ADAM10 is essential for proteolytic activation of Notch during thymocyte development

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Abstract
Notch signaling pathway has been shown to play essential roles in T lymphocyte development. Activation of Notch requires a sequential proteolytic cleavage, which converts Notch from the full-length membrane-bound form to a transcriptionally active intracellular fragment. Studies in Drosophila showed that Kuzbanian (Kuz) is responsible for the enzymatic cleavage of extracellular S2 site upon Notch binding to its ligand Delta. Both a disintegrin and metalloprotease (ADAM) 10 and ADAM17, members of the ADAM family metalloproteases, have been indicated as the mammalian counterpart of Kuz in activating Notch in mammals. Here, we investigated functions of ADAM10 in Notch signaling during thymocyte development. We show that conditional disruption of the Adam10 gene in mouse thymocytes results in a developmental defect similar to the phenotypes previously described for T lineage-specific disruption of Notch1. We further show that the activation of Notch1 and its downstream target genes Deltex-1 and Pre-Ta are impaired in Adam10-deficient thymocytes. Our study demonstrates a T cell intrinsic role for Adam10 in activation of Notch1 during thymocyte development.

Introduction
The development of T lymphoid lineage follows a series of differentiation and selection events prior to their exit of the thymus. The canonical differentiation pathway is operationally divided into CD4CD8 double-negative (DN), CD4CD8 double-positive (DP) and CD4 or CD8 single-positive (SP) stages. TCR gene rearrangement starts at the DN stage where cell fates are further divided into the αβ or γδ lineage due to expression and selection of the appropriate TCR chains (1). While a small fraction of DN cells choose the γδ fate, most DN cells enter the αβ lineage upon successful rearrangement and expression of a functional TCRβ chain (2). These TCRβ-expressing cells quickly expand and differentiate into the DP stage where TCRγ gene rearrangement occurs. DP cells are further selected for expression of a functional αβTCR before differentiating into the CD4 helper or CD8 cytotoxic SP stage. T cells expressing strong autoreactive TCR are eliminated through negative selection whereas the remaining mature CD4 and CD8 T cells leave thymus and enter peripheral lymphoid tissues.

Notch signals play critical roles in several stages of T cell development (3), including T cell-fate determination, TCRβ gene rearrangement, DN thymocyte differentiation, DP thymocyte survival, CD4/CD8 differentiation in the thymus and later on T1/δ,2 differentiation in the periphery (4). Notch receptor is a transmembrane protein, which is activated upon interaction with its ligand such as Delta-like and Jagged expressed on neighboring cells (5). The ligand binding triggers a series of proteolytic cleavage events, leading to the production of the transcriptionally active intracellular domain of Notch (ICN) (6). ICN enters nucleus to form a transcription complex with CBF-1, Suppressor of hairless and Lag-1 and together they activate Notch target genes. An important step in Notch activation is the cleavage of extracellular domain of Notch at the S2 site (7, 8). Both genetic and biochemical studies in Drosophila have demonstrated that a metalloprotease encoded by kuzbanian (kuz) is directly responsible for S2 cleavage and activation of Notch. In mammals, this cleavage is accomplished by one of a disintegrin and metalloprotease (ADAM) family members. Both ADAM10 and ADAM17 are reported as...
the potential sheddase to cleave Notch at the S2 site (9–11). Which Adam gene is responsible for activation of Notch signals in T cell development has not been firmly defined.

The specific cleavage of Notch S2 site by ADAM17 has been shown in cultured mononcytic precursor cells (9, 10). However, the phenotype of Notch1 knockout mice resembles Adam10 rather than Adam17 knockout mice (12, 13). Because Adam10 knockout resulted in embryonic lethality, further genetic study of relationship between Adam10 and different Notch genes in the lymphoid system was not possible with these conventional knockout mice. A role for Adam10 in regulating T cell development has been suggested only recently by the study of transgenic mice expressing a dominant-negative form of ADAM10 (14). The developmental defects revealed in the transgenic mice were very similar to the reported phenotypes of conditional developmental defects revealed in the transgenic mice were suggested only recently by the study of transgenic mice expressing a dominant-negative form of ADAM10 (14). The developmental defects revealed in the transgenic mice were very similar to the reported phenotypes of conditional disruption of Notch1 (14–16). However, it is not known whether Adam10 is the one and only protease inhibited by the dominant-negative transgene. In fact, a recent study has demonstrated a T cell extrinsic role for Adam17 in regulating T cell development even though Adam17 is expressed in both developing T cells and thymic stromal cells (17). To explicitly define Adam10 function in T cell development, we generated a conditional knockout allele of Adam10 and used it to test function of Adam10 in T cell development. Our study establishes a T cell intrinsic role for ADAM10 in activation of the Notch1 pathway during T cell development.

Methods

Establishment of Adam10 conditional deletion allele
Genomic DNA fragments surrounding exon 3 of the Adam10 gene were isolated from a λ phage library (Stratagene). The 5396-bp XbaI–KpnI fragment of intron 3, the 576-bp PstI–XbaI fragment containing exon 3 and the 1473-bp StuI–XbaI fragment of intron 2 from the Adam10 gene were used as the long arm, center fragment and short arm in the targeting vector, respectively. Compared with the wild-type genomic DNA, a 337-bp XbaI–PstI fragment in intron 2 was deleted in the final construct. The targeting construct was introduced into W4/129S6 embryonic stem cells (Taconic) by electroporation. PCR screen with primers KOMOCK (F) and MORC2-2 (Table 1) identified nine positive clones from 352 G418-resistant ES clones. These positive clones were further verified (Table 1) identified nine positive clones from 352 G418-resistant ES clones. These positive clones were further verified by Southern analysis. Germ line transmission of the targeting allele was obtained from two independent ES clones. The pgk-neo cassette was then deleted by crossing the positive offspring with Act-FLPe mice (Jackson Laboratory). The resulting floxed allele was labeled as Adam10fl<sup>neo</sup>. All mice were bred and maintained in Institute of Developmental Biology and Molecular Medicine Animal Facility following the general guideline of AAALAC handbook and institutional regulations.

FACS analysis and cell sorting
Single-cell suspensions from the thymus, spleen or lymph nodes were prepared in pre-cooled PBS supplemented with 5% bovine calf serum and stained for four-color FACS analysis. Data were collected and analyzed with CellQuest on a FACSCalibur (BD Biosciences). Cell sorting was performed on a FACSVintageSE (BD Biosciences). Antibodies used were FITC-conjugated anti-CD4 (Caltag and eBioscience), PE-conjugated anti-CD8b (Caltag), APC-conjugated anti-TCR<sub>b</sub> (Caltag), FITC-conjugated anti-TCR<sub>d</sub> (Caltag), PE-conjugated anti-CD19 (Caltag), PE-conjugated anti-TCR<sub>b</sub> (eBioscience), PE Cy5-conjugated anti-CD4 (Caltag), PE Cy5-conjugated anti-CD8 (eBioscience), FITC-conjugated anti-CD4 (Caltag), APC-conjugated anti-CD8b (Caltag), APC-conjugated anti-CD25 (Caltag) and PE Cy5-conjugated anti-B220 (Caltag). DN, DP, CD4 or CD8 SP thymocytes were sorted after exclusion of cells stained positive for 7-aminactinomycin D (7AAD, Invitrogen) and B220. Intracellular staining was done by 2% PFA fixation followed by permeabilizing in 0.5% saponin.

Western blotting
Thymus was dispersed into single-cell suspension in pre-cooled PBS and 5% bovine calf serum, incubated on ice for 30 s, passed through a 200-mesh size filter and spun at 4°C. Cell pellets were then homogenized in 1× RIPA buffer (Santa Cruz Biotechnology) and 1× complete protease inhibitor mixture (Roche Biochemical Laboratories). Extracted proteins were quantified with a BCA<sup>TM</sup> Protein Assay Kit (Pierce). Forty micrograms of protein per sample was used for electrophoresis on 8% SDS–PAGE gel and transferred onto polyvinylidene difluoride membrane (Millipore). Rabbit anti-mouse cleaved Notch1 antibody (1/1000; 2421s; Cell Signaling Technology) and bovine anti-rabbit HRP antibody (1/2000; Sc-2370; Santa Cruz Biotechnology) were used to detect Notch proteins. Goat anti-mouse actin antibody (1/400; Sc-1615; Santa Cruz Biotechnology) and bovine anti-goat HRP antibody (1/1000; Sc-2350; Santa Cruz Biotechnology) were used to detect actin. Enhanced chemiluminescence reagent (Pierce) was applied after antibody incubation in both experiments. For stripping, the membrane was sequentially incubated in TBST, H<sub>2</sub>O, 0.2 M NaOH, H<sub>2</sub>O

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<th>Table 1. Primers used in PCR and RT-PCR analysis</th>
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and TBST at room temperature for 5 min each step. The stripped membrane was blocked with TBST-5% milk for 3 h at room temperature before additional round of blotting.

Real-time PCR assay for gene expression

Total RNA was isolated with TRIzol (Invitrogen) and treated with RNase-free deoxyribonuclease I (TaKaRa) to eliminate genomic DNA contamination. Reverse transcription (RT)–PCR was carried out with TaKaRa RNA PCR Kit (AMV) according to the manufacturer’s instructions. Real-time PCR was performed with ABSolute QPCR SYBR Green Mixes (ABgene) or 2× HotSybr PCR Reaction Mix (NuStar Laboratory) on an Mx3000P Quantitative PCR System (Stratagene) following the manufacturer’s instructions. Expression of β-actin was used as the baseline standard for real-time PCR. The primer sequences are listed in Table 1.

Statistics

P values were obtained with two-tailed unpaired Student’s t-test on Prism 4 (GraphPad Software).

Results

Thymocyte-specific deletion of the Adam10 gene

To investigate the function of the Adam10 gene in thymocyte development, we established an Adam10 conditional deletion mouse strain. The floxed Adam10 allele, named Adam10loxP, contains two loxP sites in cis orientation flanking the third exon of Adam10 (Fig. 1A). Cre-mediated Adam10 deletion in germ line resulted in embryonic lethality around the same stage as a previous report (13) of the germ line knockout of Adam10 (data not shown). However, the Adam10loxP/loxP homozygous and the Adam10loxP/+/hemizygous mice are completely viable and fertile. A mouse line carrying the Lck-Cre transgene (18) was used to delete the genomic sequence between the two loxP sites in a T cell-specific manner. A PCR-based assay was used to evaluate the timing and efficiency of Adam10 deletion on FACS-sorted thymocytes (Fig. 1B). Three primers were used to simultaneously amplify the floxed and the deleted allele in the same PCR. Cre-mediated deletion was detected as early as the DN stage. The deleted allele became the major product in the DP cells, indicating that the majority of DP cells have lost the floxed allele. Because the size of the PCR product from the deletion allele is significantly larger than the floxed allele in this competitive PCR assay, the relative frequency of deleted allele is most likely an under estimation. No Adam10loxP allele can be detected in CD4 or CD8 SP cells by this PCR assay. Real-time RT–PCR analysis was used to further assess the extent of Adam10 deletion in total thymocytes (Fig. 1C). The exon 3 containing transcript of Adam10 in Adam10loxP/loxPLck-Cre mice was only 8% of Adam10loxP/loxP littermates, confirming that Adam10 was successfully disrupted in most thymocytes. Meanwhile, ADAM17 expression was not significantly altered in Adam10loxP/loxPLck-Cre mice (Fig. 1D).

Fig. 1. Conditional inactivation of Adam10 in developing thymocytes. (A) Schematic representations of the wide-type (WT), the floxed and the recombined Adam10 allele (deleted allele). Gray box: exon. Open arrowheads: loxP. Solid arrowheads: primers. (B) Deletion of Adam10 in thymocytes sorted from 2-month-old mice. The floxed and deleted alleles were determined by PCR with three primers [shown in (A)], which produced 1.6-kb band for the Adam10loxP allele and a 1-kb band for the deleted allele. M: 1-kb DNA ladder. (C) Real-time RT–PCR analysis of Adam10 expression. RNAs were extracted from total thymocytes of 2-month-old Adam10loxP/loxP (open bar, n = 4) and Adam10loxP/loxPLck-Cre mice (gray bar, n = 4). The average value and SEM are shown. Dots represent values from individual mice; P < 0.0001. (D) Real-time RT–PCR analysis of Adam17 expression. Data presentation is as (C); P > 0.05.
Impaired thymocyte development in Adam10 conditional knockout mice

Function of the Adam10 gene in T development was evaluated by FACS analysis of lymphocytes isolated from thymus, spleen and lymph nodes (Fig. 2A). Overall, the total thymic cellularity is decreased ~2- to 3-fold in Adam10<sub>loxP/loxP</sub>Lck-Cre mice (Fig. 2B). This decrease is primarily due to a reduction in the number of DP cells (Fig. 2C). The number of CD4 SP cells also showed a moderate decrease whereas numbers of CD8 SP cells and γδ T cells in the thymus are not affected by Adam10 disruption. To further enhance the efficiency of deletion, we also generated and tested the Adam10<sub>loxP</sub>-Lck-Cre mice, in which Cre-mediated deletion of the single Adam10<sup>loxP</sup> allele on the hemizygous background is sufficient to induce complete inactivation of Adam10. The phenotypic defects resulting from this deletion strategy is similar to those obtained with Adam10<sub>loxP/loxP</sub>Lck-Cre mice (Fig. 2B and C), confirming that the impaired thymocyte development is due to a significant loss of ADAM10. These results resemble the previously described Notch1 mutant mice, which carry homozygous Notch1 floxed allele and Lck-Cre transgene (15).

TCRβ expression is not significantly affected after Adam10 deletion

Notch signals have been shown to play a particularly important role in supporting αβ lineage development among T cell progenitors undergoing TCRβ rearrangement (2, 15). To further explore whether reduced number of DP cells is due to any impairment of TCRβ expression, we evaluated intracellular TCRβ (iTCRβ) expression in DN3 cells (Fig. 3). The percentage of iTCRβ-positive DN3 cells was found slightly increased from 29.4% (±2.1%) for the wild-type controls to 40.12% (±1.9%) for the mutant mice (Fig. 3B and C). The absolute numbers of DN3 cells for both iTCRβ-positive and -negative fractions were decreased in Adam10 conditional knockout mice (Fig. 3D). The level of iTCRβ expression in DN4 and DP cells was found indistinguishable between Adam10 mutant and Adam10 wild-type littermate controls (Fig. 3B). While this assay is not sensitive enough to detect

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**Fig. 2.** Decreased numbers of thymocytes in Adam10 conditional knockout (KO) mice. (A) FACS analysis of T cell compositions in thymi, spleens and lymph nodes. Thymocytes were isolated from 6-week-old littermate control (Adam10<sup>loxP/loxP</sup>) and Adam10<sup>loxP/loxP</sup>Lck-Cre mice, which were gated by exclusion of 7AAD-positive cells in the upper panel and by exclusion of 7AAD- and CD19-positive cells in the lower panel. Percentages of different subsets are indicated in the quadrants. (B) Reduction of thymic cellularity in 6- to 7-week old Adam10 conditional KO mice. Cell numbers from the littermate controls (including Adam10<sup>loxP/loxP</sup>, Adam10<sup>loxP/+</sup>, Adam10<sup>loxP</sup>-Lck-Cre mouse (gray bar) and Adam10<sup>loxP/loxP</sup>Lck-Cre mice (dark gray bar) are shown. The average value and SEM are shown. Values for individual mice are indicated by dots; *P < 0.0001. (C) Absolute cell numbers of different thymocyte subsets in thymi described in (A). Open bars: littermate controls (n = 19); gray bars: Adam10<sup>loxP/loxP</sup>Lck-Cre (n = 7); black bars: Adam10<sup>loxP/loxP</sup>Lck-Cre mice (n = 7). The average value and SEM are shown; *P < 0.01 and **P < 0.0001.
any subtle changes in the rearrangement status and repertoire usage involving the TCRβ locus, the result suggests that the reduced thymic cellularity, including DN3, DP and SP cells, in Adam10 conditional knockout mice is unlikely to lack of TCRβ rearrangement and expression.

**Failure of Notch activation in Adam10 conditional knockout mice**

To explore whether the impaired thymocyte development in Adam10 conditional knockout mice is due to a defect in Notch1 activation, we examined the Notch signaling pathway. A key step in Notch activation is the generation of ICN, the activated form of Notch (19). An antibody specific for ICN (20) was used in western analysis of protein extracts from total thymocytes. ICN was readily detected in Adam10loxP/loxP control mice and was significantly reduced in Adam10loxP/loxP/Lck-Cre mice (Fig. 4A). Real-time RT-PCR analysis showed that the RNA level of Notch1 is not affected by Adam10 knockout (Fig. 4B). Together, these results indicate that Adam10 knockout directly affect the Notch1 proteolysis rather than expression. To further assess Notch signaling pathway in Adam10 conditional knockout mice, we evaluated Notch1 target genes in thymocytes. Hes1, Deltex-1 and pre-Ta have been shown to be directly regulated by Notch1 in thymocyte development (21–23). Real-time RT-PCR analysis of total thymocytes showed that the transcription level of Deltex-1 was down-regulated in the mutant mice (Fig. 4C). The average mRNA level of Hes1 and pre-Ta in total thymocytes of mutant mice was not significantly altered or slightly reduced, respectively. Recent reports identified a role for Notch1 in regulating Gata3 during Tn1/Tn2 differentiation (4, 24). Given that Gata3 is also involved in CD4 SP cell development in the thymus, we examined Gata3 expression in Adam10 conditional knockout mice. Real-time RT-PCR analysis of total thymocyte RNA showed that Gata3 expression is not affected by Adam10 disruption (Fig. 4C), suggesting that Gata3 expression in thymocytes is not dependent on Notch signals.

**Discussion**

Although both ADAM10 and ADAM17 are implicated as the metalloprotease responsible for activation of Notch, which one is responsible for activation of Notch in thymocyte development remained as an outstanding issue. The
Adam10 knockout mice (open bars, n = 4). The average value and SEM are shown; *P < 0.01.

Fig. 4. Down-regulation of Notch signaling in thymocytes after deletion of Adam10. (A) Western analysis with ICN-specific antibodies (rabbit anti-mouse cleaved Notch1 antibody 2421s, Cell Signaling Technology) on total thymocytes of 2-month-old mice. β-actin was used as a loading control. (B) Notch1 transcription detected by real-time PCR. RNA samples were isolated from 2-month-old Adam10loxP/loxP (open bar, n = 4) and Adam10loxP/loxP;Lck-Cre mice (gray bar, n = 4). (C) Transcription of Notch targeting genes in thymus. Real-time PCR was performed to evaluate the expression level of Hes1, Dtx1, pre-Ta and Gata3 in total thymocytes of 2-month-old Adam10loxP/loxP (open bars, n = 4) and Adam10loxP/loxP;Lck-Cre mice (gray bar, n = 4). The average value and SEM are shown; *P < 0.01.

conditional Adam10 knockout study described here supports the idea that ADAM10 is a critical player in regulating Notch function during T cell development. Prior to the current work, the only data available to address this issue came from the transgenic study using a dominant-negative form of ADAM10 in thymocytes (14). Mice expressing the dominant-negative form of ADAM10 under the control of the Lck-proximal promoter exhibited a phenotype similar to Notch1 conditional knockout induced by the same Lck-proximal promoter (15). The overall loss of DP and SP thymocytes observed in our Adam10 conditional knockout mice are consistent with findings from these earlier reports. One noticeable difference came from the analysis of icTCRβ expression in DN3 cells. While conditional Notch1 knockout resulted in a reduced frequency of icTCRβ expression among DN3 cells (15), we find that the frequency of icTCRβ expression is slightly increased in DN3 thymocytes. Therefore, it is possible that ADAM17 which is preferentially expressed in DN thymocytes and thymic stromal cells (17) may play a compensatory role in activating Notch1 at the early stage of T cell development.

The various degrees of changes in expression of these Notch target genes could be partially explained by their expression patterns during T cell development and the Cre line used in this study. Hes1 and pre-Ta are expressed primarily in DN stage and dramatically down-regulated in DP and SP stage whereas Deltext-1 is expressed in the DN stage, moderately down-regulated in DP and up-regulated again in SP stage (25). Analysis of Adam10 deletion among sorted thymocytes showed that efficient deletion was not achieved until DP stage of development in the Lck-Cre mice. Therefore, the Notch signals may not be effectively disrupted until the DP stage in Lck-Cre-mediated conditional knockout system. In fact, the lack of significant change in pre-Ta expression is also reported in the analysis of Notch1 conditional knockout mice (15). An understanding of Adam10 function in T lineage commitment may require the use of Cre transgene which drives Cre expression earlier than Lck-Cre. Our attempt to delete Adam10 in hematopoietic progenitors with a Tie2-Cre transgene (26) resulted in embryonic lethality, presumably due to a role for Adam10 in cardiovascular development (data not shown). Therefore, further investigation of Adam10 function in DN stage development would require additional strategies other than the use of existing Cre transgenic lines.

Studies outlined above provided both genetic and biochemical evidence that ADAM10 controls Notch1 activation during T cell development. Because the phenotype is resulting from deletion of Adam10 within the T cell lineage, we conclude that cleavage of Notch S2 site is primarily accomplished by ADAM10 expressed on thymocytes instead of thymic stromal cells. Notch1 is one of four Notch genes present in mammals and is also the major Notch gene involved in T cell development in the thymus (15, 16, 27). The phenotypic similarity between Adam10 conditional knockout and Notch1 conditional knockout mice strongly implies that other Notch genes are less likely to play any major roles within the T cell lineage downstream of Adam10. Our study indicates that the T cell intrinsic interaction between ADAM10 and Notch1 is a critical step in Notch activation during thymocyte development.

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Abbreviations:
7AAD 7-amino actinomycin D
DN double negative
DP double positive
ICN intracellular domain of Notch
icTCRβ intracellular TCRβ
RT reverse transcription
SP single positive
ADAM10 activates Notch in T cell development

References