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Direct BMP2/4 signaling through BMP receptor IA regulates fetal thymocyte progenitor homeostasis and differentiation to CD4+CD8+ double-positive cell

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BMP2/4 signaling is required for embryogenesis and involved in thymus morphogenesis and T-lineage differentiation. In vitro experiments have shown that treatment of thymus explants with exogenous BMP4 negatively regulated differentiation of early thymocyte progenitors and the transition from CD4−CD8− (DN) to CD4+CD8+ (DP). Here we show that in vivo BMP2/4 signaling is required for fetal thymocyte progenitor homeostasis and expansion, but negatively regulates differentiation from DN to DP cell. Unexpectedly, conditional deletion of BMPRIA from fetal thymocytes (using the Cre-loxP system and directing excision to hematopoietic lineage cells with the *Vav* promoter) demonstrated that physiological levels of BMP2/4 signaling directly to thymocytes through BMPRIA are required for normal differentiation and expansion of early fetal DN thymocytes. In contrast, the arrest in early thymocyte progenitor differentiation caused by exogenous BMP4 treatment of thymus explants is induced in part by direct signaling to thymocytes through BMPRIA, and in part by indirect signaling through non-hematopoietic cells. Analysis of the transition from fetal DN to DP cell, both by ex vivo analysis of conditional BMPRIA-deficient thymocytes and by treatment of thymus explants with the BMP4-inhibitor Noggin demonstrated that BMP2/4 signaling is a negative regulator at this stage. We showed that at this stage of fetal T-cell development BMP2/4 signals directly to thymocytes through BMPRIA.

Introduction

BMP2/4 signaling is essential for embryonic development, and is also involved in tissue homeostasis and cancer.[1-8] It plays a role in thymus morphogenesis and T-cell differentiation.[9-16] While BMP2/4 signaling has been shown to be necessary for normal thymus organogenesis in vivo,[17,18] the evidence for BMP2/4 involvement in thymocyte differentiation is based on in vitro studies.[9-11] Thus, the physiological role of the pathway in thymocyte differentiation remains unclear.

BMP2 and 4 ligands signal to target cells via the formation of complexes with type I (BMPRIA, BMPRIB and ALK2, also known as ALK3, ALK6, and ACVR1, respectively) and II (BMPRII, ActRII, and ActRIIB) transmembrane receptors. BMP2/4 ligands bind to type II receptors, which recruit and phosphorylate any of the 3 type I receptors. Alternatively, BPM2/4 can bind first to type I receptors and then recruit type II receptors. Phosphorylated type I receptors transduce the BMP2/4 signal to the nucleus via the phosphorylation of receptor Smads (Smad-1, -5, or -8), which, in turn, recruit the co-Smad (Smad-4), translocate to the nucleus, and regulate gene expression.[19]

Suppression of BMP2/4 signaling in the epithelium and mesenchymal cells of the thymus anlage, achieved by transgenic expression of the BMP2/4 inhibitor Noggin in thymic epithelium, driven by the FoxN1 promoter, resulted in a thymus of reduced size, ectopically located in the neck. Despite this effect on thymus size and location, analysis of newborn thymocytes did not reveal a defect in thymocyte differentiation, proliferation or apoptosis.[17] Targeted deletion of BMP4 from the thymus epithelium also revealed that normal BMP4 signaling at the early stages of thymus anlage development is necessary for migration of the organ to its final location and normal organ morphogenesis and size. Loss of BMP4 signaling also resulted in delayed colonization of the organ by common lymphoid progenitors.[18] Targeted deletion of BMPRIA in the thymus epithelium, either early or late in thymus development, resulted in a morphologically normal organ of reduced size.[18]

During embryonic development, blood-borne progenitor cells of hematopoietic lineage migrate into an undeveloped thymic

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anlage. Thymocytes and thymus develop synchronously, requiring bidirectional signals for organ morphogenesis and thymocyte development. Progenitor cells migrate into the thymus and progress through tightly regulated developmental stages as they mature into αβT cells.[20] The stages of thymocyte development can be phenotypically identified by the expression of the cell surface markers CD4 and CD8. Immature $CD4^-CD8^-$ double-negative (DN) cells give rise to the $CD4^+CD8^+$ double-positive (DP) population, which differentiate into mature $CD4^+$ or $CD8^+$ single positive mature T cells that migrate out of the thymus to the secondary lymphoid organs. The DN population can be further subdivided by cell surface expression of CD44 and CD25: $CD44^+CD25^-$ (DN1) cells give rise to the $CD44^+CD25^+$ (DN2) population, which then downregulates CD44 to become $CD44^-CD25^+$ (DN3) and finally downregulates CD25 to become $CD44^-CD25^-$ (DN4).

Previous in vitro studies indicated that BMP2/4 negatively regulated thymocyte differentiation at 2 distinct developmental stages. Treatment of fetal thymus organ cultures (FTOCs) with recombinant BMP4 arrested thymocyte differentiation at the DN1 stage[9,11] and at the transition from DN to DP cell.[10,11] In addition, treatment with the BMP2/4 inhibitor Noggin accelerated development to the DP stage[9,10] and increased the percentage of $CD2^+$ DN thymocytes.[9] These experiments showed that under physiological conditions, BMP2/4 negatively regulates the transition from DN to DP cell. Tsai and colleagues[11] used thymus reaggregation experiments to investigate the contribution of stromal and thymocyte-intrinsic signaling by BMP4 to arrest differentiation at the DN1 stage and at the DN to DP transition. Their data showed that BMP2/4 signaling to both thymocytes and stroma are involved in BMP2/4's regulation of thymocyte differentiation, and suggested that while at the DN1 stage, BMP4 is exerting its effects by signaling both directly to thymocytes and indirectly via the stroma, at the DN to DP transition, the effect of BMP4 signaling is indirect and mediated by the stroma alone.

To investigate the physiological role of BMP2/4 signaling during thymocyte development, we studied the effect of targeted deletion of BMPRIA in the hematopoietic lineage, using the Cre-loxP system and the *Vav* promoter for the expression of Cre. This approach allowed us to investigate thymocyte development in the absence of direct BMP2/4 signaling to thymocytes, while indirect signaling via the stroma was not impaired.

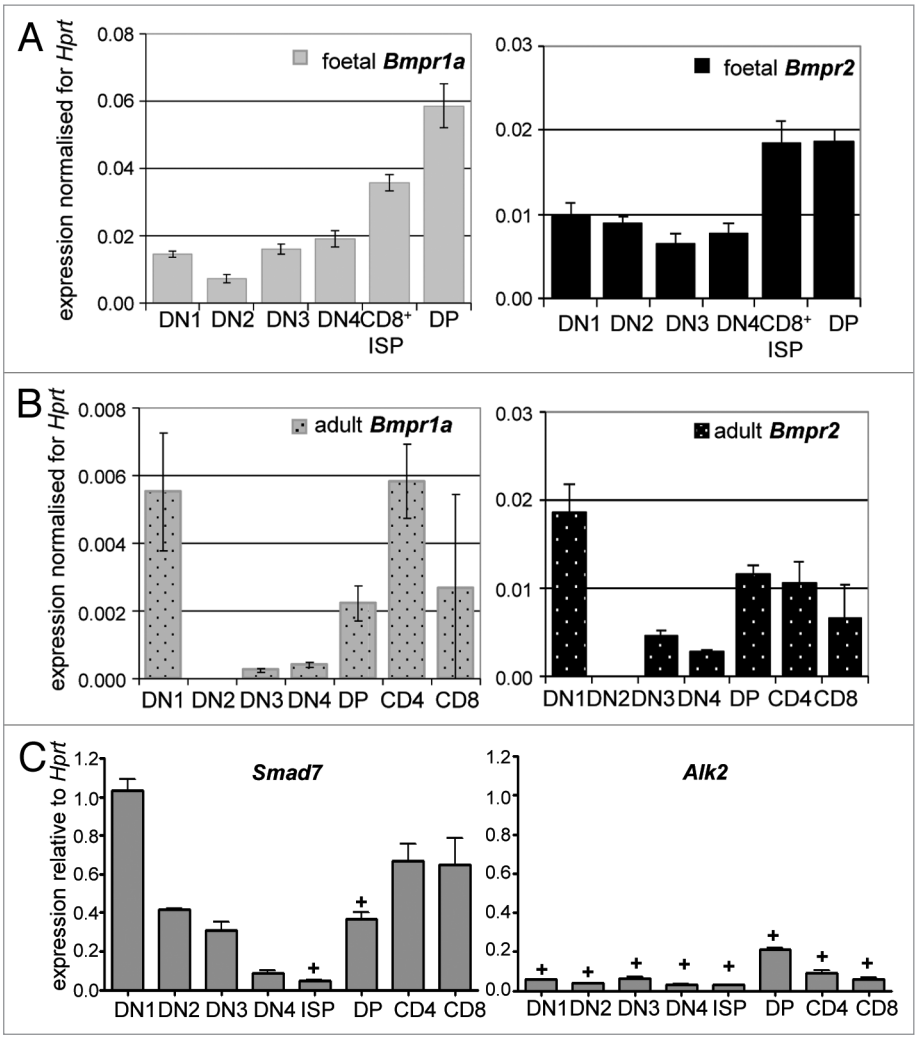


Figure 1. Expression of BMP2/4 receptors IA and II in thymocytes. (**A**) Graphs show transcription of *Bmpr1a* (left) and *Bmpr2* (right) in sorted E16.5 C57BL/6 fetal thymocyte populations. Graphs represent transcription of the 2 genes normalized by transcription of *Hprt*. (**B**) As in (**A**), but for adult thymocytes. (**C**) Expression of *Smad7* (left) and *Alk2* (right) in sorted adult thymocyte populations, derived from microarray data from ImmGen. Graphs show transcription of the genes relative to *Hprt* transcription. Values represent the unlogged hybridization intensity value for the relevant probeset for each gene divided by the corresponding value for *Hprt*. Crosses denote hybridization intensities below the ImmGen threshold value of 120, i.e., absence of significant expression of the gene of interest in the population.

Results

Expression of BMP2/4 receptors in fetal and adult thymocyte subsets

To assess the potential of thymocytes to receive and respond to BMP2/4 signal, we analyzed the expression of BMP receptors IA (BMPRIA), IB (BMPRIB) and II (BMPRII) in fetal (E16.5) and adult $CD4^-$ $CD8^-$ double-negative (DN1–4) thymocyte subsets, $CD4^+CD8^+$ double positive (DP) and $CD4^+$ or $CD8^+$ single positive (SP) thymocyte subsets by quantitative (q) RT-PCR. We detected expression of *Bmpr1a* and *Bmpr2* in both fetal (**Fig. 1A**) and adult (**Fig. 1B**) thymocytes. Both receptors were differentially expressed during thymocyte development, and their

expression profiles differed between fetal and adult thymocytes. In fetal thymocytes both receptors were expressed in all subsets, and their expression peaked at the CD8$^+$ immature SP and DP stage (**Fig. 1A**). The expression of both receptors in all subsets was lower in adult compared with fetal thymocytes. Expression of BMPRIA was detected in adult DN1 cells, was not detected in DN2 cells, was detected at very low levels in DN3 and DN4 cells, and its expression increased in DP, CD4$^+$, and CD8$^+$ SP cells (**Fig. 1B**). The *Bmpr2* expression pattern was similar (**Fig. 1B**). We were not able to detect expression of *Bmpr1b* in any thymocyte subset (data not shown). This was consistent with microarray expression data from sorted thymocyte populations available in the ImmGen database,[21] in which expression of BMPR1B was not detected in any thymocyte population. We also consulted the ImmGen database for expression of the type I receptor Alk2 and the Smad transcription factors in sorted thymocyte populations from DN1 to DP, CD4$^+$, and CD8$^+$ SP. *Smad4* and *Smad5* were highly expressed in all populations, whereas Smad1 and Smad9 were expressed at lower, but detectable levels in all populations. *Smad 6* expression was not detected. Interestingly, *Smad7*, which is known to inhibit the BMP pathway, was highly expressed in the DN1 population, and significantly downregulated step-wise from DN1 to each subsequent DN population (**Fig. 1C**). *Alk2* expression was below threshold value in all thymocyte subsets (**Fig. 1C**).

Thymus composition of adult BMPRIA conditional knockout mouse

We and others have previously published in vitro data indicating a role for BMP2/4 signaling in thymocyte development.[9-11] To assess in vivo the involvement of BMP2/4 signaling we examined the thymus of adult BMPRIA conditional knockout (BMPRIAcon-ko) mice, in which the expression of BMPRIA was defective in T-lineage cells, but normal in thymus epithelial cells. BMP2/4 signal reception and processing requires a complex of a type II BMP receptor with either one of BMPR IA, IB, or ALK2.[19] We did not detect *Bmpr1b* or *Alk2* expression in thymocyte subsets (data not shown). We can therefore safely assume that BMPRIA-deficient thymocytes are unable to transduce BMP2/4 signals, since in the BMPRIAcon-ko thymocytes none of the type I receptors are expressed, but at least one is necessary for BMP2/4 signaling. To ablate the expression of BMPRIA in thymocytes we used mice homozygous for the floxed *Bmpr1a* allele[22] expressing Cre under the control of the *Vav* promoter, directing expression to all hematopoietic lineage cells.[23] In these BMPRIAcon-ko mice exon 2 of *Bmpr1a* is flanked by loxP sites, and the exon is excised in cells expressing Cre, i.e., hematopoietic lineage cells, including even the earliest stages of thymocyte development.[23] The defective BMPRIA produced lacks the ligand binding domain and is therefore not able to activate and signal in response to the presence of BMP2/4. BMPRII can form a homodimer, but ligand binding is very poor, so BMPRIAcon-ko cells will transduce very little or no BMP signal.[24]

We found that there was no difference in the number of cells between BMPRIAcon-ko (*Bmpr1a*[flox/flox], VavCre+) adult thymus and WT (*Bmpr1a*[flox/flox], VavCre-) littermates (data not shown), and cell subset composition was normal. We observed no significant differences between BMPRIAcon-ko and WT littermate thymi in cell number or the percentages of cells at the DN, DP, CD4+ SP, or CD8+ SP (**Fig. 2A**) and DN1–4 (data not shown). We also analyzed the expression profiles of CD5, CD3 (**Fig. 2B and C**), CD24, CD2, and CD69 (data not shown) in different thymocyte cell subsets and found no difference between genotypes. The fact that conditional deletion of BMPRIA from thymocytes made no measurable impact on the composition of the adult thymus populations is consistent with the low level expression of BMPRIA in adult thymocytes (**Fig. 1B**), and absence of the alternative receptors BMPRIB (ImmGen data, and data not shown) and ALK2 (ImmGen data, **Fig. 1C**). However, to confirm that BMPRIB and/or ALK2 were not upregulated in BMPR1A-deficient thymocytes and compensating for the absence of BMPRIA, we analyzed *Bmpr1b* and *Alk2* expression in BMPRIAcon-ko and WT littermate thymocytes, compared with expression in embryo head (**Fig. 2D**). We found no expression of *Bmpr1b* and *Alk2* in WT thymocytes and no upregulation of *Bmpr1b* and *Alk2* in the absence of BMPRIA, suggesting that the pathway is not important for adult thymocytes.

Fetal development of BMPRIA-deficient thymocytes

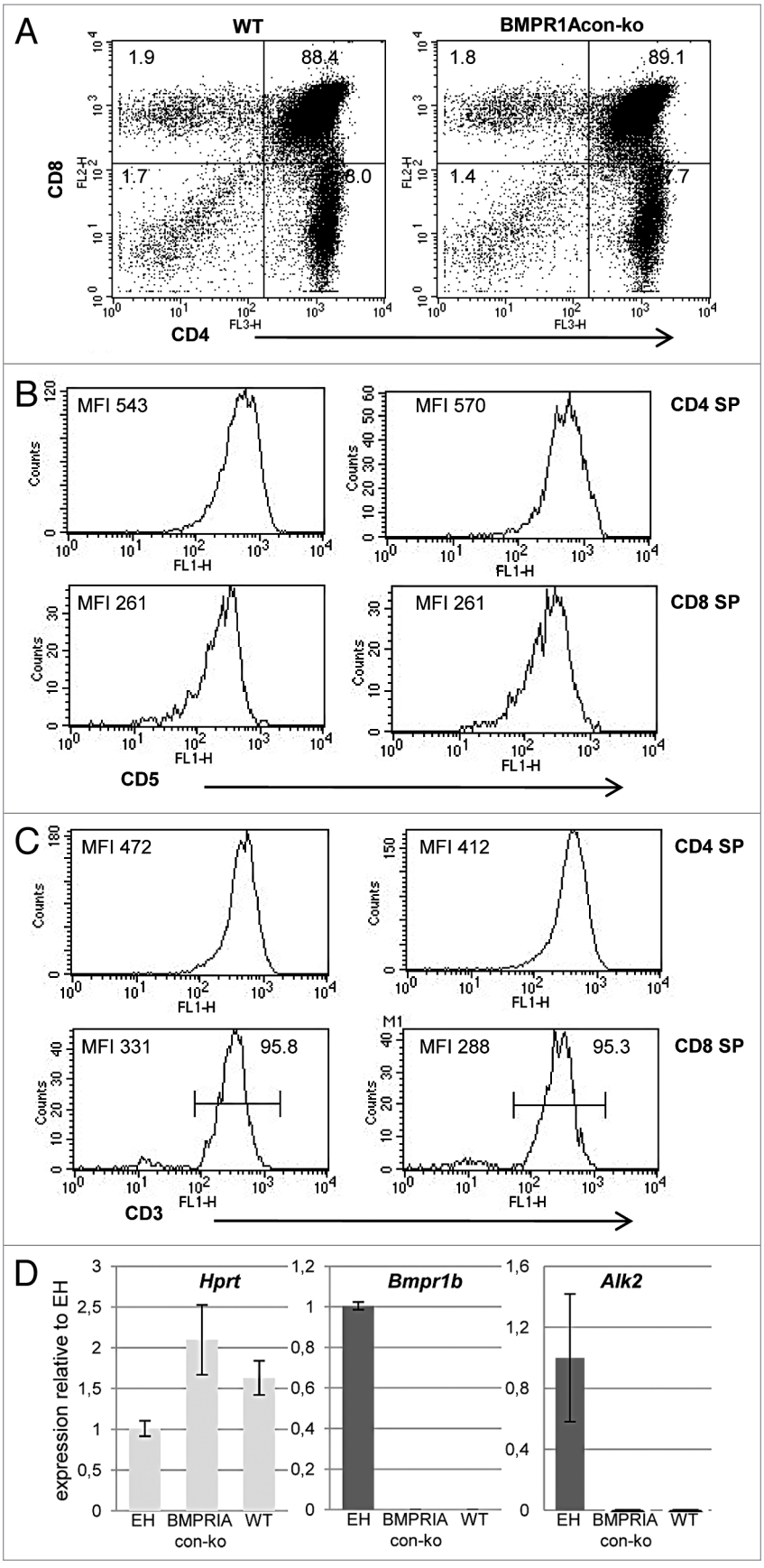
Since BMPRIA is more highly expressed in fetal thymocytes (**Fig. 1A**), we investigated the impact of absence of BMP2/4 signaling on fetal thymocyte development in vivo. To this end, we followed the development of BMPRIA-deficient fetal thymocytes at consecutive embryonic days (E), E13.5 to E16.5.

On E13.5, when the thymus is exclusively composed of DN1 and DN2 cells, we detected no differences in thymocyte number or DN1/DN2 percentages (**Fig. 3A and 3B and C**, respectively). On day E14.5, when DN3 and DN4 subsets first develop, the number of thymocytes was reduced in the BMPRIAcon-ko embryos by 60% on average compared with their WT littermates (**Fig. 3D**). While all subset cell numbers were on average reduced in the BMPRIAcon-ko thymi compared with WT, the DN3 subset showed the largest reduction (**Fig. 3E**). On average, the percentage of DN1 and DN2 cells was increased and DN3 reduced in BMPRIAcon-ko thymi compared with WT, while DN4 cell percentages did not differ between genotypes (**Fig. 3F**). On E15.5 the number of thymocytes was still reduced in BMPRIAcon-ko compared with WT (**Fig. 3G**).

At E16.5, when DP cells first appear, thymocyte cell number in BMPRIAcon-ko thymi had recovered (**Fig. 4A**), and the proportions of DN1-DN4, total DN, immature CD8+ (CD8ISP), DP, and CD4+ subsets did not differ between genotypes (**Fig. 4B and C**). Given that thymocyte number recovered between E15.5 and E16.5, we tested if BMPRIA-deficient thymocytes expand faster than WT. We cultured BMPRIAcon-ko and WT E15.5 fetal thymus explants for 7 days. The BMPRIAcon-ko fetal thymic organ culture (FTOC)s expanded on average ~5-fold, whereas WT FTOC expanded less than 2-fold, showing that BMPRIA expression by DN thymocytes is required for negative regulation of the transition to DP by BMP signaling (**Fig. 4D and E**).

Impact of BMPRIA thymocyte-specific deletion on BMP4-induced arrest of differentiation from DN1 to DN3

We have previously shown that the development of thymocytes in FTOCs treated with BMP4 is partially arrested



at the DN1 stage.[9] It remains unclear whether this effect can be attributed to BMP4 signaling directly to the thymocytes, indirectly via the thymus stroma, or to a combination of direct and indirect signaling. To address this question, we treated WT and BMPRIAcon-ko E14.5 FTOCs for 3 days with BMP4 and compared the development of the thymocytes of different genotypes to the control untreated cultures (**Fig. 5A**). As previously observed, in the WT FTOCs the percentage of DN1 cells was increased by ~6-fold on average, from 1.1% in the control cultures to 6.1% in the BMP4 treated ones. DN1 percentage increased also in BMPRIAcon-ko treated vs. control FTOCs but by approximately 3-fold, from 1.1% in the control to 3.1% in the treated cultures (**Fig. 5B**). The percentage of DN1 in the control FTOCs did not differ between genotypes, while the percentages of DN1 cells in the BMP4-treated FTOCs differed significantly between genotypes (P = 0.043). No significant differences were observed in the percentage of DN2 cells either between control and treated cultures or between genotypes (**Fig. 5B**). We observed a significant decrease in the DN3 percentage (75% to 64% in WT FTOCs and 76% to 67% in BMPRIAcon-ko FTOCs) in BMP4-treated compared with control FTOCs of both genotypes (**Fig. 5B**). No significant differences were observed for DN2-DN4 subset percentages between genotypes.

We previously showed that BMP4 treatment increases the ratio of DN1/DN3 cells.[9] Here, we found that the ratio of DN1/DN3 was significantly lower in BMPRIAcon-ko BMP4 treated FTOCs compared with their WT counterparts (**Fig. 5C**). Our data therefore shows that while BMP4 treatment arrested the development of BMPRIAcon-ko thymocytes at the DN1 stage, this effect was weaker compared with that on WT thymocytes, and therefore that BMP4 is signaling both directly to the DN1 thymocytes and indirectly via the stroma.

Figure 2. Analysis of adult BMPRIAcon-ko and WT thymi (**A**) FACS profiles of CD4 and CD8 cell surface expression in adult WT (left, *Bmpr1a*[flox/flox] VavCre-) and BMPRIAcon-ko (right, *Bmpr1a*[flox/flox] VavCre+) thymocytes. (**B and C**) Expression of CD5 (**B**) and CD3 (**C**) in CD4SP (top panels) and CD8 SP (lower panels) WT (left) and BMPRIAcon-ko (right) thymocytes. (**D**) Expression of *Hprt* (left), *Bmpr1b* (middle), and *Alk2* (right) in WT embryo head and adult thymocytes from BMPRIA-con-ko and WT, positively selected for Thy1 expression. Expression is shown relative to embryo head (EH).

Impact of thymocyte-specific BMPRIA deletion on differentiation from DN to DP

We and others have previously shown that BMP4 treatment negatively regulated the transition of DN cells to the DP stage.[9-11] We analyzed thymocyte subset composition of control and BMP4-treated WT and BMPRIAcon-ko FTOC. We found that BMP4 treatment delayed development of DN to DP cells in both WT and BMPRIAcon-ko cultures as shown by increased percentages of DN subsets in the BMP4 treated FTOCs compared with control cultures (values above 1 for DN subset, **Fig. 6A**).

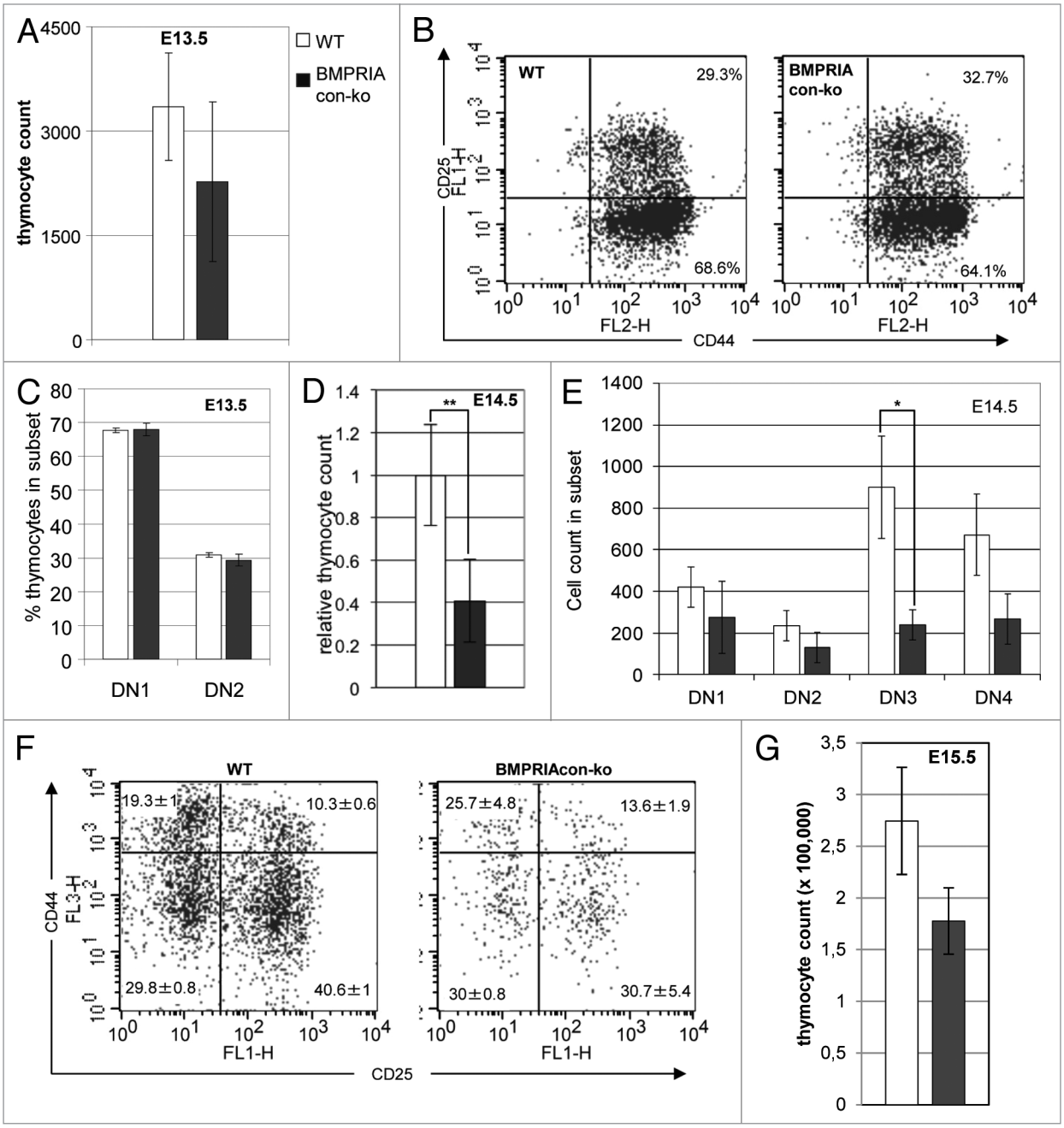


Figure 3. Analysis of E13.5 and E14.5 fetal BMPRIAcon-ko and WT thymi. (**A**) Graph representing mean number of cells recovered from E13.5 WT (open bar) and BMPRIAcon-ko (dark bar) thymi. WT n = 5, BMPRIAcon-ko n = 3. (**B**) FACS profiles of CD44 and CD25 cell surface expression in E13.5 fetal WT (left) and BMPRIAcon-ko (right) thymocytes. (**C**) Graph representing DN1 and DN2 mean percentages in E13.5 fetal WT (open bars) and BMPRIAcon-ko (dark bars) thymi. WT n = 5, BMPRIAcon-ko n = 3. (**D**) Graph representing mean relative number of cells recovered from E14.5 WT (open bar) and BMPRIAcon-ko (dark bar) thymus (WT and BMPRIAcon-ko n = 3). (**E**) Graph representing mean cell numbers at DN1 to DN4 subsets recovered from E14.5 fetal WT (open bars) and con-ko (dark bars) thymi. Standard errors of the mean shown (WT and BMPRIAcon-ko, n = 3). (**F**) FACS profiles of CD44 and CD25 cell surface expression in E14.5 fetal WT (left) and BMPRIAcon-ko (right) thymocytes. On every quadrant mean percentage values +/– standard errors are shown. (**G**) Graph representing mean number of cells recovered from E15.5 (open bar) and BMPR1Acon-ko (dark bar) thymi. Standard error of the mean shown (WT n = 10 and BMPRIAcon-ko, n = 7). On all graphs, standard errors of the mean are shown. Statistically significant differences (*$P < 0.05$, **$P < 0.10$, Student *t* test) are indicated with stars.

Nevertheless the development of BMPRIAcon-ko thymocytes by BMP4 treatment was less affected than that of their WT counterparts, as demonstrated by the statistically significant decrease in percentage of DN cells relative to control FTOCs in BMPRIAcon-ko compared with WT BMP4-treated FTOCs (**Fig. 6A**), indicating that a combination of thymocyte/hematopoietic intrinsic and stromal factors are responsible for the transduction of excess BMP signaling at this stage.

Treatment of FTOCs with BMP2/4 antagonist Noggin has revealed a physiological role for endogenous BMP2/4 signaling in thymocyte development, and we have previously shown that Noggin treatment of FTOCs accelerated DN thymocyte maturation.[9] We compared the development of thymocytes in control and Noggin-treated WT and BMPRIAcon-ko E14.5 FTOCs. We found that while Noggin significantly accelerated DN to DP transition of WT thymocytes, (**Fig. 6B**, upper panel and **Fig. 6C**), it did not affect subset composition of BMPRIAcon-ko FTOCs (**Fig. 6B**, lower panel and **Fig. 6C**). Therefore we conclude that in the thymus, under physiological conditions, endogenous BMP2/4 signals directly to developing thymocytes through BMPRIA to negatively regulate DN to DP transition.

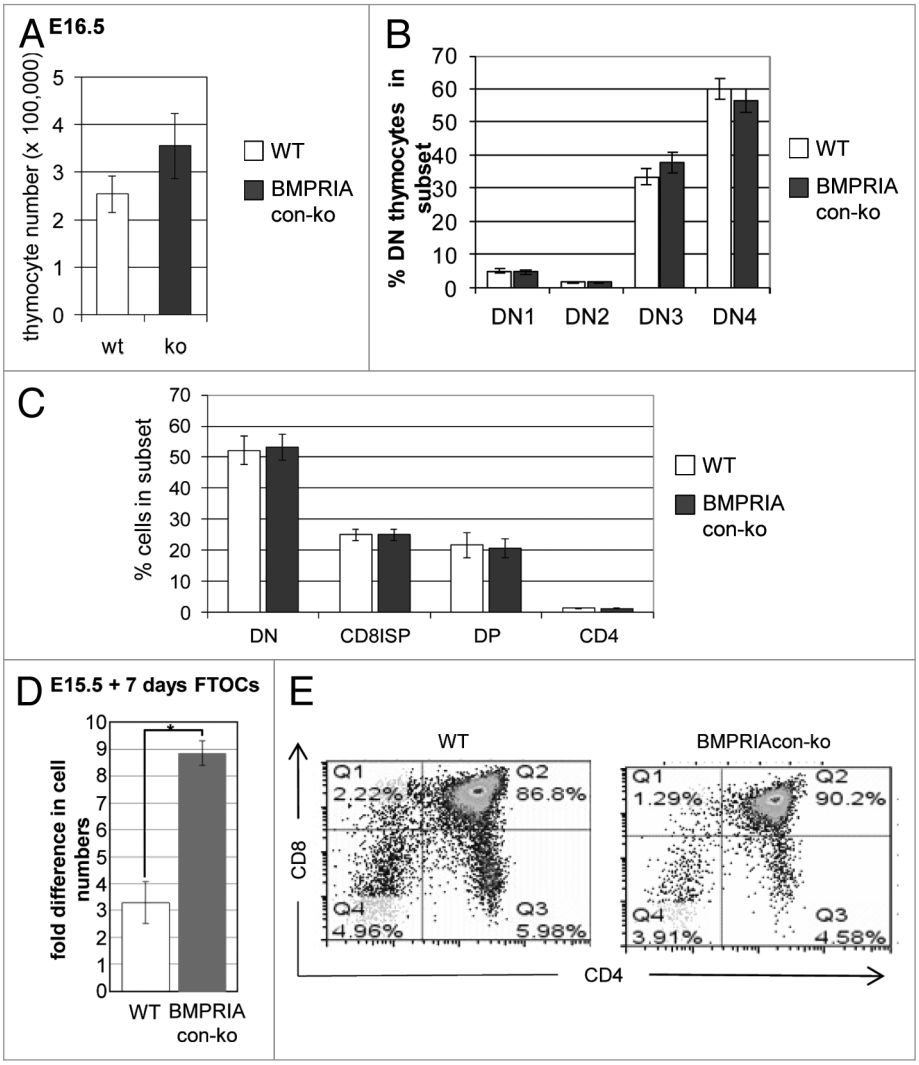


Figure 4. Analysis of E16.5 and E15.5 +7 d in culture fetal BMPRIAcon-ko and WT thymi. (**A**) Graph representing mean number of thymocytes recovered from E16.5 WT (open bar, n = 16) and BMPRIAcon-ko (dark bar, n = 14) thymi. (**B**) Graph representing DN1 to DN4 mean percentages of the DN thymocytes in E16.5 fetal WT (open bar, n = 16) and BMPRIAcon-ko (dark bar, n = 14) thymi. (**C**) Graph representing mean percentages of DN, CD8ISP, DP, and CD4SP thymocytes in E16.5 fetal WT(open bar, n = 16) and BMPRIAcon-ko (dark bar, n = 14) thymi. (**D**) Graph representing the mean fold difference in cell numbers of fresh E15.5 lobes to those cultured for 7 d (WT open bar n = 5 and BMPRIAcon-ko dark bars n = 2). (**A–D**) On all graphs, standard errors of the mean are shown. Statistically significant differences ($P < 0.05$, Student t test) are indicated with stars. (**E**) FACS profiles of CD4 and CD8 cell surface expression in thymocytes from WT (left) and BMPRIAcon-ko (right) E15.5 +7 d FTOCs. On every quadrant percentage is shown.

Discussion

We investigated the physiological role of BMP2/4 signaling during thymocyte development, via targeted deletion of BMPRIA expression in thymocytes. We showed that BMPRIB and ALK2 were not expressed in thymocytes. Deletion of BMPRIA expression therefore resulted in thymocytes that were unable to receive and respond to BMP2/4 signaling, enabling us to distinguish between the indirect effect of the signal received by the thymus stroma from the effect of signal received directly by the developing thymocytes.

Our results, in accordance with previously published studies,[9-12,19] indicate that BMP2/4 signaling is necessary for normal developmental kinetics of fetal thymocytes at 2 distinct developmental stages: the transition from DN1 to DN3, and from DN to DP cell. We show that the effects of the signaling are dose-dependent at the transition from DN1 to DN3, and that different doses can have opposing consequences on development. We showed that at the transition from DN1 to DN3, while the physiological level of signaling is required for efficient differentiation, as indicated by the reduced number of DN3 and DN4 cells in the E14.5 BMPRIAcon-ko thymi (**Fig. 3D and E**), an artificially elevated BMP4 signal has the opposite effect and results in developmental arrest at the DN1 stage (**Fig. 5** and refs. 9 and 11). The observed delay at E14.5, when DN3 cells first develop and not at E13.5 when the thymus is exclusively composed of DN1 and DN2 cells, is consistent with our previous findings that BMP2/4 signaling is involved at the DN1 to DN3 transition.[9]

At the DN to DP transition, however, physiological BMP2/4 signaling

is a negative regulator of DN to DP development, since removal of the signal by conditional deletion of BMPRIA, or by Noggin-treatment, promotes thymocyte expansion to the DP stage. In contrast, exogenous BMP4 delays development from DN to DP. Therefore, our data shows that physiological BMP2/4 signaling is necessary for normal thymocyte number at the earlier stages of thymocyte development, to maintain a normal progenitor pool, and supports previous findings[9-11] indicating that the physiological BMP2/4 signal negatively regulates the transition from DN to DP.

We did not detect an effect on thymocyte composition and cell number in the adult BMPRIAcon-ko thymus. This could be attributed either to the low level of expression of BMPRIA in adult thymocytes, meaning that BMPRIA is not important for adult thymocyte development (**Fig. 1B**), or to the opposing effects of BMP2/4 at consecutive developmental stages, masking impact of deletion in the steady-state adult thymus. In the fetus, we analyzed consecutive developmental days, and while we observed no defect in homing of BMPRIA-deficient thymocytes (day E13.5, **Fig. 3A–C**), development to the DN3 stage was initially delayed (E14.5, **Fig. 3D–F**), but thymocyte expansion and development to DP stage was accelerated, so thymocyte numbers and development caught up by E16.5 (**Fig. 4**).

Development of BMPRIAcon-ko DN1 thymocytes was arrested by BMP4 treatment, indicating that the effect of BMP4 signaling to the thymocytes is, at least partly, indirect, by signaling to other cell types in the thymus (stroma including epithelial and other non-hematopoietic cell types). Interplay between BMP2/4 regulated stromal factors and the developing thymocytes must cause the observed developmental block. We also showed that the developmental arrest of BMPRIAcon-ko thymocytes by BMP4-treatment was significantly less than in WT thymocytes, as indicated by the reduced ratio of DN1/DN3 in the BMPRIAcon-ko BMP4-treated FTOCs compared with their WT counterparts. Taken together, our data indicates that the BMPRIAcon-ko thymocytes do not respond to BMP4 treatment as strongly as their WT counterparts, and therefore that high dose BMP2/4 signaling for developmental arrest affects thymocyte transition from DN1 to DN3 both via direct signaling to the thymocytes as well as indirectly via the stroma.

Our data from Noggin treated WT and BMPRIAcon-ko FTOCs shows that, at the transition from DN to DP, physiological BMP2/4 negatively regulates differentiation by acting directly on thymocytes in a BMPRIA-dependent manner, and not via the stroma. BMPRIA-deficient thymocytes were refractory to Noggin treatment, whereas WT thymocytes differentiated faster.

In brief, our findings provide novel insight into the physiological role of BMP2/4 signaling during thymocyte development and distinguish the direct effects on thymocytes from indirect effects via the thymus stroma. We show that physiological BMP2/4 signaling positively regulates

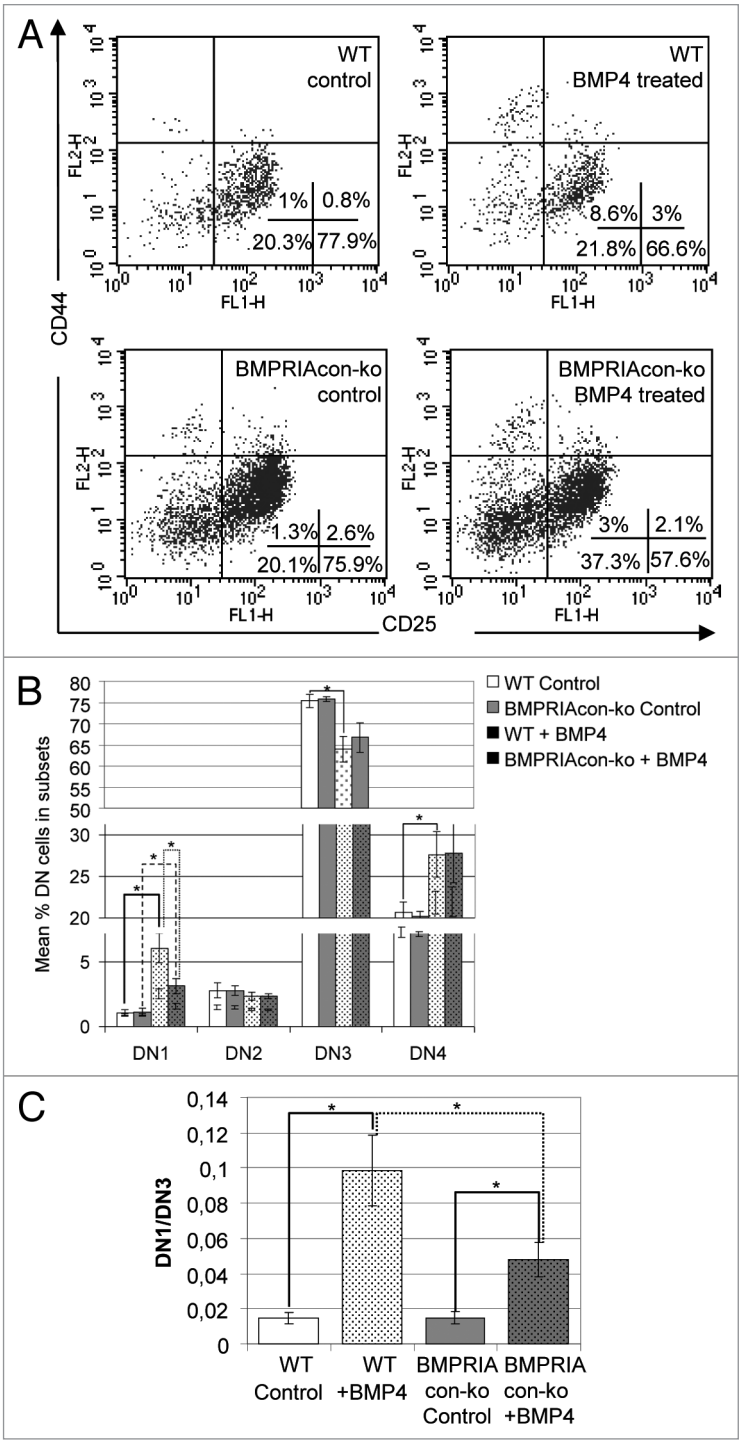


Figure 5. BMP4 treatment of WT and BMPRIAcon-ko FTOCs. (**A**) Typical FACS profiles of CD44 and CD25 cell surface expression in DN thymocytes recovered from WT (top, n = 9) and BMPRIAcon-ko (bottom, n = 5) control (left) or BMP4 treated (right) FTOCs, cultured for 3 d. (**B**) Graph representing mean percentages of DN1-DN4 thymocytes in WT control, BMPRIAcon-ko control, WT BMP4-reated, and BMPRIAcon-ko BMP4-treated FTOCs. (**C**) Graph representing DN1/DN3 cell ratios in WT control, WT BMP4 treated, BMPRIAcon-ko control and BMPRIAcon-ko BMP4 treated FTOCs (WT n = 9, BMPRIAcon-ko n = 5). On all graphs, error bars shown represent standard error of the mean. Statistically significant differences ($P < 0.05$, Student *t* test) are indicated with stars.

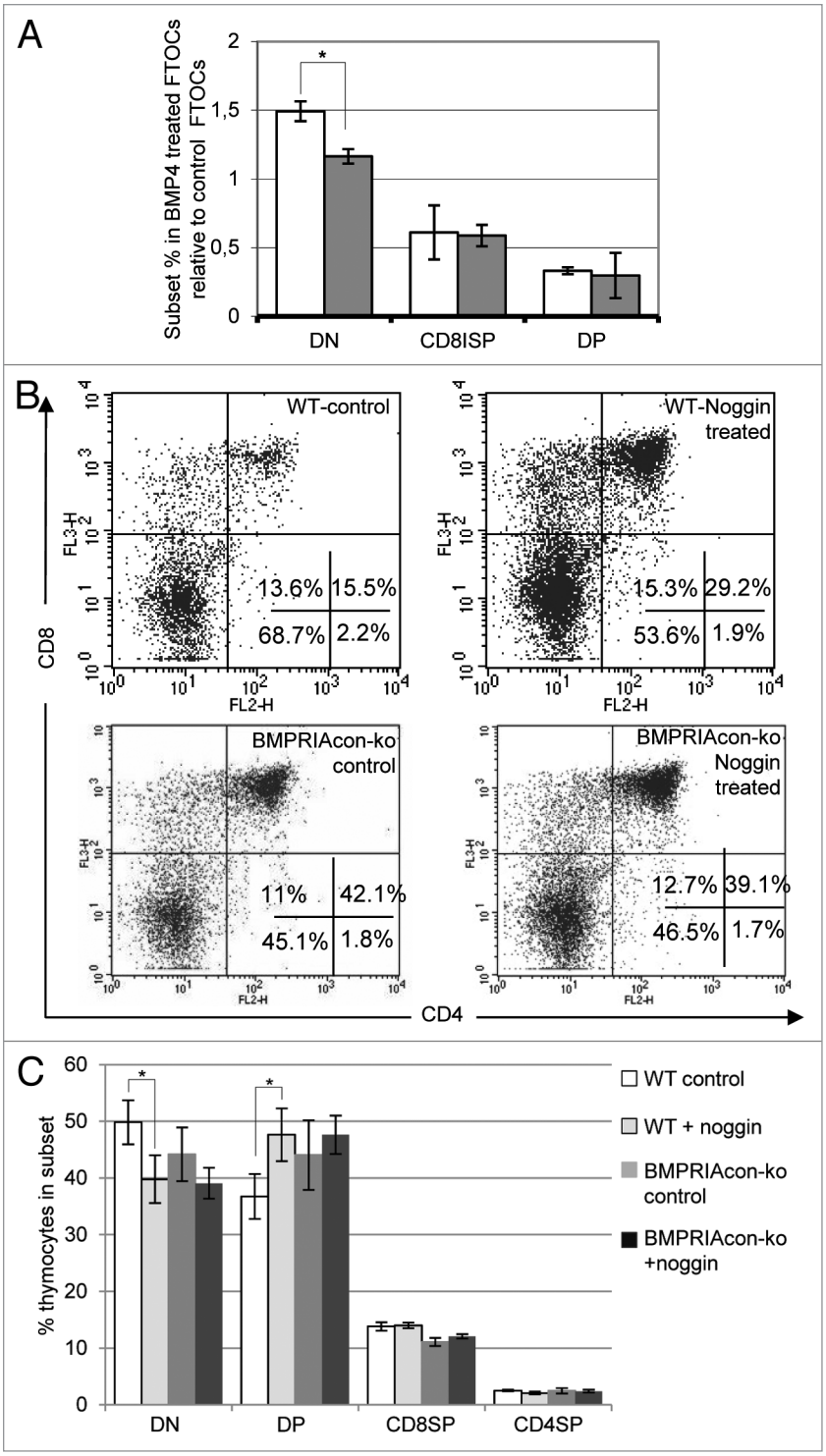


Figure 6. BMP4 and Noggin treatments of WT and BMPRIAcon-ko FTOCs. (**A**) E14.5 WT and BMPRIAcon-ko FTOCs treated with BMP4 or without (control) for 3 d. Graph representing percentages of cells in DN, CD8ISP and DP subsets in WT (open bars) and BMPRIAcon-ko (dark bars) treated relative to control FTOCs. (**B**) FACS profiles of CD4 and CD8 cell surface expression in E14.5 WT (upper) and BMPRIAcon-ko (lower) FTOCs treated with Noggin (+Noggin, right -panels) or without (control, left panel) for 3 d. (**C**) Graph representing percentages of DN, CD8ISP, DP, and CD4SP thymocytes in WT control, WT Noggin-treated, BMPRIAcon-ko control, and BMPRIAcon-ko Noggin-treated FTOCs. WT n = 5, BMPRIAcon-ko n = 4. On all graphs, error bars shown represent standard error of the mean. Statistically significant differences ($P < 0.05$, Student *t* test) are indicated with stars.

fetal thymocyte development from DN1 to DN3 stage, both directly and indirectly via the thymus stroma, and negatively regulates DN to DP transition via direct signaling to the thymocytes.

Materials and Methods

Mice

C57BL/6 mice, purchased from B and K Universal, and Vav-Cre transgenic mice, a gift from Dimitris Kioussis, backcrossed on C57BL/6, and BMPRIA-floxP mice[22] (backcrossed onto C57BL/6) were bred and maintained at Imperial and University College London, according to UK Home Office regulations. Timed mates were performed as described.[25]

Quantitative RT-PCR analysis

C57BL/6J E16.5 fetal and adult thymocytes were sorted on a Modular Flow Cytometer (MoFlo, Cytomation, Inc) at the Cancer Research UK FACS laboratory. For purification of DN1–DN4 populations, cells falling within the FSC/SSC live gate, >98% of which were CD45.2+, were sorted using antibodies directed against CD25FITC, CD44PE, and CD3/CD4/CD8Cychrome. For DP and SP populations thymocytes were stained with CD4PE and CD8Cychrome. Purity of all populations was >98%.

Selection of Thy1.2 positive thymocytes for quantitative RT-PCR analysis shown in **Fig. 2D** was performed with the EasySep™ Mouse PE Positive Selection Kit and EasySep™ Magnet (StemCell Technologies). Approximately 3×10^8 WT and BMPRIAcon-ko adult thymocytes were stained with anti-Thy1.2 antibody conjugated with PE and then Thy1.2+ thymocytes were positively selected following manufacturer's instructions. Purity of selected population was tested by flowcytometry and found to be >99.7%.

Quantitative RT-PCR was as described.[26] In brief, RNA was extracted from sorted thymocytes with the Absolutely RNA miniprep kit (Stratagene), cDNA synthesized with SuperscriptIII (Invitrogen) and random primers and the cDNA samples analyzed in triplicate by quantitative (q)PCR on an iCycler (Bio-Rad Laboratories) using the iQ™SYBR® Green Supermix (Bio-Rad) following manufacturer's instructions. The primers used for the qRT-PCR are listed in **Table 1**. All primer sets were designed to span over 2 different exons, to avoid amplification from genomic DNA contamination. For each sample, gene of interest was normalized to HPRT and standard errors were calculated.

Table 1. Primers used for quantitative RT-PCR analysis

Gene	Primer name	Sequence
Bmpr1a	1AF	TGGGAGCCTG TCTGTTCATC
	1AR	GCAAGGTATC CTCTGGTGCT AAAG
Bmpr1b	1BF	GCTGAATCAC AACCATTTGG CG
	1BR	CTCTCCATCC TCCTTCTTGG
Bmpr2	BMPRIIF	CGTGTTATGG TCTGTGGGAG
	BMPRIIR	ATTGAGGGTG GGGTGGTAG
Alk2		**QuantiTECT primer assay, QIAGEN**
Hprt	F53333	TGATTATGGA CAGGACTGAA AG
	F53334	GGTCAGCAAA GAACTTATAG CC

Mouse genotyping

Embryos and animals were genotyped by PCR as described.[27] In brief, DNA was extracted from tissue biopsies by digesting in lysis buffer containing 50 mM KCl, 1.5 mM MgCL2, 10 mM Tris HCL (pH 8.5), 0.01% gelatin, 0.45% Nonident P-40, 0.45% Tween 20, and 0.5 μg/ml proteinase K (Sigma-Aldrich) overnight at 58 °C. ~50 ng of DNA was used as a template in each PCR reaction. Primers for Vav-Cre genotyping: CreForward: AGATGCCAGG ACATCAGGAA CCTG and CreReverse: ATCAGCCACA CCAGACACAG AGATC. BMPRIAfloxP genotyping was performed with primers fx2: GCAGCTGCTG CTGCAGCCTC C and fx4: TGGCTACAAT TTGTCTCATG C.[28] BMPRIAcon-ko mice were BMPRIAflox/flox VavCre+. WT *Bmpr1a* allele produced a longer PCR product compared with *Bmpr1a* flox allele.

Flow cytometry and antibodies

Flow cytometry and staining were as described:[29] thymocyte suspensions were stained using combinations of directly conjugated antibodies obtained from BD PharMingen or e-biosciences for 30 min on ice in 50 μl Dulbecco modified medium (Life Technologies), supplemented with 2% FCS and 0.01% sodium azide. Cells were washed in this medium between incubations and prior to analysis on the FACScan (Becton Dickinson). Events were collected in list mode using CellQuest software and data analyzed using CellQuest Pro software. Live cells were gated according to their FSC and SSC profiles.

Microarray data analysis

Gene-expression microarray data sets from the Immgen Database[21] (Geo Dataseries: GSE15907, performed on Affymetrix Mouse Gene 1.0 ST Platform [GPL6246] using RNA from double sorted developmental T-cell populations from 6-week-old adult mice having- >99% purity with 3 different samples for each population) were analyzed using R. Package "affy" was used for quality control and normalization of the data set by log-scale robust multi-array analysis (RMA). Probe-set hybridization intensity values thus obtained were unlogged and used to plot expression of different genes like *Smad7 and Alk2* relative to *Hprt* in DN1-DN4, DP, and CD4+SPCD69+ and CD8+SPCD69+ thymocyte populations. Immgen Database threshold of 120 was used to indicate positive expression (at 95% confidence).

BMP4 and noggin treatments of fetal thymic organ cultures (FTOCs)

Thymi from WT and BMPRIAcon-ko E14.5 embryos were cultured on Millipore filters (0.8-μm pore size) (Millipore) in AIM-V serum-free lymphocyte medium (Life Technologies). Each FTOC consisted of one fetal thymus lobe. Human recombinant BMP4 and recombinant Noggin-Fc fusion protein were purchased from R&D Systems. For BMP4 and Noggin treatments, the 2 lobes from every fetus were separated and one was cultured in AIM-V and the other in AIM-V+BMP4 (0.1 μg/ml) or AIM-V+Noggin (0.5 μg/ml) for 72 h. Therefore each treated FTOC corresponded to a control FTOC thymus lobe from the same embryo.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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