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The *kit*-ligand (steel factor) and its receptor c-*kit/W*: pleiotropic roles in gametogenesis and melanogenesis

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SUMMARY

The c-*kit* receptor tyrosine kinase belongs to the PDGF/CSF-1/c-*kit* receptor subfamily. The *kit*-ligand, KL, also called steel factor, is synthesized from two alternatively spliced mRNAs as transmembrane proteins that can either be proteolytically cleaved to produce soluble forms of KL or can function as cell-associated molecules. The c-*kit* receptor kinase and KL are encoded at the *white spotting* (*W*) and *steel* (*Sl*) loci of the mouse, respectively. Mutations at both the *W* and the *Sl* locus cause deficiencies in gametogenesis, melanogenesis and hematopoiesis. The c-*kit* receptor is expressed in the cellular targets of *W* and *Sl* mutations, while KL is expressed in their microenvironment. In melanogenesis, c-*kit* is expressed in melanoblasts from the time they leave the neural crest and expression continues during embryonic development and in the melanocytes of postnatal animals. In gametogenesis c-*kit* is expressed in primordial germ cells, in spermatogonia, and in primordial and growing oocytes, implying a role at three distinct stages of gametogenesis.

Many mutant alleles are known at *W* and *Sl* loci and their phenotypes vary in the degree of severity in the different cellular targets of the mutations. While many *W* and *Sl* alleles severely affect primordial germ cells (PGC), several mild *Sl* alleles have weak effects on PGCs and exhibit differential male or female sterility. *Steel Panda* (Sl^{pan}) is a KL expression mutation in which KL RNA transcript levels are reduced in most tissues analyzed. In female Sl^{pan}/Sl^{pan} mice, ovarian follicle development is arrested at the one layered cuboidal stage as a result of reduced KL expression in follicle cells, indicating a role for c-*kit* in oocyte growth.

W^{sh} is a c-*kit* expression mutation, which affects mast cells and melanogenesis. While the mast cell defect results from lack of c-*kit* expression, the pigmentation deficiency appears to stem from ectopic c-*kit* receptor expression in the somitic dermatome at the time of migration of melanoblasts from the neural crest to the periphery. It is proposed that the ectopic c-*kit* expression in W^{sh} mice affects early melanogenesis in a dominant fashion. The "sash" or white belt of $W^{sh}/+$ animals and some other mutant mice is explained by the varying density of melanoblasts along the body axis of wild-type embryos.

Key words: c-*kit* receptor, *kit*-ligand, *white spotting* (*W*) and *steel* (*Sl*) mutations, melanogenesis, gametogenesis

INTRODUCTION

Receptor tyrosine kinases (RTK) and their ligands function in the transduction of extracellular signals and are known to control cell proliferation, cell survival, motility and differentiation; consequently, RTK's play key roles in embryonic development, in organogenesis and in the adult life of invertebrate and vertebrate animals. RTK's that govern developmental processes, defined by mutant phenotypes, are known in *Drosophila*, the nematode *C. elegans*, and in mammals. In mice, several mutations in RTK genes with developmental consequences have been described. The c-*kit* receptor and the *kit*-ligand, KL, are allelic with the *white spotting* (*W*) and the *steel* (*Sl*) loci respectively; the platelet derived growth factor receptor-α chain (PDGFRα) with the *patch* (*Ph*) locus, and the macrophage colony stimulating factor (CSF-1) with the *osteopetrosis* (*op*) locus (Besmer, 1991; Pawson and Bernstein, 1990). The *W* and *Sl* genes function in several unrelated cell types and have been of interest to developmental biologists for a long time. In this paper insights into c-*kit* receptor function will be discussed that arise from the molecular characterization of c-*kit* and its ligand, and from the analysis of various alleles at the *W* and *Sl* loci, with particular emphasis on gametogenesis and melanogenesis. The detailed knowledge of the functional significance of the c-*kit* receptor system in vivo, provided through analysis of mutant phenotypes, should facilitate future elucidation of mechanisms underlying cell proliferation, cell adhesion/migration, cell survival and other postmitotic functions in various cell systems during development and in adult life.

STRUCTURE OF THE c-*kit* RECEPTOR AND ITS LIGAND KL/*steel* FACTOR

The proto-oncogene c-*kit* is the cellular homolog of v-*kit*, the oncogene of the Hardy-Zuckerman 4 - feline sarcoma virus, an acute transforming feline retrovirus (Besmer et al., 1986). c-*kit* encodes a receptor tyrosine kinase of the platelet derived growth factor (PDGF) receptor subfamily. The c-*kit* protein has an extracellular domain containing five immunoglobulin repeats and a cytoplasmic kinase domain, which is divided into two subdomains by the so-called kinase insert segment (Qiu et al., 1988; Yarden et al., 1987). A normal variant of the c-*kit* protein (Kit A^+), formed as the result of alternate usage of 3′ splice sites, contains a four amino acid insert in the extracellular domain between amino acids 512 and 513 of the known murine c-*kit* sequence (Hayashi et al., 1991; Reith et al., 1991). Studies of c-*kit* signaling in several cell systems indicate that the c-*kit* receptor is autophosphorylated in response to KL and that the activated receptor binds to and phosphorylates several known cytoplasmic proteins thought to represent intermediates in signaling pathways (Rottapel et al., 1991; Funasaka et al., 1992).

The c-*kit* gene maps to human chromosome 4 and mouse chromosome 5 in the vicinity of the PDGF receptor α chain gene and the *flk1* receptor kinase gene, and consists of 21 exons covering 65 kb (Qiu et al., 1988; Yarden et al., 1987; Stephenson et al., 1991; Matthews et al., 1991; Gokkel et al., 1992; Chu, Pritzer and Besmer, unpublished data). The realization that the c-*kit* proto-oncogene resides on mouse chromosome 5 in the vicinity of the *white spotting* locus (*W*) initially raised the question of whether c-*kit* is encoded by the *W* locus. Subsequently, the identity of the c-*kit* proto-oncogene with the *white spotting* locus was established by linkage analysis (Chabot et al., 1988), the demonstration of rearrangements in the c-*kit* gene in some *W* alleles (Geissler et al., 1988), and the finding of missense *W* mutations that inactivate the c-*kit* kinase (Nocka et al., 1989; Tan et al., 1990).

The realization that c-*kit* was encoded at the *W*-locus accelerated the quest for the ligand of the c-*kit* receptor. The known function of c-*kit*/*W* in bone marrow-derived mast cells provided an assay for the isolation of a soluble form of the ligand of the c-*kit* receptor, KL (Nocka et al., 1990a,c). However, KL was also isolated as a factor that promotes the formation of colonies from early hematopoietic progenitors (Zsebo et al. 1990a,b), and as a mast cell growth factor (Williams et al., 1990). Two alternatively spliced KL RNA transcripts encode two cell-associated KL protein products, KL-1 and KL-2, that differ in their sequences N-terminal of the transmembrane segment (Flanagan et al., 1991; Huang et al., 1992). The KL-1 and KL-2 RNA transcripts are expressed in a tissue-specific fashion. The KL-2 protein lacks sequences that include the major proteolytic cleavage site for the generation of the soluble KL protein from KL-1 (Fig. 1). The KL-1 protein is efficiently processed by proteolytic cleavage to produce soluble KL; by contrast KL-2 is also processed to form soluble KL, but not as effectively. KL-2 therefore represents a differentially more stable cell-associated form of KL (Huang et al., 1992). The protease activities facilitating cleavage of KL-1 and KL-2 in COS-1 cells were shown to be distinct by using a panel of protease inhibitors (Pandiella et al., 1992). Interestingly, the protein kinase C inducer PMA accelerates proteolytic cleavage of both KL-1 and KL-2, suggesting that this process is subject to regulation (Huang et al., 1992). Consequently, differential expression of variant cell membrane associated KL molecules and their proteolytic cleavage to generate soluble forms of KL provide different means to control and modulate c-*kit* function.

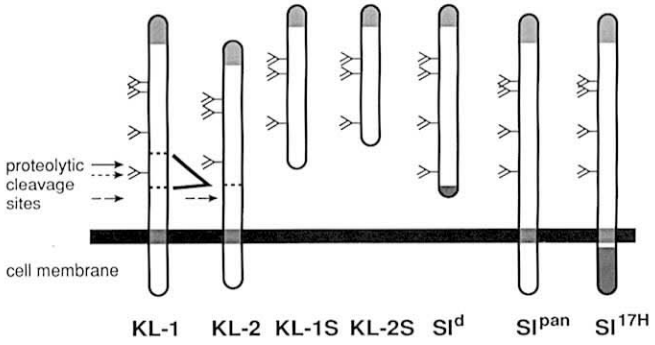

Fig. 1. Schematic representation of topological characteristics of various normal and mutant (Sl^d, Sl^{pan} and Sl^{17H}) KL protein products. Normal KL protein products: KL-1 and KL-2 (products of alternatively spliced transcripts) are membrane proteins and KL-1S and KL-2S soluble proteins, produced by proteolytic cleavage of KL-1 and KL-2. Dark shaded areas in Sl^d and Sl^{17H} proteins indicate altered protein sequences.

W AND *Sl* MUTANT PHENOTYPES

Mutations at the murine *white spotting* and *steel* loci generate deficiencies in three cell systems during embryogenesis and in the postnatal animal: the pigmentary system, germ cells and hematopoiesis (for reviews see: Russell, 1979; Silvers, 1979). During normal development, melanoblasts arise from the neural crest and migrate to the periphery where they enter the epidermis, colonize hair follicles and postnatally differentiate to become pigmented melanocytes (Silvers, 1979). *W* and *Sl* mutations affect several aspects of melanogenesis causing varying degrees of depigmentation (see below). Primordial germ cells are generated from the posterior primitive streak and migrate from the base of the allantois and the hindgut to the genital ridges; spermatogenesis and oogenesis then proceed following different developmental programs. *W* and *Sl* mutations affect the survival, migration and proliferation of primordial germ cells as well as steps in spermatogenesis and oogenesis causing impaired fertility (see below).

In hematopoiesis *W* and *Sl* mutations affect cells within the stem cell hierarchy, distinct cell populations in the erythroid cell lineage and mast cells, during early development as well as in the adult animal (for a review see Russell, 1979). Mutant animals suffer from macrocytic anemia and they lack tissue mast cells. While *W* mutations are cell autonomous, *Sl* mutations affect the microenvironment of

the cellular targets of the mutations (McCulloch et al., 1964, 1965). These findings were a strong indication that the *W* and *Sl* gene products function in the same biochemical pathway, possibly as receptor and ligand. The defects in *W* and *Sl* mutant mice are consistent with a role of the c-*kit* receptor system in facilitating cell proliferation and survival of precursor cells as well as promoting cell migration and other functions in differentiated cells (Besmer, 1991; Williams et al., 1992).

The synchronous expression of the c-*kit* receptor and its ligand in close cellular environments is a good predictor for sites at which c-*kit* functions in vivo. Therefore, the examination of c-*kit* and KL expression during embryonic development and in the adult animal using RNA blot analysis, in situ hybridization and immunohistochemistry has provided important insights into understanding c-*kit* function. In agreement with the cell autonomous nature of *W* mutations, c-*kit* is expressed in cellular targets of *W* and *Sl* mutations during embryogenesis and in the postnatal animal in melanogenesis, gametogenesis and in cells of the hematopoietic system (Nocka et al., 1989; Orr-Urtreger et al., 1990; Manova et al., 1990; Manova and Bachvarova, 1991; Ogawa et al., 1991; Nishikawa et al., 1991; Yoshinaga et al., 1991). Expression of KL has been shown to be associated with migratory pathways of melanoblasts and germ cells, and homing sites of both germ cells and hematopoietic progenitors during embryonic development, i.e. the genital ridges and the fetal liver (Matsui et al., 1990).

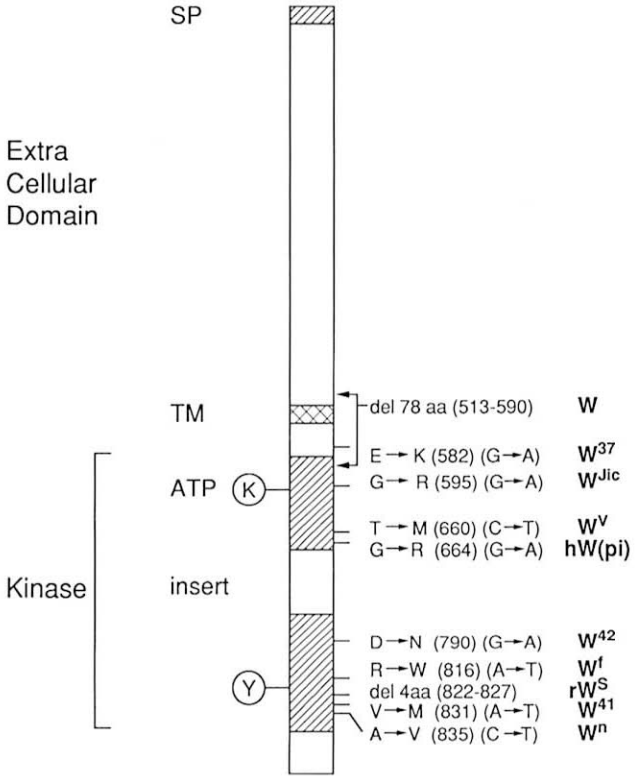

Fig. 2. Schematic representation of structural c-*kit*/*W* mutations, *W*, W^{42}, W^{n}, W^{37}, W^{Jic}, W^{v}/W^{57}, W^{41}, W^{f}, rW^{s} and *hW* (Giebel and Spritz, 1991). Missense mutations are indicated by single letter amino acid code and corresponding nucleotide changes are shown in brackets. Abbreviations: signal peptide (SP), transmembrane domain (TM), ATP binding site (ATP) and kinase insert sequence (insert).

MOLECULAR BASES OF *W* AND *Sl* MUTATIONS

An easily recognizable phenotype, coat color spotting, has made possible the isolation of many distinct mutations at the *W* and the *Sl* loci (Russell, 1979; Silvers, 1979; Lyon and Searle, 1989). These mutants provided an opportunity to characterize both the molecular basis of these mutations as well as their effects on different cell lineages and tissues, thus furthering our understanding of c-*kit* function. *W* and *Sl* mutations vary in their degree of severity on the affected cellular targets. In the homozygous state, several alleles, including the original *W* and *Sl* alleles, cause perinatal lethality while others are viable and semi-fertile. Several different *W* and *Sl* alleles have been characterized at the molecular level (see Tables 1 and 2).

W mutations

The original *W* allele W^{n} and W^{19H} are c-*kit* null or loss of function mutations (Chabot et al., 1988; Nocka et al., 1990b; Tsujimura et al., 1993). Null mutations have severe phenotypes when homozygous but display only minor effects when heterozygous, i.e. a white belly spot, no anemia, no infertility. Therefore, null mutations have primarily recessive characteristics. Several mutations of *W* ($W^{42,37,V,55,41}$) are known in which heterozygotes are affected more severely than *W*/+; they vary in severity in the homozygous state and affect the three principal cell systems to comparable degrees (Geissler et al., 1981). These alleles contain c-*kit* missense mutations (see Table 1; Fig. 2), which impair c-*kit* kinase activity to differing degrees (Tan et al., 1990; Reith et al., 1990; Nocka et al., 1990b). Work on the mechanism of activation of several tyrosine kinase receptors, including the members of the PDGF receptor family and the c-*kit* receptor, implicates receptor dimers or oligomers as intermediates (Heldin et al., 1989; Blume-Jensen et al., 1991). The dominant phenotypes of these mutations indicate that the mutant c-*kit* proteins in receptor heterodimers interfere with KL induced signal transmission, effectively reducing the number of active receptor dimer/oligomers on the cell surface. Consequently these mutations give rise to more severe heterozygous mutant phenotypes than those of null mutations and have the hallmarks of dominant negative mutations.

Sl mutations

Several severe *Sl* alleles ($Sl,Sl^{J,gb,8H,10H,12H,18H}$) have been shown to contain deletions that include the KL gene, and therefore are KL loss-of-function mutations (Copeland et al., 1990; Nocka et al., 1990c; Zsebo et al., 1990b). Homozygotes for the *Steel-Dickie* allele (Sl^{d}) are viable and less severely affected, implying some residual functional activity of KL, but they display all of the pleiotropic effects normally associated with *steel* mutations. Molecular analysis indicates that the Sl^{d} allele arose as a result of an intragenic deletion including the transmembrane domain

Table 1. Molecular bases of *W* mutations

Mutations	Homozygous phenotypes	Heterozygous phenotypes	Molecular lesion	Type of mutation	References
W^{19H}	**early lethal**	ventral spot, no effects on hematopoiesis and fertility	deletion (approx. 2cM)	**null**, loss of function	Lyon et al., 1984 Chabot et al., 1988 Geissler et al., 1988
W^{X}	**perinatal lethal** full effects on pigmentation, gametogenesis, hematopoiesis	do*	deletion	do	Russell et al., 1957 Geissler et al., 1988
W	do	do	splice donor site mutat., del. of TMS, no cell-surface protein	do	Durham, 1911 Nocka et al., 1990 Hayashi et al., 1991
W^{n}	do	some irregular spotting, no effects on hematopoiesis and fertility	Ala835 → Val no kinase act., no cell-surface protein	do	Tsujimura et al., 1993
W^{42}	do	lacks pigment anemia reduced fert.	Asp790 → Asn no kinase act.	**dominant negative**, null	Geissler et al., 1981 Tan et al., 1990
W^{37}	do	mottled fur, moderate anemia	Glu582 → Lys no kinase act.	do	Geissler et al., 1981 Nocka et al., 1990 Reith et al., 1990
W^{dic}	do	do	Gly595 → Arg no kinase act.	do	Tsujimura et al., 1993
$W^{v} = W^{55}$	**viable**, black-eyed white, anemia, sterile	coat color dil., ventral spot, mild anemia	Thr660 → Met reduced kinase act.	**partial**, dom. neg.	Little and Cloudman, 1937 Nocka et al., 1990 Reith et al., 1990
W^{41}	**viable**, mottled fur and mild anemia	mild anemia and spotting	Val831 → Met reduced kinase act.	**weak**, dom. neg.	Geissler et al., 1981 Nocka et al., 1990 Reith et al., 1990
W^{f}	viable, fertile mottled-striped fur, mild anemia	mild anemia, spotting	Arg816 → Trp	weak	Guenet et al., 1979 Larue et al., 1992 Tsujimura et al., 1993
W^{44}	little pigment, reduced fertility, but not anemic	some spotting	intron insertion, reduced RNA level	expression mutation	Geissler et al., 1981, 1988
W^{57}	mottled fur, no anemia, fertile	do	reduced RNA and protein levels	do	Reith et al., 1990
W^{sh}	black-eyed white, lack mast cells but fertile and no anemia	white sash in lower trunk	DNA rearrangement, lack of and inappropriate RNA expression	do	Lyon et al., 1982 Tono et al., 1992 Duttlinger et al., 1993
rW^{s}	viable/perinatal-lethal, red. fertil. black-eyed white, anemia, lack mast cells	mild anemia red. mast cells, dil. of coat color and spotting	del. aa 826-829	**partial**, dominant negative	Niwa et al., 1991 Tsujimura et al., 1991

* do = ditto

and C terminus, generating a secreted KL protein product with normal biological activity (Fig. 1; Flanagan et al., 1991; Brannan et al., 1991; Huang et al., 1992). The biological characteristics of homozygous Sl^{d}/Sl^{d} mice and of Sl/Sl^{d} mice suggest that, although the Sl^{d} KL protein sustains some activity, it is largely defective in facilitating proliferation and survival of target cells. This indicates that the membrane-bound form of KL plays a critical role in c-*kit* function.

ECTOPIC c-*kit* EXPRESSION AT SITES OF *kit*-LIGAND EXPRESSION IN W^{sh} MICE AFFECTS EARLY MELANOGENESIS

Melanocytes originate from neural crest cells migrating at embryonic day 9-10 (E9-10) along a dorsolateral pathway over the somites. They then spread through the dermis to all regions of the body by E12½ (see, LeDouarin, 1982; Rawles, 1947; Mayer, 1965; Serbedzija et al., 1990). Sub-

Table 2. Molecular bases of *steel* mutations

Mutations	Homozygous phenotypes	Heterozygous phenotypes	Molecular lesion	Type of mutation	References
Sl, Sl^{J}, Sl^{gb}, Sl^{SH}, Sl^{10H}	**pre-/perinatal lethal**, full effects on pigmentation, gametogenesis, hematopoiesis	dilution of color on ventral side, occasional spotting	deletion of KL coding sequences	**null**, loss of function	Sarvella and Russell, 1956 Beechey and Searle, 1985; Schaible, 1961, 1963 Copeland et al., 1990; Nocka et al., 1990; Zsebo et al., 1990
Sl^{18H}	**early lethal**	do*	do	do	Copeland et al., 1990
Sl^{d}	**viable**, black-eyed white, sterile and anemic	slight dilution of coat color, and spotting	intragenic deletion, incl. TMS and C-term.	**partial**, lacks cell membrane forms	Bernstein, 1960 Brannan et al., 1991 Flanagan et al., 1991 Huang et al., 1992
Sl^{17H}	**viable**, black-eyed white, mild anemia, females fertile, males sterile	lighter pigmentation	splice site mut. frameshift at aa 238 in cytopl. domain and term. at 265	**partial**, impaired function of cell membrane forms	Peters et al., 1987 Brannan et al., 1992
Sl^{pan}	viable, black-eyed white, mild anemia males fertile, females sterile	do	intact coding region, defect unknown	**expression mutation** reduced RNA levels	Beechy et al., 1986 Huang et al., 1993
Sl^{t}	same as Sl^{pan}, but more germ cells	do	unknown		Kuroda et al., 1988
Sl^{con}	same as Sl^{pan}, but fewer germ cells, follicle development normal	do	unknown		Beechey and Searle, 1983

*do = ditto

sequently they move from the dermis into the epidermis (Rawles, 1947; Mayer, 1973), colonize hair follicles, and differentiate to become melanocytes. The c-*kit* receptor is expressed in melanoblasts from the time they leave the neural crest throughout development, as well as in differentiated melanocytes located over the papilla in hairbulbs; KL expression has been demonstrated in the microenvironment of melanoblasts and melanocytes during development and in the postnatal animal, implying a role for c-*kit* at several stages of melanogenesis (Manova and Bachvarova, 1991; Matsui et al., 1990; Duttlinger et al. 1993; Manova, unpublished data). The unpigmented skin of *W* mutant mice is devoid of melanocytes (Silvers, 1956, 1979). The absence of melanocytes has been interpreted as a failure of the precursor cells to migrate, to proliferate, or to survive. In the postnatal animal c-*kit* function is thought to be essential for pigment formation during the cycles of active hair growth (Nishikawa et al., 1991). During embryonic development c-*kit* function is necessary at around E14½, when melanocyte precursors migrate from the dermis to the epidermis (Mayers, 1973; Nishikawa et al., 1991). An earlier function for c-*kit* was suggested by the finding of synchronous expression of c-*kit* in melanoblasts located dorsally and laterally of the somites and KL in the somitic dermatome at around embryonic day 10½ (Manova and Bachvarova, 1991; Matsui et al., 1990). This notion is supported by the evidence that melanocyte precursors in the head disappear between E11½ and E12½ in Sl^{d}/Sl^{d} embryos (Steel et al., 1992).

W-sash is a particularly interesting allele at the *W* locus that affects primarily mast cells and melanogenesis but not other cellular targets of *W* and *Sl* mutations (Lyon and Glenister, 1982). Thus, W^{sh}/W^{sh} mice are fertile and not anemic, but they lack mast cells in their skin and intestine and they are almost entirely unpigmented (Stevens and Loutit, 1982) (Fig. 3). Heterozygotes are black with a broad white *sash* or belt in the lumbar region (Fig. 3). The restricted display of *W* mutant characteristics typical of *W* mutations in W^{sh}/W^{sh} mice suggests a mutation that affects c-*kit* expression in a cell-type-specific manner. In addition, the more severe pigment defect in heterozygous $W^{sh}/+$ as compared to *W*/+ mice indicates an enhanced dominant effect of this mutation.

c-*kit* RNA and protein expression patterns in adult W^{sh}/W^{sh} mice and during embryonic development have been investigated to elucidate the basis for the phenotypes of *W-sash* mice (Duttlinger et al., 1993; Tono et al., 1992). c-*kit* expression was absent in bone marrow-derived W^{sh}/W^{sh} mast cells, the fetal and the adult lung, and the digestive tract at E13½; all of these tissues normally express c-*kit*. In addition, at E13½ W^{sh}/W^{sh} embryos lacked the c-*kit* positive melanocyte precursors normally found in the skin. However, c-*kit* was expressed normally at numerous other sites in W^{sh}/W^{sh} embryos and adults. In E10½ mutant embryos, as in normal embryos, a low number of c-*kit* positive presumptive melanoblasts are present in the skin. Unexpectedly, in E10½ W^{sh}/W^{sh} embryos, ectopic c-*kit* expression was observed in the dermatome of the somites, the mesenchyme around the otic vesicle and the floorplate of the neural tube (Fig. 4). These structures are known to express KL in wild-type embryos (Fig. 4). In $W^{sh}/+$ embryos, similar to W^{sh}/W^{sh} embryos, ectopic c-*kit* expression was observed

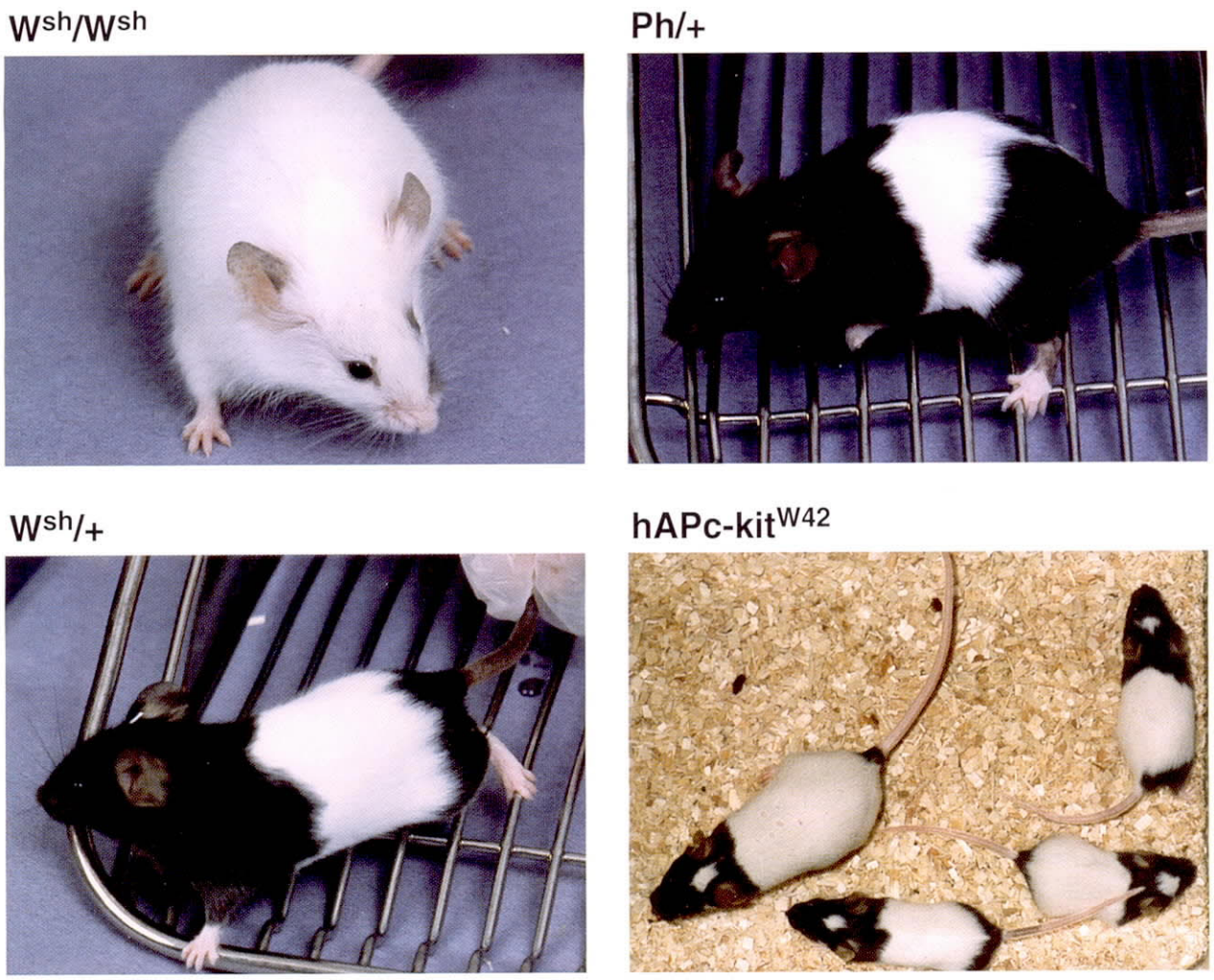

Fig. 3. Pigmentation pattern of W^{sh}/W^{sh}, $W^{sh}/+$, *Ph*/+ and transgenic line 485 hAP-c-kit^{W42} C57BL6/J mice (mother and offspring of 4th backcross generation).

in the head mesenchyme and at similar levels in all dermatomes along the axis of the embryo. This ectopic expression continues at E13½ in homozygous and heterozygous mutant embryos (data not shown).

The inappropriate c-*kit* expression in the dermatome of mutant embryos provides an explanation for the dominant pigmentation defect in adult mutant mice. c-*kit* receptor expression in cells of the dermatome of W^{sh}/W^{sh} and $W^{sh}/+$ mice may bind kit-ligand and reduce its concentration in the extracellular space. If so, the available KL may be limiting for the c-*kit*-expressing melanoblasts migrating over the dermatome, thus reducing their survival and/or proliferation. Alternatively, the co-expression of c-*kit* and its ligand may activate dermatomal cells causing changes in the extracellular matrix. In either model, the W^{sh}/W^{sh} and $W^{sh}/+$ melanocyte precursors would die or fail to proliferate between day E11½ and day E13½. In agreement with these models, migration of melanocyte precursors over the somites and through the dermis throughout the trunk region normally occurs at E10½-13½ and thus coincides with the time of inappropriate c-*kit* expression in the dermatome. Also, c-*kit*-expressing melanocyte precursors are observed in E10½-11½ W^{sh}/W^{sh} embryos, apparently on a dorsolateral pathway, whereas E13½ embryos contain no c-*kit*-positive cells in the skin. Importantly, these results imply that c-*kit* is required in melanogenesis between E10½-13½. This is a period of substantial proliferation of melanoblasts from a sparsely to a more numerously distributed cell population (Manova and Bachvarova, 1991; Steel et al., 1992).

The W^{sh} mutation blocks c-*kit* expression in mast cells and mesenchymal cells in the lung and the digestive tract at E13½, while expression in other tissues is normal. This implies that positive elements regulating c-*kit* expression in mast cells, lung and digestive tract are affected by the W^{sh} mutation. Negative elements are also affected, since c-*kit* is expressed in additional tissues; interestingly, these are tissues which normally express KL. DNA blot analysis revealed no alteration of c-*kit* exon and intron sequences in the W^{sh} allele (Duttlinger et al., 1993), in agreement with the recent demonstration that the c-*kit* coding sequence in W^{sh} is unaltered (Tono et al., 1992). However, pulsed-field gel electrophoresis showed that the W^{sh} mutation involves a

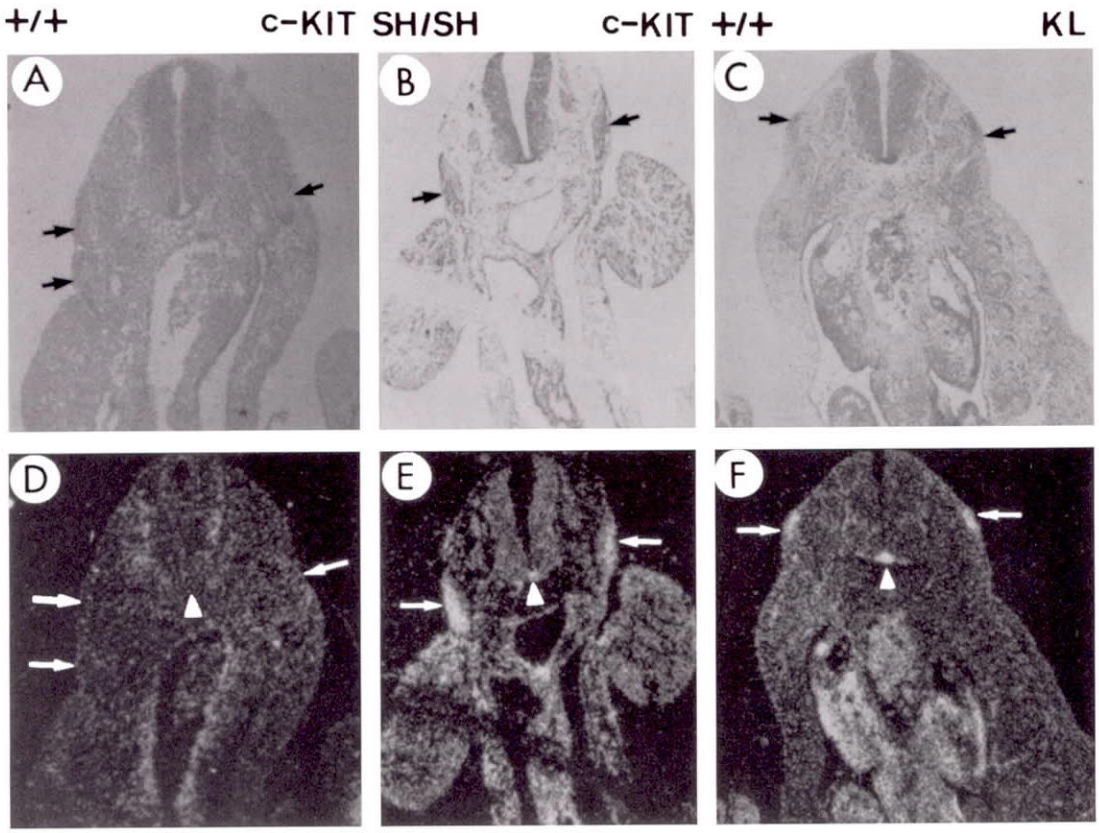

Fig. 4. Ectopic c-*kit* expression at sites of kit ligand expression in E10½ W^{sh}/W^{sh} embryos, as determined by in situ hybridization. (A,B) Bright-field images of cross sections of the trunk of +/+ and W^{sh}/W^{sh} embryos hybridized to a c-*kit* antisense probe. (D,E) Dark-field images of A and B. Arrows indicate somites and the arrowhead the floorplate of neural tube. Note labeling of these structures in the mutant. (C,F) Bright- and dark-field images of a cross section of a ICR×CB6F1 embryo hybridized to a KL antisense probe. Note labeling of somites and floorplate. Scale bar in A, 75 μm.

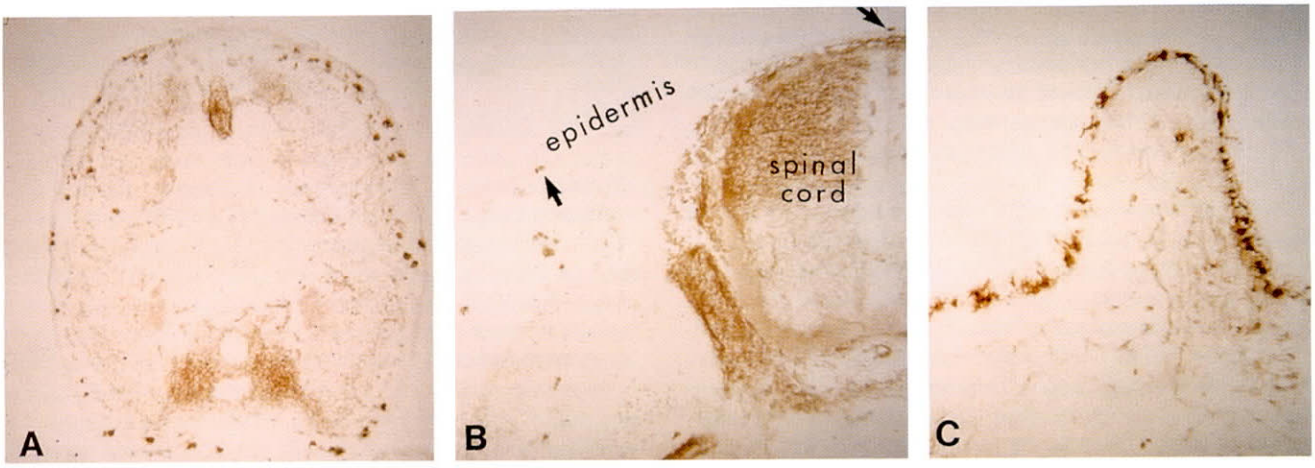

Fig. 5. Melanoblast density varies at different sites in the skin of E13½ +/+ embryos. Sections of embryos were stained with anti-kit antibody and melanoblasts identified as stained cells in the epidermis. The dermis contains both c-*kit* positive melanoblasts and potential mast cell progenitors. (A) Cross section of the proximal tail. Melanoblasts are numerous. (B) Cross section of the lower thorax. Melanoblasts (arrows) are sparse. (C) Section of the ear pinna. Melanoblasts are very abundant.

rearrangement within the vicinity of the c-*kit* gene (Duttlinger et al., 1993), consistent with the idea that several control elements are affected by this mutation. Recent experiments indicate that the W^{sh} mutation involves sequences 5′ to c-*kit*, between c-*kit* and the PDGFRα gene (Duttlinger et al., unpublished data).

sash OR BELTED PHENOTYPES REFLECT VARYING MELANOBLAST DENSITIES ALONG THE BODY AXIS DURING EMBRYOGENESIS

To investigate the formation of the '*sash*' in W^{sh}/+ mice, the distribution of melanoblasts in E11½ and E13½ normal and W^{sh}/+ embryos has been analyzed (Duttlinger et al., 1993). Interestingly, in the epidermis of normal E13½ embryos, the melanoblasts are distributed in a graded fashion with high densities in the rostral and caudal regions and a minimal density in the lumbar region (Figs 5, 6). In W^{sh}/+ embryos melanoblast numbers are reduced at all levels, with very few present in the lumbar region. In normal E11½ embryos melanoblasts are already distributed at varying densities along the body axis and their numbers are reduced in W^{sh}/+ embryos. Therefore, the melanocyte deficit in W^{sh}/+ embryos, which results in the *sash*, is established between E10½ and E13½ and may stem from a reduction of cells in the lumbar region essentially to zero, or below the minimal density required for pigmentation. Therefore, the *sash* appears to arise from a generalized effect of the mutation on melanoblast number (presumably due to ectopic c-*kit* expression) in combination with an uneven distribution of melanoblasts in normal embryos. The relatively low density of melanocyte precursors in the thorax and rump flanking the future *sash* is maintained at E15½ (unpublished observations). These cells are apparently able to colonize and contribute pigment to large contiguous areas. This is consistent with the observation that early melanocyte precursors produce clones that remain coherent during development, resulting in pigmented areas (patches) with sharp boundaries (Mintz, 1974). The low density and perhaps time-dependent restriction of migration of these melanocytes may account for their lack of colonization of the *sash*/belt region.

Further support for the proposed cellular mechanism of *sash*/belt formation in W^{sh}/+ mice comes from white spotting phenotypes in transgenic mice expressing the c-*kit* protein of the dominant W^{42} allele. In these mice the W^{42} allele was expressed ectopically under the instruction of the human actin promotor. The transgenic mice displayed irregular white dorsal and ventral spots, in agreement with the dominant negative characteristics of the c-kit^{W42} protein product (Ray et al., 1991). Presumably, these coat color patterns resulted from transgene expression in c-*kit* expressing melanoblasts as well as in the microenvironment of migrating melanoblasts. However, the coat color patterns in these mice were irregular and apparently not a stably inherited genetic trait. Since the transgenic mice had been derived in (CBA/J × C57BL6/J) F_1 mice, the variable penetrance of the pigmentation phenotype in these mice could have been the result of differences in genetic background among the offsprings. To address this issue, one transgenic line (line 485 hAP-c-kit^{W42}) was backcrossed onto a

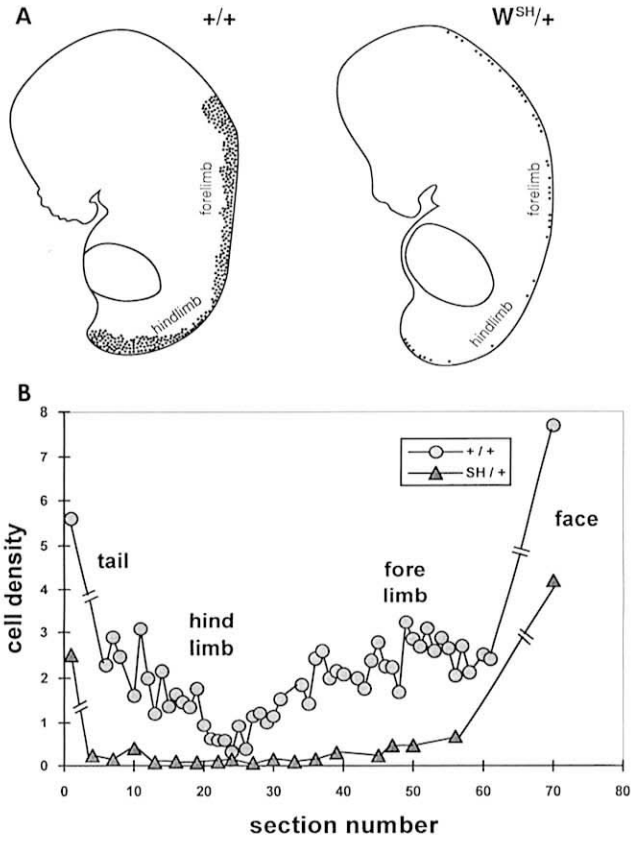

Fig. 6. Density of c-*kit*-positive cells (presumed melanoblasts) in +/+ and W^{sh}/+ E13½ day embryos determined by immunohistochemistry. (A) Density of c-*kit*-positive cells along the cranial-caudal axis assessed on sagittal sections (head not included). (B) Density of c-*kit*-positive cells along the cranial-caudal axis assessed on cross sections. Epidermal c-*kit*-positive cell bodies or processes in the dorsal half of cross sections were counted, the relative density computed, and values plotted on an arbitrary scale for sections numbered from the caudal end. The data for face and proximal tail were taken from both cross and sagittal sections, and plotted at an arbitrary position at each end. The face consisted of the region around the eyes and the dorsal surface of the snout.

C57BL6/J genetic background. A stable pigmentation pattern emerged (Fig. 3) similar to that seen in heterozygous W^{sh}/+ mice, but with a somewhat broader sash and a head spot. In these mice, the number of melanoblasts is presumably reduced all along the body axis as a result of uniform transgene expression within the melanoblasts and/or in the microenvironment of migrating melanoblasts and this effect is superimposed on the graded distribution of melanoblast precursors along the body axis. These results suggest that early melanoblasts in C57BL6/J are less numerous/viable than in CBA mice, possibly as a result of modifier genes affecting the melanoblasts or the microenvironment of melanoblasts. In agreement with this prediction, melanoblasts were more numerous at E11½ in an outbred mouse strain (ICR) than in C57BL6/J mice (K. Manova, unpublished data).

W/+ mice provide another case involving the c-*kit* receptor in which a belted phenotype is obtained. On

different genetic backgrounds, the pigmentation pattern of *W*/+ mice ranges from almost fully pigmented through belted to almost white (Dunn and Charles, 1937).

A belted pigmentation pattern similar to that in W^{sh}/+ mice is also seen in mice heterozygous for the *patch* mutation (Fig. 3). When homozygous, *Ph*/*Ph* mice die during embryonic development. The *Patch* locus encodes the PDGF receptor α-chain and is closely linked to c-*kit*/*W* (Smith et al., 1991; Stephenson et al., 1991; Duttlinger, unpublished data). The *patch* mutation consists of a deletion that includes the entire PDGF-A receptor. The 3′-deletion endpoint lies in between the 3′end of the PDGFR a gene and the 5′ end of the c-*kit* gene, but its location is not known precisely. Because of the similar pigmentation phenotypes of heterozygous W^{sh} and *patch* mice, it is reasonable to speculate that the pigmentation defect in *Ph*/+ mice results from inappropriate c-*kit* expression as in W^{sh}/+ mice, rather than from a half dose of PDGFR α and that both W^{sh} and *Ph* affect common elements in the 5′ control region of the c-*kit* gene. In agreement with these predictions, preliminary results have revealed ectopic c-*kit* expression in *Ph*/+ embryos (R. Duttlinger, unpublished data) and this ectopic c-*kit* expression may lead to an overall reduction in melanoblast numbers along the body axis of the embryo.

Taken together, the coat color patterns of W^{sh}/+, *W*/+ and *Ph*/+ mice, and of transgenic mice expressing the dominant c-kit^{W42} protein, as well as some other spotting mutations including *piebald* (Charles, 1938; Silvers, 1979), can be explained on the basis of general effects on melanoblasts and the graded melanoblast distribution at E13½; and this is in agreement with an earlier conjecture (Charles, 1938; Silvers, 1979).

An open question is why different regions of the body are unevenly populated by melanocyte precursors. First, the graded distribution may result from differential production of melanoblasts from neural crest along the body axis, i.e. more may be produced from cranial than from trunk neural crest. Second, it could arise from differential growth of body regions, i.e. the trunk region expands more than the head region from E10 to E13, or from different target sizes, i.e. the surface of the tail is smaller than that of the trunk. A third possibility is that the different melanoblast densities could be the result of differences in the cellular microenvironment through which melanocyte precursors migrate. Interestingly, the equivalent of the somitic dermatome along which melanocyte precursors migrate is absent in somitomeres and the dermis in the head originates entirely from the neural crest (Couly et al., 1992). In the tail, the neural tube and somites arise from the tail bud blastema, again raising the possibility of intrinsic and environmental differences for melanocyte precursors.

ROLE OF c-*kit* IN OVARIAN FOLLICLE DEVELOPMENT

Germ cells are formed from cells of the epiblast that move through the posterior primitive streak and first appear in the allantois at E7½ (Ginsburg et al., 1990). They then move into the hindgut, up the dorsal mesentery and laterally to the genital ridges, where they arrive by E11½. In normal mice the c-*kit* receptor is expressed highly in primordial germ cells (PGC) during their proliferative phase from E7½ to E13; thereafter, as the germ cells enter a quiescent period or meiotic prophase, c-*kit* expression is no longer detected (Manova and Bachvarova, 1991; Bachvarova et al., 1993). Early investigations by Bennett (1956) and by Mintz and Russell (1957) indicated that in mice with severe *Sl* and *W* mutations PGCs fail to increase in number during early development (E8½-E12), and that as a consequence very few are present in the fetal gonads. However, PGCs are formed in mutant animals and therefore c-*kit* does not appear to be involved in the initial determination of PGCs.

In mice, ovarian oocytes enter the diplotene stage around the time of birth and primordial follicles are subsequently formed. In a first wave, follicles in the central region of the ovary begin growth immediately and oocytes reach full size by 16 days of age. Thereafter, growing follicles are continually recruited from primordial oocytes throughout fertile life. The c-*kit* receptor is expressed in oocytes at high levels at all stages of postnatal development, starting in the diplotene stage (Manova et al., 1990). In contrast, KL is expressed in follicle cells of growing follicles and the expression increases during follicle development to high levels in the three layered follicles (Manova et al., 1993). Taken together these results suggest a role for c-*kit* in oocyte growth.

In some weak *Sl* alleles, significant numbers of germ cells are found in the gonad, but either female (Sl^{pan}, Sl^{t}, Sl^{con}) or male mice (Sl^{17H}) are sterile, implying that the c-*kit* receptor is essential for postnatal development of oocytes and spermatogonia, but that different properties of KL or its expression are important for development of female and male germ cells.

In order to elucidate the function of c-*kit* in oocyte development we investigated mice carrying the *Sl-panda* allele. Homozygous Sl^{pan}/Sl^{pan} mice are black-eyed whites with pigmented ears and scrotum and have mild macrocytic anemia; females are sterile, whereas males are fertile (Beechey et al., 1986). Molecular analysis indicated that the KL coding sequences are normal in the Sl^{pan} allele, but that the levels of the KL transcripts are consistently reduced in most tissues analyzed; therefore, the Sl^{pan} mutation affects KL gene expression (Huang et al., 1993). Histological analysis of ovaries from homozygous Sl^{pan} mice showed that the number of primordial oocytes in neonatal animals was reduced to 20% of normal, indicating an effect of the Sl^{pan} mutation on PGC's (Fig. 7). Furthermore, in juvenile and adult mice ovarian follicle development in homozygous mutant animals was arrested at the one-layered cuboidal stage (Fig. 7). Therefore, a reduced level of KL in Sl^{pan}/Sl^{pan} ovarian follicle cells appears to arrest ovarian follicle development, implying an essential role for c-*kit* in oocyte growth/maturation. Whereas KL is limiting in oogenesis, a reduced level of KL does not appear to affect spermatogenesis.

Two other *steel* alleles, Sl^{t} and Sl^{con}, specifically affect female fertility. In Sl/Sl^{t} females, follicles are arrested at a stage similar to that of Sl^{pan}/Sl^{pan}, but more germ cells are present (Kuroda et al., 1988). Unlike Sl^{t} and Sl^{pan}, in Sl^{con}/Sl^{con} females, follicle development appears to be normal, but they have only a few germ cells which are

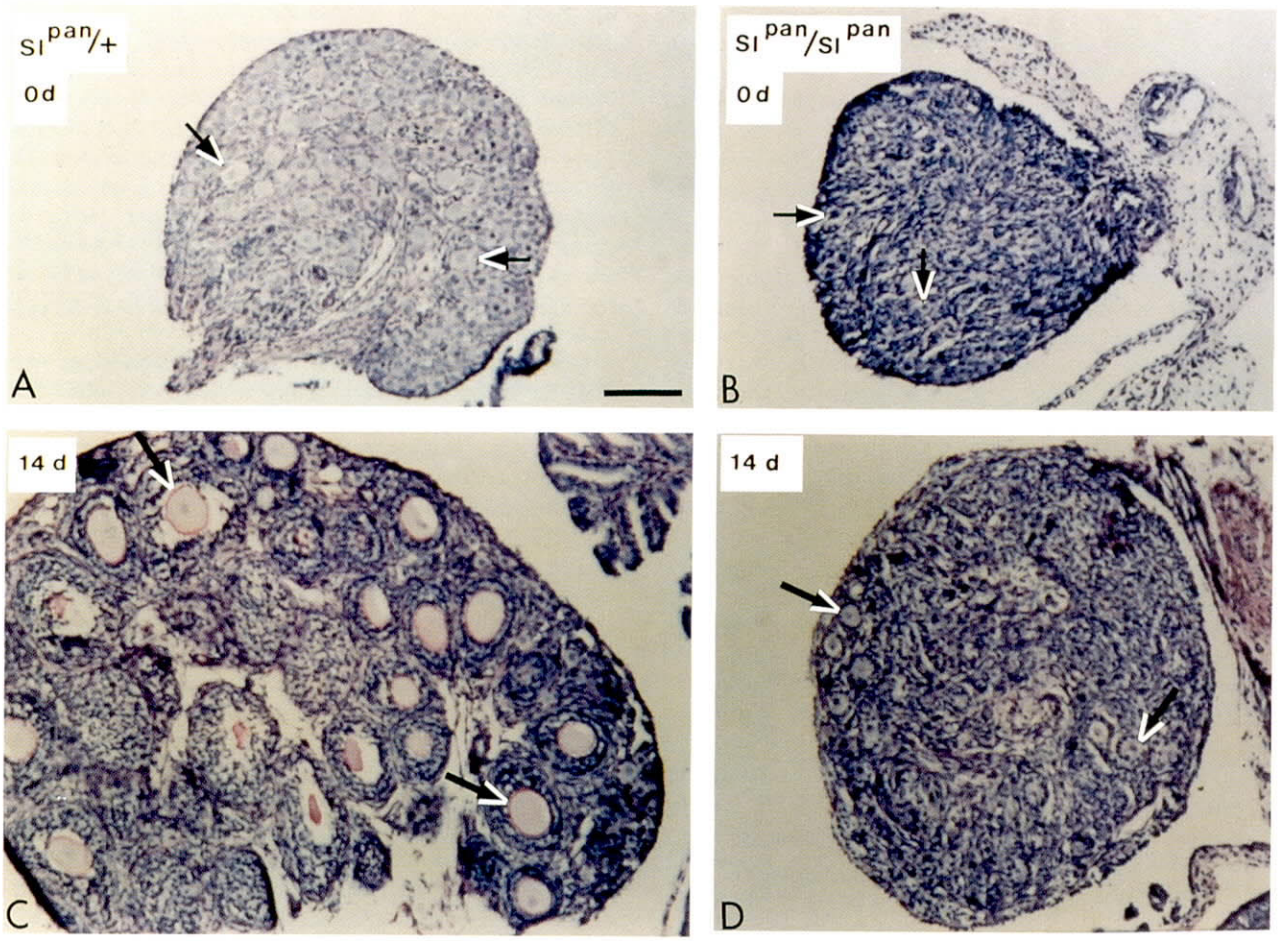

Fig. 7. Histological analysis of Sl^{pan} mutant ovaries. Sl^{pan}/+ ovaries are similar to wild-type ovaries. (A) Newborn Sl^{pan}/+ ovaries, many primordial and small growing oocytes (arrows) are present. In newborn Sl^{pan}/Sl^{pan} (B), only a few oocytes (arrows) are present. In 14 day Sl^{pan}/+ ovaries (C), most of the growing oocytes are approaching full size, each is surrounded by a narrow pink zona pellucida (arrows), and three or more layers of follicle cells. (D) In 14 day Sl^{pan}/Sl^{pan} ovaries, several follicles with small growing oocytes are present, but follicle and oocyte development is arrested early in the growth phase. Examples of oocytes in each panel are indicated by arrows. Paraffin sections of ovaries were stained with haematoxylin and periodic acid-Schiff reaction. Scale bar, 100 μm for all panels.

quickly depleted in adults, while in males spermatogenesis appears to be capable of some postnatal regeneration (Beechey and Searle, 1983).

In summary the c-*kit* receptor system is essential at several stages in gametogenesis. During development c-*kit* provides a proliferative and/or cell survival signal for primordial germ cells in a defined time period. In spermatogenesis c-*kit* is thought to facilitate proliferation/survival of spermatogonia during the first four cycles of spermatogonia proliferation/differentiation (Manova et al., 1990; Yoshinaga et al., 1991). In oogenesis c-*kit* appears to function in the diplotene stage of prophase of meiosis to facilitate oocyte growth in the early stages of follicle development (Huang et al., 1993).

CONCLUSIONS AND PROSPECTS

The discovery of the identity of the c-*kit* gene with the *W* locus has brought to light interesting pleiotropic roles of this gene in developmental processes, particularly melanogenesis, gametogenesis and hematopoiesis. Knowledge of c-*kit* function has facilitated the identification and characterization of the ligand for the c-*kit* receptor and the demonstration of allelism between *kit*-ligand and *Steel* provided a molecular basis for the relationship between *W* and *Sl* mutations in mice. A role for c-*kit* is now known in many cell types and lineages through studies of the numerous mutations that are available in this system. Interestingly, in both melanogenesis and gametogenesis, as well as in hematopoiesis, c-*kit* plays a critical role in both the earliest precursors during embryonic development, and in postnatal differentiating cells.

A corollary of recent studies of c-*kit* is the realization that the gene may function in cell types that are not known targets of *W* and *Sl* mutations (Motro et al., 1991; Manova et al., 1992). During embryonic development c-*kit* expression is seen in portions of the developing central nervous system, the olfactory epithelium and other tissues. This expression is typically observed in cells that have

ceased to divide and have begun their differentiation. In the adult animal c-*kit* and KL expression are prominent in the lung and in specific cells in the brain, in the hippocampus and the cerebellum (Manova et al., 1992; Motro et al., 1991; Mori et al., 1992). Redundant signaling mechanisms in these cell systems may compensate for the lack of c-*kit* function in these cell systems in *W* and *Sl* mutant mice. The elucidation of a role for the c-*kit* receptor system in the central nervous system is clearly an important task of the future.

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