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Development of the Thymus Requires Signaling Through the Fibroblast Growth Factor Receptor R2-IIIb

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Mice deficient for fibroblast growth factor (Fgf)R2-IIIb show a block in thymic growth after embryonic day 12.5, a stage that just precedes its detection in thymic epithelial cells. Fgf7 and Fgf10, the main ligands for FgfR2-IIIb, are expressed in the mesenchyme surrounding the thymic epithelial primordium, and Fgf10-deficient mice also exhibit impaired thymic growth. Hence, Fgf signaling is essential for thymic epithelial proliferation. In addition to the proliferative block, most thymic epithelial cells fail to progress from an immature cytokeratin 5-positive to a cytokeratin 5-negative phenotype. Nevertheless, sufficient epithelial cell differentiation occurs in the severely hypoplastic thymus to allow the development of CD4/CD8-double-positive thymocytes and a very small number of single-positive thymocytes expressing TCRs. The Journal of Immunology, 2001, 167: 1954–1961.

The thymus is the site of T cell development, where through a series of interactions with the thymic epithelium, lymphoid precursors mature and are exported to the periphery. In the mouse, the thymus primordium emerges around embryonic day (E)10.5 as an epithelial bud encompassing endoderm from the third pharyngeal pouch and possibly ectoderm of the third branchial cleft (1–3). After E11, the bilaterally formed thymic primordia with accompanying neural crest-derived mesenchyme separate from the pharynx and migrate to form a bilobed organ located above the heart (3). The process of thymic lymphopoiesis starts around E11.5–E12.5, following migration of lymphoid stem cells into the epithelial primordium, and is concomitant with organ migration and rapid epithelial cell proliferation (4, 5). Subsequently, these stem cells proliferate, rearrange their Ag receptor genes, and then differentiate into functionally mature T cells. Moreover, differentiation of the thymic epithelium and T cell development are interdependent (6, 7). Organ explant cultures have demonstrated that the E12 mesenchyme surrounding the thymic epithelial rudiment is necessary for thymic development (8). From E12.5 to birth, a high proliferation rate results in the thymic mass markedly expanding in all dimensions with the formation of numerous lobules. The late maturation of the thymus is marked by the development of the first Hassall’s bodies.

The development of the thymus has been shown to require the expression of a number of genes, including members of the Hox and Pax gene transcription factor families. Mice deficient for Hoxa3 are athymic and have several other pharyngeal organ abnormalities (9, 10). Its expression in both the neural crest mesenchyme and pharyngeal endoderm suggests that Hoxa3 could affect both these cell lineages. Pax1 and Pax9 are expressed in an overlapping pattern in the pharyngeal endodermal cells and continue throughout fetal development. Mice deficient for Pax1 have a hypoplastic thymus and show abnormal thymocyte maturation, whereas Pax9-mutant mice are athymic (11, 12).

Fibroblast growth factors (Fgfs) are a large family of intercellular signaling molecules and mediate their biological responses by binding and activating high affinity cell surface receptors (Fgfr) with intrinsic tyrosine kinase activity (reviewed in Refs. 13, 14). Fgfs have been implicated in a number of important cell activities including differentiation, proliferation, and migration (reviewed in Refs. 15, 16). The Fgfs signal through four Fgf receptor genes (Fgfr1–Fgfr4), but alternative splicing increases Fgfr diversity. Fgfr2 can generate two isoforms, Fgfr2-IIIb and Fgfr2-IIIc, with different Fgfr-binding specificity and different sites of expression (17). Fgfr2-IIIb is located in many types of epithelia and is activated by four known ligands (Fgf1, Fgf3, Fgf7, and Fgf10) that are synthesized predominantly in mesenchyme (17–22). Gene targeting studies on Fgfr2, which encompass both receptor isoforms, have given varying results with peri-implantation lethality at E4.5–E5.5 (23) to early embryonic lethality of embryos showing no limb buds and a defective placenta (24, 25). More recently, Fgfr2-IIIb isofrom-specific abrogations have shown this isoform is essential for epithelial-mesenchymal interactions during the development of several organs (26–30).

Mice deficient for Fgfr2-IIIb survive in utero to term but cannot survive after birth because they have no lungs. These mice also show a severely hypoplastic thymus of comparable size to the normal E12 thymus. Here, we show that Fgf signaling is essential for thymic epithelial cell proliferation, and although very low numbers of thymocytes are generated, differentiation occurs to the CD4/CD8-double-positive and -single-positive stages.

Materials and Methods

Gene targeting, mouse strains, and genotyping

Gene targeting of isoform IIIb of Fgfr2 has been realized by introducing an internal ribosomal entry site (IRES)-lacZ gene into exon IIIb as previously described (30). Briefly, stop codons in different reading frames were introduced upstream of the IRES. A neomycin-resistant gene, driven by the HSV-tk promoter and flanked by LoxP sites from plasmid pL2Neo (a gift of H. Gu, Institute for Genetics, University of Cologne, Cologne, Germany;
Ref. 31), was inserted into the intron between exons IIIa and IIIb. The resulting construct was electroporated into 129P2/OlaHsd embryonic stem cells (Fig. 1B). Neomycin-resistant clones were screened by Southern blotting and PCR. Five different lines of mice were used (2G1, 3G9, 2D9, 5A7, and 2G1 Cre). The 2G1 Cre line was obtained by deletion of the neomycin-resistant gene from the 2G1 line by injecting eggs derived from a 2G1-heterozygous male crossed with a wild-type B16 female with 10 ng/ml of pMC-Cre (kindly provided by H. Gu; Ref. 31). No differences in phenotype between the various lines were observed. The mice were genotyped as previously described (30). Fgf10−/− mouse embryos were kindly provided by D. Ornitz (Washington University Medical School, St. Louis, MO; Ref. 32).

**Histology**

Tissues were fixed overnight in neutral-buffered formalin, dehydrated through ethanol, embedded in paraffin, and 5-μm sections were cut for staining in H&E.

**Immunohistochemistry**

The 8-μm sections were cut, and when necessary, Ags were unmasked by prior microwaving for 10 min in 0.01 M trisodium citrate (pH 6). For detection of proliferating cells, a standard indirect immunoperoxidase protocol was used using a rabbit polyclonal Ab, NCL-Ki67p (NovoCastra, Newcastle, U.K.), and revealed by a secondary Ab, biotinylated swine anti-rabbit IgG (DAKO, Ely, U.K.) followed by streptavidin-peroxidase (DAKO) and 3,3′-diaminobenzidine (Sigma, St. Louis, MO) visualization. The cells were then counterstained with hematoxylin. Apoptotic cells were detected by the incorporation of TUNEL using the ApopTag Plus in situ apoptosis detection kit ( Oncor, Gaithersburg, MD) according to the manufacturer’s recommended protocol.

For cytokeratin 5 detection, thymic lobes were dissected from E17 embryos, embedded in OCT compound (TissueTek; Sakara Finctek Europe BV) and snap-frozen in liquid N2. Next, 5-μm cryosections were cut onto multislot slides and allowed to air-dry for 1 h before being fixed in cold methanol for 30 min. Sections were then triple-labeled for 45 min for Thy1.2-biotin, cytokeratin 5, and pan-cytokeratin, followed by two 5-min washes in PBS. These were then revealed using extravidin-tetramethylrhodamine isothiocyanate, anti-rabbit Ig-7-amino-4-methylcoumarin-3-acetic...
The generation and characterization of mice deficient for FgfR2-IIIb have been described previously (30). In brief, translational termination codons in three reading frames were placed close to the start of exon IIIb, followed by an IRES-lacZ gene (Fig. 1, A and B). These changes result in a truncated form of FgfR2-IIIb that lacks part of the ligand binding domain, the transmembrane element, and the TK domain, whereas expression of the alternatively spliced isoform, FgfR2-IIIc, remains intact.

The thymus is one of several organs showing developmental abnormality in FgfR2-IIIb<sup>-/-</sup>/lacZ<sup>+</sup> mice (Fig. 1, C and F). The dissected thoracic regions of E18.5 fetuses show that homozygous mutant mice have a profoundly smaller thymus, as well as no significant lung development, although the heart, which lies immediately posterior to the thymus, appears of normal size (Fig. 1, D, E, G, and H). To characterize thymus development in more detail, consecutive sagittal sections from E12–E17.5 fetuses were stained and examined for histological differences (Fig. 2). By E12 of gestation, the mouse thymus consists of a region of epithelium surrounded by a capsule, and the whole is embedded in a thick layer of mesenchyme. At this stage, there were no discernible histological differences in the thymus of wild-type and FgfR2-IIIb<sup>-/-</sup>/lacZ<sup>+</sup> mice (Fig. 2, A and B). However, from E12.5 to E17.5, the thymus of heterozygous and wild-type mice grew progressively larger (Fig. 2, C, E, and G), whereas the thymus of homozygous mutant mice remained the same size (Fig. 2, D, F, and H). These data suggest that FgfR2-IIIb signaling is essential for normal thymus development, and the block in thymic development occurs between E12 and E12.5.

Expression of FgfR2-IIIb, Fgf7, and Fgf10 in the thymus

The mutant FgfR2 allele contains an IRES-lacZ cassette in the disrupted IIIb exon, although β-galactosidase activity is below detectable levels in heterozygous or homozygous embryos (30). Consequently, expression of FgfR2-IIIb RNA was assessed by radioactive in situ hybridization on E13.5 heterozygote and homozygote thymus using a IIIb isoform-specific probe (lacZ) or a pan-specific FgfR2 TK probe, which detects both FgfR2-IIIb and FgfR2-IIIc (Fig. 3, A–D). The lacZ and TK probe staining were very similar, establishing the presence of FgfR2-IIIb RNA in the thymic epithelium and suggesting no discernible expression of FgfR2-IIIc in the surrounding mesenchyme. Although encoding for a defective receptor, FgfR2-IIIb RNA expression was also detected in the null mice using both the TK (Fig. 3, E and F) and lacZ (Fig. 3, G and H) probes. As a negative control for lacZ staining, the probe was shown to give no signal on sections from wild-type embryos (data not shown). The finding that FgfR2-IIIb is expressed by the thymic epithelial cells is consistent with other FgfR2 expression studies that show that this receptor is expressed specifically in several epithelial cell lineages (18, 19). FgfR2-IIIb was not detected at E12 (data not shown), but was present by E13.5 in the thymic epithelial compartment in both normal and mutant mice. Expression of FgfR2-IIIb continued in the thymic cortical region throughout fetal life, although the progressive infiltration of lymphoid and mesenchymal cells at later stages of development makes assignment of
FgfR2-IIIb to specific cell lineages ambiguous. Moreover, FgfR2-IIIb expression was not detected in the surrounding mesenchyme or on the infiltrating lymphoid cells in the mesenchyme. These data suggest a strong spatio-temporal correlation between the loss of FgfR2-IIIb expression in the epithelium and the onset of thymic hypoplasia.

FgfR2-IIIb is activated by three main ligands, Fgf3, Fgf7, and Fgf10. An in situ hybridization analysis at E13.5 showed no Fgf3 expression (data not shown), but Fgf7 and Fgf10 were both detected in the surrounding mesenchyme (Fig. 3, I–L), suggesting that they may induce thymic epithelial proliferation. However, mice deficient for Fgf7 are viable and fertile, and no thymic phenotype has been reported (34). Meanwhile, very recent data showed that mice deficient for Fgf10 have a small thymus (35). We have confirmed this observation with an analysis of similar Fgf10-deficient fetuses (Fig. 4). Embryos were assessed at E12.5 and E14.5 by standard histological staining procedures, and a significant reduction in the size of the thymus was observed (Fig. 4, B and D). As seen in the FgfR2-IIIb<sup>−/−;lacZ</sup> mice, the parathyroid appeared to be unaffected (Fig. 4, C and D). A comparison between the thymus of stage-matched Fgf10<sup>−/−</sup> and FgfR2-IIIb<sup>−/−;lacZ</sup> mice showed that those from FgfR2-IIIb<sup>−/−;lacZ</sup> mice were significantly smaller, suggesting that Fgf7 might be able to provide some compensatory signaling through FgfR2-IIIb in Fgf10-null mice. These findings support the idea that Fgf10 is the major ligand activating FgfR2-IIIb in the thymic epithelium, and this signaling is necessary for normal thymic development.

Expression of Pax1 and Pax9 are unaffected in FgfR2-IIIb<sup>−/−;lacZ</sup> mice

As an assessment of thymic endoderm differentiation into the epithelial primordium, we examined the expression of two markers of thymic epithelium, the transcription factors Pax1 and Pax9. Moreover, mice deficient for Pax1 also show a reduced thymic size, similar phenotypically to FgfR2-IIIb<sup>−/−;lacZ</sup> mice, as well as quantitative alterations in T cell maturation (11). Although structurally similar and showing a similar thymus-related expression pattern to Pax1, mice deficient for Pax9 are athymic (12). To determine whether these transcription factor genes have an altered thymic expression pattern in the FgfR2-IIIb-deficient mouse, they were analyzed using in situ hybridization at E13.5 both in the heterozygous and homozygous mutant mice. Pax1 and Pax9 were both strongly expressed by the thymic epithelial compartment of heterozygous mice (Fig. 5, A–D). Similarly, in FgfR2-IIIb-deficient mice, expression of both Pax1 and Pax9 occurs normally and at about the expected levels, consistent with a role upstream of FgfR2-IIIb receptor signaling (Fig. 5, E–H).
To investigate the mechanism behind the reduced size of the thymus, the levels of apoptosis and cell proliferation were analyzed. Sections were stained using the TUNEL method for the detection of apoptosis and for Ki67 Ag to measure proliferating cells. The TUNEL assay revealed a few apoptotic cells in the thymus of both normal and homozygous mutant mice. As a positive control, other tissues in the sections were stained for the expected level of apoptosis (Fig. 6, A and B). Staining with Ki67 showed that nearly all the stromal cells in the normal thymus stained positive, indicating a high level of proliferation, consistent with its increase in size (Fig. 6C). However, a significant decrease in staining of thymic stromal cells in the homozygous mutant was apparent, indicative of a proliferation block (Fig. 6D). A similar decrease in cell proliferation was observed in mice deficient for Fgf10 (Fig. 6F), whereas the heterozygous mice displayed normal proliferation, indicated by the extensive brown immunoperoxidase staining (Fig. 6E). Because most of the thymic stroma at E13.5 is composed of epithelial cells, this suggests that Fgf10 is the main mesenchymal inducer for the thymic epithelial cell proliferation. However, it is also likely that the reduced proliferation of epithelial cells has a secondary effect on the proliferation of the infiltrating lymphoid cells.

T cell maturation occurs despite a block of thymic growth

As a preliminary assessment of thymic development, frozen sections of the thymus from E17 heterozygous and homozygous mutant mice were stained with a pan-cytokeratin Ab to identify the epithelium, cytokeratin 5 as a marker of early epithelial cell differentiation, and Thy1.2 for thymocytes. Although the thymus fails to grow in FgfR2-IIIb/H11002/H11002lacZ mice, many of the epithelial cells remain positive for cytokeratin 5 (Fig. 7, A and B), suggesting that they remain in a precursor form and may not therefore be competent to promote T cell maturation (7). Surprisingly, flow cytometry using cells teased from E17–E18 lobes of homozygous mutant mice showed that T cell maturation is able to proceed to the CD4/CD8-single-positive stage (Fig. 7C). Because the thymuses from FgfR2-IIIb/H11002/H11002lacZ mice are extremely small, cells from E17 and E18 were pooled for the analyses; in homozygous mice at E17, there were ~3 × 10⁶ cells/lobe and, at E18, ~6 × 10⁶ cells/lobe.
shown). Similar distributions were found in thymocytes from the CD4-single-positive, and 24% were CD8-single-positive (data not subsequently, we cannot be certain that positive selection occurs nor considerably reduced from the severely hypoplastic thymus. Con-
gous mutant mice. However, the total yield of thymocytes was
detected in thymus of
FIGURE 6. Detection of proliferation and apoptosis in thymus of

but from FgfR2-IIlb−/−;lacZ− mice, only 15,500 cells/lobe were ob-
tained. With regard to TCR expression, a small proportion of TCR-
positive cells was found in the thymus of FgfR2-IIlb−/−;lacZ− mice. Analysis of αβTCR expression showed ~4 and 7% positive in the
heterozygous and homozygous mutant mice, respectively. Within the
αβTCR population from FgfR2-IIlb−/−;lacZ− mice, 8% were
CD4-single-positive, and 24% were CD8-single-positive (data not
shown). Similar distributions were found in thymocytes from the
heterozygous mice. These findings clearly demonstrate that differ-
etiation to the double-positive stage of thymocyte development
occurs in FgfR2-IIlb−/−;lacZ− mice. A similar proportion of single-
positive cells was also found in both heterozygous and homozy-
gous mutant mice. However, the total yield of thymocytes was
considerably reduced from the severely hypoplastic thymus. Con-
sequently, we cannot be certain that positive selection occurs nor-
mally, although we detect low numbers of single-positive cells that
are αβTCR positive. Further analysis of the T cell development,
which occurs in neonatal mice, was not possible because these
mice die at birth due to agenesis of the lungs.

Discussion
The mouse thymic primordium is formed around E10.5 as an ep-
ithelial bud from the third pharyngeal pouch and mesenchyme that
differentiates from neural crest cells (1–3). At about E11.5, the
epithelial primordium is first colonized by T cell progenitors that
migrate in from blood vessels in the surrounding mesenchyme. At
E12, the mesenchyme surrounds, but has not penetrated, the epi-
thelium. Removal of the mesenchyme at this critical phase pre-
vented the development of the epithelium in vitro (36). Subse-
sequently, Shinohara and Honjo showed that the mesenchyme of the
early E12 thymus influences MHC class II expression in the epi-
thelium, but they did not study the effects on lymphopoiesis (37, 38).
At E12, thymic explants show a limited lymphoid development
mental capacity, which can be enhanced by addition of fibroblasts
(39, 40).

In a recent study, E12 thymic lobes with intact mesenchyme
were shown to generate all T cell populations in vitro, but lobes
from which mesenchyme has been removed show poor lymphopoiesis (8). At E13, mesenchyme was shown to start migrating into
the epithelial primordium where it can continue to interact with
epithelial cells as well as thymocytes (8). Previous studies have
reported expression of FgfR2-IIlb in the late gestation thymus (18, 19), but here we show expression of the FgfR2-IIlb receptor on the
epithelial primordium of the E13.5 thymus, a stage when only very
few lymphoid or mesenchymal cells have infiltrated the epithelium
(41). Furthermore, the ligands for FgfR2-IIlb, Fgf7 and Fgf10, are
present in the surrounding mesenchyme, indicative of paracrine
growth regulation. Consistent with this suggestion, FgfR2 expres-
sion was not detected in surrounding thymic mesenchyme, which
also contains infiltrating lymphoid cells, nor were Fgf7 and Fgf10
detected in the epithelial compartment. Significantly, the block in
thymic growth in FgfR2-IIlb-deficient mice correlates with the
stage of development when receptor expression is first detected.
Mice deficient for Fgf10, the major ligand for FgfR2-IIlb, also
have a similar but less severe hypoplasia (Figs. 4 and 6) (35),
providing strong circumstantial evidence that Fgf10 in the mesen-
chyme acts as the paracrine inducer of stromal epithelial cell
proliferation.

Formation and migration of the thymic primordium appears to
be normal, suggesting that the role of the Hox3 paralogs is unaf-
ected (42). This view is supported by expression in the hypoplastic
thymus of the transcription factors Pax7 and Pax9, which play
important roles at this early stage of thymic morphogenesis (11, 12).
Moreover, as T cell infiltration and differentiation of the thymus
occurs in the FgfR2-IIlb−/−;lacZ− mice, the function of the whn
gene responsible for the athymic nude mouse mutation also ap-
pers unaffected (43). Therefore, we would conclude that FgfR2-
IIlb most likely functions downstream of the transcription factor
cascade involved in thymus morphogenesis, which includes Hox3
paralogs, Pax7/Pax9, and whn, although this does not exclude a
continued requirement for these transcription factors (reviewed in
Ref. 44).

Thymic epithelial cells express distinct cytokeratins that reflect
their proliferative potential and maturation status (7). Most cells
express cytokeratins 8 and 18, but a distinct subset also express
cytokeratin 5. This subset contains precursors that generate the
major population of cytokeratin 5-negative cortical epithelial cells
(7, 45). Our studies show that, in the hypoplastic thymus of mice
deficient for FgfR2-IIlb, many of the epithelial cells fail to progress from the cytokeratin 8/cytokeratin 18-positive stage (Fig.
FIGURE 7. Immunohistochemistry and FACS analyses on heterozygous and FgfR2-IIib<sup>r<sup>−/−</sup>lacZ</sup> mice. Immunohistochemistry showing sections stained with Abs to Thy1.2, pan-cytokeratin, and keratin 5 on E17 heterozygous (A) and FgfR2-IIib<sup>r<sup>−/−</sup>lacZ</sup> (B) mice. In both sections, Thy1.2-positive cells are red, pan-cytokeratin-positive cells are blue, and keratin 5-positive cells are green. Although Thy1.2-positive cells are present in both sections, a greater proportion of cytokeratin-expressing cells (blue) are keratin 5 positive (green) in the mutant thymus compared with the heterozygote thymus. C, FACS analyses of CD4/CD8-single-positive cells isolated from E17 and E18 heterozygous and pooled E17 and E18 FgfR2-IIib<sup>r<sup>−/−</sup>lacZ</sup> mice. The yields of cells were as follows: E18 heterozygotes, 6 × 10<sup>6</sup> cells/lobe; E17 heterozygotes, 3 × 10<sup>6</sup> cells/lobe; but for E18/E17 mutant mice, 15,500 cells/lobe. Note that homozygous mutant mice yield CD4- and CD8-single-positive cells.

7A and data not shown). A similar block in maturation of thymic epithelial cells has been reported in mice expressing a human CD3ε transgene (7), but in this case, the effect has been attributed to a failure to generate thymocytes that are required for epithelial maturation (46). In our studies we show that, although the epithelial phenotype is similar to that above, the thymus of FgfR2-IIib<sup>r<sup>−/−</sup>lacZ</sup> mice is capable of supporting thymocyte development. However, we cannot exclude that the small number of cytokeratin 5-negative cells are both necessary and sufficient to facilitate the observed thymocyte maturation. As well as generating CD4/CD8-double- and -single-positive cells, there is also a small proportion of TCR αβ cells in the thymus of mutant mice that includes a small subset of the single-positive populations. Although thymocytes transit the double-negative 1-double-negative 4 pathway, we cannot exclude changes in the CD25/CD44 ratios during the earlier highly proliferative phase of T cell expansion, because in line with the small size of the thymus, thymocyte numbers are severely reduced.

In summary, signaling through the FgfR2-IIib receptor on thymic epithelial cells is crucial for thymic development. Mesenchyme, which initially surrounds the epithelium, produces Fgf7 and Fgf10 capable of activating this receptor, although the genetic evidence suggests the latter is the major ligand. While in the limb, FgfR2-IIib signaling appears to be crucial for cell survival (30), and in the thymus it is required for proliferation of the thymic epithelium after E12.5. The lack of epithelial cell proliferation and the associated retention of primarily cytokeratin 5/cytokeratin 18-positive cells does not block the maturation of thymocytes to the CD4/CD8-double-positive stage, although the yield of cells is severely reduced. It is more difficult to be sure that normal positive selection occurs because of the gestational age and low cell numbers. It seems likely that the effect on thymocyte yield is secondary to the failure of epithelial proliferation, but we cannot exclude the possibility that there are direct effects on thymocytes themselves. These findings suggest that different paracrine signals may be required for growth and differentiation of the thymic epithelium. As mesenchyme also infiltrates the thymic stroma, these cells might have additional direct effects on thymocyte development, because they express an extracellular matrix able to activate thymocyte integrins (8). In this context, it will be interesting to investigate whether Fgfs can wholly substitute for the effect of removal of mesenchyme on lymphopoiesis in cultures of E12 thymus.

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