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BMP Signaling Is Required for Normal Thymus Development

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The microenvironment of the thymus fosters the generation of a diverse and self-tolerant T cell repertoire from a pool of essentially random specificities. Epithelial as well as mesenchymal cells contribute to the thymic stroma, but little is known about the factors that allow for communication between the two cell types that shape the thymic microenvironment. In this study, we investigated the role of bone morphogenetic protein (BMP) signaling in thymus development. Transgenic expression of the BMP antagonist Noggin in thymic epithelial cells under the control of a Foxn1 promoter in the mouse leads to dysplastic thymic lobes of drastically reduced size that are ectopically located in the neck at the level of the hyoid bone. Interestingly, the small number of thymocytes in these thymic lobes develops with normal kinetics and shows a wild-type phenotype. Organ initiation of the embryonic thymic anlage in these Noggin transgenic mice occurs as in wild-type mice, but the tight temporal and spatial regulation of BMP4 expression is abrogated in subsequent differentiation stages. We show that transgenic Noggin blocks BMP signaling in epithelial as well as mesenchymal cells of the thymic anlage. Our data demonstrate that BMP signaling is crucial for thymus development and that it is the thymic stroma rather than developing thymocytes that depends on BMP signals. The Journal of Immunology, 2005, 175: 5213–5221.

The adult thymic microenvironment consists of a complex mixture of epithelial cells, interdigitating dendritic cells, macrophages, and fibroblastoid cells. Whereas fibroblastoid cells derive from immigrating embryonic mesenchymal cells of the neural crest, and dendritic cells and macrophages in the adult thymus originate from the bone marrow, epithelial cells represent the resident type of the thymic microenvironment. During development, these epithelial cells form the initial organ anlage. In mice, initiation of thymus development begins around embryonic day (dE)10.5 with the outgrowth of epithelial cells from the third pharyngeal pouch endoderm into the underlying mesenchyme (1–3). This step depends on a variety of factors, such as Hoxa3 (4), Pax-9 (5, 6), Pax-1 (7), Eya1 (8), Rae28 (9), and Chordin (10), that are involved in the patterning and differentiation of the branchial arches during mouse development. The ensuing differentiation phase of thymus development is characterized by the interaction of epithelial cells, the differentiation of which depends on the transcription factor Foxn1, the product of the nude locus (11), with neural crest-derived mesenchymal cells (12, 13). How thymic epithelial and neural crest-derived mesenchymal cells communicate on the molecular level is currently unknown. Epithelial-mesenchymal interaction is a common theme that has been found in the organogenesis of a variety of organs involving pleiotropic morphogens, such as bone morphogenetic proteins (BMPs), fibroblast growth factors, and hedgehog proteins (14). The in vivo role of fibroblast growth factors and hedgehog proteins during thymus development have been reported (15, 16), but a possible role for BMP signaling in thymus development has not been investigated in vivo. However, in vitro analysis of the effect of purified BMP4 protein on thymocyte development in a fetal thymic organ culture (FTOC) indicated that BMP proteins may act directly on developing lymphocytes and cause an early developmental block (17, 18). Another group presented evidence that this effect may be due, at least in part, by indirectly affecting the thymic stroma in this experimental model (19).

In this study, we have addressed the role of BMP signals in thymus development in vivo. The analysis of the role of the BMP signaling network in this process is, however, complicated by the fact that it plays important roles much earlier in embryonic life. To be able to separate early and late effects in vivo, we used a transgenic approach. BMP signaling can be modulated by the expression of specific protein antagonists, such as Noggin (20, 21). To investigate the possible role of BMP signaling during thymus development, we used a Foxn1 promoter fragment that directs the expression of transgenes to thymic epithelial cells. The analysis of mice transgenic for Noggin under the control of a Foxn1 promoter reveals a crucial role for BMP signals for normal thymus development. Interestingly, blocking BMP signaling in the thymus interferes with proper stroma development, whereas thymocytes differentiate and proliferate normally in a thymus expressing the BMP antagonist Noggin.

Materials and Methods

Generation of transgenic mice

Foxn1::Xnoggin transgenic mice were generated by cloning a 27,970-bp Foxn1 promoter fragment (Y12488, nucleotides 5560–33650 upstream of the Xenopus laevis Noggin (Xnoggin) cDNA (M98807, nt 512-1834; a gift from Dr. R. Harland, University of California, Berkeley, CA). Downstream of the cDNA the bovine growth hormone polyadenylation sequence was inserted into pBSII-SK (Stratagene). The construct was linearized and injected into FVB pronuclei according to standard protocols. A Foxn1::Foxn1 transgene rescued the nude phenotype in skin and thymus of Foxn1+−/− mice (our unpublished observations). Foxn1::Xnoggin transgenic mice were bred with C57BL/6 mice and analyzed as F3 mice to exclude genetic background effects. Four- to six-wk-old mice were used for experiments unless indicated otherwise. Embryos were obtained from timed pregnant mice, and the day of the vaginal plug was counted as day 0.5.
0.5 of pregnancy. Mice were bred and maintained at the animal care facility of the Max-Planck-Institute for Immunobiology. All experimentation was approved by the Institute’s Review Committee.

**Immunohistochemistry**

Frozen thymus sections were processed as described (22) using mAbs directed against CD8 (clone 53-6.7; BD Biosciences), cytokeratin 8 (Tromba-1, a gift from R. Kemler, Max-Planck Institute for Immunobiology, Freiburg, Germany), *Ulex europaeus* agglutinin-1 (UEA-1; Vector Laboratories), and cytokeratin 5 (Covance Research Products). Paraffin-embedded sections were stained using an antisera directed against phosphorylated Smad1/5/8 proteins according to a protocol supplied by the manufacturer (Cell Signaling Technology). Control stainings were conducted without primary Abs. Fluorochrome-labeled Abs were detected using a Hamamatsu CCD C4880 camera mounted on a Zeiss Axioskop microscope.

**FACS**

FACS analyses were conducted as described (22) using the FITC-, PE-, or allophycocyanin-conjugated mAbs (clone names given in parentheses): anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-Vβ2 (B3.60), anti-Vβ3 (KU25), anti-Vβ6 (RR4-7), anti-Vβ7 (TR310), anti-Vβ8.1/8.2 (MR5-2), anti-Vβ11 (RR3-15), and anti-Vβ13 (MR12-3) were purchased from BD Biosciences. Apoptotic cells were detected using the Annexin V-FITC Apoptosis Detection Kit according to the manufacturer’s recommendations (BD Biosciences).

**In vivo BrdU labeling**

For detection of BrdU incorporation, 5- to 7-day-old Foxn1::Xnoggin and wild-type (WT) control mice were given injections i.p. with 0.4 mg of BrdU. Thymi were harvested 20 min after injection. Cells that had incorporated BrdU were detected using the BrdU Flow Kit according to the manufacturer’s recommendations (BD Biosciences). Stained cells were read on an LSR II FACS analyzer (BD Biosciences).

**mRNA in situ hybridization and RT-PCR analysis**

In situ hybridization of paraffin-embedded sections were conducted as described (23) using riboprobes for Foxn1 (X81593, nt 1–2200), Noggin (N98807, nt 512-1834), Bmp4 (NM_007554, nt 497-1000), Bmp2 (NM_007555, nt 721-1250), Mx1 (NM_010835, nt 996-1492), and Hoxa3 (Y11717, nt 593-1724). Control hybridizations were conducted with sense riboprobes. RT-PCR analyses on deoxyguanosine-treated lobes were conducted as before (23) using primers described earlier (18). Mock samples without reverse transcriptase did not show a PCR signal.

**Results**

**Characterization of mice that express the BMP antagonist Noggin under the control of a Foxn1 promoter fragment**

To investigate the role of BMP signaling during thymopoiesis in vivo, we generated transgenic mice that expressed the BMP antagonist Xnoggin under the control of a 27.9-kb Foxn1 promoter fragment. Four independent founder animals were generated, and the offspring of three of these founders (no. 16, no. 17, and no. 18) were analyzed in detail; their phenotypes were identical. Representative of all three founders, in situ hybridization analyses conducted for offspring of founder no. 16 revealed that the Foxn1 promoter fragment faithfully directed expression of the transgene to the Foxn1 expression domain, namely to the thymic epithelial network (Fig. 1A) and to keratinocytes in the skin as expected (data not shown). Indeed, Noggin was found to be expressed at the early stages of thymus development in a pattern that was indistinguishable from that of Foxn1. All Foxn1::Xnoggin transgenic mice derived from these three founders lacked a proper hair coat and a detectable thymus in the normal mediastinal location (Fig. 1B). Consistently, single-cell suspensions of the entire content of the mediastinum of these three mice contained only background levels of cells that fall into the CD4 and CD8 double-positive gate in flow cytometry; their number (<0.001% of normal values) is indistinguishable from that found in nude mice (Fig. 1C) under similar experimental conditions. Nevertheless, mature T lymphocytes were consistently detected in lymph nodes (Fig. 1D) and spleens (data not shown) in Foxn1::Xnoggin mice, whereas they were absent in nude mice. Splenic T lymphocytes were indistinguishable from WT cells by CD4, CD8, and TCR-β expression but reduced 25-fold in number (0.49 ± 0.23 × 10^6 vs. 12.27 ± 1.64 × 10^6 CD4-positive T cells in Foxn1::Xnoggin and WT controls, respectively). Splenic CD4-positive T cells in Foxn1::Xnoggin mice express high levels of CD44, and the fraction of cells expressing high levels of CD62L is reduced (data not shown). This marker profile is consistent with the memory phenotype of peripheral T lymphocytes found in day 3 thymectomized mice, which has been shown to coincide with lymphopenia-induced proliferation in neonates (24). These findings suggested an ectopic location of a thymus of reduced size in Foxn1::Xnoggin mice.

During embryogenesis, the bilateral thymic anlagen move caudally and ventrally to meet in the prospective mediastinum above the heart. This “descensus” of the thymus is effectively accomplished around day E15.5 in WT mice. In contrast, in Foxn1::Xnoggin mice, ectopic bilateral dysplastic thymic lobes were consistently identified at the level of the developing hyoid bone at this stage (Fig. 1E). Concomitantly, the size of Foxn1::Xnoggin thymic lobes at day E15.5 were reduced ~20-fold compared with WT thymi (see inset in the WT E15.5 panel of Fig. 2A). Thus, blocking BMP signaling by the expression of the BMP antagonist Noggin in thymic epithelial cells (TECs) interferes with normal thymus development. Thymic lobes are dysplastic, located ectopically in the neck and of strongly reduced size.

It has recently been shown that the addition of BMP4 protein to deoxyguanosine-treated FTOC increased Foxn1 expression in vitro (19). We therefore investigated the possibility that Foxn1 expression depends directly on BMP signaling. By in situ hybridization, Foxn1 expression in Foxn1::Xnoggin transgenic mice appeared unaffected at day E11.5 and day E12.5, i.e., transgene expressing TECs also expressed Foxn1 (Fig. 1A). This was considered significant, because transgenic Noggin affected the expression of several other transcripts in TECs at this point of development (see below). Consistently, expression of cytoketatin 5 and 8 in transgenic TECs at day E12.5 was normal (Fig. 2C). This ruled out that BMP signaling is immediately required for Foxn1 expression and that a significant squelching of Foxn1 promoter binding proteins by transgenic sequences had occurred. Starting around day E14.5, cystic structures formed by epithelial cells were invariably found in all investigated Foxn1::Xnoggin thymi (Fig. 2). Epithelial cells associated with these cystic structures were found to lack Foxn1 expression (Fig. 2A) and to be spatially separated from developing thymocytes (data not shown). Epithelial cells not associated with these cysts, in contrast, expressed Foxn1, and a subset of these cells expressed either cytoketatin 5 or cytoketatin 8, indicative of the development of medullary TECs and cortical TECs, respectively (Fig. 2C). Cystic dysplasia of the thymus has been found in mice affected by a cell autonomous differentiation block in developing thymocytes (25). We therefore investigated T cell development in Foxn1::Xnoggin mice.

**Phenotypical and functional analysis of T cell development in Foxn1::Xnoggin mice**

Normal thymus development requires the undisturbed development of both, thymocytes and the thymic stroma (26, 27). Also, these two components need to properly interact to allow normal thymus development. Dysplastic thymic lobes could therefore result from disturbed thymocyte differentiation or from defects in stroma development. It has recently been shown that BMP signaling directly influences the differentiation and proliferation of thymocytes at distinct stages of development in FTOC in vitro (17–
FIGURE 1. A Foxn1 promoter fragment directs Noggin expression to epithelial cells of the developing thymic anlage. A, In situ hybridizations of the thymic anlage at dE11.5 and dE12.5 are shown for the expression of Foxn1 and transgenic Noggin. Each panel is oriented such that down is ventral and left is medial. Note that transgenic Noggin expression is restricted to thymic epithelial cells and is not found in surrounding mesenchymal cells. Scale bar, 50 μm. B, A macroscopic view of a mediastinum of a 4- to 5-wk-old WT mouse (top), and a Foxn1::Xnoggin transgenic mouse of the same age (bottom) is shown. t, thymic lobe; h, heart. The thymus is absent in transgenic mice. C, Absolute number of cells that fall into the CD4 and CD8 double-positive gate by flow cytometry found in single-cell suspensions prepared from the entire contents of the mediastinum of 4- to 5-wk-old WT, nude, and Foxn1::Xnoggin mice. Data for transgenic mice derived from three independent founders are shown. Each dot represents one mouse. Cells that fall into the CD4 and CD8 double-positive gate by flow cytometry in nude mice, that lack a functional thymus, derive from autofluorescent cells, and their levels were considered background (<0.001% of normal values). D, FACs analyses of 4- to 5-wk-old WT, nude, and Foxn1::Xnoggin peripheral lymph node cells. E, Transversal, H&E-stained sections of dE16.5 Foxn1::Xnoggin embryos at the level of the developing hyoid bone. The location of dysplastic thymi in this “dwarfish” thymus appear to proceed through the well-established, developmental stages that are present at normal ratios and that proliferate with kinetics that are indistinguishable from WT mice. Finally, we investigated the possibility that BMP signaling interfered with the development of a diverse T cell repertoire and that the thymus of Foxn1::Xnoggin mice supported 19). These studies showed that developing thymocytes express BMP receptors (BMPR) and downstream signaling effectors and that the addition of BMP proteins to FTOC blocks T cell development at the double-negative (DN1) (18, 19) and the DN4 stage (17). From these studies one would predict that transgenic Noggin should lead to a de-repression of T cell development from the proposed BMP block. It is important to point out, however, that the effects of another morphogen, namely sonic hedgehog, on T cell development in FTOC in vitro has been shown to be strongly concentration-dependent and that high and low concentrations induce opposite effects (16). We therefore analyzed T cell development in 5- to 7-day-old (dP5-dP7) Foxn1::Xnoggin mice, a time point when dysplastic thymic lobes could be reproducibly isolated from their location adjacent to the hyoid bone. In older mice these dysplastic thymi were found at various locations in the neck region, which made their reliable isolation difficult. Interestingly, we did not find any evidence for a block in T cell development that could explain the drastically reduced thymus size in Foxn1::Xnoggin mice. The different subsets of T cell development are found at normal ratios in transgenic dP5-dP7 mice (Fig. 3, A and C), although total thymocyte numbers are reduced 26-fold in these mice (Fig. 3B), which is in agreement with the drastic reduction in thymus size (Fig. 2A). We then sought to rule out a significant delay in the proliferation kinetics of transgenic thymocytes. Thymocyte numbers in 5- to 7-day-old WT mice are still expanding such that differences in the proliferation kinetics of the most rapidly dividing thymocytes, the early double-positive cells (28, 29), that might be responsible for the reduced thymus size, would become obvious. We measured the in vivo incorporation of the thymidine analog BrdU and found that the fraction of cycling cells among coreceptor double-positive and DN thymocytes is the same in WT and Foxn1::Xnoggin dP5-dP7 mice (Fig. 3D). Furthermore, we find WT amounts of apoptotic cells in single-cell suspensions prepared from transgenic thymi (Fig. 3E), which makes it unlikely that increased apoptosis of developing thymocytes causes the reduction in thymus size. Thymocytes in 5- to 7-day-old mice are thought to be derived from the first wave of αβ T cell development (30), and the normal frequency of the different subsets ruled out a block in the transitions from DN to double-positive and from double-positive to single-positive thymocytes. To have a closer look at the earliest stages of T cell development of this first wave, we analyzed embryonic thymocytes at dE16.5 by flow cytometry. Again, all DN subsets were found in normal ratios in transgenic embryos (Fig. 3F). Taken together, Foxn1::Xnoggin mice have dysplastic, bilateral thymic lobes containing thymocytes that are reduced in number proportionate to the overall size of the organ. The lymphocytes in this “dwarfish” thymus appear to proceed through the well-established, developmental stages that are present at normal ratios and that proliferate with kinetics that are indistinguishable from WT mice. Finally, we investigated the possibility that BMP signaling interfered with the development of a diverse T cell repertoire and that the thymus of Foxn1::Xnoggin mice supported...
limited number of mature T cell clones in the periphery, as has been described in mice thymectomized 3 days after birth (24, 31). Collectively, we find no evidence for abnormalities in developing thymocytes that could account for the drastic reduction in thymus size found in Foxn1::Xnoggin mice.

Analysis of the developing thymic stroma in Foxn1::Xnoggin mice

To investigate the possibility that the thymic stroma rather than developing thymocytes required BMP signaling for normal development we analyzed thymic stroma by RT-PCR and detected the expression of BMPR-IA, BMPR-IB, BMPR-II, and Smad-1, -4, and -8 (Fig. 5). This finding suggested that the thymic stroma should also be able to respond to BMP signals. In an attempt to separate effects of disturbed BMP signaling on thymocytes and stroma, we concentrated on the earliest stages of thymic epithelial cell development that occur before significant numbers of thymocyte precursors have entered the thymic anlage. Indeed, we found that transgenic Noggin resulted in the aberrant expression of BMP2 and BMP4 in epithelial as well as mesenchymal components of thymic stroma at these early stages of thymus development. At dE10.75, BMP4 is strongly expressed in Foxn1-positive epithelial cells of the ventral and medial part of the third pharyngeal pouch outgrowth that gives rise to the thymus and the parathyroid (Figs. 6). At this time during development, BMP4 is also strongly expressed in the neural crest-derived mesenchymal cells that surround the thymic anlage. In contrast to Foxn1, which is from then on continuously and uniformly expressed in all epithelial cells of the thymic anlage (see Fig. 1A), strong BMP4 expression at dE11.5 was found to be restricted to the lateral aspect of the anlage and at dE12.5 mainly in mesenchymal cells that will later form the thymic capsule. Expression of the BMP target gene Mxs-1 strictly followed the BMP4 expression pattern during the observed time points, whereas BMP2 expression was undetectable by in situ hybridization. The observation that BMP4 is expressed in epithelial cells at dE10.75 and that at dE12.5 capsular mesenchymal cells, but not TECs, express BMP4 and Mxs-1 suggested a role for BMP signals in the epithelial-mesenchymal interaction that shapes the thymic stroma (32–34). Indeed, transgenic Noggin profoundly disturbed the physiological pattern of BMP expression in Foxn1::Xnoggin mice (Fig. 6). In contrast to the WT anlage, BMP4 mRNA expression was maintained in epithelial cells and absent in mesenchymal cells forming the thymic capsule at dE12.5, although these cells were present in a normal configuration (Hoxa3 panels in Fig. 6). Sustained, homogeneous BMP4 mRNA expression was observed in thymic epithelial cells without the polarization toward the lateral aspect seen in the WT dE11.5 thymic anlage. BMP2 mRNA, which was undetectable during any time point in WT mice, was aberrantly up-regulated in Foxn1::Xnoggin mice at dE12.5. Thus, diminishing expression of BMPs in epithelial cells during the analyzed time points in WT mice contrasted with increased expression of BMP4 and the additional aberrant expression of BMP2 in transgenic mice. The analysis of Msx-1 as an indicator for ongoing BMP signaling revealed expression in epithelial cells at dE11.5 and in mesenchymal cells at dE12.5 in WT mice but not in transgenic mice. This demonstrated that in WT mice, productive BMP signaling occurred early in TECs and at dE12.5 in capsular mesenchymal cells and that transgenic Noggin effectively blocked signaling in both. To confirm this conclusion, we looked for the presence of nuclear phosphorylated Smad 1/5/8 proteins in dE12.5 thymic anlagen as additional evidence for ongoing BMP signaling. This was undetectable in thymic epithelial cells at this stage in WT mice; importantly, the aberrant epithelial expression of BMP2 and BMP4 in only certain specificities that led to an oligoclonal repertoire, which might explain its reduced size. To this end, we analyzed CD4-positive splenic T cells derived from Foxn1::Xnoggin and WT mice by flow cytometry using Vβ-specific mAbs. To avoid genetic background effects, only F1, littermates of crosses to C57BL/6 were investigated. A normal TCR repertoire was found in 18- to 20-wk-old Foxn1::Xnoggin mice (Fig. 4), indicating that a polyclonal repertoire can be generated in these mice. Young WT mice by flow cytometry using Vβ-specific mAbs. To avoid genetic background effects, only F1, littermates of crosses to C57BL/6 were investigated. A normal TCR repertoire was found in 18- to 20-wk-old Foxn1::Xnoggin mice (Fig. 4), indicating that a polyclonal repertoire can be generated in these mice. Young Foxn1::Xnoggin mice revealed an under-representation of some
Foxn1::Xnoggin mice (Fig. 6, bottom panels) also did not lead to productive BMP signaling, most likely because transgenic Noggin interrupts this autocrine loop. Furthermore, evidence for non-cell-autonomous inhibition of BMP signaling via transgenic Noggin expression was detected in mesenchymal cells of the thymic capsule. In WT mice at dE12.5, mesenchymal cells that will later form the thymus capsule showed signs of ongoing BMP signaling as evidenced by the nuclear localization of phosphorylated Smad 1/5/8 proteins. No such staining was observed in Foxn1::Xnoggin mice. Similarly, Msx-1 expression in these cells was also missing.

This indicated that expression of Noggin, which in Foxn1::Xnoggin mice is exclusively of epithelial origin, specifically blocks BMP signaling also in surrounding mesenchymal cells. These experiments lead to three conclusions. First, BMP expression during the earliest steps of thymus development shows a temporally and spatially highly regulated pattern that is already suggestive of an involvement of this morphogen in the interaction between epithelial cells and mesenchymal cells. The fact that expression of Msx-1 and phosphorylation of Smad 1/5/8 proteins in capsular mesenchymal cells at dE12.5 is blocked by transgenic

**FIGURE 3.** T cell development proceeds normally in a Noggin-expressing thymus. A, FACS profiles of WT and Foxn1::Xnoggin thymocytes isolated from 5- to 7-day-old mice. The percentage of cells found in the respective quadrants is shown. B, The total number of thymocytes isolated from 5- to 7-day-old WT and Foxn1::Xnoggin mice is shown. Each dot corresponds to one mouse. C, The fractions of the different thymocyte subsets among total thymocytes (top panel) and coreceptor DN thymocytes (bottom panel) found in 5- to 7-day-old WT and Foxn1::Xnoggin mice were determined by FACS analysis. The mean and SD of at least four determinations for each subset is shown. D, The fraction of DNA-synthesizing cells was determined by BrdU pulse labeling. The results of three independent experiments are shown for 5- to 7-day-old WT and Foxn1::Xnoggin mice. E, The number of apoptotic cells as identified by annexin V staining and annexin V in conjunction with propidium iodide staining was determined in single-cell suspensions of thymocytes isolated from 5- to 7-day-old WT and Foxn1::Xnoggin mice. The numbers indicate the mean percentage and the SD of cells found in the respective gates from three determinations. F, FACS profiles of WT and Foxn1::Xnoggin thymocytes from dE16.5 embryos are shown. The percentage of cells found in the respective quadrants is shown.
Noggin demonstrates functionally that epithelial and mesenchymal cells communicate through BMP signaling. Second, transgenic expression of Noggin in epithelial cells specifically interfered with BMP signaling in epithelial and mesenchymal cells, as evidenced by the absence of Msx-1 and phosphorylated Smad proteins, and led to the aberrant expression of BMP4 at dE11.5 and BMP2 at dE12.5. In contrast, the expression patterns of tissue-specific transcription factors like Foxn1 and Hoxa3 are undisturbed; these findings made the possibility of a mere toxic effect of the transgene on TECs highly unlikely. Third, a disturbed expression pattern of BMP4 and Msx-1 in TECs of transgenic mice as a hallmark of aberrant differentiation was already found at dE11.5, well before the arrival of significant amounts of thymocytes. Together with the observation that thymocytes develop with normal kinetics in Foxn1::Xnoggin thymi, our data indicate that it is the thymic stroma rather than developing thymocytes that requires BMP signals for proper development. Consistently, Noggin transgenic TECs at later stages of development formed cystic structures of nonfunctional epithelial cells. These structures were first detected around dE14.5 (Fig. 2B), i.e., long after epithelial as well as surrounding capsular mesenchymal cells showed clear signs of disturbed BMP signaling. Because Foxn1 expression does not directly depend on BMP signaling (see above), the data suggest that cyst formation and loss of Foxn1 expression in some TECs is secondary to abnormal epithelial-mesenchymal interaction. Cystic dysplasia is found in all later stages of development (Figs. 2A and 7).
Every lobe of dP5–7 thymi contained at least one large epithelial cyst. Such cysts were invariably found right next to phenotypically normal medullary areas characterized by UEA-1-positive, cytokeratin 5-positive, cytokeratin 8-negative epithelial cells and low lymphocyte density and normal cortical areas, characterized by UEA-1-negative, cytokeratin 8-positive, cytokeratin 5-negative epithelial cells and high lymphocyte density (Fig. 7).

**Discussion**

We show that BMP signaling is required for normal thymus development in vivo. Blocking BMP signals by the transgenic expression of Noggin results in the formation of dysplastic thymic lobes of drastically reduced size that do not descend to the mediastinum but remain in their embryonic location on the level of the hyoid bone. Interestingly, the small numbers of thymocytes in these dysplastic lobes show a normal phenotype and develop with normal kinetics. In contrast, the physiological temporal and spatial pattern of BMP2 and BMP4 expression in epithelial and mesenchymal cells of the developing thymic stroma is lost in Noggin transgenic mice even before significant numbers of thymocyte precursors populate the anlage. These findings indicate that it is the thymic stroma that requires undisturbed BMP signaling for normal development rather than the thymocytes that develop with normal kinetics in a thymus expressing the BMP antagonist Noggin.

The analysis of BMP signaling in thymus development, as with other pleiotropic morphogens, is complicated by the fact that loss of function mutations of BMP proteins or their extracellular antagonists lead to early embryonic lethal phenotypes as in the case of BMP2 (35) and BMP4 (36) or to defects that prevent the proper patterning of the pharyngeal endoderm as in the case of chordin (10). The use of the Foxn1 promoter to direct expression of the BMP antagonist Noggin into differentiating thymic epithelial cells is an approach that will be applicable to the in vivo analysis of the effects of other pleiotropic morphogens.

The potential role of BMPs on T cell development has previously been studied in vitro using a FTOC assay (17–19). We developed the transgenic approach with the view that morphogens such as BMPs are thought to function as extracellular signaling molecules that form gradients (20), which cannot easily be mimicked by the addition of BMP protein into the culture medium of an FTOC. Furthermore, the effect of BMP signals on the thymic stroma cannot be properly analyzed in this system. Evidence for the notion that BMPs may indeed work by forming gradients also in the thymus comes from the observation that at dE11.5, only TECs of the lateral aspect of the thymic anlage, those in close proximity to BMP4-positive mesenchymal cells, express BMP4 and the BMP target gene Msx-1 (Fig. 6). Although the physiological relevance of this finding is unclear at this point, we are currently screening for other transcripts that are also asymmetrically expressed in the thymic anlage (our unpublished data). This observation indicates that there are clear differences between TECs positioned in the medial vs lateral thymic anlage at this point of thymus development. According to their position within the thymic anlage, epithelial cells may therefore require distinct levels of BMP signaling for proper development. This could explain why some TECs form nonfunctional cysts and others a thymic microenvironment that is capable of supporting T cell development when asymmetry of BMP signals is lost as in Foxn1::Xnoggin transgenic mice (Fig. 6). However, we cannot rule out the possibility that transgenic Noggin is limited, resulting in sufficient BMP signaling for proper development in some TECs but not others. Collectively, our data demonstrate complex effects of blocked BMP signaling on thymic stroma cells that cannot be properly modeled in vitro.

BMP signals have been suggested to be involved in the organogenesis of a number of organs, e.g., kidney, lung, heart, and tooth (20). In particular, tooth development is a well-studied model for the interaction between epithelial layers and underlying mesenchyme, the interactions of which have been shown to be regulated by BMP signals (37, 38). In tooth development, expression of BMPs shifts together with the odontogenic potential from epithelial cells to the underlying mesenchyme. For the thymus, the finding that expression of Msx-1 and phosphorylation of Smad 1/5/8 proteins in capsular mesenchymal cells at dE12.5 is abolished by transgenic Noggin demonstrates functionally that epithelial and mesenchymal cells communicate through BMP signaling. The requirement for mesenchymal cells in thymus organogenesis is well documented (32–34), but how these tissues communicate on the molecular level has not been determined. Our own work shows that the reduction of migrating neural crest mesenchymal cells also results in ectopically located, hypoplastic thymic anlagen (34). Therefore, the observed phenotype in Foxn1::Xnoggin mice may in large part be due to the inability of the thymic epithelium to properly induce the activity of surrounding capsular mesenchymal cells. To our knowledge, this is the first demonstration of a signaling mechanism that operates between epithelial and mesenchymal cells during early thymus development.

Although epithelial-mesenchymal communication is a complex process that is likely to involve factors other than BMPs, our data clearly demonstrate that Foxn1::Xnoggin thymi at dE11.5 lack the signal that down-regulates BMP4 and suppresses BMP2 expression in differentiating epithelial cells. This signal could be part of a negative feedback loop in which epithelial BMP inhibits its own

**FIGURE 7.** Thymic lobes of 5- to 7-day-old Foxn1::Xnoggin transgenic mice contain cystic areas of dys-differentiated epithelial cells next to phenotypically normal thymic stroma. A, Immunohistochemical staining for cytokeratin 8, UEA-1, and CD8 reveals the thymus architecture in WT and Foxn1::Xnoggin mice. Scale bar, 100 µm. B, Panels show fluorescence microscopic images of stains for cytokeratin 5 (green) and cytokeratin 8 (red). Note that the cyst, which is invariably found in Foxn1::Xnoggin thymic lobes, contains epithelial cells that stain for either cytokeratin 5 or cytokeratin 8 as well as a few double-positive cells (yellow). Scale bar, 50 µm. C, cortex; M, medulla.

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expression. Alternatively, it could be a BMP-dependent signal that comes from the surrounding mesenchyme involving morphogens distinct from BMPs, transgenic Noggin in Foxn1::Xnoggin would block its induction in mesenchymal cells as it inhibits BMP4 expression in these cells (Fig. 6). The interpretation of later stages of thymus development are even more complicated because the signals produced by capsular mesenchymal cells that completely surround TECs starting around dE12.5 are unknown. Therefore, we can only speculate what causes the formation of epithelial cysts that are first observed at dE14.5 together with the occurrence of Foxn1-negative epithelial cells. From the in situ hybridization studies on WT thymic anlagen it is clear that Foxn1 is equally expressed in BMP4-positive as well as BMP4-negative epithelial cells (compare the dE11.5 panels in Figs. 1A and 6), ruling out direct regulation of Foxn1 by BMP4. Consistently, Foxn1 expression in Foxn1::Xnoggin thymi is unaffected at dE11.5 and dE12.5, whereas BMP2, BMP4, and Msx-1 expression is clearly dysregulated. Therefore, we favor the notion that loss of Foxn1 expression in epithelial cells associated with cysts is a result of the disturbed interaction with mesenchymal cells that do not receive the epithelial-derived BMP signals required for their normal differentiation. The fact that epithelial cells at dE11.5 located along the mediolateral axis of the thymic anlage appear to be exposed to different levels of BMP signals, as mentioned above, may explain why not all epithelial cells degenerate into cystic structures. Further work will be necessary to elucidate the differences between epithelial cells that go on to support T cell development and those that lose Foxn1 expression. Irrespective of which mechanism applies, our data demonstrate that normal stroma development depends on unhindered BMP signaling.

The previously reported experiments using FTOCs treated with exogenous BMP protein would have predicted that blocking BMP signals to thymocytes by transgenic expression of Noggin in vivo leads to a de-repression of thymocyte development (17–19). Although it remains formally possible that a specific effect of BMPs on thymocyte maturation was exactly compensated for by alterations in the thymic microenvironment, our data do not show any evidence for a qualitative change in T cell development. Bulk populations of transgenic thymocytes showed normal levels of proliferation and apoptosis, and, at the same time, all four DN stages were represented at WT ratios. From this constellation, we conclude that even small subsets, e.g., DN1s, turn over at a normal rate. Thus, the low number of developing thymocytes cannot be explained by aberrant T cell development, which is why we favor the interpretation that the disturbed development of the thymic stroma in Foxn1::Xnoggin mice limits the niches available for developing thymocytes. To reconcile in vivo and in vitro data, one should keep in mind that the in vivo phenotype of Foxn1::Xnoggin mice represents the sum of most likely multiple effects of blocked BMP signaling during thymus development. The present experiments were designed to dissect as much as possible in vivo the effects on thymocyte development on the one hand and stroma differentiation on the other, although we and others (22, 26, 39) have shown that these two processes are tightly connected. Concerning the reported in vitro experiments, it is important to point out that the effective BMP concentration in vivo is unknown, which is of importance because high and low doses of another morphogen, namely sonic hedgehog, have opposing effects on T cell development in FTOC (16). In our in vivo experiment, the amount of the transgene-derived BMP inhibitor Noggin was clearly sufficient to block BMP signaling in the thymic anlage.

The presented experiments show that BMP signals are involved in the physiological descensus of the thymic anlage to the mediastinum during embryogenesis. This adds to our previous work implicating Pax9 in epithelial cells (6) and BMP signals in neural crest cells (34) as important determinants for the descensus of the thymus. Although Foxn1::Xnoggin mice regularly lack a medias
tinal thymus, a single mediastinal dysplastic thymus was isolated only from one Foxn1::Xnoggin mouse, which suggested that the dysplastic lobes observed at dE15.5 in the neck region do descend in rare cases to the mediastinum (data not shown). This was confirmed by microdissection of newborn Foxn1::Xnoggin mice. The fact that the thymic rudiment of nude mice is found at the correct anatomical location above the heart suggests that Foxn1 is not necessary for the BMP-mediated descensus of the thymus.

Transgenic expression in epithelial cells of the inhibitory Smad protein Smad7 under the control of the cytokerin 5 promoter resulted in a milder thymus phenotype (40) than that observed in this study. Compared with Foxn1::Xnoggin mice, this is surprising because biochemical studies suggest that Smad7 inhibits TGF-β and activin signaling and BMP signaling at the same time (41). Mice transgenic for k5::Smad7 typically die at day 3 after birth and possess a thymus with cortex and medulla at the correct anatomical location that is about one third the size of a WT mouse and that contains many apoptotic thymocytes. Clearly, further analysis of k5::Smad7 mice is required, foremost the demonstration that the thymus phenotype is independent of the generally poor health condition of these mice, to demonstrate the specificity of the thymus phenotype. The phenotype of mice deficient for the extracellular modulator of BMP signaling twisted gastrulation (Tsg) has recently been investigated (42). Tsg-deficient mice are born with normal thymocyte numbers, suggesting that BMP signals during the differentiation phase of thymus development are normal in the absence of Tsg. Taken together, our study is to our knowledge the first report that specifically looks at the role of BMP signaling for thymus development in vivo and allows the analysis of both thymocytes and stroma.

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Disclosures

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