Mastocytosis in mice expressing human Kit receptor with the activating Asp816Val mutation

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Mastocytosis is a rare neoplastic disease characterized by a pathologic accumulation of tissue mast cells (MCs). Mastocytosis is often associated with a somatic point mutation in the Kit protooncogene leading to an Asp/Val substitution at position 816 in the kinase domain of this receptor. The contribution of this mutation to mastocytosis development remains unclear. In addition, the clinical heterogeneity presented by mastocytosis patients carrying the same mutation is unexplained. We report that a disease with striking similarities to human mastocytosis develops spontaneously in transgenic mice expressing the human Asp816Val mutant Kit protooncogene specifically in MCs. This disease is characterized by clinical signs ranging from a localized and indolent MC hyperplasia to an invasive MC tumor. In addition, bone marrow–derived MCs from transgenic animals can be maintained in culture for >24 mo and acquire growth factor independency for proliferation. These results demonstrate a causal link in vivo between the Asp816Val Kit mutation and MC neoplasia and suggest a basis for the clinical heterogeneity of human mastocytosis.

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Kit was originally identified as the viral oncogene (*v-kit*) responsible for the transforming activity of the Hardy-Zuckerman IV feline sarcoma virus (1). The Kit receptor, which binds mouse Kit ligand (Kitl; stem cell factor [SCF]), is a type III tyrosine kinase receptor belonging to the platelet-derived growth factor and macrophage colony-stimulating factor–1 receptors subfamily. This subfamily is characterized by the presence of five Ig-like domains in the extracellular region and by a cytoplasmic kinase domain split into an adenosine triphosphate–binding region and a phosphotransferase region separated by an insert of variable length (2). Kit and Kitl, which are encoded by the murine dominant *White spotting* (*W*) and *Steel* (*Sl*) loci, respectively, play major roles in hematopoiesis and in the generation of at least four cell lineages: melanocytes, primordial germ cells, interstitial cells of Cajal, and mast cells (MCs) (3).

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Human mastocytosis, first described in 1869 (4), constitutes a heterogeneous group of disorders characterized by the abnormal growth and accumulation of MCs in diverse tissues. Most cases begin in childhood and are benign and confined to the skin (cutaneous mastocytosis, the most frequent form being *urticaria pigmentosa*). In contrast, systemic mastocytosis (SM) is characterized by the accumulation of neoplastic MCs in multiple organs and can exhibit either an indolent or an aggressive clinical course (5, 6). The organs most frequently affected are bone marrow, skin, liver, spleen, and the gastrointestinal tract (5, 6). Mastocytosis is often associated with somatic mutations in the Kit protooncogene, which cause its ligand-independent constitutive phosphorylation and activation (7–9). By far, the most frequent Kit mutation in mastocytosis patients is the Asp816Val substitution (7–9). This mutation has also been reported in other human cancers (10–12) and in several leukemic MC lines (13–15). Despite the fre-

quent presence of this activating Asp816Val Kit mutation in mastocytosis patients, its specific contribution to MC pathogenesis and tumor development remains unclear. Indeed, the same mutation has been reported in several forms of the disease, ranging from mild skin MC hyperplasia to severe MC leukemia (7, 16, 17). How could such a heterogeneous clinical outcome be associated with a single mutation? Several hypotheses can be proposed. First, the activating Asp816Val Kit mutation may contribute to MC hyperplasia, but genetic polymorphisms could influence the clinical outcome. In this context, it is interesting to note that a polymorphism in the IL-4R α chain gene has been recently associated with indolent skin mastocytosis (18). Second, the timing of appearance of the somatic mutation with respect to MC ontogeny (bone marrow hematopoietic progenitor vs. more committed MC precursor) could be critical in determining the severity of the disease (19). Finally, the Asp816Val mutation could be seen as a "permissive" mutation for MC transformation, but additional "hits" would be required to completely transform the cell. The number and nature of these additional hits may also determine the severity of the disease (20).

To decide between these various hypotheses, we have generated transgenic mice expressing the human D816VKit transgene in MCs. The development of spontaneous mastocytosis in these mice demonstrates a direct role for Asp816Val mutant Kit in the development of MC neoplasia. In addition, the high variability of clinical signs observed in inbred animals derived from the same transgenic line suggests a basis for the clinical heterogeneity of mastocytosis.

RESULTS AND DISCUSSION

Asp816Val Kit mutant is causally associated with mastocytosis development

To directly evaluate whether the Asp816Val Kit mutation is causally linked to mastocytosis development in vivo, we have generated transgenic mice harboring a fusion transgene consisting of the 571 bp primate chymase gene (Δ571-bchm) promoter fragment and the human Kit protooncogene cDNA with the codon 816 Asp→Val substitution (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050807). The Δ571-bchm promoter was used to direct specific expression of the transgene to MCs, as previously described by Liao et al. (21). Seven founders were identified by Southern blot analysis on genomic DNA obtained from the tail (Fig. S1). They appeared phenotypically normal and were backcrossed onto the C57BL/6 strain to establish stable transgenic lines. Among these lines, four expressed the transgene-specific transcripts in different tissues and in MCs and were selected for subsequent experiments (Fig. S1).

Given the variable and nonspecific clinical manifestations of mastocytosis, we killed transgenic and nontransgenic lit-

Table I. Mastocytosis in Bchm/Asp816Val Kit transgenic mice

Age of mice at analysis	Genotype (# of mice)	MC pathology		
		None	Hyperplasia	Tumor
≤6 mo	non-Tg (n = 16)	16	0	0
	Bchm/D816VKit (n = 29)	29	0	0
≥12 mo	non-Tg (n = 22)	22	0	0
	Bchm/D816VKit (n = 28)	20	7	1

The frequency of MC disease is increased in the old (≥12 mo) transgenic mice as compared to both old nontransgenic (non-Tg) littermates ($P = 0.006$ using the two-tailed Fisher's exact test) and young (≤6 mo) transgenic mice ($P = 0.002$). MC disease is also more frequent ($P = 0.02$) in the entire transgenic group (n = 57) as compared with nontransgenic controls (n = 38).

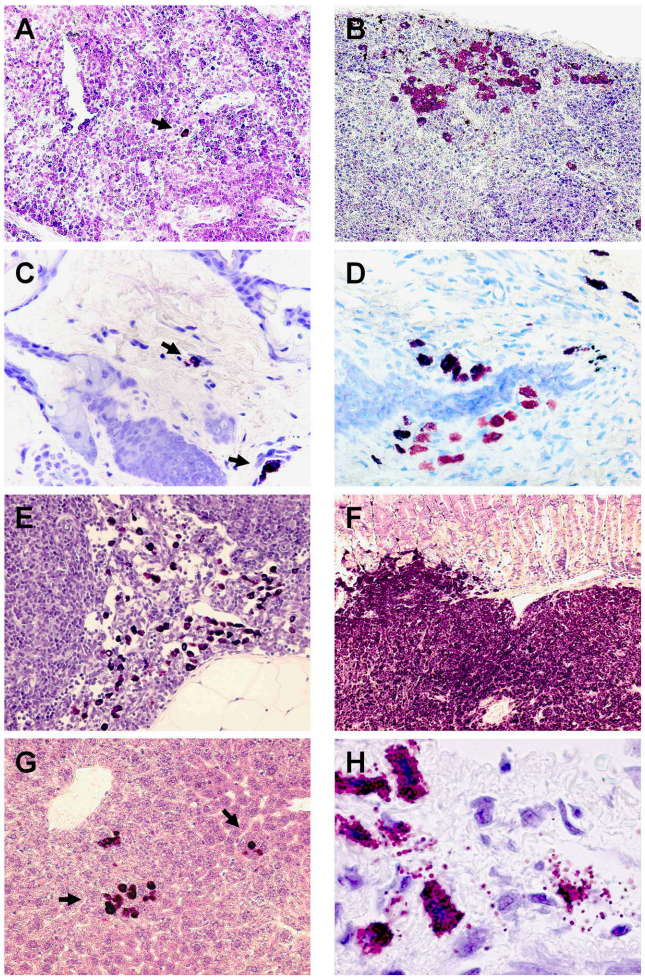

Figure 1. Histological studies of Bchm/Asp816Val Kit transgenic mice. Histopathological findings assessed by toluidine blue staining in four distinct transgenic mice. In the spleen of transgenic mice, MCs formed aggregates (B), whereas very few scattered MCs are seen (arrow) in nontransgenic controls (A). Skin involvement is characterized by a striking increase of MCs (D) compared with nontransgenic littermates (C, arrows). (E) Accumulation of MCs in lymph node. A large MC tumor (F) infiltrating the mucosae was detected in the intestine of a transgenic mouse, which also presented with multiple MC foci in the liver (G, arrows). (H) Observation of extracellular granules in the vicinity of MCs. Magnifications: (A, B, E, F, and G) 200; (C and D) 320; (H) 1,000.

termates from each expressing line at different ages to evaluate the histopathological changes associated with Asp816Val Kit expression. None of the nontransgenic mice and none of the young (4–6 mo) transgenic animals showed any signs of MC pathology (Table I). In addition, the percentages of peritoneal MCs were similar in 4–9-mo-old nontransgenic (mean ± SD = 2.87 ± 1.23%; $n = 13$) and transgenic (mean ± SD = 2.95 ± 1.04%; $n = 10$) animals. An abnormal accumulation of MCs was noted in 8 out of 28 (28.6%) old (12–18 mo) transgenic animals in the four lines studied. Two of these mice exhibited isolated skin involvement (Fig. 1, C and D; Fig. 2 B; and Table II). Similar to SM patients, the other six mice presented with MC aggregates involving different tissues, and all but one presented an indolent disease (Table II). These diseased animals therefore fulfilled the World Health Organization criteria for human SM (5, 6). The spleen, which has been reported as a primary site of MC disease in SM (22), was the most frequently affected tissue (Fig. 2 A). Affected spleens exhibited multiple randomly distributed foci of 5–30 MCs, with large MC aggregates predominating in the subcapsular area (Fig. 1, A and B). In one animal, a striking increase of MCs was also detected in the subcapsular sinus of a lymph node, showing morphological features comparable to those described in the spleen (Fig. 1 E). Moderate increase in MC density in the heart or in the stomach, not fulfilling the criteria for SM, was also detected in three additional transgenic mice (Fig. 2, C and D). Of interest, extracellular granules were observed in the vicinity of MCs in some skin and gastrointestinal tract lesions, suggesting that these cells were actively degranulating (Fig. 1 H). No bone marrow involvement was noted, which was consistent with the lack of transgene expression in this tissue (Fig. S1). This likely results from the use of the chymase promoter, which specifically targets differentiated MCs expressing mouse MC protease 5 (21).

Table II. Pathological abnormalities observed in Bchm/Asp816Val Kit transgenic mice

Animal	Transgenic line	Sex/age (mo)	MC disorder	Affected tissues
23369	2	M/12	Hyperplasia	Skin
23370	2	M/12	SM[a]	Heart, spleen
23161	2	F/18	SM, tumor	Intestine, spleen, liver
23034	8	M/13	SM	Spleen
23342	8	M/12	SM	Lymph node
23039	11	M/13	SM	Esophagus/stomach, spleen
23044	16	F/13	Hyperplasia	Skin
23356	16	F/12	SM	Spleen, skin

[a]According to the recent World Health Organization criteria applicable to human SM (references 5, 6).

In addition to this abnormal, but clinically silent, accumulation of MCs, a large tumor (10 mm in diameter) was detected in the intestine of an 18-mo-old transgenic mouse (Fig. 1 F). Microscopically, it consisted of MCs, which appeared enlarged and showed abundant basophilic granular

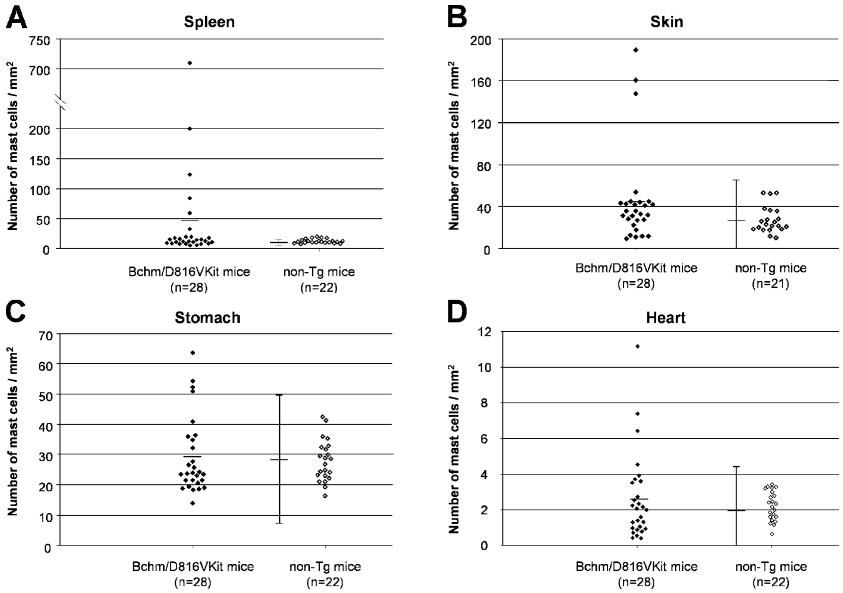

Figure 2. Increased number of tissular MCs in Bchm/Asp816Val Kit transgenic mice. Paraffin-embedded sections of spleen (A), skin (B), stomach (C), and heart (D) from old (≥12 mo) transgenic ($n = 28$) and nontransgenic ($n = 22$) littermates were stained with toluidine blue. MCs were counted in 13–40 randomly chosen microscopic fields by a researcher unaware of the genotype of the mice. The horizontal bar represents the mean MC density in each group. Values indicate ±3 SD for the control group. The number of splenic MCs is significantly increased in the transgenic mice as compared with nontransgenic littermates ($P = 0.018$ using the two-tailed Mann-Whitney U test). In addition, nine transgenic mice, but none of the control mice, have increased density of MCs in the skin, stomach and/or heart ($P = 0.003$ using the two-tailed Fisher's exact test).

cytoplasm and excentric, slightly atypical nuclei. These neoplastic MCs were strongly positive for toluidine blue stain, which revealed numerous metachromatic cytoplasmic granules, some of a remarkably large size. This mouse also exhibited multiple microscopic foci of MCs in the spleen and the liver (Fig. 1 G), two organs that are often affected in human SM (5, 6).

Asp816Val Kit mutant confers growth factor independency to bone marrow–derived MCs (BMMCs) from transgenic mice

To further analyze the impact of the Asp816Val Kit mutation on MC proliferation, we produced BMMCs from transgenic and nontransgenic littermates. As expected, only BMMCs from transgenic animals expressed the transgene (Fig. S1). Interestingly, we were repeatedly able to maintain transgenic, but not nontransgenic, BMMCs in continuous culture for >24 mo. Furthermore, after several months in culture, transgenic BMMCs became independent of growth factors and displayed equivalent proliferation rates with or without Kitl (SCF) or IL-3 (Fig. 3 B). However, exogenous IL-3 was still required during the initial phases of the culture of transgenic BMMCs, suggesting that the expression of Asp816Val Kit is necessary, but not sufficient, to confer factor-independent proliferation. Sommer et al. (23) recently reported similar findings for BMMCs derived from KitV558Δ/+ mutant mice. This observation correlated well with the fact that transgenic BMMCs exhibited a constitutive phosphorylation of Kit (Fig. 3 A), which was noted even before the acquisition of IL-3 independence (unpublished data). The characteristic phosphorylated mature (145

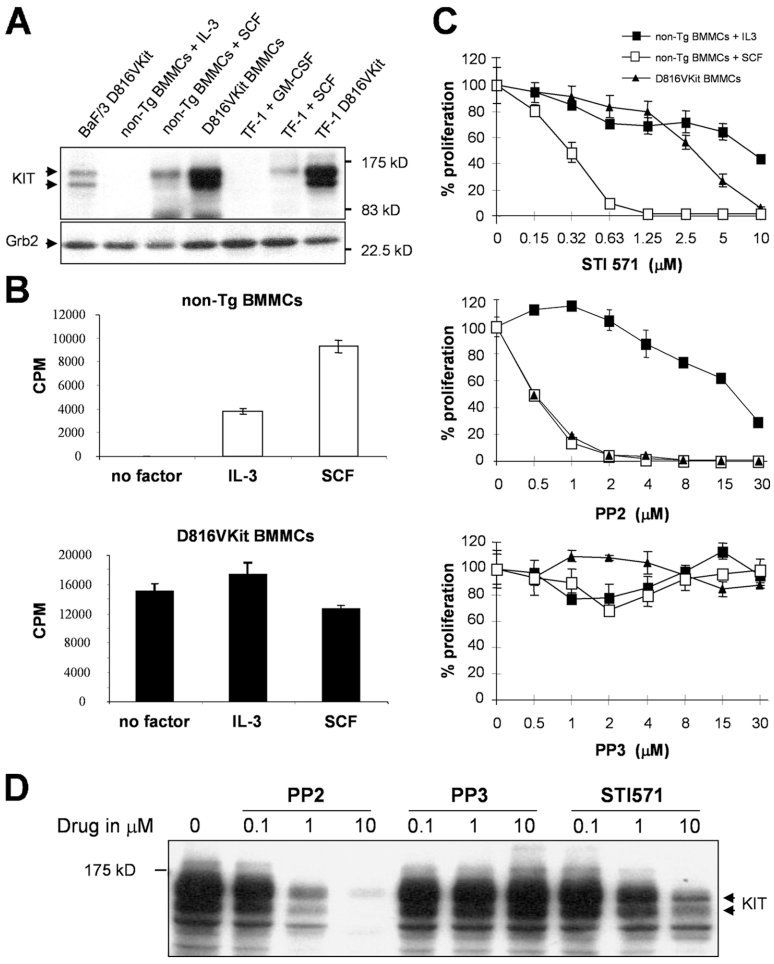

Figure 3. Growth factor–independent Kit phosphorylation and proliferation of BMMCs from transgenic mice. (A) Total proteins from Asp816Val Kit and nontransgenic BMMCs, TF-1 cells, and Asp816Val Kit–transfected BaF/3 and TF-1 cells were resolved by SDS-PAGE and probed with an antiphosphotyrosine antibody (top) and with an anti-Grb2 antibody (bottom) to assess the amount of protein loaded. (B) Proliferation of Asp816Val Kit and nontransgenic BMMCs assessed by [^{3}H]thymidine incorporation. Data are expressed as means ± SD of triplicate cultures. (C) Effect of increasing concentrations of tyrosine kinase inhibitors on the spontaneous proliferation of transgenic BMMCs and on growth factor–induced proliferation of nontransgenic BMMCs. Results are shown as percentages of the proliferation obtained without addition of drugs. Data are expressed as means ± SD of triplicate samples. (D) Kit phosphorylation in BMMC lysate from transgenic mice treated or not with the indicated tyrosine kinase inhibitors.

kD) and immature (125 kD) forms of Kit detected in transgenic BMMCs were similar to those observed in transfected BaF/3 or TF-1 cells expressing the human Asp816Val Kit mutant receptor (Fig. 3 A). This phosphorylation of Kit in transgenic BMMCs was associated with intense and constitutive activation of STAT3 but not of ERK1,2 or AKT (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050807/DC1), a pattern that was not observed in Kitl-stimulated nontransgenic BMMCs. This altered signal transduction pattern suggests that STAT3 plays an important role in Asp816Val Kit–induced MC transformation.

The ability to generate long-lived BMMCs expressing the mutant receptor prompted us to assess their in vitro sensitivity to a range of tyrosine kinase inhibitors. STI571 efficiently inhibits wild-type but not Asp816Val Kit (24). In line with these data, we observed that STI571 inhibited the Kitl-induced proliferation of nontransgenic BMMCs with a cellular IC_{50} of 300 nM, whereas factor-independent transgenic BMMCs were considerably more resistant (Fig. 3 C). In sharp contrast, transgenic and Kitl-stimulated nontransgenic BMMCs displayed identical sensitivity to PP2 (Fig. 3 C), a kinase inhibitor active on both wild-type and Asp816Val Kit (not depicted) (25). The inactive analogue PP3 had no important effect, nor did PP2 and STI571 inhibit IL-3–induced proliferation of nontransgenic BMMCs (Fig. 3 C). Collectively, these data strongly suggest that the factor-independent proliferation of the transgenic BMMCs is the consequence of the expression of the mutant Kit. This was further supported by the fact that inhibition of transgenic BMMC proliferation correlated closely with the inhibition of Kit autophosphorylation (Fig. 3 D).

Clues on the pathogenesis and clinical heterogeneity of human mastocytosis

Our finding that the expression of Asp816Val human Kit in MCs can recapitulate essential features of mastocytosis in mice is direct proof that this mutation plays an essential role in the abnormal accumulation and/or growth of MCs characteristic of this disease. Moreover, this transforming event can occur late in MC differentiation, an observation that correlates well with the fact that patients suffering from indolent SM can harbor the mutation without bone marrow involvement (26). Interestingly, mice originating from the same transgenic line (Table II) can develop all clinical forms of the human disease (MC hyperplasia selectively affecting the skin, SM, or MC tumor) while sharing the same site of transgene integration, timing of expression of the gain-of-function Kit mutation, genetic background, and environmental influences. This result therefore suggests that the expression of Asp816Val Kit is necessary for the neoplastic transformation of MCs but that transformation requires the involvement of additional somatic mutations. This hypothesis is strengthened by the fact that even though this mutation confers oncogenic properties in growth factor–dependent cell lines (27–29), it is unable to transform primary hematopoietic cells (30). In the same line of evidence, two other transgenic mice expressing different constitutively active murine Kit mutants, in particular the Val558del mutant that is sufficient to cause gastrointestinal stromal tumors, did not develop sign of mastocytosis, except for a mild increase in skin MCs (23, 29).

Collectively, our results are in agreement with the Knudson model (20), which proposes that cancer is rarely associated with a single mutation but has to be seen as a multistep process in which each step correlates with one or more distinct mutations in major regulatory genes. The nature, the accumulation, and/or the timing of appearance of these additional events would be crucial in determining the clinical outcome of the disease. Our transgenic mouse model offers a new tool to investigate in vivo the molecular requirements for the progression of mastocytosis. Moreover, it provides both a cellular and an animal model for preclinical testing of protein tyrosine kinase inhibitors potentially useful for patients with SM.

MATERIALS AND METHODS

Transgene construction. Site-directed mutagenesis was performed on the human Kit cDNA encoding the isoform lacking the GNNK sequence and containing Ser 715, to replace the aspartic acid at position 816 by a valine. The mutant Kit cDNA was cloned into a pBluescript vector that contained the SV40 late polyA region. A 571 bp fragment of the 5′ flanking sequences of the baboon chymase promoter (Δ571-bchm; a gift from A. Husain, Victor Chang Cardiac Research Institute, Sydney, Australia) (21) was cloned upstream of the mutated human Kit cDNA.

Generation of Bchm/Asp816Val Kit transgenic mice. Transgenic mice were generated by pronuclear injection of the linear Bchm/Asp816Val Kit transgene into fertilized (C57BL/6 × DBA2) F2 zygotes. Founder animals were identified by Southern blot analysis (Fig. S1). The French Ministry of Research approved these genetically modified animal studies. All animal studies were performed in accordance with the European Union guidelines and had local committee approval. Mice were backcrossed up to nine times on the C57BL/6 genetic background.

RT-PCR analysis. To assess transgene expression, 3 μg of total RNA was treated with RNase-free DNase (Roche) transcribed with Superscript (Invitrogen), using Oligo dT as a primer, and amplified in a 40-cycle PCR using the transgene-specific oligonucleotides 5′-CCGCTTCCTCCTCCCAGCCTC-3′ and 5′-GTTTCAGGTTCAGGGGGAGGTGTG-3′. Primers used for β-actin cDNA amplification were 5′-CCATCGTGGGCCGCCCTAGGCAC-3′ and 5′-CCGGCCAGCCAGGTCCAGAC-3′.

Tissue preparation and histological analysis. Mice were killed, and multiple tissues (skin, spleen, liver, lymph nodes, femur, stomach, jejunum, ileum, colon, lung, heart, brain, kidney, and genitals) were dissected, fixed in formalin for 24 h, and embedded in paraffin. 5 μm–thick tissue sections were stained by hematoxylin and eosin or periodic acid Schiff. Acid toluidine (0.5% toluidine blue in 0.5 M HCl) staining was performed to identify MCs. Tissue MCs were enumerated in 13–40 independent microscopic fields from 3–4 toluidine blue–stained sections for each mouse.

Culture of BMMCs. BMMCs were derived from femurs of 2-mo-old male mice. Bone marrow cells were cultured at a starting density of 2×10^5 cells/ml in Optimem medium supplemented with L-glutamine, penicillin, streptomycin, 10% fetal calf serum (Invitrogen), and 2 ng/ml murine recombinant IL-3 (Immugenex). The medium was renewed every 5–7 d.

Cells were used after their differentiation into MCs, which occurred after at least 28 d in culture. At monthly intervals, BMMCs were deprived of IL-3 to assess the acquisition of growth-factor independency.

Proliferation assays. BMMCs were washed twice in PBS, plated in triplicates at 5×10^4 cells/well in 96-well plates, and stimulated with either 250 ng/ml Kitl (SCF), 10 ng/ml IL-3, or without added growth factors. After 2 d of culture, 1 μCi [^{3}H]thymidine was added for 6 h. For the inhibition of BMMC proliferation, the drugs were added at the beginning of the culture at the concentrations indicated in the figures. STI571 was provided by E. Buchdunger (Novartis Pharma); PP2 and PP3 were purchased from Calbiochem-Novabiochem.

Western blotting analysis. For each assay, 5×10^6 cells were lysed, and the equivalent of 5×10^5 cell lysate was loaded per assay, run on a 7.5% SDS-PAGE gel, and transferred to a nylon membrane. Anti-PS^{476}-AKT, anti-PY^{705}-STAT3, and anti-STAT3 (Cell Signaling Technology), 4G10 antiphosphotyrosine (UBI), anti-active-(pTEpY)ERK1,2, and anti-Grb2 antibodies (Santa Cruz Biotechnology, Inc.) were used in immunoblotting according to the manufacturers' instructions. Membranes were incubated either with horseradish peroxidase–conjugated goat anti–mouse or anti–rabbit IgG antibodies (Immunotech). HRP revelation was performed by incubation with ECL reagent (GE Healthcare). For drug-induced inhibition of Kit phosphorylation, Asp816Val Kit BMMCs were serum-starved for 3 h and treated with or without tyrosine kinase inhibitors for an additional 90 min before cell lysis. Membranes were blotted with the 4G10 anti-phosphotyrosine antibody.

Online supplemental material. Fig. S1 contains detailed information about the construct and shows transgene integration in founder mice DNA and its expression in established transgenic lines. Fig. S2 characterizes the downstream signaling pathways induced by Asp816Val Kit receptor and likely implicated in MC transformation. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050807/DC1.

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