c-kit Gene Was Not Transcribed in Cultured Mast Cells of Mast Cell-Deficient Wnh / Wnh Mice That Have a Normal Number of Erythrocytes and a Normal c-kit Coding Region

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The Wnh is a mutant allele at the W (c-kit) locus of mice. Mice of Wnh / Wnh genotype have white hairs and black eyes. Although adult C57BL/6-Wnh / Wnh mice were not anemic, they showed a remarkable depletion of mast cells. Most homozygous or double heterozygous mutant mice at the W (c-kit) locus, of which mast-cell depletion was comparable to that of Wnh / Wnh mice, are deficient in germ cells. However, male and female Wnh / Wnh mice have an appreciable number of germ cells in their gonads. We investigated the mechanism of specific depletion of mast cells in Wnh / Wnh mice. Cultured mast cells (CMC) derived from the spleen of Wnh / Wnh mice neither attached to normal (+/+ ) fibroblasts nor survived in coculture with + / + fibroblasts. The c-kit messenger RNA (mRNA) was strongly expressed in + / + CMC, but not detectable in Wnh / Wnh CMC. Despite the lack of c-kit mRNA in Wnh / Wnh CMC, the c-kit mRNA was normally detectable in the cerebellum and weakly detectable in the testis and spleen of Wnh / Wnh mice. No significant changes were found in the nucleotide sequence of the c-kit transcripts obtained from the cerebellum of Wnh / Wnh mice. Development of mast cells, erythrocytes, and germ cells in Wnh / Wnh mice appeared to be parallel with the magnitude of the c-kit gene expression in each cell type.

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THE DOMINANT SPOTTING (W) locus influences the coat color of mice.1-2 Mice of W/+ or W/+ genotypes have one or two well-defined white spots. Although the rest of the coat of W/+ mice is normal, that of W+/+ mice is apparently diluted. Many mutant alleles other than W and W have been reported at the W locus. The Wnh mutant allele reported by Lyon and Glenister3 resulted in a broad sash of white around the body in the lumbar region. Mice of specific depletion of mast cells in Wnh / Wnh mice. In addition to the depletion of melanocytes in the skin, Wnh / Wnh mice show hypoplastic anemia and depletion of mast cells in the skin of the former is smaller than the value obtained in the skin of the latter.5,6 As in contrast to the extracellular domain of c-kit protein expressed,13 the extracellular domain is normally expressed.34,35 Wnh / Wnh mice also showed hypoplastic anemia and depletion of mast cells, but not in Wnh / Wnh mice. The W locus was shown to be identical with the c-kit proto-oncogene.7,8 The c-kit gene is the normal cellular homologue of the v-kit oncogene and encodes a receptor tyrosine kinase.9-12 Recently, a ligand for the receptor encoded by the W locus was identified and was shown to be encoded by the steel (Sl) locus of mice.13-16 The molecular nature of some W (c-kit) mutant alleles has been analyzed.17-20 The W mutant allele produces a truncated c-kit protein without the transmembrane domain due to a point mutation of the noncoding region.20 Some point mutations in the tyrosine kinase domain of the W (c-kit) locus have been characterized.17-19 Both W and W mutant alleles completely abolish the kinase activity19-20; in the extracellular domain of c-kit protein encoded by the W allele is expressed normally, although the W mutation appears to destabilize the mature c-kit protein.18 As a result, the extracellular domain of c-kit protein is expressed only weakly on the surface of cultured mast cells (CMC) derived from W / W mice. The W and W (W3) mutant alleles result in the decrease of the kinase activity, but the extracellular domain is normally expressed.18,19 Both erythrocytes and mast cells are derived from the multipotential hematopoietic stem cell.1,12,22 Generally, the magnitude of anemia in various W mutant mice parallels that of mast-cell depletion.1,2,4,5 For example, W / W mice are more anemic than W / W mice,1,2 and the number of mast cells in the skin of the former is smaller than the value observed in the skin of the latter.4,5 However, this rule is not applicable to W / W and W / W mice. They showed an apparent deficiency of mast cells, but they were not anemic.2,3 Although W / W and W / W mice are sterile,1,2 both male and female W / W mice have been reported to be fertile.2,3 In the present study, we attempted to study the mechanism of mast-cell depletion in W / W mice that can produce a considerable amount of erythrocytes and germ cells.

MATERIALS AND METHODS

Mice. The original stock of Wnh mice was obtained from Dr J.F. Loutit (MRC Radiobiology Unit, Harwell, Didcot, UK). The Wnh mutant gene was maintained by repeated backcrosses to our own inbred colony of the C57BL/6-+/+ strain. At the time of the present experiment, the backcrosses exceeded more than 20 generations. C57BL/6-Wnh /+ and WBN-Wnh /+ mice were also maintained in our laboratory, and C57BL/6-Wnh /Wnh, Wnh / Wnh, +/+}

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and WB-(W/W, +/+) were raised. The genotype of mice was identified by their coat color.

**Numbers of erythrocytes and mast cells.** Mice were killed by decapitation after ether anesthesia. Blood samples were obtained, and the number of erythrocytes was counted by using a hemocytometer. Pieces of dorsal skin were removed, smoothed onto a piece of filter paper to keep them flat, fixed in Carnoy’s solution, and embedded in paraffin. Sections (4-µm thick) were stained with Alcian blue. Mast cells between epithelium and panniculus carnosus were counted under the microscope, and the number was expressed as mast cells per centimeter of skin.4

**Spleen colony assay.** Bone marrow cells of C57BL/6-HK/Hy and -/+ +/ + mice were obtained as described previously.23 Mice of C57BL/6-+/-/- were used as recipients and received x-ray body irradiation (8.0 Gy) before the injection of bone marrow cells (BMC). BMC (5 × 10⁹) were suspended in 0.2 mL Eagle’s medium (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) and were injected into the late tail vein of recipient with 3 hours after irradiation. The recipients were killed by overinhalation of ether 8 days after the injection of BMC. Spleens were removed and fixed in Bouin’s fluid; the number of spleen colonies was counted under the dissecting microscope (× 10).

**CMC and fibroblast cell line.** To obtain CMC, mice of 5 to 10 days of age were used. Spleens were removed, and cells were suspended as described previously.23 Pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCM) was prepared according to the method described by Nakahata et al.25 Culture flasks (Nunc, Roskilde, Denmark) containing 2 × 10⁹ spleen cells and 5 mL α-minimal essential medium (α-MEM; Flow Laboratories, Irvine, UK) supplemented with 10⁻⁴ mol/L 2-mercaptoethanol, 10% PWM-SCM were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. One-half of the medium was replaced every 7 days, and more than 95% of cells were CMC at 4 weeks after the initiation of the culture.23 The WCB6F-/-/-/+ + 3T3-1 fibroblast cell line was established from WCB6F1 mouse embryos in our laboratory.26

**Maintenance of CMC by coculture with fibroblasts.** Coculture of CMC with fibroblasts was performed as described previously.24,25 CMC (1.0 × 10⁵ cells) were suspended in 2 mL α-MEM containing 5% fetal calf serum (FCS; Hyclone Laboratories Inc, Logan, UT) and antibiotics and were inoculated to a confluent culture of fibroblasts in a 35-mm dish (Corning, New York, NY). The coculture was performed at 37°C in a humidified atmosphere containing 5% CO₂ in air. The culture medium was aspirated and replaced with 2 mL fresh culture medium without PWM-SCM every 2 days. Mast cells were identified by staining cytocientrifuge preparations of trypsinized cultures with Alcian blue, and the number of mast cells were counted under the microscope. Results were expressed as the mean ± SE of four dishes.

**Evaluation of attachment with fibroblasts.** Attachment of CMC to fibroblasts was investigated as described previously.23 CMC (1.0 × 10⁵ cells/well) were suspended in 0.4 mL α-MEM containing 5% FCS and antibiotics, and were inoculated to fibroblast cultures (1.0 × 10⁵ cells/well) in wells of 24-well tissue culture plates (Corning). At 1, 3, and 6 hours after initiation of the coculture, the microwells were washed twice with warmed (37°C) α-MEM to remove nonattaching CMC. Fibroblasts and attaching CMC were harvested after trypsin (0.1%) treatment (37°C for 15 minutes). Cytocentrifuge preparations were fixed with Carney’s solution and stained with Alcian blue and nuclear fast red. The number of remaining mast cells per fibroblast was calculated.

**Flow cytometry.** The preparation and specificity of the ACK2 rat monoclonal antibody (MoAb) against the extracellular domain of the c-kit receptor have been described in detail.30,31 CMC were incubated first with ACK2 MoAb at 4°C for 30 minutes, and then rinsed and stained with fluorescein isothiocyanate (FITC)-conjugated monoclonal antirat k antibody prepared at Kumamoto University Medical School.30,31 CMC were rinsed and analyzed on a FACScan (Becton Dickinson, Los Angeles, CA).

**Immunoprecipitation analysis.** Testes were removed from C57BL/6-HK/Hy and -/+ +/ mice and were decapsulated. Seminiferous tubules were unraveled by forceps and gentle pipetting in ice-cold phosphate-buffered saline (PBS). The dispersed tubules were allowed to sediment, and supernatant was filtered through a nylon mesh and collected as a crude extratubular cell population. The viability of this fraction was more than 95% when determined by Trypan blue dye exclusion test, and the proportion of contaminated germ cells (sperm and spermatid) was about 5%.

For metabolic labeling, 4 × 10⁶ CMC and 5 × 10⁶ testicular cells were washed with prewarmed PBS and incubated for 2 hours in methionine-free Eagle’s minimal essential medium containing 5 mmol/L glutamine, 1 mmol/L sodium pyruvate, and 10% dialyzed fetal bovine serum (Hazleton, Lenexa, KS). Incubation temperatures were 37°C and 32.5°C in CMC and testicular cells, respectively. After the medium change, [³⁵S]-methionine (American Radiolabeled Chemicals, Inc, St Louis, MO: 100 μCi/mL in CMC and 200 μCi/mL in testicular cells) was added to the fresh medium and incubation continued for 6 hours. The labeled cells were collected, washed with ice-cold PBS, and lysed in TDS buffer (10 mmol/L Na₂HPO₄ [pH 7.2] and 160 mmol/L NaCl containing 1% Triton-X-100, 1% deoxycholic acid, and 0.3% sodium dodecyl sulfate [SDS]). After 30 minutes on ice, the extracts were centrifuged at 50,000 rpm for 30 minutes at 4°C to remove insoluble cell debris. Lysates were precleared with protein-G Sepharose beads (Pharmacia, Uppsala, Sweden) and then precipitated with ACK2 MoAb,30,31 or class-matched control antibody conjugated to protein-G Sepharose beads in TDS buffer for 12 hours at 4°C. Immunoprecipitates were washed with TDS buffer three times and with 10 mmol/L phosphate buffer (pH 7.0) containing 0.5% Tween 20 four times, resuspended in sample buffer (10 mmol/L Tris-HCl [pH 7.4] containing 4% SDS, 10% β-mercaptoethanol, and 10% glycerol), and boiled for 5 minutes. Electrophoretic separation was performed on 7.5% SDS-polyacrylamide gels, according to the method of Laemmli?2 Gels were fixed with 10% acetic acid and 30% methanol and treated with 1 mol/L sodium salicylate before autoradiography.

**Northern blotting analysis.** Total RNA was prepared from +/+ CMC and W/K/Hy CMC, spleens of C57BL/6-+/-/- +/+ and -/- W/K/Hy mice at 10 days of age, and testes and cerebellums of C57BL/6- +/+ and -/- W/K/H mice at 60 days of age by using the guanidine isothiocyanate method, and poly(A) RNA was purified by Oligotex-dT30 (Japan Synthetic Rubber/Nippon Roche, Tokyo, Japan). Northern blot analysis was performed by using the mouse c-kit cDNA and β-actin34 cDNA labeled with [³²P]-α-deoxycytidine triphosphate (dCTP) by random oligonucleotide priming as probes.

**Oligonucleotide primers.** Polymerase chain reaction (PCR) was performed by using eight complementary oligonucleotide primers. The following oligonucleotide primers were used: primer 1, GAGCTTACAGTTTAACCCAGC (1 through 20); primer 2, CATATATATCTACCTGTGCGTGCTC (866 through 1090); primer 3, GTTTGAATACGAGGCCTACC (1039 through 1058); primer 4, AACTTGCGCTTTGTTGGG (1779 through 1798); primer 5, AGGGAGAAAAAACAAATATTG (1707 through 1731); primer 6, CTTCTCTGTACATCCTTGAGAACCTT (2654 through 2678); primer 7, TAGCCCCAGACACATCGAAATG (2460 through 2481); and primer 8, TTGTCTAAGCGTTTACACAATG (3401 through 3419) (odd number primers are sense primers and even number primers are antisense primers;
sequences are written from 5' to 3', and the number in parentheses refers to the region of the sequence as described previously11).

cDNA synthesis and isolation of PCR amplified products. Total RNA (5 μg) from cerebellums, testes, spleens, and CMC of C57BL/6-Wsh/Wsh and +/+ mice was used as a template and the single-strand cDNA was synthesized by using reverse transcriptase and downstream antisense primers. cDNA was amplified in a 100 μL reaction mixture by PCR using Tag DNA polymerase in 30 cycles of 1 minute of denaturation at 94°C, 2 minutes of annealing at 55°C, and 3 minutes of synthesis at 72°C.5,6 Reverse transcriptase modification of PCR (RT-PCR) products were examined on the 1% agarose gel. For the analysis of DNA sequence, RT-PCR products from RNA of cerebellums of C57BL/6-Wsh/Wsh and +/+ mice were gel-purified, treated with T4 polynucleotide kinase, and then treated with Klenow fragment of DNA polymerase I. The products were subcloned into the EcoRV site of Bluescript KS(-) (Stratagene, La Jolla, CA). DNA sequencing reaction was performed by the method of Sanger et al6 using [32P]-dCTP.

RESULTS

The number of erythrocytes and skin mast cells were reexamined in C57BL/6-Wsh/Wsh mice. Because the effect of the W mutant allele on erythropoiesis is more severe in growing mice than in adult mice,1,2 we counted erythrocytes in mice of 10 days of age. C57BL/6-Wsh/Wsh mice were slightly but significantly anemic at this age, but the anemia was not detectable in C57BL/6-Wsh/Wsh mice of 60 days of age (Table 1). In contrast, a severe anemia was observed in C57BL/6-Wsh/Wsh mice of both 10 and 60 days of age. The decrease in the number of skin mast cells was not so severe in both C57BL/6-Wsh/Wsh and -/+ mice at 10 days of age. The decrease became remarkable in the skin of C57BL/6-Wsh/Wsh mice at 60 days of age, but did not become remarkable in the skin of C57BL/6-Wsh/Wsh mice (Table 1). As a result, 60-day-old C57BL/6-Wsh/Wsh mice were not anemic but showed remarkable mast-cell deficiency. Although C57BL/6-Wsh/Wsh mice of 60 days of age were not anemic, their BMC produced very few macroscopic colonies in the spleen of irradiated mice (Table 2).

Mast-cell deficiency of various W mutant mice can be reproduced by the in vitro coculture system.27,28 We attempted to evaluate the fibroblast-dependent proliferation of Wsh/Wsh CMC by using this coculture system. Spleen cells of C57BL/6-(+/+, Wsh/Wsh, Wsh/Wsh) mice and WB-Wsh mice were cultured in the presence of PWSM. The resulting CMC of various genotypes were cocultured with WCBGF1-Wsh/Wsh fibroblasts without PWM-SCM. The numbers of remaining CMC were counted 2 weeks after the initiation of the coculture. As reported previously,29 +/+ CMC were maintained, but W/W CMC were not. The number of Wsh/Wsh CMC decreased, but a significant number of Wsh/Wsh CMC remained 2 weeks after initiation of the coculture (Table 3). Wsh/Wsh CMC disappeared by that time, as in the case of W/W CMC.

Table 1. Number of Erythrocytes and Mast Cells in the Skin of C57BL/6-Wsh/Wsh and -/+ Mice at 10 and 60 Days of Age

<table>
<thead>
<tr>
<th>Age (d)</th>
<th>Genotypes</th>
<th>Erythrocytes (per liter × 10^12)</th>
<th>Mast Cells (per centimeter of skin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>+/+</td>
<td>5.09 ± 0.14* (13)</td>
<td>325 ± 15* (9)</td>
</tr>
<tr>
<td></td>
<td>Wsh/Wsh</td>
<td>4.04 ± 0.13 (8)</td>
<td>74 ± 4 (10)</td>
</tr>
<tr>
<td></td>
<td>Wsh/Wsh</td>
<td>3.27 ± 0.24 (6)</td>
<td>58 ± 5 (9)</td>
</tr>
<tr>
<td>60</td>
<td>+/+</td>
<td>8.62 ± 0.29 (12)</td>
<td>359 ± 29* (11)</td>
</tr>
<tr>
<td></td>
<td>Wsh/Wsh</td>
<td>8.13 ± 0.35 (6)</td>
<td>7 ± 2 (7)</td>
</tr>
<tr>
<td></td>
<td>Wsh/Wsh</td>
<td>5.94 ± 0.33* (7)</td>
<td>59 ± 8* (8)</td>
</tr>
</tbody>
</table>

Number of mice is shown in parentheses.

*P < .01 when compared with values of Wsh/Wsh mice by the t-test.

Table 2. Number of Colonies Produced in the Spleen of X-irradiated C57BL/6-Wsh/Wsh Mice by the Injection of BMC (5 × 10^6) From C57BL/6-Wsh/Wsh Mice

<table>
<thead>
<tr>
<th>Genotypes of Donors</th>
<th>No. of Colonies (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.1 ± 0.1 (13)*</td>
</tr>
<tr>
<td>+/+</td>
<td>9.4 ± 1.2 (11)*</td>
</tr>
<tr>
<td>Wsh/Wsh</td>
<td>0.9 ± 0.2 (14)</td>
</tr>
</tbody>
</table>

Number of spleens is shown in parentheses.

*P < .05 when compared with values of Wsh/Wsh mice by the t-test.

Both W/W and Wsh/Wsh CMC are not maintained in the coculture with fibroblasts,17,19 but the interaction of W/W CMC with fibroblasts is different from that of Wsh/Wsh CMC when evaluated in 6 hours.29 The attachment of W/W CMC to WCBGF1+/+ fibroblasts was apparently impaired, but the attachment of Wsh/Wsh CMC to the same fibroblast cell line was comparable to that of +/+ CMC.29 We examined whether Wsh/Wsh CMC attached to WCBGF1+/+ fibroblasts. As shown in Fig 1, the attachment of Wsh/Wsh CMC was impaired, as in the case of W/W CMC. Because we showed that the extracellular domain of c-kit receptor is necessary for attachment,29 the expression of c-kit protein on the surface of Wsh/Wsh CMC was examined by using ACK2 MoAb. Wsh/Wsh CMC did not express the extracellular domain of c-kit protein (Fig 2).

Even if the c-kit protein is not expressed on the surface, there is a possibility that truncated c-kit protein is produced within CMC. We metabolically labeled Wsh/Wsh and control +/+ CMC with [35S]-methionine, and the presence of labeled c-kit protein was examined by immunoprecipitation. The c-kit protein was detectable in +/+ CMC, but not in Wsh/Wsh CMC (Fig 3). In testicular cells of Wsh/Wsh mice, c-kit protein was weakly detectable.

In the next experiment, we examined whether c-kit messenger RNA (mRNA) was transcribed in various tissues of Wsh/Wsh mice. The 5.5-kb c-kit transcripts were...
DEFICIENT C-KIT TRANSCRIPTION

Fig 1. Impaired attachment of CMC derived from C57BL/6-Wsh/ Wsh mice. CMC of C57BL/6-(+/+), Wsh/Wsh) and WB-W/W were cocultured with WCB6F1+/+3T3-1 fibroblasts without PWM-SCM. Each point represents mean ± SE of four wells.

found to be unaltered in size in the cerebellums of Wsh/Wsh mice. The magnitude of the c-kit gene expression in the cerebellums was comparable between Wsh/Wsh and +/+ mice (Fig 4A). In the testis and spleen, the message level of c-kit gene in C57BL/6-Wsh/Wsh mice was lower than that of C57BL/6-+/+ mice (Fig 4A). Although the message was very faint in the spleen of C57BL/6-Wsh/Wsh mice, its presence was convincingly shown by the RT-PCR technique (Fig 4B). A marked difference of c-kit gene expression was found between Wsh/Wsh CMC and +/+ CMC. The c-kit gene was strongly expressed in +/+ CMC, but was undetectable in Wsh/Wsh CMC (Fig 4A). Moreover, we failed to detect c-kit transcripts in Wsh/Wsh CMC even by using RT-PCR (Fig 4B).

The c-kit mRNA was expressed in the cerebellum, testis, and spleen of Wsh/Wsh mice. But it does not imply that no point mutations are present in the coding region c-kit cDNA. The c-kit cDNA was cloned from the cerebellum of Wsh/Wsh mice with RT-PCR by using a series of c-kit-specific oligonucleotide pairs, and the nucleotide sequence was determined. When compared with the published sequence,11 an AT → CG transversion at nucleotide 648 and a CG → TA transition at nucleotide 1618 were found in the c-kit cDNA of Wsh/Wsh mice. These substitutions have been reported as strain-specific polymorphisms.19 Although the GCG codon encoding Ala777 at nucleotide 2357-2359, which had been reported by Qiu et al.,11 changed to a GGC codon encoding Gly777 in the c-kit cDNA of Wsh/Wsh mice, this change was observed in the control C57BL/6-+/+ mice as well. No other changes were detected in the coding sequence of the c-kit cDNA of Wsh/Wsh mice.

DISCUSSION

C57BL/6-Wsh/Wsh mice showed a slight but significant anemia at 10 days of age, but no significant anemia was detectable at 60 days of age. Because the effect of W mutation on erythropoiesis is known to be remarkable in growing mice,12 the present result may not be surprising. Despite the recovery of anemia in adult C57BL/6-Wsh/Wsh mice, their BMC did not produce biologically significant numbers of macroscopic spleen colonies. This is consistent with the recent result of Barker et al.37 that C57BL/6-Wld/Wld mice are not anemic despite the defective spleen colony-forming potential of their BMC. Probably, some compensation mechanisms may work in hematopoietic tissues of C57BL/6-Wld/Wld and -Wsh/Wsh mice. In fact,
were reverse transcribed with primer 6 and then PCR-amplified using primers 5 and 8 for 30 cycles. RT-PCR products were analyzed on 1% agarose gel with pHY size marker (Takara Shuzou, Kyoto, Japan). Cer, cerebellum; Tes, Testis; Spl, spleen.

the number of burst-forming units-erythroid (BFU-E) increases in the bone marrow of C57BL/6-W/W mice. Further investigations are necessary to clarify the compensation mechanism in the hematopoietic tissues of C57BL/6-W/W mice. Because the magnitude of the anemia decreased and that of the mast-cell deficiency increased in adult C57BL/6-W/W mice, this mutant is potentially useful as a mast-cell deficient mouse without anemia.

A remarkable mast-cell deficiency observed in the skin of adult C57BL/6-W/W mice was consistent with the result of the in vitro experiments. W/W CMC that contained no detectable levels of c-kit mRNA did not survive on the monolayer of WCB6F1-+/+ 3T3-1 fibroblasts. W/W CMC did not attach to the same fibroblasts either. This confirmed our recent result that the extracellular domain of the c-kit receptors played an important role for attachment of CMC to fibroblasts.

The c-kit gene was strongly transcribed in +/+ CMC, but not in W/W CMC. On the other hand, the expression of the c-kit gene was weak but detectable in the testis and spleen of C57BL/6-W/W mice. The expression of the c-kit gene in the cerebellum of C57BL/6-W/W mice was comparable to that of C57BL/6-+/+ mice. Moreover, the production of c-kit protein was recognizable in the testis of C57BL/6-W/W mice. The expression of the c-kit gene in C57BL/6-W/W mice appeared to be influenced by cell types. In other words, mice of W/W genotype may be useful to obtain a clue for understanding the mechanism of cell type-specific regulation of c-kit gene expression. We are now attempting to identify the mutation(s) in c-kit gene affecting the phenotype of C57BL/6-W/W mice. The mutation(s) should lead us on a path towards the clarification of the regulation, organization, and origin of receptor tyrosine kinase genes.

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c-kit Gene was not transcribed in cultured mast cells of mast cell-deficient Wsh/Wsh mice that have a normal number of erythrocytes and a normal c-kit coding region

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