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原著論文

Genetic and Phenotypic Changes of Thymus Tissue in Mice during Pregnancy

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キーワード：thymus, gene expression, pregnancy, IGFBP5

Abstract：The maternal immune system during pregnancy is largely changed to maintain immune tolerance against the embryo. Although thymus tissues, which generate T cells, are also dynamically altered during pregnancy, the precise phenotype of thymic T cells and gene alteration in thymus tissues remain to be completely elucidated. In this study, we performed a comprehensive analysis of gene expression and assessed T-cell phenotypic changes in the thymus of mice during pregnancy. Thymus tissues of female C57BL/6 mice during pregnancy were resected to investigate T-cell phenotypes using flow cytometric and immunofluorescence analyses and gene expression using a DNA microarray. Decreased weight of thymus tissues was observed during gestation. Cell numbers of total populations of thymic T cells in pregnant mice were significantly decreased compared with those in non-pregnant mice. DNA microarray and RT-PCR analyses revealed several upregulated genes in the thymus tissues of pregnant mice. Among them, the insulin growth factor-binding protein 5 (IGFBP5) was the most upregulated gene, and its increased protein expression of macrophages was confirmed by immunofluorescence analysis. In summary, a unique change in gene expression was observed in transient atrophy of thymus tissues during pregnancy. The change in gene expression, including that in IGFBP5 of macrophages, may influence thymic differentiation of T cells to maintain an immunological tolerance during pregnancy.

Introduction

The female immune system during pregnancy is precisely controlled to tolerate paternal alloantigens expressed in fetal tissues and its immune response against foreign pathogens[1, 2]. The immunological mechanisms employed to maintain pregnancy with a semiallogeneic fetus are regulated by a cytokine balance at the maternal-fetal interface on the basis of the change in sex hormones during pregnancy[3]. An appropriate alteration in T-cell subsets, including T-helper cells (Th)1, Th2, and Th17, γδT, regulatory T (Treg) cells, provides the cytokine balance to maintain pregnancy[4-11]. In contrast, an impertinent change in T-cell subsets during pregnancy may induce miscarriage.

Treg cells play an essential role in promoting fetal survival by avoiding the recognition of paternal semiallogeneic antigens by the maternal immune system[9]. The IL-10 and Bcl-2 genes of peripheral Treg cells in pregnant women are significantly upregulated compared with those in non-pregnant women[12]. Various factors, including cytokines, sex hormones, and seminal fluids, influence pregnancy success by increasing the number of Treg cells[9]. During pregnancy, the levels of sex hormones, such as estrogen, are largely changed. Pregnancy-induced thymic atrophy is a phenomenon that occurs in various mammals, including mice, rats, and humans[13, 14]. It is well known that maternal immune system during pregnancy is largely modulated to maintain immune tolerance against

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an embryo[1, 2]. However, the physiological significance and immunological mechanism of the reduction of thymus weight during pregnancy remain unclear.

In this study, the gene expression in thymus tissues of mice during late pregnancy was comprehensively analyzed using a DNA microarray. In addition, we focused on upregulated genes to confirm the protein expression in thymus tissues during pregnancy. Moreover, the possible mechanism of pregnancy-induced thymic atrophy was discussed, suggesting that the thymus plays a potent role in immunological tolerance during pregnancy.

Materials and Methods

Animals

Animal studies were conducted according to the Animal Care and Use Committee guidelines of Tokushima University (Permit number: T27-7). Female C57BL/6 mice were obtained from the Japan SLC Laboratory (Shizuoka, Japan) and were maintained in a specific pathogen-free colony with food and water provided *ad libitum*. After mating with male mice, vaginal plugs of female mice were checked as being pregnant or not. On pregnant day 0, 5, 10, 15, and 20, thymus tissues of pregnant and non-pregnant (control) B6 mice were resected under anesthesia. For each group, five mice were used.

Flow cytometric analysis

Antibodies against FITC-CD4 and PE-CD8 (eBioscience, San Diego, CA, USA) were used for staining suspended thymic T cells. A FACSCanto flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to determine the cell populations, and the data were analyzed using the FlowJo FACS Analysis software (Tree Star, Ashland, OR, USA).

DNA microarray

Total RNAs were purified using an RNeasy Mini kit (Qiagen, Baltimore, MD) from thymus tissues in pregnant (day 15) and control mice. The quality of extracted RNA was checked with a Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA). The synthesis and labeling of target cRNA probes were performed using the Agilent Low RNA Input Linear Amplification kit PLUS (Agilent Technologies) according to the manufacturer's instructions. Labeled cRNA samples were hybridized for 17 hours at 65℃ with Mouse Gene Expression v2 Microarray Kit (Agilent Technologies). Hybridized DNA samples were scanned using an Agilent Microarray Scanner (Agilent model G2505A). Scanned images were analyzed with the Feature Extraction Software (Agilent Technologies). All data normalization and selection of fold-changed genes were performed using GeneSpring GX 7.3 (Agilent Technologies). Logged gene expression ratios were normalized by LOWESS regression. Four mice in each group were used for the cDNA microarray experiments (one-channel method), and the average of the gene expression values for each gene was used for further analysis.

Quantitative RT-PCR

Total RNA was extracted from thymus using Isogen (Wako Pure Chemical Industries, Osaka, Japan) and the reverse transcription was performed. The levels of mRNA encoding Orm2, Igfbp5, Aqp3, Igfbp3, Cyp2g1, Angpt2, IL-33, Chrdl1, Fgf10, Lyve1, Adamts1, Cav2, Krt19, Figf, and β-actin genes were determined using a PTC-200 DNA Engine Cycler (Bio-Rad Laboratories) with the SYBR Premix Ex Taq (Takara Bio, Shiga, Japan). The primer sequences used were as follows: Orm2, forward, 5'-GAATGGGACCCTCTCCAAGT-3' and reverse, 5'-TCAAAGGCAAGCATGAAGG-3'; Igfbp5, forward, 5'-GGCGAGCAAACCAAGATAGA-3' and reverse, 5'-AGGTCTCTTCAGCCATCTCG-3'; Aqp3, forward, 5'-CTGGGGACCCTCATCCTT-3' and reverse, 5'-TGGTGAGGAAGCCACCAT-3'; Igfbp3, forward, 5'-GACGACGTACATTGCCTCAG-3' and reverse, 5'-GTCTTTTGTGCAAAATAAGGCATA-3'; Cyp2g1, forward, 5'-AGGAGGAGGCTGGCTACC-3' and reverse, 5'-TCAGGTATAAGGTGGGATCTATGG-3'; Angpt2, forward, 5'-CTCACCACCAGTGGCATCTA-3' and reverse, 5'-CCCACGTCCATGTCACAGTA-3'; IL-33, forward, 5'-GGTGAACATGAGTCCCATCA-3', and reverse, 5'-CGTCACCCCTTTGAAGCTC-3'; Chrdl1, forward, 5'-CTCAAGACTTGCCCCAAACT-3' and reverse, 5'-TTCCCACGATAATTCTGCATC-3'; Fgf10, forward, 5'-CGGGACCAAGAATGAAGACT-3' and reverse, 5'-GCAACAACTCCGATTTCCAC-3'; Lyve1, forward, 5'-GGTGTCCTGATTTGGAATGC-3' and reverse, 5'-AGGAGTTAACCCAGGTGTCG-3'; Adamts1, forward, 5'-CACATGCAAGAAGATGTCAGG-3' and reverse, 5'-CCCTTTGATTCCGATGTTTC-3'; Cav2, forward, 5'-CCTCACCAGCTCAACTCTCA-3' and reverse, 5'-CACATATTTGCTGATTTCAAAGAGA-3'; Krt19, 5'-TGACCTGGAGATGCAGATTG-3' and reverse, 5'-CCTCAGGGCAGTAATTTCCTC-3'; Figf, 5'-GCAACTTTCTATGACACTGAAACAC-3' and reverse, 5'-TCTCTCTAGGGCTGCATTGG-3'; β-actin, forward, 5'-GTGGGCCGCTCTAGGCACCA-3' and reverse, 5'-CGGTTGGCCTTAGGGTTCAGGGGG-3'.

Immunofluorescence analysis

Frozen sections of thymus tissues were fixed with a cold acetone and methanol (1:1) mixture. The sections were incubated with rabbit anti-IGFBP5 (Bioss Inc, Boston, MA), subsequently developed using Alexa® 568-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA). For double staining,

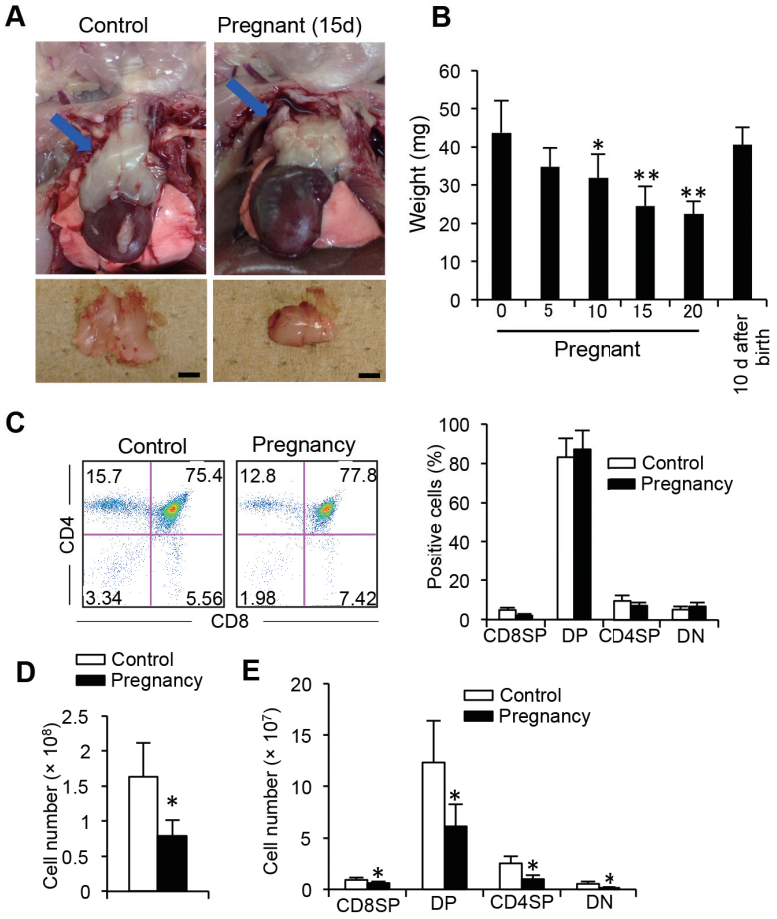

fig.1 Pregnancy-induced thymic atrophy.
(A) Thymic atrophy was observed in pregnant mice. Scale bar = 2 mm. (B) Weight change of thymus tissues during pregnancy. The weight of thymus tissues gradually reduced depending on the gestation day. The results represent the mean ± SD of five mice from each group. *$p < 0.05$, **$p < 0.005$ (vs. Day 0). (C) CD4 and CD8 T-cell subsets of thymus in control and pregnant (day 15) mice were analyzed using flow cytometry. Results are representative of each group. Data are the mean ± SD of five mice from each group. (D) Number of total thymocytes is shown as the mean ± SD of five mice from each group. (E) Number of CD4 and CD8 T-cell subsets was calculated, and data are the mean ± SD of five mice from each group. *$p < 0.05$ (vs. control).

the sections were stained with biotinylated anti-mouse EpCAM, Thy1.2, CD11c and F4/80 antibodies (eBioscienes) and Alexa® 488-conjugated streptavidin as the secondary antibody. The nuclear DNA was stained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probe Inc., Eugene, OR, USA). The images were acquired using an LSM 5 PASCAL confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

Data analysis

Statistical analysis was assessed by one way analysis of variance, and $p < 0.05$ was considered to be statistically significant.

Results

Pregnancy-induced atrophy and thymic T-cell subsets

Atrophy of thymus tissues in B6 mice on day 15 of pregnancy was observed (Fig. 1A). In addition, thymus tissue weight in pregnant mice was measured from day 0 to 20 and on day 10 after birth. The weight of pregnant mice gradually decreased until day 20 (Fig. 1B). At 10 days after birth, the weight recovered to that at day 0 (Fig. 1B). T cell-subsets of the thymus in the control and pregnant mice (day 15) were detected using flow cytometry. There were no differences in the proportion of T-cell subsets, including $CD4^-CD8^-$ (DN), $CD4^+CD8^+$ (DP), $CD4^+CD8^-$ (CD4SP), and $CD4^-CD8^+$ (CD8SP), between the control and pregnant mice (Fig. 1C). However, the numbers of thymocyte in pregnant mice

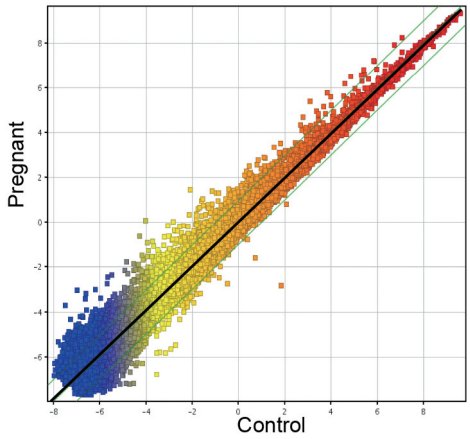

fig.2 DNA microarrays of thymus tissues of pregnant mice (day 15). Up-and downregulated genes of thymus tissues in pregnant mice compared with those in control mice were plotted. Green line indicates a two-fold change in gene expression. Signal intensity is shown as blue to red color.

Table 1 Upregulated genes of thymus tissue in pregnant mice

ProbeName	FC (abs)	Regulation	GeneSymbol	Description
A_51_P141546	5.203756	up	Orm2	Mus musculus orosomucoid 2 (Orm2), mRNA [NM_011016]
A_52_P281702	5.104808	up	Igfbp5	Mus musculus insulin-like growth factor binding protein 5 (Igfbp5), mRNA [NM_010518]
A_51_P245090	3.923982	up	Aqp3	Mus musculus aquaporin 3 (Aqp3), mRNA [NM_016689]
A_52_P253179	3.667473	up	Igfbp3	Mus musculus insulin-like growth factor binding protein 3 (Igfbp3), mRNA [NM_008343]
A_51_P137452	3.4213557	up	Cyp2g1	Mus musculus cytochrome P450, family 2, subfamily g, polypeptide 1 (Cyp2g1), mRNA [NM_013809]
A_51_P201982	3.2864256	up	Angpt2	Mus musculus angiopoietin 2 (Angpt2), mRNA [NM_007426]
A_55_P1964960	3.2312324	up	Il33	Mus musculus interleukin 33 (Il33), transcript variant 1, mRNA [NM_001164724]
A_66_P124091	3.2102666	up	Chrdl1	Mus musculus chordin-like 1 (Chrdl1), transcript variant 2, mRNA [NM_031258]
A_52_P42269	3.2056398	up	Fgf10	Mus musculus fibroblast growth factor 10 (Fgf10), mRNA [NM_008002]
A_55_P2364768	3.139236	up	Mcpt-ps1	Mus musculus mast cell protease, pseudogene 1 (Mcpt-ps1), non-coding RNA [NR_028284]
A_52_P686785	3.121155	up	Lyve1	Mus musculus lymphatic vessel endothelial hyaluronan receptor 1 (Lyve1), mRNA [NM_053247]
A_51_P157083	3.0592566	up	Gas1	Mus musculus growth arrest specific 1 (Gas1), mRNA [NM_008086]
A_52_P489295	2.9858954	up	Adamts1	Mus musculus a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1 (Adamts1), mRNA [NM_009621]
A_51_P279552	2.933013	up	Cav2	Mus musculus caveolin 2 (Cav2), mRNA [NM_016900]
A_51_P356642	2.8929784	up	Krt19	Mus musculus keratin 19 (Krt19), mRNA [NM_008471]
A_51_P117739	2.8568933	up	Figf	Mus musculus c-fos induced growth factor (Figf), mRNA [NM_010216]
A_51_P145132	2.8136868	up	Mcpt4	Mus musculus mast cell protease 4 (Mcpt4), mRNA [NM_010779]
A_52_P175242	2.777105	up	Irs1	Mus musculus insulin receptor substrate 1 (Irs1), mRNA [NM_010570]
A_55_P1971889	2.7715304	up	F3	Mus musculus coagulation factor III (F3), mRNA [NM_010171]
A_55_P1990121	2.7109623	up	Aqp5	Mus musculus aquaporin 5 (Aqp5), mRNA [NM_009701]
A_51_P436652	2.6999686	up	Ccl7	Mus musculus chemokine (C-C motif) ligand 7 (Ccl7), mRNA [NM_013654]

(day 15) were significantly decreased compared with those in control mice (Fig.1 D), and the numbers of all T-cell subsets in the thymus of pregnant mice were reduced compared with those of control mice (Fig.1 E). Therefore, the differentiation of total T-cell subsets in the thymus was suppressed during pregnancy.

Expression profiles of genes regulated during pregnancy in thymus tissues

To elucidate the expression profiles of genes in thymus tissues between the control and pregnant mice, DNA microarray analysis was performed (Fig. 2). We found 1369 upregulated genes, with at least a two-fold change, in pregnant mice compared with control mice (Fig. 2). In contrast, 367 downregulated genes with at least a half-fold change were observed in pregnant mice compared with control mice (Fig. 2). Among the up- and downregulated genes, endocrine- or immune response-related genes were identified by the gene ontology pathway analysis (Tables 1 and 2). We confirmed the expression levels of upregulated genes using quantitative RT-PCR. Among them, mRNA expression levels of insulin-growth factor-binding protein 5 (IGFBP5) were most increased in thymus tissues of pregnant mice (Fig. 3).

IGFBP5 expression in thymus tissues

To confirm the protein expression and distribution of IGFBP5 in thymus tissues, immunofluorescence analysis was performed. The expression of main component cells, including thymic T cells, thymic epithelial cells, dendritic cells, and macrophages, was analyzed by double staining using an anti-IGFBP5 antibody and an antibody against each marker protein. EpiCAM$^+$ thymic epithelial cells, Thy1.2$^+$ thymic T cells, and CD11c$^+$ did not express IGFBP5 (Fig. 4A). By contrast, F4/80$^+$ macrophages expressed IGFBP5 (Fig. 4B).

Table 2　Downregulated genes of thymus tissue in pregnant mice

ProbeName	FC (abs)	Regulation	GeneSymbol	Description
A_55_P2003034	2.0148218	down	P4ha3	Mus musculus procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide III (P4ha3), mRNA [NM_177161]
A_55_P2205186	2.0611012	down	5830415G21Rik	Mus musculus adult male thymus cDNA, RIKEN full-length enriched library, clone: 5830415G21 product:unclassifiable, full insert sequence. [AK017932]
A_51_P195598	2.4063785	down	Olfr259	Mus musculus olfactory receptor 259 (Olfr259), mRNA [NM_146770]
A_55_P1952304	2.0833826	down	Usp44	Mus musculus ubiquitin specific peptidase 44 (Usp44), transcript variant 1, mRNA [NM_001206851]
A_55_P1986781	3.0202398	down	Gm10694	PREDICTED: Mus musculus predicted gene 10694 (Gm10694), mRNA [XM_003084785]
A_52_P248013	2.0406182	down	Chsy3	Mus musculus chondroitin sulfate synthase 3 (Chsy3), mRNA [NM_001081328]
A_51_P267278	24.46408	down	Slc15a2	Mus musculus solute carrier family 15 (H+/peptide transporter), member 2 (Slc15a2), transcript variant 1, mRNA [NM_021301]
A_66_P125787	2.033159	down	Gm11292	PREDICTED: Mus musculus predicted gene 11292 (Gm11292), mRNA [XM_003084913]
A_55_P2082215	2.7741027	down	Ttbk1	Mus musculus tau tubulin kinase 1 (Ttbk1), mRNA [NM_001162864]
A_55_P2015949	2.2984664	down	Cpa5	Mus musculus carboxypeptidase A5 (Cpa5), mRNA [NM_144537]
A_66_P109080	2.0036366	down	Lrguk	Mus musculus adult male corpora quadrigemina cDNA, RIKEN full-length enriched library, clone:B230320P20 product:hypothetical L domain-like structure containing protein, full insert sequence. [AK045903]
A_55_P2073567	2.4099538	down	Epb4.1l4b	erythrocyte protein band 4.1 -like 4b [Source:MGI Symbol;Acc:MGI:1859149] [ENSMUST00000095076]
A_52_P667960	2.553187	down	Tcte2	Mus musculus t-complex-associated testis expressed 2 (Tcte2), mRNA [NM_022311]
A_55_P2089362	2.3977284	down	Wfdc13	Mus musculus WAP four-disulfide core domain 13 (Wfdc13), mRNA [NM_001012704]

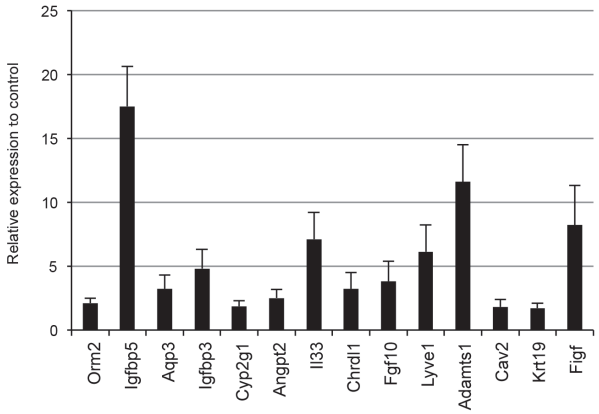

fig.3　mRNA expression of selected, upregulated genes of thymus tissues in pregnant (day 15) mice was confirmed using quantitative RT-PCR.
Data represent the ratio of the relative expression value, normalized with β-actin, from pregnant mice to the average of those from control mice. Data are the mean ± SD of three mice from each group. The expression is shown as relative to mRNA expression of control thymus tissues.

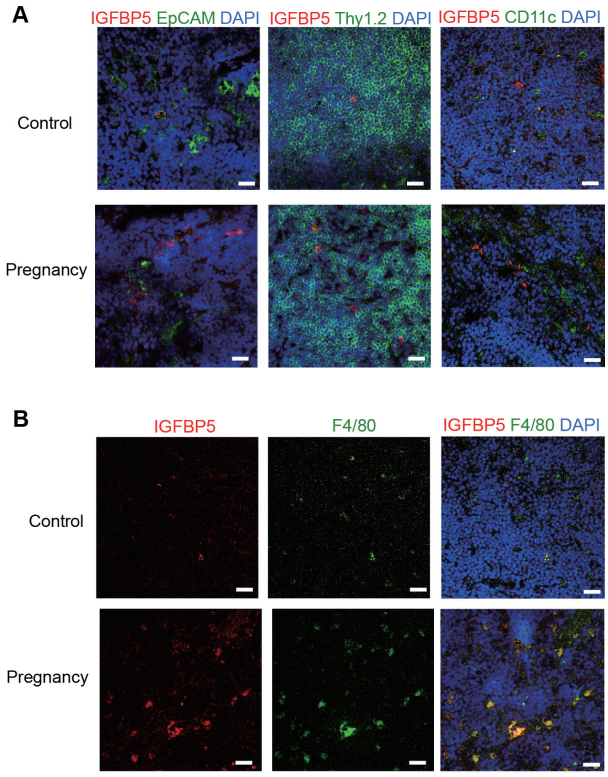

fig.4　IGFBP5 expression in thymus tissues of pregnant mice.
(A) IGFBP5 expression on EpiCAM$^+$ thymic epithelial cells, Thy1.2$^+$ T cells, and CD11c$^+$ dendritic cells was analyzed by confocal microscopy using frozen thymus sections in pregnant (day 15) and control mice.
(B) IGFBP5 expression on F4/80$^+$ macrophages was analyzed. The results are representative of three mice from each group. Scale bar: 20 μm.

Discussion

Pregnancy-induced thymic atrophy is a well-known physiological phenomenon in various mammalian species, and the number of cortical thymocytes is dramatically reduced during pregnancy[13, 14]. In addition, thymic atrophy is linked to a steroid-sensitive cortical reduction[15]. Thymic atrophy is believed to be a regulatory mechanism to maintain immunological tolerance between the fetus and mother[9]. In this study, although the total numbers of thymocytes in

pregnant mice were significantly reduced compared with those in control mice, there was no change in the proportion of T-cell subsets. T-cell differentiation in the thymus may be largely influenced by pregnancy. Moreover, it is possible that the generation of T cell precursor in the bone marrow might be suppressed during pregnancy. A previous study demonstrated that the number of peripheral regulatory T cells increased during pregnancy[3]. The T-cell immune response during pregnancy is regulated by machinery even in central or peripheral immune tolerance.

Insulin-like growth factors (IGFs) play potent roles in many physiological and pathological functions of various cells[16]. Six IGFBPs are known to bind to IGFs to control bioactivity[16]. IGFBP5 is the most conserved of all IGFBPs and has been reported as a key factor during cellular development[17]. IGFBP5 is expressed in the embryo during early development in the central nerve system and myotomes of somites[17, 18]. Furthermore, IGFBP5 is increased in several neoplasms, such as pediatric cancer, prostate cancer, rhabdomyosarcoma, glioma, osteosarcoma, and leiomyoma[19, 20]. A previous study indicated that IGFBP5 stimulates bone formation through an IGF-independent mechanism[21]. In this study, we focused on thymus tissues of pregnant mice. A previous report described that IGFBP5 mRNA expression in the thymus of mice changes with age[22]. In addition, IGFBP5 expression is regulated by pregnancy-associated plasma protein-A[22]. Therefore, IGFBP5 may play a role in the functioning of the thymus during pregnancy to maintain an immunological tolerance between the fetus and mother. Moreover, a previous report demonstrated that activated macrophages increased in the thymus tissue of pregnant mice[23]. In this context, IGFBP-5-expressing macrophages in the thymus may control T-cell survival or T-cell differentiation during pregnancy.

In this study, there were many up- and downregulated genes in the thymus tissues of pregnant mice. This finding suggested that the expression of many genes affects the maintenance of immunological tolerance in the thymus during pregnancy. We also focused on immune response- or endocrine-related genes among the upregulated genes. The drastic change in sex hormones during pregnancy affects the gene expression in various organs. In conclusion, pregnancy-induced thymic atrophy is a physiological phenomenon to maintain the immune system during pregnancy. Genomic change in the thymus during pregnancy controls T-cell immune tolerance between the fetus and mother, and among the upregulated genes, IGFBP5-expressing macrophages may be important for T-cell differentiation and/or survival in the thymus during pregnancy.

Conflicts of interest

The authors state that they have no conflicts of interest.

Acknowledgments

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