Control of KIT signalling in male germ cells: what can we learn from other systems?

Sridurga Mithraprabhu¹ and Kate L Loveland^{1,2}

¹Monash Institute for Medical Research, Monash University, Clayton, Victoria 3168, Australia and ²Australian Research Council Centre of Excellence in Biotechnology and Development, Callaghan, NSW 2308, Australia

Correspondence should be addressed to K L Loveland who is now at Department of Biochemistry and Molecular Biology and Department of Anatomy and Developmental Biology, Monash University, Building 77, Level 1, Wellington Road, Clayton, Victoria 3800, Australia; Email: kate.loveland@med.monash.edu.au

Abstract

The KIT ligand (KITL)/KIT-signalling system is among several pathways known to be essential for fertility. In the postnatal testis, the KIT/KITL interaction is crucial for spermatogonial proliferation, differentiation, survival and subsequent entry into meiosis. Hence, identification of endogenous factors that regulate *KIT* synthesis is important for understanding the triggers driving germ cell maturation. Although limited information is available regarding local factors in the testicular microenvironment that modulate *KIT* synthesis at the onset of spermatogenesis, knowledge from other systems could be used as a basis for identifying how *KIT* function is regulated in germ cells. This review describes the known regulators of *KIT*, including transcription factors implicated in *KIT* promoter regulation. In addition, specific downstream outcomes in biological processes that *KIT* orchestrates are addressed. These are discussed in relationship to current knowledge of mammalian germ cell development.

Reproduction (2009) **138** 743–757

Significance of research on the KIT/KITL-signalling pathway

KIT receptor expression and interaction with KIT ligand (KITL) are crucial for proliferation, migration, survival and maturation of germ cells in embryonic and postnatal gonads (Yoshinaga *et al.* 1991, Dym *et al.* 1995, Packer *et al.* 1995, Orth *et al.* 1997, Vincent *et al.* 1998, de Rooij *et al.* 1999, Ohta *et al.* 2000, Yan *et al.* 2000a, Guerif *et al.* 2002, Prabhu *et al.* 2006, Runyan *et al.* 2006, Gu *et al.* 2009). In addition to serving essential functions in gametogenesis, KIT/KITL signalling is required for the development and function of haematopoietic cells, melanocytes, mast cells and interstitial cells of cajal, among other cell types. This information has been gained from studies of mice with mutations in the white spotting (*W*) locus (encoding KIT) and the *Steel* (*Sl*) locus (encoding KITL). Some homozygous *Kit* mutations are embryonic lethal, while other homozygous and heterozygous mutations have differential effects on fertility, anaemia and pigmentation (Besmer *et al.* 1993). In the pre-cancerous human testis, KIT is a marker of carcinoma *in situ* cell (CIS), the pre-invasive precursor gonocyte (Rajpert-De Meyts & Skakkebaek 1994) that eventually develops into germ cell tumours. Genetic modifications in the locus encoding KITL in humans have recently been linked with testicular cancer risk in two screens of two

independent population cohorts (Kanetsky *et al.* 2009, Rapley *et al.* 2009), firmly implicating KIT signalling as a key contributor to the development of germ cell tumours. Gain-of-function mutations in *KIT* contribute to specific human cancers, including small lung cell carcinoma, gastrointestinal stromal tumour (GIST) and prostate cancers (Kitamura & Hirota 2004, Sattler & Salgia 2004). Therefore, identification of endogenous factors that modulate *KIT* synthesis is important for understanding disease development. Such knowledge can in turn be applied to understand the fundamental biology of germ cell maturation. This review focuses on recent developments in knowledge that applies to regulation of KIT and addresses its potential to enhance our understanding of germ cell maturation.

Structures of KIT and KITL

KIT

The *Kit* gene is allelic to the *W* locus on mouse chromosome 5 (Chabot *et al.* 1988). The 21-exon gene encodes a 5.5 kb transcript that is translated into a product of ~145 kDa (Yarden *et al.* 1987). KIT belongs to a family of growth factor receptors with intrinsic tyrosine kinase activity that transduces growth regulatory signals across the plasma membrane. The KIT receptor has three main functional regions: the outer extracellular

domain, a transmembrane region and intracellular domain (Fig. 1). The outer domain consists of five immunoglobulin-like repeats required for ligand binding and dimerization (Blechman *et al.* 1995). The transmembrane region is a 23 amino acid hydrophobic domain, which anchors the receptor to the cell membrane. The 433 amino acid intracellular domain consists of three domains, with a proximal kinase region for ATP binding, a 70–100 amino acid non-conserved insert and a distal phosphotransferase kinase region (Blechman *et al.* 1993). Tyrosine (Tyr) residues in the

intracellular juxtamembrane domain serve as docking sites for signal transduction molecules that undergo activation (Roskoski 2005).

Two KIT receptor isoforms arise from alternative splicing in the extracellular juxtamembrane region (Fig. 1) distinguished by the presence or absence of a four amino acid region consisting of GNNK (Hayashi *et al.* 1991, Reith *et al.* 1991). Both isoforms bind with equal affinity to KITL (Caruana *et al.* 1999); however, KITL induces faster activation of the GNNK– form, while the GNNK+ remains active for longer in myeloma cells (Montero *et al.* 2008). This is in accord with the more rapid SRC signalling kinetics of the GNNK– compared with the GNNK+ isoform (Voytyuk *et al.* 2003).

Truncated forms of the KIT protein originate from 2.3 and 3.2 kb transcripts synthesized uniquely in spermatids (Sorrentino *et al.* 1991). The 3.2 kb transcript, TR-KIT, encodes part of the non-conserved insert from the C-terminal tail region and the distal phosphotransferase kinase region, and it lacks the entire extracellular and transmembrane domain (Albanesi *et al.* 1996). TR-KIT cannot interact with KITL, so spermatids are independent of KITL stimulation. This KIT isoform is also found in the residual sperm cytoplasm and there is evidence for it serving a function in mouse oocyte activation at fertilization (Sette *et al.* 1997). A soluble KIT receptor (S-KIT) isoform has also been identified, which is proteolytically cleaved from the surface of haematopoietic cells, mast cells and endothelial cells and also circulates in human plasma (Wypych *et al.* 1995, Tajima *et al.* 1998, Ishiga *et al.* 2000, Nakamura *et al.* 2004). The cleavage truncates S-KIT at the junction of the transmembrane and extracellular domains, and this isoform binds KITL with affinity comparable with the membrane-anchored protein. It blocks KITL stimulation of haematopoietic colony growth *in vitro*, and thus one function of S-KIT is to modulate KITL bioactivity (Dahlen *et al.* 2001).

KIT ligand

The ligand for KIT, KITL, also known as stem cell factor and mast cell growth factor, is encoded on chromosome 10 in mice. The gene consists of nine exons and is syntenic with the *Sl* locus (Zsebo *et al.* 1990a, 1990b). This 30 kDa glycoprotein is a member of the short-chain subgroup of helical cytokines, which includes macrophage colony-stimulating factor (CSF1), FLT3 ligand and platelet-derived growth factor (Zhang *et al.* 2000). The protein has four functionally important regions: a signal sequence, an extracellular domain, a transmembrane domain and a cytoplasmic domain (Fig. 2; Martin *et al.* 1990, Zsebo *et al.* 1990a, 1990b, Okada & Suda 1992). The 25 amino acid N-terminal signal sequence is removed during intracellular trafficking and is similar to that of many other proteins targeted for the cell surface. The extracellular domain contains

Figure 1 Schematic illustration of KIT receptor protein domain structures. The KIT extracellular domain has five immunoglobulin-like repeats (green) required for ligand binding. The transmembrane domain (dark blue) anchors the receptor to the cell membrane. The intracellular domain (light blue) consists of a proximal kinase region for ATP binding, a non-conserved kinase insert (yellow) and a distal phosphotransferase kinase region. Isoforms of KIT are created by differences in the extracellular juxtamembrane domain of KIT (GNNK+ and GNNK–). Soluble KIT is proteolytically cleaved near the junction of the extracellular and transmembrane regions and binds KITL with affinity comparable with that of membrane-bound KIT. TR-KIT is comprised of the catalytic kinase domain and part of the non-conserved insert from the C-terminal of the mature full-length KIT, but lacks the entire extracellular domain (Albanesi *et al.* 1996).

Figure 2 Schematic representation of the production of the two KITL isoforms. The *Kitl* mRNA encodes an intracellular trafficking domain (purple), which is cleaved when protein is targeted to the cell surface. The mature protein consists of an extracellular domain (blue), hydrophobic transmembrane domain (red) and cytoplasmic domain (green). Exon 6 (yellow) of *Kitl* encodes a site at which proteolytic cleavage occurs to generate a truncated soluble KITL (s-KITL). Cleavage also occurs with lower efficiency at a site encoded within exon 7 (pale blue) to generate a second s-KITL isoform. The membrane-bound KITL (m-KITL) lacks the primary cleavage site (exon 6) and remains as a membrane-bound protein (adapted from Loveland & Schlatt (1997)).

the major structural elements of KITL, including sequences that bind to KIT with high specificity. A hydrophobic domain of 26 amino acids functions as the transmembrane domain. When KITL binds to the KIT extracellular domain, it induces receptor dimerization and thus mediates autophosphorylation by the now adjacent tyrosine kinase domains. This generates a signal transduction cascade to the cell interior that ultimately leads to altered gene transcription (Lev *et al.* 1992), as discussed later in this review.

Alternative splicing at exon 6 of murine *Kitl* yields two distinct isoforms encoding membrane-bound (m-KITL) and soluble (s-KITL) proteins (Miyazawa *et al.* 1995). A 248 amino acid transmembrane precursor is processed at the primary cleavage site within the 84 bp exon 6 to produce a soluble 165 amino acid isoform (s-KITL; Flanagan *et al.* 1991, Huang *et al.* 1992). This was initially demonstrated by the transfection of stromal cells with human cDNA that contained or lacked exon 6 (Toksoz *et al.* 1992). The alternate isoform, m-KITL, lacks the primary cleavage site and encodes a predominantly membrane bound form of 220 amino acids. An alternate form of the s-KITL protein can also be produced following cleavage at a site on exon 7; however, the rate of cleavage is much lower than that incurred within exon 6 sequences (Huang *et al.* 1992).

Expression and function of KIT during gametogenesis

In the mouse, *Kit* mRNA is detected in primordial germ cells (PGCs) at embryonic day 7.5–8.0 (E7.5–8.0) and during their subsequent migration to the genital ridge (Fig. 3). This expression persists through their period of proliferation (Orr-Urtreger *et al.* 1990, Manova & Bachvarova 1991), while somatic cells along the migratory pathway and genital ridges synthesize *Kitl* (Matsui *et al.* 1990, De Felici *et al.* 1996, Runyan *et al.* 2006, Gu *et al.* 2009). Presentation of KITL to PGCs is required for their adhesion to somatic cells, proliferation, migration and survival prior to E9.0 after which down-regulation of KITL is associated with switching on the intrinsic apoptotic pathway in ectopic germ cells (Dolci *et al.* 1991, Godin *et al.* 1991, Runyan *et al.* 2006). In the absence of somatic cells, PGC migrates in response to a gradient of KITL (Farini *et al.* 2007). From E15 to day 3 in the mouse, KIT protein expression is markedly reduced, concordant with a period of germ cell quiescence (Prabhu *et al.* 2006).

Figure 3 KIT expression and function during gametogenesis. The timing of first appearance for different types of germ cells and Sertoli cell maturation status during testicular development are illustrated. Dark blue boxes represent specific germ cell stages when KIT is expressed. Dark green boxes illustrate the expression of KITL in Sertoli cells. The interactions of KIT and KITL yield distinct cellular outcomes depending on the stage of germ cell development (represented in light blue rectangles). Purple, germ cell nucleus; red, Sertoli cell nucleus. The timing of germ cell and Sertoli cell development is adapted from McCarrey (1993).

In the postnatal rodent testis, between days 0 and 5, during the period of gonocyte migration from the centre to the periphery of the seminiferous cord, *Kit* mRNA is not readily detected in the mouse testis (Hasthorpe *et al.* 1999, Prabhu *et al.* 2006). Synthesis of *Kit* mRNA and protein during testicular development is concordant with the first appearance of differentiating spermatogonia, which occurs at approximately day 7; *Kit* mRNA persists at relatively lower levels in meiotic pachytene spermatocytes (Prabhu *et al.* 2006). It is intriguing that *Kit* mRNA can be detected in undifferentiated spermatogonia, while KIT protein expression is associated only with differentiating spermatogonia (Prabhu *et al.* 2006), as this suggests separate mechanisms exist to regulate *Kit* transcription and translation during spermatogenesis. The presence of KIT protein in spermatogonia has been routinely used as a marker to identify differentiating spermatogonia (Shinohara *et al.* 1999, 2000). KIT is also present in Leydig cells of the postnatal and adult testis and is implicated in their steroidogenic capacity and survival (Yan *et al.* 2000b, Rothschild *et al.* 2003, Prabhu *et al.* 2006). KITL protein is present in Sertoli cells starting from ages day 6 to 8 in the mouse testis (Rossi *et al.* 1991, Tajima *et al.* 1991a, 1991b). Culture of germ cells and immature Sertoli cells from day 2 and day 5 mice with the ACK2 antibody to block the interaction of the KIT receptor with KITL caused dose-dependent inhibition of spermatogonial proliferation only in the day 5 mice, indicating that spermatogonial proliferation is directly associated with the onset of KIT receptor expression and its subsequent interaction with KITL (Tajima *et al.* 1994). The KIT and KITL interaction is required for maintenance and proliferation of differentiated KIT-positive spermatogonia (Fig. 3), but not for the preceding initial step of spermatogonial cell differentiation (Ohta *et al.* 2000, 2003). KIT signalling is also critical for progression to and survival during the meiotic pachytene stage of spermatogenesis (Packer *et al.* 1995, Vincent *et al.* 1998, Yan *et al.* 2000a). Transcriptome analysis of spermatogonia from day 7 mouse testes stimulated with s-KITL for 24 h indicated that KITL modifies the expression of genes known to be up-regulated or down-regulated in spermatogonia during the transition from the mitotic to the meiotic cell cycle. This is consistent with a role for KIT activation by KITL in male germ cell entry into meiosis (Rossi *et al.* 2008).

Regulators of *KIT* mRNA synthesis

Growth factors

The biological significance of *KIT* expression at the onset of spermatogenesis relates to its essential role in spermatogonial differentiation (de Rooij *et al.* 1999). Hence, the timing of mRNA up-regulation is consistent with the current understanding that KIT is a marker of

differentiating spermatogonia. However, limited data are available regarding the endogenous factors that modulate *KIT* at this crucial time point. A summary of the growth factors that are known to regulate *KIT* in the testis and other systems is provided in Table 1 and are discussed below.

Transforming growth factor β superfamily

Ligands of the transforming growth factor β (TGFB) superfamily of growth factors regulate many cellular functions including cell growth, adhesion, migration, differentiation and apoptosis. Their actions are essential for embryonic development, in particular for germ layer specification and patterning during embryogenesis (Chan & Etkin 2001). The mammalian TGFB superfamily contains ~40 known ligand subunits, each forming a dimer to affect signal transduction via a serine/threonine kinase receptor in target cells (Loveland *et al.* 2007, Schmierer & Hill 2007). This includes 3 TGFB isoforms, 4 activin β subunits, nodal, 10 bone morphogenetic proteins (BMPs) and 11 growth differentiation factors (Schmierer & Hill 2007). Of particular relevance to male reproductive physiology are the inhibins, activins and BMPs (Itman *et al.* 2006).

The only TGFB superfamily member known to control *Kit* mRNA and protein synthesis in the testis to date is BMP4. In one study, northern-blot analysis of *Bmp4* expression indicated that this factor is produced exclusively in Sertoli cells from day 4 testis and is subsequently down-regulated in older pre-pubertal Sertoli cells (Pellegrini *et al.* 2003). In other reports, the *Bmp4* mRNA was detected in germ cells in day 7 and older ages (Hu *et al.* 2004, Baleato *et al.* 2005). Its type I receptor, BMPRI1A, and one of its downstream transcription factors, SMAD5, are present both in proliferating PGCs and in postnatal spermatogonia (Pellegrini *et al.* 2003). Germ cell-enriched cultures from day 4 mouse testes exhibit increased KIT levels and display a mitogenic response to KITL following BMP4 treatment. Thus, the commitment of spermatogonia to differentiate may require the expression of BMP4 during the first spermatogenic wave (Pellegrini *et al.* 2003); however, this has not been tested *in vivo*. In a similar manner, BMP4 stimulation of *Kit* mRNA levels was also observed in the immature neural crest cell line, NCCmelb4m5, in which BMP4 was suggested to programme melanoblasts to enter a KIT-dependent phase of melanogenesis, required for their progressive maturation (Ito *et al.* 2004, Kawakami *et al.* 2008).

Other members of this superfamily, TGFB1 and activin A, are known to modulate *KIT* in systems other than the testis. Treatment with TGFB1 elevates *KIT* mRNA levels in T-leukaemia cells and in melanoblasts (Tomeczkowski *et al.* 1998, Kawakami *et al.* 2002). In contrast to these two studies that reported a stimulatory impact, the capacity of TGFB1 to reduce *KIT* levels

Table 1 Summary of regulators of *KIT* synthesis. Modulation of *KIT* by growth factors, hormones, cytokines and vitamins is indicated.

Type	Factor	Outcome	Cell type	References
Growth factors/hormones	BMP4	↑	Undifferentiated spermatogonia culture	Pellegrini <i>et al.</i> (2003)
	Activin A	↓	Immature melanocytes cell line NCCmelb4M5	Kawakami <i>et al.</i> (2008)
	TGFB1	↑	Murine erythroleukaemia cell line	Hino <i>et al.</i> (1995)
			T-leukaemia cell lines	Tomeczkowski <i>et al.</i> (1998)
			Melanoblast cell line	Kawakami <i>et al.</i> (2002)
	TGFB1	↓	Mouse peritoneal mast cells and cultured human mast cells	Norozian <i>et al.</i> (2006)
			Human colorectal carcinoma cell line HT-29 CRC	Bellone <i>et al.</i> (1997)
			Human acute myeloid leukaemia (AML) blast cells	Heinrich <i>et al.</i> (1995) and Hassan & Zander (1996)
			Human CD34+ progenitor cell population	Heinrich <i>et al.</i> (1995) and Sansilvestri <i>et al.</i> (1995)
	FGF2	↑	Murine erythroleukaemia cell line	Burger <i>et al.</i> (2002)
Cytokines	IL1A	↓	Inflammatory model of human umbilical vein endothelial cells	Konig <i>et al.</i> (1997)
	IL1A	↑	Bone marrow cells of irradiated mice	Neta <i>et al.</i> (1994)
	TNFA	↓	Human vascular endothelial cells	Buzby <i>et al.</i> (1994)
			Haematopoietic cell line	Jacobsen <i>et al.</i> (1995)
			Normal and acute myeloid leukaemic cells of human	Khoury <i>et al.</i> (1994)
Cytokines	TNFA	↑	Human acute myeloid leukaemic cells	Hassan & Zander (1996)
	IL4	↓	Human acute myeloid leukaemic cells	Hassan & Zander (1996)
			Human mast cell line HMC-1	Sillaber <i>et al.</i> (1991)
			Human colorectal cancer cell line	Lahm <i>et al.</i> (1995)
	IL1B	↓	Human vascular endothelial cells	Buzby <i>et al.</i> (1994)
Vitamins	IL10	↓	Human mast cell line HMC-1	Mirmonsef <i>et al.</i> (1999)
	CSF2	↓	Human mast cells	Welker <i>et al.</i> (2001)
	Retinoic acid (vitamin A)	↑	Spermatogonia in vitamin A-deficient mice	Schrans-Stassen <i>et al.</i> (1999)
			Gonocytes	Wang & Culty (2007) and Zhou <i>et al.</i> (2007)

↑, up-regulation of *KIT*; ↓, down-regulation of *KIT*. References for each factor implicated in *KIT* regulation are provided.

has been demonstrated in several systems (Heinrich *et al.* 1995, Sansilvestri *et al.* 1995, Hassan & Zander 1996, Bellone *et al.* 1997, Norozian *et al.* 2006). This repressive effect of TGFB1 on *KIT* may occur through accelerated degradation of *KIT* mRNA, rather than through a direct impact on mRNA synthesis (Dubois *et al.* 1994, Heinrich *et al.* 1995). Activin A represses *Kit* mRNA and protein synthesis over 48–72 h in a murine erythroleukaemia cell line (Hino *et al.* 1995). This erythroblast cell line has relatively high levels of *KIT*; with addition of activin, these cells undergo differentiation to form erythroid cells.

Retinoic acid

Vitamin A, or dietary retinol, is required for normal spermatogenesis, and its function has been extensively studied using *in vivo* models and culture systems. Adult vitamin A-deficient mice (VAD; deprived of dietary vitamin A) exhibit seminiferous tubules containing only Sertoli cells, type A spermatogonia and few pre-leptotene spermatocytes. These type A spermatogonia are almost all arrested before differentiation into A₁ spermatogonia, and *Kit* mRNA and protein levels are significantly reduced in VAD testis. Administration of vitamin A to these animals results in synchronized

spermatogenesis emerging from type A spermatogonia and enhanced expression of *Kit* (Schrans-Stassen *et al.* 1999). More recently, gonocyte culture studies have established that retinoic acid (RA) stimulated *Kit* levels by acting directly on germ cells (Wang & Culty 2007, Zhou *et al.* 2007). Besides increasing *Kit* expression, RA also up-regulated *Kitl* levels in Sertoli cells, resulting in increased levels of the early meiotic cell markers stimulated by RA gene 8 (STRA8) and DMC1. This activation is independent of germ cell viability and occurs through the phosphatidylinositol 3-kinases (PI3K) and MAP kinase (MAPK) pathways (Pellegrini *et al.* 2008). Thus, RA may control the timing of meiosis by influencing both somatic and germ cell compartments of the postnatal testis at least partially through activation of the KITL/KIT system.

Hormones and cytokines

KIT expression is modulated by various pro-inflammatory signals. Differential effects are induced by some cytokines depending on the model or type of cell system used for investigation. Among these, interleukin 1 α (IL1A), lipopolysaccharides, tumour necrosis factor α (TNFA), IL4, IL1B, granulocyte-macrophage CSF2, IL10 and basic fibroblast growth

factor 2 have been reported to modulate *KIT* synthesis (Sillaber *et al.* 1991, Buzby *et al.* 1994, Khoury *et al.* 1994, Neta *et al.* 1994, Jacobsen *et al.* 1995, Lahm *et al.* 1995, Hassan & Zander 1996, König *et al.* 1997, Mirmonsef *et al.* 1999, Welker *et al.* 2001, Burger *et al.* 2002). A summary of these published reports listing the cell type tested is provided in Table 1.

***KIT* promoter region**

The expression pattern of *KIT* is dynamic and thus highly regulated during development; however, the molecular basis for *KIT* transcriptional regulation remains largely unknown. *KIT* promoter analyses have been performed for mouse and human genes; in general, these studies indicate that interacting mechanisms may coordinately regulate *KIT* expression.

The transcription initiation site of the *KIT* promoter is located at 58 bp upstream of the initiation codon in both mice and humans. Analysis of the sequence upstream of the transcription initiation site reveals a TATA-less and a non-GC-rich promoter region. The 2.7–5.0 kb upstream sequences appear to be required for transcription inhibition (Yamamoto *et al.* 1993, Chu & Besmer 1995, Vandenbark *et al.* 1996). Investigation of six DNase I-hypersensitive sites within the promoter and first intron of the mouse *Kit* gene in haematopoietic cells, in PGCs (Cairns *et al.* 2003) and in mast cells (Berrozpe *et al.* 2006) revealed that DNase I-hypersensitive site 2 acts as an enhancer, integrating transcriptional signals in PGCs and haematopoietic cells. Potential binding sites for transcription factors pertinent to *KIT* expression, such as specificity protein 1 (SP1), stem cell leukaemia (SCL), putative binding sites for activator protein 2 (AP-2), GATA1, MYB, ETS and POU domains, have been identified (Yamamoto *et al.* 1993, Yasuda *et al.* 1993, Chu & Besmer 1995).

Upstream transcription factors

The upstream transcription factors that enhance or repress promoter region activity ultimately determine the cellular expression profile of *KIT*, and thus influence the cellular functions that *KIT* signalling facilitates. Regulation of *KIT* promoter activity is tissue specific and complex. Studies of these factors during normal testicular development and testicular pathologies will provide valuable clues about the mechanisms underlying development and maintenance of germ cell tumours in which surface *KIT* is consistently present.

Testicular transcription factors

Promyelocytic leukaemia zinc finger (PLZF)

The only transcription factor known in the testis to directly bind to *Kit* and affect its transcription during spermatogonial differentiation is PLZF, renamed as

ZBTB16. Male mice lacking *Zbtb16* exhibit progressive germ cell depletion due to exhaustion of the spermatogonial stem cell pool (Costoya *et al.* 2004). ZBTB16 directly represses transcription both of endogenous *Kit* and of a reporter gene controlled by the *Kit* promoter. A significant increase in *Kit* levels is also observed in spermatogonia isolated from *Zbtb16* null mice (Filipponi *et al.* 2007). ZBTB16 has also been suggested to affect the expression of *KIT* and aid haematopoietic stem cell mobilization from the bone marrow to the peripheral blood (Quaranta *et al.* 2006). ZBTB16-mediated negative control of *Kit* was demonstrated in normal haematopoietic progenitor cells and in acute myeloid leukaemias (Spinello *et al.* 2009).

AP-2G

The *KIT* promoter region has three putative AP-2-binding sites (Chu & Besmer 1995) within the 1.2 kb region upstream of the promoter (Yamamoto *et al.* 1993). AP-2 protein binds directly to the *KIT* promoter in melanoma cell lines, when tested using a luciferase reporter gene (Huang *et al.* 1998). Human foetal gonocytes coexpress *KIT* and AP-2G, suggesting that they play linked roles in maintaining the proliferative state of foetal germ cells (Pauls *et al.* 2005). With relevance to the development of germ cell tumours, *KIT* is a marker of intratubular germ cell neoplasia that is coexpressed with AP-2G (Hong *et al.* 2005). However, to date there has been no examination of whether AP-2G directly promotes *KIT* transcription in germ cells of the normal postnatal and adult testis.

SOHLH1 and SOHLH2

Spermatogenesis and oogenesis specific basic helix-loop-helix 1 and 2 are transcription factors essential for early spermatogenesis (Ballow *et al.* 2006). Both SOHLH1 and SOHLH2 proteins are present in the differentiating spermatogonia subtypes (Ballow *et al.* 2006, Toyoda *et al.* 2009). Since mice lacking either SOHLH1 or SOHLH2 show disturbed spermatogonial differentiation resulting in testicular phenotypes similar to *Kit*-mutant mice, it was suggested that both these proteins promote the differentiation of *KIT*-positive cells in testes (Ballow *et al.* 2006, Toyoda *et al.* 2009). This possibility remains to be formally tested.

SIX5

SIX5 belongs to the *SIX* family of transcription factors containing the SIX domain and the SIX homeodomain required for specific DNA binding (Kawakami *et al.* 2000). Loss of SIX5 results in male infertility and a reduction in testicular mass, as both survival and maturation of germ cells are affected. In addition, Leydig cells exhibit hyperproliferation, and increased FSH levels are detected in postnatal *Six5*^{-/-} mice.

KIT protein levels are decreased in *Six5*^{-/-} mice relative to wild-type mice leading to the suggestion that this reduction in KIT could underlie the disturbed spermatogenic viability and enhanced Leydig cell proliferation (Sarkar *et al.* 2004), although no direct link between *SIX5* and *KIT* gene has been established.

Non-testicular transcription factors

Specificity protein-1

The *KIT* promoter region contains several potential SP1-binding sites within the highly GC-rich region between 83 and 124 bp upstream of the 5' transcription initiation site in mice and humans. SP1 selectively binds to a single site within this region, and site-directed mutagenesis of the -93 or -84 promoter region reduces promoter-reporter activity to basal levels in *KIT*-expressing cells. These results indicate that SP1 binding is a selective process that is essential for core promoter activity, despite the presence of multiple additional SP1-binding sites in the *KIT* promoter (Park *et al.* 1998). Silencing of SP1 during erythroid differentiation resulted in elevated *Kit* expression, indicating a repressive role for SP1 in *Kit* regulation in haematopoietic progenitor cells (Hu *et al.* 2007).

Stem cell leukaemia

SP1 forms a complex with the SCL protein to maintain pluripotency of haematopoietic stem cells (Lecuyer *et al.* 2002). SCL, also known as T-cell acute lymphocytic leukaemia-1 is a basic helix-loop-helix transcription factor, first shown to function upstream of *KIT* in a haematopoietic cell line (Krosl *et al.* 1998). In normal haematopoietic cells, a motif containing overlapping SCL, SP1 and E2A sites within the promoter region regulates *KIT* transcription (Lecuyer *et al.* 2002). Appropriate activation of the *KIT* promoter depends on the combined interaction of all complex members. Since SCL is down-regulated in maturing cells, it has been suggested that loss of SCL inactivates the SCL complex with the resulting down-regulation of KIT expression, an important event in pluripotent haematopoietic cell differentiation (Lecuyer *et al.* 2002).

MYB and ETS

Of the several MYB and ETS-binding domains identified in the *KIT* promoter region (Chu & Besmer 1995), two MYB-binding motifs, MYB1, a partial repressor, and MYB2, a positive element, appear essential for the regulation of *KIT* expression (Vandenbark *et al.* 1996). A reporter construct containing the *Kit* promoter was activated when cotransfected with a MYB expression vector, providing further evidence of a role for Myb in the regulation of *KIT* (Hogg *et al.* 1997). Overexpression of the proto-oncogene c-MYB in avian

neural crest cells increased KIT and subsequent KITL-KIT signalling, transforming these cells into melanocytes and thereby establishing a role for c-MYB in KIT regulation (Karafiat *et al.* 2007). Similarly, c-MYB knockdown in normal erythroid progenitor cells demonstrated that c-MYB is required for KIT expression in erythroid cells (Vegiopoulos *et al.* 2006). It has also been shown that c-MYB and ETS2 coexpression augmented transactivation of *KIT* promoter constructs in contrast to cells transfected with either construct alone (Ratajczak *et al.* 1998). A role for the c-MYB in activation of germ cell *KIT* expression has not been investigated yet.

GATA1

Evidence that GATA1 represses *Kit* synthesis was demonstrated in a study employing a *GATA1*-null erythroblast cell line that proliferates in a KITL-dependent fashion. Upon restoration of GATA1 function, these cells undergo G₁ cell cycle arrest concordant with decreased levels of *Kit* (Munugalavadda *et al.* 2005). In the testis, GATA1 is expressed in Sertoli cells and in Leydig tumour cells (Ito *et al.* 1993, Yomogida *et al.* 1994, Onodera *et al.* 1997, Feng *et al.* 1998). Therefore, whereas GATA1 may regulate *Kit* levels in Leydig cells, it most likely does not play a role in regulating *Kit* levels in spermatogonia. This observation highlights the need to define the cell-type-specific mechanisms that affect KIT levels.

MicroRNAs

MicroRNAs are small non-coding RNAs that act as post-transcriptional regulators of gene expression (He & Hannon 2004). *Kit* has been described as a target of *Mir221* and *Mir222* during erythropoiesis, melanogenesis and in cancer progression (Felli *et al.* 2005, Felicetti *et al.* 2008). MicroRNAs are also prevalent during testicular germ cell tumour development (Looijenga *et al.* 2007), although the contribution of microRNAs that specifically affect *KIT* mRNA levels in the testis is yet to be determined.

KITL/KIT-signalling pathways

The first three immunoglobulin-like extracellular domains of KIT are required for ligand binding, while the fourth immunoglobulin domain is required for receptor dimerization (Blechman *et al.* 1995). Bivalency of the KITL facilitates the dimerization of KIT that induces rapid intracellular Tyr autophosphorylation (Reith *et al.* 1991, Lev *et al.* 1992). The residues that initially undergo phosphorylation are within the intracellular juxtamembrane segment, and these include Tyr residues Tyr567 and Tyr569 in the murine protein. Other phosphorylation targets are Tyr701, Tyr719 and Tyr728, located in the kinase insert domain (Roskoski 2005). The resulting

phosphotyrosine residues serve as docking sites for particular signal transduction molecules, which typically bind and phosphorylate a specific cytoplasmic protein during the initiation of the cellular signal transduction cascade (Fig. 4).

The activation and recruitment of different downstream-signalling pathways in response to the KIT/KITL interaction are critical for generating distinct developmental outcomes. Each orchestrates distinct cell functions, highlighting the need to study each of the different KIT-signalling pathways to understand how germ cell biology is modulated by KIT activity. In spermatogenesis, the KIT/KITL interaction is critical at the onset of spermatogenesis, while down-regulation of KIT signalling during spermiogenesis is also important. Evidence for the need to control KIT function was provided by generating mice with a mutation (D814V) that renders the KIT kinase constitutively active (Piao *et al.* 1996). Abnormalities emerge in spermiogenesis, with the transition from round into elongating spermatids interrupted (Schnabel *et al.* 2005). These observations demonstrate that KIT signalling must be reduced at later stages of germ cell development for normal sperm formation.

The KIT/KITL interaction is known to trigger five types of signalling pathways; the type of pathway selected appears to be unique to the cell type or developmental stage at which this interaction is invoked (Fig. 4). The key mediators of these pathways are PI3K, v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homologue (SRC) kinases, JAK/STATs, RAS/MAPK and the

phospholipase-C γ (PLC γ ; Sattler & Salgia 2004, Reber *et al.* 2006). Each pathway contributes to critical cell functions that include migration, proliferation and survival, as discussed below. For some of these, we have little to no information about their importance at each phase of testis development and spermatogenesis when KIT is active.

Phosphatidylinositol 3-kinase

The best characterized pathway downstream of KIT/KITL signalling of these with regard to structure–function relationships involves the PI3K heterodimer, composed of an 85 kDa regulatory subunit and a catalytic subunit of 110 kDa. Its potential substrates include phosphatidylinositol, phosphatidylinositol-4-phosphate and phosphatidylinositol-4, 5-bisphosphate (Duronio *et al.* 1998, Shepherd *et al.* 1998). Tyr719 of murine KIT (Y721 of human KIT) associates with the 85 kDa subunit of PI3K. This pathway has functions that vary with the cell lineage in KITL-mediated responses, including adhesion (via c-JUN and c-FOS activation), proliferation (via AKT and p70S6K) and survival (via AKT and BAD regulation; Serve *et al.* 1994; Fig. 4). Studies of the contribution of PI3K in KIT signalling employ a mutant form of KIT in which the Tyr at position 719, normally capable of recruiting PI3K when phosphorylated, is changed to a phenylalanine residue, termed Y719F.

KITL/KIT activation of the PI3K/AKT pathway appears to be crucial exclusively in postnatal stage spermatogenesis, since the only obvious phenotype in the Y719F

Figure 4 Signalling pathways and downstream cellular functions activated by KITL-mediated phosphorylation of KIT. Five pathways are known to be activated in response to KITL stimulation. The PI3K pathway results in cell survival, adhesion and proliferation (blue circles). The SRC kinase pathway is required for receptor internalization, chemotaxis and proliferation (purple circles). The MAP kinase pathway (green) mediates gene transcription and proliferation; JAK/STAT (yellow) signalling is essential for proliferation and differentiation. The PLCG pathway (orange) mediates KITL-independent sperm–egg fertilization and KITL-dependent proliferation in haematopoietic cells (adapted from Roskoski (2005) and Reber *et al.* (2006)). Double-line circles represent the pathways that are active during spermatogenesis.

mutant is sterility in homozygous males, arising from reduced proliferation and subsequent extensive apoptosis in spermatogonia (Blume-Jensen *et al.* 2000). These mice exhibited no impact of the mutation in embryonic gametogenesis (Kissel *et al.* 2000). The signalling pathway involving the recruitment of PI3K and p70S6 kinase has also been demonstrated to promote cell cycle progression during the initiation of type A spermatogonial proliferation (Feng *et al.* 2000). KITL-induced PI3K recruitment also leads to AKT activation (a substrate in PI3K pathway) and to subsequent phosphorylation of the pro-apoptotic factor BAD. This phosphorylation inhibits BAD activity, thereby promoting cell survival (Blume-Jensen *et al.* 1998). PI3K is also active during foetal germ cell migration, activating SRC kinase/AKT autophosphorylation. LY294002, a specific inhibitor of PI3K, abolished KITL-dependent PGC migration and inhibited AKT phosphorylation *in vitro* (Farini *et al.* 2007).

SRC kinase pathway

KITL induces the activation of multiple SRC family members, including SRC, TEC, LYN and FYN (Blume-Jensen *et al.* 1994, Tang *et al.* 1994, Linnekin *et al.* 1997). These SRC proteins associate with phosphorylated murine Y567 (Y568 of human) and murine Y569 (Y670 of human) in the intracellular juxtamembrane domain of KIT (Roskoski 2005, Reber *et al.* 2006). SRC protein kinases are required for (a) normal internalization of the KIT receptor via phosphorylation of the CBL proteins (Shivakrupa & Linnekin 2005) and (b) cell spreading and chemotaxis via the RAC1, RAC2 and MAPK p38 pathway (Samayawardhena *et al.* 2007). Mutations at Y567 and Y569 affect cell migration in IL3-dependent murine pro-B cell line (Ueda *et al.* 2002). In contrast to the PI3K function in the postnatal testis, mutation at Tyr 567 (Y567F), does not affect fertility of homozygous mutant males; instead, it affects only precursors of T-cell and B-cell development (Agosti *et al.* 2004). This demonstrates that KIT downstream-signalling pathways are specific for different cellular and developmental contexts *in vivo*. SRC kinase signalling also converges with other signalling pathways to affect cell-specific functions. SRC kinase signalling affects cell migration and is active during PGCs migration *in vitro*. The SRC kinase inhibitors, PP2 and SU6656, caused significant reduction in KITL-dependent PGC migration and AKT phosphorylation in mouse PGCs (Farini *et al.* 2007).

JAK/STAT pathway

Conventionally associated with cytokine signalling, Janus kinase family members play critical roles in haematopoietic lineages and in cell proliferation, as

illustrated by animals deficient in JAK1, JAK2 and JAK3 (Linnekin 1999). The key signalling molecules that activated downstream of JAK proteins are STATs. KITL addition to the human megakaryoblastic cell line MO7e and to normal haematopoietic progenitor cells induces phosphorylation of JAK2 and subsequently activates STAT1 (Deberry *et al.* 1997). KITL also stimulates STAT3 activation in a human myeloid cell line (Gotoh *et al.* 1996) and STAT5 in bone marrow-derived mast cells (Ryan *et al.* 1997). KITL-induced JAK/STAT activation is suggested to play a crucial role in foetal liver haematopoietic progenitor cell proliferation and differentiation (Linnekin *et al.* 1996). The JAK/STAT pathway is also activated in response to phosphorylation at Y567 (which also recruits SRC) and Y719 (recruits PI3K) for mast cell proliferation (Timokhina *et al.* 1998). In the *Drosophila* testis, in the absence of JAK/STAT signalling, germ line stem cells differentiate but fail to self-renew (Tulina & Matunis 2001). There is yet no evidence of a role for JAK/STAT activation during KIT/KITL signalling in the mammalian testis.

MAP kinases

Phosphorylation at Y703 in the KIT intracellular region binds the growth factor receptor-bound protein-2 (GRB2; Thommes *et al.* 1999). In this position, GRB2 associates with the son-of-sevenless protein, and this complex interacts with and activates the small G-protein, RAS. Activated RAS regulates the MAPK cascade first through recruitment of RAF1 and then MEK1/2, p38 and MAPK3/1 (Reber *et al.* 2006). These MAPKs directly influence transcription factor activity and thereby regulate gene transcription. MAPK signalling is also activated in mouse PGCs in response to recombinant KITL treatment (Farini *et al.* 2007). KITL-induced murine spermatogonial cell cycle progression is also partly mediated by the MAPK pathway, through transient activation of MAPK3/1 (Dolci *et al.* 2001).

Phospholipase-C γ

PLCG activation occurs in response to stimulation by m-KITL, but not s-KITL, through phosphorylation at KIT Y728 in myeloid cells (Gommerman *et al.* 2000). This demonstrates that there are different signalling outcomes mediated by distinct KITL isoforms. In addition, transfection of COS cells with the murine TR-KIT leads to activation of PLCG (Sette *et al.* 1998). TR-KIT-mediated PLCG activation is implicated in the resumption of meiosis in mouse eggs (Sette *et al.* 1998, 2002). Thus, this pathway is used in both KITL-dependent and KITL-independent signalling (Fig. 4).

Downstream factors and functions

Identification of KIT signalling targets is required to understand the cell development fates that KIT and KITL orchestrate. This section describes some of the downstream target proteins of KIT signalling, which may provide insight for research directed towards clinical application and understanding human diseases. There is currently little information available to link KIT signalling with direct transcriptional targets during spermatogenesis, and this remains an important avenue for future studies in relationship to male fertility.

SLUG (SNAI2)

SLUG is a member of the Snail family of zinc finger transcription factors, evolutionarily conserved proteins found in vertebrates and invertebrates, which have been implicated in the generation and migration of mesoderm and neural crest cells in several vertebrate species (Nieto *et al.* 1994). *Snai2*-mutant mice, like *Kit*- or *Kitl*-defective mice, have a complex phenotype including pigmentation, gonadal and haematopoietic defects (Perez-Losada *et al.* 2002). Testes from 6-week-old *Snai2*-deficient mice exhibit testicular atrophy and an overall reduction in seminiferous tubule size, which is also characteristic of *W/W^v* and *Sl/Sl^d* mouse testes. Activation of KIT by KITL was demonstrated to directly induce SNAI2 expression in the bone marrow of wild-type animals (Perez-Losada *et al.* 2002). SNAI2 is also required for melanocyte development, effected by KIT activation through the *mi/MITF* transcription factor in melanoblasts (Sanchez-Martin *et al.* 2002). These studies reveal that SNAI2 is a molecular target in the KITL and KIT pathway activation. No direct evidence for a role of SNAI2 in the testis at the onset of spermatogenesis, when the KIT/KITL interaction is crucial, is currently available.

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is one of the most important mediators of tumour angiogenesis, and a relationship between VEGF and the KITL/KIT-signalling pathway has been established over the past decade. VEGF levels were suppressed following KIT inactivation by treatment with the tyrosine kinase inhibitor, imatinib mesylate/ST1571, in GIST (Bono *et al.* 2004, Jin *et al.* 2006), small lung cell cancer (Litz & Krystal 2006) and neuroblastoma cell lines (Beppu *et al.* 2004). Use of the PI3K inhibitor, LY294002, has implicated KIT-mediated activation of PI3K in the regulation of VEGF mRNA, since the PI3K inhibitor led to reduced VEGF mRNA and protein levels (Litz & Krystal 2006). In the testis, the relationship of VEGF to the KITL/KIT pathway has not yet been investigated.

Cyclins

The D-type cyclins, cyclin D1, D2 and D3 are required for progression of cells through the G₁/S transition of the cell cycle (Feng *et al.* 2000). Expression studies indicate that cyclin D3 is present in spermatogonia of the immature mouse testis (Zhang *et al.* 1999), while cyclin D2 is required for spermatogonial differentiation in the adult (Beumer *et al.* 2000). KITL/KIT acts through the PI3K pathway to facilitate the up-regulation and nuclear accumulation of cyclin D3, thus inducing spermatogonial cells isolated from juvenile testis to proliferate (Feng *et al.* 2000, Dolci *et al.* 2001). The contribution of KITL/KIT to mediating cyclin D2 expression and subsequent spermatogonial differentiation in the adult testis is yet to be determined.

Summary and prospects

As knowledge of KIT-signalling biology expands, so does our potential to understand the intricate and interdependent manner in which testicular development and spermatogenesis occur. We know that KIT signalling drives many processes that are central to a wide variety of cellular activities, and each of these is, in some way, important to a particular stage of male germ cell maturation. It is therefore surprising that very little is known about the regulation of developmental-specific KIT expression in the testis. Control of KIT regulation in the testis has been relatively underinvestigated in comparison with the number of studies in haematopoiesis and melanogenesis (summarized in Table 1). However, recent studies have identified several factors, including TGFB superfamily ligands, ILs and RA, which are each independently implicated in transcriptional and translational regulation of KIT; among these only RA and BMP4 have been shown to positively regulate KIT in germ cells from a juvenile testis. It is increasingly evident from the studies of haematopoiesis that TGFB1 and activin are likely to play roles in negative regulation of KIT. Identifying factors in the testicular niche, which enhance or suppress *KIT* levels, is paramount for understanding the complex molecular network that is active during the first wave of spermatogenesis and addressing how spermatogenesis is sustained through adulthood.

The transcription factors SCL, SP1 and GATA1 are each implicated in non-testicular transcriptional regulation of *KIT*, while to date only ZBTB16 has been shown to be relevant for this in the testis. Transcriptional control of *KIT* in PGCs and spermatogonia is poorly understood, and understanding how this is achieved should reveal key components in the transcriptional network, which are deregulated in testicular germ cell tumours. Among the KIT downstream-signalling pathways, PI3K performs a broad repertoire of functions in the testis, affecting adhesion proliferation, survival

and maturation of germ cells. Since KIT is present in both CIS and seminoma cells, it would be useful to identify the signalling pathways that are activated during testicular tumour progression. This review has intended to provide an up to date overview of the upstream regulators and downstream effectors of KIT signalling in testis and non-testicular tissues, with the objective of enabling future discoveries that will aid the treatment of male infertility and disease, and to provide new avenues for fertility control.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research/work reported.

Funding

This work was supported by grants from the NHMRC of Australia (#545916, #545917) and a Strategic Grant from the Monash University Faculty of Medicine Nursing and Health Sciences.

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Received 29 December 2008

First decision 9 February 2009

Revised manuscript received 10 June 2009

Accepted 29 June 2009