year)[10–16] that independent confirmation is warranted. In particular, we wanted to investigate the possibility that these increased mutation/substitution rates resulted from immigration of heterogeneous colonists after the meltdown.

We therefore sequenced the cytochrome *b* gene from a Chernobyl-captured female *M. arvalis* which contained five embryos (Fig. 1; 'm' identifies the mother in the tree of *M. arvalis*). To control for polymerase error and to check for mosaicism, DNA was isolated from the mother's liver and heart, and two clones from each tissue were sequenced in both directions. No variation was found among these four clones. For each embryo, two clones were sequenced, and if any site differed from that of the mother (a potential mutation), a third independent amplification of that region from a new aliquot of DNA was sequenced to verify the presence of this base-pair substitution. No variation was detected among different clones from individual embryos. Two embryos had cytochrome *b* genes with nucleotide sequences identical to the mother. Three embryos were distinguished from their mother by one nucleotide change each; two individuals shared a third-position synonymous substitution, and one individual had a first-position non-synonymous substitution. Two independent (twinning, or a single mutation in the germ line before the two oocytes developed could account for the shared mutation) substitutions in 5,715 bp provide a substitution rate estimate of 3.5×10^{-4}, which corroborates the estimates from the populational comparisons above. Although our data are limited to a single female and her embryos, using the highest annual mutation rate normally accepted for the mitochondrial genome[10–16] (1×10^{-6}), the probability of two mutations being observed by chance in 5,715 base pairs is 3.3×10^{-5}. Results from embryos suggest that the high substitution rate within the Chernobyl populations is ongoing, and contradict the hypothesis that the observed populational polymorphisms are the result of immigration. If this elevated mutation rate in cytochrome *b* extends to the entire mitochondrial genome (~17,000 bp), such a rate would translate into 3–5 substitutions per mitochondrial genome per generation. It is probable that such a high mutation rate does not extend to the nuclear genome because such a mutation rate across three billion base pairs would result in 600,000 mutations per gamete.

It must be understood that this increased substitution rate may reflect the presence of mutagens other than, or in addition to, radioactivity, or some synergistic effects of mutagens[17]. If these changes were caused solely by ambient levels of radiation, then the substitution rate should decline as the radioactivity decays. Although the level of ambient radiation has declined substantially since the accident[18], our limited analyses of embryos suggest that the substitution rate is still vastly elevated over controls and may be similar to that which led to the current levels of genetic variation in the populations around the reactor. Unlike many of the radioisotopes released at Chernobyl, heavy metals and other mutagenic chemicals can persist indefinitely in the environment. In this and other respects, the environmental pollution resulting from the Chernobyl accident is different from that resulting from nuclear weapons. It does not appear that the biological consequences of the Chernobyl accident can be adequately predicted from results of previous laboratory studies[19,20] or from the extensive investigations of the effects of Hiroshima and Nagasaki[21,22]. □

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Essential role of the posterior morphogen nanos for germline development in *Drosophila*

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In many animal groups, factors required for germline formation are localized in germ plasm[1], a region of the egg cytoplasm. In *Drosophila* embryos, germ plasm is located in the posterior pole region and is inherited in pole cells, the germline progenitors. Transplantation experiments have demonstrated that germ plasm contains factors that can form germline[2–4], and germ plasm also directs abdomen formation[5]. Genetic analysis has shown that a common mechanism directs the localization of the abdomen and germline-forming factors to the posterior pole[6–12]. The critical factor for abdomen formation is the nanos (*nos*) protein (nanos)[13–15]. Here we show that *nos* is also essential for germline formation in *Drosophila*; pole cells lacking nanos activity fail to migrate into the gonads, and so do not become functional germ cells. In such pole cells, gene expression, which normally initiates within the gonad, begins prematurely during pole-cell migration. Premature activation of genes in germline precursors may mean that these cells fail to develop normally. A function for *nos* protein in *Drosophila* germline formation is compatible with observations of its association with germ plasm in other animals[16–18].

Localized *nos* messenger RNA is translated *in situ* to form a gradient of *nos* protein with its highest concentration in germ plasm[11,12,15,19]. The *nos* protein is only transiently present in the abdominal anlagen, and becomes undetectable by the cellular blastoderm stage. In contrast, in germ plasm it is incorporated into pole cells and remains readily detectable throughout pole-cell migration until they reach the embryonic gonads[15]. These observations led us to speculate that *nos* protein may also be essential for germline formation. We investigated whether pole cells lacking nanos activity can develop into germ cells. Females homozygous for the maternal-effect *nos* mutation produce progeny that lack nanos activity[13–15] and form pole cells[13,14]. However, it is not clear whether these pole cells can condense in the embryonic gonads, and give rise to functional germ cells. This is because

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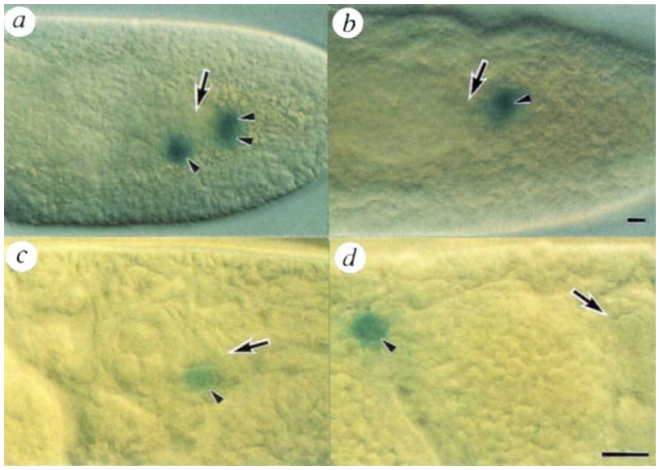

FIG. 1 The developmental fate of transplanted *nos* pole cells. In the text, we refer to embryos from nos^{BN}/nos^{BN} females as *nos* embryos, and to the pole cells formed by these embryos as *nos* pole cells, irrespective of zygotic genotype, because they lack nanos activity[15]. Pole cells formed in embryos from $nos^{BN}/+$ females are designated as control pole cells. During normal embryogenesis, pole cells are formed at the posterior pole of cellularized embryos and migrate through the posterior midgut epithelium into the haemocoel, where they separate into two bilateral groups, and condense in the embryonic gonads (in the fifth abdominal segment) to differentiate as germ cells[27,28]. To determine whether *nos* pole cells can differentiate as germ line, we examined the developmental fate of pole cells transplanted from *nos* embryos into ovo^{D1} host embryos. To identify the transplanted pole cells in the host embryos, a marker construct, PLHΔ23, which contains the *lacZ* gene under the control of a heat-shock promoter[24], was introduced into the donor embryos. The fate of the transplanted pole cells was followed by heat treatment, fixation and staining for β-gal at appropriate stages. Host embryos carrying *nos* pole cells (*b* and *d*) and control pole cells (*a* and *c*) were stained with X-gal. *a*, *b*, Embryos at stage 10 (stages as ref. 28). The *nos* and control pole cells (arrowheads) were located outside the midgut rudiments (arrows). *c*, *d*, Embryos at stage 15. The control pole cell was incorporated into the gonads (*c*), but none of the transplanted *nos* pole cells were incorporated in the host gonads. The *nos* pole cells were scattered in the midgut and hindgut lumen and in the haemocoel of the posterior half of the embryos (*d*). Arrowheads and arrows point to the transplanted pole cells and the gonads, respectively. Scale bars, 10 μm.

METHODS. Pole-cell transplantation was as described in Table 1. After transplantation, hosts were kept at 25 °C until stage 10 or 15. The hosts were heat treated at 36 °C for 30 min, followed by incubation at 25 °C for 1 h, then were stained with X-gal as previously described[24]. Transplanted pole cells were stained green with X-gal. Gonads were identified by morphological features: two dorsolateral distinctive cell clusters located in abdominal segment 5 (refs 27,28).

embryos lacking nanos activity have no abdominal mesodermal cells, which are required to assemble with pole cells to form gonads; one of the effects of *nos* mutation is the inability to form abdominal segments.

To determine whether *nos* pole cells can differentiate as germ line, we examined the developmental fate of pole cells transplanted from *nos* embryos into host embryos that can form normal abdomens. The transplanted *nos* pole cells migrated into the haemocoel, just as control pole cells did (Fig. 1*a*, *b*). However, none of the transplanted *nos* pole cells were incorporated in the gonads of the hosts (Table 1, Fig. 1*d*). In contrast, pole cells taken from the control embryos were found in the gonads, the midgut lumen and the haemocoel (Table 1, Fig. 1*c*). These results indicate that *nos* pole cells cannot penetrate the gonads.

To test whether *nos* pole cells contribute to egg production in adult females, we transplanted them into embryos carrying the dominant female sterile mutation ovo^{D1}. All female progeny are expected to be sterile unless they have received functional pole cells. None of the females that were transplanted with *nos* pole cells produced any progeny, whereas 16.4% of females that had received control pole cells were fertile and produced gametes derived from the transplanted pole cells (Table 2). Thus the autonomous deficiency of nanos activity in pole cells leads to their inability to penetrate the gonads and, consequently, results in their failure to become functional germ cells.

These results suggest that removal of nanos activity results in some change in gene regulation in pole cells, rendering them unable to penetrate the gonads. To understand this better, we examined the expression of three independent enhancer-trap markers, which show β-gal staining in pole cells incorporated in the gonads (see Fig. 2 for details). In control embryos, β-gal expression in pole cells became detectable at stage 13–14, when pole cells are incorporated in the gonads (Fig. 2*a*, *c*, *e*). The proportions of embryos with stained pole cells reached a maximum at stage 16, when maternal *nos* protein becomes undetectable in pole cells[15]. In contrast, in *nos* embryos these markers were prematurely expressed in pole cells during the course of their migration (Fig. 2*a*, *d*, *f*).

The above experiments show that the absence of nanos activity leads to the premature activation of marker genes normally expressed in pole cells within the gonads. In the pathway leading to abdominal segmentation, graded *nos* protein in the posterior half of embryos generates abdomen by repressing translation of maternal *hb* transcript distributed throughout the embryo[20,23]. Similarly, the premature expression of the marker genes in *nos*

TABLE 1 The ability of transplanted pole cells to enter gonads

Genotype of females producing donor	Transplants	Surviving embryos	Embryos with labelled cells	Embryos with labelled cells in gonads (%)
nos/TM3	505	130	66	24 (36.4)
nos/nos	309	129	45	0
With *hb(Δ)*	186	76	45	19 (42.2)
Without *hb(Δ)*	162	75	44	16 (36.4)
Control		78	0	0

Genetically marked pole cells were transplanted into ovo^{D1} embryos. The full genotype of the nos^{BN} stock was nos^{BN} *e/TM3 e Sb Ser*. The nos^{BN} allele is caused by a P-element insertion into the promoter region of the *nos* gene[15]. Nos^{BN} produces a strong abdominal phenotype, but does not display the oogenesis defects associated with some *nos* alleles. Females homozygous for nos^{BN} produce embryos lacking *nos* RNA and *nos* protein[15]. Pole-cell donor embryos were derived from nos^{BN}/nos^{BN}, $nos^{BN}/TM3$, *w/w* females carrying the *hb(Δ)* construct[23], or *w/w* females without the *hb(Δ)* construct mated with PLHΔ23 males. Because all embryonic cells with the PLHΔ23 construct express β-gal after heat treatment[24], we can follow the fate of the transplanted pole cells during embryogenesis by heat treatment and staining for β-gal. To avoid contamination with somatic cells, we used late-syncytial blastoderm-stage embryos as donors. Pole-cell transplantation was performed basically as previously described[25]. Pole cells (10–12) were transplanted from late-syncytial blastoderm donors to early-syncytial blastoderm hosts. The host embryos were derived from a cross between wild-type (Oregon-R) females and ovo^{D1}/Y males. After transplantation, the hosts were kept at 25 °C until stage 15, were heat-treated at 36 °C for 30 min, followed by incubation at 25 °C for 1 h, then were stained for β-gal as described[24]. The stained embryos were devitellinized manually, dehydrated in an ethanol series, and mounted in Eukitt. The embryos were observed under a compound microscope (DMRB, Leica) with Nomarski optics. The average number of labelled cells in a host that was ascertained to have stained pole cells was 2.6 ($n = 57$) when *nos* pole cells were transplanted, and was 3.1 ($n = 21$) when control pole cells were transplanted. The inability of *nos* pole cells to condense within the gonads does not result from selective death of these cells, as *nos* pole cells were as efficiently transplanted as control pole cells, and were present in comparable numbers in transplanted hosts. For controls, pole cells were not transplanted.

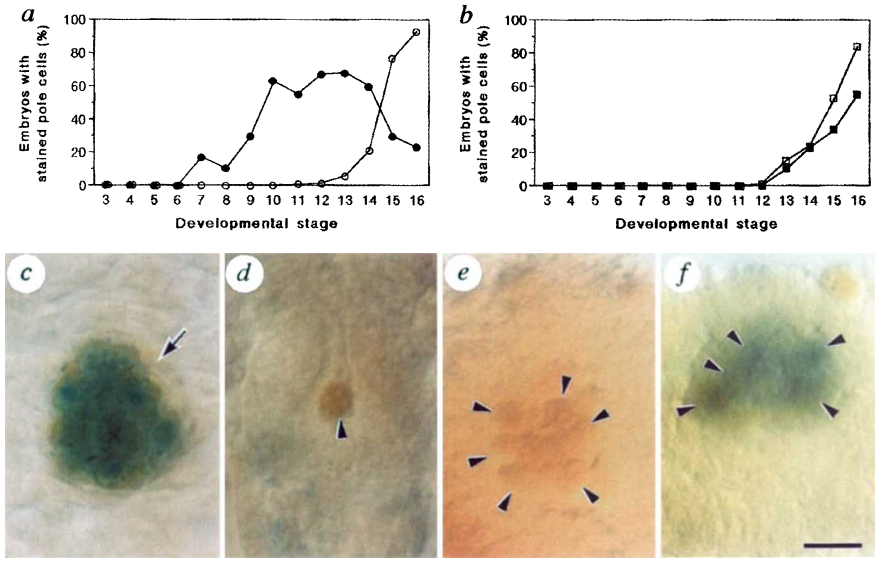

FIG. 2 Removal of nanos activity results in the premature expression of late pole-cell marker, which normally appears in pole cells within the gonads. We examined the expression of an enhancer-trap marker, 198, which shows β-gal staining only in pole cells incorporated in gonads (Y. Obara and S.K., unpublished data). This marker was crossed to nos^{BN}/nos^{BN} females and its pattern of expression determined in the progeny. *a*, *b*, Stage-dependent expression of β-gal in pole cells in embryos produced from nos^{BN}/nos^{BN} (filled circles), $nos^{BN}/TM3$ (open circles) females and *w/w* females with (filled squares) and without *hb(Δ)* (open squares) mated with the 198 enhancer-trap line. The frequency of embryos with stained pole cells is plotted against the stage. Embryos (10–157) of each genotype were examined at each developmental stage. In control embryos produced from $nos^{BN}/TM3$ females, β-gal expression in pole cells was detected at stage 13–14, when pole cells were incorporated in the embryonic gonads, compared to stage 7–8, when pole cells begin moving into the embryo with the proctodeal invagination, in *nos* embryos. Similar results were obtained with two other independent enhancer-trap lines (data not shown). *c*–*f*, Embryos derived from $nos^{BN}/TM3$ (c and e) and nos^{BN}/nos^{BN} females (*d* and *f*) mated with the 198 enhancer-trap line were double stained with X-gal (green) and an antibody against *vasa* protein, a marker protein specific to pole cells (orange). *c*, Pole cells incorporated in the gonad (arrow) of a stage-15 embryo were double stained. In the embryo, almost all pole cells in gonads were stained. *d*, A pole cell (arrowhead) in the hindgut lumen of a stage-15 embryo was not stained with X-gal. *e*, Pole cells (arrowheads) in the midgut rudiment of a stage-9 embryo were not stained with X-gal. *f*, Pole cells in the midgut rudiment of a stage-9 embryo were double stained (arrowheads). Scale bar, 10 μm.

METHODS. Three enhancer-trap lines (198, 640 and 4351) were screened out from over 1,600 enhancer-trap lines carrying single P-lwB constructs (Y. Obara and S.K., unpublished data). These enhancer-trap lines express β-gal in pole cells incorporated in gonads; 198 and 640 are homozygous viable and fertile, but 4351 is homozygous lethal. Embryos produced from nos^{BN}/nos^{BN}, $nos^{BN}/TM3$ females and *w/w* females with and without *hb(Δ)* construct mated with the 198 enhancer-trap line were double stained with X-gal and an anti-vasa antibody as described previously[29]. The dechorionated embryos were fixed in heptane saturated with 4% formaldehyde, then in heptane and 50% methanol. Fixed embryos were devitellinized with a tungsten needle and were processed for immunoperoxidase staining with an anti-vasa antibody. Immediately before peroxidase colour development, embryos were stained with X-gal, then peroxidase staining was resumed. The stained embryos were dissected and mounted in Eukitt.

pole cells can be explained by a failure of *nos* protein to suppress a regulatory pathway that is responsible for the expression of the late pole-cell markers. By analogy, this pole-cell-specific regulatory pathway could be mediated by a regulatory interaction between *nos* protein and *hb* RNA; however, the following observations demonstrate that this is not the case.

The ability of *nos* protein to regulate *hb* is mediated by discrete target sites, or *nos* response elements (NREs), in the 3′ untranslated region of the *hb* transcript[23]. Deletion of NREs from *hb* mRNA overrides *nos*-mediated repression, and embryos carrying this altered transcript *hb(Δ)* express *hb* protein throughout their entire length, including pole cells, and develop as embryos lacking abdominal segments[23]. We examined whether premature expression of the late pole-cell markers is observed in the embryos expressing *hb(Δ)* transcript; no premature expression was discernible (Fig. 2*b*). During late embryogenesis, the pole cells expressed the markers without being enclosed by gonadal mesoderm. These observations show that *hb* protein could not direct the premature expression of the late pole-cell markers. Furthermore, pole cells derived from *hb(Δ)* embryos condensed in the gonad just as well as the normal pole cells (Table 1) if they were transplanted into a host that produced abdominal mesoderm.

Our results imply that *nos* protein in pole cells is not required for expression of the late pole-cell markers, but is essential to define the stage of expression. Another regulatory factor may be required to activate expression of the late pole-cell markers, with *nos* protein acting to repress expression. Although we cannot exclude the possibility that *nos* protein in pole cells directly represses the expression of the late pole-cell markers, we suggest that *nos* protein represses the production of the activator of marker expression, presumably by a translational control mechanism. Our results also show that ectopic overexpression of *hb* protein in pole cells does not result in premature expression of the late pole-cell markers, suggesting that maternal *hb* mRNA is not the only regulatory target of *nos* protein in pole cells. It is likely that regulatory factor(s) other than *hb* protein, which are responsible for the activation of late pole-cell markers, are stored in pole cells as mRNA(s) whose translation is repressed by *nos* protein. Once maternal *nos* protein is degraded in pole cells within the gonads, the mRNA(s) are translated to produce protein(s), which in turn activate late pole-cell enhancers.

It will be interesting to determine whether *nos* protein acts as part of an evolutionarily conserved mechanism of germ line

TABLE 2 Contribution of transplanted pole cells to progeny production

Genotypes of females producing donor	Transplants	Surviving females	Fertile females producing progenies derived from donor pole cells (%)
nos/TM3	857	67	11 (16.4)
nos/nos	702	78	0
Control	–	109	0

Pole-cell transplantation was as described in Table 1. The ovo^{D1} females are sterile and do not produce eggs[26]. The hosts were kept at 25 °C. The hatched larvae were transferred to a standard culture medium and allowed to develop to adult flies; they were mated and their fertility was examined. All female progeny are expected to be sterile, unless they have received functional pole cells. To confirm that the progeny of the fertile host females were derived from the transplanted pole cells, the host females were crossed to e/e males, and the genotype of their progeny determined. Because the genotype of the transplanted pole cells was nos^{BN} *e/TM3 e Sb Ser* or nos^{BN} e/nos^{BN} *e*, they produce the progeny homozygous for e. For controls, pole cells were not transplanted.

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

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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*$^{-/-}$-

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