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Thymic epithelial cell–specific deletion of *Jmjd6* reduces *Aire* protein expression and exacerbates disease development in a mouse model of autoimmune diabetes

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ABSTRACT

Thymic epithelial cells (TECs) establish spatially distinct microenvironments in which developing T cells are selected to mature or die. A unique property of medullary TECs is their expression of thousands of tissue-restricted self-antigens that is largely under the control of the transcriptional regulator Aire. We previously showed that Jmjd6, a lysyl hydroxylase for splicing regulatory proteins, is important for Aire protein expression and that transplantation of Jmjd6-deficient thymic stroma into athymic nude mice resulted in multiorgan autoimmunity. Here we report that TEC-specific deletion of *Jmjd6* exacerbates development of autoimmune diabetes in a mouse model, which express both ovalbumin (OVA) under the control of the rat insulin gene promoter and OT-I T cell receptor specific for OVA peptide bound to major histocompatibility complex class I K^b molecules. We found that Aire protein expression in mTECs was reduced in the absence of Jmjd6, with retention of intron 2 in *Aire* transcripts. Our results thus demonstrate the importance of Jmjd6 in establishment of immunological tolerance in a more physiological setting.

1. Introduction

Thymic epithelial cells (TECs) establish spatially distinct microenvironments in which developing T cells gain the ability to distinguish self from nonself through interaction with the epithelial cells [1,2]. Cortical TECs, a major stromal cell type in the cortex, contribute to the selection of thymocytes that are able to recognize complexes of self-antigens and major histocompatibility complex (MHC) molecules, whereas medullary TECs (mTECs) play a key role in induction of self-tolerance through elimination of self-reactive T cells.

Autoimmune regulator (Aire), an important transcriptional regulator [3,4], contributes to self-tolerance by promoting the expression of thousands of tissue-specific self-antigens in mature mTECs [5–8]. Mutations of the *AIRE* gene have thus been identified as the cause of an autoimmune disease known as autoimmune polyendocrinopathy-candidiasis–ectodermal dystrophy (APECED) [9,10]. Despite its important role in central tolerance induction, however, the mechanism underlying the regulation of Aire expression has remained unclear.

Jmjd6 is a member of the JmjC domain–containing family of proteins that participate in a wide range of oxidation reactions [11]. Jmjd6 catalyzes lysyl hydroxylation of multiple substrates, including splicing regulatory proteins, and its deficiency in mice results in perinatal mortality due to abnormal development of multiple organs during embryogenesis [12]. We previously showed that transplantation of Jmjd6-deficient thymic stroma into athymic nude mice led to multiorgan autoimmunity as a result of impaired expression of the *Aire* gene in mTECs [13]. Bioinformatics analysis

Abbreviations: TEC, thymic epithelial cell; MHC, major histocompatibility complex; mTEC, medullary TEC; Aire, autoimmune regulator; mOVA, membrane-bound form of ovalbumin; TCR, T cell receptor; UEA-1, *Ulex europaeus* agglutinin-1; PE, phycoerythrin; PCR, polymerase chain reaction; RT, reverse transcription.

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revealed that in the absence of Jmjd6, intron 2 of *Aire* was not efficiently spliced out of the pre-mRNA due to the unique 3′ splice site sequence of the gene.

Here we show that TEC-specific deletion of *Jmjd6* exacerbates development of autoimmune diabetes in a mouse model (OT-I^{+}RIP-mOVA^{+} mice), which express both ovalbumin (OVA) under the control of the rat insulin gene promoter and OT-I T cell receptor specific for OVA peptide bound to major histocompatibility complex class I K^{b} molecules. Jmjd6 deficiency resulted in retention of *Aire* intron 2 and a consequent decrease in Aire protein expression. Our present results thus demonstrate the importance of intronic regulation in the control of Aire expression in a more physiological setting compared with our previous transplant model.

2. Materials and methods

2.1. Animal treatment

Mice were maintained under specific pathogen–free conditions in the animal facility of Kyushu University. This study was approved by the Committee on Ethics Regarding Animal Experiments of Kyushu University and was performed according to the guidelines of the American Physiological Society. *Jmjd6*$^{tm1a(EUCOMM)Wtsi}$ mice were obtained from Helmholtz Zentrum München. B6(Cg)-Foxn1tm3(cre)Nrm/J (Foxn1-Cre) mice and C57BL/6-Tg(Ins2-TFRC/OVA)296Wehi/WehiJ (RIP-mOVA) mice were obtained from The Jackson Laboratory. B6-Tg(CAG-FLPe)37 mice (RBRC01835) were provided by RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

2.2. Histopathology and immunofluorescence analysis

Histopathology and immunofluorescence analysis were performed as described previously [13]. For histopathology, tissue was fixed with 4% formaldehyde, embedded in paraffin, sectioned at a thickness of 3 μm, stained with hematoxylin-eosin, and examined with a light microscope. For immunofluorescence analysis, tissue was embedded in OCT compound (Sakura Finetech), frozen at −80 °C, sectioned with a cryostat at a thickness of 8 μm, fixed by immersion for 10 min in ice-cold acetone, and exposed to 10% horse serum (Sigma-Aldrich) before incubation first with biotinylated *Ulex europaeus* agglutinin-1 (UEA-1) (Vector Laboratories) and then with Alexa Fluor 488–conjugated antibodies to mouse Aire (5H12, eBioscience), phycoerythrin (PE)–conjugated antibodies to mouse CD80 (16-10A1, eBioscience), and Alexa Fluor 647–conjugated streptavidin (Jackson Immuno Research).

2.3. Evaluation of lymphocytic infiltration in pancreatic islets

Islets were evaluated according to the level of lymphocyte infiltration in a double-blinded manner with the use of a bright-field microscope at a magnification of 100 × . The number and size of islets were taken into account in the evaluation. The pancreatic tissue was thus scored from 0 to 3 as follows: 0, normal islets; 1, infiltration of lymphocytes in the peri-insular area; 2, infiltration of lymphocytes in islets; or 3, islets are reduced in number and size and manifest a disturbed architecture.

2.4. Flow cytometry

Flow cytometry was performed with fluorescein isothiocyanate–conjugated antibodies to mouse CD3ε (145-2c11), PE-conjugated antibodies to mouse CD45 (30-F11), and PE-conjugated antibodies to mouse MHC class II (I-A/I-E) (M5/114.15.2) from eBioscience as well as with PE-conjugated antibodies to mouse CD8α (53-6. 7) and fluorescein isothiocyanate–conjugated antibodies to mouse CD45 (30-F11) from BD Biosciences. Before staining with these antibodies, cells were incubated for 10 min on ice with antibodies to Fcγ III/II receptor (2.4G2, BD Biosciences). Separated CD8^{+} T cells were also stained with allophycocyanin-conjugated OVA/H-2K^{b} tetramer (TS-5001-2, MBL). Flow cytometry was performed with a FACS Calibur instrument (BD Biosciences).

2.5. Sorting of TECs

TEC sorting was performed as described previously [13]. In brief, thymic lobes isolated from 4-week-old mice were cut into small pieces and digested for 1 h at 37 °C with 0.125% collagenase D–dispase (Roche) and 0.1% DNase I (Roche) in RPMI 1640 medium. TECs were enriched by depletion of CD45^{+} hematopoietic cells with the use of CD45 MicroBeads (Miltenyi Biotec) and were then stained with the relevant antibodies and reagents for sorting as CD45^{-}MHC class II^{+} cells with a FACS Aria instrument (BD Biosciences).

2.6. Diabetes assessment

Mice were screened for diabetes by testing of urine for glucose. with test strips (UA-PIG3, Terumo). Glycosuria was scored as recommended by the strip manufacturer.

2.7. Genomic PCR analysis

Genomic DNA was isolated from sorted TECs and subjected to polymerase chain reaction (PCR) analysis with the use of KOD plus polymerase (Toyobo) and the *Jmjd6*-specific primers 5′-TCGGAAGAAGGATTCCATGGG-3′ and 5′–CCCTGAAATCCAACAAATGAAGAA-3′ (forward and reverse, respectively).

2.8. RT-PCR analysis

Reverse transcription (RT) and PCR analysis was performed as described previously [13] with the following PCR primers (forward and reverse, respectively): *Aire* (exon 1 to exon 3), 5′-TAGACAGTGCCTTTCCGCTGCTGC-3′ and 5′-GGAGACGCTCTTTGAGGCCAGAGTTG-3′.

Band intensity was quantified with the use of a BAS-2500 image analyzer and Image Gauge 4.0 software (Fujifilm). The identity of all detected bands was confirmed by sequencing.

2.9. Statistical analysis

The lymphocytic infiltration score for the pancreas as well as the glycosuria ratio and score were compared among groups of mice with one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. The mature/immature transcript ratio for *Aire* was compared between groups with the two-tailed unpaired Student's *t*-test. A P value of <0.05 was considered statistically significant. Statistical analysis was performed with the use of Prism 6 software (SAS Institute).

3. Results

3.1. TEC-specific deletion of Jmjd6

To develop mice with TEC-specific deletion of *Jmjd6* (*Jmjd6*$^{\Delta/\Delta}$ mice), we crossed mice with a floxed allele of *Jmjd6* (*Jmjd6*$^{flox/flox}$) with Foxn1-Cre transgenic mice, which express Cre recombinase