Ensemble gene ID
Hp	Haptoglobin precursor	0.93	341.363	–8.52	0	ENSMUSG00000031722
Fcgbp	Putative uncharacterized protein	0.24	83.29	–8.43	2.31×10^{-246}	ENSMUSG00000078777
Fcgbp	Fc fragment of IgG binding protein	0.61	190.130	–8.29	0	ENSMUSG00000047730
Pdk4	Pyruvate dehydrogenase	0.08	12.46	–7.24	1.47×10^{-41}	ENSMUSG00000019577
Inhbb	Inhibin beta B chain precursor	0.07	9.03	–7.07	1.68×10^{-37}	ENSMUSG00000037035
Saa1	Serum amyloid A-1 protein precursor	0.47	38.34	–6.35	2.75×10^{-24}	ENSMUSG00000074115
Ckm	Creatine kinase M-type	0.61	49.20	–6.340	7.59×10^{-69}	ENSMUSG00000030399
Aoc3	Membrane primary amine oxidase	0.31	24.90	–6.33	1.66×10^{-112}	ENSMUSG00000019326
Igfbp5	Insulin-like growth factor-binding protein 5 precursor	1.81	141.70	–6.29	0	ENSMUSG00000026185
Pck1	Phosphoenolpyruvate carboxykinase	0.76	56.57	–6.21	1.73×10^{-8}	ENSMUSG00000027513

From lactation to involution (down-regulation DEGs)

Gene	Description	La	In	$\log_2$(FC)	*P*-value	Ensemble gene ID
Csn1s2b	Alpha-S2-casein-like B precursor	4245.66	2.356	10.82	0	ENSMUSG00000061388
Wap	Whey acidic protein precursor	71884.99	162.93	8.78	0	ENSMUSG00000000381
Lao1	L-amino acid oxidase 1	977.83	4.08	7.91	0	ENSMUSG00000024903
Atp2b2	Plasma membrane calcium-transporting ATPase 2	18.43	0.11	7.36	1.05×10^{-127}	ENSMUSG00000030302
Olah	S-acyl fatty acid synthase thioesterase	92.23	0.69	7.07	2.07×10^{-133}	ENSMUSG00000026645
Csn1s2a	Alpha-S2-casein-like A precursor	18507.04	140.59	7.04	0	ENSMUSG00000061937
Slc34a2	Sodium-dependent phosphate transport protein 2B	128.09	1.06	6.91	0	ENSMUSG00000029188
Csn1s1	Alpha-S1-casein precursor	16689.11	266.44	5.97	0	ENSMUSG00000070702
Csn2	Beta-casein precursor	17160.06	274.38	5.97	0	ENSMUSG00000063157
Lpo	Lactoperoxidase	3.71	0.07	5.69	3.37×10^{-12}	ENSMUSG00000009356

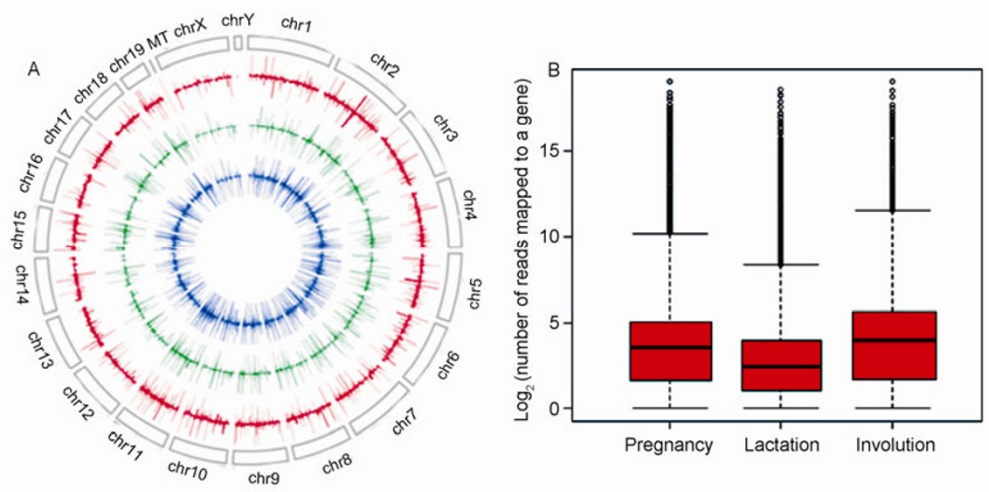

Figure 1 Read counts distribution of mouse chromosomes and read counts for each gene. A, Circos plot of the transcriptomic expression profiles. The expression profiles of the transcripts with RPKM values (read count is normalized against the total number of mapped reads and the gene length) up to 20 (we set the cutoff value to 20 to see the overall transcriptional profiles of most genes) in all three mouse samples. The expression profile of each stage is represented as a single circle. RPKM abundance for individual genes is depicted as peaks. The outside and inside peaks of a single circle represent the positive and negative strands of the expressed DNA, respectively. The order of the transcriptional expression profiles from the outer to inner circles is as follows: pregnancy (red), lactation (green), and involution (blue). Lactation-associated gene expression in most of the genomic loci appears to be weaker and shorter in length than that of the other two stages. B, Box plot of the read counts for each gene during the three stages of mammary gland development.

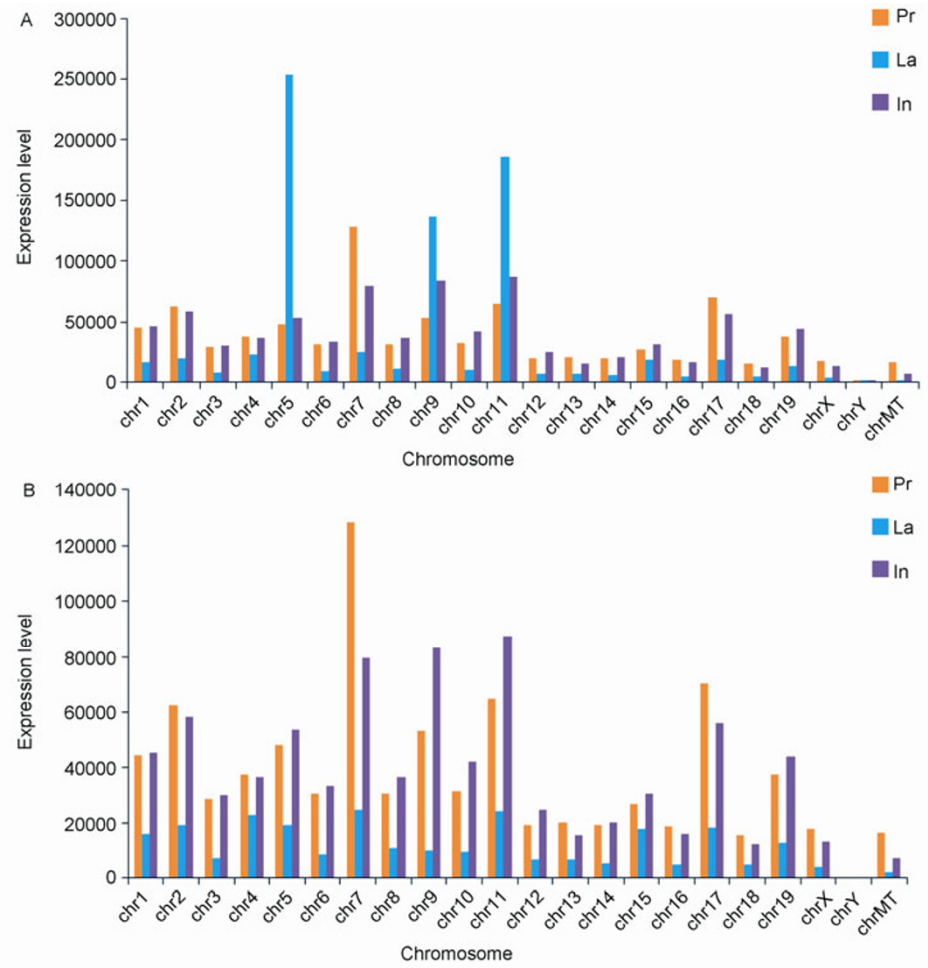

Figure 2 mRNA expression intensity on each chromosome. A, Expression intensities of mRNAs in exons on each chromosome. Orange, blue, and purple bars denote pregnancy (Pr), lactation (La), and involution (In), respectively. B, The expression intensities of mRNAs in exons on each chromosome after the removal of sequencing reads from nine (chr5: casein family 5 member; chr9: Lars2 and Trf; chr11: Wap and Fasn) highly expressed lactation-associated genes.

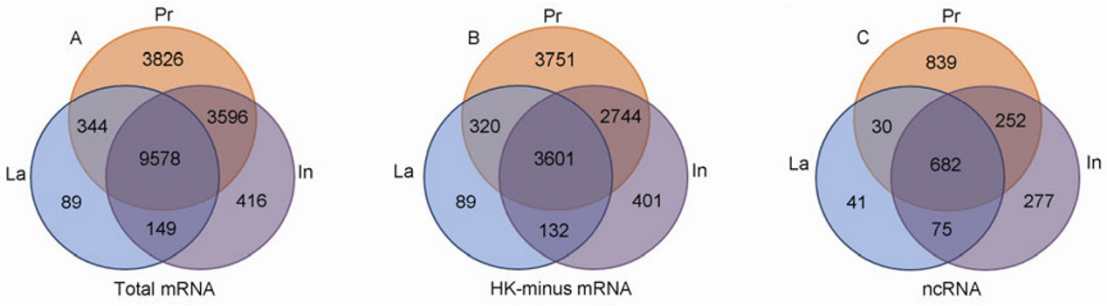

Figure 3 Venn diagrams of annotated genes in each stage of mammary gland development. A, B, and C are annotated total mRNAs, HK-minus mRNAs, and ncRNAs, respectively.

2.3 DEGs associated with cellular signaling pathways

To investigate the interrelationship between HK- and MS-specific DEGs and to interpret their transcriptional profiles, we performed metabolic and signaling pathway analyses and focused on three pathways. First, from pregnancy to lactation, we found that most lysosomal acid hydrolases and lysosomal membrane proteins are activated during lactation; these include proteases, glycosidases, sulfatases, lipases, phosphatases, sphingomyelinases, and minor lysosomal membrane proteins (Figure 4). For example, *Abcb9* expression increases to nearly four times the level observed before lactation. We identified 18 up-regulated and five down-regulated DEGs that are related to lysosomes. Second, 43 cell cycle related genes, which include nine MS genes, are all down-regulated DEGs while only two DEGs were up-regulated from pregnancy to lactation; all of the down-regulated DEGs had statistically significant, >2-fold changes (Figure 5). Cyclin D1 is unusual in that it is down-regulated 19-fold. During S phase of the cell cycle, four of the DNA polymerase subunits (α1, α2, pri1, and pri2) and six of the MCM complex which possess DNA helicase activity, exhibited down-regulated expression to varying degrees. Third, comparisons of DEGs between lactation and involution for the Notch signaling pathway identified 27 up-regulated DEGs including ligands, receptors, and modifier proteins of the pathway. Among them, seven MS genes were up-regulated and both activation and suppression of the Notch signal pathway genes were involved. Among the up-regulated genes, two suppressors, *Jag1* and *Jag2*, showed increased expressions of five and 12 times, respectively. The expression levels of two other negative regulators, *Numb* and *Dvl*, which reside in the cytosol and prevent the notch intracellular domain translocating to the nucleolus, were also increased, while the ligand Delta-like1 gene, which activates the Notch signal pathway, has an expression level during involution, which is nine times that of its expression level during lactation. In addition, among those components of the enzyme complex that catalyzes schizolysis of the notch receptor, expression of *Ncstn* had increased. Likewise, the ubiquitination transmembrane protein gene Deltex (*Dtx*), which activates Notch, was elevated 3–10 times that of its normal level, while the expression levels of *Maml1*, *Maml2*, *Maml3*, and the DNA-binding protein genes *Csl* and *Crebbp* (that have intrinsic histone acetyltransferase activity) were all up-regulated (Figure 6).

2.4 Expression of transcription factors relevant to the PL cycle

We extracted the expression profiles of 1675 mouse transcription factors from the Riken genome-wide and non-redundant mouse-transcription-factor database [17]. This process identified 375 down-regulated and 36 up-regulated DEGs between pregnancy and lactation, whereas 363 DEGs were up-regulated, but only nine were down-regulated between lactation and involution. Firstly, during pregnancy, *Trps1*, *Foxp1*, *Gtf2i*, and *Tcf7l2*, which have not been identified in previous studies, were all highly expressed. Secondly, during lactation, higher expression levels of *Atf4*, *Nupr1*, *Rab15*, and *Rab18* were observed compared with those in pregnancy and involution, thus indicating their potential contributions to lactation. Thirdly, during involution, the secretary epithelium undergoes a striking programmed cell death, and many potential apoptosis-related transcription factors are DEGs. For example, the expression levels of *sox4* and *Rb1* increase approximately 40-fold and 15-fold, respectively, during involution as compared with that during lactation. Another example is *Aebp1* that promotes macrophage inflammatory responsiveness when encountering large-scale apoptosis. Additionally, *Runx1* may perform important immunity-related function(s) in the mammary gland (Figure 7).

2.5 ncRNA expression

Our analysis of ncRNA expression identified some potential regulatory ncRNAs, such as miRNA, snRNA, snoRNA, rRNA, MT-RNA, and miscRNA (including unprocessed and processed pseudogenes). Focusing on miRNAs, we detected 231, 160, and 226 miRNAs during pregnancy, lactation, and involution, respectively. Our results agree with a previous report, where nine miRNAs were verified [30]. Among the miRNAs that were identified, 21 were up-

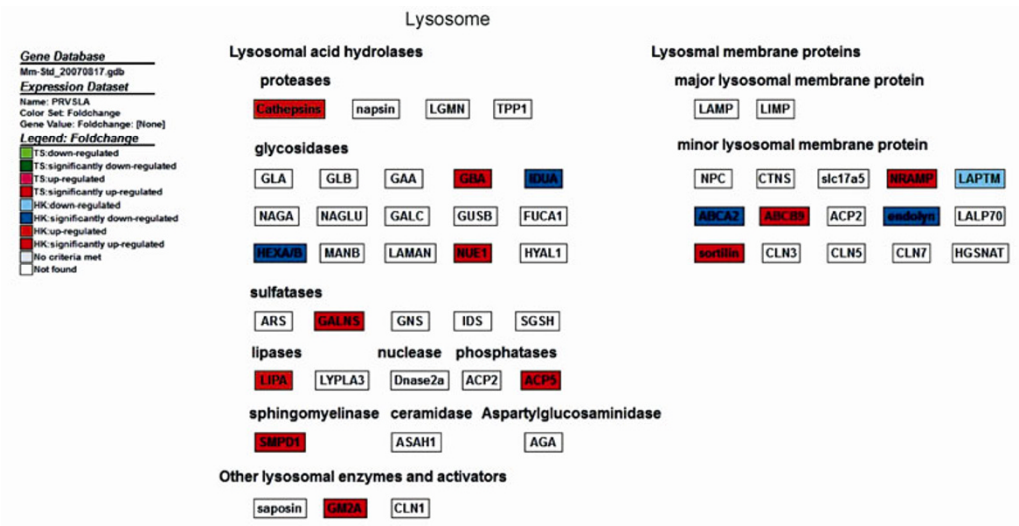

Figure 4 DEGs associated with lysosomes. DEGs are colored according to their fold change. Blue and orange denote HK DEGs. Colored shading denotes various fold changes: dark color, fold change>2; light color, fold change≤2. Green and red represent HK-minus DEGs.

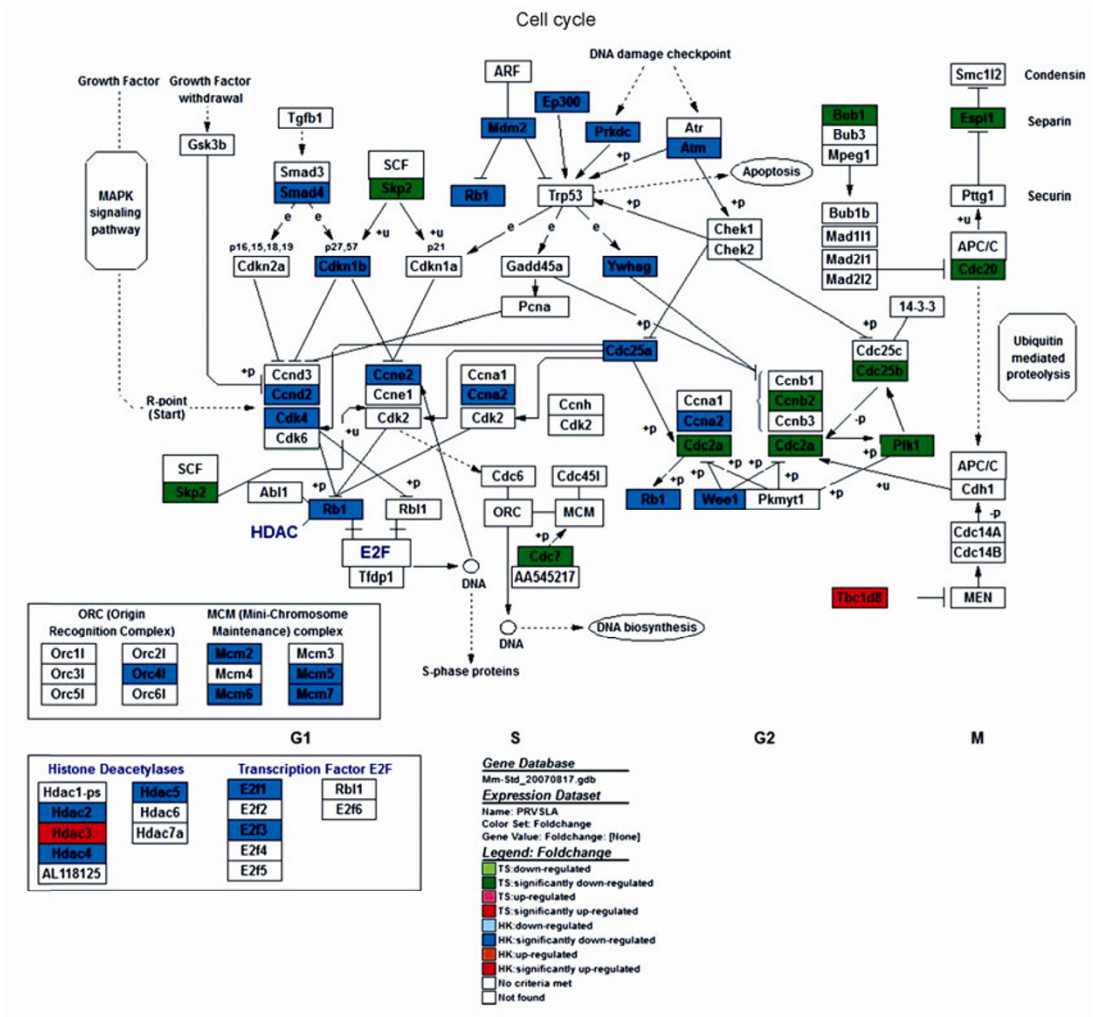

Figure 5 DEGs involved in the cell cycle. DEGs are colored according to their fold change. Blue and orange denote HK DEGs. Colored shading denotes various fold changes: dark color, fold change>2; light color, fold change≤2; green and red represent HK-minus DEGs.

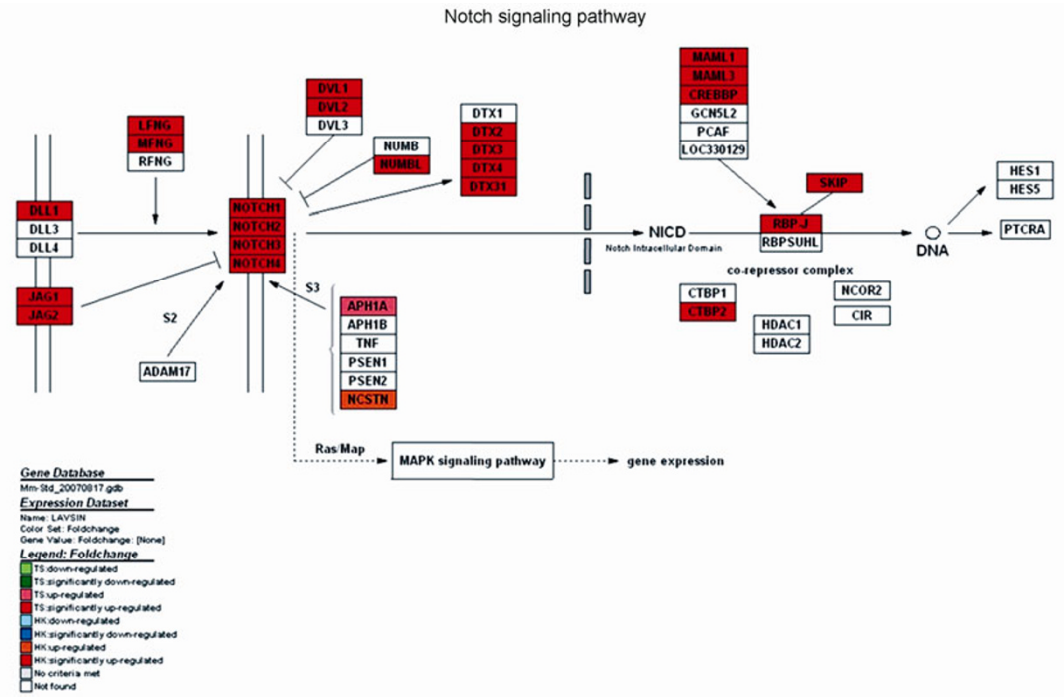

Figure 6 DEGs of the Notch signaling pathway. DEGs are colored according to their fold change. Blue and orange denote HK DEGs. Colored shading denotes various fold changes: dark color, fold change>2; light color, fold change≤2; green and red represent HK-minus DEGs.

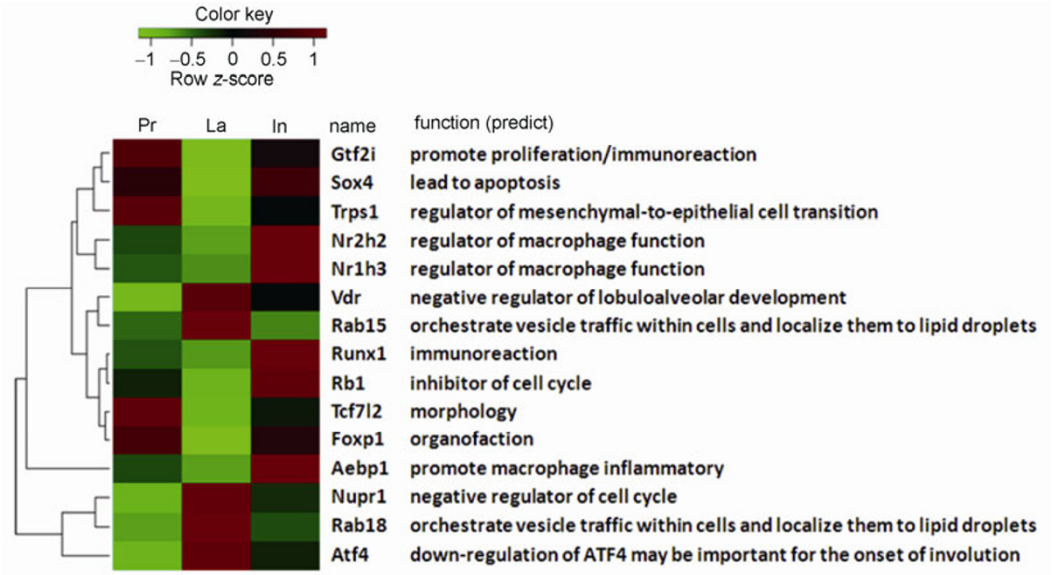

Figure 7 Heat map of key transcription factors. Columns, developmental periods; rows, genes. The color key indicates gene expression values; green, lowest; red, highest. The functions of the genes shown on the heat map are listed after the gene symbols.

regulated and 72 were down-regulated from pregnancy to lactation, while 69 that were up-regulated and 12 that were down-regulated from lactation to involution were defined as DEGs. The large number of down-regulated miRNAs during lactation indicates that these molecules play important lactation-related roles that merit further investigations. This large group is not only composed of most of the *let7* family members (i.e., *let7a*, *b*, *d*, and *I*), but also contains other miRNAs, such as *mir-223*, *mir-744*, *mir-433*, and *mir-877*. Also, it is worth noting that *mir-181a* and *mir-181b* appear to have higher expression levels during lactation than those during pregnancy or involution. Focusing on the 69 DEGs that were up-regulated from lactation to involution, *mir-17*, *mir-21*, *mir-24-1*, *mir-29*, *mir-93*, *mir-125*, and *mir-150* are reported to be related to immunoreactions [6,31–33]. The data suggest, therefore, that mir-146a, the *mir-17-92* cluster and *mir-92a-1*, which are all highly expressed during involution, may also participate in immune reaction. We also found several apoptosis-associated up-regulated miRNAs;

these include *mir-195*, *let-7a*, *mir-15* and *mir-34a*, the levels of which were differentially raised, and *mir-15a*, which was 15-fold up-regulated (Figure 8).

3 Discussion

Our study is unique in two ways with respect to its methodology. First, we used next-generation sequencing technology coupled with an RNA-seq protocol [13], which allowed deep coverage of the sequence reads. The combined methodology is becoming increasingly popular because it increases the power of transcriptomics analyses. Second, we used the so-called ribo-minus RNA-seq for library construction, where non-protein coding RNAs are included in the sampling. As a result, we acquired data for both mRNAs and ncRNAs. Another point we would like to emphasize is our use of mouse as a mammalian model. The mouse genome is the most fully assembled and annotated of the mammalian genomes, thus making sampling easier and more precise than that of the other mammalian genomes. Finally, our work provides comprehensive insight into regulatory genes expression during development of the mammary gland, which includes 1675 transcription factors and ncRNAs (especially miRNAs) in addition to the protein-coding genes.

3.1 Decisive changes in both HK and MS gene regulation during the PL cycle

The PL cycle, in essence, promotes two biological changes in female mammals: from pregnancy to lactation and from lactation to involution. During the first change, we observed a global suppression of HK gene expression and MS genes activation. For example, among the mapped reads, those of the five casein family members, *Lars2*, *Trf*, *Wap*, and *Fasn* accounted for >80% of the total reads on the chromosome that these genes reside on, whereas the remaining reads were minimal for all other HK genes. We also observed striking reductions in the number of genes and in the expression of protein-coding and ncRNAs. If only the defined numbers of DEGs during the two physiological changes are taken into account, 84.53% are suppressed during pregnancy to lactation while 95.53% are activated during lactation to involution. For the second stage change, partial recovery of the expression profiles of genes that were suppressed in the first change was apparent, but more than 50% of the suppressed genes did not recover their normal expression levels. Such genes merited further functional scrutiny.

To explore the functional and biological alternations that occur during the PL cycle, we examined the top 10 DEGs identified between pregnancy and lactation in more details. Aside from the abundant milk protein genes, other relevant functions corresponding to the synthesis, secretion and transport of milk fats, act as protease inhibitors or have antibacterial functions. For instance, *Wfdc3*, a protease encoding gene, increases about 800-fold from pregnancy to lactation. Another interesting gene is *Slc34a2* (also named NaPi-II b), which is expressed primarily during lactation [34]. Although the function of *Slc34a2* in the mammary gland is not clear, it is important for the production of surfactant fluids in lung tissue [35]. We speculate that *Slc34a2* may play a role as a solute transporter in the production and

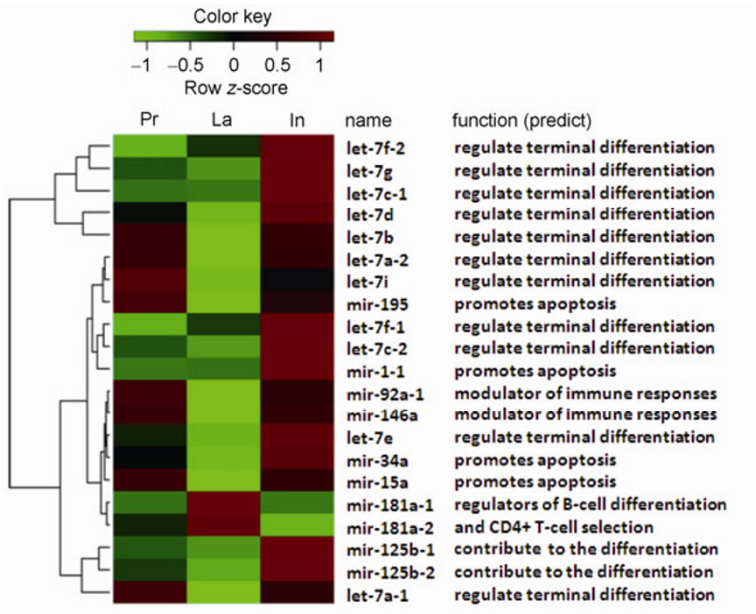

Figure 8 Heat map showing the abundance of key miRNAs in each developmental stage. The color key indicates gene expression levels from low (green) to high (red). Gene functions of the miRNAs are shown next to the gene symbols.

secretion of milk [36].

Several of the top lactation-associated genes are related to fatty acid metabolisms, in agreement with the results of research conducted on porcine mammary gland development [3]. One example is *Cidea*, which regulates lipolysis through the negative control of tumor necrosis factor-alpha (TNF-alpha) transcription in human adipocytes [37] and possibly perhaps via monitoring of lipid droplet size [38]. Another example is oleoyl-ACP hydrolase, otherwise known as *Olah*, which shows an 86-fold up-regulation during lactation and is involved in fat accumulation. The third example is the gene encoding carboxyl ester lipase (*Cel*), previously named cholesterol esterase or bile salt-stimulated (or dependent) lipase. It has increased expression levels in the human lactating mammary gland and pancreas [39]. Here, the expression level of *Cel* during lactation is found to increase 135-fold compared with the expression level during pregnancy. *Cel* participates in chylomicron assembly and secretion in a mechanism mediated through its ceramide hydrolytic activity [40]. Other up-regulated genes such as *Lao* and *Qsox* encode antibacterial activity in their respective products through the production of hydrogen peroxide [41] and via a secretory function during mammary cell differentiation [42,43]. For the most significant top 10 down-regulated genes, alveolar ductal proliferation and cell differentiation were apparently inhibited, while cell motion and cell communication were also less active during lactation. *Prune2*, the drosophila homologous gene, is number one of the top 10. This gene regulates formation of the terminal branches of the tracheal (respiratory) system [44]. Therefore, we suspect that this gene may play a role in terminal branching of the mammary glands. *Arhgap29*, a member of the Rho GTPase-activating protein family, had a 54-fold decrease in expressions. The higher expression level of *Arhgap29* in pregnancy, which is characterized by massive proliferation of ductal branching, suggests that this gene may play a role in mammary ductal morphogenesis, so do P190A RhoGAP and P190B RhoGAP as we have noticed [45,46]. Another related gene is the Mast/stem cell growth factor receptor known as Kit, which is also involved in cell differentiation as indicated by phenotypic analysis of mouse mutants [47]. Other function of the prominently down-regulated genes, *Kank4*, *Pde8b*, and the *Gli3* transcription factor during development of the mammary gland will be the topic of further investigation, together with three hypothetical proteins.

As to the top 10 genes that were down-regulated during lactation to involution, five (*Csn1s2b*, *Wap*, *Lao1*, *Slc34a2*, and *Olah*) genes were identical to those top 10 genes that were up-regulated during pregnancy to lactation. The remaining three, which are members of the Casein gene family, are also major proteins during lactation. As for the 7-fold down-regulated gene, *Atp2b2*, whose structure is similar to those of the SERCA calcium pumps, this function as a calcium transporter that moves calcium across the apical surface of cells into the milk [48,49]. The *Lpo* gene encodes an oxidoreductase that acts as a natural antibacterial agent in the innate immune system by killing bacteria in milk. Activation of this gene during lactation may explain the well-established observation that a long lactation history reduces the risk of breast cancer [50]. In addition to milk protein synthesis, the major biological functions during lactation are the transportation of calcium and the synthesis of antibacterial materials.

The top 10 up-regulated genes during lactation to involution are related to immunoreactions, cell apoptosis, and stromal membrane reconstruction. *Hp*, which increased 368-fold, is the top up-regulated gene during lactation to involution. It belongs to the group of acute phase proteins (APPs) that come into play during the acute phase reaction (APR), which comprises a complex cascade of reactions that prevent further tissue damage, eliminate any infective organisms, and enhance the healing process to restore homeostasis. Huntoon et al. suggest a major regulatory activity for *Hp* in supporting proliferation and functional differentiation of B and T cells as part of homeostasis and in response to antigen stimulation, as based on the phenotype of Hp-deficient mice [51]. Similar to *Hp*, *fcgbp* and *Saa1*, which are involved in immunoreactions, are also dramatically up-regulated as acute-phase reactants during inflammation [52,53].

As to the apoptosis-related genes, the function of insulin-like growth factor binding protein 5(*Igfbp5*) during involution has been known. It promotes apoptosis through binding insulin-like factor. It was reported that *Inhbb* regulates apoptosis based on its interactions with *Wnt4* [54] and it may be involved in apoptosis of the mammary gland. However, the present study shows that *Wnt4* expression levels were not as pronounced as we expected and *Wnt4* may have different target genes for regulating mammary epithelial apoptosis. *Pdk4* is related to apoptosis and may be a regulatory gene during mammary epithelial cell apoptosis [55]. The up-regulated creatine kinase, known as *Ckm*, is reported in several studies to be elevated during muscle or heart damage [56]; our data identified that it was up-regulated. Another gene from our top 10 list, which increased 223-fold during involution, is the secreted phosphoprotein 1 (*Spp1* or *Opn*). This finding is in agreement with the results from related studies [57]. *Opn* is reported to act as an immune modulator in a variety of ways.

3.2 Many functional pathways are closely related to the PL cycle

Here, we investigated a most interesting subject: lysosomal biogenesis. The major function of acid hydrolases in lysosomes is to degrade a wide variety of substrates. Lysosomes must be involved in involution, although relevant studies

have been directed toward the expression of lysosome enzyme genes and the role of lysosomes during lactation. One such study found heavier aryl sulfatase (a lysosomal marker enzyme) staining in the endoplasmic reticulum of the gland during lactation than that during pregnancy [58], while another reported prolactin receptor complexes in the lysosomes of mammary epithelia [59]. At the transcriptome level, we are the first group to have observed that lysosomal-related genes are up-regulated during lactation. It is worth noting that cathepsin D activity during lactation is about 4-fold higher than in pregnancy; this is thought to be related to the intense turnover of the biochemical compounds that constitute different organelles and cellular compartments [60]. Therefore, lysosomes may be heavily involved in regulating milk synthesis and release.

We found that cell-cell signal communication and transduction are highly activated in pregnancy. In addition, the most significantly enriched pathways were related to cell cycle control and proliferation. One of the key regulators of the cell cycle, cyclinD1, shortens the G1 period of the cell cycle and promotes swift entry into S phase: it is reported to be an essential protein for the completion of alveolar development in the later stages of pregnancy [61]. The Notch signaling pathway in involution regulates cell apoptosis. Genes associated with both activation and suppression of the Notch signal pathway tend mostly to be up-regulated, but among those genes, the activation-related genes are of a higher amount. Some of the other DEG-enriched pathways are also associated with apoptosis, such as the glycerophospholipid metabolism pathway [62], the chemokine signaling pathway, and the B and T cell receptor signaling pathways.

3.3 Many key transcription factors as DEGs in regulating mammogenesis are identified

In mammogenesis, mammogenic hormones act on target cells, leading to activation of transcription factors that in turn stimulate the development of mammary alveoli, and form the mammary hierarchy structure. Previous work identified several important transcription factors that work during development of the gland. In pregnancy, *Stat5* is an obligate mediator of epithelial cell differentiation, secretory alveolar cell development, and milk protein gene expression [63,64]. Working cooperatively, nuclear factor 1(*Nf1*) regulates transcription of milk protein genes such as whey acidic protein (*Wap*), *β-Casein*, and *lactoglobulin*, and its functional roles are similar to that of *Stat5* [65–68]. *Elf5*, *AP2*, and *AP-1* significantly influence over the morphogenesis in pregnancy [69–74]. In lactation, *Thrsp* is involved in lipid biosynthesis and activates genes encoding lipid-synthesizing enzymes [75]. In involution, nuclear factor 1 C (NF1C) members of the *Nf1* gene family [76], *AP-1* [77], C/EBP-α [78], C/EBP-β [79,80] and *Elf3* [81] are involved in cell apoptosis, immune reactions, and tissue remodeling. Although all the factors mentioned above play important roles in mammary gland development, no systematic research has been conducted on transcription factors associated with development of the mammary gland. To discover as-yet unknown transcription factors involved in mammogenesis, we investigated their possible roles in details in our study.

First, in pregnancy, we found that *Trps1*, *Foxp1*, *Gtf2i*, and *Tcf7l2* are all highly expressed. *Trps1* is a critical regulator of mesenchymal-to-epithelial cell transition [82]. Studies on renal tubule epithelial differentiation and the roles of *Trps1* in other tissues pave the way for elucidating a potential role for *Trps1* in mammary gland development [82–84]. The FOX family regulates genes involved in cell growth, proliferation and differentiation. Some of the FOX proteins are downstream targets of the hedgehog-signaling pathway that has an important function in organofaction. Two other highly expressed genes in pregnancy are *Gtf2i* and *Tcf7l2*; the former is known to induce cell proliferation [85], while the latter is a key factor in the Wnt signaling pathway.

Second, we discovered that *Atf4*, *Nupr1*, *Rab15*, and *Rab18* are highly expressed DEGs during lactation, unlike pregnancy and involution. According to transgenic studies on *Atf4*, its down-regulation is important for the onset of involution in the mammary gland [86]. *Nupr1* is involved in negative regulation of the cell cycle [87], which could explain why cell cycle related genes are more active in pregnancy and involution. *Rab18*, a small GTPase that orchestrates vesicle traffic within cells and localizes vesicles to lipid droplets, has an elevated expression level which is 11-fold higher compared to its level during pregnancy, and 4-fold higher compared to its level during involution [88–90]. Two other Rab family transcription factors, *Rab15* and *Rab25*, are also highly expressed and may be involved in differentiation of the secretory epithelium and milk secretion. In addition, the vitamin D3 receptor is also up-regulated (3.4-fold) in lactation as compared with its level during pregnancy. Several studies suggest that vitamin D plays a central role in calcium homeostasis, which may be mediated by the *Vdr* receptor; however, *Vdr* ablation does not disturb calcium homeostasis, thus *Vdr* does not affect milk protein or calcium content in the mammary gland. Transgenic mice lacking *Vdr* exhibit accelerated lobuloalveolar development; therefore, *Vdr* may be a negative regulator of lobuloalveolar development, which is in consistent with lobuloalveolar development during the reproductive cycle. The targets of *Vdr* and the downstream molecules during mammary gland development require further investigation [91–93].

In involution, we identified transcription factor candidates by referencing tissue damage-repair pathways. As expected, some potential apoptosis-related transcription factors were up-regulated DEGs. For example, *Sox4* and *Rb1* expression increased approximately 40-fold and 15-fold in involution as compared with lactation, respec-

tively. *Sox4* and *Rb1* may function in the apoptosis pathway, which leads to cell death, as one known function for *Rb1* is preventing excessive cell growth by inhibiting cell cycle progression [94]. During involution, Fox subfamily members (*Foxi1*, *Foxp1*, *Foxp4*, *Foxk1*, and *Foxo3*) exhibit elevated expression levels to various extents. These members play important roles in apoptosis regulated by PI3K-PKB signaling [95].

Accompanied by cell apoptosis, macrophages are assumed to phagocytize milk and cell debris, thus allowing remodeling of the gland. In our study, we found that the liver X receptors alpha and beta are highly expressed; both are key regulators of macrophage function. Another up-regulated gene in involution is adipocyte enhancer-binding protein 1 gene or *Aebp1*, whose function has been elucidated recently, and appears to promote macrophage inflammatory responsiveness in ablation attenuated atherosclerosis [96]. Cell apoptosis also leads to immunoreactions and we found that *Runx1*, which is also known as acute myeloid leukemia 1 gene (*Aml1*), and *Gtf2i* both exhibits higher expression levels in involution than in pregnancy. *Runx1* has been shown to have important functions in the mammary gland immune system [97]. *Gtf2i* regulates the synthesis of two proteins, BAP-135 and TFII-I. BAP-135 is involved in normal immune system function, whereas TFII-I is involved in coordinating cell growth and division. Therefore, we hypothesize that *Gtf2i* plays major roles in controlling cell differentiation in pregnancy and immune system activity in involution.

3.4 miRNAs play significant roles in regulating the PL cycle

ncRNAs are now recognized as key participants in the control of many biological processes [98], and are also important regulators of mammogenesis [99,100]. The differentially expressed miRNAs we identified herein provide clues for finding potential miRNA regulators of mammogenesis. Firstly, among the 72 down-regulated miRNAs from pregnancy to lactation, the let7 family members, including *let7a*, *let7b*, *let7d*, and *let7I*, are all down-regulated during lactation. Despite a direct role for the let-7 family in vertebrate development not being clearly demonstrated, changes in the expression level of let-7 members are associated to cell cycle timing in *Caenorhabditis elegans*. Since the function of the let-7 family is highly conserved from *C. elegans* to humans, we suspect that the major function of the let-7 genes may be regulating terminal differentiation during mammary development [101]. Another known down-regulated miRNA is *mir-125b*, which has been described in two studies as contributing to mammary epithelial cell differentiations during pregnancy [102,103]. As to some of the other down-regulated miRNAs, *mir-223*, *mir-744*, *mir-433*, and *mir-877* may play important roles in mammary gland development during pregnancy, and are worth considering for functional analysis in future research. Secondly, some of the miRNAs were up-regulated during lactation, such as *mir-181a* and *mir-181b*, are hypothesized to be important for immune system development during infancy [104]. Thirdly, in involution, we identified the potential involvement of *mir-146a* in the PL cycle, which is known to be a key modulator of immune responses [105,106] and the *mir-146a* knock-out mice inhibit Treg (the T regulator cell) function, leading to impaired immune response [107]. Another member of the *mir-17-92* cluster, *mir-92a-1*, may also be involved in immunoreactions. *Mir-15a*, *let-7a*, and *mir-34a* are proapoptotic miRNAs in neural differentiation, including mouse embryonic stem cells, PC12, and NT2N cells [108]. In addition, *mir-195* promotes apoptosis in mouse podocytes [109]. In the present study, we found that the expressions of these four miRNAs were all elevated, especially *mir-15a*, which is reported to be involved in inducing apoptosis in chronic lymphocytic leukemia through reacting with its target gene *Bcl2*. However, *Bcl2* is not significantly down-regulated during involution according to our expression data, which indicates that *mir-15a* may be sensitive to the cellular environment when regulating its target genes [110]. Similarly, our data suggest that *mir1-1* may play a role in regulating apoptosis in mammogenesis [111].

4 Conclusion

When drastic changes occur in the morphology and functions of the mammary gland during the PL cycle, gene expression at each of the turning points of the PL cycle should naturally display obvious differences. Based on Gene Ontology and KEGG annotations, we extracted a list of relevant biological information and identified a group of critical protein coding genes, transcription factors and miRNAs involved in these processes from our RNA-seq gene expression data. During lactation, most of the down-regulated genes that were identified appear to be enriched in terms of their diverse functions relating to tissue reconstruction, such as cell cycle, cell adhesion, and ubiquitin-mediated proteolysis. Only a small portion of the genes specific for synthesis and transportation of lactose, milk fats, and milk proteins were up-regulated. In addition, genes that were down-regulated from pregnancy to lactation restored their expression levels during involution. The most striking result relates to the discovery that genes related to the lysosome were significantly up-regulated during lactation compared with pregnancy. The high expression levels of lysosomal enzyme-coding genes in lactation may be responsible for casein synthesis and release, and contribute to the regulation of milk secretion. Our data thus provide categories for the expressed genes, including the miRNAs, as well as basic understanding of molecular events during mammogenesis.

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Supporting Information

Figure S1 Real-time PCR assay. We randomly selected 22 genes irrespective of their expression levels for real-time PCR-based validation of their expression levels. In each graph, the *X*-axis corresponds to the ratio of genes whose RPKM values represent pregnancy to lactation, or lactation to involution, while the *Y*-axis corresponds to the ratio obtained from the real-time PCR results. The related coefficient of the two methods from pregnancy to lactation, and from lactation to involution, is 0.91 (P=2.183×10^{-9}) and 0.90 (P=8.08×10^{-9}), respectively.

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