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Maintenance of Thymic Epithelial Phenotype Requires Extrinsic Signals in Mouse and Zebrafish

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Thymopoiesis strictly depends on proper differentiation of the thymic epithelial anlage. Differentiation of thymic epithelial cells (TECs) is controlled by Foxn1 transcription factor. The in vivo signals initiating and maintaining Foxn1 expression in the future thymus anlage are unknown. In the mouse, bone morphogenetic protein (BMP) signaling is required for the maintenance of Foxn1 expression in TECs, as shown here by lineage tracing using a Foxn1-driven Cre transgene. Loss of Foxn1 expression after BMP inhibition reverts TECs to a basal state of pharyngeal epithelium unable to support T cell development; it does not divert them into a parathyroid fate. In zebrafish larvae, BMP inhibition likewise causes loss of foxn1 expression in the thymic anlage and subsequent impairment of thymopoiesis. These results indicate an evolutionarily conserved role of BMP signaling in the maintenance of Foxn1 expression. The Journal of Immunology, 2008, 181: 5272–5277.

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hemopoiesis strictly depends on the differentiation of the thymic epithelial anlage. In mouse, rat, and human, this process depends on the activity of the Foxn1 transcription factor (1). Accordingly, the expression of Foxn1 in the thymic anlage begins before the immigration of the first T cell progenitors, and, in the absence of normal Foxn1 function, progenitor cell homing does not occur (2). Although functional evidence is still lacking, it has been suggested that Foxn1 homologues in other vertebrates possess the same function. Indeed, a Foxn1 homologue, initially termed whnb and now called foxn1, was identified and shown to be expressed in the larval thymic anlage of zebrafish (3). It is currently unknown how Foxn1 expression is initiated in the prospective thymic anlage and which signal(s) maintain its expression. Earlier studies have indicated how the Foxn1 expression domain might be established in the epithelium of the third pharyngeal pouch. In mice lacking Shh, the expression domain of Foxn1 is broadened at the expense of that marked by Gcm2 expression (the future parathyroid) (4). It is therefore possible that Shh expression initiates specification of the parathyroid that requires Gcm2 for further development. Based on in vitro studies, it has also been suggested that Wnt signaling plays a role in activating Foxn1 in the prospective thymic rudiment (5). Interestingly, the future thymic anlage is marked by bone morphogenetic protein (BMP) expression, while that of the future parathyroid is marked by Noggin expression (6), suggesting that BMP signaling might be involved at least in maintaining the reciprocal specification of parathyroid and thymus anlagen in the pharyngeal pouch. This hypothesis predicts that BMP signaling is required for the maintenance of Foxn1 expression and that loss of BMP signaling in the thymic anlage might convert it into parathyroid tissue. Our earlier observations indicated that, in the absence of BMP signaling, thymus development was drastically impaired but the mechanism remained unclear (7), in particular the possible emergence of ectopic parathyroid tissue was not investigated. In this study, we describe an evolutionarily conserved function of BMP signaling in the maintenance of Foxn1 expression in the thymic epithelium; in the absence of BMP signaling, the thymic epithelium reverts to a basal state of foregut epithelium but is not converted into parathyroid tissue.

Materials and Methods

Mice

Foxn1::EGFP (8), Foxn1::Xnocggin (7), and Foxn1-tDT9 (9) mouse strains have been described. The Foxn1::Cre line was created using the previously described Foxn1 promoter fragment (7) (contained in plasmid pAHB14) appended to the Cre sequence (plasmid pAHB59; the original Cre-encoding DNA fragment was a gift of K. Rajewsky); details of construction are available on request. [4-(2-Piperidin-1-yloethoxy)phenyl]-3-pyridin-4-ylprazolol[1,5-a]pyrimidine (Dorsomorphin) was obtained from Sigma-Aldrich (Catalog No. P5499), dissolved in DMSO, and used at a final concentration of 50 µM.

Other methods

Conditions and probes for RNA in situ hybridizations on mouse tissue sections have been described (7, 10). Additional probes used were mouse E-cadherin (accession number NM_009864, nt. 2333 to 2759), mouse P-cadherin (accession number X06340, nt. 2153 to 2576), mouse Gcm2 (accession number AF 081556, nt. 581 to 1506), and mouse IL 7 (accession number X07962, nt. 847 to 1415). Thymic anlagen of deE12.5 embryos of wild-type and nude mice were isolated by laser capture microdissection, RNA was prepared and analyzed by RT-PCR as described (11) using the following primers for Cre/l25 (Teck) (5'-TCCGCCAGAAAAGTAGTGTG-3' and 5'-TTTCCTCCCTCCTCAGAACTCA-3'), for Il-7 (5'-GCACCTGAACTGTTCTGCA-3'), for Tbp (5'-AGGGTTAGACGTTCAATC-3'), and for Rag1 (5'-ATAGATGAGTGTTCTGTA-3'). Zebrafish foxn1, ikaros, and ragl probes and conditions for whole mount RNA in situ hybridizations of zebrafish larvae have been described (12). Sense probes did not give specific signals (not shown). Flow cytometric analysis of splenic T cells has been described (9). The analysis of sections for EGFP and EYFP fluorescence was essentially done as described (13). Immunohistochemistry using anti-GFP Abs (AbD Serotec 4745-1051; 1/500 dilution) and anti-E-cadherin...
Abs (BD 610182; 1/200 dilution) were done as described (7) using biotinylated donkey anti-sheep IgG F(ab’)_2, and Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories; 1/1000) and AlexaFluor 594 goat anti-mouse IgG (Invitrogen A11005; 1/200) for detection. The volume (v) of thymic anlagen in zebrafish larvae was estimated by converting the morphometrically determined area (a) of the 2-dimensional projection of hybridization signals into a pseudo-spheroid according to the formula v = (3/8πa^2) / 3.

Results

Inhibition of BMP signaling in thymic epithelial cells (TECs) causes loss of Foxn1 expression

We have previously shown that specific expression of the BMP inhibitor Noggin in the epithelial anlage of the thymus dramatically impairs T cell development (7). This is a quantitative rather than qualitative effect, indicating that the amount of stroma functionally competent to support T cell development is limited rather than affecting the process of T cell development per se. Indeed, the thymus in Foxn1::Xnoggin transgenic mice is small, but residual thymopoiesis is clearly detectable. Although the interference with thymic epithelial differentiation by use of Foxn1-promoter-driven transgenesis is the best option currently available, transgenes cannot be activated before the onset of endogenous Foxn1 gene expression. Therefore, TECs are exposed to the effects of activation or inhibition of a signaling pathway only after a certain period of time following initiation of the differentiation program. This problem complicates the interpretation of phenotypes obtained with this particular strategy. For instance, from E14 onwards, the thymus of Foxn1::Xnoggin mice consists of two distinct epithelial compartments: one that expresses Foxn1 and another that does not. This finding raised the question whether the Foxn1-negative epithelium had expressed Foxn1 at earlier stages of thymus development and had lost Foxn1 expression at a later time point. Alternatively, expression of the BMP antagonist Noggin could have caused recruitment into the thymic anlage of endodermal epithelial cells not normally destined to become TECs, such as those giving rise to the parathyroid gland. To examine this, a lineage tracing system was developed. First, we verified that the mosaic epithelial structures could be visualized by expression of EGFP under the Foxn1 promoter. Fig. 1A demonstrates that the results previously obtained by RNA in situ hybridization (7) (see also Fig. 2) are confirmed in this reporter system as indicated by direct observation of EGFP fluorescence and immunohistochemical analysis with anti-GFP Abs. Next, we generated mouse strains that expressed Cre recombinase under the Foxn1 promoter (Foxn1::Cre). This allowed us to activate fluorescent reporter expression specifically in Foxn1-expressing tissue in mice transgenic for the Rosa26 reporter strain, in which a floxed stop cassette prevents expression of EYFP (14). We then compared the fluorescence in thymic epithelial compartments of Foxn1::EGFP; Foxn1::Xnoggin mice vs those of Foxn1::Cre; Rosa26CreYFP; Foxn1::Xnoggin mice. As shown in Fig. 1B, the epithelium lining the cystic structure in the malformed thymi expresses YFP. This indicates that the undifferentiated epithelium had expressed Foxn1 at one stage during their development. This formally shows that interference with BMP signaling causes the reversion of Foxn1-expressing cells to a Foxn1-negative state.

Phenotypic differences between Foxn1-positive and Foxn1-negative thymic epithelium

To determine the phenotype of the reverted epithelial cells, we examined the expression of several additional markers (Fig. 2). Conveniently, the chimeric structure of the Foxn1::Noggin thymi allowed us to directly compare the phenotypic consequences of the loss of Foxn1 in epithelial cells as opposed to Foxn1-expressing epithelium. The absence of thymopoiesis in Foxn1-negative epithelial areas was confirmed by lack of Granzyme A, which is highly expressed in immature thymocytes (7). It was of particular interest for us to determine whether Foxn1-negative epithelial cells adopted a parathyroid fate, the alternative option of epithelia in the third pharyngeal pouch. However, no evidence for this was found, as Gcm2 expression in these cysts was absent (Fig. 2A). This indicates that Noggin expression in the third pharyngeal pouch per se does not induce Gcm2 expression. By contrast, these cells express Pax9 and E-cadherin, which are characteristic general features of parathyroid endoderm. This supports their embryological origin as suggested from the lineage tracing experiment above. The expression of E-cadherin, but not P-cadherin, is particularly notable, as epithelial cells in the normally developing thymus and parathyroid fields acquire P-cadherin expression as part of their normal differentiation pathway (Fig. 2B); the differential expression of the E-cadherin gene is confirmed at the protein level using anti-E-cadherin Abs (Fig. 2C). Furthermore, expression of the IL-7 gene, as a distinguishing feature of TECs, is no longer detectable in cystic epithelia. Although IL-7 expression is Foxn1-independent (15) (Fig. 2D), it is nevertheless associated with an early stage of TEC specification. Collectively, these results indicate that the
FIGURE 2. BMP signaling is required to support the differentiated phenotype of TECs. A, Schematic of the mosaic thymus tissue in E15.5 Foxn1::Xnooggin transgenic mice (upper panel) and RNA in situ hybridizations on consecutive transversal sections for Foxn1 (marker for TECs), Granzyme A (marker for thymocytes), Gcm2 (marker for developing parathyroid), and Pax9 (marker for foregut epithelium). The parathyroid rudiment is indicated by red circles. The results are representative of eight mice for Foxn1-, five mice for Gcm2-, and three for Pax9-specific hybridizations. Scale bar 50 mm. B, RNA in situ hybridizations for E-cadherin and P-cadherin of transgenic Foxn1::Xnoggin (upper panel) and wild-type (lower panel) E 15.5 mice; sections are shown to scale to demonstrate the smaller size of transgenic thymic anlagen. Scale bar 50 mm. Data representative of three embryos each. C, Immunohistochemistry for E-cadherin expression in Foxn1::XNoggin induced cysts. Increased E-cadherin signal is seen in the area surrounding the two cysts (white arrows) where the epithelium appears columnal; the hematopoietic part of the thymus (white asterix) expresses less E-cadherin. Results representative of three embryos. D, Loss of IL 7 expression in Foxn1-deficient epithelium of Foxn1::Xnooggin transgenic mice (left panel); data representative of three embryos. IL 7 is normally expressed in thymic rudiments genetically deficient for Foxn1 (right panel).

Foxn1-negative epithelial cells reverted to a basal state of pharyngeal epithelium that is unable to attract and sustain lymphocyte progenitors.

Noggin sensitivity and TEC differentiation

Although the above experiments clearly demonstrate that BMP signaling is required for the maintenance of Foxn1 expression, the reason(s) for the occurrence of the epithelial somatic mosaic remain unclear. Why do some TECs maintain their Foxn1 expression while others lose it? One possible explanation is that the expression of Foxn1 might be sensitive to Noggin-mediated BMP inhibition just before or at the onset of TEC differentiation. Thus, because of the inevitable lag in time before the manifestation of transgenic interference, some epithelial cells might have already differentiated to the point where they are insensitive to the effects of BMP inhibition. If this hypothesis is true, BMP blockade before the onset of Foxn1 expression and the subsequent initiation of TEC differentiation should lead to complete abrogation of T cell development. This possibility was examined using our previously described system of conditional postnatal differentiation of TEC progenitors in the mouse (9). This system is based on mice homozygous for a Foxn1 allele (Foxn1SAS) that can be reactivated by the action of Cre recombinase. In the absence of the Cre transgene, virtually no normal T cells are found in the periphery of young mice. In the presence of the hK14::CreERT2 construct, a functional Foxn1 gene can be generated postnatally in individual epithelial cells. This initiates TEC differentiation and leads to normal thymopoiesis in such a way that the peripheral T cell pool contains large numbers of T cells expressing high levels of TCRαβ. In the additional presence of the Foxn1::Noggin transgene, however, an inhibitory environment with respect to BMP signaling is established at least 2 wk before differentiation commences. This occurs because the Foxn1::Xnooggin transgene is active from E11.5 onwards, and, in Foxn1-deficient epithelium, the endogenous Foxn1 gene and the promoter fragment are still actively transcribed (16). Thus, if the hypothesis of rapid differentiation leading to a Noggin refractory state is correct, then failure of thymopoiesis should be observed in the composite genotype. However, this is not the case. When we compared the absolute numbers of TCRβhigh T cells in the spleen of cohorts of Foxn1SAS/Foxn1SAS; hK14::CreERT2 vs mice additionally carrying the Foxn1::Noggin transgene (whose expression begins at E11.5), these mature cells were still observed (Fig. 3). Note that the reduction in peripheral T cell numbers caused by the Foxn1::Xnooggin transgene is the same in both situations (2.2-fold for Foxn1SAS/Foxn1SAS vs 2.1-fold for Foxn1SAS/Foxn1SAS; hK14::CreERT2). This indicates that the effect of BMP inhibition is independent of initial Foxn1 activity. It also suggests that a possible differentiation of TECs per se does not prevent the Noggin-induced loss of Foxn1 expression in Foxn1::Xnooggin mice. Rather, it appears that the amount of transgenic Noggin emanating from the epithelial anlage is insufficient to completely block BMP signaling. Since transgenic mouse lines expressing higher Noggin levels were not available among our collection of transgenic founders, we chose another experimental system, the zebrafish, to examine this possibility. At the same time, this approach was expected to address the issue of evolutionary conservation of the BMP requirement for Foxn1 expression.

BMP signaling maintains foxn1 expression in the zebrafish thymic anlage

To use the zebrafish to examine the above question, we first needed to establish the precise timing of foxn1 expression in relation to thymus colonization. Therefore, we conducted an RNA in situ expression analysis at various stages of larval development. This was done by comparing the expression patterns of foxn1 as a marker for the thymic epithelium, of the ikaros transcription factor gene as an

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Interestingly, 8/27 transgenic fish show an abnormal distribution of rag1-positive cells on both sides (Fig. 5B, middle, lower panel); three fish exhibited this pattern only on one side, with no rag1-positive cells in the contra-lateral thymic anlage, one additional fish was considered to have a normal pattern on the contra-lateral side. At present, it is not clear whether this is indicative of rag1-positive cells exiting from the thymus after loss of foxn1 expression in the thymic epithelium or of cells that are prevented from entering the thymus. It nevertheless underscores the severe consequences of loss of foxn1 expression in the thymus.

As a further measurement of thymopoiesis, we determined the sequence complexity of TCRβ rearrangements in wild-type and transgenic fish for Vβ12-Cβ2 rearrangements as previously described (12). In wild-type fish, an average of five different sequences was found, whereas in transgenic fish the average was one sequence only (data not shown). This suggests quantitatively impaired thymopoiesis. To independently confirm the role of BMP signaling on foxn1 expression in zebrafish embryos, we treated wild-type fish with dorsomorphin, a recently identified inhibitor of BMP signaling (18). Fig. 5E shows that foxn1 expression is diminished in the treatment group. Of 17 embryos in the control group, 16 showed a normal expression (1 had reduced foxn1 expression according to the above criteria). In contrast, only 11 of 33 embryos of the treatment group had normal foxn1 expression (p < 0.001); two fish showed no foxn1 expression. A significant effect was observed also for rag1 expression (data not shown).

Taken together, our results in the zebrafish system indicate an evolutionarily conserved requirement of BMP signaling for maintenance of foxn1 expression in the thymic epithelium. They also

FIGURE 3. Incomplete inhibition of postnatal thymopoiesis in rescued Xnoggin-expressing thymic epithelium. The absolute number of splenic T cells in 4-to 6-wk-old mice of the indicated genotypes is shown. Abbreviations: SA2, Foxn1SA2 allele (9); wt: Foxn1 wild-type allele; Cre: hK14::CreERT2 transgene (22); Xnoggin: Foxn1::Xnoggin transgene. SDs are indicated, as are the number of mice analyzed for the different genotypes.
strongly suggest that in our transgenic mice, lack of sufficient Noggin in the thymic anlage of Foxn1::XNoggin strains prevents the complete loss of Foxn1 expression in the epithelium.

Discussion

In this study, we demonstrate an evolutionarily conserved requirement of BMP signaling for the maintenance of Foxn1 expression in TECs. This conclusion is based on the observed effects in two model systems, mouse and zebrafish. Our finding reaffirms the similarities between these two evolutionarily distant species and demonstrates the value of comparative analyses to address important questions in the biology of lymphoid organ development.

Our results have several important implications. First, they are the first indication that foxn1 in zebrafish might have similar functions to those of Foxn1 in mammals. Assuming that BMP inhibition does not affect in vivo T cell development per se, as shown previously in the mouse, our data suggest that loss of foxn1 expression in zebrafish drastically affects thymopoiesis possibly by regulating entry to and differentiation in the thymic rudiment. To formally prove this function, genetic inactivation of foxn1 in fish is required.

Second, our results support the notion that Noggin in the Gcm2-expressing epithelia of the third pharyngeal pouch serves to suppress Foxn1 expression, suggesting that Foxn1 expression in the third pharyngeal pouch is a default pathway and needs to be actively suppressed to allow the adoption of an alternative developmental fate. This is compatible with our observation of lack of Gcm2 expression in Foxn1-negative epithelium in thymic cysts. This has an evolutionary parallel in that the thymus evolves earlier than the parathyroid gland, which appears as a distinct anatomical structure only in tetrapods (19). The observation that Foxn1 expression does not resume after the silencing of the Noggin transgene (as a consequence of down-regulation of Foxn1) can be explained in at least two ways. First, at this stage of development BMP signaling might be insufficient to re-initiate Foxn1 expression, whereas it would suffice during earlier stages. Second, BMP signaling might always require additional factors for the initiation of Foxn1 expression, such as the Wnt signaling pathway (5), irrespective of gestational age. This latter prediction could be tested by an attempt to inhibit Wnt signaling in the developing thymic anlage.

Third, our results might have implications for future attempts to develop in vitro culture systems for the analysis of thymic epithelial stem cells. It has recently been shown that thymic epithelial progenitor cell differentiation crucially depends on Foxn1 function (9); at the same time, TECs tend to lose Foxn1 expression when maintained in tissue culture (20). Our study has shown that loss of Foxn1 expression by lack of BMP signaling causes the reversion of once Foxn1-expressing epithelia to uncommitted pharyngeal epithelial cells. Interestingly, this state of differentiation is different and right (r) thymic anlagen at 108 hpf as determined by foxn1 and rag1 in situ hybridizations. Wild-type fish are indicated in black or gray bars, transgenic in red and pink bars, respectively. SDs are shown. The volume of the left thymic lobes of wild-type fish was set to 1. The data are derived from the experiments summarized in D. D. Lack of foxn1- and rag1-positive thymic anlagen in a large proportion of transgenic fish. Reduced thymic anlagen were assumed if the calculated volume was smaller than the wild-type average minus two SDs. E. The BMP inhibitor dorsomorphin reduces foxn1 expression in zebrafish embryos. Wild-type fish were treated with dorsomorphin from 82 to 108 hpf and analyzed by RNA in situ hybridization for foxn1 expression as above. Most embryos showed reduced foxn1 expression (middle panel) as compared with solvent control (left panel); few embryos showed no foxn1 expression (right panel). The growth hormone probe was used as an internal control.
from that caused by genetic inactivation of Foxn1, as the latter preserves some aspect of thymic epithelial commitment, namely IL 7 expression.

Fourth, the Foxn1::Cre transgenic line described here allows the specific targeting of Cre activity to all TECs (21). These mice will thus be an essential tool for genetic gain-of-function and loss-of-function studies using the Cre/loxP system.

In conclusion, our results strongly suggest that Foxn1 expression in the thymic anlage requires extrinsic factors (i.e., BMP). Why Foxn1-negative cells line the cysts observed in the Foxn1::Noggin thymi is unclear. One possibility is that the lack of thymopoietic capacity leads to loss of thymocytes in these areas and collapse of the reticular meshwork of the epithelial compartment, resulting in epithelial and lymphocytic voids. It will be interesting to determine whether lack or diminished activity of such signals underlies the process of thymus evolution during ageing, often accompanied by the appearance of epithelial cysts.

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Disclosures

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References