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Phosphatase Wip1 Is Essential for the Maturation and Homeostasis of Medullary Thymic Epithelial Cells in Mice

Lina Sun,*1 Hongran Li,*1 Haiying Luo,* Lianjun Zhang,* Xuelian Hu,* Tao Yang,* Chenming Sun,* Hui Chen,* Jianfeng Zhang,† and Yong Zhao*

Thymic epithelial cells (TECs) are a key cell type in the thymic microenvironment essential for T cell development. However, intrinsic molecular mechanisms controlling TEC differentiation and activities are poorly defined. In this study, we found that deficiency of p53-induced phosphatase 1 (Wip1) in mice selectively caused severe medullary TEC (mTEC) maturation defects in an intrinsic manner. Wip1 knockout (KO) mice had decreased mature epithelial cell adhesion molecule

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L.S. designed and performed the experiments with cells and mice, analyzed data, and contributed to writing the manuscript; H. Li designed and performed the experiments, analyzed histology data, and contributed to managing the mouse colonies; H. Luo performed flow cytometry; L. Zhang performed TEC isolation; X.H. performed flow cytometry and mouse breeding; T.Y. performed the cell signal assays; C.S. performed the CD40 expression assays; H.C. performed real-time PCR assays; L. Zhang provided animal models and revised the manuscript; and Y.Z. designed experiments, analyzed data, wrote the manuscript, and provided overall direction.

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Abbreviations used in this article: BMC, bone marrow cell; CK, cytokeratin; cTEC, cortical thymic epithelial cell; Cy, cyclophosphamide; EptCAM, epithelial cell adhesion molecule; FGF, fibroblast growth factor; FTOC, fetal thymus organ culture; KO, knockout; LTβ, lymphotixin β; MHC II, MHC class II; mTEC, medullary thymic epithelial cell; RANK, receptor activator for NF-κB; TEC, thymic epithelial cell; TNRFSF, TNFR superfamily; UEA-1, Ulex europaeus agglutinin-1; WT, wild-type.

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Materials and Methods

Mice

Wip1KO mice and GFP-transgenic mice were provided by the Key Laboratory of Human Diseases Comparative Medicine, Ministry of Public Health (Beijing, China). Wip1KO mice have been described previously (15, 19) and were backcrossed to the C57BL/6 genetic background in our laboratory (20). BALB/c nude mice were purchased from Beijing University Experimental Animal Center (Beijing, China). All mice were bred and maintained in specific pathogen-free conditions. Sex-matched littermate mice 4–6 wk of age were mainly used for experiments. All animal experiments were performed in accordance with the approval of the Animal Ethics Committee of the Institute of Zoology, Beijing, China.

Abs and flow cytometry

The following biotinylated or fluorochrome-conjugated Abs were used in flow cytometry detection. Biotinylated Ulex europaeus agglutinin-1 (UEA-1) (clone 5H12; eBioscience) and Ki-67 (BD Pharmingen) were performed according to the manufacturer’s protocol. Intracellular staining for Foxp3-PE (BD Pharmingen) or Alexa Fluor 610-R-PE (Invitrogen). Anti–CD4-PE (clone G04352), anti–CD80-PE (clone 16-10A1), anti–CD40-PE (clone 3/23), anti–CD8-PE/Cy5 (clone M5/144.15.2), anti–CD8βPE (clone 16-10A1), anti–CD40-PE (clone 3/23), anti–CD8-PE/Cy5 (clone 53-6.7), anti–RANK-PE (clone R12-31), and anti–LTβR-PE (clone SG11). Anti–MTS24 Ab was offered by Prof. Richard Boyd (Monash University). Surface staining of cell suspensions was performed in PBS/0.1% BSA/0.02% NaN3 solution at 4˚C. For flow cytometric analysis of phosphorylated p38 MAPK, Alexa Fluor 488 anti–p-p38 MAPK (pT180/ y182) (Cell Signaling Technology) and anti–p38 MAPK (BD Pharmingen) were performed according to the manufacturer’s protocol. Intracellular staining for Foxp3-PE (eBioscience), Aire–FITC (provided by Prof. Francois-Xavier Hubert, Walter and Eliza Hall Institute of Medical Research), or Aire-Alexa Fluor 647 (clone 5H12; eBioscience) and Ki-67 (BD Pharmingen) were performed using fixation buffer (eBioscience) and permeabilization buffer (eBioscience) according to the manufacturer’s protocols. Apoptosis of TECs was quantified by an in situ cell death detection kit (Roche) by flow cytometry following the manufacturer’s instructions.

Immunohistology and immunofluorescence

For analysis of thymic medulla and cortex by immunohistology, thymi from Wip1KO mice and age-matched WT control mice were fixed in 4% formalin and embedded in paraffin blocks. Sections (5 µm) were stained with H&E and examined by light microscopy. For immunofluorescence, serial section in 1 µm were embedded in Tissue Tek OCT compound and fixed in cold acetone and blocked in PBS/1% BSA, washed in PBS/0.05% Tween 20, and incubated with optimal dilutions of anti–cytokeratin 5 (Abcam) and anti–cytokeratin 8 Troma–I; Developmental Studies Hybridoma Bank, Iowa City, IA), biotinylated UEA-1, and Alexa Fluor 488 goat anti–I-A/E for 2 h at room temperature before washing and incubating with secondary reagents, that is, Alexa Fluor 546 goat anti-rabbit IgG (H+L), Alexa Fluor 488 goat anti-rat IgG (H+L) (Invitrogen), and streptavidin-PE. Control slides were incubated with isotype-matched Ig. Images were acquired with two-photon microscopy (Carl Zeiss). For calculating thymic area, images from immunofluorescence and H&E staining were captured with the CRI Nuance v2.8 multispectral imaging system (Cambridge Research and Instrumentation, Woburn, MA). The resulting images were mathematically unmixed into individual cytokeratin (CK5 substrate, CK8 substrate using spectra deduced from control specimens to remove nonspecific fluorescence. In-Form v1.2 software (CRI) was used to calculate area size and ratio.

Thymic stromal cell isolation and in vitro culture of TECs

Thymic stromal cells from postnatal thymi were isolated as previously described (21). In brief, freshly dissected thymi were cut into 1 mm3 pieces and washed with DMEM medium with 2% FBS several times to remove most thymocytes. The thymic fragments were then incubated at 37˚C for 10 min in 0.2 ml solution of dispase II collagenase D (equivalent to 0.1% w/v) with 20 U/ml DNase I (Sigma-Aldrich). Enzymatic treatment was repeated three times (the final incubation with collagenase/dispace enzyme mixture) until all fragments were dispersed. Gentle agitation was performed periodically at mid- and endpoints of each digestion. Cell suspensions from each digestion were pooled in PBS containing 1% FBS and 5 mM EDTA to neutralize digestion and remove cell aggregates. Cells were centrifuged, resuspended in DMEM with 2% FBS medium, and filtered to remove clumps. Phenotypes of TECs were analyzed by surface FACS staining.

For TEC culture, thymi from WT neonatal mice were digested as mentioned above. Small thymic fragments from each step were collected and pooled. Fragments were allowed to settle and washed twice with PCT medium (CnT07; CellTEC). The remaining thymic explants were plated in 48-well plates with CnT07 medium and cultured at 37˚C and 5% CO2 for several days, during which TECs outgrew other stromal cells. To determine the effect of p38 MAPK and STAT1 signaling on the expression of CD40, cultured TECs were treated with 50 µM p38 MAPK inhibitor (SB203580; Sigma-Aldrich) or 400 µM STAT1 inhibitor (MTA; Sigma-Aldrich). After 48 h of treatment, TECs were collected with trypsin (Sigma-Aldrich) digestion and analyzed for CD40 expression by FACS.

Fetal thymus organ culture

Fetal thymus organ culture (FTOC) was performed as described previously (7). Briefly, thymic lobes were isolated from embryos 16.5 d after coitus and were cultured for 4 d on the top of Nucelopor filters (Whatman) placed in DMEM medium supplemented with 10% FBS (Life Technologies), 2 mM l-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 mM 2-ME containing 1.35 mM 2-deoxyglucose (Sigma-Aldrich). To test the effect of cytokine treatment using FTOC, 2-deoxyglucose–treated fetal thymic lobes were cultured in DMEM plus 10% FBS with recombinant CD40L (2.5 µg/ml; PeproTech) or RANKL (1 µg/ml; Sigma-Aldrich). Four days after stimulation, the lobes were harvested for flow cytometric analysis.

Brdu incorporation assays

For BrdU (BD Biosciences) labeling, 4-wk-old Wip1KO and WT mice were injected with 1 mg/day BrdU in PBS i.p. for 2 consecutive days. Twenty-four hours after the secondary injection, thymic lobes were digested, and thymic stromal cells were enriched for flow cytometric analysis as mentioned above. BrdU incorporation was detected with a FITC–BrdU flow kit (BD Biosciences) according to the manufacturer’s protocol.

Cyclophosphamide treatment

Mice were injected i.p. with cyclophosphamide (Cy) at a dose of 100 mg/kg body weight per day for 2 consecutive days. The first day after Cy treatment was considered to be recovery day 1.

Quantitative RT-PCR

RNA was purified from WT and Wip1KO mTECs sorted by flow cytometry and characterized as CD45+EpCAM+UEA-1+. mRNA was prepared using an RNeasy Mini kit (Qiagen) and the cDNA library was generated with an RNeasy Mini kit (Qiagen) and the cDNA library was generated with an RNeasy Mini kit (Qiagen) and the cDNA library was generated with an RNeasy Mini kit (Qiagen) and the cDNA library was generated with an RNeasy Mini kit (Qiagen) and the cDNA library was generated with an RNeasy Mini kit (Qiagen). The mRNA was amplified using primers specific to genes of interest, and the resulting amplicons were sequenced using a BigDye Terminator cycle sequencing kit (Perkin-Elmer) according to the manufacturer’s instructions.

Bone marrow chimeras and fetal thymus transplantation models

Bone marrow cells (BMCs) from 12-wk-old B6-GFP mice were prepared. BMCs (1 x 107) were injected into the tail vein of lethally irradiated 6- to 8-wk-old WT and Wip1KO recipients to set up full chimeras as described (22, 23). Mice were sacrificed 8 wk after reconstitution, and the percentages of donor-derived cells were revealed as GFP+ by flow cytometry.

Thymic lobes from E16.5 Wip1KO and WT embryos were used for transplantation. Thymic lobes were surgically transplanted under the kidney capsule of adult nude recipients (24). Mice were sacrificed after 6 wk transplantation. Grafts of thymus were harvested and analyzed for TEC phenotype.

Statistical analysis

All data are presented as the means ± SD. A Student unpaired t test for comparison of means was used to compare groups. A p value of <0.05 was considered statistically significant.

Results

Wip1 deficiency selectively impairs mTEC maturation

Wip1KO mice, in which Wip1 expression was eliminated via gene targeting as reported previously (15), were bred back to have a B6
genetic background (20). These Wip1KO mice were fertile and displayed no gross abnormalities within 3 mo after birth. However, Wip1KO mice had a thymus defect as evidenced by the small thymus size, the low ratio of thymus weight to body weight, and decreased cell number of thymocytes compared with age-matched WT mice (p < 0.01, Fig. 1A–C), which is consistent with the previous report (18). To assess the effect of Wip1 deficiency on TECs, we analyzed the levels of CD45+ EpCAM+ TECs of Wip1KO and WT mice using FACS. The percentage and cell number of CD45+ EpCAM+ TECs in the thymus of Wip1KO mice were significantly lower than those in WT mice (p < 0.001, Fig. 1D–F). In addition to the decreased thymic size and cell number, thymus of Wip1-deficient mice had significantly smaller medulla compared with WT mice, as shown in the thymic sections stained with H&E or stained with mAbs against CK5 and CK8 (6, 9). Functional maturation of UEA-1+ mTECs affects the development and maturation of EpCAM+ mTECs, including MHC IIhigh, CD80+, CD40+, and Aire+ cells, was markedly decreased in Wip1KO mice (p < 0.001, Fig. 2D–F). In line with the impaired maturation phenotypes of mTECs, the expressions of Aire-dependent and -independent tissue-restricted Ags, such as spl1, insulin 2, Tff3, Mup1, nAchr-1, GAD67, and CRP in sorted Wip1-deficient mTECs, were significantly decreased as determined by real-time PCR assays (Fig. 2G, Table I). It has been reported that mTECs might further differentiate into terminally differentiated involucrin+ mTECs and form Hassall’s corpuscles (31). Indeed, Wip1KO mice had defective Hassall’s corpuscles as determined by H&E staining (Fig. 2H) and a significantly decreased cell number of involucrin+ UEA-1+ mTECs in the thymus as assayed by flow cytometry (p < 0.001, Fig. 2I). Thus, Wip1 deficiency caused a defect of mTEC maturation in mice.

In contrast to mTECs, the total cell numbers of cTECs including EpCAM+BP-1+ and EpCAM+BP-1+CD40+ cells were indistinguishable in Wip1KO mice compared with WT mice (p > 0.05, Fig. 3A, 3B). The percentage of EpCAM+BP-1+ cells in EpCAM+ cells was increased in Wip1KO mice compared with WT mice (Fig. 3A), possibly due to the decreased mTEC components (p < 0.001, Fig. 2B). Thus, Wip1 deficiency selectively affects the development and maturation of EpCAM+ UEA-1+ mTECs in the thymus.

The distribution of thymocyte subsets, including CD4+CD8−, CD4+CD8+, CD4−CD8−, and CD4−CD8+ thymocytes, was altered in Wip1KO mice (p < 0.05, Fig. 3C) as reported previously (18). The total cell numbers of these thymocyte subsets were decreased in Wip1KO mice (p < 0.001, Fig. 3C), mainly due to the dramatic decrease of the total cell number of thymocytes (Fig. 1C). These changes also indirectly support the functional alter-

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**FIGURE 1.** Selectively decreased thymic medullary region was detected in Wip1KO mice. (A) Representative photographs of thymus organs in WT and Wip1KO mice is shown. (B) Ratios of thymus weight to body weight in Wip1KO mice (●) were significantly lower than in WT mice (○). (C) Total cell numbers of thymocytes in WT (open bar) and Wip1KO (filled bar) mice are presented. (D) Representative FACS analysis of CD45+ EpCAM+ TECs in the isolated thymic cells is shown. The percentage (E) and the total cell number (F) of CD45+ EpCAM+ TECs in WT and Wip1KO mice are shown. (G) H&E staining of the thymus of 7-d-old WT and Wip1KO mice is shown. (H) The ratio of thymic medullary region to cortical region in 7-d-old Wip1KO mice was less than in WT mice. (I) The CK5/CK8 staining of the thymus of 7-d-old WT and Wip1KO mice is presented. (J) The area of thymic medullary region in 7-d-old Wip1KO mice was smaller than in WT mice. (K) The ratio of CK5/CK8 area in the thymus of 7-d-old Wip1KO mice was less than in WT mice. Data presented are means ± SD (n = 6). Representative results are shown from one of three independent experiments performed. **p < 0.01, ***p < 0.001 (WT versus Wip1KO mice).
ation of thymic epithelium in Wip1-deficient mice. The percent-
age of Foxp3+CD4+CD8^-thymocytes, identified as a phenotype of natural regulatory T cells (32, 33), was significantly increased in the thymi of Wip1KO mice (p < 0.001, Fig. 3D). However, the total cell number of Foxp3+CD4+CD8^-thymocytes in Wip1KO mice was similar to those found in WT mice (Fig. 3D). These data indicate that the development of Foxp3+CD4+CD8^-natural regulatory T cells in Wip1KO thymus might not be impaired and the increased percentage of Foxp3+CD4+CD8^-natural regulatory T cells in Wip1KO thymus was likely caused by the decreased Foxp3+CD4+CD8^-thymocytes.

Wip1 intrinsically regulates mTEC development

Does Wip1 affect the EpCAM+UEA-1^+ mTEC population intrinsically? When we adoptively transferred WT or Wip1-deficient BMCs, respectively, into lethally irradiated syngeneic recipients to establish full hematopoietic chimeras, identical thymocyte numbers, percentages of CD45^+EpCAM^+TECs and EpCAM^+UEA-1^+ mTECs, and cell numbers of EpCAM^+UEA-1^+ mTECs were observed in recipients receiving Wip1-deficient BMCs and those receiving WT BMCs (p > 0.05, Supplemental Fig. 1), indicating that Wip1 deficiency in hematopoietic-derived cells, such as thymocytes, B cells, and dendritic cells, does not significantly impact mTEC development. Reversely, Wip1-deficient mice grafted with WT BMCs showed smaller thymus size, decreased cell numbers of TECs, and decreased percentages of EpCAM^+UEA-1^+MHC II^high^, CD40^+^, and Aire^+^ cells in the gated thymic EpCAM^+UEA-1^+ cells of WT and Wip1KO mice were summarized. (F) Total cell numbers of EpCAM^+UEA-1^+MHC II^high^, CD40^+^, and Aire^+^ cells in the gated thymic EpCAM^+UEA-1^+ cells of WT and Wip1KO mice are summarized. (G) The expression of Aire-dependent and -independent tissue-restricted Ags in sorted WT and Wip1KO mTECs were determined by real-time PCR. Data are representative of two to three independent experiments. Data presented are means ± SD (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001 compared with WT controls. (H) H&E staining of WT and Wip1KO thymus is shown. The poor Hassall’s corpuscle in Wip1KO thymus is noted (upper panel, original magnification ×20; lower panel, original magnification ×40). (I) Cell number of involucrin^+UEA-1^-cells in the thymi of WT and Wip1KO mice. Data presented are means ± SD (n = 5). ***p < 0.001 compared with WT mice.

FIGURE 2. Mature mTECs were significantly decreased in Wip1-deficient mice. (A) FACS analysis of CD45^+EpCAM^+UEA-1^- cells of 8-wk-old WT and Wip1KO mice. (B) Percentage of UEA-1^-cells among the gated CD45^+EpCAM^+cells in WT and Wip1KO mice. (C) The cell number of CD45^+EpCAM^+UEA-1^- cells in the thymus of WT and Wip1KO mice is shown (mean ± SD, n = 7 mice of each genotype). (D) Phenotypic characterization of MHC II, CD40, and Aire expressions in the gated thymic EpCAM^+UEA-1^-cells of WT and Wip1KO mice. (E) Percentages of MHC II^high^, CD80^+^, and Aire^-^ cells in the gated thymic CD45^+EpCAM^+UEA-1^-cells of WT and Wip1KO mice are summarized. (F) Total cell numbers of CD45^+EpCAM^+UEA-1^-MHC II^high^, CD40^+^, CD80^+^, CD40^+^, and Aire^-^ cells in the gated thymic CD45^+EpCAM^+UEA-1^-cells of WT and Wip1KO mice are summarized. (G) The expression of Aire-dependent and -independent tissue-restricted Ags in sorted WT and Wip1KO mTECs were determined by real-time PCR. Data are representative of two to three independent experiments. Data presented are means ± SD (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001 compared with WT controls. (H) H&E staining of WT and Wip1KO thymus is shown. The poor Hassall’s corpuscle in Wip1KO thymus is noted (upper panel, original magnification ×20; lower panel, original magnification ×40). (I) Cell number of involucrin^+UEA-1^-cells in the thymi of WT and Wip1KO mice. Data presented are means ± SD (n = 5). ***p < 0.001 compared with WT mice.

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Meanwhile, the decreased percentages and cell numbers of EpCAM+UEA-1+ mTECs and mature EpCAM+UEA-1+ mTECs such as CD80+, Aire+ cells in Wip1KO thymic grafts were detected (p < 0.05, Fig. 4K). Functionally, the cell numbers of thymocyte subsets, including CD4+CD8−, CD4+CD8+, CD4−CD8−, and CD4−CD8+ cells in Wip1KO thymic grafts, were significantly lower than in WT thymic grafts (p < 0.001, Fig. 4L). Based on these data, we concluded that Wip1 is important for regulating the EpCAM+UEA-1+ mTEC maturation compartment pool in an intrinsic manner.

To determine whether Wip1 is involved in thymic epithelium regeneration after thymus damage, we detected the recovery efficiency of TEC subsets in 8-wk-old Wip1KO and WT mice treated with Cy. Because Wip1KO mice had a smaller thymus than did WT mice before Cy treatment, we chose the recovery ratio of cell number as a parameter to determine the thymic regeneration ability in these assays. As shown in Fig. 5, by 14 d after Cy treatment, WT thymus weight and thymocyte subsets were recovered at 70–80%.
of normal levels, but Wip1KO mice were recovered at <50% (p < 0.001, Fig. 5A–C). Meanwhile, the CD45<sup>+</sup> EpCAM<sup>-</sup> TECs including MHC II<sup>high</sup> and MHC II<sup>low</sup> subsets recovered 70% of normal levels in WT mice, but these cell populations reached only ∼35% in Wip1KO mice (p < 0.01, Fig. 5D, 5E). Whereas the recovery of cTECs was identical in WT and Wip1KO mice (p > 0.05, Fig. 5F), mTECs including relatively mature mTECs such as MHC II<sup>high</sup>, CD80<sup>+</sup>, CD40<sup>+</sup>, and Aire<sup>+</sup> cells were unable to be recovered efficiently in Wip1KO mice after Cy treatment (p < 0.01, Fig. 5G, 5H). These data suggested that Wip1 deficiency decreased the regenerative potentiality of mTECs after tissue damage.

The thymus originates from the third pharyngeal pouch endoderm during embryogenesis. Thymus organ development progresses from initial thymus cell fate specification through multiple stages of cTEC and mTEC differentiation (2). Does Wip1 deficiency impact thymus organogenesis and TEC development in the fetus? We studied the embryonic 16.5-d-old fetus and failed to detect any significant alteration of the fetal thymus with respect to thymus size, thymocyte number, as well as percentage and cell number of mTECs and cTECs when analyzed by multicolor flow cytometry (Supplemental Fig. 2). Thus, Wip1 is unlikely involved in fetal thymus development, but it participates in postnatal homeostatic maintenance of the thymic medullary epithelium.

Wip1 deficiency decreases mTEC proliferation and differentiation kinetics
The mTEC defect of Wip1KO mice might be due to an increase of cell death and/or a decrease in the differentiation of mTECs from progenitors. To address the first possibility, we detected the cell death rate of TECs, mTECs, and cTECs as assayed by TUNEL staining. TECs in Wip1KO mice showed similar cell death kinetics as those of WT mice (Supplemental Fig. 3). Alternatively, the expression of apoptosis-related genes such as Bcl-x<sub>L</sub>, Bcl-2, and Bax in TECs and mTECs was also identical in WT and Wip1KO mice (data not shown). Thus, it is unlikely that the decreased frequency and cell numbers of mTECs in the absence of Wip1 are due to the altered survival ability of mTECs.

It is speculated that the turnover time for mature CD80<sup>+</sup> mTECs in postnatal thymus is between 2 and 3 wk (34, 35). We next
FIGURE 5. Delayed recovery of mature mTECs was observed in Wip1KO mice after Cy treatment. (A) The recovery ratio of thymocyte cell number in Wip1KO mice was lower than in WT mice after Cy treatment. (B) Flow cytometry analysis of CD4 and CD8 expressions in thymocytes of WT and Wip1KO mice treated with Cy or not is shown. (C) The delayed recovery of thymocyte subsets in Wip1KO mice was observed. (D) Representative FACS staining of thymic CD45 EpCAM− cells of WT and Wip1KO mice is shown. (E) The recovery ratio of CD45 EpCAM−, CD45 EpCAM−MHC II80, and CD45 EpCAM+MHC II80 cells in Wip1KO mice were slower than in WT mice after Cy treatment. (F) The recovery ratios of CD45 EpCAM+BP-1+, CD45 EpCAM+BP-1+MHC II80, and CD45 EpCAM+BP-1+MHC II80 cells in WT and Wip1KO mice after Cy treatment were identical. (G) Representative FACS profiles of MHC II, CD40, and Aire expressions in the gated thymic CD45 EpCAM− cells of WT and Wip1KO mice are shown. (H) The recovery of CD45 EpCAM−UEA-1+MHC II80, CD45 EpCAM−UEA-1+CD40−, CD45 EpCAM−UEA-1+CD80+, and CD45 EpCAM−UEA-1+ Aire− cells in Wip1KO mice was slower than in WT mice after Cy treatment. Data are means ± SD (four to seven mice per group) from one of two independent experiments. ***p < 0.001, **p < 0.01, *p < 0.05 (WT versus Wip1KO mice).

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It was reported that bipotent progenitor cells for both mTECs and cTECs displayed an MTS24+ phenotype (3, 4). To see whether Wip1 deficiency impacts thymic epithelial precursor/progenitor cells, we detected MTS24+EpCAM+ cells in the thymus of Wip1KO and WT mice. Although the percentage of MTS24+EpCAM+ cells was increased in Wip1KO mice (p < 0.001, Fig. 6A, 6B). Similarly, by BrdU incorporation assay in vivo, the percentage and absolute cell number of BrdU+ cells were significantly lower in mTECs but not in TECs and cTECs of Wip1KO mice compared with the controls (p < 0.01, Fig. 6C, 6D).

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The upregulation of MHC II, CD80, and CD40 molecules and the transcriptional regulator Aire occur during UEA+ mTEC differentiation and maturation (26–30). Expression levels were used to identify the developing stages of mTECs. Mature TECs express the highest levels of CD80 and Aire to be a CD80−Aire− phenotype (35, 36), although some controversy exists in this respect (30). Precursor-product analysis studies have shown that progenitors of MHC II80CD80−Aire− mTECs were contained within the MHC II80CD80−Aire− mTEC subset (35, 36). The percentage of CD80−Aire− mature mTECs decreased whereas the percentage of CD80−Aire− immature mTECs increased in the fraction of UEA-1+EpCAM+ mTECs in Wip1KO mice (p < 0.01, Fig. 6H, 6I). Importantly, the cell numbers of CD80−Aire− mature mTECs and CD80−Aire− intermediate mature mTECs were significantly decreased (p < 0.001, Fig. 6J) and the cell numbers of CD80−Aire− immature mTECs were slightly but significantly decreased in Wip1KO mice compared with WT mice (Fig. 6J). Thus, the ratio of CD80−Aire− mature mTECs to CD80−Aire− immature mTECs was markedly lower in Wip1KO mice than in WT mice (Fig. 6K), indicating the disrupted process kinetics of mTEC differentiation in Wip1KO mice. Therefore, these data collectively illustrate that decreased cell proliferation and/or maturation during progenitor cell differentiation into mature mTECs may contribute to poor medullary epithelial compartment of Wip1-deficient mice.

Wip1 regulates mTEC differentiation through a p38 MAPK/CD40 signal pathway

To date, three TNFRSF members, that is, RANK, CD40, and LTβR, have been shown to play a crucial role in thymus medulla development. Mice deficient of RANK, CD40, and LTβR, respectively, displayed absence or abnormality of mTECs (7, 36–38). Additionally, fibroblast growth factors (FGFs) and Wnt4 signaling could also boost thymopoiesis as well as promote differentiation by working on both thymocytes and TECs (39, 40). To identify the molecular mechanisms that mediate the function of Wip1 in
mTEC proliferation and differentiation, we examined the expression of TNFRSF members Wnt4 and FGF receptor (FGFR2IIIb) in Wip1KO mTECs by protein and mRNA levels. Wip1KO UEA-1+ mTECs expressed similar levels of RANK and LTβR as did WT cells as determined by FACS (Fig. 7A). Real-time PCR assays showed that the mRNA levels of RANK, LTβR, Wnt4, and FGFR2IIIb in Wip1KO and WT mTECs were not significantly different (Fig. 7B). Consistent with decreased CD40 protein expression on mTECs as described above (Fig. 2), the CD40 mRNA expression in sorted Wip1KO mTECs was significantly lower than in WT mTECs (Fig. 7B). Therefore, Wip1 may control mTEC development and differentiation by governing CD40 expression.

The molecular signaling pathways that regulate CD40 expression in mTECs, and even in macrophages, have not been well defined. To understand the molecular mechanisms that mediate Wip1 function in the differentiation of mTECs, we assessed the potential involvement of intracellular downstream pathways of Wip1, including p38 MAPK and STAT1 (20), in regulating CD40 expression. The freshly isolated mTECs, but not cTECs, from Wip1KO mice expressed higher levels of p38 MAPK activity than that of WT mice (p < 0.001, Fig. 7C). Interestingly, in the in vitro TEC culture system, inhibition of p38 MAPK but not STAT1 increased CD40 expression on mTECs (p < 0.01, Fig. 7D). In the FTOC system, inhibition of p38 MAPK significantly increased the percentage of CD80+Aire+ mTECs (Fig. 7E). Adding CD40L significantly enhanced the percentages and cell numbers of CD80+Aire+ mTECs in WT thymi (Fig. 7F, 7G). However, CD40L failed to induce CD80+Aire+ mTECs in Wip1KO thymi (p < 0.01, Fig. 7F, 7G). Addition of RANKL in the FTOC system induced more CD80+Aire+ mTECs in WT and Wip1KO thymi without significant difference (p > 0.05, Fig. 7F, 7G). All of these data demonstrated that the CD40 signaling pathway was impaired in Wip1KO mice. Therefore, p38 MAPK inhibits CD40 expression on mTECs and Wip1 deficiency increases p38 MAPK activity to downregulate
CD40 expression, which subsequently blocks mTEC differentiation and maturation.

**Discussion**

In the present study, we provide evidence that Wip1 plays a key role in the control of mTEC development and maturation in both physical and pathological situations in an intrinsic manner. Wip1 deficiency in thymic epithelium selectively caused a severe defect of mTEC differentiation, as indicated by the decreased mTEC/cTEC ratio, medullary thymic zone, and the percentage and total cell number of mTECs in Wip1KO mice, WT bone marrow cell–transplanted Wip1KO mice, and fetal Wip1KO thymus-grafted mice. The significantly decreased mTECs including CD80^+ Aire^+ and involucrin^+ Aire^+ mTECs in unchallenged postnatal Wip1KO mice, as well as the poor mTEC recovery in Cy-treated adult Wip1KO mice, support that Wip1 not only controls mTEC turnover and homeostasis in physiological situation but also impacts mTEC regenerative ability in pathological conditions.

The lineage commitment and differentiation process and the relevant mechanisms for mTEC development are still poorly understood. Accumulated studies suggest that CD80^+ MHC II^low or CD80^+ Aire^+ immature mTECs are derived from CD80^− MHC II^high or CD80^− Aire^− immature mTECs (36). The decreased mTEC lineage differentiation in Wip1-deficient mice is likely to be the key reason for reduced thymic medullary region in these mice, as supported by the following evidence. First, Wip1KO mice had a normal thymic epithelial precursor MTS24^+ cell pool and unimpaired early developing mTECs such as CD80^+ Aire^− cells, indicating that Wip1 is not involved in the early stages of mTECs. The ratio of mature-to-immature mTECs (CD80^+ Aire^+ mTECs of WT and Wip1KO thymus in FTOC cultured with CD40L and RANKL for 4 d is shown. (G) The percentage and cell number of CD80^+ Aire^+ mTECs of WT and Wip1KO thymus cultured with CD40L and RANKL are shown. Data presented are the means ± SD (n = 5 mice/group) from one of two independent experiments. **p < 0.01, ***p < 0.001 between the indicated groups.
also causes thymus abnormality (8). Additionally, FGFs and Wnt4 could boost thymopoiesis as well as promote differentiation of TECs (39, 40). Wip1KO UEA-1− mTECs expressed similar levels of RANK, LTβR, Wnt4, and FGFR2IIIb as WT cells as determined by FACS and real-time PCR assays. However, CD40 expression in Wip1KO mTECs was significantly lower than in WT mTECs. Therefore, Wip1 may regulate CD40 expression on mTECs to control mTEC development. A recent study has shown that CD40L/CD40 signaling controlled proliferation, but not the rate of apoptosis, within the mTEC compartment (41). These findings are consistent with our results in that Wip1 deficiency caused decreased proliferation but not apoptosis in mTECs.

To identify the molecular mechanisms that mediate the function of Wip1 in mTEC differentiation, we examined signaling pathways potentially activated by Wip1 in mTECs. We found that Wip1 deficiency caused the enhanced activation of p38 MAPK in mTECs. Notably, inhibiting p38 MAPK by SB203580, a specific pharmacological inhibitor of p38α and p38β MAPK, remarkably enhanced CD40 expression on mTECs, indicating that Wip1 regulated mTEC differentiation via the p38 MAPK/CD40 pathway. Note that the defects of LTβRKO, CD40KO, or TNFRSF11KO mouse lines in establishing the thymic medullary microenvironment appear to be substantially milder compared with TRAF6KO and RelBKO mice (7, 38). Thus, the decreased CD40 expression caused by Wip1 deficiency may be one of the altered pathways relevant to the severe defects of mTECs in Wip1KO mice. Other molecular pathways involved in the impaired mTEC development and maturation in Wip1KO mice should also be explored in the future. It was demonstrated that the activity of p53, which plays a central role in preserving genomic integrity, is one of the key target molecules attenuated by Wip1 in many different type of cells (42, 43). However, our preliminary study failed to detect the alteration of p53 activity in mTECs of Wip1-deficient mice. It was reported that the positive regulating role of Wip1 in T cell development in the thymus is dependent on the presence of p53 and its oncogenic activity (12, 18). The potential involvement of p53 in mTECs needs to be determined in the future.

Identical numbers and phenotypes of TECs were observed in the thymus of 16.5-d-old WT and Wip1KO fetus. Thus, Wip1 may not be involved in regulating mTEC development at the fetal stage. The different roles of Wip1 on mTEC maturation and homeostasis in fetal and postnatal stages may be explained by the distinct CD40 expression on mTECs, indicating that Wip1 regulated mTEC development via the p38 MAPK/CD40 pathway. Wip1, as the phosphatase identified to be an intrinsic positive master for TECs, may be a promising target for therapeutic intervention to modulate mTEC function.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Hemopoietic cell-specific Wip1 deficiency failed to cause mTEC alteration in bone marrow transplantation model.

A, Full chimeric mice were generated by transplanting either B6 WT or Wip1KO bone marrow cells (BMCs) to lethally irradiated B6-GFP mice. By 12 weeks after transplantation of BMCs, the TEC subsets were assayed. B, Representative photographs of thymus organs in recipient mouse received WT and Wip1KO BMCs. C, Total cell numbers of thymocytes in thymus of mice received WT (open bar) and Wip1KO (black bar) BMCs are presented. The percentage of CD45-EpCAM+ TEC cells (D), and the percentage (E) and cell number (F) of CD45-EpCAM+UEA-1+ cells in the thymus of mice reconstituted with WT and Wip1KO BMCs. Data were shown as mean±SD (n=6), which were one representative of two independent experiments.
Supplementary Figure 2. Wip1 is not involved in regulating fetal thymus development.

(A), Representative photographs of thymic lobes from E16.5 WT and KO embryos. (B), Total cell numbers of thymocytes in WT (open bar) and Wip1KO (black bar) mice are presented. (C), FACS analysis of MHCII expression in the gated thymic CD45-EpCAM+ TECs. (D), The percentages of UEA-1+ and BP-1+ cells in TECs, and MHCII+, CD80+, and Aire+ cells in the gated thymic CD45-EpCAM+UEA-1+ cells of WT and Wip1KO mice were presented. The percentage (E) and cell number (F) of TECs, MHCII$^{high}$ (TEC$^{hi}$) and MHCII$^{low}$ (TEC$^{lo}$) TECs were shown. (G) and (H), the percentage of indicated cell subsets were analyzed. Data were shown as mean±SD (WT n=4, KO n=5), which were one representative of three independent experiments.
Supplementary Figure 3. Apoptosis of TECs from WT and Wip1KO mice were analyzed by FACS. 
(A), Individual thymi from WT and Wip1KO mice were analyzed for apoptosis by the TUNEL assay. TUNEL+ cells in TECs, mTECs, and cTECs were gated. 
(B), Summary of the percentage of apoptotic TECs in indicated TECs from WT and KO mice. 
(C) and (D), Percentage of apoptotic cells in mTEC^{hi} (CD45-EpCAM^+UEA-1^+MHCII^{hi}) and mTEC^{low} were presented. 
(E) and (F), Percentage of apoptotic cells in CD80^{+}mTEC (CD45-EpCAM^+UEA-1^+CD80^{+}) and CD80^{+}mTEC were shown. Data were shown as mean±SD (n=4), which were one representative of two independent experiments.