development, as *nos*-related mRNAs are associated with germ line in dipteran insects[16] and the frog[17,18]. We propose that localized nanos activity has a widespread role in germ-line development, in addition to its role in the establishment of embryonic asymmetry. □

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CORRESPONDENCE and requests for materials should be addressed to S.K. (e-mail: skob@sakura.cc.tsukuba.ac.jp).

Defects in cardiac outflow tract formation and pro-B-lymphocyte expansion in mice lacking *Sox-4*

Marco W. Schilham*, Mariëtte A. Oosterwegel†, Petra Moerer, Jing Ya‡, Piet A. J. de Boer‡, Marc van de Wetering, Sjef Verbeek§, Wouter H. Lamers‡, Ada M. Kruisbeek†, Ana Cumano‖ & Hans Clevers

Department of Immunology and § Transgenic Mouse Facility, University Hospital, PO Box 85500, 3508 GA Utrecht, The Netherlands
† Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
‡ Department of Anatomy and Embryology, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105A2 Amsterdam, The Netherlands
‖ Unité de Biologie Moléculaire du Gène, Institut Pasteur, 28 rue du Dr Roux, 75024 Paris Cedex 15, France

A STRIKING example of the relationship between regulation of transcription and phenotype is the central role of the Y-chromosomal gene *Sry* in mammalian sex determination[1,2]. *Sry* is the founding member of a large family of so-called *Sox* genes[1,3]. During murine embryogenesis, the transcriptional activator *Sox-4* is expressed at several sites, but in adult mice expression is restricted to immature B and T lymphocytes[4]. Using targeted genedisruption, we have found that *Sox-4*$^{-/-}$ embryos succumb to circulatory failure at day E14. This was a result of impaired development of the endocardial ridges (a specific site of *Sox-4* expression) into the semilunar valves and the outlet portion of the muscular ventricular septum. The observed range of septation defects is known as 'common arterial trunk' in man. We studied haemopoiesis in lethally irradiated mice reconstituted with *Sox-4*$^{-/-}$ fetal liver cells and found that a specific block occurred in B-cell development at the pro-B cell stage. In line with this, the frequency and proliferative capacity of IL-7-responsive B cell progenitors in fetal liver were severely decreased *in vitro*.

In the heart at embryonic day E13, *Sox-4* is expressed exclusively in the endocardial cushions and ridges (Fig. 1). After disruption of the *Sox-4* gene (Fig. 2), no homozygous mutant offspring were born. At E13, *Sox-4*$^{-/-}$ embryos were macroscopically indistinguishable from their littermates. They then rapidly developed generalized oedema and died at E14. In moribund mutant embryos, heart rate increased while the blood was oscillating, suggesting a valvular insufficiency. Histological analysis of 12 mutant embryos consistently revealed dysplasia of the semilunar valves (Fig. 3). Furthermore, a large septation defect affected the entire outlet portion of the ventricles and, to a variable extent, the great arteries. This spectrum of cardiac development defects was indistinguishable from common arterial trunk type I (Fig. 3*c–e*) and type II (Fig. 3*a*, *b*) in man[5].

The semilunar valves develop from the upper part of the endocardial ridges. Neural crest tissue is thought to be an important contributor to the proper development of the arterial pole of the heart, including the endocardial ridges[6]. The lower part of the endocardial ridges of the outflow tract is invaded by cardiomyocytes to form the outlet portion of the muscular interventricular septum[7]. To separate left and right circulation, the endocardial ridges fuse. Common arterial trunk type II will result, when the ridges remain unfused[5]. When additionally the aortopulmonary septum is not formed, common arterial trunk type I will result[5].

In E13/14 *Sox-4* mutant embryos, the myocardium was not primarily affected by the mutation (Fig. 3*f*, *g*). The endocardial cushions were present and yielded normal atrioventricular valves (Fig. 3*f*). The endocardial ridges, however, never fused and failed to develop into proper semilunar valves. The absence of functional semilunar valves explains the oscillations of the blood observed in dying E14 embryos.

We conclude that the cardiac phenotype is the consequence of a primary defect in endocardial ridge development. This conclusion is based on two observations. (1) The ridges are specific (although not exclusive) sites of *Sox-4* expression, and (2) development of the semilunar valves from the ridges and fusion of these ridges is impaired. We suggest that the impaired development of the neural-crest-derived aortopulmonary septum is a consequence of the defect in the endocardial ridges, possibly due to the disturbance of the inductive processes between the ridges and the ingrowing neural crest-derived cells. Although several other gene disruptions interfere with cardiac septation[8–13], the features of the *Sox-4* mutation are unique in that they are confined to the arterial pole of the heart.

To investigate *Sox-4*$^{-/-}$ haemopoiesis, fetal liver cells of E13 embryos (H–2^{b}) were injected into lethally irradiated MHC disparate recipients (H–2$^{b/d}$). In all mice, normal numbers of granulocytes and monocytes of donor origin were observed (not shown). After 8 weeks, lymphoid tissues were analysed. Donor-derived T lymphocytes (60–75%) were present, independent of the *Sox-4* genotype of the transplant (Fig. 4*a*, *b*). In contrast, CD43^{-}B220^{+} pre-B cells were almost completely absent, whereas numbers of CD43^{+}B220^{+} pro-B cells were reduced in *Sox-4*$^{-/-}$-

* Present address: Department of Pediatrics, University Hospital, PO Box 9600, 2300 RC Leiden, The Netherlands.

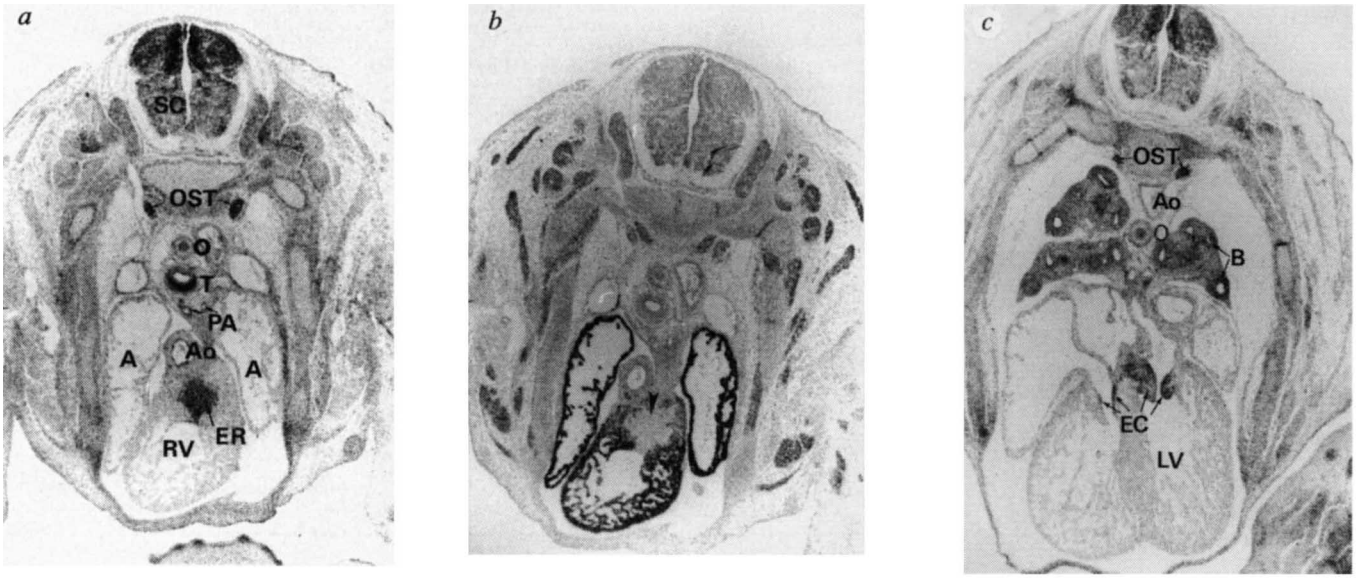

FIG. 1 Expression of Sox-4 in E13 embryos. Transverse sections of E13 embryos probed for Sox-4 (*a*, *c*) or calcium-dependent ATPase (*b*, *d*)[27]. *a*, The *Sox-4* gene is expressed in the endocardial ridges (ER) of the outflow tract, cartilage of the trachea (T), orthosympathic trunk (OST) and spinal cord (SC). Indicated as well are oesophagus (O), aorta (Ao), pulmonary arteries (PA), atria (A) and right ventricle (RV). *b*, (Ca^{2+})ATPase expression in the myocardium of atria and ventricles in a consecutive section. Endocardial ridges are identified by the specific lack of (Ca^{2+})ATPase expression (arrowhead). *c*, *Sox-4* expression in the (EC) endocardial cushions of the atrioventricular canal, and in the cartilage of the bronchi (B) of the lungs. Indicated are aorta (Ao), orthosympathic trunk (OST), oesophagus (O) and left ventricle (LV). *d*, (Ca^{2+})ATPase expression in a consecutive section to *c*. Endocardial cushions of the atrioventricular canal specifically lack (Ca^{2+})ATPase expression.
METHODS. *In situ* hybridization has been described[27]. The RNA probe was transcribed from linearized *Sox-4* cDNA[4] in pBluescript SK using T3 RNA polymerase and ^{35}S-UTP (Stratagene). The probe (300 bp) contained unique untranslated sequences from the 5′ end of the mRNA (*Eco*RI–*Sac*I).

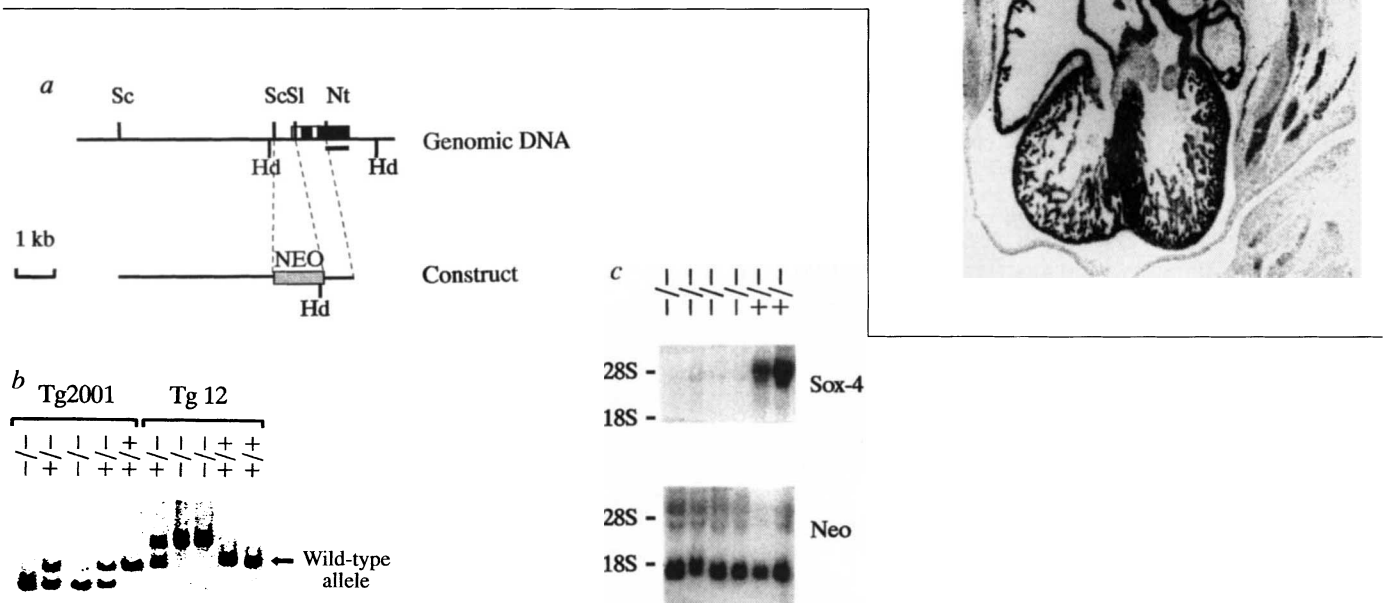

FIG. 2 Targeting of the *Sox-4* gene. *a*, Structure of gene and construct used for homologous recombination. The coding sequence of the gene (top) comprises about 1,300 bp (boxed region). Positions of the HMG box (dark shading) and serine-rich stretch (lighter shading) are indicated. The construct lacks the initiator methionine. The PGK-neo^r cassette was inserted in two orientations. A cDNA probe 3′ of the *Not*I site (underline bar) was used as flanking probe to detect homologous recombination on DNA digested with *Hin*dIII. Restriction sites: Sc, *Sac*I; Nt, *Not*I; SI, *Sal*I; Hd, *Hin*dIII. *b*, Identification of *Sox-4*$^{-/-}$ mutant embryos by Southern blotting. Strain Tg12 contains the *neo* cassette in the sense orientation of the *Sox-4* gene and in strain Tg2001 it is in the opposite orientation. Examples of wild-type, heterozygous and homozygous mutant embryos of both strains are shown. *c*, Northern blotting of RNA isolated from *Sox-4*$^{+/-}$ and *Sox-4*$^{-/-}$ embryos. RNA was isolated from the heads of E13 embryos, electrophoresed, blotted and hybridized with a 5′ untranslated cDNA probe (Fig. 1). Lower panel shows hybridization of the same samples with a *neo*^{r}-coding probe as a control. Migration positions of RNA size markers (28S, 16S) are indicated.
METHODS. The *Sox-4* gene was cloned from a mouse (129/Ola) genomic library[28]. As the long arm of homology, a 4-kb *Sac*I fragment was cloned in the *Sac*I site of the pGEM-11 (Promega) vector. An *Xho*I–*Sal*I digested PGK-neo cassette, containing the PGK promoter and the bovine growth hormone polyadenylation signal (from P. Soriano), was ligated in the *Sal*I site of pGEM-11 in two orientations. Finally, an 800-bp *Sal*I–*Not*I fragment from the *Sox-4* coding sequence was ligated between the *Xho*I and *Not*I sites of pGEM-11 as the short arm of homology (oligonucleotides from Isogen, Amsterdam). After linearization with *Not*I, the construct was electroporated in E14 ES cells. G418 (300 μg ml^{-1})-resistant clones were selected in the presence of buffalo rat liver cell conditioned medium. Germline transmission was obtained from two ES cell lines, with the PGK-neo cassette in opposite orientations, yielding identical phenotypes. Heterozygous mice were backcrossed to C57BL/6 for consecutive generations. Southern and northern blots were prepared according to standard procedures.

reconstituted bone marrow (Fig. 4*c*). During differentiation in the pro-B cell compartment, progenitors express increasing levels of heat-stable antigen (HSA)[14]. Pro-B cells are divided in fraction A (HSA^{low}), where D-J rearrangement starts to occur[15], fraction B + C (HSA^{+}), and fraction C′ (HSA^{++}), in which most cells have rearranged the heavy chain gene[14,15]. In the *Sox-4*$^{-/-}$ bone marrow, fraction C′ was virtually absent. Fraction B + C was decreased, whereas cells of fraction A were present in normal numbers. We also noted low numbers of $CD43^{-}B220^{++}$ cells in *Sox-4*$^{-/-}$ bone marrow (Fig. 4*c*), spleen and lymph nodes (Fig. 4*a*, *b*). These cells were donor-derived, surface-immunoglobulin-positive and represented mature B cells, indicating that the block in B-cell differentiation was not absolute.

In vitro, B-cell precursors in fetal liver that were responsive to interleukin-7 (IL-7) were reduced at least 10-fold in *Sox-4*$^{-/-}$ embryos compared to littermates (Fig. 4*d*). Furthermore, *Sox-4*$^{-/-}$ clones contained 10^{2}–10^{3} cells after two weeks of culture, whereas the control clones contained 10^{4}–10^{5} cells. 29/30 heterozygous clones secreted immunoglobulin in response to lipopolysaccharide (LPS), indicating that B cells were present. None of seven mutant clones responded to LPS (Fig. 4*e*). Some *Sox-4*$^{-/-}$ clones grew more rapidly after 6 weeks with IL-7 and became LPS-responsive (results not shown). Taken together *Sox-4*$^{-/-}$ fetal livers not only contain less IL-7-responsive precursors, but these cells also expand much more slowly than control cells.

Where should the *Sox-4* gene be positioned in the molecular hierarchy controlling B-cell development? The hallmark of B-lineage development is the ordered rearrangement of the immunoglobulin genes and the assembly of functional receptor complexes on the cell surface. Mutations that interfere with surface expression of the immunoglobulin heavy chain[15,16] result in an accumulation of cells in fraction B + C, and normal frequencies of IL-7 clonable precursors[14,17,18]. In contrast, *Sox-4*$^{-/-}$

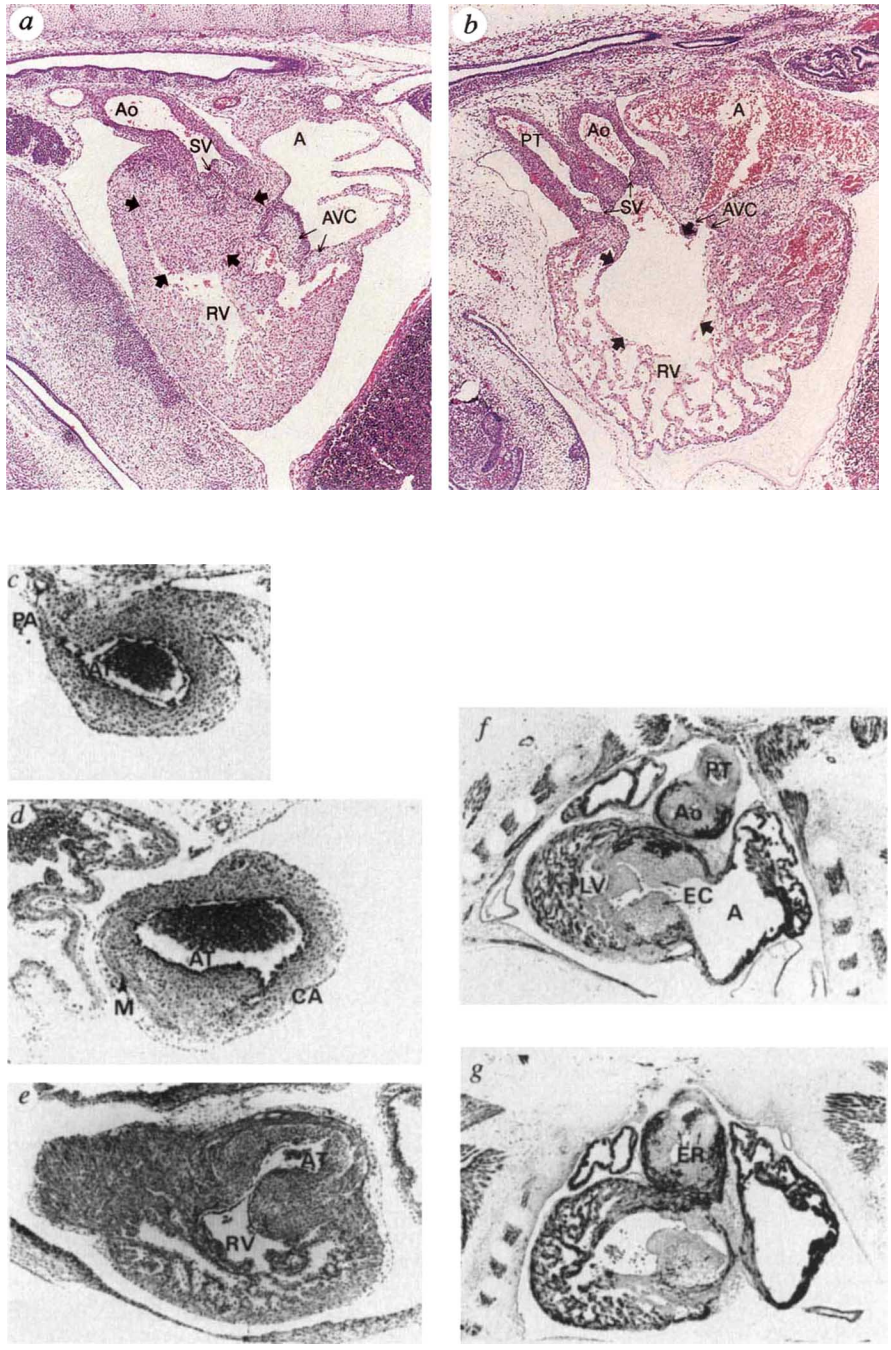

FIG. 3 Cardiac abnormalities in *Sox-4*$^{-/-}$ embryos. *a*, *b*, Sagittal sections (haemotoxilin–eosin (HE) staining) of normal (*a*) and mutant (*b*) E14 embryos show that in this *Sox-4*$^{-/-}$ embryo the outlet portion of the muscular ventricular septum has failed to develop from the endocardial ridges (thick arrows). The semilunar valves are severely dysplasic. This particular heart represents an example of common arterial trunk type II. Aorta and pulmonary trunk are present, but both connect to the right ventricle. The atrioventricular valves (tricuspidalis) are normal. The thinning of the myocardium (sometimes present at E14) was never observed at E13 and therefore probably represented a secondary effect of the mutation. *c*–*e*, Transverse sections (haematoxylin–azophloxine (HA) staining) at different levels of a mutant heart (E14) representing a common arterial trunk type I. There is only one arterial trunk. It originates from the right ventricle (*e*), giving rise to both coronary arteries (*d*) as well as pulmonary arteries (*c*), and proceeds as aorta. *f*, *g*, Frontal sections of a mutant heart (E13) stained for smooth muscle α-actin, demonstrating that endocardial cushions (*f*) as well as the myocardium are normal (*f*, *g*) at E13. Although the endocardial ridges (*g*) will not develop into semilunar valves, the available mass of the ridges prevents regurgitation of the blood at this stage, explaining why circulatory failure occurs as late as E14. A, atrium; Ao, aorta; AT, common arterial trunk; EC, endocardial cushions of atrioventricular canal (AVC); CA, coronary artery; DA, ductus arteriosus; ER, endocardial ridges; LV, left ventricle; M, myocardium; PA, pulmonary artery; PT, pulmonary trunk; SV, semilunar valves; RV, right ventricle.

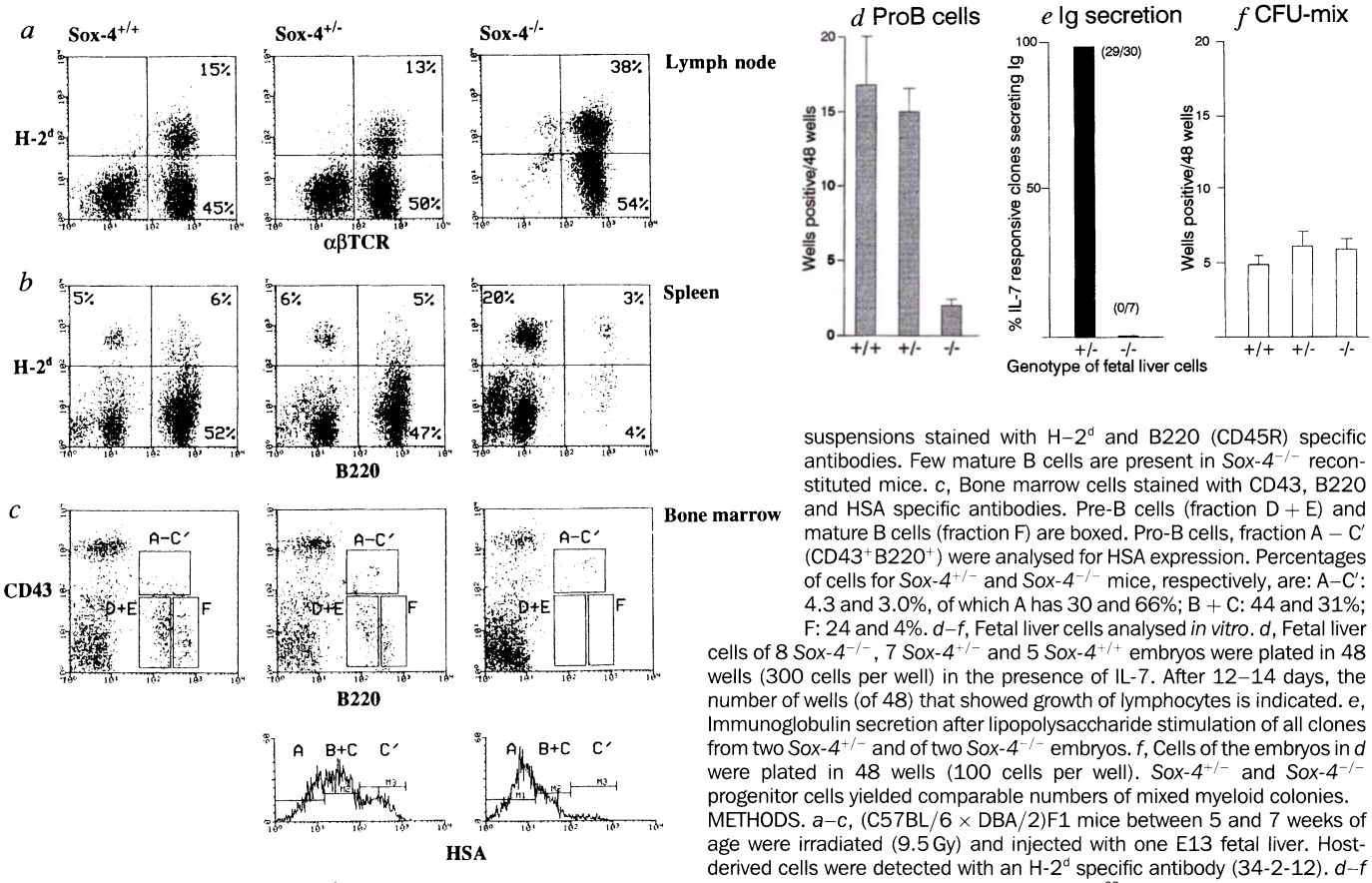

FIG. 4 B-cell development of *Sox-4*$^{-/-}$ progenitor cells is impaired *in vivo* and *in vitro*. *a–c*, Lymphoid cells of bone marrow irradiation chimaeras analysed by FACS eight weeks after reconstitution. *a*, Lymph node cell suspensions stained with H-2^d and αβ T-cell receptor specific antibodies. T cells are generated in nearly normal numbers from *Sox-4*$^{-/-}$ progenitor cells. Note the virtual absence of non-T cells in the mutant. *b*, Spleen cell suspensions stained with H-2^d and B220 (CD45R) specific antibodies. Few mature B cells are present in *Sox-4*$^{-/-}$ reconstituted mice. *c*, Bone marrow cells stained with CD43, B220 and HSA specific antibodies. Pre-B cells (fraction D + E) and mature B cells (fraction F) are boxed. Pro-B cells, fraction A – C′ (CD43$^+$B220$^+$) were analysed for HSA expression. Percentages of cells for *Sox-4*$^{+/-}$ and *Sox-4*$^{-/-}$ mice, respectively, are: A–C′: 4.3 and 3.0%, of which A has 30 and 66%; B + C: 44 and 31%; F: 24 and 4%. *d–f*, Fetal liver cells analysed *in vitro*. *d*, Fetal liver cells of 8 *Sox-4*$^{-/-}$, 7 *Sox-4*$^{+/-}$ and 5 *Sox-4*$^{+/+}$ embryos were plated in 48 wells (300 cells per well) in the presence of IL-7. After 12–14 days, the number of wells (of 48) that showed growth of lymphocytes is indicated. *e*, Immunoglobulin secretion after lipopolysaccharide stimulation of all clones from two *Sox-4*$^{+/-}$ and of two *Sox-4*$^{-/-}$ embryos. *f*, Cells of the embryos in *d* were plated in 48 wells (100 cells per well). *Sox-4*$^{+/-}$ and *Sox-4*$^{-/-}$ progenitor cells yielded comparable numbers of mixed myeloid colonies.

METHODS. *a–c*, (C57BL/6 × DBA/2)F1 mice between 5 and 7 weeks of age were irradiated (9.5 Gy) and injected with one E13 fetal liver. Host-derived cells were detected with an H-2^d specific antibody (34-2-12). *d–f* Primary cultures were prepared as described[29]. For the detection of mixed myeloid colonies, 100 cells per well were plated in 96-well plates with MGF (Genetics Institute), IL-3 (from F. Melchers), IL-11 and EPO (1 U ml^{-1}). For the detection of clonable B-cell precursors, 300 cells per well were plated on irradiated (30 Gy) S17 stromal cells (from K. Dorshkind) with IL-7 (A. Rolink) and IL-11 (R. Hawley[30]).

bone marrow shows a decrease in fraction B + C (Fig. 3*c*), and the responsiveness of B-cell progenitors to IL-7 in *Sox-4*$^{-/-}$ fetal liver is strongly diminished. The proliferative capacity of *Sox-4*$^{-/-}$ pro-B cells is affected at a stage not yet dependent on surface expression of immunoglobulin heavy chain. This defect resembles the B-lineage defect in IL-7 receptor and IL-2R γ-chain mutant mice[19,20] and suggests that the *Sox-4* gene product is a component of the pathway that controls pro-B-cell expansion.

Disruption of several other transcription factor genes has affected the B lineage. A tentative hierarchy of such factors can be proposed. Mutation of the *Ikaros* zinc-finger gene results in the absence of all lymphoid lineages[21]. Absence of functional EBF or E2A results in a B-lineage block, apparently preceding that of the *Sox-4* mutation, before or in the pro-B cell stage, as mice with this mutation generate no[22,23] or only very few[24] B220$^+$ cells. Mutation of the *Pax-5* gene[25] has yielded a B-lineage block comparable to that of *Sox-4*.

We have previously reported that disruption of the T-lymphocyte-specific HMG-box gene *Tcf-1* specifically blocks thymocyte expansion at the transition of CD4$^-$CD8$^-$ cells to the CD4$^+$CD8$^+$ stage[26]. *Sox-4* and *Tcf-1* might thus be controlling similar processes in B- and T-lineage development, respectively. □

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