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POSSIBLE ROLE OF *MSX1* IN MURINE HEMATOPOIESIS*

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ABSTRACT

The *Msx1* gene is expressed at sites of epitelium-mesenchymal interaction throughout development and is important in morphogenesis since *Msx1* null mice die 24 h. after birth and show defects in craniofacial bones. The *Msx1* gene code for a transcription factor activated by BMP4 and, when active, maintains progenitor cells in an undifferentiated state. BMP4 is a crucial factor for hematopoietic development in the murine embryo and although *Msx1* is activated by BMP4, there have been no reports relating *Msx1* to hematopoiesis. To investigate the role of *Msx1* in murine hematopoiesis, samples of hematopoietic tissues (spleen, liver, thymus, bone marrow) and blood collected from 18.5 days *post coitum* (dpc) fetuses of *Msx1* mutant and wild-type embryos were analyzed histologically. Blood cell counts as well as erythropoietic clonogenic assays for liver and bone marrow cells were also done. Histological analysis of the spleen suggested the presence of fewer erythrocytes but more hematopoietic progenitors in mutant embryos. While the bone marrow of wild-type mice had mesenchymal and hematopoietic components, in mutant mice only the hematopoietic component was seen. Hematopoietic progenitors and more mature cells, as well as extracellular matrix, filled the entire bone marrow in heterozygous mutants. In homozygous null mice, this phenotype was enhanced with a poor bone marrow. In hematopoietic colony assays, the liver and bone marrow of *Msx1* knockout embryos had a higher number of erythropoietic progenitors whereas peripheral blood had a lower number of erythrocytes compared to wild-type mice. These results suggest that *Msx1* plays a role in erythropoietic differentiation since low or null gene expression increased the level of progenitors and decreased the number of differentiated erythroid cells. *Msx1* appears to act in mesenchymal cells since in $Msx1^{-/-}$ embryos the hematopoietic cells were abnormal and the cells that supported hematopoietic development in bone marrow were missing.

Key words: Bone marrow, embryonic development, hematopoiesis, knockout mice, *Msx1*

INTRODUCTION

Msx genes regulate crucial steps in morphogenesis throughout vertebrate development, especially epithelium-mesenchymal interactions during organogenesis. In the mouse, three genes have been identified [10] and two of them (*Msx1* and *Msx2*) have been characterized and extensively studied.

Msx1 and *Msx2* are expressed in many embryonic regions, including the neural tube, limb buds, and derivatives of the cranial neural crest [9,18,20-22,29-31,33,34]. Despite this wide spread expression, the only abnormalities described in *Msx1* null mutants are cranio-facial malformations, such as cleft palate, reduced mandibular length, abnormalities of the nasal, frontal and parietal bones, and arrested tooth development [11,31]. This phenotype cannot explain the neonatal lethality of *Msx1* mutants ($Msx1^{-/-}$ mutant mice die within 24 hour after birth) or the role of *Msx1* at several sites of expression.

The analysis of *Msx1* gene expression at sites of epithelium-mesenchymal interactions has revealed a particular set of diffusible factors involved in *Msx1* regulation, namely the proteins BMP2, BMP4 and BMP7, which are co-expressed with *Msx1* at numerous locations in vertebrate embryos [14,19]. During vertebrate tooth development, epithelially expressed BMP4 and 7 activate *Msx1* expression in the tooth mesenchyme, which is responsible for the transfer of BMP4/7 expression to this tissue [3-5]. The induction of *Msx1* by BMP proteins has also been observed during neural tube patterning and limb bud growth [17,28].

BMP2/4 is required for mesoderm patterning and hematopoietic cell generation during early embryogenesis in *Xenopus*, zebrafish, and mice [16,23,36]. Injection of XBMP4 RNA into the animal hemisphere of *Xenopus* eggs has revealed a role for BMP4 in ventralization of the mesoderm during development [6, 13]. Similarly, the over expression of *Msx1* in early

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Xenopus embryos leads to their ventralization [32]. In mice, a lack of BMP4 retards embryonic development, with posterior structural disorganization and a reduction in the extraembryonic mesoderm, including blood islands [36]. A role for BMP4 in hematopoiesis has also been demonstrated by the differentiation of Embryonic Stem Cells (*ES*) cells into an erythroid lineage after addition of BMP4 to serum-free cultures [2,12]. During murine embryogenesis, the pattern of *Msx1* expression follows that of BMP4, including the sites where the first blood precursor cells will emerge, although so far *Msx1* has not been implicated in this process. Considering that *Msx1* is required to maintain cells in an undifferentiated state, any implication of this gene in hematopoiesis could be indicative of an important role in the multipotentiality of the hematopoietic stem cell.

Based on these considerations, the aim of this work was to investigate a possible role of *Msx1* in hematopoiesis in definitive hematopoietic tissues (liver, spleen, thymus, bone marrow and peripheral blood) of *Msx1* mutant mice. Because of the neonatal lethality of *Msx1* homozygous mutants, this analysis was done in 18.5 dpc embryos, a developmental stage when all hematopoietic tissues have already been colonized by hematopoietic colony forming units (CFU) [37]. Results strongly suggest a role for *Msx1* in erythropoietic differentiation and in the generation of bone marrow stromal cells.

MATERIAL AND METHODS

Mice

All of the experimental procedures were done in accordance with the guidelines for the care and use of laboratory animals (CAUAP) of the Carlos Chagas Filho Biophysics Institute, which conform to those of the National Institutes of Health (Bethesda, MD, USA).

The breeding stock of C57Bl6 mice (*Msx1*$^{+/-}$) was a kind gift from Dr Benoit Robert (Pasteur Institute, Paris, France) [11]. The mice were maintained in a heterozygous state. Two colonies were used. One colony was derived from crosses between a heterozygous C57Bl6 male and a wild-type C57Bl6 female, while the other resulted from crosses between a heterozygous C57Bl6 male and a wild-type Swiss female. The latter colony was established to obtain more resistant knockout mice that provide more representative offspring. The C57Bl6Sw*Msx1*$^{-/-}$ mice had the same phenotype as the original knockout, as shown by the presence of a palatine cleft in homozygous mutant embryos (data not shown). Embryos were obtained from crosses between heterozygous mice of the same colony (C57Bl6*Msx1*$^{+/-}$ x C57Bl6*Msx1*$^{+/-}$ or C57Bl6Sw*Msx1*$^{+/-}$x C57Bl6Sw*Msx1*$^{+/-}$). We used three embryos from one C57Bl6*Msx1*$^{+/-}$ cross and 64 embryos from seven C57Bl6Sw *Msx1*$^{+/-}$ crosses.

Embryos and embryonic tissues

To obtain age-defined embryos, female mice 12-24 weeks old were housed individually overnight with an adult male mouse. The next morning (defined as day 0.5 of gestation), the females were removed and examined for the presence of a vaginal plug. Pregnant mice were sacrificed by cervical dislocation at 18.5 days of gestation and the fetuses were surgically excised and examined with a Nikon SM2660 stereomicroscope (USA) to certify the developmental stage according to Kaufmann & Bard [15]. The fetuses were subsequently washed in BSS (NaCl, in 138 mM; KCl, in 5 mM; $CaCl_2$, in 0.08 mM; $MgSO_4$, in 0.63 mM; Na_2HPO_4, in 3 mM and KH_2PO_4, in 1 mM) and a fragment of the tail of each fetus was excised to determine the genotype. The fetuses were then decapitated and blood was collected in microcapillary tubes washed with 10% EDTA to prevent coagulation. Liver, spleen, thymus and bones (tibiae and femurs) were dissected from each embryo and used as described below.

Isolation of cells and clonogenic assays

Fetal livers were disrupted with a 1000 µL pipette (Eppendorf, Germany) and then filtered through a 40µm mesh filter. Bone marrow cells were obtained by homogenizing the bones and then filtering the suspension.

Aliquots of filtered cells (2.5 x 10^4 fetal liver cells and 5 x 10^4 fetal bone marrow cells) were plated in triplicate in 35-mm tissue culture plates in Methocult M3434 (StemCell Technologies, Canada) containing 1% methylcellulose, SCF (50 ng/mL), IL-3 (10 ng/mL), IL-6 (10 ng/mL) and Epo (3 U/ml). Colony formation was assessed 7-10 days later.

Blood cell analysis

Blood samples were diluted 1:200 in BSS containing 10% EDTA and the cell viability was determined using a hemacytometer (American Optical Corporation, USA). Blood smears prepared from undiluted blood samples were stained with hematoxylin-eosin (HE) and the cells were counted using a Nikon Eclipse E600 microscope (Japan). Differential counts were done based on the characteristic morphology of each blood cell type when stained with HE. The relative proportion of lymphocytes, monocytes and granulocytes was determined by counting all cells until 100 lymphocytes had been counted.

Histological analysis

Liver, spleen, thymus and bones (femurs) were fixed in a buffered 4% formol-saline solution for 72 h before embedding in paraffin. Sections 4 µm thick were stained with HE or Lennert's Giemsa and examined by light microscopy.

Embryonic genotyping

For DNA extraction, a tail fragment from each fetus was lysed in 20 mM Tris-EDTA pH 8.0, 50 mM KCl, 0.1% SDS and 0.1 ng of proteinase K/mL (Gibco BRL, USA) and digested overnight at 56ºC. DNA was prepared by phenol-chloroform extraction and ethanol precipitation, as described elsewhere [7], and redissolved in ultrapure water. PCR was done in a thermocycler (Hybaid Omn-E, UK) using 3 mM $MgCl_2$, 200 µM dNTP mix, 1.25 U *Taq* DNA polymerase (Gibco BRL, USA), 5-10 ng of DNA and 0.4 µM each oligonucleotide [11]. Three oligonucleotides were used: one sense primer *Msx1* D730-5'-

GCG GAA TTC TCC AGC TCG CTC AGC CTC ACC-3' and two antisense primers, *Msx1*-3'-TGC AGG ACC GCC AAG AGG AAA AGA GAG GCC-5' and *lacZ*-3'-GGC AAA GCG CCA TTC GCC ATT CAG GC-5'. This set of primers allowed the amplification of the wild-type (177 bp fragment) and mutant (250 bp fragment) alleles. The PCR products were analyzed in 1.5% agarose gels containing 0.5 µg/mL of ethidium bromide.

PCR conditions were: one initial cycle of 3 min at 94°C and 5 min at 80°C; 40 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C; 1 cycle of 10 min at 72°C.

Statistical analysis

The results were expressed as the mean ± S.E.M. The blood cell counts and clonogenic assay results were analyzed using the non-parametric Kruskal-Wallis test, with a value of $p<0.05$ indicating significance.

RESULTS

Confirmation of mutant embryos by PCR

Msx1 mutant embryos were obtained from crosses between two heterozygous mice of the same colony and were genotyped by PCR. Figure 1 shows the characteristic eletrophoretic profile of PCR products in which wild-type embryos had a 177 bp fragment that corresponded to the *Msx1* wild-type allele, and homozygous mutant embryos showed a 250 bp fragment that corresponded to the *Msx1* mutant allele. Heterozygous embryos had both fragments since they contained the wild-type and mutant *Msx1* alleles. All of the embryos used in this work are listed in Table 1.

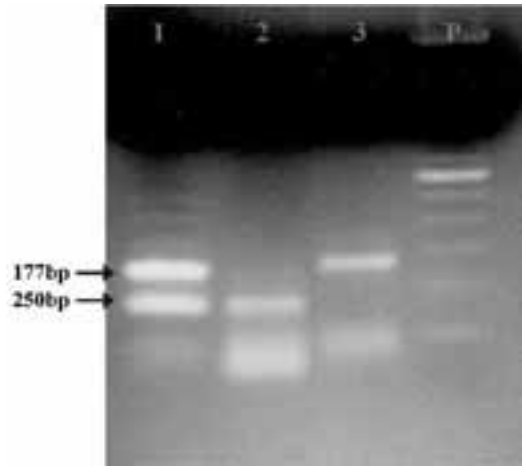

Figure 1. Analysis of PCR products in 1.5% agarose gels containing ethidium bromide (0.5 µg/mL). The DNA used was from crosses between *Msx1* heterozygous null mice. **1** – two fragments (177 bp and 250 bp) from heterozygous embryo DNA, **2** – a 177 bp fragment from wild type fetus DNA, **3** – a 250 bp fragment from homozygous embryo DNA, **P** –100 bp DNA ladder (GIBCO/BRL, USA).

Number of hematopoietic cells in mutant mice

Figure 2 shows that there were no significant differences in the total (Fig. 2A) and differential (Fig. 2B) counts of hematopoietic cells in the peripheral blood of fetuses from the three groups of mice studied.

Homozygous mutant mice had fewer differentiated hematopoietic cells and a higher number of precursor cells in the spleen

Histological analysis of the spleen from embryos in all groups showed the same morphological appearance that included various hematopoietic cell types such as macrophages, megakaryocytes, plasma cells, lymphocytes and granulocytes. However, the massive presence of erythrocytes seen in the spleen parenchyma of wild-type embryos was considerably reduced in mutant embryos. A further difference between wild-type and knockout embryos was the

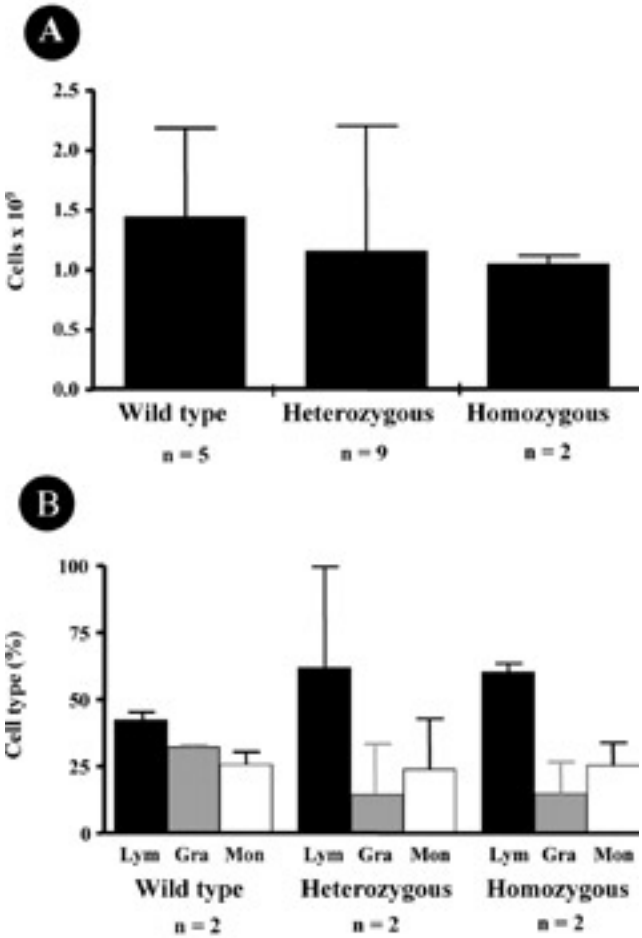

Figure 2. Total blood cell counts (**A**) and differential counts of mononuclear cells (**B**) in peripheral blood from 18.5 dpc embryos. The columns are the mean ± S.E.M. of the number of embryos examined. There were no significant differences among the groups.

Table 1. Offspring obtained from crosses between heterozygous mutant mice. All of the mice were used in one or more assays.

Crosses	Wild-type embryos	Heterozygous embryos	Homozygous embryos	Total
1 C57Bl6SW*Msx1* x C57Bl6SW*Msx1*	3	5	0	8
2 C57Bl6*Msx1* x C57Bl6*Msx1*	1	2	0	3
3 C57Bl6SW*Msx1* x C57Bl6SW*Msx1*	5	8	1	14
4 C57Bl6SW*Msx1* x C57Bl6SW*Msx1*	3	4	1	8
5 C57Bl6SW*Msx1* x C57Bl6SW*Msx1*	0	1	1	2
6 C57Bl6SW*Msx1* x C57Bl6SW*Msx1*	0	0	3	3
7 C57Bl6SW*Msx1* x C57Bl6SW*Msx1*	1	4	1	6
8 C57Bl6SW*Msx1* x C57Bl6SW*Msx1*	4	4	1	9
9 C57Bl6SW*Msx1* x C57Bl6SW*Msx1*	3	5	3	11

presence of more precursor cells in spleens from mutant mice (Fig. 3).

In contrast to the spleen, there were no morphological differences between the thymic tissue of wild-type and mutant embryos (Fig. 4). This finding suggested the *Msx1* gene was not related to T cell development.

Hepatic erythroid progenitors in mutant mice

Figure 5A shows that there were no significant differences in the numbers of progenitor cells from the livers of wild-type and mutant (cross 3 C57Bl6Sw*Msx1*$^{+/-}$) mice. Similarly, histological analysis of tissue sections stained with HE showed no marked difference between wild-type and mutant fetuses (Fig. 4).

Alterations in the bone marrow of mutant mice

As with liver, there were no significant differences in the numbers of erythroid progenitors in bone marrow from mutant and wild-type mice, although the levels in *Msx1* mutants tended to be higher (Fig. 5B). Histological analysis of the bone marrow revealed marked morphological differences among embryos expressing different levels of *Msx1*. In wild-type embryos, the bone marrow consisted of two compartments: 1) a red compartment containing hematopoietic progenitors and more mature hematopoietic cells that formed numerous erythopoietic centers in contact with a loose extracellular matrix, and 2) a yellow compartment with mesenchymal progenitors and some adipocytes and stromal cells that supported the hematopoietic development in bone marrow (Fig. 6A,B and G). In heterozygous mutants in which a copy of the wild-type gene was preserved, no mesenchymal component was observed but the extracellular matrix had expanded and erythropoietic foci were evident (Fig. 6C,D). In homozygous null embryos, the changes were more dramatic, with the loss of at least half of the cellular component, including erythropoietic foci (Fig. 6E,F and H).

DISCUSSION

The *Msx1* gene codes for a transcription factor, the fundamental role of which is maintaining progenitor cells in a proliferating and undifferentiated state. *Msx1* is expressed at several sites of the mouse embryos, including limb buds, craniofacial bones, teeth and somites; in adult mice, this expression is detected only in cells capable of self-renewal such as mammary, uterine and basal dermal epithelium [8,26,27]. However, the phenotype of *Msx1* null mice is restricted to defects in craniofacial development. These alterations are not sufficient to explain the role of *Msx1* at several sites of expression or the neonatal lethality of homozygous null mice. The absence of defects at some sites of *Msx1* expression could be related to *Msx2* functional redundancy, although an analysis of *Msx1/Msx2* double knockout mice did

not confirm this hypothesis [3]. So far, there has been no detailed investigation in *Msx1* knockout mice to determine the causes of premature death in null mutants.

The mechanisms of hematopoietic commitment and differentiation in mice have been extensively studied and several molecular and cellular aspects have been identified. However, little is known about the molecular regulation of the transition of the mesodermal cells to cells committed to hematopoietic lineages. Soluble factors such as fibroblast growth factors (FGFs) and bone morphogenetic protein-4 (BMP4) induce commitment of the ventral mesoderm to hematopoiesis. These factors also activate *Msx* gene expression at many sites of epithelium-mesenchymal interaction, such as in dental buds.

Since hematopoietic tissue is derived from the ventral mesodermal layer and is induced by BMP4, we examined the relationship between *Msx1* and hematopoiesis. We used *Msx1* knockout mice to assess possible hematopoietic defects in the absence of functional Msx1 protein. Various hematopoietic

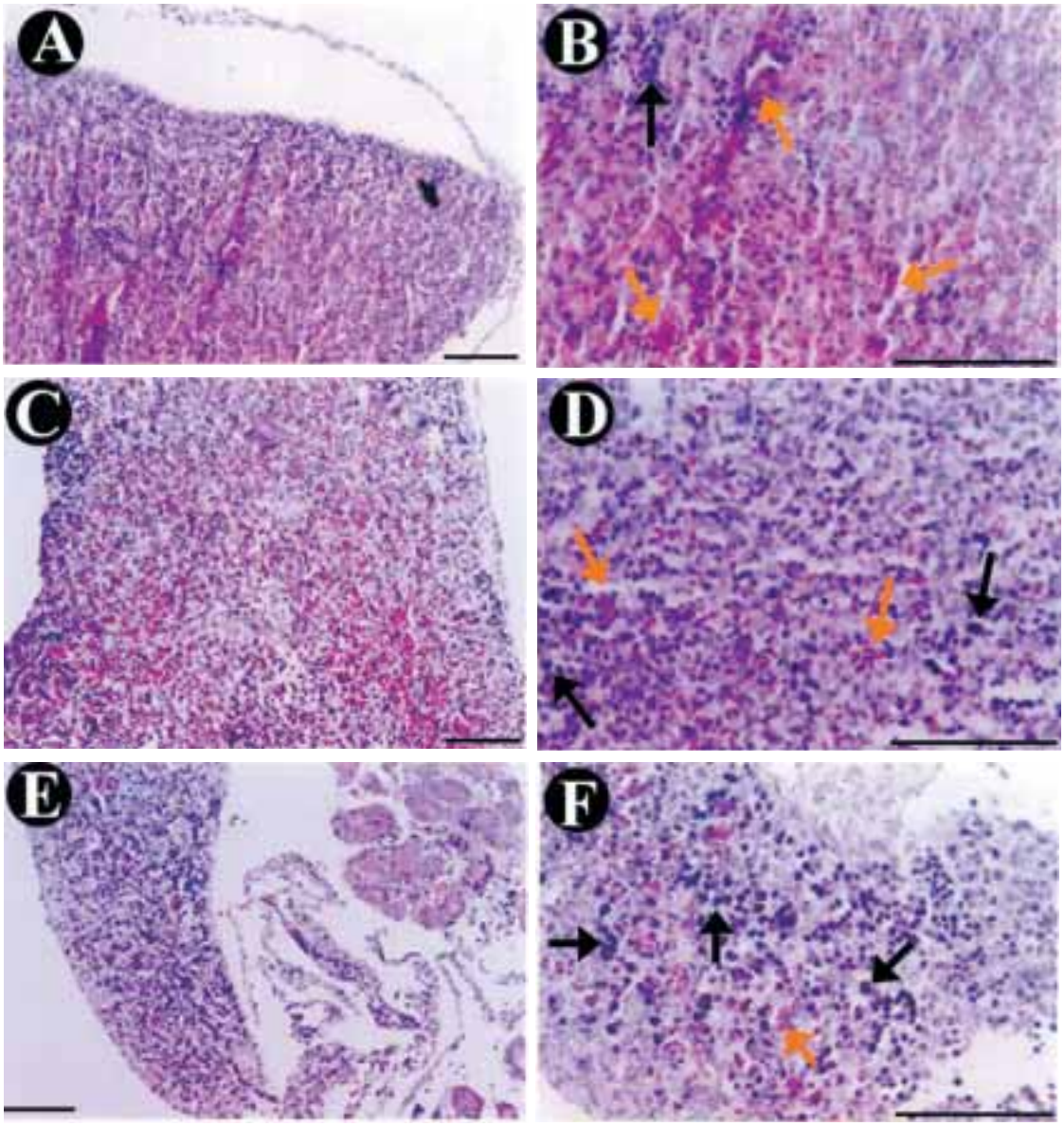

Figure 3. Histological analysis of spleen from 18.5 dpc embryos, **A** and **B** – wild type embryos, **C** and **D** – *Msx1* heterozygous embryos, **E** and **F** – *Msx1* homozygous embryos Note the massive presence of putative erythrocytes (red, anucleated cells) in wild type embryos compared to the low number in mutant embryos. Note also the presence of more precursor cells (probably hemopoietic) in the spleen parenchyma of mutant mice. Orange arrows – putative erythrocytes and black arrows – putative hemopoietic precursors. Bars = 100 μm.

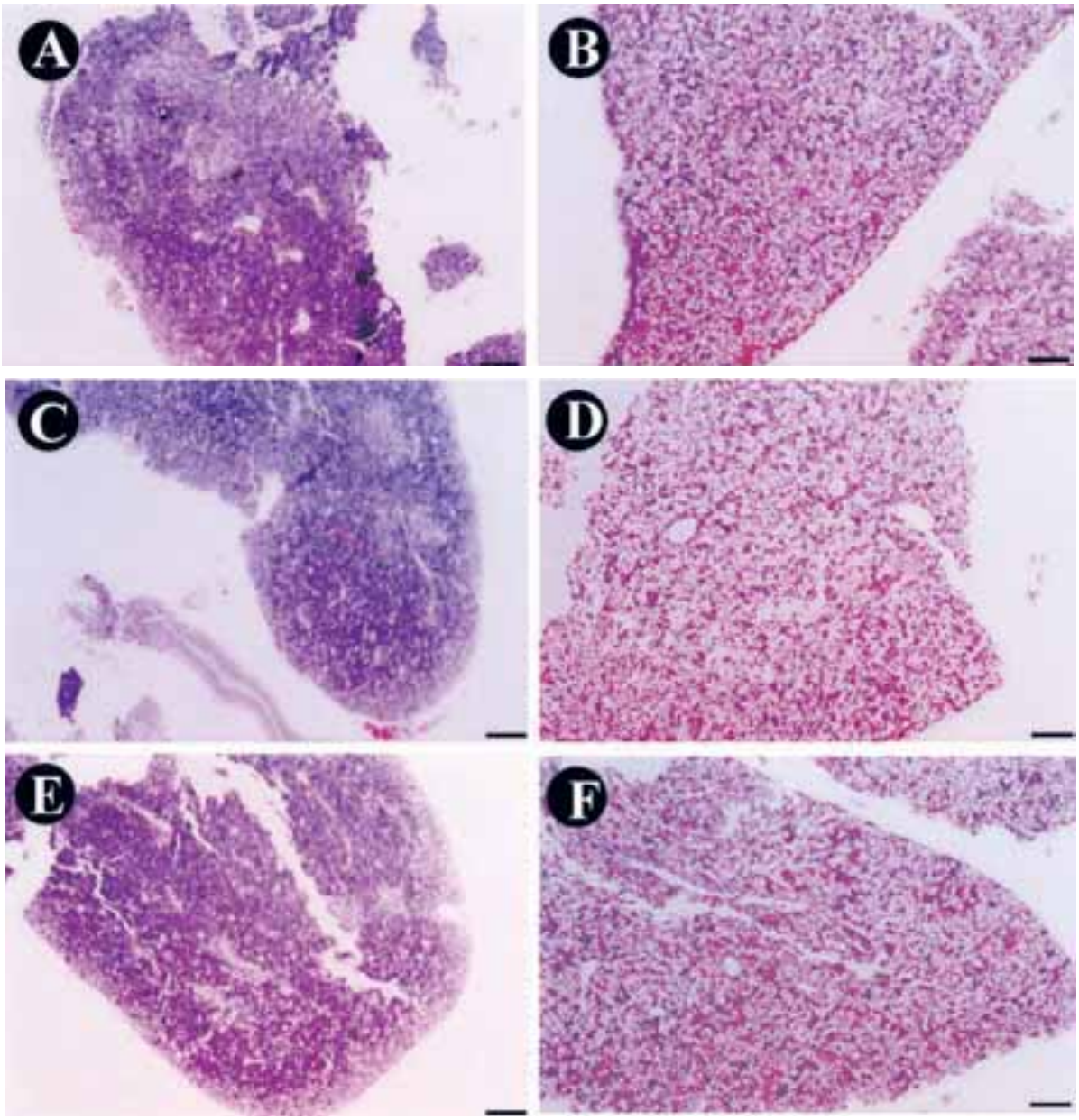

Figure 4. Histological analysis of thymus (**A**, **B** and **C**) and liver (**D**, **E** and **F**) from 18.5 dpc embryos, **A** and **D** – wild type embryos, **B** and **E** – *Msx1* heterozygous embryos, **C** and **F** – *Msx1* homozygous embryos. Bar = 100 μm.

tissues of *Msx1* null mice (spleen, thymus, liver and bone marrow, as well as peripheral blood) were examined histologically and using hematopoietic progenitor cell assays *in vitro*. Because neonatal homozygous mice die within 24 h of birth, this study was done using 18.5 dpc embryos, when all of the tissues analyzed had already been colonized by hematopoietic stem cells and progenitors.

The mouse spleen develops from the mesenchyme in the dorsal mesogastrium at day 13 of gestation. The mesenchyme forms the structural network of the spleen that is later populated by stem cells from the aortha-gonad-mesonephrons (AGM) region and from the liver by day 15. The spleen shows erythropoiesis and myelopoiesis and is the first fetal site of myelopoiesis [15]. Here, histological analysis of spleen revelead a lower number of erythroid cells and a higher number of progenitor (probably hematopoietic) cells in the parenchyma of mutant mice. These progenitor cells may contain a defect that prevents them from maturing and/or differentiating into erythrocytes.

The thymus is the primary lymphoid organ for the development of T cells of the αβTCR (αβ T-cell receptor) lineage. The thymic primordium of mice arises bilaterally by fusion of the third pouch endoderm with ectoderm from the corresponding branchial clefts on day 10 of gestation [1]. By day E11.5, hemopoietic precursor cells seed the thymus anlage although its epithelial cells are not yet competent to fully support T cell development [1] The first signs of thymocyte differentiation appear by E13.5, although the first mature CD4 and CD8 double and single positive thymocytes do not appear until E17 [24,25]. Morphological analysis is insufficient for evaluating thymic development, as also shown here since we detected no histological differences between mutant and wild-type embryos. Preliminary results based on the analysis of double positive cells by FACS (fluorescence activated cell sorter) suggest that T cell development in mutant embryos is unaffected by a lack of Msx1 (data not shown), although this remain to be confirmed.

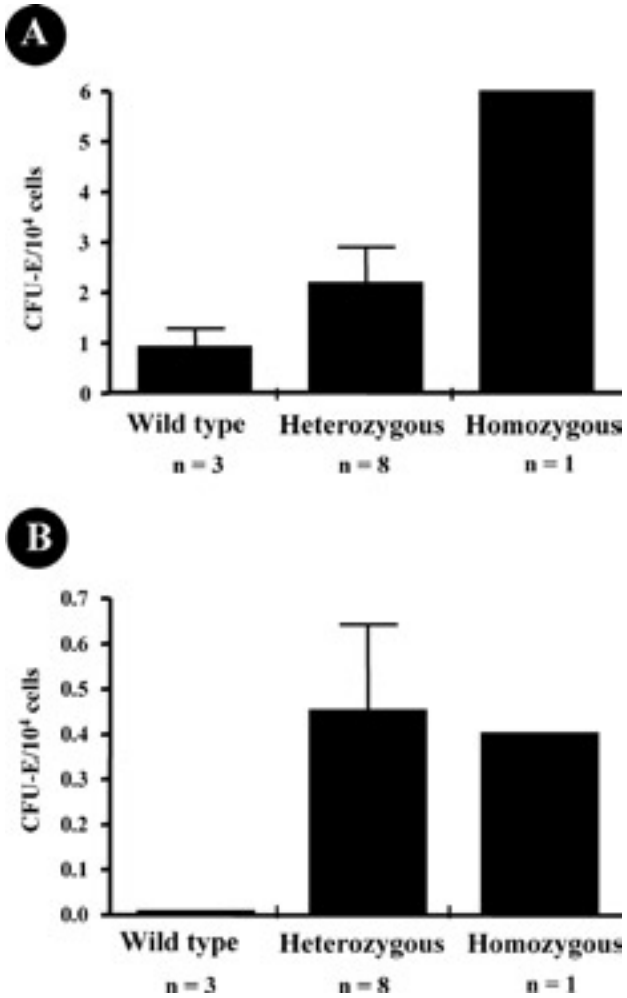

Figure 5. Erythroid progenitor cells in the liver (**A**) and bone marrow (**B**) of 18.5 dpc embryos. The columns (except for homozygous embryos) are the mean ± S.E.M. of the number of embryos examined. There were no significant differences among the groups.

The mouse liver develops from the hepatic diverticulum at the foregut-midgut junction of the primitive gut tract on day 8 of gestation. Thereafter, the parenchymal tissue is invaded by large numbers of venous channels to form the hepatic sinusoids and the liver rapidly enlarges as it replaces the yolk sac as the main source of hematopoietic activity [35]. *Msx1* knockout embryo liver showed an apparently normal morphology in histological analysis when compared to wild type embryos. Also, in clonogenic assays no significant differences were observed in the number of erythroid progenitor cells from livers of wild type and mutant embryos.

Otherwise, the bone marrow results obtained in this work were unexpected. Bone marrow is the main hematopoietic organ in adult life and the last one to be colonized during development. Hematopoietic stem cells and progenitor cells migrate from the liver to bone marrow as birth approaches. The bone marrow of fetal pups immediately prior to birth contains very few CFUs [37]. Hence, the important morphological and structural differences seen in the bone marrow of 18.5 dpc mutant embryos compared to wild-type embryos were unexpected. While in wild-type fetuses the bone marrow was organized into two distinct compartments (mesenchymal and hematopoietic), this organization was absent in mutant mice. The bone marrow stroma consists of several cell types (fibroblasts, endothelial cells, adipocytes, and macrophages) with a mesenchymal origin. These cells are responsible for producing extracellular matrix and hematopoietic growth factors. This environment is essential for the growth and differentiation of hematopoietic cell precursors. Despite the lack of mesenchymal tissue in heterozygous mutant embryos, the hematopoietic compartment expanded throughout the bone marrow to produce reticular cells and hematopoietic precursors. In homozygous mutant embryos, the hematopoietic compartment was also expanded but was much more disorganized and less dense.

The results of this study indicate that *Msx1* deficient mice have an enhanced ethropoietic activity, with more progenitors in histological analysis of the

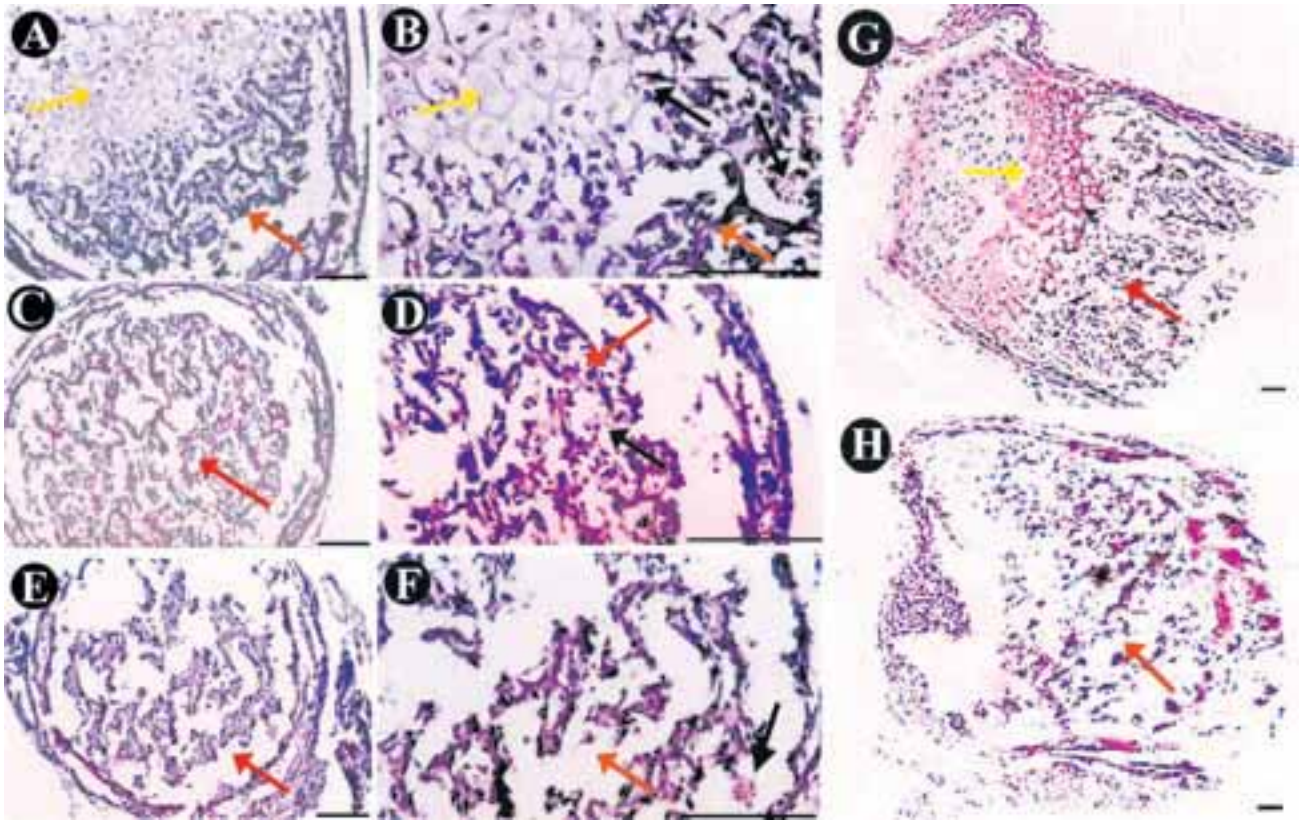

Figure 6. Histological analysis of transversal sections from the lower one-third of the femur from 18.5 dpc embryos: **A** and **B** – wild type embryos, **C** and **D** – *Msx1* heterozygous embryos, **E** and **F** – *Msx1* homozygous embryos (HE staining), **G** and **H** – longitudinal sections from the lower end of the femur from 18.5 dpc wild type and homozygous null embryos, respectively (Lennertz Giemsa staining). Red arrows – hematopoietic compartment, yellow arrows – mesenchymal compartment, black arrows – putative hematopoietic progenitors. Note the disappearence of mesenchymal cells in homozygous and heterozygous embryos, and the differences in the localization of erythropoietic foci in the bone marrow of mutant embryos. Bars = 100 µm.

spleen, while differentiated hematopoietic cells were present at lower levels in the spleen (diminished erythrocyte number) of the same embryos. These results suggest that *Msx1* has a role in the differentiation of erythroid progenitors, with a lack of Msx1 protein inhibiting erythrocyte differentiation and resulting in the accumulation of progenitor cells. The histological defects seen in the bone marrow of *Msx1* knockout mice also suggested a role for *Msx1* in the development of cells derived from the mesodermal layer. If this is true, then the *Msx1* gene also influences mesenchymal progenitor cells that are the precursors of bone marrow stromal cells.

This is the first study to demonstrate a role for the *Msx1* gene in the development of hematopoietic and bone marrow mesenchymal cells. The alterations in hematopoiesis in homozygous null mutants could contribute to the premature death seen in these mice.

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