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Bone Morphogenetic Protein 2/4 Signaling Regulates Early Thymocyte Differentiation

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Bone morphogenetic protein (BMP)2 and BMP4 are involved in the development of many tissues. In this study, we show that BMP2/4 signaling is involved in thymocyte development. Our data suggest that termination of BMP2/4 signaling is necessary for differentiation of CD44−CD25−CD4−CD8− double negative (DN) cells along the T cell lineage. BMP2 and BMP4 are produced by the thymic stroma and the requisite BMP receptor molecules (BMPR-1A, BMPR-1B, BMPR-II), and signal transduction molecules (Smad-1, -5, -8, and -4) are expressed by DN thymocytes. BMP4 inhibits thymocyte proliferation, enhances thymocyte survival, and arrests thymocyte differentiation at the CD44−CD25− DN stage, before T cell lineage commitment. Neutralization of endogenous BMP2 and BMP4 by treatment with the antagonist Noggin promotes and accelerates thymocyte differentiation, increasing the expression of CD2 and the proportion of CD44−CD25− DN cells and CD4+CD8+ double-positive cells. Our study suggests that the BMP2/4 pathway may function in thymic homeostasis by regulating T cell lineage commitment and differentiation.
The earliest CD44\(^{+}\)CD25\(^{-}\) DN cells also express CD117 and are not fully committed to the T cell lineage. This population contains cells that are capable of differentiating into T, B, NK, and dendritic cells (26, 29, 30). However, the majority of CD44\(^{+}\)CD25\(^{-}\) DN thymocytes do differentiate into T cells and their fate is probably influenced by the fact that the thymic micro-environment is not only necessary for normal T cell development (31), but also favorable to it. Little is known about the factors that control T cell lineage commitment of CD44\(^{+}\)CD25\(^{-}\) cells and their differentiation to CD44\(^{+}\)CD25\(^{-}\) cells. A recent study has shown that inactivation of the Notch1 gene arrests thymocyte development at the CD44\(^{+}\)CD25\(^{-}\) stage and prevents T cell lineage commitment (32). In this study, we show that BMP4 and BMP2 and their extracellular inhibitors Noggin, Chordin, and Tsg are expressed in the thymus, and that BMP2/4 signaling regulates differentiation of early CD44\(^{+}\)CD25\(^{-}\) DN thymocytes along the T cell lineage.

Materials and Methods

**RT-PCR**

RT-PCR was performed as described (33). RNA was prepared using a StrataPrep Total RNA kit (Stratagene, La Jolla, CA), including a DNase I digestion step. Primers were purchased from Genosys (The Woodlands, TX). The following primer pairs were used: hypoxanthine guanine phosphoribosyl transferase (HPRT), (forward) F:TCGG TGA AAT AGC GAG ACC; reverse (R):CTGGCAACATCAAGAGC ACTCC; BMP2, F:TGGTGAAGATGATGACGCCAGC, R:AGGTCA GGTGGTCAGCAAGG; BMP4, F:TTCCTGTCAAGACATGATTG, R: AACTCTCTCAACGTTGGCTGCTG; BMP4 primers were selected to span exon-exon boundaries); BMPR-IA, F:CCCTATGGAAGAAATGATGTTGG, R:GGTCAGCAATACAGCAACTTCC; BMPR-IB, F:CCCTAGAGCACCACTCC; BMPRII, F:CATTCCTGTGGCTTCCACAA, R:GACTGATGGCTGGAGCTATT; BMP2 signal peptide and propeptide fused to the human BMP4 mature sequence (BMP4 primers were selected to span exon-exon boundaries); BMPR-IA, F:CGCTATGGAGAAGTATGGATGG, R:CATTCCTGTGGCTTCCACAA, all of which contained specific elements in the published size of transcribed (34). Primers used to detect Noggin and Chordin were F:ATTGTGCCTTGGCTGGCTTACC, R:GAGTCTAATGGAGCATCGAA. Smad-8, F:CCATACCATTACCGCAGAGTGG, R:TGAAGC TTATCTACACA, Chordin, F:ACCAAGAAGAAATGGGGTTCAGC, R:GCAGGAACACTTACACTCG; Noggin in Fig. 2, F:CG GCCAGCACGTCTATGAGCACG; Noggin in Fig. 1, F:AAGGATCTGAACGAGACG, R:GGTGGTCAGCAAGG; BMP4, F:TTTCTGTCAAGACATGATTG, R:GGTGGTCAGCAAGG; BMP4, F:TTTCTGTCAAGACATGATTG, R:GGTGGTCAGCAAGG; BMP4, F:TTTCTGTCAAGACATGATTG, R:GGTGGTCAGCAAGG; BMP4, F:TTTCTGTCAAGACATGATTG, R:GGTGGTCAGCAAGG; BMP4, F:TTTCTGTCAAGACATGATTG, R:GGTGGTCAGCAAGG; BMP4, F:TTTCTGTCAAGACATGATTG, R:GGTGGTCAGCAAGG; BMP4, F:TTTCTGTCAAGACATGATTG, R:GGTGGTCAGCAAGG; BMP4, F:TTTCTGTCAAGACATGATTG, R:GGTGGTCAGCAAGG; BMP4, F:TTTCTGTCAAGACATGATTG, R:GGTGGTCAGCAAGG; BMP4, F:TTTCTGTCAAGACATGATTG, R:GGTGGTCAGCAAGG; BMP4, F:TTTCTGTCAAGACATGATTG, R:GGTGGTCAGCAAGG; BMP4, F:TTTCTGTCAAGACATGATTG, R:GGTGGTCAGCAAGG; BMP4, F:TTTCTGTCAAGACATGATTG, R:GGTGGTCAGCAAGG; BMP4, F:TTTCTGTCAAGACATGATTG, R:GGTGGTCAGCAAGG.

Components of the BMP2/4 signaling pathway are expressed in the thymus, and that BMP2/4 signaling regulates differentiation of early CD44\(^{+}\)CD25\(^{-}\) DN thymocytes along the T cell lineage.

**Histology and immunofluorescence**

Thymus cryosections (7 μm) were air dried for 2 h at room temperature and fixed in acetone for 10 min. Non-specific binding of Abs was blocked by incubation with diluted donkey serum (Santa Cruz Biotechnology, Santa Cruz, CA) and avidin-biotin (Vector Laboratories, Burlingame, CA). Sections were immunostained to detect the expression of Abs against the following: anti-B cell, F(ab\(^{2}\)) of donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), FITC-conjugated streptavidin (Amersham Pharmacia Biotech, Buckinghamshire, U.K.), and anti-cytokeratin (BioGenex Laboratories, San Ramon, CA) followed by Texas Red-conjugated mouse absorbed F(ab\(^{2}\)) of donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories). To analyze the expression of BMP2/4 receptor, human thymic sections were incubated with anti-BMPR-IB Abs (R&D Systems, Minneapolis, MN), biotin-conjugated F(ab\(^{2}\)) of donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories), FITC-conjugated streptavidin (Amersham Pharmacia Biotech), and anti-CD2 (Beckman Coulter, Brea, CA) followed by Texas Red-conjugated F(ab\(^{2}\)) of donkey anti-rat IgG (Jackson ImmunoResearch Laboratories). Slides were mounted in Vectashield (Vector Laboratories) and examined on a Zeiss Axioplan-2 microscope (Zeiss, Oberkochen, Germany).

**Mice**

BALB/c mice were purchased from B&K Universal (Grimston, U.K.) and Rag1\(^{-}\)/ mice and TCR\(^{\delta}\)/ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Timed matings were conducted as described (36).

**Cell flow cytometry and Abs**

Suspensions of thymocytes were prepared by crushing the thymus between two pieces of ground glass. Cells were stained as described (36) using combinations of the following directly conjugated Abs purchased from BD Pharmingen (San Diego, CA): anti-CD4\(^{+}\), anti-CD4\(^{-}\)CyChrome, anti-CD8\(^{+}\), anti-CD8\(^{-}\)CyChrome, anti-CD25\(^{+}\), anti-CD25\(^{-}\)CyChrome, anti-CD117\(^{+}\), and anti-CD117\(^{-}\)CyChrome. Data were analyzed on a FACSscan (BD Biosciences, Mountain View, CA). Data are representative of at least six experiments. To analyze composition of the DN subsets, cells were stained with anti-CD4, anti-CD3, and anti-CD8 conjugated to the same fluorochrome, and gated to exclude those cells that stained positive with these Abs. For the experiment in Fig. 2, C and D, purified DN cells were sorted by flow cytometry on a MoFlo. Final purity was in excess of 98%.

Annexin V staining was conducted using an annexin V-FITC apoptosis detection kit (BD Pharmingen), according to manufacturer’s instructions. Before annexin V staining, cells were stained with CD44-PE and CD25-CyChrome. Data shown represent annexin-positive cells that fell into a “live gate” defined by FSC and SSC.

Propidium iodide (PI) staining of cells permeabilized in 0.1% Triton X-100 in PBS was conducted according to the manufacturer’s instructions using a BD Pharmingen apoptosis detection kit. Data were acquired on a linear scale using a doublet discrimination module on the FACSscan.

**Fetal thymus organ culture (FTOC)**

Fetal thymi were dissected on E14.5 or E13 and cultured on Millipore filters (0.8-μm pore size) (Millipore, Bedford, MA) in AIM-V serum-free lymphocyte medium (Life Technologies, Grand Island, NY). Human recombinant BMP4 was purchased from R&D Systems. This recombinant human BMP4 was generated from DNA sequence encoding the human BMP2 signal peptide and propeptide fused to the human BMP4 mature chain and expressed in a mouse myeloma cell line. The manufacturer assessed the activity of the protein by its ability to induce alkaline phosphatase production by mouse ATDC-5 chondrogenic cells, and its ED50 was typically 10–30 ng/ml. The recombinant BMP4 was used within the manufacturer’s recommended dose range.

The recombinant Noggin–Fc fusion protein was purchased from R&D Systems and was used at the concentration recommended by the manufacturer to neutralize 0.1 μg/ml BMP4.

Thymic stroma was prepared by culturing E14.5 thymi for 7 days with 0.36 mg/ml 2′,4′-deoxyguanosine (Sigma-Aldrich, St. Louis, MO).

**Results**

Components of the BMP2/4 signaling pathway are expressed in the mouse thymus

To assess the expression of RNAs encoding the secreted factors, BMP2 and BMP4 and their receptor molecules, BMPR-IA, BMPR-IB, and BMPRII, we performed RT-PCR on RNA prepared from E15 thymus. RNA isolated from E15 head was used as a positive control for expression of these molecules (Fig. 1A). We detected both BMP2 and BMP4 in embryo thymus as well as their requisite receptor molecules, BMPR-IA, BMPR-IB, and BMPRII (Fig. 1B).

The specific downstream effector molecules for the BMP signaling pathway are Smad-1, -5, and -8 (37). Upon activation by phosphorylation, these molecules form complexes with the common Smad (Smad-4). RNAs for these four Smad proteins (Smad-1, -4, -5, and -8) were detected in the thymus (Fig. 1B). In the case of Smad-8 and Smad-4, two different-size transcripts were detected and the band corresponding to the published size of transcript is indicated by an arrow.

As the BMP2/4 signaling pathway is modified by extracellular antagonists, we analyzed expression of the antagonists Noggin,
Chordin, and Tsg in RNA isolated from E15 head, thymus, and adult Rag1<sup>1<sup>-/-</sup> thymus. The levels of cDNA used in each reaction were equivalent as judged by expression of HPRT (Fig. 1C). Noggin was easily detected in E15 head, but only a weak band was amplified from E15 thymus and Rag1<sup>1<sup>-/-</sup> thymus (Fig. 1D). Tsg and Chordin was detected in all tissues analyzed (Fig. 1, E and F). The presence of these RNAs in adult Rag1<sup>1<sup>-/-</sup> thymus indicates that these molecules are expressed in adult thymus as well as during fetal ontogeny, and that they do not require pre-TCR signaling for their expression.

To analyze expression of BMP4 at the protein level the histological localization of BMP4-expressing cells was analyzed by double immunofluorescent stainings on thymic tissue sections. BMP4 was detected in cytokeratin-positive epithelial cells that appeared in the subcapsular and cortical areas (Fig. 2A), but not in the medullary region (data not shown). The expression of BMP4 receptor protein was revealed with anti-BMPR-IB Abs and was found in most thymocytes throughout the thymic parenchyma (Fig. 2B) and also in some epithelial cells (data not shown). In this case, stainings were made on human thymic sections to avoid nonspecific staining generated by the use of mouse Abs on mouse tissue sections. To confirm expression of the BMP2/4 receptor molecules in mouse thymocytes we analyzed expression of BMPR-IA,
BMP4 arrests early thymocyte development in a dose-dependent manner

As expression of all components of the BMP2/4 signaling pathway were found in the E15 thymus, we tested the effect of BMP4 on thymocyte development in FTOC. We treated E14.5 FTOC for 3 days with 0.1 μg/ml recombinant BMP4. The thymocytes were then harvested and analyzed by flow cytometry. Fig. 3A, upper panel, shows CD4 and CD8 staining in control and BMP4-treated cultures. Analysis of the DN cells revealed an increased percentage of CD44<sup>+</sup>CD25<sup>-</sup> DN cells in BMP4-treated cultures compared with control cultures, whereas the percentage of CD44<sup>-</sup>CD25<sup>-</sup> DN cells was significantly lower in BMP4-treated compared with control cultures. In a typical experiment, in BMP4-treated cultures 47% of DN thymocytes were CD44<sup>+</sup>CD25<sup>-</sup> and 15% CD44<sup>-</sup>CD25<sup>-</sup>, compared with 6% CD44<sup>-</sup>CD25<sup>-</sup> and 53% CD44<sup>+</sup>CD25<sup>-</sup>, respectively, in control cultures (Fig. 3A). Thus, addition of BMP4 caused accumulation of CD44<sup>+</sup>CD25<sup>-</sup> precursor cells. The percentage of cells expressing CD2, a molecule that is up-regulated on the cell surface following pre-TCR signaling (38, 39), was reduced in BMP4-treated cultures (Fig. 3B). Only 5% of cells cultured in the presence of BMP4 expressed high cell surface levels of CD2, compared with 26% of cells in control cultures. The reduction in CD2 expression is consistent with the accumulation of CD44<sup>+</sup>CD25<sup>-</sup> cells, indicating an accumulation of immature thymocytes. To show that the CD44<sup>+</sup>CD25<sup>-</sup> DN cells that accumulated in the BMP4-treated cultures were the earliest precursor population, we analyzed the expression of the developmentally regulated marker CD117 (c-kit) in the cultures (Fig. 3C). In a typical experiment in which 47% of DN cells expressed CD44 after 5 days of treatment with BMP4, compared with 10% panel, Staining with anti-CD25 and anti-CD44, gated on DN cells. Lower panel, The composition of the DN subsets, stained with anti-CD117 and anti-CD25. BMP4 treatment increased the proportion of cells in the CD117<sup>-</sup>CD25<sup>-</sup> subset. Cell recoveries were 4.5 × 10<sup>4</sup> and 5.5 × 10<sup>4</sup> in control and BMP4-treated cultures, respectively. D, The scatter plot shows the cell recovery from E14.5 FTOC cultures for 5 days. The total number of cells recovered in each experiment from BMP4-treated cultures was divided by the number of cells recovered from the control cultures, to give the relative cell number from six individual experiments. E, The scatter plot shows the relative number of CD44<sup>+</sup>CD25<sup>-</sup> DN cells recovered from the same E14.5 FTOC used in the scatterplot of D. For each experiment, the number of CD44<sup>+</sup>CD25<sup>-</sup> DN cells recovered from BMP4-treated cultures was divided by the number of CD44<sup>-</sup>CD25<sup>-</sup> DN cells recovered from control cultures to give the relative number of CD44<sup>-</sup>CD25<sup>-</sup> DN cells from six different experiments. F, E14.5 FTOC were cultured for 6 days in diluting concentrations of BMP4 (0.1, 0.01, or 0.001 μg/ml) and analyzed by flow cytometry. Bars show the mean and SDs from six separate experiments. Left panel, The percentage of DN cells in the four DN subsets. Right panel, The ratio of CD44<sup>-</sup>DN cells to CD44<sup>+</sup>DN cells. The ability of BMP4 treatment to arrest thymocyte differentiation at the CD44<sup>-</sup>CD25<sup>-</sup> DN stage was dose-dependent.

FIGURE 3. BMP4 arrests thymocyte development at the CD44<sup>+</sup> CD25<sup>-</sup> DN stage in a dose-dependent manner. A, E14.5 FTOC were cultured for 3 days with 0.1 μg/ml BMP4 and analyzed by flow cytometry. Upper panel, Staining with anti-CD4 and anti-CD8. Lower panel, The composition of the DN subsets, stained with anti-CD44 and anti-CD25. BMP4 treatment increased the proportion of cells in the CD44<sup>-</sup>CD25<sup>-</sup> subset and decreased the proportion of cells in the CD44<sup>+</sup>CD25<sup>-</sup> subset. B, Cells from the cultures in A were stained with anti-CD2. BMP4 treatment reduced cell surface CD2 expression. C, E14.5 FTOC were cultured for 5 days with 0.1 μg/ml BMP4 and analyzed by flow cytometry. Upper panel, shows CD4 and CD8 staining in control and BMP4-treated cultures. Lower panel, Staining with anti-CD44 and anti-CD8. E14.5 FTOC were cultured for 6 days with 0.1 μg/ml BMP4 and analyzed by flow cytometry, respectively.
in the control cultures, 50% of DN cells were CD117+CD25−, compared with only 5.7% in the control. To confirm that these CD117+ DN cells also expressed CD44, we stained the DN cells with both anti-CD117 and anti-CD44, having gated out CD4+ CD8+, CD3−, and CD25+ cells. In both control and BMP4-treated cultures, the CD44+ cells also expressed CD117 (Table I).

The number of cells recovered from the BMP4-treated cultures was not significantly different from the control cultures (Fig. 3D), and more CD44+ CD25− DN cells were recovered from BMP4-treated cultures than from control cultures (Fig. 3E).

The inhibition of thymocyte development by BMP4 was dose-dependent, as demonstrated by the percentage of DN thymocytes in the four populations defined by cell surface CD25 and CD44 expression (Fig. 3F, left panel), and by the ratio of CD44+ CD25− to CD44− CD25+ cells in the cultures (Fig. 3F, right panel).

Noggin neutralizes the effect of BMP4 in FTOC

One explanation for the accumulation of immature thymocytes in the presence of BMP4 is that BMP4 is toxic for all more mature thymocytes. To assess this possibility we tested whether the addition of recombinant Noggin, in the form of a Noggin-Fc fusion protein, would neutralize the effects of exogenous BMP4. Recombinant Noggin should bind directly to BMP4 and thereby prevent it from binding its receptor. E14.5 FTOC were treated with BMP4 and Noggin for 5 days and then stained with anti-CD44, anti-CD25, anti-CD4, anti-CD3, and anti-CD8 Abs. In the BMP4-treated cultures, 33% of DN cells were CD25− CD44+, compared with 61 and 70% in the control cultures and the cultures treated with both BMP4 and Noggin, respectively. Likewise, the increase in the percentage of CD44+ CD25− DN cells in the BMP4-treated cultures was neutralized by addition of Noggin from 20% (BMP4-treated) to 1.5% (BMP4- and Noggin-treated), compared with 4% in control cultures (Fig. 4A). The fact that addition of Noggin to the BMP4-treated cultures neutralizes the action of BMP4 on thymocyte development indicates that BMP4 is not nonspecifically toxic to developing thymocytes but is mediating a biological effect.

BMP4 enhances survival of thymocytes

BMP2/4 signaling affects apoptosis and cell cycle progression in many developmental systems (3–5, 7, 16). Therefore, it seemed possible that addition of BMP4 to the FTOC was increasing the percentage of CD44+ CD25− DN thymocytes by inducing apoptosis specifically in more mature thymocyte populations. To test this, we measured apoptosis in cultured thymocytes by annexin V staining. FTOC were treated with BMP4 for 5 days and then cells were stained with annexin V and anti-CD44 and anti-CD25 Abs. The percentage of dying cells was significantly lower in BMP4-treated cultures compared with control cultures for all of the DN populations (Fig. 4B). Thus, addition of BMP4 does not induce cell death, but enhances survival of cultured thymocytes, suggesting that BMP4 acts directly to prevent the differentiation of CD44+ CD25− DN cells along the T cell lineage, rather than indirectly, by causing the death of the subsequent thymocyte populations.

BMP4 inhibits thymocyte proliferation

The cell cycle status of thymocytes in BMP4-treated cultures was assessed by PI staining. Cells harvested from the same FTOC used for the cell death assay were stained with PI and anti-CD44 Ab. FACS analysis revealed a reduction in the proportion of cells in S and G2 in the BMP4-treated cultures compared with control cultures (Fig. 4C). There was an ~2.5-fold reduction in the percentage of CD44+ cells in S and G2 in the BMP4-treated cultures compared with the control cultures. This indicates that the enrichment of the CD44− CD25− DN population in the BMP4-treated culture is not a result of an increase in their rate of proliferation relative to other populations in the culture, but rather is due to an arrest in thymocyte differentiation at that stage of development. Thus, BMP4 acts both to arrest early thymocyte differentiation and to inhibit thymocyte proliferation, thereby negatively regulating progression of developing cells along the T cell lineage.

Table I. Expression of CD117 on CD44+ CD25− DN cells from E14.5 FTOC treated with 0.1 µg/ml BMP4 for 5 days

<table>
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<tr>
<th>Control</th>
<th>BMP4</th>
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<tr>
<td>Percentage of CD44+ CD25− DN cells that express CD117</td>
<td>97.3</td>
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*Cells were stained with Abs against CD3, CD4, CD8, and CD25 labeled with the same fluorochrome, and with anti-CD44 and anti-CD117. Cells that stained positive for CD3, CD4, CD8, and CD25 were excluded, and the percentage of CD44+ cells that stained with the anti-CD117 Ab in control and BMP4-treated cultures is given.

FIGURE 4. BMP4 treatment is not toxic. BMP4 enhances thymocyte survival and inhibits thymocyte proliferation. A, The effect of BMP4 treatment on DN thymocytes is neutralized by addition of the BMP4 antagonist Noggin. E14.5 FTOC were treated for 6 days with 0.1 µg/ml BMP4 alone (left dot plot), 0.1 µg/ml BMP4 and 0.5 µg/ml Noggin (middle dot plot), or no treatment (right dot plot). Cells were analyzed by flow cytometry, and anti-CD25 and anti-CD44 staining on DN cells are shown. Noggin inhibited the ability of BMP4 to arrest thymocyte differentiation, indicating that BMP4 is not nonspecifically toxic in the cultures. Cell recoveries were 1.3 × 10^6 for BMP4-treated cultures, 1.3 × 10^6 for BMP4 and Noggin treated cultures, and 1.8 × 10^6 for control cultures. B, BMP4 treatment enhances thymocyte survival. E14.5 FTOC were treated for 5 days with 0.1 µg/ml BMP4. Apoptosis was measured by annexin V staining. The graph shows the percentage of annexin-positive cells in the DN subsets and in the whole thymocyte population. Bars show data from six individual experiments. BMP4 treatment reduced the percentage of apoptotic cells in all subsets relative to control cultures. This reduction is statistically significant. C, BMP4 treatment inhibits thymocyte proliferation. Cells from the same cultures as in B were stained with PI and anti-CD44. The graph shows the percentage of cells in the S and G2 in all cells, the CD44+ fraction and the CD44− fraction. Data are from six individual experiments. BMP4 treatment significantly reduced the proportion of cells in S and G2 relative to the control.
The BMP2/4 antagonist Noggin accelerates maturation of DN thymocytes

To study the function of endogenously produced BMP2 and BMP4 in the thymus, we neutralized endogenous BMP2/4 activity in FTOC by addition of recombinant Noggin alone. E14.5 FTOC were treated with Noggin for 6 days. The thymocytes were then analyzed by flow cytometry. In the Noggin-treated cultures, the percentages of DP cells (Fig. 5A) and CD44+CD25+ DN cells (Fig. 5, B and C) were higher than in control cultures. In addition, the percentage of CD2-expressing cells was increased in Noggin-treated cultures compared with control cultures (Fig. 5, D and E). In a representative experiment, 42% of cells stained brightly with anti-CD2 Ab, compared with 25% of thymocytes in the control culture. The increase in CD2 expression is in accordance with the increased percentage of the most mature CD44+CD25+ population of DN cells, because cell surface CD2 expression is up-regulated on pre-TCR signaling (38, 39).

FIGURE 5. Noggin accelerates the development of DN thymocytes. A–E, E14.5 FTOC were cultured for 6 days with 0.5 μg/ml Noggin and analyzed by flow cytometry. Cell recoveries in A, B, and D were 1.6 × 10^6 for the control cultures and 3 × 10^6 for the Noggin-treated cultures. A, CD4 and CD8 staining showed that Noggin increased the percentage of DP thymocytes. B, Composition of DN subsets, stained with anti-CD44 and anti-CD25. C, Proportion of DN cells in the four subsets defined by CD44 and CD25. Data are the mean and SD of six individual experiments. The increase in the percentage of CD25+CD44+ DN cells in the Noggin-treated cultures is statistically significant, relative to the control. D, CD2 expression in Noggin-treated and control cultures. Noggin increased cell surface CD2 expression. E, The graph shows mean CD2 expression in Noggin-treated and control cultures. Data are from six individual experiments. F, The scatter plot shows the cell recovery from E14.5 FTOC cultures for 5 days. The total number of cells recovered in each experiment from Noggin-treated cultures was divided by the number of cells recovered from the control cultures to give the relative cell number from 13 individual experiments.

FIGURE 6. Noggin accelerates the development of DN thymocytes and enhances thymocyte survival. A–C, E13 FTOC were treated for 5 days with 0.5 μg/ml Noggin or 0.1 μg/ml BMP4 and analyzed by flow cytometry. A, Analysis of DN cells, stained with anti-CD25 and anti-CD44. B, Cell surface CD2 expression in the cultures. C, Cell surface CD4 and CD8 expression in the cultures. D and E, E14.5 FTOC were cultured for 5 days with 0.5 μg/ml Noggin. D, The graph shows the percentage of cells from these cultures in the S and G2 phase of the cell cycle in all cells, and in the CD44+ and CD44+ fractions. There were no significant differences in cell cycle status between the Noggin-treated and control cultures. Data are derived from six individual experiments. E, Noggin treatment enhances thymocyte survival. Apoptosis was measured by annexin V staining. The graph shows the percentage of annexin-positive cells in the DN subsets and in the whole thymocyte population. Histograms show data from six individual experiments. Noggin treatment reduced the percentage of apoptotic cells, relative to control cultures. This reduction is statistically significant.
The number of cells recovered from Noggin-treated cultures was greater than in the control cultures as shown for individual experiments in Fig. 5F.

In some experiments, we treated E13 FTOC for 5 days with Noggin or BMP4. Addition of Noggin at this earlier stage of ontogeny reduced the proportion of CD44⁺CD25⁻ DN from 9% in control cultures to 2.9% in Noggin-treated cultures, whereas 42% of cells were CD44⁺CD25⁻ DN in BMP4-treated cultures (Fig. 6A). In agreement with the data from E14.5 FTOC, the proportion of CD2⁺ cells was increased in the Noggin-treated cultures and decreased in the BMP4-treated cultures relative to the control (Fig. 6B). As in the E14.5 FTOC, Noggin treatment increased the percentage of DP cells, which were just beginning to appear in the control cultures (Fig. 6C). BMP4 treatment blocked the production of DP thymocytes in E13 FTOC (Fig. 6C). We did not observe this absence of DP cells in the E14.5 BMP4-treated cultures (Fig. 3A), and this difference is probably because the BMP4 was added at a later time point when some cells were irreversibly destined to become DP.

Noggin enhances the survival of cultured thymocytes

Thymocytes from Noggin-treated FTOC were stained with PI and anti-CD44 Ab. There was no statistically significant difference in the proportion of CD44⁺ or CD44⁻ thymocytes in the S and G₂ stages of the cell cycle between Noggin-treated and control cultures (Fig. 6D). Thus, the increased expression of CD2 in the DN cells of Noggin-treated cultures was not due to increased proliferation of this population, but rather resulted from an increase or acceleration in thymocyte differentiation. Thymocytes from the same cultures were stained with annexin V and anti-CD44 and anti-CD25 Abs. The percentage of annexin V-positive dying cells was significantly lower in Noggin-treated cultures compared with control cultures (Fig. 6E). The fact that both BMP4 and its inhibitor Noggin reduced thymocyte apoptosis in FTOC seems surprising, but may reflect the different cellular composition of the BMP4-treated and Noggin-treated cultures, and differences in the survival signals required by thymocytes at different stages in their development. Alternatively, the heterogeneity of cells within each thymocyte population, with regards to developmental state and lineage commitment, could mean that within a given subset there may be both cells that survive better in response to BMP4 treatment and cells that survive better in response to BMP4 neutralization (Noggin treatment).

FIGURE 7. Noggin cannot promote differentiation of DN thymocytes in the absence of pre-TCR signaling. A, E17.5 TCRβ⁻/⁻/S⁻/⁻ FTOC were cultured for 5 days with 0.5 μg/ml Noggin and analyzed by flow cytometry for CD4 and CD8 expression. Noggin did not induce differentiation to the DP stage. Cell recoveries were 1.3 × 10⁵ for the control culture and 2 × 10⁵ for the Noggin-treated culture. B–D, E14.5 TCRβ⁻/⁻/S⁻/⁻ FTOC were cultured for 5 days with 0.1 μg/ml BMP4, both 0.1 μg/ml BMP4 and 0.5 μg/ml Noggin, or 0.5 μg/ml Noggin alone. B, DN cells were analyzed for CD25 and CD4 expression. C, Apoptosis in DN subsets was assessed by annexin V staining. The graph shows the percentage of annexin-positive cells in the DN subsets and in the whole thymocyte population. Bars show data from six individual experiments. Neither Noggin nor BMP4 treatment induced apoptosis. D, PI and anti-CD44 staining. The graph shows the percentage of cells from these cultures in the S and G₂ phases of the cell cycle in all cells, and in the CD44⁺ and CD44⁻ fractions. The decrease in the percentage of CD44⁺ cells in S and G₂ in the BMP4-treated cultures was statistically significant compared with the other cultures. E, The scatter plot shows the cell recovery from 5-day FTOC cultures. The total number of cells recovered in each experiment from BMP4, BMP4 and Noggin, and Noggin-treated cultures was divided by the number of cells recovered from the control cultures, to give the relative cell number from nine individual experiments.
Bmp2/4 regulation in TCRβ−/−δ−/− FTOC

Thymocyte development in TCRβ−/−δ−/− mice is arrested at the CD25− DN stage, due to the inability of the developing thymocytes to express and signal through the pre-TCR complex (40). As Noggin treatment accelerated thymocyte differentiation in wild-type FTOC and increased populations that arise after pre-TCR signaling, we asked whether neutralization of endogenous BMP2/4 signaling by Noggin treatment would be sufficient to overcome the block in thymocyte development in TCRβ−/−δ−/− FTOC. We treated TCRβ−/−δ−/− FTOC with Noggin for 5 days. Noggin was unable to overcome the developmental arrest, and 95% of thymocytes recovered from the Noggin-treated cultures were DN cells, compared with 93.6% in control cultures (Fig. 7A). When we treated TCRβ−/−δ−/− FTOC for 5 days with BMP4 alone, both BMP2 and Noggin, and Noggin alone, and analyzed the DN populations, we found that BMP4 increased the percentage of CD44+CD25− cells compared with control cultures (Fig. 7B). In contrast to the effect of Noggin treatment in wild-type FTOCs, there was no increase in the percentage of CD44+CD25− DN cells in Noggin-treated TCRβ−/−δ−/− FTOC compared with control cultures (Fig. 7B), confirming that neutralization of endogenous BMP2/4 signaling in the FTOC is insufficient to overcome the developmental arrest caused by the lack of pre-TCR signaling. In cultures treated with both BMP4 and Noggin, the effect of BMP4 was neutralized and no difference was observed in comparison to control cultures (Fig. 7B).

Neither BMP4 nor Noggin treatment of TCRβ−/−δ−/− FTOCs induced cell death in comparison to control FTOCs as assessed by annexin V staining (Fig. 7C). PI staining showed that BMP4 inhibited cell proliferation in TCRβ−/−δ−/− FTOCs. The percentage of CD44+ cells in the S and G2 phases was lower in BMP4-treated cultures compared with control cultures and cultures treated with Noggin, and both Noggin and BMP4 (Fig. 7D). The cell recovery from the cultures are consistent with these findings (Fig. 7E).

These data suggest that the ability of BMP4 to arrest thymocyte differentiation is independent of pre-TCR signaling, and that neutralization of BMP2/4 signaling is insufficient to overcome the need for a pre-TCR signal. This is consistent with the finding that in wild-type FTOC, BMP4 arrested thymocyte development at the CD44+CD25− stage, before TCRβ rearrangement and pre-TCR signaling.

Discussion

Our data suggest that the morphogens BMP2 and BMP4 are made by the thymus epithelium and that they signal to DN thymocytes to negatively regulate T cell differentiation. During the development of many tissues, the action and local concentration of BMPs are controlled by specific antagonists and cell fates are specified by these molecules in a concentration-dependent manner (4, 5, 41). In vertebrate development, BMP4 is an important negative regulator of neural induction, whereas its antagonists Noggin and Chordin promote neural induction and are essential for patterning of the neural tube (5, 16). As the BMP2/4 antagonists Chordin, Tsg, and Noggin are expressed in the thymus, it is possible that BMP2/4 signaling might work in a similar way to regulate T cell development. BMP2/4 signaling might act to specify the fate of lymphocyte precursors, the antagonists promoting T cell lineage commitment and thymocyte differentiation, and BMP4 and BMP2 inhibiting T cell lineage commitment and differentiation, and perhaps also maintaining some precursors in a pluripotent state. In this way, BMP2/4 signaling might be involved in thymic homeostasis, regulating the number of cells in the thymus and representation of the different cell types.

Both TGFβ and sonic hedgehog (Shh) negatively regulate thymocyte development and inhibit the transition from DN to DP cell (24, 42). Shh arrests thymocyte development at the CD25+ DN stage, after TCRβ gene rearrangement (24), whereas BMP4 treatment arrests thymocyte development at the earliest CD44+CD25− DN stage. The Shh and BMP4 pathways interact during the organization of many developing tissues, and in human hematopoietic precursors the Shh pathway is upstream of BMP4 signaling (21).

As BMP4 arrests thymocyte development at an earlier developmental stage than Shh, it seems likely that Shh signaling is downstream of BMP4 signaling in the regulation of thymocyte development, although the relationship between these two pathways remains to be elucidated.

We found expression of both BMP2 and BMP4 in the thymus. BMP2 and BMP4 are >92% identical at the amino acid level and they have the same biological activity (6). However, they are encoded by different genes and they have unique essential nonredundant roles during mouse embryogenesis (3, 7), which presumably reflect differences in the timing and tissue specificity of their expression. Our study does not distinguish between the function of BMP2 and BMP4 in the thymus, as both gene products are expressed, and the increased thymocyte differentiation in FTOC on Noggin treatment could be due to neutralization of the activity of either or both these BMPs. It is possible that BMP2 and BMP4 have overlapping functions in the thymus, or that the two genes are expressed at different times and in different locations, thereby effecting different target cells.

BMP4 treatment arrested thymocyte development at the earliest CD44+CD25− stage. This population is not committed to the T cell lineage but contains cells that have multipotent activity, and can give rise to T, B, NK, and dendritic cells (26, 29). Neutralization of endogenous BMP2/4 signaling promoted thymocyte differentiation. Taken together these findings suggest that BMP2/4 signaling might be involved in the specification of T cell lineage commitment. Local concentrations of positive (Chordin, Noggin, Tsg) or negative (BMP2/4) regulators could signal to individual thymocyte precursors, determining their lineage commitment, and the timing and speed of their differentiation along the T cell lineage.

In the future it will be interesting to assess this model for the function of BMP2/4 in the thymus using genetic models, and to study the interactions between BMP signaling and the other patterning genes that have been shown to function during T cell development (24, 32, 43, 44).

References


