The survival of differentiating embryonic stem cells is
dependent on the SCF-KIT pathway

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Summary
The stem cell factor (SCF)-KIT signal transduction pathway plays a role in the proliferation, differentiation
and survival of a range of stem and progenitor cell types but little is known about its function in embryonic stem
(ES) cells. We generated ES cells carrying a null allele of Kit as well as a knock-in allele that encodes an SCF-
independent hybrid KIT receptor that can be activated by the FKBP binding drug, AP20187. KIT null ES cells die
when induced to differentiate upon withdrawal of leukaemia inhibitory factor in monolayer culture. This phenotype is recapitulated in wild-type ES cells treated
with a KIT-neutralising antibody and reversed in mutant cells by activation of the hybrid KIT receptor.

Differentiating KIT null ES cells exhibit elevated levels of DNA laddering and reduced BCL2 expression, indicative
of apoptosis. We conclude that mouse ES cell differentiation in vitro is dependent on the SCF-KIT
pathway contrasting with the apparently normal differentiation of KIT null inner cell mass or epiblast cells
in vivo. This discrepancy could be explained by the presence of compensatory signals in the embryo or it could lend support to the idea of a phenotypic relationship
between ES cells and early germ cells.

Key words: KIT, Survival, ES cells, Differentiation, Apoptosis

Introduction
The stem cell factor (SCF) receptor KIT is a member of family
III of receptor tyrosine kinases (RTK) that activates a number
of signalling pathways leading to a multitude of biological
responses (Ashman, 1999). Mutations in the genes encoding
either KIT or SCF in mice result in pigmentation defects,
anæmia and reduced fertility (Ashman, 1999) and loss- and
gain-of-function mutations in KIT have been associated with
human diseases including piebaldism and cancer (Kitamura
and Hirota, 2004; Pullarkat et al., 2000; Spritz, 1994). The
SCF-KIT pathway has been reported to be an important
survival factor for many progenitor cell types including
primordial germ cells (PGCs) (Dolci et al., 1991; Guerif et al.,
2002; Mauduit et al., 1999; Yan et al., 2000), haematopoietic
stem cells (HSCs) (Caceres-Cortes et al., 1999; Engstrom et
al., 2003; Galli et al., 1995; Keller et al., 1995; Young et al.,
2006), neuronal stem cells (Erlandsson et al., 2004) and
melanocyte precursors (Ito et al., 1999; Wehrle-Haller and
Weston, 1995) and it has been proposed that the SCF-KIT
pathway promotes survival of these cell types by suppressing
apoptosis. A correlation between Kit expression and
pluripoeticy of embryonic stem (ES) cells has been reported
(Palmqvist et al., 2005) which indirectly suggests that this
signalling pathway may be important in a system with
immense therapeutic potential (Keller, 2005). We therefore
aimed to determine directly whether the SCF-KIT pathway
played a role in the survival or differentiation of murine ES
cells.

Differentiating mouse ES cells express varying levels of
SCF and KIT so it was difficult to control the activation of this
signalling pathway using standard methods of cell starvation
followed by addition of the ligand. We therefore developed a
novel strategy to specifically activate a form of KIT in ES cells
that is independent of the endogenous receptor and ligand. A
gene-targeting strategy was used to knock out the Kit gene in
ES cells and to knock in an SCF-independent form of the KIT
receptor. We used a pharmacologically activatable form of KIT
(FKB-KIT) where two FK506-binding domains (FKBP12) are
fused to KIT-signalling domains (Jin et al., 1998). The FKB12
domains allow intracellular protein dimerisation and reversible
activation in response to a lipid soluble dimeric form of the
drug FK506, called AP20187. Using this system, we reveal that the
SCF-KIT pathway plays a crucial role in the survival of
differentiating ES cells in vitro by suppressing apoptosis
through the pro survival protein, BCL2.

Results
Generation of mutant Kit ES cell lines
To produce a null allele at the Kit locus, E14 ES cells were
electroporated with the targeting vector pGNΔ-kit (Fig. 1A)
To generate homozygous multiplex PCR (Fig. 1D) and Southern blotting (Fig. 1E). We identified from 36 G418-resistant colonies using codons of KitW-lacZ/W-FKB cytometry using an anti-KIT antibody. Hygromycin-resistant cell lines were first screened by flow neomycin and hygromycin to identify ES cell clones in which KitW-lacZ/H9004 and KitW-lacZ/H11032 respectively. (E) Southern blot analysis of DNA isolated from KitW-lacZ/+/H9251. From 216 neomycin- and hygromycin-resistant colonies, the genotype was confirmed by multiplex PCR (Fig. 1D) and by Southern blotting (Fig. 1E). To produce an allele encoding the KIT receptor with an FKB domain fused to the KIT intracellular domain, the CK35 ES cell line (Bernex et al., 1996) and a heterozygous KitW-lacZ/W-FKB cell line was electroporated with the ΔFKB-kit vector (Fig. 1C). Colony formation was scored as mixed or differentiated generated from KIT null ES cells. In limiting concentrations (1 U) of LIF, a significantly (P<0.05) difference in the number of colonies that were scored as mixed or differentiated from KitW-lacZ/W-FKB ES cells compared with KitW-lacZ/+/H9251. However, in contrast to KitW-lacZ/+ cells, only 40% of colonies scored as undifferentiated, stem cell colonies when compared with KitW-lacZ/+/H9251. ES cells showed a less-flattened morphology in 100 U/ml LIF there was no significant (P>0.05) difference in the number of stem cell colonies generated from KitW-lacZ/ZW-lacZ and KitW-lacZ/ZW-FKB ES cells which indicated that the lack of KIT signalling did not alter the self-renewal capacity of ES cells. However, there was a significant (P<0.05) difference in the number of colonies that were scored as mixed or differentiated generated from Kit null cells (Fig. 2D), which suggests that the apparently reduced growth rate was due to the loss of spontaneously differentiating cells. These data provided the first hint that the KIT was involved in the production or survival of differentiating cells. A more dramatic effect was observed when LIF was withdrawn. KitW-lacZ/+ ES cells produced a high number (>200) of differentiated or mixed colonies but no colonies were detected when LIF was withdrawn from KitW-lacZ/ZW-lacZ and KitW-lacZ/ZW-FKB ES cells. In limiting concentrations (1 U) of LIF, a significantly (P<0.05) lower number of colonies were produced from KitW-lacZ/ZW-lacZ and KitW-lacZ/ZW-FKB ES cells compared with KitW-lacZ/+ cells. However, in contrast to KitW-lacZ/+ cells in 1 U/ml LIF where none of the colonies were scored as undifferentiated, a relatively high proportion (60%) of colonies produced from KitW-lacZ/ZW-lacZ and KitW-lacZ/ZW-FKB cells displayed a stem cell phenotype.
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KitW-lacZ/W-lacZ cell line isolated using high G418 concentrations from that line (data not shown).

Blocking the KIT pathway in wild-type ES cells results in a comparable phenotype

Wild-type E14 ES cells were assayed for their self-renewal capacity in the presence of a monoclonal anti-Kit antibody, ACK2, an antagonistic blocker of KIT function (Nishikawa et al., 1991). There was a slight (but not significant $P>0.05$) reduction in the total number of colonies generated in high concentrations of LIF in the presence of the antibody (Fig. 2E). However in the absence of LIF, no colonies were generated in the presence of the antibody and at limiting concentrations of LIF a higher proportion of colonies had an undifferentiated phenotype compared to control cells (Fig. 2E).

Differentiating KIT null cells die by apoptosis

We assessed whether the loss of colonies derived from KitW-lacZ/W-lacZ and KitW-lacZ/W-FKB ES cells on LIF withdrawal was due to programmed cell death. There was no significant ($P>0.05$) difference between the cell lines in the proportion of cells in G0-G1 and G2 phase of the cell cycle immediately after LIF withdrawal indicating that disruption of KIT signalling does not induce inappropriate cell-cycle arrest (Fig. 3A). There was, however, a significant ($P<0.05$) difference in the pre-G1 peak between the KitW+ (30%) and KitW-lacZ/W-FKB (80%) cells 3 days after LIF withdrawal, which could suggest that the differentiating KitW-lacZ/W-FKB cells are dying by apoptosis. To support this finding we performed a DNA fragmentation assay to detect the characteristic DNA ladder formation that is well documented to denote internucleosomal cleavage of DNA (Cohen and Duke, 1984). We detected a ladder in differentiating KitW-lacZ/W-FKB cells that had been grown in the absence of LIF for 3 days but not in undifferentiated KitW-lacZ/W-FKB cells nor in undifferentiated or differentiated KitW+/+ cells (Fig. 3B). This further supports our hypothesis that in the absence of KIT signalling, differentiating ES cells die by apoptosis. It is interesting to note that a fragmentation ladder was detected in DNA from wild-type cells after LIF withdrawal when ten times as much DNA had been analysed (Duval et al., 2004) indicating that there is a quantitative rather than a qualitative difference between wild-type and KIT null cells.
Our efforts to further support this finding using the standard Annexin V and propidium iodide (PI) double staining revealed another interesting phenotype in KitW-lacZ/W-FKB and KitW-lacZ/W-lacZ cells. In a representative experiment, 87% of undifferentiated KitW-lacZ/W-FKB and KitW-lacZ/W-lacZ cells grown in the presence of LIF stained positive for Annexin V compared with only 13% in Kit+/+ cells (Fig. 3C). We tried to exclude the possibility that this staining represented an autofluorescent artefact of the null cells by comparing autofluorescent profiles of unstained cells (data not shown) and by minimising differences in staining or holding times between cell lines. Furthermore we performed a number of control experiments including the depletion of apoptotic cells using an Annexin V microbead kit (Miltenyi Biotec) before analysis that further supported the fact that this represented true Annexin staining. However, if this staining accurately reflected the levels of apoptosis in KitW-lacZ/W-FKB and KitW-lacZ/W-lacZ cells we would have expected a substantial reduction in their growth rate and plating efficiency in the presence of LIF but we observed only a slight (20%) reduction in cells that were assayed in parallel (Fig. 2B). We therefore propose that the binding of Annexin V in the KitW-lacZ/W-FKB and KitW-lacZ/W-lacZ cells might be due to higher levels of expression of acidic phospholipids, such as phosphatidylserine (PS) on the cell surface. (Normally PS is found on the inner leaflet of the plasma membrane bilayer.) This could suggest that Kit is involved in the regulation of anionic lipid asymmetry on the plasma membrane but further studies are required to confirm this prediction.

The SCF-KIT signalling pathway promotes the survival of a number of progenitor cell types by upregulation of the pro-survival protein BCL2 (Carson et al., 1994; Dhandapani et al., 2005; Kimura et al., 2005; Zeuner et al., 2003). This mechanism has also been implicated in the prevention of apoptosis in differentiating ES cells upon LIF withdrawal so we tested the expression of BCL2 in wild-type and KIT null cells (Fig. 3D). BCL2 protein was detected at comparable levels in wild-type and KIT null cells grown in the presence of LIF and in wild-type cells after LIF withdrawal. However, no BCL2 was detected in KIT null cells growing in the absence of LIF which supports the hypothesis that KIT promotes the survival of differentiating ES cells and this is mediated at least in part via the BCL2 pathway.

KitW-lacZ/W-FKB mutant phenotype is rescued upon pharmacological activation

When maintained for two passages in LIF and in the presence of AP20187 KitW-lacZ/W-FKB cells reverted to the more flattened morphology (data not shown) and growth rate (Fig. 4A) characteristic of the parental Kit+/+ cell line. Similarly, in the self-renewal assays addition of AP20187 reverted the morphology and number of colonies derived from the KitW-lacZ/W-FKB cell line comparable with that of Kit+/+ (Fig. 4B,C). Importantly, the KitW-lacZ/W-lacZ cells did not show this phenotype reversal, eliminating any non-specific effect of AP20187.

To associate the observed phenotypic rescue with specific activation of KIT at the biochemical level, we performed western
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Fig. 4. (A) Growth rates of Kit\textsuperscript{+/+} and Kit\textsuperscript{W-lacZ/W-FKB} cells in the presence and absence of AP20187 during routine passage, expressed as the total number of cells present 2 days after plating 1×10\textsuperscript{5} cells. The solid bars within the individual scatters represents the median of six independent passages. (B) Total numbers of stem cell (SC), differentiated (DIFF) or mixed (MIX) colonies produced from Kit\textsuperscript{+/+}, Kit\textsuperscript{W-lacZ/+}, Kit\textsuperscript{W-lacZ/−} and Kit\textsuperscript{W-lacZ/W-FKB} (−/FKBKit) in 100 or 0 U/ml of LIF in the presence of AP20187. The results shown are representative examples of three assays carried out in duplicate. (C) Combined number of mixed and differentiated colonies produced from Kit\textsuperscript{+/+}, Kit\textsuperscript{W-lacZ/+}, Kit\textsuperscript{W-lacZ/−} and Kit\textsuperscript{W-lacZ/W-FKB} ES cells 5 days after plating at low density in 100 U/ml LIF and 10 nM AP20187. The graphs show the mean (± s.e.m.) calculated from three independent experiments carried out in duplicate. (D) Western blot analysis of protein lysates isolated from Kit\textsuperscript{+/+}, Kit\textsuperscript{W-lacZ/+}, Kit\textsuperscript{W-lacZ/−} and Kit\textsuperscript{W-lacZ/W-FKB} ES cells grown in the presence (+) or absence (−) of AP20187 as indicated. Blots were sequentially probed with an antibody specific to KIT phosphotyrosine 730 (pY730) then an anti-GAPDH (GAPDH) antibody as a loading control.

Discussion

We have developed an inducible system that allows the activation of the SCF-KIT signalling pathway in ES cells in an environment devoid of complicating endogenous signalling and promiscuous ligands. We knocked out the endogenous Kit gene and subsequently knocked in the FKB-Kit fusion cDNA to the Kit locus by homologous recombination in ES cells. We report that differentiating ES cells devoid of KIT signalling die by apoptosis. This phenotype was partially recapitulated in wild-type ES cells using a blocking antibody and reversed when cells expressing FKB-Kit were grown in the presence of the dimerising agent, AP20187. It has been reported that up to 30% of wild-type cells undergo apoptosis when induced to differentiate on withdrawal of LIF (Duval et al., 2000) and we show that this increases to over 80% in cells devoid of KIT.

Interestingly we observed that a significant proportion of colonies were scored as ‘undifferentiated’ in limiting concentrations of LIF in self-renewal assays whereas wild-type cells produced only mixed or differentiated colonies under these conditions. This is possibly explained by the fact that ‘mixed’ colonies produced by Kit\textsuperscript{W-lacZ/W-FKB} and Kit\textsuperscript{W-lacZ/W-FKB} cells might be scored as ‘stem cell’ colonies owing to the death of differentiated cells associated with these mixed colonies. It may also reflect a phenomenon comparable with that described in stem cell selection strategies whereby the continuous removal of differentiated cells by drug selection enhanced the derivation of pluripotent stem cells (McWhir et al., 1996).

It has been proposed that LIF-deprived ES cells undergo apoptotic crisis preceding differentiation because the apoptotic signals are a prerequisite for triggering ES cell differentiation (Duval et al., 2000). Several pathways are induced in response to various apoptotic stimuli (Beere, 2005; Cho and Choi, 2002; Kim, 2002; Lavrik et al., 2005; Strasser et al., 2000) including the pro- and anti-apoptotic genes from the BCL2-BAX family (Green and Kroemer, 2005). SCF-KIT has been reported to upregulate the expression of BCL2, a pro-survival protein, thereby preventing apoptosis in a number of cell types (Carson et al., 1994; Dhandapani et al., 2005; Jin et al., 2005; Zeuner et al., 2003) and overexpression of BCL-2 prevents LIF-withdrawal-induced cell death of differentiating ES cells (Duval et al., 2004). We observed a high level of expression of BCL2 in both undifferentiated and differentiating wild-type ES cells and in
undifferentiated KIT null ES cells but no BCL2 expression was detected in KIT null cells 4 days after LIF withdrawal. BCL2 expression is maintained and regulated by a number of different factors and mechanisms (Fan et al., 2005; Shore and Viallet, 2005) depending on the cell type and apoptotic signal. (Domen and Weissman, 2000; Kimura et al., 2005). Our data indicate that BCL2 expression becomes KIT dependent upon induction of differentiation, whereas in the undifferentiated state it is controlled by other survival factors, probably including LIF signalling.

The fact that there is no apparent pre-implantation defect in mice lacking either KIT or SCF function (Geissler et al., 1981) suggests that the SCF-KIT signalling pathway is not essential for the survival of the differentiation products of the inner cell mass (ICM) from which ES cells are derived. This apparent discrepancy could be explained by genetic redundancy and the well-documented promiscuity between different receptor-ligand systems (Dubreuil et al., 1991; Waskow et al., 2004; Yu et al., 1998). An alternative explanation for the difference in phenotype between the ICM cell in vivo and ES cells in vitro could be the fact that ES cells do not accurately reflect their in vivo counterpart (Chambers and Smith, 2004) and in fact more closely resemble primordial germ cells (PGCs) (Zwaka and Thomson, 2005). This has been primarily based on the comparison of marker expression between ES cells, PGCs and ICM (Zwaka and Thomson, 2005). Interestingly KIT is expressed in ES cells and PGCs, but not ICM (Horie et al., 1991) and has been shown to play a crucial role in the survival and differentiation of primordial germ cells (Dessypris, 1994; Godin et al., 1991; Kissel et al., 2000; Matsui, 1998). The phenotypic analysis of mice carrying a specific point mutation in KIT suggested that a block in KIT signalling could be compensated for by other pathways in hematopoiesis, melanogenesis and PGC development but not during the PGC differentiation processes of spermatogenesis and oogenesis (Kissel et al., 2000). The fact that KIT null ES cells can survive and self renew but show an absolute requirement for KIT signalling for their survival during differentiation therefore provides another biological similarity between the ES cells and the germ cell lineage.

In conclusion, we have, for the first time, successfully used the pharmacologically inducible system to demonstrate a role for the SCF-KIT signal transduction pathway in ES cell survival during monolayer differentiation in vitro. As AP20187 can rescue the monolayer ES cells in vitro from death during the initial stages of differentiation we can now use this system to assess the role of SCF-KIT at later stages of differentiation into therapeutic cell populations. Furthermore proteomic approaches will provide a powerful strategy to define in detail the SCF-KIT signalling pathway in ES cells (Unwin et al., 2005). As human ES cells are being considered as a source of mature cell types that could be used in regenerative medicine a more detailed understanding of the signalling pathways involved in ES cell survival and differentiation will aid in the development of protocols to produce optimal quantities of therapeutic cell types (Keller, 2005).

Materials and Methods

Construction of the ΔFKB-Kit targeting vector

To generate the ΔFKB-Kit plasmid targeting vector, a 1200 base pair (bp) 5’ arm of homology comparable to that used in the pGNΔ-kit targeting vector (Bernex et al., 1996) was PCR amplified from DNA isolated from the E14 ES cell genomic DNA, using the primer pair OJB1-TGATACCAAAGGATCCCTCTTCTTGGA and OJB2-GGATCAAAGGCATCTTCTGTCTGT. The resulting amplicon was cloned into the pCR4-TOPO vector (Invitrogen), and an EcoR1 fragment from that was subsequently cloned into the multiple cloning site (MCS) of Vitality-phrGFP2a vector (Stratagene). The unnecessary IRES-irGFP fragment was removed from this vector by Xhol-PsiI digestion and a 4122bp Xhol-PsiI fragment from pBFJ3KIT, corresponding to the FKBP-KIT chimeric cDNA (Jin et al., 1998), was cloned in-frame with the 1200 bp 5’ arm of homology to generate pKPF3K3. After removing the 667 bp CMV promoter from pKPF3K3 by Nsi-I-NorI digestion, a 6100bp Nsi-I-NorI fragment from pGNΔ-kit was cloned into pKPF3K3, to generate pKPF3K3.1. The Vitality-phrGFP2a vector allows for directional insertion of eukaryotic resistance genes, contained in pre-fabricated modules, into the core vector, by Cre-mediated recombination. A 3100 bp pExchange module EC-Hyg (Stratagene) was introduced into pKPF3K3.1 by Cre mediated site-specific recombination to introduce a floxed hygromycin resistance cassette into the final ΔFKB-kit targeting vector that was linearised at the unique 3’yl site before electroporation.

ES cell maintenance

The feeder-independent mouse E14 parental ES cell line was maintained in Glasgow’s minimal essential medium (GMEM) as described (Jackson et al., 2002) without feeders and in the presence of leukaemia inhibitory factor (LIF). LIF was produced as described previously and immobilising the conditioned medium from COS7 cells transfected with the pB10 LIF-DIA expression construct (a gift from Austin Smith) (Smith, 1991). LIF-containing supernatant was tested and the concentration that showed detectable inhibition of ES cell differentiation was defined as 10 U/ml then 100 U/ml was used in standard maintenance medium.

Electroporation of targeting vectors

To generate the KitWlacZ/KitWlacZ+ cell line, 1×10^5 E14 ES cells were electroporated with 150 μg Norf-linearised pGNΔ-Kit vector by applying a single pulse at 0.8 mV, 3 μF in a Bio-Rad Gene Pulser. Cells were plated on gelatin at 3×10^5 cells/100 mm dish and selected after 24 hours in 150 μg/ml G418 for 10 days. To generate KitWlacZ/KitWlacZ+ KitWlacZ/KitWlacZ+ cells, the KitWlacZ/KitWlacZ+ cell line was plated at 1×10^5 cells/100 mm plate then selected with 2000 μg/ml G418 for 15 days. To obtain the KitWlacZ/KitWlacZ+ KitWlacZ/KitWlacZ+ cell line, KitWlacZ/KitWlacZ+ cells were electroporated with the Syl-linearised ΔFKB-Kit vector and selected with 150 μg/ml G418 and 175 μg/ml hygromycin for 10 days. In all cases, resistant colonies were picked, replicated in 96-well dishes and genomic DNA was isolated from expanded colonies for screening, using established protocols (Laird et al., 1991).

Screening of homologous recombinants

Multiplex PCR was used to screen for correctly targeted KitWlacZ/KitWlacZ+ and KitWlacZ/KitWlacZ+ colonies using primer set: OJB56, AGTTGGCCGATGACTTTAAT; OJB57, AAAAGCCAACGATCCTACCTC; OJB58, ACAGATGAAACGCCGAAGAG; OJB59, AGTTTGCGCATGACTTTAAT; OJB60, AAAAGCCAACGATCCTACCTC; OJB58, ACAGATGAAACGCCGAAGAG; OJB59, AGTTTGCGCATGACTTTAAT. Potential KitWlacZ/KitWlacZ+ colonies were screened by flow cytometry using the tricolor conjugate KIT-specific antibody (α-CD117) (Caltag Medsystems) then confirmed by Southern blotting (Glasbrenner et al., 2005) of EcoRI-digested genomic DNA, hybridised using an external probe (Bernex et al., 1996). Self-renewal assays

ES cells were plated in duplicate onto 35-mm gelatin-coated wells at a density of 1×10^5 cells per well in ES cell medium containing 100, 10, 1 or 0 U/ml LIF. After 5 days, colonies were stained for alkaline phosphatase activity using the AP leukocyte kit (Sigma, Poole, Dorset, UK) then examined microscopically for ‘differentiated’ (Jackson et al., 2004). Statistical analysis was carried out by Mann-Whitney U test using the GraphPad Prism software (GraphPad Software), P<0.05 was considered statistically significant.

Blocking KIT activity using ACK2

E14 ES cells were cultured for 2 days in ES cell medium with LIF and 10 ng/ml ACK2 (Insight Biotechnology) then plated in self-renewal assays as above in the presence or absence of ACK2.

Pharmacological activation using AP20187

AP20187, a synthetic dimer that can be used to induce dimersization of mutant FKBPI2 domains was a gift from ARIAD Pharmaceuticals (Cambridge, MA; www.ariad.com/regulation kits). ES cell survival and self-renewal assays determined the optimal dose of 10 nM (data not shown). To activate the FKB-KIT fusion protein, ES cells were cultured in ES cell medium with 100 U/ml LIF and 10 nM AP20187 for two passages then plated in the standard self-renewal assays with or without AP20187.

Western blotting

Western blotting was performed as previously described using the anti-KIT phospho-antibody (pY730) (Biosource), anti-GAPDH monoclonal antibody.
DNA fragmentation assay
DNA was isolated as described previously from cells grown for up to 4 days in the presence or absence of LIF, separated by electrophoresis (2 μg/lane) then visualised by ethidium bromide staining.

Cell cycle analysis
Cells (1×10^6) were washed in PBS, resuspended in 0.3 ml PBS containing 50% FCS then fixed by addition of 0.9 ml cold (4°C) 70% ethanol. Staining with propidium iodide (PI) and flow cytometry was carried out following established protocols (Crompton et al., 1992).

AnnexinV-PI staining
Cells (1×10^6) were resuspended in binding buffer, labelled with FITC-conjugated AnnexinV (Bender MedSystems), washed and resuspended in binding buffer before the addition of a 488 nm laser and analysed using CellQuest software (Becton Dickinson).

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