Spatial and temporal patterns of c-kit-expressing cells in \( W^{lacZ/} + \) and \( W^{lacZ}/W^{lacZ} \) mouse embryos

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SUMMARY

In the mouse, the Kit receptor and its ligand, the stem cell factor (SCF), are encoded at the W/Kit and Steel loci, respectively. The Kit/SCF transduction pathway is involved in promoting cellular migration, proliferation and/or survival of melanoblasts, hematopoietic progenitors and primordial germ cells. Furthermore, a functional Kit/SCF pathway is required for the development of interstitial cells of Cajal (ICC) in the small intestine. Whereas all c-kit-expressing cells in embryogenesis were not identified, previous studies clearly demonstrated that the c-kit expression pattern extends well beyond cells known to be affected by W mutations. To investigate further Kit function, we specifically marked the c-kit-expressing cells and followed their fate during embryogenesis. A mutation was introduced by gene targeting at the W/Kit locus in mouse embryonic stem cells. The lacZ reporter gene was inserted into the first exon of c-kit, thus creating a null allele, called \( W^{lacZ} \).

The lacZ expression reflects normal expression of the c-kit gene in \( W^{lacZ/} + \) embryos. The comparison of the patterns of lacZ-expressing cells between \( W^{lacZ/} + \) and \( W^{lacZ}/W^{lacZ} \) embryos allowed us to detect where and when melanoblasts, primordial germ cells and hematopoietic progenitors failed to survive in the absence of Kit. We also observed that ICC express c-kit during embryogenesis. ICC are found identically in \( W^{lacZ/} + \) and \( W^{lacZ}/W^{lacZ} \) embryos. Therefore, ICC do not depend on Kit expression during embryogenesis. These results indicate that the function of the c-kit gene is only required for the postnatal development of the ICC.

Unexpected sites of c-kit expression were uncovered in embryos, including endothelial, epithelial and endocrine cells. None of these cells are dependent on Kit expression for their migration, proliferation and/or survival during embryogenesis. Nevertheless, we assume that the Kit/SCF pathway could be involved in the growth of transformed endothelial, epithelial and endocrine cells.

Key words: homologous recombination, ES cells, W locus, c-kit proto-oncogene, stem cell factor, receptor tyrosine kinase, mouse

INTRODUCTION

The c-kit proto-oncogene encodes Kit, the receptor tyrosine kinase for stem cell factor (SCF). In the mouse, c-kit is allelic with the W/Kit locus, and SCF is encoded at the Steel (Sl) locus (for review, see Galli et al., 1994; Witte, 1990). Because of the receptor-ligand relationship, both W and Sl mutant phenotypes are characterized by similar pleiotropic effects affecting three cellular lineages during embryogenesis. In the absence of Kit, migration, proliferation and/or survival are impaired in primordial germ cells (PGC) from E9.5 onwards, in melanoblasts from E11 onwards, and in hematopoietic precursors from E11-E12 onwards (Buehr et al., 1993; Mintz and Russell, 1957; Cable et al., 1995; Russell et al., 1968). As a result of the absence of hematopoietic erythroid cells, newborn mutant homozygotes die of anemia.

We hypothesized that anemia and the resulting death could mask the existence of other cellular lineages, which would be dependent on the Kit/SCF system. It is notable that northern and RNA in situ hybridization studies showed that c-kit and Steel are expressed in several other organs, such as the embryonic lung, kidney, gut and brain (Orr-Urtreger et al., 1990; Keshet et al., 1991; Matsui et al., 1990). Moreover, hybridization studies on W and Sl mutant embryos revealed that both c-kit and Steel are contiguously expressed in a wide variety of anatomical sites (Motro et al., 1991). The hypothesis that the Kit/SCF pathway may play a role at some of these sites is supported by the report that the interstitial cells of Cajal (ICC), considered to be responsible for the pacemaker activity of the intestine, express c-kit and are absent in the ileum of W/W° adult mice. Despite the absence of notable morphological abnormalities, these mice display irregular patterns of electrical activity in the small intestine (Huizinga et al., 1995).

To identify other Kit-dependent cellular lineages during embryogenesis, we generated a null W mutant mouse using gene targeting. In the \( W^{lacZ} \) mutation, the c-kit gene was inactivated and replaced by the Escherichia coli lacZ gene. A nuclear localisation signal sequence fused to the lacZ gene...
allowed an unambiguous blue staining of the nuclear membrane. As the lacZ expression in WlacZ/+ embryos recapitulates the known Kit expression, we compared the spatial and temporal expression patterns of the lacZ gene in WlacZ/+ and WlacZ/WlacZ embryos. The identity of Kit-positive cells was assessed, and sites of Kit expression were uncovered. Two types of β-galactosidase (β-gal)-positive cells were identified. (1) β-gal-positive cells of heterozygous embryos absent in homozygous mutants: among these cells, which depend on Kit for their migration, proliferation and survival, only melanoblasts, PGC and hematopoietic progenitors were identified. (2) β-gal-positive cells found at identical sites and stages of development in WlacZ/+ and WlacZ/WlacZ embryos; these Kit-expressing cells, including endothelial cells and various endocrine cells, do not require Kit function. Interestingly, ICC were present in WlacZ/WlacZ newborn mice and thus do not depend on Kit for their migration, proliferation and/or survival during embryogenesis.

MATERIALS AND METHODS

Construction of the targeting replacement vector pGNΔ-kit

The pGNA-Kit plasmid targeting vector was prepared from 129/Sv strain mouse DNA. To construct the plasmid, a single ligation involving three restriction fragments was conducted with (1) the 3486 bp Sall-Ixho fragment (position 2497-5983) from pGNA vector (Le Mouellec et al., 1990); (2) the 5.5 kb BglII-Xbal fragment from pPROKkitlacZ, containing successively 1.2 kb of c-kit flanking sequences together with the 5′-untranslated region and the first 18 coding nucleotides of c-kit exon 1, the E. coli lacZ gene containing the SV40 nuclear localisation signal (nis), the neo gene and a SV40 polyadenylation site (De Sepulveda et al., 1995), after the addition of Xhol and Kpnl linkers at Xbal and BglII sites respectively; and (3) the 6.1 kb SalII-Kpnl fragment from cosmid pCOS.F, corresponding to the c-kit 3′ flanking sequences and containing the 5′ end of the c-kit first intron after filling-in the internal NotI site (De Sepulveda et al., 1995).

Embryonic stem cell culture, detection of homologous recombinants and generation of chimeric mice

A new line of embryonic stem (ES) cells, CK35, was established from a 129/Sv male embryo. CK35 ES cells were cultured on feeder layers of G418 embryonic fibroblasts with ES medium. The NotI linearized pGNA-Kit plasmid was electroporated as previously described (Colucci-Guyon et al., 1994). DNA from surviving colonies was digested with EcoRI, and screened by Southern blotting using a 300 bp Ahal probe. Homologous recombinant clones contained a wild-type band (9.1 kb) and a mutant band (6.4 kb). Correct targeting was confirmed following a EcoRI/EcoRV digestion, which gave a wild-type (9.1 kb) band and a mutant (4 kb) one. Germ line transmission of the mutation was determined by Southern blot analysis.

RNA extraction and RT-PCR

Total RNA was extracted from livers of E15.5 and E16.5 embryos. c-kit mRNA was amplified using specific oligonucleotides K3 and K6 (Reith et al., 1990). The RT-PCR reaction was done as previously described (De Sepulveda et al., 1994).

Histology, β-gal activity detection and histochemical detection of PGC

 Noon of the day of the plug was considered E0.5. Post-implantation embryos were recovered, rinsed in cold phosphate-buffered saline (PBS), fixed for 30 to 90 minutes, depending on their developmental stage, in PBS containing 4% paraformaldehyde (PFA) at 4°C. The embryos were rinsed twice in PBS and stained overnight in X-Gal buffer (0.4 mg/ml 5-bromo-4-chloro-3-indolyl-D-galactoside, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide and 4 mM MgCl2 in PBS) at 32°C. Early stage embryos (E7-E10), once postfixed in 4% PFA for at least two days, were first embedded in gelose and then in paraffin. For more advanced stages (from E11 onwards), embryos were frozen in Tissue Tek (OCT Medium, Miles Scientific), cryostat-sectioned, and mounted onto microscope slides (SuperFrost Plus, Fisher) as described previously (Kress et al., 1990). The embryo sections were stained for β-gal, counterstained with neutral red and mounted. After staining for β-gal, some slides were processed to detect the presence of intracellular alkaline phosphatase activity (Zambrowicz et al., 1994). The appropriate reagents were supplied by Sigma, and the sections counterstained with Mayer’s haematoxylin.

Immunofluorescence and immunohistochemistry

The primary antibodies used were the rat anti-Kit (ACK2) monoclonal antibody (Ogawa et al., 1991), a rabbit anti-β-gal (Cappel), and the rabbit αPEP8 (TRP-2) polyclonal antibody (Tsukamoto et al., 1992). The secondary antibodies were used as a goat anti-rat IgG-FITC, anti-rabbit IgG-biotin or -TRITC conjugates (Sigma). Kit and TRP-2 were detected as previously described (Ogawa et al., 1991; Pavan and Tilghman, 1994). For the immunodetection of the β-gal, the primary antibody was diluted at 1/1000 in PBS, and the slides were then processed as described for TRP-2.

RESULTS

Generation of a new null mutation at the W/Kit locus

To disrupt the c-kit gene, we used a replacement-type targeting vector, which contained successively 1.2 kb of 5′ homology region extending from the upstream region of c-kit to the first exon, a nlslacZ-neo expression cassette, and 6.1 kb of 3′ homology region corresponding to the proximal part of c-kit first intron. Homologous recombination resulted in a deletion removing codons 7 to 22 and 200 pb of the first intron, and their replacement by the coding region of nlslacZ and the neo gene (Fig. 1). The lacZ sequence was inserted in frame with the first six codons of c-kit. The CK35 ES cell was electroporated with the targeting vector. Five independent homologous recombinant clones were identified by Southern blotting, giving a frequency of homologous recombination of 2.5% per G418-resistant colonies. Four chimaeric males, obtained from two independent clones, transmitted the disrupted allele when mated with either C57BL/6 or 129/Sv females. The new mutation at the W/Kit locus was called WlacZ.

129/Sv WlacZ/+ mice appeared healthy. They had white feet and a white tail tip. When heterozygotes were intercrossed, about 25% of progeny carried two copies of the novel 6.4 kb EcoRI targeted fragment (Fig. 2). These homozygous newborn mice were pale and rapidly died of a severe macrocytic and hyperchromic anemia. To ensure that the targeting event produced a truly null phenotype, WlacZ/WlacZ embryos were examined for both residual mRNA and protein. Neither c-kit mRNA nor Kit were detected (data not shown). Thus, as predicted, the introduced cassette prevents c-kit expression. We have therefore generated a null allele, WlacZ, at the W/Kit locus.

lacZ expression in known target cells of W mutations

lacZ expression pattern was studied in +/+ , WlacZ/+ and
c-kit targeting scheme. A partial map of a mouse genomic DNA fragment containing c-kit exon 1 is shown. Restriction enzymes: B, BamHI; EI, EcoRI; EV, EcoRV; K, KpnI. The targeting vector, pGNAKit, includes the nlslacZ-neo cassette flanked by a 1.2 kb fragment located 5' of exon 1 and a 6.1 kb fragment of 3' homology. The structure of the targeted allele is indicated, with the c-kit exon 1 replaced by the cassette nlslacZ-neo. The probe used for Southern blots is shown below the map of the targeted allele.

Wild type c-kit allele

Targeting vector pGN<sub>+</sub>-Kit

Targeted c-kit allele

**Fig. 1.** c-kit targeting scheme. A partial map of a mouse genomic DNA fragment containing c-kit exon 1 is shown. Restriction enzymes: B, BamHI; EI, EcoRI; EV, EcoRV; K, KpnI. The targeting vector, pGNAKit, includes the nlslacZ-neo cassette flanked by a 1.2 kb fragment located 5' of exon 1 and a 6.1 kb fragment of 3' homology. The structure of the targeted allele is indicated, with the c-kit exon 1 replaced by the cassette nlslacZ-neo. The probe used for Southern blots is shown below the map of the targeted allele.

**Neural-crest-derived melanocytes**

To test that lacZ expression in the skin was identifying melanocytes, we first examined the histochemical staining for β-gal activity in neonatal skin of W<sup>lacZ</sup>/+ and W<sup>lacZ</sup>/W<sup>lacZ</sup> newborn mice. In the heterozygous mice, melanocytes were found in hair follicles, in the dermis and in the basal layer of the epithelium; they displayed high β-gal activity (Fig. 3A). The cells were also stained with both anti-Kit and anti-β-gal antibodies. Their identity as melanocytes was confirmed by TRP-2 expression (data not shown). TRP-2, a melanogenic enzyme with DOPAchrome autotransferase activity, is a melanoblast marker (Tsukamoto et al., 1992; Pavan and Tilghman, 1994). Furthermore, melanocytes were absent from the skin of W<sup>lacZ</sup>/W<sup>lacZ</sup> newborn mice (Fig. 3B).

We therefore looked for lacZ expression in melanoblasts of W<sup>lacZ</sup>/+ and W<sup>lacZ</sup>/W<sup>lacZ</sup> embryos. During embryogenesis, melanoblasts were identified on the basis of their location, close association with endothelial cells. From E11 onwards, melanoblasts appeared to migrate in the mesenchyme. At E10.5, rare clusters of three to five melanoblasts were detected in the head near the pineal bud and the nasal pit. Few isolated melanoblasts were found closely associated with endothelial cells. From E11 onwards, melanoblasts were absent from the surface ectoderm of W<sup>lacZ</sup>/W<sup>lacZ</sup> embryos (see in Fig. 3F), and no β-gal-positive cells were ever observed in the trunk section of W<sup>lacZ</sup>/W<sup>lacZ</sup> embryos.

These data specify that both the migration and survival of melanoblasts are severely impaired from E15.5 onwards, and they allow melanoblasts to colonize the distal parts of the limb buds and of the tail tip, resulting in the dominant phenotype observed in W<sup>lacZ</sup>/+ adults. From E16 onwards, melanoblasts began to colonize the hair follicles, and as a consequence the surface of embryos became unstained.

In W<sup>lacZ</sup>/W<sup>lacZ</sup> embryos, from E9.5 to E13.5, β-gal-positive cells were still found on the dorsal part of the neural tube (Fig. 3E). The consequence of the absence of Kit on melanoblasts was observed as early as E10.5, i.e. when melanoblasts begin to migrate in the mesenchyme. At E10.5, rare clusters of three to five melanoblasts were detected in the head near the pinal bud and the nasal pit. Few isolated melanoblasts were found closely associated with endothelial cells. From E11 onwards, melanoblasts were absent from the surface ectoderm of W<sup>lacZ</sup>/W<sup>lacZ</sup> embryos (see in Fig. 3F), and no β-gal-positive cells were ever observed in the trunk section of W<sup>lacZ</sup>/W<sup>lacZ</sup> embryos.

These data specify that both the migration and survival of melanoblasts are severely impaired from E15.5 onwards, lacking one functional allele of c-kit, and from E10.5 onwards, in the null mutant embryos. Furthermore, we suggest that melanoblast migration begins at E10.5 and consists of a single and continuous wave, impaired by Kit absence.

**Germ cell lineage**

As Kit is expressed in germ cells of neonatal ovaries, we first examined the lacZ expression patterns in ovaries of W<sup>lacZ</sup>/+
were found in ovaries of W lacZ in the gonadal ridges of b staining with anti-AGM (for aorta, gonad and mesonephros), in the dorsal aorta and uro-genital ridges, the so-called intraembryonic mesodermal region, including the and later in the fetal liver. However, additional place in two embryonic sites: first in the yolk sac Mammalian hematopoiesis was believed to take onwards.

We looked for β-gal activity in PGC of WlacZ/ and WlacZ/WlacZ embryos. During embryogenesis, PGC were identified on the basis of their location and intracellular alkaline phosphatase enzyme activity (Manova et al., 1990; Buehr et al., 1993; Mintz and Russell, 1957). These cells were also detected with the anti-Kit and anti-β-gal antibodies. Finally, alkaline phosphatase-positive cells showed β-gal activity. Before E9.5, no difference in the PGC distribution was observed between WlacZ/+ and WlacZ/WlacZ embryos. At E9.5, PGC migrated differently, depending on the presence or absence of Kit. β-gal-positive PGC were mostly seen dispersed in the wall of the gut in WlacZ/+ embryos. In contrast, β-gal-positive PGC were focused in the ventral half of the gut in WlacZ/WlacZ embryos (Fig. 4C), and were sometimes grouped in clumps located at ectopic sites such as inside the vitelline artery (data not shown). These clumps were never found in WlacZ/+ embryos. From E10.5 onwards, a marked diminution of β-gal activity of PGC was observed in WlacZ/+ and WlacZ/WlacZ embryos. Hence, the migration of PGC towards the gonadal ridges was difficult to follow on histological sections. From E11.5 to E12.5 the PGC reach the gonadal ridges. At E12.5 a very faint labelling, following histochemical staining for β-gal activity or immunostaining with anti-β-gal antibodies, was detected in the gonadal ridges of WlacZ/+ embryos. This faint expression was not found in homozygous mutant embryos.

Differences in the expression level of the lacZ gene product and the endogenous c-kit gene were detected in PGC from E10.5 to E13.5. β-gal activity was weak in PGC, in contrast with the strong expression of the endogenous c-kit gene, as determined after immunofluorescence staining with the anti-Kit antibody. The deviation in the level of expression of β-gal from that of the endogenous Kit protein might derive from the deletion of a regulatory region from the targeting construct.

Altogether, our results confirm that in the absence of Kit, migration and survival of PGC are impaired in WlacZ/WlacZ embryos from E9.5 onwards.

Hematopoiesis

Mammalian hematopoiesis was believed to take place in two embryonic sites: first in the yolk sac and later in the fetal liver. However, additional hematopoietic activity was recently located in the intraembryonic mesodermal region, including the dorsal aorta and uro-genital ridges, the so-called AGM (for aorta, gonad and mesonephros), in the E8 to E11.5 mouse embryos (Godin et al., 1995; Dzierzak and Medvinsky, 1995). As Kit is expressed on fetal and adult murine hematopoietic cells (Ogawa et al., 1993), we looked for lacZ expression in WlacZ/+ and WlacZ/WlacZ embryos in the three sites where an hematopoietic stem cell activity had been found. Immunoreactions using either anti-Kit or anti-β-gal antibodies stained the same cells (data not shown).

From E7.5 to E9.5, β-gal-positive cells were detected in the blood islands of the yolk sac of WlacZ/+ and WlacZ/WlacZ embryos (Fig. 5A). From E9.5 to E13.5, we observed β-gal-positive cells in the AGM of both heterozygous and homozygous mutant embryos. The number of β-gal-positive cells in

Fig. 3. LacZ expression in neural-crest-derived melanocytes. (A) WlacZ/+, newborn mice. Head section: β-gal activity in hair follicles is restricted to melanocytes with a pigmented cytoplasm. In the dermis, β-gal-positive cells probably include melanoblasts and mast cells, the latter first appearing at this site between E15 and E16. (B) WlacZ/WlacZ, newborn mice. A similar section showing the absence of melanocytes and mast cells within the skin. (C) WlacZ/+ embryo, E11.5. In situ detection of β-gal activity: the arrows indicate melanoblasts, visualized as blue cells distributed in the mesenchyme and surface ectoderm. (D) WlacZ/+ embryo, E12.5. Melanoblasts (blue) are present in the surface ectoderm and underlying mesenchyme of the pinna. (E) WlacZ/WlacZ embryo, E9.5. Whole-mount staining showing the β-gal-positive cells along the dorsal midline of the neural tube (white arrow). (F) WlacZ/WlacZ embryo, E12.5. Melanoblasts are absent from the section of the pinna. Scale bars: A, B, D and F, 30 μm; C, 0.35 mm; E, 0.25 mm.
the AGM increased from E9.5 to E11.5 and dropped at E12.5. The cells were observed inside the aortic wall of the dorsal aorta and in the umbilical arteries. Cells were mostly distributed on the ventral part of the aorta (Fig. 5B). As exemplified in Fig. 5C, β-gal-positive cells seemed to cross the ventral aortic endothelium and directly enter the blood: these cells would probably seed the liver and the thymus. In the liver of \( \text{WlacZ}^{+/+} \) and \( \text{WlacZ}^{+/WlacZ} \) embryos, β-gal-positive cells were first detected at E10.5; their number increased until E11.5 (Fig. 5D), and decreased from E12 onwards in embryos of both genotypes. At E15.5, a difference was observed between the liver of the embryos depending on their genotype: the number of stained cells in the liver of \( \text{WlacZ}^{+/WlacZ} \) embryos was considerably lower than was found in the liver of \( \text{WlacZ}^{+/+} \) embryos. Finally at birth, the spleen of homozygous mutant mice lacked the β-gal-positive cells found in the spleen of heterozygotes and was threefold smaller.

Due to the \( \text{lacZ} \) expression in hematopoietic stem and committed cells, our study allowed the spatial and temporal visualization of the Kit-positive cells of the AGM, as well as the migration of these cells in the blood. The cells were detected in \( \text{WlacZ}^{+/WlacZ} \) embryos, indicating that \( c\text{-kit} \) is not required for the hematopoietic cells of the AGM.

**Interstitial cells of Cajal**

The interstitial cells of Cajal (ICC) are believed to be responsible for the electrical activity of the intestine muscle layers (Thuneberg, 1982; Langton et al., 1989). Recently, it was reported that ICC express \( c\text{-kit} \), and are dependent on its expression. Indeed, adult \( \text{W/W}^V \) mice lack the network of ICC, and their ileum fails to display any slow-wave-type action.

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**Fig. 4.** \( \text{LacZ} \) expression in the PGC. (A) \( \text{WlacZ}^{+/+} \), newborn mice. β-gal-positive oocytes are present in ovary. (B) \( \text{WlacZ}^{+/WlacZ} \), newborn mice. β-gal-positive cells are absent from ovary. (C) \( \text{WlacZ}^{+/WlacZ} \) embryo, E9.5. PGC, identified as alkaline phosphatase-positive cells, are visualized by a red cytoplasmic staining (arrows). β-gal-positive PGC are found in the ventral part of the hindgut (hg) endoderm. Scale bars: A and B, 30 μm; C, 15 μm.

**Fig. 5.** \( \text{LacZ} \) expression observed in the embryonic hematopoietic sites. (A) \( \text{WlacZ}^{+/WlacZ} \) embryo, E8.5. β-gal activity is present in the yolk sac. The expression was observed in the hematopoietic progenitors of the blood islands (arrow). (B) Sagittal section of a \( \text{WlacZ}^{+/WlacZ} \) embryo, E11.5. β-gal activity is observed in the AGM, and the activity is essentially concentrated in the ventral part of the aorta. On the sections of homozygous mutant embryos, no PGC, as detected by alkaline phosphatase activity, is observed at this site. (C) The same embryo, at a higher magnification. (D) \( \text{WlacZ}^{+/+} \) embryo, E11.5. β-gal activity in the liver. ao, aorta; l, liver. Scale bars: A, 25 μm; B, 120 μm; C, 30 μm; D, 10 μm.
We analyzed the Kit expression in the digestive tract of W lacZ/+ newborn mice. Kit-positive cells were found in the outer longitudinal and in the inner circular smooth muscle layers of the small intestine. The cells were elongated, and parallel to the axis of the intestine. Few of these surrounded the myenteric ganglia. Both anatomical criteria and Kit expression allowed us to identify these cells as ICC (Faussone-Pellegrini, 1985; Huizinga et al., 1995). A high β-gal immunoreactivity was also detected in the ovoid nucleus of ICC of the small intestine (Fig. 6A). Kit- and β-gal-positive ICC were found from the oesophagus to the anus. This difference in ICC number, depending on the digestive tract part, was observed as early as lacZ is expressed. Indeed, during embryogenesis, lacZ expression in ICC was detected from E12.5 and E13.5 onwards in all the digestive tract sections of W lacZ/+ embryos. Fig. 6C shows ICC in the stomach at E15.5. β-gal activity in ICC persisted until birth.

In W lacZ/W lacZ embryos, the ICC lacZ expression pattern was identical to the pattern observed in W lacZ/+ embryos, as exemplified on Fig. 6D in the colon at E16.5. Therefore, Kit function is not required for ICC migration, proliferation and/or survival during embryogenesis.

Taken together, these results indicated that during embryogenesis, (1) ICC express Kit and (2) ICC do not require c-kit for their migration, proliferation and/or survival.

**LacZ expression in lineages not known to be affected by W mutations**

The fidelity of the lacZ gene introduced into the c-kit gene was established in the W lacZ/+ embryos by the identity between the patterns obtained after histochemical staining for β-gal activity and immunostaining with the anti-Kit antibody (data not shown). Thus lacZ expression reflected normal expression of the c-kit gene.

Many cells with β-gal activity were found in W lacZ/W lacZ as well as in W lacZ/+ embryos, indicating that these cells do not require c-kit function during embryogenesis.

**Early development**

Faint β-gal activity first appeared at the late 2-cell stage, and persisted in some blastomeres at later stages. In the blastocyst, isolated β-gal-positive cells were observed in the trophectoderm and the inner cell mass (not shown). We searched for lacZ expression in the targeted ES cells. About 30% of the c-kit mutated ES cells showed a high β-gal activity.

**Skeleton**

Chondrocytes expressing the lacZ gene were detected for the first time at E13.5. More β-gal-positive chondrocytes were found from E15.5 onwards (see at E16.5 on Fig. 7A). In newborn mice, β-gal activity was clearly observed in mature caecum. By contrast, ICC were less numerous in the pyloric part of the stomach and scattered in the ileum. This difference in ICC number, depending on the digestive tract part, was observed as early as lacZ is expressed. Indeed, during embryogenesis, lacZ expression in ICC was detected from E12.5 and E13.5 onwards in all the digestive tract sections of W lacZ/+ embryos. Fig. 6C shows ICC in the stomach at E15.5. β-gal activity in ICC persisted until birth.

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and proliferating chondrocytes of few ossification centers. This activity was not detected in all the cells, and varied from faint to strong.

Tooth development

β-gal-positive cells were found in the primordia of the teeth bud from E16.5 to birth (not shown).

Brain and neural tube

Analysis of the developing brain showed an extensive staining, particularly in the cerebellum, hippocampus, choroid plexus and telencephalon. In the neural tube, β-gal activity was found in the dorsolateral marginal zone, and the level of lacZ expression was higher in dorsal than in ventral parts.

β-gal-positive cells were also found in several cranial ganglia. The trigeminal (V), facial (VII), cochlear (VIII), glossopharyngeal (IX) and vagal (X) ganglia contained many β-gal-positive cells from E9.5 to birth. β-gal-positive cells were observed at E9.5 in neural-crest-derived spinal ganglia, whose cells give rise to the sensory cells of dorsal root ganglia (DRG). Indeed, some β-gal-positive cells were found in DRG, from E11.5 to newborn animals, together with other DRG cells that were not stained (see at E15.5, on Fig. 7B).

Sensory organs

In the eye, β-gal-positive cells were found in two distinct locations from E14.5 onwards: in the lens and in the neural layer of retina, in both the inner and outer nuclear layers (see Fig. 7C at E16.5). In the ear, β-gal-positive cells were found in the first branchial membrane at E9.5. This labelling persisted at birth in the epithelium of the future tympanic membrane and in the pharyngo-tympanic (Eustachian) tube (not shown).

The respiratory system

β-gal activity was found in the endodermal lining of the respiratory tube. The epithelial cells of the olfactory epithelium were detected as expressing the lacZ gene from E10.5 onwards. After E15.5, a high expression level of the lacZ gene appeared in several other locations: in all the cells of the nasopharynx (Fig. 7D), in a few cells of the trachea and bronchi, and in most cells of the lungs. The lacZ expression pattern persisted until birth.

The digestive system

From E7.5 onwards, the foregut cells expressed the lacZ gene. At E10.5 β-gal activity appeared in the caudal half of the stomach epithelium and in the hind- and mid-gut epithelium. From E14.5 onwards, β-gal-positive cells were found in all sections of the gut epithelium. In newborn mice, β-gal activity was found in epithelial cells of each portion of the digestive tract. In the anterior part of the digestive system, β-gal-positive epithelial cells were seen in the bucco- and oro-pharynx, the larynx, the oral epithelium, the tongue and the oesophagus (Fig. 7E). In the stomach, most β-gal-positive cells were found in the pyloric half of the epithelium (see above, Fig. 6B). Many β-gal-positive cells were found in the intestine, but only scattered cells expressed β-gal activity in the rectum. Intestinal epithelial cells are a mixed population of enterocytes, Paneth cells, Goblet cells and neuroendocrine cells. Each of these cell types expressed the lacZ gene, although not all cells were stained.

β-gal activity was also observed in the endodermal lining of

Fig. 7. Analysis of lacZ expression patterns in various sites of WlacZ/WlacZ embryos.

(A) E16.5. β-gal activity (blue) in chondrocytes of a head bone. (B) E15.5. β-gal activity in dorsal root ganglia in the thoracic region. (C) E16.5. β-gal activity in the eye is detected in both the inner (arrow) and outer (arrowheads) nuclear layers of the retina. (D) E15.5. β-gal activity is seen in the epithelial cells of the oesophagus, the nasopharynx and in a few epithelial cells of the trachea. (E) Newborn mice. β-gal activity is found in all the cells constituting the epithelium of the oropharynx and on the superficial lining of the tongue. drg, dorsal root ganglia; l, lens; n, nasopharynx; oe, oesophagus; o, oropharynx; p, pharynx; pre, pigmented retina epithelium; r, retina; ri, rib; t, trachea; to, tongue. Scale bars: A, 30 μm; B, 75 μm; C, 120 μm; D, 235 μm; E, 120 μm.
digestive tract-derived glands. In digestive salivary glands, such as the submandibular gland, a few collector tube cells and glandular cells showed β-gal activity from E15.5 onwards (see at E 16.5 on Fig. 8A). The β-gal activity persisted in newborn mice. In the liver, few endodermal β-gal-positive cells were detected in newborn mice. In the biliary ducts and in the gall bladder of newborn mice, few epithelial cells were β-gal-positive. In the pancreas, at E12.5 few epithelial collector tube cells were β-gal-positive while most pancreatic cells were unstained. In newborn mice, this β-gal activity was restricted to some collector tube cells and to most cells of the Langhamers islets (Fig. 8B).

**Endocrine organs**

β-gal-positive cells were found in several endocrine tissues. In the thyroid gland, cells with β-gal activity were first detected at E14.5. This activity persisted through to birth and was limited to cells scattered within the thyroid gland, in close association with the epithelial cells (Fig. 8C). The description closely corresponds to that of calcitonin-producing cells. In the pituitary gland primordium, scattered β-gal-positive cells were found from E15.5 onwards. At E16.5 many cells of the pars intermedia were β-gal-positive. The β-gal activity was still observed in newborn mice, restricted to most cells of the pars intermedia (Fig. 8D). The β-gal activity was still observed in newborn mice, restricted to most cells of the pars intermedia (Fig. 8E). The Kit expression in the pineal gland was assessed by immunofluorescence at this stage (Fig. 8F). β-gal activity was found in most cells of the gland in the newborn mice. From E14.5 onwards, β-gal-positive cells were found in the adrenal gland; at birth, β-gal activity was still found in a few adrenal medulla cells.

**The circulatory system**

At E7.5, the mesenchymal aggregates forming the blood islands of the yolk sac were stained. The lacZ expression was not restricted to the hematopoietic progenitors, but was also found in vitelline veins. At E9.5, the lacZ expression was observed in the blood islands of the yolk sac vasculature (Fig. 9A), in the endocardium, in the endothelium of the dorsal aorta and in cells lining the intersomitic vessels (Fig. 9B). β-gal activity was detected in the endothelium of vessels from E9.5 onwards (for example, see at E13.5 in Fig. 9C). The lacZ expression was detected as early as E11 in the endothelial cells of the lung primordium, and persisted in newborn mice. At birth, all the cells lining vascular lumen were stained. The β-gal activity was higher in the endocardium, the arterial and capillary endothelium than in the veins, veinules and lymphatic endothelium.

**The genitourinary system**

β-gal-positive cells were found in the urogenital system. No β-gal-positive cells were detected in the pronephros. At E11.5, β-gal activity was detected in the mesonephric tubules. From E13 onwards, β-gal-positive cells were found in the mesonephric duct (Wolffian duct) of male embryos and, in newborn males, β-gal activity was observed in cells of the ductus deferens and of the epididymis. From E14.5 onwards, β-gal-positive cells were found in the paramesonephric duct (Müllerian duct), and lacZ expression persisted at birth in the oviduct and in few uterine horn cells. From E12.5 onwards, β-
gal-positive cells were observed in the metanephros, and in the ureters. In newborn mice, β-gal-positive cells were also found among the epithelial cells of collector and distal tubes. The bladder and ureter epithelial cells exhibited β-gal activity in newborn mice (not shown).

**DISCUSSION**

In situ hybridization of 35S-labelled RNA probes to embryo sections was previously used to investigate the tissue distribution of c-kit transcripts (Orr-Urteger et al., 1990; Keshet et al., 1991; Motro et al., 1991). However, conventional in situ hybridization has severe limitations, including the fact that a clear identification of the individual cell expressing the gene is not always possible. Immunohistochemistry has been successfully used to detect Kit expression (Ogawa et al, 1991; Cable et al., 1995; Huizinga et al., 1995). However, Kit expression cannot be followed in embryos homozygous for a null mutation in the c-kit gene.

We have taken advantage of the convenient nlslacZ reporter gene since it provides an easily detectable, highly sensitive and resolutive marker. Furthermore, its product is non-deleterious for the embryo. The replacement of the first exon of c-kit by the lacZ gene enables β-gal expression to faithfully recapitulate the endogenous c-kit expression. Through a cell by cell observation, the marker enabled us to follow the fate of cells that express and require c-kit during embryogenesis. Indeed, the disappearance of lacZ-expressing cells in embryos homozygous for the null mutation allowed the determination of where and when the cells of the affected lineage fail to survive in the absence of c-kit. Furthermore, novel sites of c-kit expression were identified during embryogenesis; these include ICC, but also endothelial, epithelial and endocrine cells.

**ICC are Kit-independent during embryogenesis**

ICC generate electrical rhythmicity and mediate neural inputs in the gastrointestinal tract. In vitro, freshly dispersed and cultivated ICC are excitable and spontaneously rhythmic. ICC are thought to be responsible for the autonomic intestinal motility, characterized by a continuous slow-wave activity (Langton et al., 1989). ICC were identified in young and adult mice, but not in embryos (Faussone-Pellegrini, 1984, 1985). Recently, Kit was found to be expressed in ICC in newborn mice and in adults (Huizinga et al., 1995). Our analysis reveals that c-kit is expressed in ICC from E13.5 onwards. Thus, the WlacZ allele provides a useful marker to study the ICC lineage. In the stomach, ICC were found to form part of the inner muscle cell bundles, in accordance with experiments showing a mesodermal origin of ICC (Lecoin et al., 1996).

Two observations indicate that, in postnatal life, ICC survival requires c-kit expression. First, anti-Kit antibody injections between 0 to 4 days post partum abolish highly autonomous phasic contractions. Second, ilea of W/WV adults lack the network of ICC (Huizinga et al., 1995). W and WV mutations have been analyzed at the molecular level. The W mutation is responsible for the synthesis of a non-functional protein, which is not expressed at the cell membrane, while WV is associated with a reduced kinase activity of the receptor (Nocka et al., 1990). Hence, in W/WV double heterozygotes only the WV mutant Kit is found on the cell surface. By contrast, WlacZ/WlacZ embryos completely lack Kit protein. Thus the effects of W mutations on ICC is expected to be increased in WlacZ/WlacZ mice compared to W/WV animals. Interestingly, the comparison of digestive tract sections of WlacZ/+ and WlacZ/WlacZ mutants revealed that ICC were present in homozygous newborn mice, implying that in the absence of Kit, ICC migration, proliferation and/or survival are not impaired until birth.

In support of this conclusion, transmission electron microscopic studies have revealed that ICC differentiate at birth and are fully developed in 30-day-old animals (Faussone-Pellegrini, 1984, 1985). Therefore, c-kit is crucial for the ICC survival and/or differentiation in postnatal life. Nevertheless, it is worth noting that out of six W/W adult mice cured of anemia following an injection of hematopoietic stem cells during embryogenesis, only one suffered intussusception causing blockade in the telescoped intestinal region (Fleischmann and Mintz, 1979). This result may suggest that another receptor is able to promote ICC survival in W/W mice and/or that the functional necessity of ICC for the intestinal motility has been overestimated.

**Endothelial cells express c-kit**

In birds, the hemangioblast lineage (hematopoietic and endo-

![Fig. 9. LacZ expression in endothelial cells of WlacZ/WlacZ embryos. (A) E9.5. β-gal activity (blue) is observed in the yolk sac vasculature. (B) E9.5. lacZ expression is found in endothelial cells of the intersomitic arteries (arrows), as well as in endothelial cells of larger vessels. (C) E13.5. β-gal activity in the endothelium of vessels. s, somite; ao, aorta. Scale bars: A, 150 μm; B, 60 μm; C, 30 μm.](image)
thelial cells) can be traced by means of Mab MB1 (Dieterlen-Liévre and Le Douarin, 1993). In mice, it is unclear at present whether hematopoietic and endocrine cells arise from the same putative hemangioblast precursor. CD34 and GATA2 are expressed in hematopoietic stem cells and in EC (Young et al., 1995; Dorfam et al., 1992). Here, we show that c-kit is also expressed in both hematopoietic stem cells and EC. Therefore, we assume that the hemangioblast precursors could be considered as CD34+ GATA2+ Kit+ cells.

During embryogenesis, vasculogenesis is achieved through two different processes: vasculogenesis and angiogenesis. Vasculogenesis, i.e. the formation of blood vessels from in situ differentiating angioblasts, is considered to account for the formation of the heart, the dorsal aorta, the cardinal and vitelline vessels, as well as the extraembryonic vessels of the yolk sac. Angiogenesis, i.e. the formation of blood vessels that sprout from pre-existing vessels, is thought to be responsible for the formation of vessels such as the intersomitic arteries (Yamaguchi et al., 1993). c-kit expression was found in the intersomitic vessels as well as in the endothelium of the dorsal aorta, and in vessels of the yolk sac. Therefore c-kit is expressed during both vasculogenesis and angiogenesis.

Several RTK are important for the transduction of angiogenic stimuli. Some of them are widely expressed in several tissues and cell types, whereas others are endothelial cell-specific, including flk-1, flt-1, tek, tie-1 and tie-2. Specific RTK play a crucial role in endothelial cell differentiation, and are necessary for the normal development of the mouse embryo vasculature (Shalaby et al., 1995; Fong et al., 1995; Dumont et al., 1994; Sato et al., 1995). In contrast, c-kit expression, although detected early in embryogenesis, is not required for the endothelial cell lineage, as revealed by the fact that the endothelial cell migration, proliferation and/or survival were not impaired in the absence of Kit. In human, SCF is expressed in dermic endothelial cells (Weiss et al., 1995), and both Kit and SCF are expressed by umbilical vein and adult aortic endothelial cells (Broudy et al., 1994; Buzby et al., 1994). Therefore, the Kit/SCF system may be involved in the adult in cell adhesion and migration, in vascularisation of normal or wounded tissues. Further studies should clarify the functions of the Kit/SCF system in endothelial cells and the potential involvement of the Kit/SCF system in tumor angiogenesis.

**Epithelial and endocrine cells express c-kit**

In the mouse, c-kit expression was found in several epithelial cells of the digestive, respiratory and genital tracts, and in several endocrine cells, including cells of the Langherans islets, adrenal medulla cells, thyroid, pineal and pituitary cells. None of these cells have been identified as c-kit-expressing cells before. Interestingly, the Kit-positive endocrine cells do not share the same embryological origin: neural crest for cells before. Interestingly, the Kit-positive endocrine cells do not share the same embryological origin: neural crest for

thyroid (Tsuzuki et al., 1995), c-ret mutations are associated with multiple endocrine neoplasia (MEN) types 2A and 2B, with familial medullary thyroid carcinoma, and with sporadic thyroid carcinomas (Goodfellow and Wells, 1995). A c-ret mutation does not account for all the MEN-2A cases, nor does it explain all cases of sporadic medullary thyroid carcinoma and of MEN 2B (Goodfellow and Wells, 1995). Because c-kit and c-ret share the same transduction pathways, as shown by the fact that c-ret can compensate for the c-kit melanoblast deficiency in W/Wv mice (Iwamoto et al., 1992), and because activation of c-kit has been reported in association with neoplasia (Kitayama et al., 1995), we suggest that gain-of-function mutations of c-kit could be involved in endocrine neoplasia.

We assumed that cellular lineages other than known target cells of W mutations could be dependent on the Kit/SCF system. Our data show that no other cell is dependent on this system during embryogenesis. Nevertheless, it is worth noting that known target cells of W mutations, i.e. melanoblasts, PGC and hematopoietic progenitors, are derived from precursors that express c-kit, but are not dependent on its expression. Thus, several c-kit-positive cells, not affected by the lack of Kit in W/Wv embryos during embryogenesis, may be precursors of cells that depend on Kit for their survival, as exemplified by the ICC. To test further this hypothesis, rescue of W/Wv embryos by microinjection of wild-type hematopoietic stem cells will be necessary.

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