Short paper

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Sox-4 facilitates thymocyte differentiation

The mouse *Sry*-like transcription factor Sox-4 is expressed in thymus, bone marrow, and gonads of adult mice. Sox-4-deficient mice die at embryonic day E14 due to cardiac malformation. In transfer experiments to irradiated recipients, B cell development was shown to be severely impaired in Sox-4-deficient progenitor cells. However, no drastic effects on T lymphocyte development were noted, despite the high level expression of the Sox-4 gene in the thymus of normal mice. Here, we report a detailed analysis of T cell development from Sox-4-deficient progenitors. Explanted fetal thymic organ cultures (FTOC) of Sox-4-deficient thymi yielded 10–50-fold fewer CD4 CD8 double-positive and single-positive cells than FTOC of littermates. This effect was T cell-autonomous, since similar observations were made when FTOC were performed by culturing of Sox-4-deficient progenitors in wild-type thymus lobes. When Sox-4-deficient fetal liver cells were injected together with normal cells intrathymically, they did not compete efficiently for reconstitution. It is concluded that Sox-4 facilitates thymocyte development.

1 Introduction

Developmental processes are orchestrated by a complex pattern of expression of transcription factors. The application of gene knockout strategies has revealed that a single transcription factor can be critically involved in more than one differentiation process [1]. These processes can occur in different organs and at different time points in development. Often, early embryonic-lethal effects of the introduced mutations obscure effects on organ systems that develop later, such as the immune system. Several strategies have been employed to circumvent early embryonic lethality, e.g. the transplantation of mutant hemopoietic precursors to recipient mice [1]. The high mobility group-1 (HMG) box containing transcription factor gene *Sox-4* is expressed in the thymus and gonads of adult mice [2, 3]. During embryonic development, Sox-4 is expressed in multiple organs, such as neural tube, heart, lung, thymus, tooth buds, and mesonephros. Mutation of the gene in the germ line of mice leads to premature death at embryonic day 14 (E14) due to improper development of the valves in the outflow tract of the heart [4]. This result is in agreement with the cardiac expression of Sox-4, which is restricted to the endocardial cushion tissue at E13. Using fetal liver cells from homozygous mutant embryos, radiation bone marrow chimeras were produced. In these chi-

meras, B cell development was severely impaired at the stage of the pro-B cells. The block, occurs around fraction B/C as defined by Hardy and coworkers [5] but it is not absolute, since chimeras do accumulate low numbers of B cells in peripheral lymphoid organs in the course of time. *In vitro*, low numbers of IL-7-responsive clonable B precursors were detected in fetal livers of mutant embryos. The frequency of these precursors was about tenfold lower than in control embryos. Even more striking was the reduced growth capacity of these B lineage clones: the number of pro-B cells was 100-fold lower in colonies from Sox-4^{-/-} fetal liver progenitors compared to those of littermates. Some LPS-responsive B cells did arise from these clones after extended culture. Thus, expansion of Sox-4^{-/-} early B cell progenitors does occur at a very low rate, both *in vivo* and *in vitro*.

While donor-derived T lymphocytes were present in the lymph nodes of these radiation chimeras after 2 months, we always noted a delay in the reconstitution of the Sox-4^{-/-} T cell compartment of these mice. In the present study, we address the development of Sox-4^{-/-} T lymphocytes in more detail. Fetal thymic organ cultures (FTOC) from mutant embryos yielded less thymocytes expressing CD4, CD8, or both after 2 weeks of culture. In competition reconstitution experiments, it was shown that mutant fetal liver cells gave rise to less progeny after intrathymic injection than control cells.

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Abbreviations: DP: Double positive E14: Embryonic day 14
FTOC: Fetal thymic organ culture HMG: High mobility group-1 SP: Single positive

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2 Materials and methods

2.1 Mice

Mice were kept at the transgenic mouse facility of the central laboratory for experimental animals, University of Utrecht. C57BL/6 (Charles River, Iffa Credo) and C57BL/6.Ly-5.1 (Jackson Laboratory, Bar Harbor, ME) mice were purchased. (C57BL/6 × DBA/2)F1 females were bred locally. Sox-4-deficient mice have been described [4] and were backcrossed to C57BL/6 mice for 4–8 generations. For timed pregnancies, the day of vaginal plug was day 0.

2.2 Monoclonal antibodies

PE-labeled anti-CD4, PE-labeled anti-TCR $\alpha\beta$, PE-labeled anti-NK1.1, and FITC-labeled anti-CD3 were purchased from PharMingen (San Diego, CA). CD8 (53/6.7) [6], TCR $\alpha\beta$ (H57-597) [7], H-2D^d (34-2-12) [8], Ly-5.1 (A20), and Ly-5.2 (104)-specific hybridomas were grown and supernatants collected. Antibodies were FITC-conjugated or biotinylated according to standard procedures. For flow cytometry, cell suspensions were incubated with respective antibodies, washed in PBS/FCS/sodium azide and subsequently analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

2.3 Irradiation chimeras

(C57BL/6 $\times$ DBA/2)F1 mice (H-2^{b/d}) were irradiated (9.5 Gy) and within 24 h, one fetal liver (H-2^{b/b}) was injected i.v. as a cell suspension. The donor embryos were genotyped by Southern blotting [4]. All uninjected and 20–40 % of injected mice died after 12–18 days. Host-derived cells were detected by staining with an H-2D^d specific antibody (34-2-12) [8]. Donor cells always contributed more than 90 % of the myeloid cells in the blood after 3 weeks. Animals were killed and analyzed after 8 weeks.

2.4 Fetal thymic organ cultures

Thymic lobes were dissected from E13–E14 embryos and cultured on floating filters (0.8 μ m pore size, Nuclepore polycarbonate, Costar, Cambridge, MA) in supplemented medium (Opti-MEM or Iscove's modified Dulbecco's medium, Gibco, Grand Island, NY) for 14 days. For reconstitution experiments, E14 embryonic lobes from Ly-5.1 mice were isolated and irradiated (25 Gy). These lobes were cultured for 24 h in hanging drop cultures (Terasaki plates) together with 10^5 fetal liver cells and subsequently transferred to floating filters. After staining with mAb, cells positive for Ly-5.2 (fetal liver-derived) were analyzed.

2.5 Intrathymic injections

C57BL/6.Ly-5.1 mice (2–4 months old) were irradiated (7.5 Gy) and anesthetized. The thorax was opened and fetal liver cell suspensions were injected into the thymus (10^5 cells/10 μ l). The thorax was closed immediately and thymocyte subpopulations were analyzed 4 weeks later by flow cytometry.

3 Results and discussion

3.1 Sox-4^{-/-} T lymphocytes develop in long-term irradiation chimeras

Since Sox-4-deficient embryos die at E14 due to cardiac failure, the development of the Sox-4^{-/-} hemopoietic system and in particular of the lymphoid lineages was studied in radiation chimeras [4]. Sox-4^{-/-} fetal liver progenitors give rise to all hemopoietic lineages except the B lineage. In the lymph nodes and spleen, donor-derived (60–75 % of total) T lymphocytes were detectable, independent of the

Figure 1. Sox-4^{-/-} T cells and NK cells develop in bone marrow chimeras. Fetal liver cells of Sox-4^{+/+} (A and C) and Sox-4^{-/-} embryos (B and D), H-2^{b/b}, were injected into lethally irradiated hosts (H-2^{d/b}). After 2 months, spleens were removed and single cells were stained with antibodies specific for TCR $\alpha\beta$, NK1.1, CD3, and H-2D^d. (A and B) TCR $\alpha\beta$ ⁺ cells of donor (H-2D^d) derived from both Sox-4^{+/+} and Sox-4^{-/-} embryos were detected in the spleens of recipient animals. (C and D) Spleen cells of donor origin (gated to be H-2D^d) were stained with anti-NK1.1 and anti-CD3 antibodies. Sox-4^{-/-} NK cells were present in numbers comparable to Sox-4^{+/+} cells.

Sox-4 genotype of the transplanted fetal liver cells (Fig. 1). In addition, natural killer (NK) cells defined as NK1.1⁺ CD3⁻ cells from donor origin were detected in these chimeras (Fig. 1).

The development of T lymphocytes in these radiation chimeras was surprising, given that the thymus is a major site of Sox-4 expression in adult mice. To study T cell differentiation more directly, thymi from the chimeric mice were analyzed. Staining with mAb specific for CD4 and CD8 confirmed that reconstitution had occurred (Fig. 2). All subpopulations were present and staining with mAb specific for TCR $\alpha\beta$ and markers of the double-negative (DN) subpopulation, CD25 and CD44, revealed no discrepancies (not shown).

3.2 Sox-4^{-/-} thymocyte development is impaired in FTOC

These observations did not rule out the possibility that a more subtle defect in Sox-4^{-/-} thymocyte development exists. To analyze the developmental potential of thymocytes beyond day E14, thymi were removed from E13

Figure 2. Thymocyte development of long-term *Sox-4*^{−/−} bone marrow chimeras is normal. Thymi from chimeras similar to those described in Fig. 1 were isolated 2 months after reconstitution and stained with antibodies specific for CD4 and CD8. All mature thymocytes were donor-derived because they were not stained with H-2D^d-specific mAb (immature thymocytes could not be analyzed because they do not express sufficient MHC class I antigens). (A) Mice reconstituted with *Sox-4*^{+/−} progenitors and (B) reconstituted with *Sox-4*^{−/−} progenitors, were examined.

embryos and maintained in FTOC for 14 days. Thymocytes were then counted, stained with mAb, and analyzed by flow cytometry. Thymi from *Sox-4*^{−/−} embryos typically yielded two- to fourfold fewer cells than thymi from wild-type or heterozygous embryos. Furthermore, maturation in fetal thymic organ culture of mutant thymi (as indicated by the numbers of cells expressing CD4, CD8, or both) was impaired. Fig. 3A gives the sum of the total numbers of CD4/CD8 double-positive (DP) and CD4 and CD8 single-positive (SP) cells per cultured thymus lobe. The yield of maturing thymocytes (defined as DP + SP cells) was decreased 10- to 50-fold in *Sox-4*^{−/−} thymus lobes.

To determine whether the observed effect was cell-autonomous, irradiated thymic lobes (E14) of C57BL/6.Ly-5.1 mice were reconstituted in hanging drop cultures with fetal liver cells from mutant or control embryos. After 14 days of culture, cells were stained with mAb and analyzed by flow cytometry. As in the standard FTOC described above, maturation of DN thymocytes from *Sox-4*^{−/−} fetal liver cells into CD4/CD8 expressing cells was severely impaired (Fig. 3B). Nevertheless, it should be stressed that in both types of FTOC, *Sox-4*^{−/−} T cell maturation occurred to some extent, because small numbers of $\alpha\beta$ TCR-expressing cells were detected. After prolonged culture periods (3 weeks or more), the development of *Sox-4*^{−/−} thymocytes recovered to the same level as the controls. This is in agreement with the observation that in 2-month-old bone marrow chimeras, the numbers of peripheral T cells are virtually normal (Fig. 1).

3.3 *Sox-4*^{−/−} progenitors in competitive intrathymic reconstitution experiments

The organ culture experiments demonstrated a retardation of T cell development from *Sox-4*^{−/−} progenitors. However, this was in apparent contrast to the reconstitution experiments in the bone marrow chimeras. To study T cell development *in vivo*, competitive reconstitution experiments were designed. Timed pregnancies between *Sox-4*^{+/−} parents were set up and embryos removed at E14.

Figure 3. Development of *Sox-4*^{−/−} thymocytes is inhibited *in vitro*. (A) Fetal thymi of E13 embryos were removed and cultured on floating filters for 14 days. Thymocytes were then counted and stained with CD4- and CD8-specific antibodies. The absolute number of cells expressing CD4, CD8, or both, per thymic lobe is shown. Each dot corresponds to an individual embryo. (B) Reconstitution of irradiated (25 Gy) Ly-5.1 fetal lobes (E14) with fetal liver cells of *Sox-4*^{+/−} and *Sox-4*^{−/−} embryos. Thymocytes were analyzed after 14 days of culture with conjugated mAb specific for CD4, CD8, and TCR $\alpha\beta$. Staining with Ly-5.1-specific antibodies demonstrated that there were less than 3% recipient-derived thymocytes.

Fetal liver cell suspensions of heterozygous and wild-type (Ly-5.2) embryos were prepared and cells were counted. These cell suspensions (Ly-5.2) were mixed in a one-to-one ratio with fetal liver cells from Ly-5.1 (normal) E14 embryos. The mixtures of Ly-5.2 (*Sox-4*^{+/−} or *Sox-4*^{−/−}) and Ly-5.1 (normal) fetal liver cells were injected intrathymically into sublethally irradiated hosts (Ly-5.1). The mice were killed 4 weeks later and thymi analyzed. Staining of the cells with Ly-5.1-specific mAb distinguished between cells derived from the *Sox-4* embryos and the normal Ly-5.1 competitor cells. When 1:1 mixtures of liver cells from Ly-5.2 *Sox-4*^{−/−} embryos and normal Ly-5.1 embryos were injected, 10–40% of the thymocytes were *Sox-4*^{−/−} (*i.e.* Ly-5.2), while the remainder was Ly-5.1 (Fig. 4). When cells were mixed in a 5:1 ratio (*Sox-4*^{−/−}:Ly-5.1), the *Sox-4*^{−/−} progenitors yielded 40–70% of the

Figure 4. *Sox-4*^{-/-} progenitor cells do not compete as well as wild-type cells in thymus reconstitution. Fetal liver cells from embryos heterozygous (A and C) or homozygous (B and D) for the *Sox-4* mutation, were co-injected with normal (BL/6.Ly-5.1) fetal liver cells in the ratios 1:1 (A and B) and 5:1 (C and D) in the thymi of BL/6.Ly-5.1 recipient mice. Thymi were removed 4 weeks after injection and stained with anti-TCRαβ and anti-Ly-5.1 antibodies.

thymocytes. When *Sox-4*^{+/-} fetal liver cells were injected together with normal cells, the contribution to reconstitution was 80–98 % (Fig. 4). These data indicate that the *Sox-4*^{-/-} progenitor cells are less able to compete for reconstitution than normal cells.

The data presented show that *Sox-4*-deficient progenitor cells have a reduced capacity to reconstitute the T cell lineage. This is in agreement with the expression of *Sox-4* in adult thymus [2]. As has been reported before, *Sox-4* is required for early B cell lymphopoiesis and proper formation of the cardiac outflow tract [4]. The defect in B cell differentiation appeared to be primarily the lack of expansion of very early B cell precursors, which occurs before rearrangement of the immunoglobulin genes. However, the block in differentiation is not absolute, since small numbers of *Sox-4*^{-/-} B cells are able to mature. The block in T cell differentiation as described in this report is more subtle. We observe a decrease in maturing thymocytes in FTOC of *Sox-4*^{-/-} genotype and a delay in reconstitution after intrathymic injection which only becomes apparent in a competitive experimental setup.

4 Concluding remarks

The experiments demonstrate that *Sox-4* is required for optimal progression along the T cell differentiation pathway. Although the nature of the molecular events that are disturbed by the absence of *Sox-4* is unknown, we believe that they are comparable between the B and T lineage; the difference in phenotype between the *Sox-4*^{-/-} B and T cell lineages might only reflect a quantitative effect.

Recently, we demonstrated that the T cell-specific HMG-box transcription factor Tcf-1, which is related to *Sox-4*, is required for the expansion of adult thymocytes [9]. This suggests a common function for these two HMG box-containing proteins in the expansion of early lymphocytes.

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