THE ROLE OF THE SCF/KIT SIGNALLING PATHWAY IN EMBRYONIC STEM CELLS

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I DECLARE THAT THE WORK PRESENTED IN THIS THESIS IS MY OWN UNLESS OTHERWISE STATED

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ABSTRACT

Murine embryonic stem (ES) cells are derived from the inner cell mass of the developing embryonic blastocyst. These cells can self renew which allows them to be propagated indefinitely in the laboratory and they can differentiate into cell types derived from all three germ layers. Manipulation of the mouse genome using gene targeting techniques in conjunction with ES cell technology has provided valuable insights into embryonic development and cell lineage specification.

KIT is a trans-membrane receptor tyrosine kinase (RTK) that dimerises upon binding to its ligand, stem cell factor (SCF) resulting in the auto-phosphorylation of intracellular kinase domains. This activity is crucial for the transmission of signals from the cell surface to the nucleus. KIT is expressed on stem and progenitor cells of many lineages and defects in the SCF/KIT signaling pathway causes detrimental effects at both the cellular and physiological level. This project aimed to investigate the role of the SCF/KIT signalling pathway during murine ES cell differentiation and survival.

To assess the role of SCF/KIT signalling in ES cell proliferation and survival, we knocked out the *c-kit* gene in mouse ES cells to produce heterozygous ($Kit^{W\text{-}lacZ/+}$) and KIT Null ($Kit^{W\text{-}lacZ/W\text{-}lacZ}$) cell lines. The self renewal and differentiation profile of these cell lines revealed an auxiliary role for SCF/KIT during ES cell self renewal and an absolute role in survival upon *in vitro* differentiation. This phenotype of apoptosis upon differentiation was recapitulated in wild type E14 ES cells treated with a KIT neutralising antibody (ACK2). Wild type cells that were treated with the JNK inhibitor, SP600125 had a comparable phenotype to KIT null cells indicating that this could be one of the mediators of KIT signalling that has a protective role in the survival of differentiating ES cells.

We hypothesised that blocking classical apoptotic pathways might prevent the death on differentiation observed in KIT null cells. However, neither blocking the pro-apoptotic P38 pathway with the chemical inhibitor PD169316 nor over-expressing the pro-survival

protein BCL2 in KIT Null cells could prevent their apoptosis upon differentiation phenotype. This strongly suggests that these pathways are not involved in KIT mediated survival of differentiating ES cells.

Although compensatory mechanisms are thought to exist for defective KIT signaling *in vivo*, an absolute role is assigned to KIT during ES cell differentiation. Further analysis of micro array data comparing gene expression from wild type E14 and KIT Null cell lines may reveal the specific mechanisms of KIT mediated survival during differentiation onset.

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ABBREVIATIONS

α = anti-
AML= Acute myeloid leukemia
APC= allophycocyanin
BAC = bacterial artificial chromosome
BCL2= B-Cell Lymphoma 2
bHLH = Basic Helix-Loop-Helix
c-Kit= Stem cell factor receptor
DNA= deoxyribonucleic acid
EGF= Epidermal growth factor
ERK = extracellular signal-related kinase;
ERK= Extracellular Regulated Kinase (Erk)
FGF = fibroblast growth factor
Flt3= Fms-like tyrosine kinase 3
GIST= gastrointestinal stromal tumors (GIST);
HSC= Hematopoietic stem cells (HSC);
ICC= Interstitial cells of Cajal
ICM = inner cell mass
IL = interleukin
IP_3 = inositol-1, 4, 5-trisphosphate
JAKs= Janus kinases (JAKs)
JMD = Juxtamembrane domain
JNK = c-Jun N-terminal kinase
Kb = kilo base
kDa= kilo Dalton
KO= knockout
LB = Luria-Bertani bacterial culture broth
LIF = leukaemia inhibitory factor
MAPK = mitogen-activated protein kinase
M-CSF= macrophage colony stimulating factor
MEK= MAPK/ERK Kinase
MMP = matrix metalloproteinase
P38= P38α MAPK
PBS = phosphate-buffered saline
PCR= polymerase chain reaction
PDGF-B = Platelet-Derived Growth Factor-B
PI= Propidium iodide
PI3-kinase= phosphoinositide 3-kinase
$PIP_{3=}$ phosphatidylinositol-3,4,5-trisphosphate

PKC=protein kinase C
PLC-γ= phospholipase C-γ
PTB= phosphotyrosine binding (PTB) pleckstrin
Puro = puromycin
qPCR = quantitative polymerase chain reaction
RA= retinoic acid
RNA = ribonucleic acid RNA ribonucleic
RT= room temperature
SCF= stem cell factor
SCLC= small cell lung cancer (SCLC)
SDS= sodium dodecyl sulfate
SDS-PAGE= sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFK= Src family kinase (SFK)
SH2= Src homology 2
SHP-1 and 2 = Protein tyrosine phosphatases 1 and 2
SOC= superoptimal culture broth
SOCS= suppressors of cytokine signaling
SSC =saline-sodium citrate buffer
TBS = tris-buffered saline buffer
TE = Tris-EDTA buffer
TM = transmembrane domain
tr-KIT= truncated form of c-Kit

Chapter 1

Introduction

Chapter 1

Introduction

1.1 THE DERIVATION OF EMBRYONIC STEM CELLS

The first pluripotent cell lines, named Embryonic Carcinoma (EC) cells, were derived from the undifferentiated compartment of germ cell teratocarcinomas (Kleinsmith et al. 1964; Martin et al 1981; Rippon et al. 2004). These cells were employed as the early *in vitro* model for murine development (Kleinsmith et al. 1964) due to their ability to self renew and contribute to all tissues of the adult mouse after blastocyst injection (Illmensee et al. 1976). However, as EC cells are often aneuploid and harbor genetic abnormalities due to their teratocarcinoma origins (Andrews et al. 2005; Rippon et al. 2004), their contribution to medicinal therapy was severely compromised. This emphasised the requirement for a source of non- cancerous, pluripotent cells for clinical use.

Pluripotent, murine embryonic stem (ES) cells were first derived from the inner cell mass of the pre-implantation blastocyst (Evans et al. 1981; Martin et al. 1981). Similarly to EC cells, ES cells have the ability to self renew (and therefore can grow continuously in culture) and differentiate into derivatives of all three embryonic germ layers; endoderm, ectoderm and mesoderm as well as contributing to the germ line as demonstrated by Bradley et al (1984) following blastocyst injection. ES cells differ from transformed tumour cell lines as they retain normal karyotypes, even after extensive passage (Evans et al 1981) and differ from adult stem cells as their self renewal potential allows the generation of large quantities of specific cells without the need for intrusive, surgical procedures.

The ability of embryonic stem cells to differentiate into the three main germ layers, or pluripotency, has been of particular importance in many areas of research such as tissue engineering, drug discovery, regenerative medicine and the study of embryonic development and cellular differentiation (Keller et al. 1995; Keller 2005; Rippon et al.

2004; Ronnstrand 2004; Thomson et al. 1998; Smith 2001). Manipulation of the mouse genome using gene targeting techniques in conjunction with ES cell technology has provided valuable insights into embryonic development, tissue lineage specification and specialised cells for therapeutic application. (Thomas et al. 1987; Beddington et al. 1989)

1.2 THE MAINTENANCE OF UNDIFFERENTIATED ES CELLS

Although pluripotent cells from the inner cell mass (ICM) are short lived in the developing embryo *in vivo*, murine ES cells can be maintained in their undifferentiated state for prolonged passage *in vitro*. Initially, their self renewal capacity and successful prolonged growth was achieved upon co-culture with mouse embryonic fibroblast (MEFs) feeder layers (Evans et al. 1981; Martin 1981) indicating that MEFs not only acted as a matrix for ES cell growth but provided a source of growth factors and cytokines to maintain their proliferation and self renewal potential. Further to this, analysis of MEF conditioned media and molecular examination of the ES cells themselves identified several key extrinsic and intrinsic factors for pluripotency maintenance. Identified extrinsic factors include the regulatory factor of Differentiation Inhibiting Activity (DIA) which is identical to the pleiotropic cytokine Leukaemia Inhibitory Factor (LIF) (Smith et al. 1988; Williams et al. 1988), Bone Morphogenic Proteins (BMP) Ying et al. (2003) and Wnts (Aubert et al. 2002; Sato et al. 2004) whereas intrinsic factors include OCT4 and NANOG (Chambers et al. 2004).

1.2.1 Extrinsic factors

LIF, a member of the IL6 family of cytokines, signals through the transmembrane receptor gp130. Here, LIF binds to the LIF receptor and forms a trimeric complex with gp130 (Zhang et al. 1997) causing receptor dimerisation and signal transduction. The signal transducer and activator of transcription (STAT3) then phosphorylates to its active, dimeric form, locates to the nucleus to activate gene transcription for ES cell self-renewal (Yoshida et al. 1994; Niwa et al. 1998)

In the absence of serum, Bone Morphogenic Protein (BMP4) inhibits ES cell differentiation by the activation of SMADs which in turn, induce Id factors (Nakashima, Takizawa et al.2001; Ying et al. 2003). As BMP4 signalling primarily inhibits the differentiation of neuronal lineages and LIF signalling inhibits non-neural lineage development, both are required to maintain ES cell pluripotency in serum free ES cell cultures (Nakashima et al. 2001; Ruzinova et al. 2003). WNTs are also implicated in short term ES cell pluripotency by suppressing neural differentiation (Aubert et al. 2002; Sato et al. 2004).

1.2.2 Intrinsic factors

OCT4 is a transcription factor which is expressed at various stages during embryonic development including the unfertilised egg, the embryo before ICM / trophoectoderm segregation, the ICM, the epiblast, the migratory germ cell and the gonadal ridge (Pesce et al. 2001; Chambers et al. 2004). Although OCT 4 alone can not prevent ES cell differentiation, continuous expression of OCT4 (Niwa et al. 2000) in conjunction with gp130 pathway activation (Chambers et al. 2004) is required in order to maintain pluripotency. In addition, the transcription factor SOX2 has been shown to enhance pluripotency by working in conjunction with OCT4 to synergistically activate the transcription of target genes E.g. FGF4 (Ambrosetti et al. 2000).

Nanog is also a determinant of ES cell pluripotency (Chambers et al. 2003) and is only observed in cells that continually express *Oct4* (Niwa et al. 2002). Within the developing embryo, *Nanog* is first observed in the morulae, then highly expressed in the early blastocyst but reduces prior to embryo implantation. Following implantation, *Nanog* is then re-expressed in epiblast cells and E9-13 migrating PGCs and PGCs of the genital ridge (Chambers et al. 2003). Although *Nanog* works separately form the STAT3 pathway, it works in conjunction with LIF to provide ES cells with maximum self-renewal potential (Chambers et al. 2004).

1.2.3 Intrinsic factors and iPs cells

The importance of intrinsic factors becomes evident when considering the derivation of induced pluripotent stem (iPs) cells. iPs cells are generated from non-pluripotent adult somatic cells via the forced expression of retrovirally delivered transgenes involved in the intrinsic regulation of self renewal. During their derivation, iPs cells are cultured under conditions for self renewal and upon reaching a reprogrammed pluripotent state, forced transgene expression becomes silenced (Takahashi et al. 2007). iPs cells are similar to ES cells as they are able to self renew, express stem cell markers (murine ES cells SSEA-1 Takahashi et al. 2006) (human ES cells SEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, (Yu et al. 2007) and can be directed to differentiate into cell lineages of all three germ layers.

Takahashi et al (2006) derived the first murine iPs cells by the forced expression of *Oct-3/4*, *Sox2*, *c-myc*, and *Klf4* by retrovirus transfection of mouse fibroblasts. Following this, human iPs cells were generated by 2 independent groups. Takahashi et al (2007) induced pluripotency with the genes *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC* with a retroviral system whereas Yu et al (2007) used *OCT4, SOX2, NANOG*, and *LIN28* using a lentiviral system. (Takahashi et al 2007; Yu et al 2007)

Although human ES cells differ form murine ES cells on several accounts *e.g*. human ES cells require bFGF for self-renewal (Amit. 2007) and differentiate in the presence of BMP whereas murine ES cells depend on the LIF/STAT 3 pathway in conjunction with BMP in order to maintain pluripotency (Matsuda et al 1999 ; Niwa et al 1998;Ying et al. 2003) Takahashi et al (2007) have demonstrated that the transcription factors required to induce pluripotency in both human and murine models are consistent (OCT3/4, SOX2, KLF and *c*-MYC). Therefore, the system of transcription factors required for pluripotency maintenance is conserved between human and mouse. Interestingly, even though the same transcriptions factors can induce pluripotency in both species, transgene induced human cells grown under the culture conditions for murine ES cell/ iPs cell self renewal do not become reprogrammed. Therefore, extrinsic factors and signals maintaining pluripotency are unique for each species. (Takahashi et al. 2007)

1.3 ES CELL DIFFERENTIATION

Murine ES cells are directed to differentiate upon the withdrawal of LIF (Smith et al. 1988; Williams et al. 1988) and lineage specific differentiation is achieved with a recognised differentiation system, defined culture conditions and the addition of known growth factor and cytokine cocktails. Universally employed differentiation systems include monolayer induction, co-culture with cell or matrix proteins, methylcellulose assays or the formation of embryoid bodies (EBs).

When directed to differentiate in monolayers (LIF-), ES cells form mixed populations of cells from endodermal and mesodermal lineages (Mummary et al. 1990). Under serum free conditions, the addition of retinoic acid initiates the formation of parietal endoderm (Smith et al. 1987) and in the presence of FGF-supplemented N2b27 media; neural precursors develop (Ying et al. 2003).

Haematopoietic lineages can be induced *via* EB generation or by co-culture of ES cells with stromal cell lines (Nakano et al. 1994) or with explanted cells from the Aorta-Gonad-Mesonephros (AGM) region (Medvinsky et al. 1996; Krassowska, Gordon-Keylock *et al*. 2006). Common stromal lines employed include OP9 and AM20-1B4 (Krassowska, Gordon-Keylock et al. 2006) where both cell lines function to provide a microenvironment for haematopoietic differentiation and the development of multipotential haematopoietic lineages. Haematopoietic differentiation has also been observed with co-culture upon type IV collagen-coated dishes where haematopoietic lineages were derived from mesodermal precursors (Nishikawa et al 1998).

EBs are aggregates of ES cells formed during their growth within a density rich medium such as methylcellulose or by ‘hanging drop’ culture. As methylcellulose formulation does not determine the number of cells per aggregate, the EBs produced by this system are often irregular in shape and size whereas, EBs generated from ‘hanging drop’ cultures involve a defined cell number upon initial culture resulting in uniform aggregates. EBs uniformity is often required when comparing differentiation potential between cell lines or genetic modifications. EBs can generate cells expressing markers from all three germ

layers, (Itskovitz-Eldor et al. 2000) including hematopoietic cell lineages (Doetschman et al. 1985; Nakano et al. 1994; Nishikawa et al. 1998), endothelial cells (Risau et al. 1988; Yamashita et al. 2000), neuronal cells (Fraichard et al. 1995; Strubing et al. 1995) cardiomyocytes (Boheler et al. 2002) and bone derivatives (Buttery et al. 2001; Kramer et al. 2000). The 3D structure of EBs provides enhanced cell-cell and signalling interactions that are key to the generation of certain cell lineages that are otherwise unattainable by monolayer systems alone. (Doetschman et al. 1985; Keller 1995).

1.4 GENETIC MANIPULATION OF ES CELLS

Modifications to the mouse genome arising from randomly integrated DNA or targeted DNA mutations in ES cells *in vitro* and subsequent generation of transgenic mice *in vivo* has greatly advanced our understanding of gene function, specifically in regard to mammalian development and disease progression. **(**Capecci et al. 1989, Beard et al. 2006)**.** The mutagenesis of ES cells has several advantages. Firstly, ES cells can be screened *in vitro* for the desired genetic alteration before being reintroduced into the embryo. Secondly, it is possible to study the effect of the genetic manipulation on ES cell differentiation using defined differentiation models *in vitro*. The third being their ability to contribute to the germline and thus allow direct analysis of the genetic modification *in vivo* by the generation of a mutant mouse.

Both randomly inserted and targeted DNA can be introduced into the genome of ES cells by transfection, viral transfer or electroporation techniques (Capecci et al. 1989). A random insertion event results in the indiscriminant incorporation of DNA (cloned or genomic) into the ES cell genome. This method is generally employed to observe the effect of over expressing a desired gene during cellular differentiation or development. These sequences contain a selection cassette such as neomycin, puromycin or hygromycin to convey antibiotic resistance to resulting clones or contain reporter genes such as EGFP or LacZ to identify clones during selection (Gossler et al. 1989). Expression of these genes is driven by promoters such as the CMV promoter for constitutive expression (Schimidt et al. 1990) or an inducible promoter such as (CArG) for expression regulation (Worthington et al. 2005).

As ES cells are pluripotent *in vivo* and *in vitro* (Evans et al, 1981; Martin, 1981) and undergo homologous recombination, Thomas et al (1987) demonstrated that DNA could be integrated into the murine ES cell genome *via* homologous recombination and Bradley et al (1984) demonstrated that manipulated ES cells could contribute to the formation of chimeras and the germ line when reintroduced into murine blastocysts (Bradley et al., 1984). Gene targeting relies on the site specific introduction of exogenous DNA into chromosomal DNA via homologous recombination. Gene alterations in this process include gene deletion, gene addition or subtle base pair alterations. Generally, vectors comprise of a gene or reporter sequence of interest flanked by 5'- and a 3- regions of homology to the chromosomal loci of interest. As targeting frequency is low, a positive selectable marker such as neomycin (neo) which confers resistance to the antibiotic kanamycin in bacteria and G418 in mammalian cells is employed to identify potential clones during selection. Other positive selection cassettes include hygromycin B (hygro)-phosphotransferase and the puromycin (puro) gene. (Thomas et al. 1987; Lev. et al. 1994; Keller et al. 1995)

1.5 THE IMPORTANCE OF SCF/KIT IN ES CELLS

One signalling pathway associated with ES cell pluripotency (Palmqvist et al., 2005; Lu et al 2007) and survival upon differentiation (Bashamboo et al. 2006) is the tyrosine kinase receptor KIT and its ligand SCF. Although there is some debate over the role of KIT signalling in ES cell self-renewal (Lu et al 2007, Palmqvist et al. 2005; Bashamboo et al. 2006) the research by Bashamboo et al associated the KIT receptor with a key role in murine ES cell survival upon differentiation and was the founding paper on which this study was based. By employing a gene targeting strategy they ablated the *Kit* gene in ES cells and although the self renewal capacity of these cells was unaffected by the knock-out mutation, they demonstrated that KIT Null ES cells perished by apoptosis upon differentiation in parallel with a reduced expression of the pro-survival protein BCL2. This research, for the first time, demonstrated an absolute requirement for KIT during ES cell differentiation via the pro-survival protein BCL2 and defined apoptosis as the

mechanism of cell death mediated by KIT ablation. To investigate the specific mechanisms by which SCF and KIT may promote ES cell self renewal as well as survival upon differentiation, it is of importance to understand the biology of this receptor-ligand pair including specific signalling events downstream and relate these events to cell survival upon differentiation, lineage specification and disease progression

1.6 THE DISCOVERY OF THE KIT RECEPTOR AND ITS LIGAND STEM CELL FACTOR.

The discovery of *c-kit*, its ligand SCF and their related function was nearly 70 years in the making. Primarily, investigations into spontaneous murine pigmentation defects lead to the discovery of the White spotting (*W*) and Steel (*Sl*) loci in 1920 and 1957 respectively (Russel 1979). These mutations were named in response to their resulting phenotypes where (*W*) heterozygotes displayed a characteristic white belly spot due to an inability of melanocyte migration from the neural crest to the ventral ectoderm and *Sl* heterozygotes were grey in colour and often white belly spots due to their overall deficiency of melanocytes (Mintz et al. 1957; Russel, 1979; Geissler et al. 1988). Following from this, many other *W* and *Sl* mutations were discovered and it was shown that these mutations affected three independent stem cell lineages; melanocytes, hematopoietic stem cells and primordial germ cells where phenotype severity depended on the nature of the mutation within each gene (Reith et al. 1991; Russell, 1979) (Table 1).

The striking phenotypic similarities between *W* and *Sl* mutations, even though located on two separate loci lead to the belief that these mutations were in some way related and could possibly encode a receptor ligand pair (Geissler et al. 1988). This hypothesis was supported by transplantation studies where *W* mutants were rescued by neural crest (Mayer et al. 1968) (site of melanocyte precursors) and bone marrow cells from wild-type embryos upon transplantation. However, neural crest and bone marrow cells from wild-type embryos did not proliferate or migrate in the *Sl hosts*. Similarly, when melanocyte and haematopoietic stem cells from *W* embryos were transplanted into irradiated wild-type embryos, no stem cell proliferation or migration was observed whereas transplanted

Sl stem cells could both proliferate and migrate in wild-type environment (Bernstein et al. 1968; Mayer et al. 1968). Therefore, it was concluded that the *W* mutation affected the cellular constituent (receptor) whereas the *Sl* mutation affected the microenvironment in which the cells divided and migrated (ligand).

c-kit was originally identified as the viral oncogene *v-kit* which provided Hardy-Zuckerman IV feline sarcoma virus with transforming activity (Besmer et al. 1986). Following from this, c-*kit* was cloned, sequenced and mapped to mouse chromosome 5 and found to have genetic similarity not only to a tyrosine kinase region of v-*kit* but shared major structural features of with CSF1 and PDGF receptor subfamilies (Yarden et al. 1987). Geissler et al (1988) then demonstrated an absence of c-*kit* expression in the W^{19H} cell line, a mutation in which the entire *W* locus has been deleted. In comparison c-kit expression was strong in the wild-type control mapping the location of c-*kit* to the *W* locus on chromosome 5. Further analysis of 2 other *W* mutants revealed gene rearrangements within c-*kit* gene (Geissler et al. 1988). In 1990, Williams et al isolated the ligand for c-*kit* via immuno-precipitation techniques and reverse translation of this protein determined its identity as Stem Cell Factor (SCF). Following from this, the gene encoding, SCF was cloned, sequenced and mapped to murine chromosome 10 (Copeland et al. 1990)

Mutant	Mutation	Homozygous phenotype	Heterozygous phenotype	Molecular mutation	Reference
W	78 aa deletion 513 - 590	Perinatal lethal	Defects in melanogenesis	Deletion of transmembrane domain	*Nocka et al 1990* *Reith et al 1990*
W^{37}	E-582-K	Perinatal lethal	Mottled fur anemia	No kinase activity	*Nocka et al 1990* *Reith et al 1990*
W^{v}	T-660-N	White Severe anemia Sterile	Defects in melanogenesis Mild anemia	Reduced kinase activity	*Reith et al 1990* *Nocka et al 1990*
W^{42}	N-790-D	Perinatal lethal	White Anemic Reduced fertlity	No kinase activity	*Geissler 1981* *Tan 1990*
W^{41}	V-831-M	Mottled fur Mils anemia	White spotting Mild anemia	Reduced kinase activity	*Reith et al 1990* *Nocka et al 1990*

Table 1. Phenotypes of *W* mutations.

(aa=amino acid; E=Glutamine; K=Lycine; T = Threonine; N=Asparagine; D = Aspartic acid; V=Valine; M= Methionine)

1.7 KIT- A RECEPTOR WITH TYROSINE KINASE (RTK) ACTIVITY

The development of many multicellular organisms is regulated by a combined arrangement of polypeptides, growth factors, differentiation factors, cytokines and hormones. These factors act by binding to and activating cell surface receptors with protein tyrosine kinase activity (Ullrich et al. 1990). There are approximately 20 RTK subgroups (Fantyl et al. 1993; Lemmon et al. 2010) with conserved molecular topology comprising of a signal sequence, a large glycosylated, extracellular ligand binding region, a hydrophobic transmembrane domain a juxtamembrane regulatory unit, a cytoplasmic domain with tyrosine kinase activity and a carboxy (C-) terminal. The evolutionary conserved structural components of RTKs and their associated signalling pathways, even between species, demonstrates their fundamental role in development and gene regulation (Lemmon et al. 2010).

RTKs are activated upon specific ligand binding where upon binding, the extracellular region undergoes a conformational change causing two adjacent receptor monomers to dimerise. After dimerisation, ATP binds to the receptor where tyrosine kinase enzymes transfer a phosphate group from ATP to tyrosine residues on the receptor. The receptor is then autophosphorylated and allows the phosphorylation of other cellular substrates downstream. The conformation of the receptor's cytoplasmic domains determines molecule binding and these molecules are usually components of pre-existing signalling complexes that are found within the cytoplasm or located at the cell membrane. (Riedel et al. 1989). The activity of ligand binding, receptor dimerisation and auto-phosphorylation is crucial for the transmission of a signal from the cell surface to the nucleus (Majumder, et al. 1988). After ligand binding and auto-phosphorylation, the receptor ligand complex is internalised by endocytosis and will continue to signal (Bergeron et al. 1995) until they enter into cell sorting pathways for degradation.

1.7.1 KIT receptor structure

KIT (also referred to as stem cell factor receptor or CD117) is a member of the RTK type III subfamily which also includes platelet-derived growth factor receptor (PDGFα and β),

the macrophage colony-stimulating-factor receptor (CSF-1), and the Fl cytokine receptor (Flk2/Flt3) (Fantyl et al. 1993). As well as containing the conserved features of RTKs, the Type III receptors are a distinctive subgroup with an extracellular region comprising of five immunoglobulin domains and an intracellular kinase domain separated into a proximal and distal regions by a kinase insert (Ullrich et al. 1990). Crystal analysis of KIT reveals that SCF binds to the first three extracellular, immunoglobulin regions of KIT and the 'helix bundle' conformation of SCF facilitates dimerisation of the receptor upon binding (Lui et al. 2007). The importance of the fourth extracellular immunoglobulin region was reported by Blechman et al (1995) who demonstrated that a deletion of this region destabilised receptor dimerisation and inhibited subsequent downstream signalling events (Blechman et al. 1995). No definitive role has yet been assigned to the 5^{th} immunoglobulin region (Lui et al. 2007) but it is thought to be involved in proteolytic cleavage of the receptor from its membrane to its soluble form (Broudy et al. 2001).

Alternative splicing in both human and murine mRNA can give rise to four different KIT isoforms in human and two in mouse. Isoforms may lack a Gly-Asn-Asn-Lys (GNNK) sequence in the extracellular juxtamembrane segment (human and mouse) or lack a serine reside in the kinase insert region (human only) (Reith et al. 1991; Ashman, 1999). The isoforms have distinct characteristics where phosphorylation of KIT and ERK1/2 activation was more efficient in $GNNK^{-}$ than for the $GNNK^{+}$ isoform and $GNNK^{-}$ promoted anchorage-independent growth, loss of contact inhibition and increased tumorogenicity (Ashman, 1999) The function of the serine $^{(-)}$ isoform in the human is undefined but may be associated with PI3K signalling due its close proximately to the P13K binding site on the inter kinase site. (Crosier et al. 1993). A truncated 3.3kb alternative- transcript of c-*kit* arising from a second promoter region in intron 16 and comprising of only the second part of the kinase domain (Rossi er al. 1992; Lennartson et al. 2005) has been detected in murine spermatozoa (Sakamoto et al. 2004) and murine hematopoietic stem cell and multipotent progenitors (Zayas et al. 2008). Although tr-KIT lacks the ATP binding pocket, activation of the truncated form is thought to arise from interaction with the intracellular membrane protein FYN and activation is via kinase

phosphorylation and subsequent phosphorylation of PLCγ and mobilization of calcium from intracellular stores (Sette et al. 2002).

The cytoplasmic region of KIT comprises of a juxtamembrane Region (JMR) and a tyrosine kinase domain that is split into a N lobe (proximal) and a C lobe (distal) by a kinase catalytic site (Figure 1). The catalytic site is essential for enzyme function and stimulation of this site is regulated by an 'activation loop' (A loop). This A loop is 20-25 amino acid residues in length and inhibits kinase activity of the receptor by its location within the ATP binding pocket on the catalytic site. Upon ligand binding, dimerisation and conformation change of the receptor, the JMR becomes autophosphorylated on tyrosine residues (Tyr568 andTyr570), the activation loop moves from its inhibitory position and the conformation of the intracellular kinase domains extend and open allowing access to ATP to the ATP binding pocket in the kinase insert region. The receptor is now primed for phosphorylation on tyrosine residues and subsequent downstream signalling events. (Zou et al. 2008)

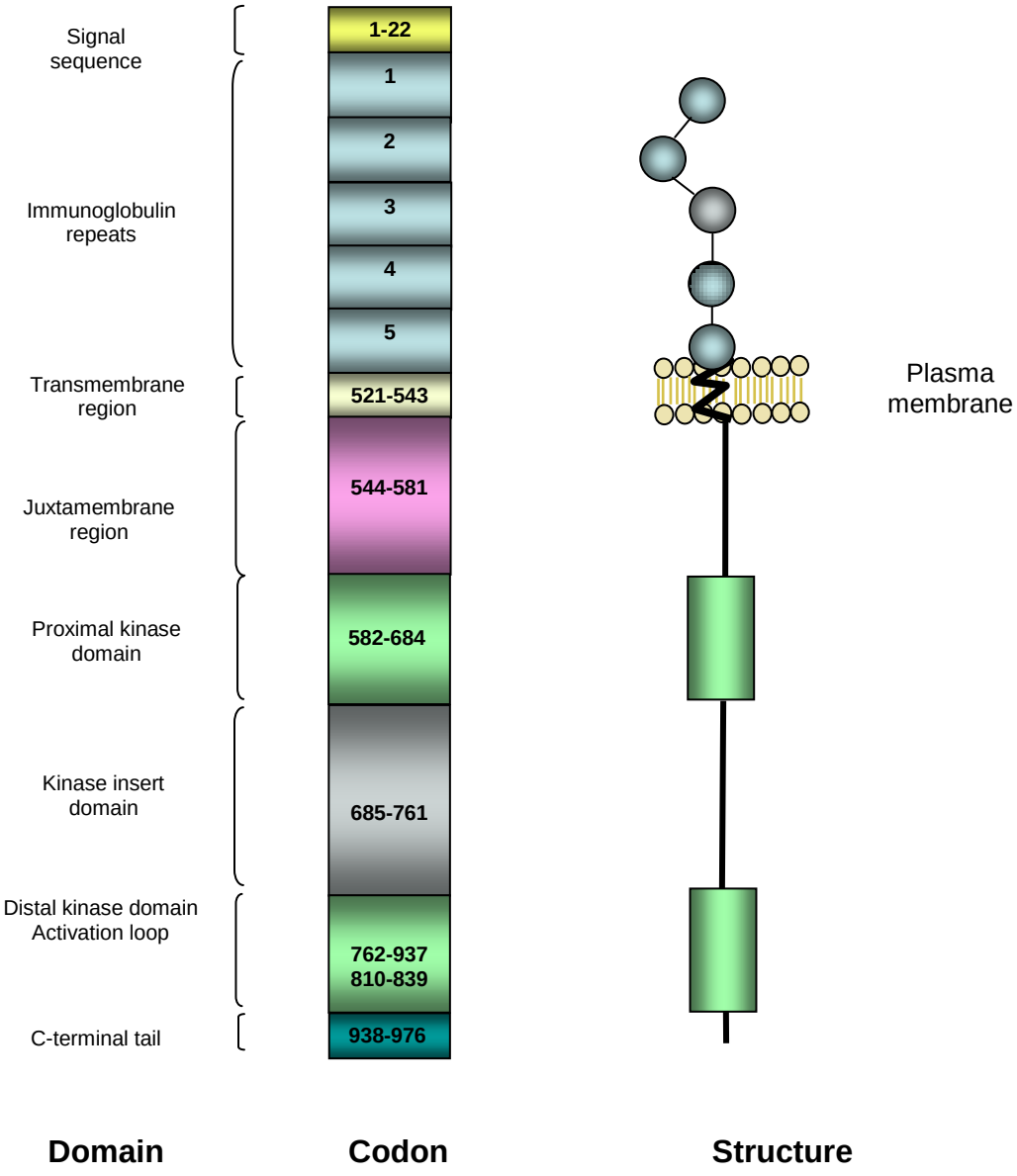

Figure 1. A diagram of the KIT receptor outlining the main structural domains and corresponding amino acid codons. (adapted from Edling et al. 2007; Lennartsson et al. 2005)

1.7.2 Regulation of c-kit expression

The *Kit* promoter is located approximately -124 and -83 base pairs (bp) from the initiation of translation site and contains binding sites for many transcription factors such as basic helix loop helix binding regions (bHLH) SP1, MITF AP-2, ETS, MYB, SCL and GATA-1. The major transcription start sites are located 62 and 58bp upstream of the translation site (Yamamto et al. 1993) and more distal positive and negative regulatory elements are found 5.5 kb upstream (Vandenbark et al. 1996; Ratajczak et.al 1998).

Stem Cell Leukaemia (SCL also known as TAL 1) is a member of the basic bHLH family of transcription factors and plays a central role during haematopoiesis (Shivdasani et al. 1995). SCL regulates gene expression by hetero-dimerising with E2A gene products (Hu et al. 1992) then binding to E-box DNA sequences (CANNTG) **(**Lécuyer et al. 2002)**.** Using antisense *Scl* c-DNA or dominant negative SCL in TF-1 haematopoietic cell lines Krosel et al (1998) linked impaired SCL DNA binding to a reduction in c-kit expression and an increase in apoptosis progression. Therefore, c-*kit* was identified as a potential down stream target of SCL. SCL and c-*kit* are co-expressed in primary haematopoietic precursors and an increase in SCL increases c-*kit* expression in B-cell precursors. Activation of the c-*kit* promoter during haematopoiesis requires a multifactorial ensemble of proteins including SCL, E2A, LMO2, LDB-1, and GATA1/2 and as assembly is driven by SCL, it is termed the SCL complex. Activation of the c-*kit* promoter involves direct binding of the complex to promoter DNA *via* SCL and E2A and the assembly is secured to promoter DNA by specificity protein 1 (SP1**)** (Lécuyer et al. 2002)

Huang et al (1998) demonstrated a direct correlation between AP-2 and c-*kit* expression over-expressing AP-2 in melanoma cells that were negative for both AP-2 and c-*kit*. Here, AP2 over-expression caused the induction of c-*kit* mRNA and protein with a concurrent reduction in metastatic potential of the cells indicating KIT mediated proliferation inhibition in this cell lineage.

During erythropoiesis, the survival of early erythroid precursors is dependent on c-*kit* expression. Upon maturation, c-*kit* is down regulated by the transcription factor GATA 1 and the mechanism of cell survival switches from c-*kit* to *Epo* expression (Pevny et al. 1991**;** Munugalavadla et al. 2005)**.** Although there are putative GATA 1 binding sites on the promoter region of c-*kit,* Munugalavadla *et al* (2005) did not relate the control of c-*kit* by GATA 1 to these sites on the promoter. However, Spinello et al (2009) have reported that GATA 1 and 2 associate with promyelocytic leukemia zinc-finger protein (PLZF) at position 281 to 305 on the *Kit* promoter to directly down regulate c-*kit* expression upon erythroid maturation.

1.7.3 The structure of Stem Cell Factor (SCF)

The ligand for KIT, stem cell factor (SCF; also known as KIT ligand, mast cell growth factor, or steel factor) is expressed at varying stages during embryonic development and is expressed along the migratory pathways of primordial germ cells (De Felici et al. 1994), melanocytes (Wehrle-Haller et al. 1995) and in sites of the developing haematopoietic system including the yolk sac, fetal liver, and bone marrow (Kallianpur et al. 1994 ; Silver et al. 1997 ; Bernex et al. 1996) as well as the brain, ovary, testis and retina (Toyooda et al. 2009; Ballow et al. 2006; McClanahan et al 1996; Wehrle-Haller 1995; Blackshaw et al 2004; Wehrle-Haller et al. 2003) . Although SCF exists as a monomer under physiological conditions, dimerisation of SCF has been associated with KIT receptor activation and subsequent signal transduction. (Hsu et al. 1997; Zhang et al. 2000; Xu et al. 2000). Crystal analysis has determined that the SCF dimer complex comprises of two SCF monomers with head-to-head interaction to form an elongated homo-dimer stabilised by both polar and non-polar interactions (Xu et al. 2000; Zhang et al. 2000). SCF exists as both soluble and membrane bound forms arising from alternative mRNA splicing and proteolysis (Broudy et al. 1997). Both membrane and soluble isoforms have an extracellular region, a transmembrane component and an intracellular domain (Zhang et al. 2000). The soluble form of SCF is created when the enzyme metalloprotease-9 cleaves after an alanine residue (ala189) in the extracellular region of SCF. The membrane bound form is a result of alternative splicing within exon 6 which omits the cleavage site for metalloprotease (Kridel et al. 2001). The soluble form of SCF

causes the rapid activation and internalisation of the KIT receptor before it is endocytosed and degraded. In contrast membrane bound SCF results in sustained receptor signalling possibly due to anchorage of the KIT/SCF complex and delayed ubuiquitination (Kissel et al. 2000).

Deletion of both transmembrane and cytoplasmic regions of SCF has been observed in the steel mutant Sl^{d}. Mice with this mutation only produce the soluble form of SCF and although viable, suffer from severe macrocytic anemia, reduced mast cell numbers, reduced melanocytes and are sterile. This implies that the soluble form of the ligand alone is insufficient for normal development. (Broudy et al. 1997)

1.8 THE ROLE OF C-KIT DURING DEVELOPMENT

As well as promoting ES cell self renewal and survival upon differentiation, the SCF/KIT pathway has been implicated in the survival of many stem and progenitor lineages including primordial germ cells (Dolci et al. 1991 ; Guerif et al. 2002) haematopoietic stem cells (HSCs) (Caceres-Cortes et al. 1999 ; Engstrom et al. 2003) neuronal stem cells (Erlandsson et al. 2004) and melanocyte precursors (Ito et al. 1999; Wehrle-Haller and Weston, 1995). Using a $W^{lacZ/+}$ transgenic mouse model, Bernex et al (1996) demonstrated that KIT was expressed on a plethora of tissues throughout embryonic development which extended beyond those initially identified by *W* mutations. Following from this, specific point mutations in the KIT receptor identified downstream signalling proteins and events that were crucial to haematopoiesis (Agosti et al. 2004; 2009), gametogenesis (Kissel et al. 2000), melanogenesis and mast cell development (Kimura et al. 2004).

Observing the natural (wild type) progression of a developmental process in *vivo* and comparing these processes to developmental defects caused by aberrations in KIT signalling may provide insight into the specific periods at which Kit signalling is important during development. This may be of importance when designing lineage specific ES cell differentiation strategies.

1.8.1 c-kit and melanogenesis

In the dorsal region of the developing embryonic neural tube resides a population of pluripotent cells that have the potential to give rise to multiple lineages such as neurons, smooth muscle, bone, cartilage and melanocytes of the skin, inner ear and iris. This population of cells is termed the neural crest (Bronner-Fraser et al. 1995). Cells from this region undergo rapid proliferation and migrate to specific locations within the developing embryo dependent on cell lineage and environmental cues. Melanocytes are the pigment producing cells of the skin and melanoblast precursors are the first cells from the melanocyte lineage to appear from the neural crest. Melanoblast precursors are defined from other cell populations in this region as they express c-*kit*, DOPA chrome tautomerase (Dct) and tyrosinase-related protein-2 (tryp-2) (Steel et al., 1992). These cells first appear at E9.5 on the neural crest (Opdecamp et al. 1997) and by E10.5 migrate to the basal layer of epidermis, hair follicles, the inner ear, uveal tract tail bud and leptomeninges. Melanocytes migrate from dorsal to ventral regions through the dermis to occupy the epidermis from E12.5-14 (Yoshida et al 1996). Epidermal melanocytes also expand in number and migrate through the epidermal basal layer between E13.0 and E15.5 (Yoshida et al. 1996). Following birth, melanocytes are found as mature pigment cells in the skin, hair follicles, the choroid layer of the eye, the Harderian gland, the anal canal and the inner ear (Goding et al. 2000).

Mice with *W* or *S1* mutations or wild type mice treated with a KIT neutralising antibody lack coat pigmentation due to the failure of neural crest derived melanoblasts to survive and migrate to the skin and developing hair follicles (Bernex et al 1996; Nishikawaet al 1991; Yoshida et al.1996; Hou et al. 2000). Melanocyes from *Sl/Sl* animals with no SCF are able to migrate from the neural crest but migration is terminated before reaching the dermis. In contrast, melanocytes from *Sl/Sl*^{d} animals with no membrane bound SCF are able to migrate to the dermis but apoptose in this region soon after. This indicates that KIT signalling is not required for the initial stages of melanocyte migration from the neural crest but requires soluble SCF for their migration to the dermis where the membrane bound form become necessary for melanocyte survival (Wehrle-Haller et al, 1995).

Inoculation of a KIT neutralising antibody (ACK2) into pregnant mice at different periods throughout gestation have shown that KIT signalling becomes important at specific time points during melanogenesis. KIT is expressed on melanoblasts during their emergence to the neural crest at E9.5 and are required for dorso-lateral migration (Yoshida et al. 1996). Kit expression is not required for melanocyte entry to the epidermal layer at E12.5 but is regained at E13-15 (Yoshida et al. 1996; Bernex et al. 1996; Nishikawa et al. 1991) during a proliferation stage in the epidermal layer where they become integrated into the hair follicles. Postnatal KIT dependence arises in proliferating and differentiating melanoblasts in hair follicles and proliferation is coordinated with the postnatal hair cycle (Nishikawa et al. 1991 Yoshida et al.1996). Neutralisation of KIT *via* administration of ACK2 into the peritoneal cavity of post natal animals resulted in loss of hair pigmentation that was not restored to the animal even 72 days post injection. Inhibition of KIT signalling in melanoblasts located within the hair follicles interrupted their survival and differentiation potential and caused their demise by apoptosis.

KIT mediated survival of migrating and differentiating melanoblasts to melanocyte lineages is thought to arise from the activation of the Microphthalmia transcription factor (MITF) and the melanin catalising enzyme tyrosinase (Tyr), a down stream target of MITF (Gooding et al. 2000). MITF was also shown to promote survival of pigment cells by up-regulation of the major anti-apoptotic protein BCL2 (McGill et al. 2002). MITF has a basic helix loop helix structure and can bind to the E-box sequence contained within the KIT promoter to control transcription (Tsujimura et al. 1993). During the early stages of melanogenesis MITF up-regulates c-*kit* expression in Melanoblasts (Opdecamp et al. 1997) but in later stage melanocytes and melanoma cells, KIT signaling leads to the activation of ERK 2 and (Hemesath et al. 1998) an increase in MITF phosphorylation. Analysis of neural crest cells from *Kit* $^{W\text{-}LacZ/KitW\text{-}LacZ}$ mice indicated that MITF alone is not sufficient for *Tyr* expression and that KIT signaling is needed not only for the proliferation and survival of melanoblasts but also later in development for *Tyr* induction and the transition of melanoblasts to mature Melanocytes (Hou et al. 2000)

1.8.2 c-kit and haematopoiesis

Haematopoiesis arises from the continuous proliferation of stem cells, which upon differentiation form various cells of the haematopoietic system. These cell types have specialised function and include lineages such as erythrocytes, megakaryocytes granulocytes, monocytes and mast cells. (Bernstein et al. 1993; Paulson et al. 1995), Haematopoiesis occurs in two waves during embryonic development. The first wave is termed primitive haematopoiesis and occurs within the yolk sac at E7.5. Following gastrulation, blood islands appear within the yolk sac which contains primitive erythrocytes that are morphologically distinct from their definitive adult counterparts. Although haematopoietic precursors found within the yolk sac have the potential to form definitive lineages they are unable to generate lymphocytes or HSCs prior to circulation onset (Cumano et al. 2001). The main function of the progenitors located at this site is to sustain the immediate requirements of the early embryo during early development (Medvinsky et al. 1996; Palais 1995; Palis et al. 1999; Keller, 2005)

The second wave, termed definitive haematopoiesis, produces blood cell lineages that will create the adult haematopoietic system Definitive haematopoiesis originates in the para-aortic splanchnopleura (P-Sp), which later becomes the developing aorta, gonads, and mesonephros (AGM) (Godin 1995; Medvinsky et al. 1996; Palis et al. 1999). Dissections of E8-9 embryos have attributed progenitors from this region with multipotent activity producing both lymphocytes and myeloid cells (Godin et al. 1995) and after E10 produces HSCs with long term repopulating potential (Medvinsky et al. 1996). Subsequently, hematopoietic stem cells (HSCs) migrate from these regions to the fetal liver E11, thymus, and spleen. Around the time of birth, the major site for haematopoiesis becomes the bone marrow, and this is maintained during adult life (Lennartson et al. 2005)

Haematopoietic progenitors expressing KIT are primarily detected at E7.5 to E 9.5 in the blood islands of the embryonic yolk sac, from E9.5-E11.5 in the AGM (Palacious et al. 1992; Bernex et al 1996; Silver et al. 1997) and by day E10.5 to E11.5 in the fetal liver (Palacious et al. 1992; Bernex et al 1996; Silver et al. 1997; Broudy et al. 1997) where

they progressively increase until day E15 and then decrease, (Palacious et al. 1992) paralleling the transition from yolk sac to fetal liver. Fetal thymus also contains KIT positive primitive T-lymphocyte and B-lymphocyte progenitors (Palacious et al. 1992 Hoang et at 1996; Broudy et al. 1997) where expression is down regulated upon terminal differentiation of the granulocyte-macrophage, B-, and T-lymphoid lineages **(**Lécuyer et al.2002) (Figure 2). Although KIT expression is down regulated during haematopoietic cell differentiation to definitive blood lineages found in the adult, some haematopoietic cells (e.g. mast cells) maintain expression.

W mutant mice display several haematopoietic defects which are restricted to erythroid and mast cell lineages which result in severe macrocytic anemia and mast cell deficiency respectively. In particular, W/W^{v} mice are profoundly mast cell deficient containing no mast cells in the peritoneal cavity, gastrointestinal tract, respiratory system heart, brain or spleen. In addition mast cell numbers in the skin are severely depleted in comparison to their wild type counterparts (Kitamura et al. 1978) and upon the removal of SCF from mast cells *in vitro* and *in vivo*, cells undergo rapid apoptosis (Iemura et al. 1994). Sl/Sl^{d} mutants contain reduced numbers of pluripotent haematopoietic stem cells contained within the yolk sac and fetal liver and mice homozygous for the *W* mutation die perinatally due to severe anemia (Russell, 1979). In the adult, anemia is induced when wild type mice are treated with the KIT blocking antibody ACK2 resulting in complete abolishment of haematopoietic progenitors from the bone marrow (Ogawa et al. 1991).

In the absence of KIT signalling other receptors have been shown to compensate for KIT e.g.The EPOr was shown to partially rescue erythroid formation in *W/W* mice (Waskow et al. 2002) and hepatocyte growth factor was shown to promote proliferation and differentiation of haematopoietic stem cells and progenitors from *W/W* mice (Yu et al. 1998)

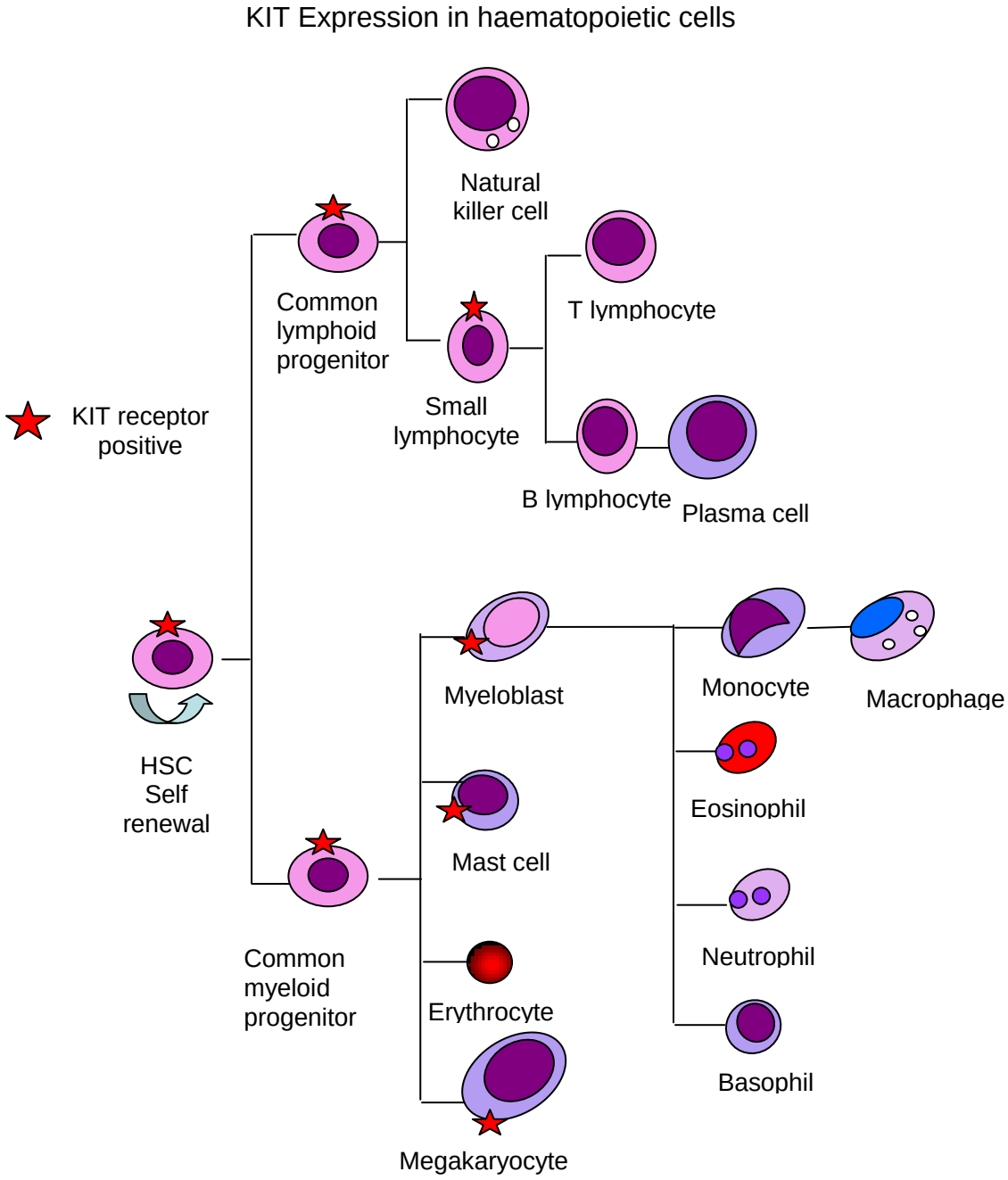

Figure 2. KIT expression during the differentiation of haematopoietic stem cells. Kit expression decreases upon terminal differentiation but expression remains high in megakaryocytes and mast cells (Lennartsson 2005). Cell lineages that express KIT are depicted with a star.

1.8.2 c-kit and germ cell development

Primordial germ cells (PGCs) are the founding population of the germ cell lineage and develop concurrently with endoderm, mesoderm and ectoderm. PGCs are first detected as alkaline phosphatase staining cells (MacGregor et al. 1995) at E7 in the posterior primitive streak where they migrate to the allantois and then directly into the adjacent embryonic endoderm and the hindgut at E8, to reach the gonadal ridge at E9.5-11 (Anderson et al. 2001).

In the male, PGCs begin to rapidly proliferate within the developing testis and begin to differentiate to gonocytes which are mitotically inactive until a week after birth (Mclaren et al. 2003). After this time they migrate to the seminiferus tubuli and differentiate to A spermatogonia (De Rooij et al. 2001). During their development, spermatogonia pass through stages of differentiation from A1-A4, and then differentiate to type b spermatogonia which are spermatocyte pre cursors. Spermatocytes then enter meiosis and differentiate into mature haploid spermatozoa by day 20 post partum. Males retain their type 1 stem cell like spermatogonia throughout life (DeRoojg et al. 1998)

During female PGC development, PGCs begin to differentiate in the developing ovary upon reaching the gonadal ridge. They lose their motility, cease to proliferate and are termed oocytes which become enveloped in somatic granulosa cells. At E13.5, female germ cells initiate meiosis and pass through leptotene, zygotene and pachytene stages of meiotic prophase before birth, then arrest in the meiotic prophase diplotene stage until adulthood. Cells that are not surrounded by granulosa cells perish by apoptosis (Hutt et al. 2006; Pepling et al. 2001). Upon reaching puberty a small number of primordial follicles are recruited into the growth and maturation phase of folliculogenesis whilst the remaining oocytes remain quiescent within the primordial follicles. Maturation onset is activated by a surge of pituitary Luteinizing hormone (LH) which recommences meiosis in the oocyte to metaphase II stage and the oocyte is ready for ovulation (Arnault et al. 2008)

c-*kit* is expressed in PGCs when they arrive at the base of the allantois at E7 (Manova et al. 1991). c-*kit* mRNA is still present at E12·5 upon reaching the gonadal ridge in both

males and females (Orr-Urtreger *et al*. 1990). In the post natal mouse testis, c-*kit* is expressed in mitotic spermatogonia (Dolci et al. 2001), proliferating diploid spermatogonia with a reduction of expression in primary spermatocytes and is absent in post meiotic cells (Sorrentino et al., 1991). Round spermatids express a truncated version of c-kit during their differentiation into spermatoza and is also localised in the subacrosomal region of mature sperm (Sette et al. 1997). Injection of tr-kit into MII phase mouse oocysts results in reactivation of meiosis and triggers the calcium oscillations required for successful fertilisation onset (Sette et al. 2002)

In the female, c-kit and SCF are expressed in zygotene/pachytene oocytes of the fetal mouse ovary at E 16.5- 17.5 (Doneda et al. 2002) and by E18.5, SCF expression is restricted to ovarian somatic tissue. At birth SCF is high in the ovary then expression becomes associated with the granulosa cells (Manova et al. 1993). Although present in migrating and mitotic PGCs, c-*kit* protein or mRNA is not present in germ cells undergoing first stage meiosis during fetal life (Manova et al. 1991). c-*kit* expression is restored in diplotene oocytes (Manova et al. 1990), is expressed uniformly in primordial follicles and later stage follicles but expression decreases in antral follicles (Horrie et al 1991; Hutt et al. 2006)

SCF is expressed concurrently along the migratory pathways of the PGCs and is clearly expressed in the hind gut, ventral wall of the dorsal aorta, the mesentery and the genital ridge whereas c-*kit* is only expressed upon germ cells (Matsui et al 1990). At this stage of migration and proliferation, the membrane bound form of SCF is most predominant (Manova et al 1993). In the testis SCF is produced by the sertoli cells (Ohta et al 2000) whereas SCF expression is found in granulosa cells and ovarian epithelial cells of the ovary.

Mice homozygous for severe *W* and *Sl* mutations are sterile as the rapid proliferation of PGCs usually observed during E7-13.5 embryonic development fails to occur. Observations of mice with *W* or *Sl* mutations in one allele only has allowed the analysis of SCF/KIT signalling and their effects on germ cell development. W/W^{v} and W^{v}/W^{v} mice

have significantly reduced germ cell numbers and reduced germ cell development rates in both oocytes and spermatogonia (Reith et al. 1990). Using E8.5-10.5 pre-gonadal PGCs Dolci et al (1991) demonstrated the requirement for KIT signalling, with specific emphasis on membrane bound SCF, during PGC survival. Following this, Guerif et al. 2002 evaluated the role of *Kit* during spermatogenesis onset and maintenance using a $Kit^{W\text{-}lacZ/+}$ murine mode. In comparison to their wild type counterparts, they found that $Kit^{W\text{-}lacZ/+}$ mice had a reduction of several germ cell lineages ranging from type B spermatogonia to spermatozoa suggesting not only a smaller founding PGC population but also reduced proliferation rates in these mice. They also demonstrated that apoptosis was enhanced upon the first wave of spermatogenesis in $Kit^{W\text{-}lacZ/+}$ mice resulting in delayed meiosis, severely reduced sperm numbers and compromised fertility. A similar result of reduced PGC number and inhibited sperm development was observed in Sl^{17H} mutants during the first wave of spermatogenesis. Although there was a significant reduction in female primordial and mature oocytes, female Sl^{17H} mutants remained fertile (Brannan et al. 1992). Yan et al., 2000 showed that spermatogonia, spermatocytes and spermatids survival was positively correlated with increasing levels of SCF whereas blocking the KIT receptor with ACK2 was negatively correlated to cell survival where apoptosis was the mechanism of cell death.

1.9 KIT MEDIATED SIGNALLING PATHWAYS

Bashamboo et al (2006) reported a fundamental role for the SCF/KIT signalling pathway during ES cell differentiation and survival. Although they highlighted BCL2 as a survival protein expressed during the differentiation process, they did not identify a specific down stream pathway or mechanism that was responsible for this survival. An understanding of the signalling pathways and function of effectors mediated by KIT signalling would allow the identification and investigation of potential survival pathways involved in ES cell differentiation.

1.9.1 SRC

The SRC family of tyrosine kinases, named after its founding member c-*src*, includes members such as BLK, FGR, HCK, LCK, and LYN which are primarily expressed in hematopoietic lineages (Abram et al. 2000). Linnekin et al 1997 demonstrated that LYN is the dominant SRC family member expressed in SCF responsive haematopoietic progenitor cells and treatment of these cells with an antisense *Lyn* specific oligonucleotide or a SRC inhibitor (PP1) reduced SCF induced proliferation, indicating a critical role for LYN in KIT mediated proliferation. Shivakrupa et al 2005 also showed that LYN deficient mice had reduced mast cell SCF induced proliferation rates when compared with the wild type control. Using a KIT fusion protein Linnekin et al 1997 demonstrated that the SRC Kinase interacts with phosphotyrosine residues 568 and 570 in the KIT Juxtamembrane segment via a SH2 domain (Figure 3). Using a mutated KIT receptor that had point mutations at 7 different residues, Hong et al demonstrated that mitogenic potential, survival and migration properties of the 32D myeloid cell line was only rescued when tyrosines 568 and 570 were restored to the receptor. Restoration also reactivated the Ras/Erk and Rac/JNK pathways.

1.9.2 Adapter proteins

Adapter proteins have the ability to interact with multiple proteins simultaneously due to containing multiple binding domains. APS is an adapter protein that binds to KIT at tyrosine 568, a residue that also binds CHK, SHC and SRC (Wollberg et al 2003). Upon phosphorylation APS binds to the ubiquitin E3 ligase CBL whose action links ubiquitin to the KIT receptor resulting in their internalization and degradation (Hu et al. 2005; Yokouchi et al. 1999)

1.9.3 SHP1/2 phosphatase

SHP1 and SHP2 are phosphotyrosyl phosphatases which are primarily expressed in haematopoietic and epithelial cells and comprise of two SH2 domains and a phosphatase domain. Shp1 and Shp2 phosphatase bind to tyrosine 570 and 568 within the Juxtamembrane segment of KIT and have the ability to inhibit KIT signalling by

dephosphorylation of the receptor directly or by dephosporylating its downstream protein tyrosine kinases (Kozlowski 1998)

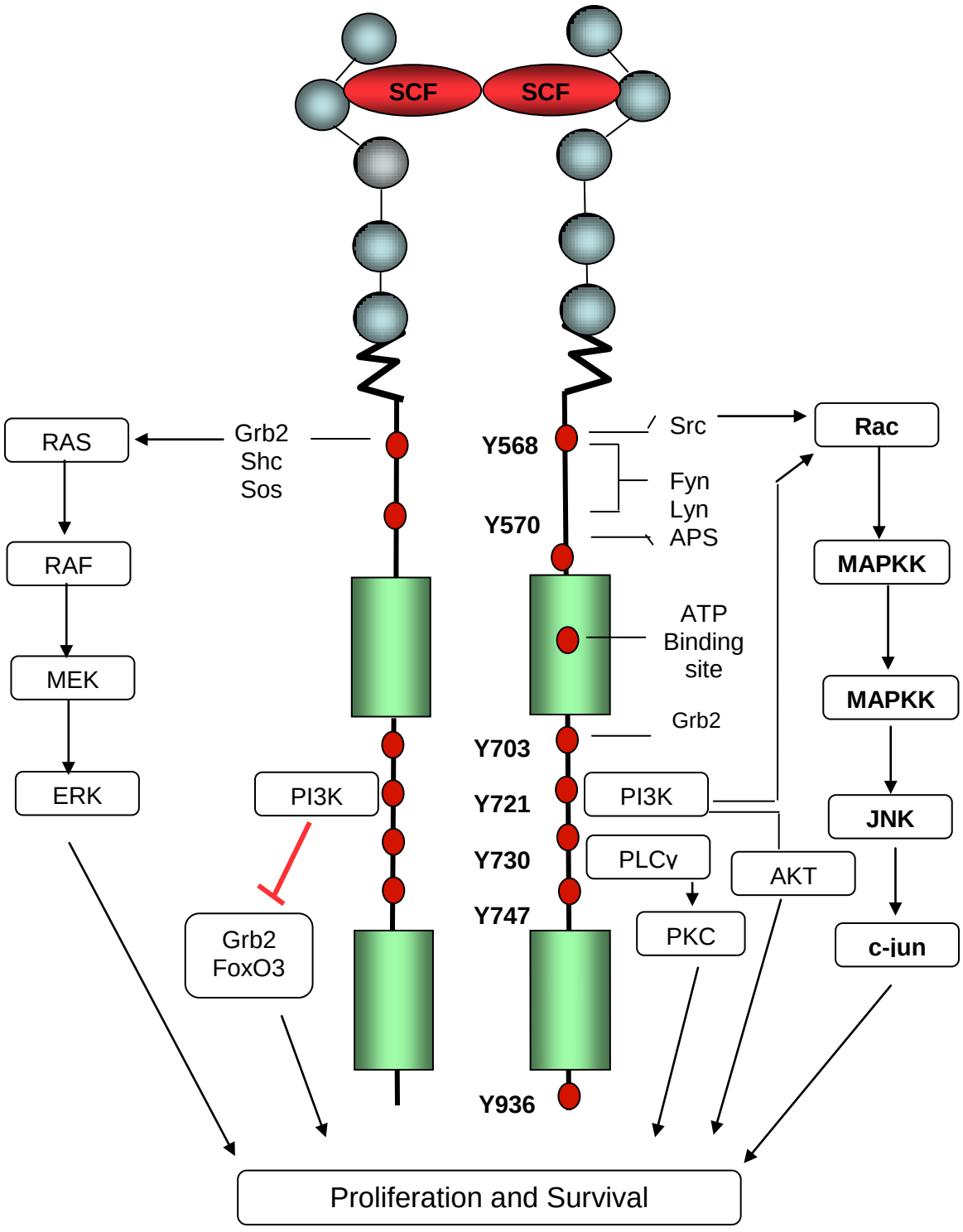

Figure 3. The SCF/KIT receptor signalling pathway and associated downstream effectors (adapted from Lennartsson et al. 2005; Rönnstrand et al 2004)

1.9.4 Phosphatidylinositol 3-kinase activation (PI3K)

Phosphatidylinositol 3-kinase (PI3K) represents a class of lipid kinases that phosphorylate the 3- hydroxyl group of phosphatidylinositol-4,5-bisphosphate (PIP2) to create phosphatidylinositol-3,4,5-trisphosphate(PIP3). PIP3 is able to bind with proteins containing a pleckstrin homology (PH) domain, resulting in their recruitment to the plasma membrane and subsequent activation (Foster et al. 2003). There are three classes of PI3K (I,II and III). Class I PI3K is most associated with intracellular RTK signalling and consists of a regulatory subunit (p85) and a catalytic subunit (P110). The regulatory subunit contains a SH3 domain, a RHO-GTPase domain and two SH2 domains (Fruman et al. 1998) whereas the catalytic subunit contains a RAS binding domain and a phosphatidylinositol kinase accessory (PIKa) domain. The regulatory subunit binds to target proteins with SH2 homology causing PI3K to change conformation and become activated (Carpenter et al. 1993) as well as binding with adapter proteins CBL, CRKII and CRKL (Ronnestrand et al. 2004). The p110 subunit contains enzymatic activity to phosphorylate phosphoinositides (Fruman et al. 1998).

PI3K associates with activated RTKs by binding to phosphorylated tyrosine residues by one or both SH2 domains and interacts with KIT on tyrosine 721 (mouse 719) (Lev et al. 1994). Binding and activation of PI3K occurs at the plasma membrane where PI3K substrates such as AKT (also referred to as protein kinase PKB) are located. AKT is a serine threonine kinase downstream of PI3K and is associated with cell survival. Blume et al (1998) demonstrated that binding of PI3K to KIT promotes survival in human embryonic kidney (HEK) cells *via* PI3K dependent activation of AKT resulting in phosphorylation and inactivation of the pro-apoptotic protein BAD. KIT mediated activation of PI3K and AKT has also been implicated in the survival of haematopoietic progenitors by the phosphorylation and inactivation of pro-apoptotic forkhead transcription factors (Engstrom et al. 2003) as well as the survival and proliferation of primordial germ cells (Miguel et al. 2002)

A role for PI3K was first observed in ES cells *via* the generation of a PTEN (phosphatase and tensin homologue) null cell line. PTEN dephosphorylates PIP3 to negatively regulate PI3K signalling. PTEN null ES cells displayed increased levels of phosphorylated AKT with enhanced ES cell proliferation rates even under reduce serum conditions. Survival in these cells was attributed to the phosphorylation and inactivation of the pro-apoptotic protein BAD (Sun et al, 1999). Using LY294002, an inhibitor of PI3K, Paling et al. 2004 demonstrated a reduction of AKT phosphorylation alongside a reduction in ES cell self renewal potential. Concurrently, an up-regulation of ERK, a protein linked with ES cell differentiation in reduced levels of LIF (Burdon et al 1999), was observed. When LY294002 was used in conjunction with the MEK inhibitor U0126, inhibition of self renewal was reversed associating the mechanism of PI3K mediated self renewal with ERK regulation. In particular, the p110 catalytic sub unit of P13K appears to have a role in both the self renewal and proliferation of ES cells that is isoform dependent where the p110α isoform was involved with proliferation whereas the p110β subunit regulates self-renewal (Kingham et al. 2009). Upstream regulation of PI3K during self renewal has been associated with both LIF and BMP4 (Welham et al., 2007).

1.9.5 PLC-γ

Phospholipase C-*γ* (PLC-*γ)* exists as two isoforms (1 and 2) which contain two SH2 domains, a PH domain and a catalytic domain. Isoform 1 is expressed ubiquitously whereas as 2 is expressed predominantly in the haematopoietic system (Carpenter et al. 1999). Activation of PLC-*γ* hydrolyses phosphoinositide PIP2, to create diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). DAG activates Protein Kinase C whereas IP3 interacts with the endoplasmic reticulum (ER) to release stored Ca2+ (Putney 2002). Using a mutant KIT receptor Gommerman et al (2000) demonstrated that PLCγ interacts with tyrosine 730 in 32D cells. Interestingly, tr-KIT which is present in haploid spermatozoa, associates with the SH2 domain of PLCγ and is essential for tr-KIT–induced egg activation (Sette et al. 1998)

1.9.6 The Ras/Erk pathway

RAS is a G-protein that switches from its inactive form when bound to GDP to an active form when bound to GTP (Lewis et al. 1997). This phosphorylation exchange and activation of RAS occurs at the inner cell membrane and is mediated by the guanine nucleotide exchange factor SOS, which exists in the cell as a protein complex with GRB2 (Hancock et al. 2003). The SOS-GRB2 protein complex binds to KIT via a SHP2 domain at tyrosine residues 703 and 936 (Thömmes et al. 1999). Upon phosphorylation, RAS activates RAF-1 which in turn, activates MEK1 and MEK2 whose downstream targets include ERK1 and ERK2. Activated ERKs dimerise upon phosphorylation then translocate to the nucleus to phosphorylate transcription factors and modulate gene transcription (Ronnesstrand et al. 2004; Khokhlatchev et al. 1998).

1.*9.7 The JAK/STAT pathway*

The Janus kinases (JAKs) are cytoplasmic tyrosine kinases that activate the Signal Transducers and Activators of Transcription (STATS). STATS are transcription factors with DNA binding domains and SH2 domains that dimerise upon phosphorylation initiating translocation to the nucleus for gene expression and regulation (Rawlings et al. 2004). Stimulation of the KIT receptor with SCF leads to the association of JAK2 with KIT, its subsequent phosphorylation then activation of STAT1α, 3, 5a and 5b (Gotoh et al. 1996). SCF induced JAK/STAT activation is reported to play a crucial role in the proliferation and differentiation of fetal liver haematopoietic progenitor cells. . (Linnekin et al, 1996; Radosevic et al. 2004).

1.10 MAPK SIGNALLING

Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinases that regulate cellular response to extracellular stimuli such as mitogens, heat shock and pro-inflammatory cytokines to elicit alterations in gene expression, mitosis, differentiation, proliferation, and apoptosis (Pearson et al. 2001; Davis et al. 2000; Kyriaki eet al. 2001). Signaling via the MAPK pathway involves an intracellular and

sequential phosphorylation cascade where upstream MAPKKKs phoshorylate and activate MAPKKs then MAPKs downstream (Davis et al. 1993; Seger et al. 1993) Several MAPKs have been identified in mammalian cells including extracellular signal-regulated kinases (ERK -ERK 1/2 or p44/p42), c-Jun N-terminal kinases (JNK/SAPK 1-3) and P38 (α, β, γ and δ,) (Davis et al. 2000; Kyriakis et al. 2001) (Figure 4)

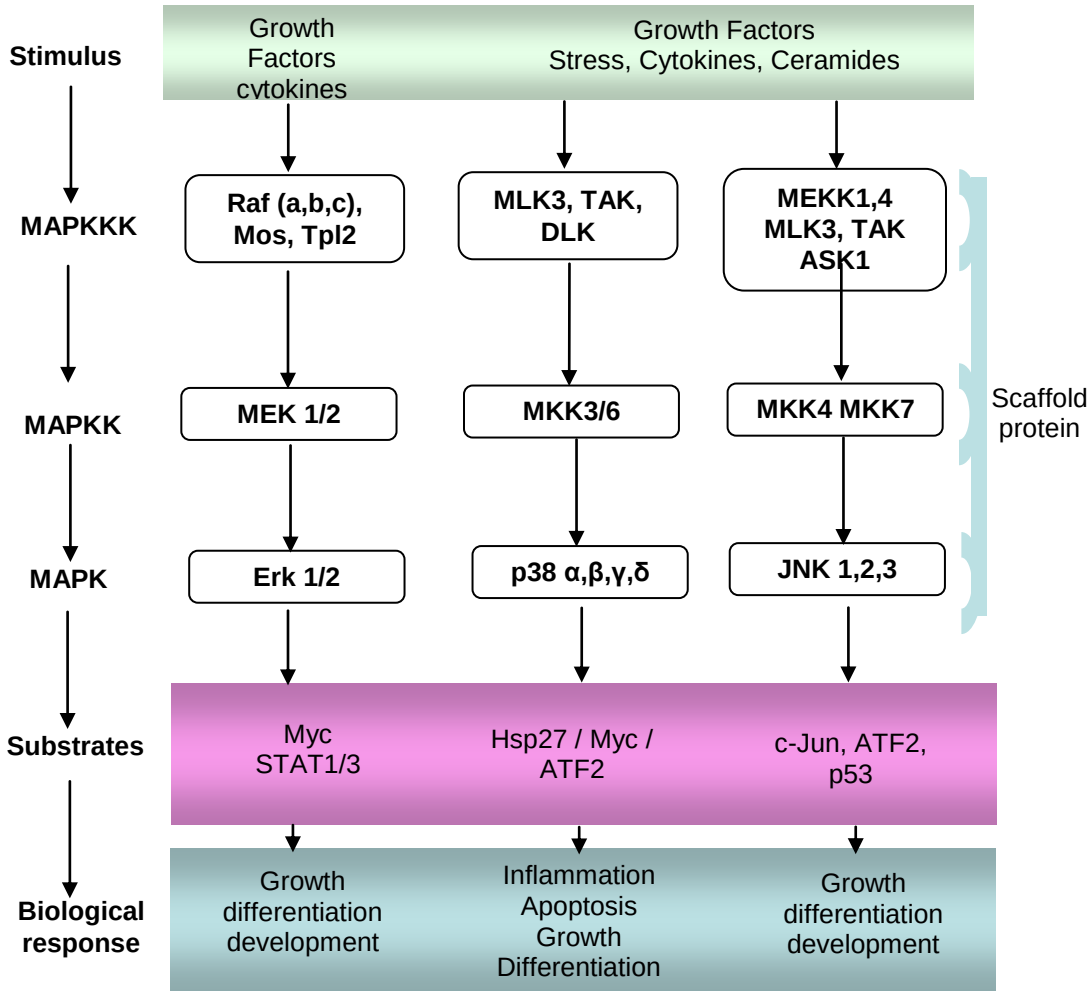

Figure 4 After stimulation by growth factors, cytokines or stress, MAPK signalling pathways progress as three sequentially activated proteins; a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK. Pathways are organised by scaffold proteins to activate substrates downstream. Adapted from Aouadi et al 2006.

1.10.1 ERK

ERKs 1/2 are activated by dual phosphorylation on threonine 183 and tyrosine 185 residues on their conserved Thr-Glu-Tyr motif (Chong et al. 2003). Upon activation, ERKs phosphorylate membrane proteins such as CD120a, Syk, and calnexin; the nuclear substrates SRC-1, PAX6, NF-AT, ELK-1, MEF2, c-FOS, c-MYC, and STAT3 and cytoskeletal proteins (Chen et al. 2001; Aouadi et al. 2006). However, ERK signalling is not required for the self renewal or proliferation of ES cells (Niwa et al. 1998; Burdon et al. 1999; Burdon et al. 2002). Furthermore, by deregulating SHP2 activation of ERK or using the MEK inhibitor PD098059, Burdon et al (1999) showed that inhibition of ERK activity enhanced ES cell self renewal and proliferation (Burdon et al. 1999). Upon differentiation, both ERK1 and ERK2 have distinct roles during development and Pages et al (1999) demonstrated that ERK1 knockout mice, although viable and fertile, were defective in thymocyte maturation with reduced cell expression of CD4+CD8+ and T cell receptors. Mice with ablated ERK 1 also displayed reduced adipose tissue development, were resistant to a high fat diet and were protected against insulin resistance (Bost et al. 2005). Conversely, ERK2 knockout mice were embryonic lethal at E6.5due to trophoblast and placental tissue malformation in C57BL/6 mice (Saba-El-Leil et al. 2003) whereas a lack of ERK2 caused embryonic lethality in E6.5 BALB/C mice due to defective mesoderm formation.

1.10.2 P38

P38 is predominantly activated by MKK3 and MKK6 by dual phosphorylation of threonine 180 and tyrosine 182, which are separated by a glycine residue in the sequence Thr-Gly-Tyr (TGY) (Enslen et al. 1998). The activation of p38 is initiated by cytokines, hyperthermia, osmotic stress and radiation and upon activation contributes to inflammation, apoptosis and cellular transformation. (Zarubin et al. 2005). *In vivo*, p38α-deficient mice die at E10.5–11.5 due to of defective placenta formation and deficient

vascularisation. In addition angiogenesis was abnormal in the yolk sac, the embryo proper myocardium and encephalon (Mudgett et al. 2000).

Of the four p38 (α,β,γ and δ) isoforms only p38α (which will now be referred to as p38) is expressed in ES cells (Allen et al. 2000) and is associated with early apoptotic events during ES cell differentiation. Inhibition of this pathway in differentiating ES cells increases cell survival with an up regulation of BCL2 (Duval et al. 2004) and a reduced level of apoptosis. This phenotype is of particular importance as differentiating KIT Null ES cells apoptose upon differentiation with a down regulation of BCL2 (Bashamboo et al. 2006). This correlative phenotype may attribute KIT signalling with a p38 inhibitory effect during ES cell survival.

As well as mediating apoptosis in ES cells, p38 has been associated with lineage specification and its expression occurs in two waves during ES cell differentiation. One wave occurs between 2-5 days LIF withdrawal (Aouadi et al.2006) and controls a switch between cardiomyogenesis and neurogenesis. Using retinoic acid to induce neuronal differentiation and consequently inhibit cardiomyogenesis, Aouadi et al (2006) showed that RA inhibited the wave of p38 α activity at days 2-5 with neuronal lineage formation. They also showed that $p38^{-/-}$ ES and p38 inhibitor (PD169316) treated cells spontaneously differentiated into neurons and did not form cardiomyocytes. The second wave of p38α occurs between days 12 and 16 and is involved in the inhibition of adipogenesis.

1.10.3 JNK

JNK, also known as stress related kinase (SAPK) is activated specifically upstream by MKK4 and MKK7 and participates in complex signaling pathways which result in a plethora of cellular processes from cell proliferation to differentiation and cell survival to programmed cell death (IP et al. 1998; Liu, 2004). MKK4 and MKK7 phoshorylate the Threonine -Proline-Tyrosine (Thr-Pro-Tyr) motif of JNK and dual phosphorylation is required to activate JNK and substantiate embryogenesis and cellular development (Wada

et al. 2004). Once activated, JNK translocates to the nucleus to regulate transcription through phosphorylation of several transcription factors such factors such as JUN, MYC and ATF2; AP-1 proteins, including JUNB, JUND (IP et al. 1998) as well as members of the BCL2 family (Davis et al. 2000; Nishina et al. 2003; Wada et al. 2004).

JNK is encoded by 3 genes *Jnk1, Jnk2* and *Jnk3. Jnk1* and *Jnk2* are expressed ubiquitously in many tissues but *Jnk3* is expressed selectively in neurons (Davis 2000). Similarly to the ERK and p38 MAPK pathways, JNK is not required for the self-renewal or maintenance of ES cells *in vitro* (Xu 2010). Using ES cells derived from *jnk*1, *jnk*2 or *jnk*3 knockout mice Amura et al (2005) reported a requirement for JNK1 during RA induced neurogenesis. $JNK1^{-/-}$ ES cells failed to differentiate into neuronal lineages but were associated with enhanced epithelial induction as determined by increased expression of E-cadherin Wn*t*-4, Wnt-6 and BMP4. Therefore during ES cell differentiation, JNK1 activity enhances neuronal lineages by repressing epithelial lineage signalling pathways. (Amuraet et al. 2005)

The action of JNK throughout development is varied contributing to the differentiation of primitive endoderm (Kanungo et al. 2000; Lee et al. 2004), ectoderm and cardiac lineages (Xu, 2010). Although a single knock out of *Jnk1, Jnk2 or Jnk 3* does not affect embryonic viability, double knock out of both *Jnk1* and *Jnk2* causes early embryonic lethality due to abnormal neuronal apoptosis (Davis et al. 2000; Kuan et al. 1999; Sabapathy et al.1999). Here, murine embryos lacking both *Jnk1* and *Jnk2* had reduced cell death during hindbrain development but increased cell death during forebrain formation indicating multiple roles for JNK in regard to apoptosis.

1.10.4 KIT and MAPK

c-kit has been associated with JNK expression via Src activation, where Src members bind to phosphorylated residues at positions 567 and 569, 567, and 569 (Hong et al, 2004). Here FYN activates JNK in the sequence; FYN-GAB2-SHP2-VAV-PAK-RAC-JNK and plays an essential role in mast cell migration (Samayawardhena et al. 2008). JNK activation has also been observed down stream of KIT-mediated PI3K activation in the survival of 32D tumourigenic myeloid cell lines (Chian et al. 2001). ERK1/2 and p38 also associate with the KIT receptor *via* the SRC binding site tyrosine 567 to enhance Ca^{2+} influx and cell migration in the pro-B cell line BAF3 and human embryonic kidney cells 293T (Ueda et al. 2002). Additionally ERK is activated via KIT-mediated recruitment of SOS - GRB2 protein complex which binds to KIT via a SHP2 domain at tyrosine residues 703 and 936 (Thömmes 1999).

1.11 APOPTOSIS

1.11.1 The anti-apoptotic role of SCF/KIT

Relating KIT signalling with anti-apoptotic proteins has been demonstrated in several studies. Kapur et al (2001) demonstrated that SCF/KIT was essential for the expression of BCL2, EPOr and STAT 5 in GATA1 deficient ES cells. SCF-mediated up-regulation of STAT 5 results in enhanced BCL2 and BCL-xl expression in immature erythroid cells (Socolovsky et al. 1999). Endo et al (2001) demonstrated that KIT-induced the phosphorylation of AKT which in turn phosphorylated and therefore inactivated the binding of BAD to BCL-XL. BCL-XL is an important anti-apoptotic molecule in haematopoietic progenitors as it inhibits apoptosome formation and subsequently inhibits caspase cleavage and activation and mitochondrial pore formation (Jofesen et al. 2000). Hu et al 2008 associated a decrease in hepatocyte apoptosis with an increase in KIT-mediated expression of BCL2 and BCL-XL

Upon differentiation, ES cells are reported to under go a period of apoptotic crisis where only cells maintaining within a certain, undefined threshold are able to survive to differentiated lineages. It has also been proposed that the signals required for ES cell survival upon differentiation are equivalent but require balance for survival (Duval et al. 2000). Bashamboo et al (2006) reported an apoptotic phenotype in differentiating KIT Null ES cells; with a concurrent down regulation of BCL2 indicating that SCF-KIT is essential for the survival of differentiating ES cells *in vitro* by suppressing apoptosis via the pro survival protein, BCL2. To further investigate the role of KIT in ES cell survival it is of importance to understand the mechanisms of cell death, their consequences and means of detection.

1.11.2 Distinguishing the process of apoptosis from necrosis

Apoptosis is a distinct and ordered program of energy-dependent, caspase-mediated cell death. Morphological features of apoptosis include cell shrinkage, membrane blebbing, organelle packing, nuclear fragmentation and chromatin condensation (Kerr et al. 1972.) Apoptosis can be distinguished from necrosis in several ways. Necrosis is an irregular energy-independent, passive form of cell death where degradative processes include cell swelling, dissolution of chromatin, cell rupture and the recruitment of inflammatory cells (Manjo et al. 1995).

Unlike necrosing cells, apoptotic cells do not rupture but form membrane bound cell fragments (apoptotic bodies) that express phagocytic markers for macrophages, parenchymal cells, or neoplastic cells resulting in their subsequent engulfment and degredation within phagolysosomes (Elmore, 2007). As apoptotic cells do not release cellular constituents but are quickly engulfed by phagocytic cells without the production of anti-inflammatory cytokines, the process of apoptosis occurs independently from inflammation (Kurosakaet al. 2003). Although there are specific differences between the two processes, an apoptotic process has the potential to convert to necrosis if the apoptotic stimulus is extended over a long time period in conjunction with a decrease in

caspase availability or depleted intracellular ATP (Leist et al. 1997; Denecker et al. 2001 Zeiss. 2003)

1.11.3 Biochemical features of apoptosis

Caspases are cysteine proteases whose activities arise via protein cleavage of specific aspartate residues (Thornberry et al. 1998). Caspases are ubiquitously expressed but are maintained as zymogens under physiological conditions. Caspases have been categorised according to function where "initiator" caspases such as caspase-8 and caspase-9 proteolytically cleave and activate "executioner" caspases (caspase-3, caspase-6, and caspase-7) which subsequently target protein substrates for degradation (Shi et al. 2002). Caspase 12 is another mediator of apoptosis but is specifically involved with the stress response mediated by the endoplasmic reticulum (Nakagawa et al. 2000). At the cellular level both inactive pro-caspases and cleaved activated caspases can be detected in a number of ways including Western blot, immuno-histochemistry or fluorescently conjugated caspase inhibitors such as FAM-VAD-FMK (Grabarek et al. 2002).

Apoptosis is often characterized by DNA fragmentation caused by calcium and magnesium ion dependent endonucleases which fragment DNA into segments of 180-200 base pairs (Kerr et al 1972; Wyllie et al. 1980). Endonucleases selectively cleave DNA at sites located between nucleosomal units (linker DNA) to produce DNA fragments that form a distinctive ladder pattern when resolved on agarose gel during electrophoresis. Another detection method involves the TUNEL (Terminal dUTP Nick End-Labeling) assay where DNA end breaks are labeled with probe conjugated UTP to allow detection by light microscopy, fluorescence microscopy or flow cytometry. (Kressel et al. 1994). The apoptotoic phenotype of chromatin condensation and nuclear fragmentation can be envisaged using the fluorescent dye 4'-6-Diamidino-2-phenylindole (DAPI) and light microscopy. This dye forms fluorescent complexes with natural double-stranded DNA and stains brightly in small, fragmented apoptotic nuclei whereas staining in viable cells is diffuse (Hamada et al. 1983).

Although membrane integrity is maintained during the early onset of apoptosis, disruption of membrane asymmetry occurs where exposure of phosphatidylserine (PS) from the inner to the outer lipid bilayer acts as a recognition and engulfment ligand to phagocytes (Bratton et al. 1997). As Annexin V binds to PS in a Ca2+ dependent manner, Annexin V can be conjugated to fluorescent dyes and used in conjunction with Flow Cytometry to detect early apoptotic cells within a population. Loss of membrane integrity is a feature of necrotic cells and because of this, apoptotic cells can be distinguished from necrotic cells by exposing the cell population to a double stain of Propidium Iodide (PI) and Annexin V. PI is a cell membrane impermeable dye that can only enter compromised cell membranes. Upon double staining, cells in the early phase of apoptosis will express Annexin V only, cells under late phase apoptosis will express both Annexin V and PI; whereas necrotic cells with damaged cell membranes will be PI positive only.

1.11.4 Mechanisms of Apoptosis

There are three main pathways that a cell follows during the process of apoptosis; the 'extrinsic' pathway which recruits death receptors; the 'intrinsic' pathway involving the mitochondria; and the 'perforin/granzyme A' pathway. All three processes converge to activate executioner caspase 3 to initiate cellular apoptotic degradation and debris clearance by phagosomes (Figure 5). The extrinsic pathway involves the recruitment of transmembrane death receptors and their corresponding ligands such as FASL/FASR, TNF-α/TNFR1, APO3L/DR3, APO2L/DR4 and APO2L/DR5. These receptors contain a cytoplasmic death domain that is key for signal transduction and activation of the caspase cascade (Wajant et al. 2002).

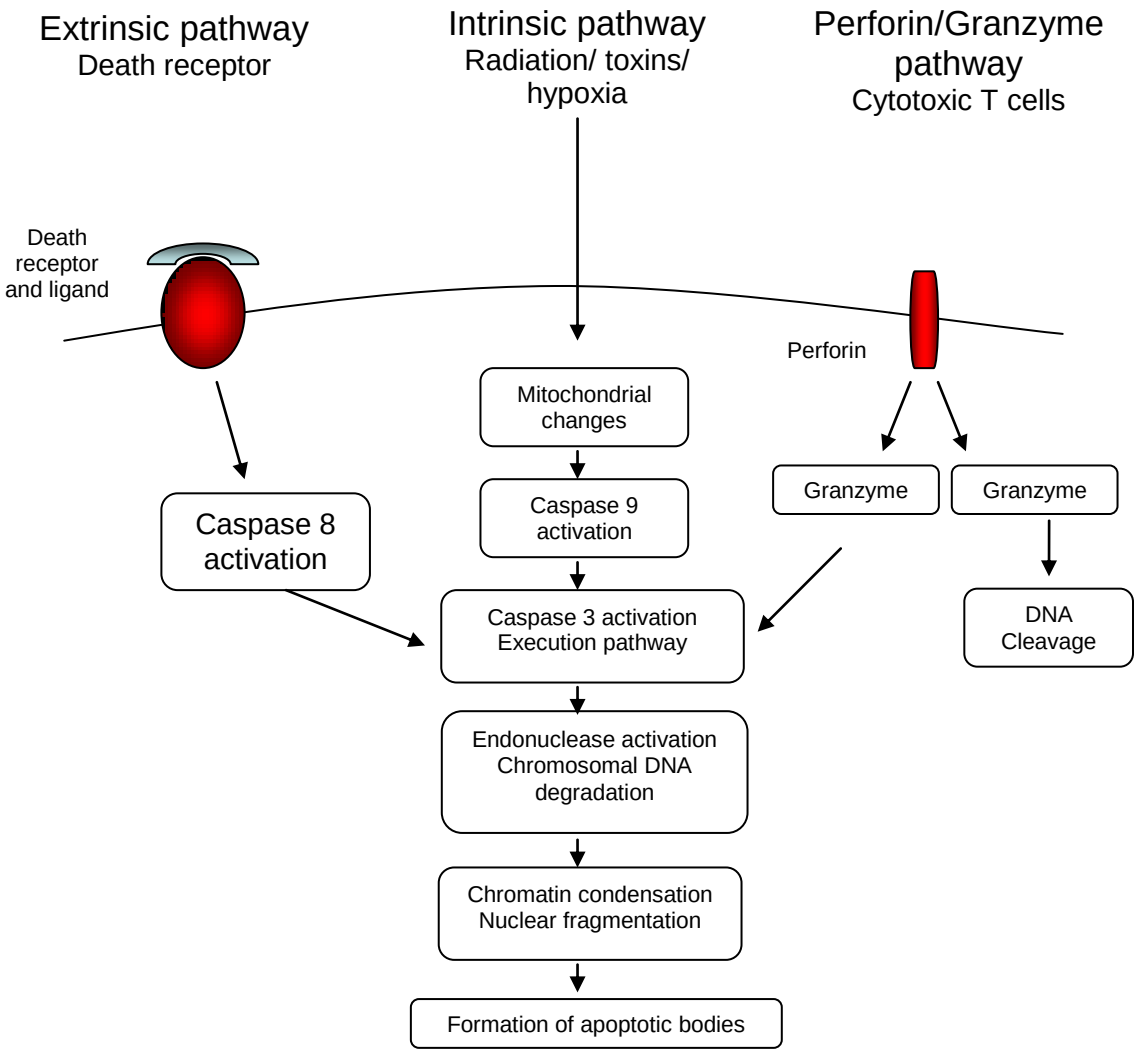

Figure 5 Schematic showing the 3 pathways associated with apoptosis; extrinsic, intrinsic as well as the perforin / granzyme pathway. Upon stimulation by specific ligands (extrinsic), cell stress (intrinsic) or Cytotoxic T cells, energy-dependent caspase cascades are triggered to activate the executioner pathways resulting in the morphological features associated with apoptosis. Adapted from Elmore, 2007

The intrinsic pathway of apoptosis is triggered in several ways including withdrawal of growth factors, loss of anti-apoptotic signal, hypoxia, radiation, hyperthermia or infection. As a result of intrinsic initiation, mitochondrial membrane potential is lost allowing the permeation of pro-apoptotic proteins from the mitochondrial inter-membrane space to the cytosol (Saelens et al. 2004). Cytochrome c, Smac/DIABLO and HtrA2/Omi released from the mitochondria then cause the interaction and clustering of APAF1 and caspase 9 to form an apoptosome which triggers the caspase cascade (Adams 2003; Wang et al. 2001). Interestingly, the membrane potential of the mitochondria is also thought to be regulated, in part, by the Endoplasmic reticulum (ER). The ER is a major store of calcium ions and disruption of the endoplasmic reticulum results in an efflux of calcium ions into the cytosol. Uptake of these ions by the mitochondrion results in loss of mitochondrial membrane potential and cell death initiation. Hawkins et al (2000) have shown that Ca^{2+} signaling occurs between the mitochondrion and the ER and that the ER may provide a critical signal for mitochondrial disruption.

In late apoptotic events, other pro-apoptotic proteins are released from the mitochondria and include AIF, endonuclease G and CAD. AIF and endonuclease G translocate to the nucleus to cleave nuclear chromatin and cause DNA fragmentation independently from the caspase cascade (Susin et al. 2000) whereas CAD relies on caspase cleavage before translocation to the nucleus causing DNA fragmentation (Susin et al. 2000).

Cytotoxic T -lymphocytes (CTLs) employ the perforin/granzyme pathway of apoptosis in order to eliminate virus-infected or transformed cells. CTLs use perforin to form a pore in target cells through wich they secrete the apoptosis inducing serine proteases Granzyme A and B (Trapani et al. 2002). Granzyme B has the ability to cleave proteins at aspartate residues, activate both initiator and executioner caspases and the ensuing caspase cascade. (Goping et al. 2003). On the other hand, Granzyme A activates caspase independent pathways and instead initiates apoptosis by activating NM23-H1, a chromosomal DNA damaging DNase (Fan et al. 2003; Martinvalet et al. 2005).

The execution pathway marks the activation of the execution caspases and the final stages of apoptosis. Here, execution caspases initiate nuclear degradation and cleavage of cytoskeletal proteins such as PARP, cytokeratins and alpha fodrin *via* endonucleases and proteases. (Slee et al. 2001). In particular caspase 3 activates the endonuclease CAD to degrade chromosomal DNA, cause chromatin condensation as well as the reorganization of cellular components into apoptotic bodies (Sakahira et al. 1998). The exposure of PS on the cell surface of apoptotic cells and apoptotic bodies is key for phagocytic recognition and degradation and marks the final sage of the apoptotic process.

1.11.5 The action of BCL2

The control of apoptosis within the cell and the regulation of mitochondrial events occur *via* the Bcl2 family. The Bcl2 family consists of 20 apoptotic regulators that both contribute to and oppose apoptosis (Figure 6). Members of this family fall into three categories, BCL2, BH3 or BAX (Figure 6) (Lindsten et al. 2000; Cuconati et al. 2002). The BCL2 group promotes cell survival by inhibiting pro-apoptotic members of the BAX group via inactivation of initiator caspases as well as inhibition of BAX and BAK mediated mobilization of Ca^{2+} from ER stores and its uptake into mitochondria (Nutt et al. 2002; Zong et al. 2003). BCL2 is located on the cytoplasmic face of the mitochondrial membrane, the endoplasmic reticulum and the nuclear envelope. Upon apoptotic stimuli, pro-and anti-apoptotic members of the Bcl2 family congregate at these areas in order to mediate apoptotic events (Hsu et al. 1997). The BH3 group, in particular BAD, is activated upon dephosphorylation (Zha, et al. 1996) in response to developmental cues, insufficient growth factors and intracellular damage (Huang et al. 2000). BAD elicits cell death by directly binding to and antagonising the action of BCL2 members as well as enhancing the activity of the BAX group. Both BAX and BAK form aggregates upon activation and have the ability to activate initiator caspases and pemeablise the outer mitochondrial membrane. This permeabilisation allows the flow of apoptotic proteins and the release of cytochrome c from the mitochondrion to the cytosol (Wang et al. 2001; Adams. 2003).

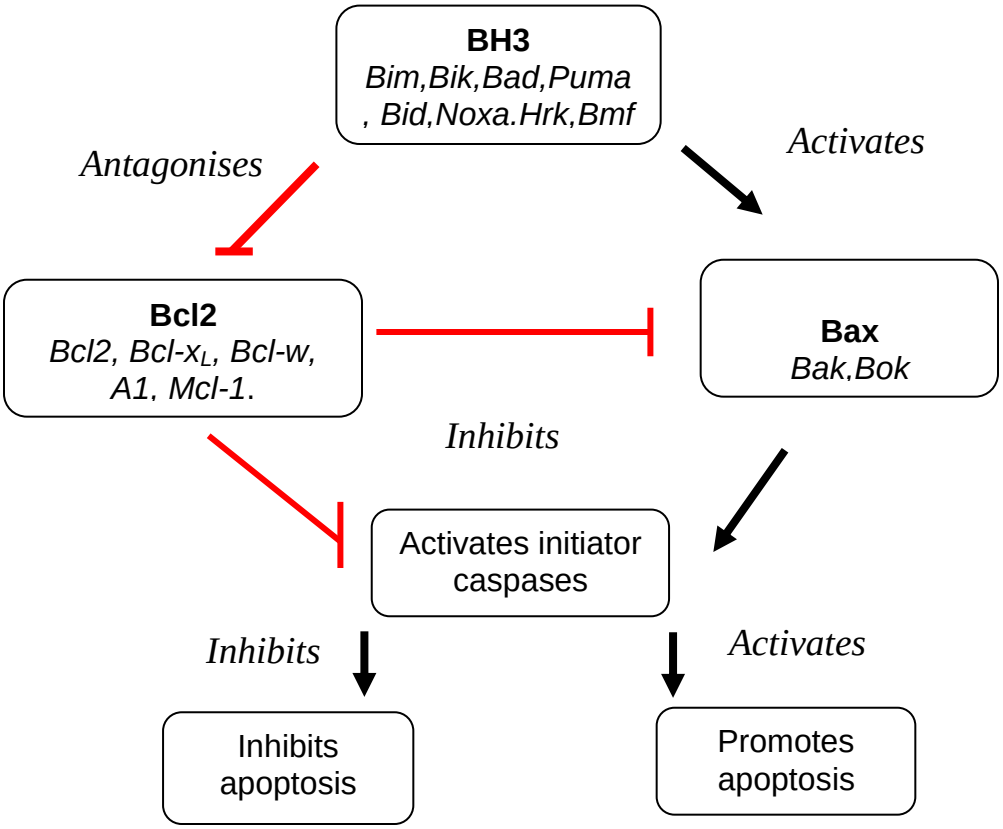

Figure 6 A simplified flow diagram indicating the relationship between anti-apoptotic and pro-apoptotic proteins of the BCL2 family (Lindsten et al. 2000; Adams 2003).

1.11.6 Apoptosis in development

Apoptosis not only proceeds in order to eliminate defective cells but is essential in maintaining homeostasis during normal development. Apoptosis is required during the process of spermatozoid and oocyte maturation and acts to remove germ cells that have undergone DNA damage or mutations during DNA replication Rodriguez et al. 1998) There are two waves of apoptosis during germ cell development. The first being at E13 during PGC migration to the gonadal ridge (Larsen et al. 1993) and the second at day 13 post partum when the spermatogonia undergo a phase of rapid proliferation. Apoptosis during oogenesis involves two main meiotic processes; an early phase involving the oogonia and oocytes in the preleptotene stage and a later phase concerning the oocytes in the pachytene stage (De Pol et al. 1998). Apoptosis is present in the mammalian blastocyst and distortions of apoptosis during blastocyst formation results in embryonic death or fetus abnormalities (Brison et al. 1997; Brill et al. 1999). In the post implantation embryo apoptosis functions to eliminate redundant cells, control cell proliferation and sculpt structures such as the fingers (Zakeri et al. 1997) and removal of the pronephric duct (Larsen et al. 1993; Hu et al. 1998)

1.12 KIT AND HUMAN DISEASE

The association of KIT with human disease arises from receptor gain or loss of function mutations (Figure 7) and their resulting downstream signalling abnormalities. Loss of function mutations present human syndromes, such as autosomal-dominant piebaldism, displaying phenotypes in man that are similar to the *W* mutations described in mice. These defects include skin and hair pigment anomalies, deafness, megacolon and defects in the development of the interstitial cells of Cajal (ICC) (Spritz et al.1994)

c-*kit* gain of function mutations can be divided into two classes; mutations that reside in the juxtamembrane region of KIT and those that reside in the distal kinase region (Sattler et al. 2004). X-ray crystallography indicates that the juxtamembrane domain negatively regulates KIT by inserting into and inactivating the proximal kinase domain by blocking formation of the active receptor conformation (Chan et al. 2003). Mutations in the JMD

are responsible for disrupting the inhibitory effect of the JMD/kinase domain interaction allowing constitutive c-kit activation and dimerisation. Human disease that arise from this mutation include GIST (Furitsu et al. 1993; Duensing et al. 2004) sinonasal lymphomas (Hongyo et al. 2000) and mastocytosis (Buttner et al. 1998)

The second region that generates activating mutations is the distal kinase and codon 816 which causes ligand independent activation of the receptor (Moriyama et al. 1996). Disease associated with gain of function in the distal kinase domain include mastocytosis (Nagata et al. 1995), mast cell leukemia, acute myeloid leukemia (Ashman et al. 1999; Ning et al. 2001), testicular germ cell tumors (Tian et al. 1999) and ovarian dysgerminomas (Pauls et al. 2004). Additionally, Core binding factor (CBF)-acute myeloid leukemia (AML) arises through mutations of exon 8 which corresponds to the immunoglobulin loop 5 in the extracellular region. Mutation within this region cause hyperactivity of the receptor when stimulated with SCF (Kohl et al. 2004)

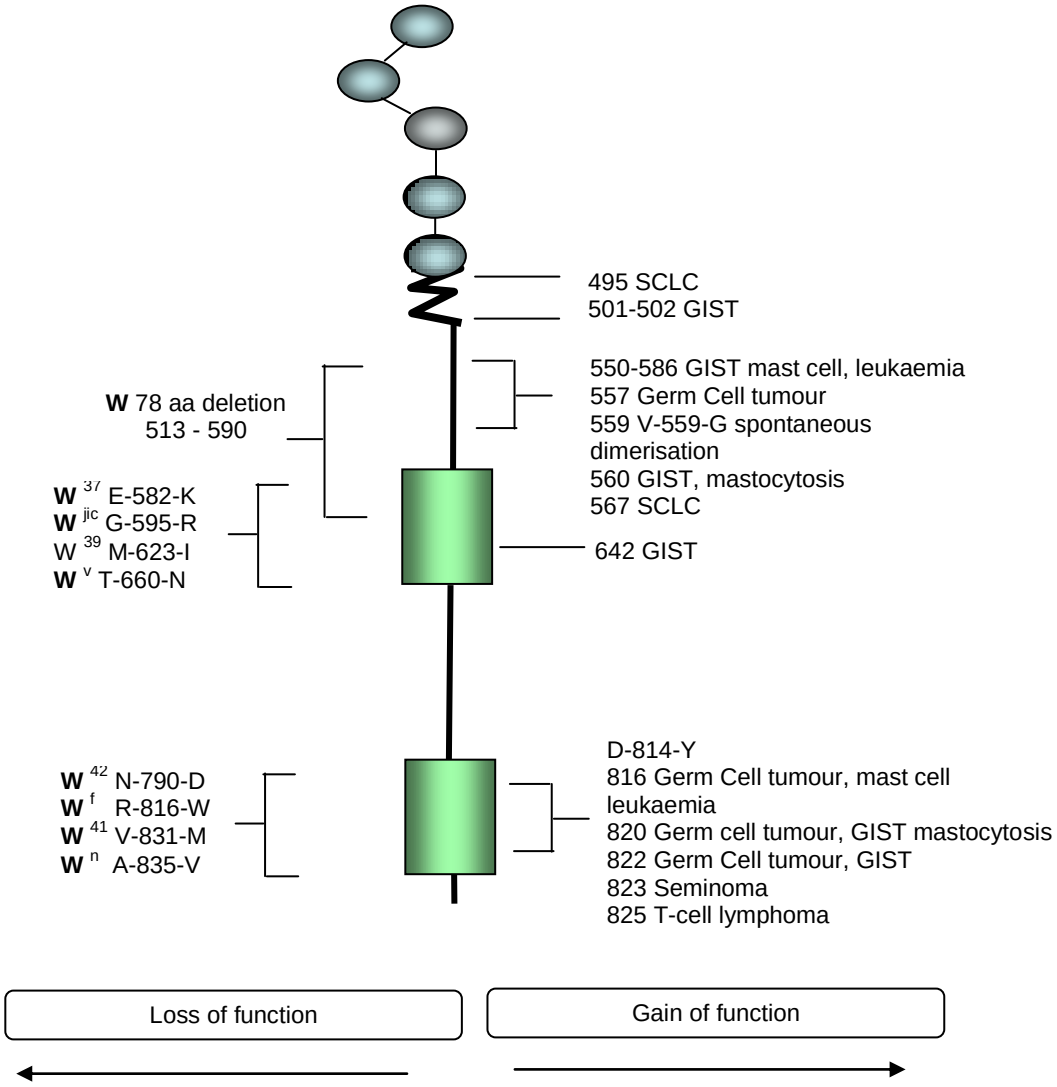

Figure 7. A diagram of the KIT receptor and point mutations causing loss or gain of receptor function. (Nocka et al. 1991; Tsujimura et al. 1993; Reith et al. 1990; Geissler et al. 1981; Lennartsson et al. 2006)

Gain of function mutations are typically treated with the chemical inhibitor Imatinib. Imatinib binds to the proximal kinase domain of the KIT receptor and locks the receptor in its inactive conformation. Prolonged exposure of KIT to Imatinib can result in acquired resistance due to secondary, activating mutations in the distal kinase. Severe side effects have also been reported with this inhibitor as Imatinib is not KIT specific but concurrently inactivates other receptors such as PDGFßR, BCR-ABL, ABL and ARG. (Demetri et al. 2002; Buchdunger et al. 2002). Although Imatinib can inhibit both wild type and JMD mutant receptors (Frost et al. 2002), it is functionless in the treatment of constituently active mutations such as codon 816 in the distal kinase. This mutant receptor is always in its activated conformation which impedes Imatinib binding.

Distal kinase mutations have been alleviated by the KIT activation loop specific ATP based inhibitors AP23464 and AP23848. Although these drugs can also inhibit the function of JMD mutant receptors, efficacy was only reached at extremely high inhibitor concentrations (Corbin et al. 2005) Other drugs effective against both JMD and distal kinase mutations of KIT include the indolinone chemicals SU6577, SU11652, SU11654 and SU11655 (Ma et al. 2000; Lennartsson et al. 2006) . These drugs are currently under investigation to alleviate the problems of side effects and secondary receptor mutations. However, none of these drugs are effective against Loss of function mutations.

ACK2 is a KIT specific monoclonal antibody that functions by binding to and inactivating the extracellular ligand binding region of the receptor and therefore inhibits receptor dimerisation and subsequent downstream signalling events (Yoshinaga et al. 1991). At the cellular level and in experimental animal models ACK2 has been employed to demonstrate the effect of KIT inhibition during the development of the blood system and self renewal of haematopoietic progenitors (Ogawa et al. 1991), during peristaltic movement of the ICC within the gut (Maeda et al. 1992), apoptosis induction in c-Kit positive melanoblasts (Ito et al. 1995), melanocyte development (Nishikawa et al. 1991) and during spermatogenesis (Yoshinaga et al. 1991). Therefore, gaining insight of KIT mediated development and disease progression at the molecular level will ensure optimal treatment determination and identification of potential targets for novel drug therapies.

1.13 PROJECT RATIONALE

The SCF/KIT signalling pathway has been implicated in the survival of many stem and progenitor lineages during their proliferation, differentiation and survival. Bashamboo et al (2006) also revealed a critical role for KIT during ES cell survival upon differentiation via the pro-survival protein BCL2. However the specific signalling events involved with differentiating ES cell survival had not been elucidated. Characterisation of the signalling pathways regulated by KIT during ES cell differentiation will not only reveal essential pathways required during ES cell survival upon differentiation but also identify key signalling events during lineage specification. This is of particular importance for understanding the process of embryogenesis and the generation of a consistent supply of defined cell lineages for regenerative medicine and cellular therapy. Understanding the consequences of inappropriate KIT signalling will also identify targets for novel drug therapies in the treatment of KIT related tumours and leukaemias.

1.14 AIMS

Three key hypotheses were addressed in this study to further the research of Bashamboo et al and elucidate the role of KIT during ES cell self renewal, proliferation and survival upon differentiation.

1.14.1.

Hypothesis 1:

KIT is not an essential factor in ES cell self renewal but becomes an essential survival factor upon ES cell differentiation

- Bashamboo et al (2006) claimed that although KIT was an essential factor for ES cell differentiation it was not essential to the process of ES cell self renewal whereas Lu et al (2007) reported that KIT was involved in the maintenance of

undifferentiated ES cells and could serve as a marker of ES cells with optimal differentiation. Can this discrepancy be addressed in this study?

- Is the death upon differentiation phenotype specific to the Bashamboo KIT Null clone? Can this phenotype be recapitulated in a newly derived ES cell model?
- How does a KIT Null mutation / KIT inhibition by ACK2 affect the self renewal and differentiation profile of ES cells?
- How does enriching for KIT expression affect the ability of ES cells to self renew and differentiate?

1.14.2

Hypothesis 2:

KIT Null cells die by apoptosis upon differentiation and inhibition of these apoptotic processes may support cells through the apoptotic crisis to allow survival upon differentiation.

- The mechanism of KIT Null ES cell death was previously reported as apoptosis. Can this be confirmed?
- Can inhibitors of apoptotic pathways prevent the KIT Null death upon differentiation phenotype?
- KIT survival upon differentiation has been associated with the pro-survival protein BCL2. Will over-expression of BCL2 prevent the KIT Null death phenotype?

1.14.3

Hypothesis 3:

KIT mediated survival of differentiating ES cells may act through activation of the JNK pathway.

- JNK activity has been associated with both pro and anti apoptotic effects depending on cell lineage and stage of development. What is the effect of JNK inhibition during ES cell self renewal and differentiation?
- What downstream effectors are affected by a block in JNK?
- How do these effectors associate with KIT signalling and the KIT Null phenotype?

Chapter 2

Materials and Methods

Chapter 2

Materials and Methods

2.1 MOLECULAR BIOLOGY TECHNIQUES

Standard molecular biology techniques and associated solutions were carried out and produced in accordance with the Molecular Cloning Laboratory Manual (Sambrook et al., 1989) unless otherwise stated.

2.1.1 Transformation of bacterial cells

Chemically competent DH5α cells (TOP10, Invitrogen) were thawed and incubated on ice with 1µl of plasmid DNA or 5-20µl of ligation product for 20 minutes. The heat 'shock technique' facilitated DNA entry into DH5α cells where the cell/DNA mixture was incubated at 42ºC for 45 seconds and then directly transferred to ice and incubated for 2 minutes. The resulting transformation reaction was placed into a 15ml Falcon tube containing 500µl SOC medium (Super optimal culture broth - supplied with the competent cells, Invitrogen) and incubated at 37ºC for 1 hour in an orbital shaker at 200rpm. A 50µl and 450µl aliquot of the transformation product was spread onto LB plates with an appropriate antibiotic and left to grow at 37ºC overnight.

2.1.2 Plasmid preparation (Miniprep and Maxiprep)

Single colonies picked from an overnight LB plate culture (described in 2.1.1) were grown in individual 5ml (Miniprep) or 200 to 400ml (Maxiprep) antibiotic containing medium and incubated at 37ºC overnight in an orbital shaker (200rpm). After 12-16 hours the cells were harvested by centrifugation (Miniprep – 6, 000g for 5 minutes; Maxiprep - 3000 x g for 20 minutes at 4°C) and plasmid DNA was isolated from the cells

in accordance with the QIAgen kit Miniprep/Maxiprep manufacturers recommendations and protocol.

2.1.3 Preparation of glycerol stocks

5mls of LB (Luria-Bertani) broth was inoculated with one bacterial colony from an overnight LB plate culture (described in 2.1.1). The bacterial cells were allowed to incubate for 8-12 hours at 37ºC at 200rpm in an orbital shaker then 800µl of the cells were added to 200µl of 80% autoclaved glycerol (in water). The cell and 80% glycerol mixture was vortexed briefly before immediately freezing and storing at -80°C in cryo-tubes. To grow the cells from glycerol stocks, a pipette tip was gently touched onto the surface of the glycerol stock and placed immediately into 5mls of LB broth where it was incubated for 8 -12 hours at 37ºC at 200rpm in an orbital shaker.

2.1.4 DNA extraction from animal tissue and ES cells

Cells under investigation were grown to confluence in 25cm^2 tissue culture flasks, washed twice with PBS before adding 2 mls of genomic DNA extraction buffer (4M Urea, 10mM CDTA, 0.5% sarcosyl, 0.1M Tris pH8 and 0.2M NaCl) supplemented with 1mg/ml Proteinase K and incubated with gentle agitation overnight at 55ºC. To precipitate the genomic DNA from the lysis reaction, 2mls of isopropanol was added directly to the flask and gently agitated at ambient temperature for 5 minutes. When visible, the white DNA precipitate was spooled from the flask using a pipette tip and placed into a 1.5ml eppendorf then resuspended in 500µl EB. To extract genomic DNA from ES cells grown on a 96-well plate, the cells were washed once with 100µl of PBS before adding 50µl of ES cell lysis buffer (10mM Tris pH7.5, 10mM EDTA, 10mM NaOH, 0.5% sarcosyl) and 1mg/ml Proteinase K to each well. The plate was incubated in a pre-humidified chamber at 55ºC with gentle agitation overnight. After incubation, DNA was precipitated by adding 200µl of 7.5mM NaOH in 95% ethanol to each well of the plate using a multi-channel pipette. The plates were then incubated at ambient temperature for 4-12 hours

before discarding the supernatant by up turning the plate onto absorbent material. The wells were then washed 3 times with 100µl of 70% ethanol and then air-dried 30 minutes. At this stage the DNA was enzymatically digested in preparation for Southern Blot (2.3)

2.1.5 Polymerase Chain Reaction (PCR)

PCR was used for the production of the Southern probe, generation of the EGFP-Flag-BCL2 fusion protein PCR product isolated from the P513-kEGFP-Flag-BCL2 vector (Duval, Malaise et al. 2004), cDNA analysis and multiplex PCR for WT E14, heterozygous and KIT Null clones. A representative PCR reaction generally contained 10 to 100ng template DNA along with 10µM of 2 primers each in 2x PCR buffer (Promega) containing MgCl2 (1.5mM) dNTPs (10µM) and 1unit of Taq DNA polymerase (Invitrogen) to a final volume of 25µl in nuclease free water (supplied with the Promega 2 X buffer). The template DNA was amplified in a HYBAID PCR-Express machine. A typical PCR proceeded as follows: 2 minutes denaturing at 94ºC followed by 30-32 cycles of 94ºC for 15 seconds, 55ºC for 30 seconds, 72ºC for 1 minute and a final extension cycle of 72 ºC for 10 minutes.

2.1.5.1Multiplex PCR

Genomic DNA was extracted from 96 well plates cells are described above (2.1.4) and PCR was carried out using the parameter described (2.1.5). Multiplex PCR was carried out using the following primer sets;

LacZ1 (5'-GCATCGAGCTGGGTAATAAGCG-3', forward);

LacZ2 (5'-GACACCAGACCAACTGGTAATGGTAGCG-3', reverse);

Kit1 (5'-TCCTGTTGGTCCTGCTCC-3', forward);

Kit2 (5'-GTGGTAGGCATGGGAAAAG-3', reverse)

The *LacZ* primer pair amplified an 800 bp-fragment of the *LacZ* gene and the *Kit* primer pair amplified a 148 bp-fragment of *Kit*. Heterozygotes displayed both bands (Hou, Panthier et al. 2000) . Resulting PCR fragments were run on a 0.75% gel with Ethidium bromide and visualized under UV illumination.

2.1.6 RT PCR

Cells or Embryoid bodies (EBs) were harvested by trypsinisation or sedimentation respectively and given two washes in PBS before being snap frozen at -80 ºC. The pellet was either stored at this stage or employed directly in the Quiagen RNeasy mini Kit for 'mammalian cell RNA isolation' according to manufacturer's instructions (Quiagen). Simply, 10^7 cells were disrupted with buffer RLT and homogenised using a pipette tip (cells) or passed through a 20-gauge (0.9 mm) needle attached to a sterile plastic syringe. An equal volume of 7% ethanol was added to the homogenate before addition to the spin colum and spun at 8000 x g for 15 seconds. The supernatant was discarded and buffer RW1 was added to the spin column and centrifuged for 15 seconds at 8000xg. Following this the supernatant was discarded and the column was washed a further two times with buffer RPE. RNA was eluted from the column using RNAse free water. Keeping the resulting samples on ice, the concentration of the eluted RNA was determined by the nanodrop spectrophotometer (ND-1000) and associated software. 1U of DNAse 1 (Roche) and 1 µl of DNAse buffer were added to 1 µg of RNA to make a final volume of 10 µl. This mixture was heated at 37°C for 15 minutes or 75°C or 10 minutes to inactivate the enzyme. This was carried out in duplicate in order to produce a cDNA and a –reverse transcriptase (-RT) control. Using the high capacity cDNA reverse transcriptase kit from AB Applied Biosystems, 1 µg of RNA was transcribed to cDNA according to manufacturer's instructions. –RT samples included RNAse free water to replace the reverse transcriptase enzyme and cycle conditions were as follows; 25°C for 10 minutes; 37°Cfor 120 minutes; 85°C for 5 minutes and 4°C holding stage. 10ng of the resulting cDNA or –RT control was added to 2X Promega master mix with 10µM of 5' and 3' primers for c-kit and β-*Tubulin* to a final volume of 25µl. The primers for c-*Kit* and β-*Tubulin* were as follows;

c-*Kit* 1 (5'- TCC TCA CTC ACG GGC GGA TC, 3' forward)
c-*Kit* 2 (5'-TGG AGG TGG GGT GGG GAA CT 3', reverse)
β-*Tubulin* 1(5'- GGAACATAGCCGTAAACT 3', forward)
β-*Tubulin* 2(5'- TCACTGTGCCTGAACTTA 3' reverse)

The *c-kit* primer pair amplified an 668 bp-fragment of the *Kit* gene and the β-*Tubulin* primer pair amplified a 300 bp-fragment of β-*Tubulin* gene. Cycle conditions were 95°C 5 min denature step; 25-35 cycles: 95°C 30 seconds denature step; 55°C 30 seconds annealing primer with single strand DNA template; 72°C 30 seconds extension step and 72°C 8 minutes final extension step. Resulting cDNA samples and controls were added to ethidium bromide stained 1% agarose gels and separated and visualized by gel electrophoresis. Band lengths were made in reference to a 1KB plus ladder (Invitrogen)

2.1.6.1 Q-RTPCR

RNA was isolated from cells as described (2.1.6) from heterozygous and KIT null clones developed by Bashamboo *et al* (2006) (Het1 and Null 1) and with newly derived Heterozygous and KIT null clones (Het 2 and Null 2). RNA was isolated from three independent experiments grown in the presence of LIF. TaqMan analysis was carried out by Gillian Cowan (2009 PhD thesis) and performed using the Roche Universal Probe Library with the primers

Oct4 1 (5'- TGAGGCTACAGGACACCTTTC 3'forward)
Oct4 2 (5'-TGCCAAAGTGGGGACCT 3' reverse)
Nanog 1 (5' –AGCCTCCAGCAGATGCAA 3'forward)
Nanog 2 (5' GGTTTTGAAACCAGGTCTTAACC 3' reverse)
Relative expression, with error bars, was presented as bar charts.

2.1.7 Restriction digest

DNA was cut with an appropriate restriction enzyme and buffer then incubated for 2-16 hours at an enzyme dependent optimal temperature. If restriction reaction required two enzymes, the buffer allowing maximum activity to both enzymes was selected. Efficient restriction was visualized by fragment separation by gel electrophoresis.

2.1.8 Sequencing

A sample (5µl) of purified plasmid DNA isolated by Miniprep (2.1.2) was combined with 1µl of 10mM uni-direction primer then submitted for automated sequence analysis by the University of Edinburgh's School of Biological Sciences Sequencing Service. Sequencing reactions were carried out according to the manufacturer's specifications, using the ABI PRISM Big-dye terminator premix sequencing kit (Perkin-Elmer) where thermocycle-sequencing was run on an ABI sequencer (Applied Biosystems).

2.2 ES CELL ELECTROPORATION

2.2.1 DNA preparation for electroporation

20µg of targeting vector was linearised with an appropriate enzyme overnight (2.1.7) at a temperature optimal to the enzyme. The restriction reaction was subsequently purified using an equal volume of 50:50 phenol: chloroform solution. The mixture was vortexed, spun at full speed in an Eppendorf 5415D microfuge for 10 minutes before recovering the aqueous phase. This purification step was repeated. The purified and linearised vector DNA was then precipitated with ammonium acetate and isopropanol, washed with 70% ethanol and resuspend in 20µl of sterile, tissue culture grade PBS ready for electroporation.

2.2.2 Electroporation Technique

$3x10^7$ cells were pelleted by centrifugation (1000rpm for 5 minutes) then resuspended in 600µl of sterile PBS. This suspension was then gently mixed with 20µg of linearised and purified targeting vector DNA (as described 2.2.1) and transferred into an electroporation cuvette (Invitrogen, 0.4cm gap). The cells were electroporated in a GenePulser™ (Biorad) with one pulse of 250V set at a capacitance of 500µF. After a recovery period of 5

minutes on ice, the electroporated cells were resuspend in 110mls (10mls per plate) of LIF containing ES cell growth media and placed into 11 pre-gelatinised 10cm tissue culture grade Petri-dishes. An additional plate containing only WT E14 cells was set up concurrently as a non-electroporated cell control. The cells were allowed to adhere to the plates overnight before replacing the media with antibiotic and LIF containing media. Of the 11 electroporated plates, 10 were treated with antibiotic/LIF containing media to identify resistant cell clones. The remaining plate was treated with LIF containing media only, to identify any contaminants arising from the electroporation process that would be masked on addition of the antibiotic. The non-electroporated control cells were grown in the presence of the antibiotic to validate the efficacy of the antibiotic. Cells were treated for approximately 9 days with the antibiotic with daily fluid and media changes after which time individual resistant clones were picked and placed into individual wells of a 96 well plate in the presence of Antibiotic /LIF containing media. Upon reaching 70-80% confluency the cells were trypsinised and re-plated in triplicate, into fresh pre-gelatinised 96 well plates using a multichannel pipette with a fresh tip per clone. When reaching 70-80% confluency, 2 plates were cryopreserved for further expansion upon identification of correctly targeted clones. To cryopreserve, cells were trypsinised with 50µl of trypsin and then agitated into single cells suspension on addition of 50µl of 20% DMSO in fetal calf serum. The plate was sealed with parafilm, placed into an insulated polystyrene container and stored at -80ºC. The remaining plate was allowed to grow to 100% confluency for DNA extraction and Southern blot analysis.

2.2.3 Generation of heterozygous and KIT Null clones

To generate the heterozygous (*KitW*$^{lacZ/+}$) cell line, wild type ES cells were electroporated (as 2.2.2) with 20µg of N*ot*I- linearised and purified pGN Δ-Kit targeting vector DNA. To generate the KIT Null (*Kit* $^{W\text{-}lacZ/W\text{-}lacZ}$) cells, the heterozygous *Kit* $^{W\text{-}lacZ/+}$ cell line was plated at $1x10^3$ cells/$10cm^2$ tissue culture grade Petri-dishes then selected with 2mg/ml G418 for 15 days (Lefebvre, Bai et al. 2001). Resistant colonies were picked, replicated in 96-well dishes and genomic DNA was isolated from expanded colonies for screening, using established protocols (Laird, Zijderveld et al. 1991)

2.2.4 X-gal stain of targeted clones.

This assay was designed to envisage the in *situ* detection of cells carrying the *lacZ* gene. *lacZ* encodes the β-galactosidase enzyme which catalyses the hydrolosis of the β-galactoside, X-gal. This reaction results in the release of galactose and 5-bromo-4-chloro-3-hydroxyindole. 5-bromo-4-chloro-3-hydroxyindole is then oxidised to 5,5'-dibromo-4,4'-dichloro-indigo, a blue insoluble product that easily distinguishes the presence of a functional *lacZ* gene by light microscopy.

Media was aspirated from targeted clone monolayers grown in 96 well plates (as 2.2.2). 100µl of 0.05% glutaraldehyde in PBS (Sigma) was added to each well using a multichannel pipette and incubated for 5 minutes at RT. The supernatant was discarded by aspiration and the monolayers were gently washed twice with 100µl of PBS. On the final wash, the PBS was left to contact the cells for 10minutes at RT. After the incubation the PBS was aspirated and replaced with 200µl of X-gal solution. (sigma). Cells were incubated at 37°C at 5% CO_2 for 2-24 hours. Positive clones with functional *lacZ* turn blue.

2.2.5 ACK2- APC detection of targeted clones.

KIT Null (*Kit* $^{W\text{-}lacZ/W\text{-}lacZ}$ *)* were screened for KIT protein expression by flow cytometry using the ACK2-APC conjugated antibody (eBioscience), an APC- conjugated Rat IgG2b isotype control and unstained cells. $6x10^5$ WT E14 and KIT Null cells were resuspended in 450µl of PBS. 150 µl of each cell line was divided between 3 separate 15ml falcon tubes and labeled 'stained'(ACK2-APC), 'unstained' or 'isotype control' (APC-Isotype control). 5 µl of the appropriated antibody (0.015µg) or PBS (for unstained sample) was added to each tube and incubated under foil at +4°C for 20 minutes. After this incubation period, 5mls of PBS was added to each tube and centrifuged at 1200rpm for 5 minutes. The supernatant was discarded and 400µl of PBS was added to each tube then transferred to fresh appropriately labeled FACS tubes. Samples were then analysed using Flow cytometry with the FACS Caliber cytometer

(633 nm laser) and analysed using CellQuest software (Becton Dickinson). Resulting data was displayed as flow cytometry histograms.

2.3 SOUTHERN BLOT

2.3.1. The Southern Blot Technique

Genomic DNA (10µg) was digested with an appropriate enzyme overnight *(Eco*RI) at a temperature optimal to the enzyme. DNA was precipitated with ammonium acetate and isopropanol then resuspended in 30µl of nuclease free water.

Genomic DNA isolated from a 96-wellplate was restricted using a cocktail containing 1x buffer, 1mM spermidine, 100µg/ml RNAse and 10units of restriction enzyme. 30µl of this restriction cocktail was added per well using a multi-channel pipette then incubated overnight in a pre-humidified chamber at a temperature optimal to the restriction enzyme. 10X loading buffer was added to each well before adding the restriction fragments to a 0.7% agarose gel alongside a 1KB+ genomic ladder (Invitrogen). The fragments were resolved on gel at 20V overnight and the resulting gel picture was captured using a UV-doc system (Uvitec). A ruler placed next to the DNA ladder allowed sizing of the DNA fragments upon Southern Blot development. Following this, the gel was depurinated for 5 minutes in depurination solution (10.25ml HCl in 500ml H_2O) and denatured for 45 minutes in 0.5M NaOH and 1.5M NaCl then further 15 minutes incubation in 10XSSC neutralisation solution. DNA from the denatured gel was then transferred Hybond N+ membrane (Amersham) by wet Southern Blotting Capillary Transfer as described in (Sambrook et al., 1989). The DNA was blotted onto the membrane overnight and upon disassembly, the location of each well was marked lightly onto the membrane with pencil. The membrane was rinsed in 2XSSC and air-dried then the DNA was cross linked to the membrane by exposing the membrane to UV light for 2 minutes using the UVdoc System (Uvitec).

2.3.2 Probe generation, radioactive labeling and purification

Procedures involving radioactive materials and reagents were carried out in a designated radioactivity area in accordance with The University of Edinburgh's *Working with Radioactivity* guidelines. The Southern probe DNA was generated by high fidelity PCR of the DNA from WT E14 genomic DNA using the primer pair (Bernex et al., 1996).

Probe 1 (5'- CAAGAGGAAAGTGTAGCTCGATG 3' forward)
Probe 2 (5' CAAGAGGAAAGTGTAGCTCGATG 3' reverse)

The probe was generated by PCR amplification (Invitrogen, Platinum Taq Polymerase High Fidelity kit) with the following settings: 94C for 5min then 30 cycles of 94C for 15sec, 55C for 30sec, and 72C for 1min followed by 72C for 10min. The resulting PCR fragment was gel purified with a Quiagen gel purification kit in accordance with manufactures instructions and 100ng of purified DNA was denatured by adding nuclease free water to a final volume of 11µl and incubated at 100ºC for 5 minutes. The resulting DNA was then was snap-cooled on ice and 4µl of HighPrime mix (Roche) containing 1 unit/µl Klenow polymerase; 125µM dATP, dGTP and dTTP each in 50% glycerol was added. Following this, 5µl of radioactively labeled [α-^{32}P] dCTP (10mCi/ml) was added to the labeling mix (on ice) and the reaction was incubated at 37ºC for 1 hour. On incubation completion, the reaction mix was applied to a 1.5ml Sephadex G50 (Sigma) column. The column was washed with 1ml dH_2O and the labeled probe was eluted from the column into an eppendorf and diluted with 800µl dH_2O. Finally, the probe DNA was denatured for 5 minutes at 100ºC.

2.3.3 Pre-hybridisation and hybridisation

The Southern blot membrane was pre-hybridised for 30 minutes at 65ºC in an evenly rolling Techne hybridisation bottle in a Techne Hybridiser HB-1D oven. The denatured radioactively labeled probe was added to 10mls of pre-warmed (65ºC) ExpressHyb

solution (Clontech) pre-hybridisation solution and the blot was further incubated at 65ºC overnight.

2.3.4 Washing

After incubation with the probe, the pre-hybridization solution was discarded from the blot in accordance with the University of Edinburgh's radioactivity disposal guidelines. The blot was then washed three times with 100ml of pre-warmed (65ºC) 2XSSC/ 1% SDS and incubated for 15 minutes in a rolling Techne hybridisation bottle in a Techne Hybridiser HB-1D oven at 65ºC. The blot was then washed a further 3 times in 0.2XSSC/ 1%SDS for 15 minutes at 65ºC. The washing procedure was terminated when the level of background radioactivity, measured by Geiger counter, reached a low localised radioactive level. If the signal remained high, the washing procedure (0.2XSSC/ 1%SDS) was repeated. The blot was then mounted in Saran-wrap inside an X-ray cassette and exposed to X-ray film (Kodak) at -80ºC for 2 to 10 days.

2.4 CELL AND TISSUE CULTURE

2.4.1 Culture conditions

The feeder-independent mouse WT E14 tgt2a parental ES cell line was obtained from Smith (1991) and grown in ES cell media comprising of; Glasgow's Minimal Essential Medium (GMEM) supplemented with 20% fetal calf serum, 0.25% sodium bicarbonate (Gibco), 0.1% non-essential aminoacids, 2mM L-glutamine (Gibco), 1mM sodium pyruvate (Gibco), 0.1mM β-mercaptoethanol (Sigma) and 100 U/ml Leukaemia Inhibitory Factor (LIF) (Jackson et al., 2002). Cells were grown on tissue culture grade plastic pre-coated with 0.1% gelatin and incubated at 5% CO2 in a 37ºC humidified incubator (Galaxy S, Wolf Laboratories). LIF was produced on site at the John Hughes Bennett Laboratories and involved the collection of conditioned medium from COS7 cells transfected with the pDR10 LIF-DIA expression construct (a gift from Austin Smith) (Smith. 1991) LIF-containing supernatant that detectably inhibited ES cell

differentiation via clonal assay was defined as 10 U/ml then 100 U/ml was used in standard maintenance medium.

2.4.2 Thawing cells

Cryopreserved cells stored at -140ºC were thawed rapidly in a 37ºC water bath and transferred to a universal tube containing 10mls of pre-warmed growth media. The cells were pelleted by centrifugation at 1000rpm for 3 minutes, the supernatant was removed and the cell pellet was resuspended in fresh culture medium and transferred into a pre-gelatinised 25cm2 culture flask with 10ml culture medium. The culture medium was replaced after 4 to 12 hours upon cell adherence to the flask.

2.4.3 Passage and expansion of ES cells

Cells were seeded into pre-gelatinised flasks at a seeding density of $1x10^6$ cells per 10ml and were passaged every 2 days upon reaching 70-80% confluency. Cells were never allowed to overgrow. To passage, medium was aspirated from the flask then the cells were washed twice with PBS and incubated (5% CO2 at 37ºC) with 2mls of trypsin solution (1% trypsin, 1% chick serum and EDTA in PBS). After 5 minute incubation in trypsin solution, the cells lifted off the tissue culture plastic and were gently disaggregated into a single cell suspension using a 10ml pipette. Trypsinised cells were then neutralised with a 5X volume of culture medium in a Falcon tube and cells were centrifuged at 1000rpm for 5 minutes. The cell pellet was resuspended in 10ml fresh medium and cell number was determined using a haemocytometer on a Leitz Labovert microscope. $1x10^6$ cells per ml in 10ml were transferred to a new pre gelatinized 25cm2 flask, $3x10^6$ cells per ml in 30ml to a $75cm^2$ flask or $8x10^6$ cells in 80ml of culture medium to a $225cm^2$ flask and incubated at 5% CO2 in a 37ºC until required or reaching 70-80% confluency.

2.4.4 ES cell clonal assay

500 ES cells per well were plated in pre-gelatinised 6-well plate with ES cell medium containing LIF. Cells were added to the plates in a swirling motion in order to avoid clumping and gain an even distribution of cells in the wells. The following day the wells were gently rinsed with PBS and the medium was replaced to the wells. LIF was only added to wells if stem cell colony growth was required. Cells under differentiation were grown without LIF. After 5 days in these culture conditions the ES cell colonies were fixed and stained for alkaline phosphatase activity in accordance with the Alkaline Phosphatase Kit (Sigma-Aldrich). Primarily, cells were fixed in the fixative solution (67% acetone, 25% citrate solution, 8% formaldehyde) for 30 seconds, then gently rinsed in water and subsequently incubated with the colour substrate solution for 15 minutes in the dark. After a final rinse, the plates were left to air dry and alkaline phosphatase staining of individual colonies was analysed using an inverted Zeiss Axiovert25 microscope. Colonies were scored as stem cell (pink), mixed (dense central core of pink surrounded by a periphery of unstained cells) and differentiated (unstained) (Figure 2.1). Statistical analysis was carried out by t-test using the Minitab software $P<0.05$ was considered statistically significant.

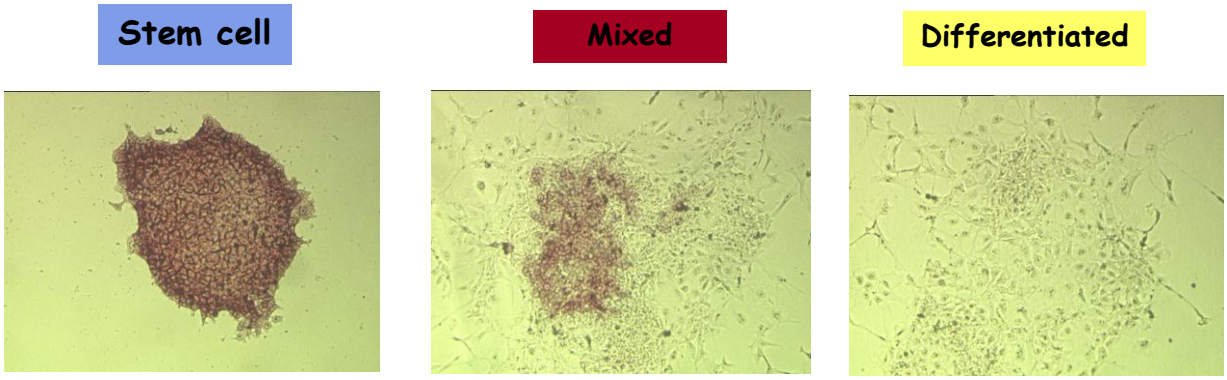

Figure 2.1 Classical morphologies of Stem cell, Mixed and Differentiated colonies. The colour coding above the pictures represents stem cell (blue); mixed (red) and differentiated (yellow) represent the bar colours in subsequent clonal assay histograms.

2.4.5 Preparation of embryoid bodies (EB) and cardiomyocyte assay

A suspension of ES cells at $6x10^5$/20mls of growth media (LIF+) was poured into the lid of a square Petri dish ($120mm^2$) an used as suspension reservoir. Using multichannel pipette 10µl drops of this ES cell suspension was pipetted onto the lid of a fresh square Petri dish ($120mm^2$) to produce a sequence of equally sized and distributed 'hanging drops'. The petri dish lid, containing the hanging drops was gently placed onto the base of the sterile water filled (10mls) petri dish. The drops were then incubated at 37ºC in a humidified incubator for 2 days and then harvested by tapping the dishes and collecting the EBs in solution with a pipette. EBs from all dishes were pooled into a universal then spun at 800 rpm for 3 minutes. After discarding the supernatant, the EBs were resuspended fresh medium without LIF and supplemented with Penicillin/Streptomycin solution (Sigma) at 1:100. Following this, the EBs were cultured in suspension in a bacteriological $100mm^2$ dish for a 5 days with medium changes every two days. After 5 days, single EBs were transferred into a pre-gelatinised 24 well plate and analysed for beating foci.

2.4.6 Growth rate determination

WT E14 and KIT Null cells were seeded into $25cm^2$ tissue culture grade flasks at $1x10^6$/10mls in ES cell growth media with the inclusion of LIF. After 2 days in culture, cells were trypsinised, pelleted and counted using a haemocytometer. Resulting cell numbers were presented as histograms and statistical analysis was carried out using a student's t-test.

2.4.7 Colony size determination

WT E14 and KIT Null cells were cultured in a clonal assay as described (2.4.4) . After 5 days, the colonies were, fixed stained and colony size was determined using a calibrated Zeiss Axiovert microscope scale**.**

2.4.8 Karyotyping of ES cell clones

ES cells were seeded into 25cm^2 flask at 2x10^6 cells and allowed to adhere to the flasks overnight. The next day 100µl of KaryoMAX (Invitrogen) was added to the flask and the cells were incubated at 37ºC for 2 hours. Cells were then trypsinised, placed into 15ml falcon tubes and pelleted by centrifugation (1000 rpm for 3 minutes). The supernatant was removed and 8mls of 0.56% KCl solution was added drop by drop to resuspend the pellet. The pellet was being continually and gently vortexed throughout this time to avoid clump formation. Following this, the cell suspension was incubated in a 37ºC water bath for 12 minutes then fixed with 2ml of freshly prepared fixative (methanol 3:1 acetic acid). After gently mixing by inversion, the cells were then spun at 1000 rpm for 5 minutes (1000rpm), the supernatant was removed and the cells were again resuspended in 8mls of fixative. This spin and re-suspension was repeated 3 times. After the final centrifugation, step the cells were resuspended in 1ml of fixative and one drop of this cell fixative mixture was dropped onto a sterile slide which had been pre-cooled at 4ºC in ethanol. To stain the karyotype spreads, DAPI (Sigma) was used at a concentration of 1:2,500 dilution, then 10µl was added to the slide and cover slip mounted. 20-30 karyotyping spreads were obtained for each clone and images were captured under fluorescent light on a Zeiss Axioskop2 microscope using a 63x oil emulsion objective. The camera used was a ProgRes C14 from Jenoptik and images modified and saved using Open*lab*TM software. The number of chromosomes in each of the spreads was counted manually using Adobe Photoshop by marking each counted chromosome on the image with a red dot.

2.4.9 G418 Concentration Kill Curve

Heterozygous ES cells were plated at 4x10^3 cells into each well of a pre gelatinized 6-well plate and left to adhere to the plastic overnight in an incubator at 5% CO2 /37ºC in 5ml of LIF containing ES cell medium. After approximately 12 hours, the growth medium was discarded and replaced with 5mls of fresh, antibiotic (G418 ; Geneticin, PAA) containing medium. Several antibiotic concentrations (0, 100, 150, 200, 250, 300, 350 and 400µg/ml) were employed to determine the concentration at which WT E14 cells

perished over the course of 7-9 days. Media from each antibiotic condition was replaced on a daily basis and cell death was monitored. The optimal G418 concentration at which all heterozygous cells perished within 9 days was 260-280µg/ml.

2.4.10. Puromycin concentration Kill Curve

KIT Null and WTE14 cells were plated at $4x10^3$ cells into each well of a pre gelatinized 6-well plate and left to adhere to the plastic overnight in an incubator at 5% CO2 /37ºC in 5ml of LIF containing ES cell medium. After approximately 12 hours, the growth medium was discarded and replaced with 5mls of fresh, antibiotic containing medium. Puromycin was added at various concentrations 0, 0.5, 0.75, 1.00, 1.25 or 1.5 µg/ml to determine the concentration at which KIT Null/ WT E14 cells perished over the course of 7-9 days. Media from each antibiotic condition was replaced on a daily basis and cells death was monitored. The optimal Puromycin concentration at which all WT E14 / KIT Null cells perished within 9 days was *0.7 µg/ml.*

2.5 WESTERN BLOT PROCEDURE

2.5.1 Protein extraction from cells

Media was carefully removed from ES cell monolayers and washed twice with ice cold PBS containing protease (Sigma) and phosphatase inhibitors (Sigma). 300µl of ice cold, RIPA buffer (Pierce scientific), containing protease and phosphatase inhibitors, was added to 25cm2 flasks monolayers and cells were harvested on ice using a rubber policeman. Harvested cells were placed into an appropriately labeled eppendorf and centrifuged at +4ºC for 15 minutes at 14,000g. Protein yields were increased by sonicating the pellet for 30 seconds with a 50% pulse. The supernatant was collected in a fresh tube (on ice) and protein concentration was determined using a Bradford Assay (Bio-Rad).

2.5.2 Gel electrophoresis and Western blotting procedure

Running buffer (including antioxidant) and transfer buffer (including 10% methanol) were prepared in accordance with the Invitrogen NU Page 1D electrophoresis protocol. Extracted protein samples were also prepared in accordance with the Invitrogen NuPage 1D electrophoresis protocol. In brief 50µg of protein sample was prepared with NuPage LDS sample buffer, reducing agent and glycerol then denatured by boiling at 70ºC for 10 minutes. Samples and molecular weight ladder (Prestained Marker-Invitrogen) were then loaded onto pre-cast gels 10% (BIS-Tris) gels (Invitrogen) and run in the NuPAGE Xcell surelock Mini cell electrophoresis module at 200V for 35 minutes (Mes) or 200V for 50 minutes (Mops). Mops running buffer was used for proteins between 14-200 kD whereas Mes running buffer was used for proteins 2.5 – 60kD. After running the proteins on the gel, proteins were transferred (wet transfer) onto nitrocellulose membranes (Whatmann) in accordance with Invitrogen Xcell surelock Mini cell electrophoresis manufacture's instructions and using the Xcell surelock Mini cell electrophoresis equipment. Transfer was carried out at 30V for 1 hour.

To eliminate background staining, unbound sites on the membrane were blocked for 1 hour at RT or at 4ºC overnight in 5% milk or 5% BSA in 1X TBS -0.05% Tween20 with gentle rocking. Primary antibody at an appropriate dilution (0.5% BSA in 1XTBS with 0.05% Tween) was then added to the membrane and incubated for 2 hours at RT or overnight at 4ºC depending on antibody binding efficacy. Following this, the membranes were washed in three times for 10 minutes in 1XTBS with 0.05% Tween then incubated for 1 hour in secondary antibody conjugated to HRP diluted in 0.5% BSA in TBS with 0.05% Tween according to manufacturer's instructions. The membrane was then washed a further 3 times to remove unbound antibody in 1XTBS with 0.05% Tween. All incubations and washes were performed on a shaking platform. Protein bands were visualised by incubation with ECL reagents for I minute (Luminol-Fluka / Coumarc acid Sigma) then mounted in Saran-wrap inside an X-ray cassette and exposed photographic film (Kodak) at RT for 1 minute, 2minutes and 5 minutes. Primary antibodies include anti-GAPDH monoclonal antibody (AbCam) anti-Bcl2 (N-19) Anti JNK1 and anti p-c-

Jun (Santa Cruz Biotechnology), anti-Flag (UBI Company, 2EL-1B11 mouse monoclonal), anti-EGFP (BD company, JL-8 mouse monoclonal); Secondary antibodies include Horseradish-peroxidase (HRP) conjugated goat anti-rabbit and sheep anti-mouse (Biosource).

2.6 ASSAYS

2.6.1 Sorting cell populations

WT E14 cells were sorted into populations of KIT $^{hi, lo}$ and ^{neg} based on their level of KIT expression using the ACK2-APC KIT antibody (e-Bioscience). Cells falling between categories were eliminated from analysis. Cells were sorted using FACS analysis on the FACSVantage cytometer (laser 633nm) and gated using unstained and isotype control stained cells. Sorted cells were then grown in their individual categories for 3 passages (6 days) with the inclusion of LIF then re-analysed for KIT expression with an ACK2-APC antibody via flow cytometry with the FACS Calibur cytometer (laser 633 nm) analysis. WT E14 and KIT Null cells were tested concurrently as controls. This was carried out in triplicate and KIT expression was recorded as a percentage and presented a histogram.

To observe the effect of KIT expression, sorted cells, WT E14 and KIT Null cells were placed into a clonal assay and their ability to form stem, mixed or differentiated colonies was recorded. Cells were grown under both self renewal (+LIF) and differentiation (LIF-) conditions and fixed, stained and analysed as described (2.4.4).

2.6.2 ACK2 Dose dependency

500 WTE14 ES cells were plated per well in a pre-gelatinised 6-well plate with ES cell medium containing LIF. The following day the wells were gently rinsed with PBS and the medium replaced LIF+/- ; ACK2 +/- (Insight Biotechnology) or isotype control +/-. ACK2/isotype control was added at a final concentration of 0, 5 10 and 15μg. After 5

days the assay fixed and stained for alkaline phosphatase activity, and colonies were scored as stem mixed or differentiated as described above. The assay was carried out in triplicate and analysed by student's t-test.

2.6.3 Prolonged exposure to ACK2

WT E14 cells were passaged once (2 days) twice (4 days) or > 10 times (>20days) in the presence of the KIT neutralising antibody ACK2. Following from this, clonal assays were performed (2.4.4), fixed stained and analysed (2.4.4). To observe the effect of neutralisation reversal, cells previously exposed to the antibody were placed into assay without antibody or given one clear passage (2 days) without antibody before being placed into assay.

2.6.3.1 Assessing ACK2 induced apoptosis

WT E14 cells were cultured ACK2 +/- or isotype control +/- for one passage (2days) in the presence of LIF. Following this, cells were re-seeded at 0.5 x 10^6 in fresh 25cm^2 tissue culture grade flasks with ACK2 +/- or isotype control +/- in the presence or absence of LIF. Flasks were incubated at 37°C at 5% CO^2 for 3 days before staining with Annexin V-FITC as described (2.6.4).

2.6.4 AnnexinV- FITC / PI staining

Annexin-PI staining was carried with the BD Pharmingen Annexin V-FITC Apoptosis KIT II in accordance with BD Bioscience's manufacturer's instructions. Briefly, 1x10^6 KIT Null cells, WTE14cells, BCL2 overexpressing clones or PD169316/ACK2 /ZVAD/SP600125/ isotype control treated cells were resuspended in 1X Annexin V binding buffer (Ca^{2+}). 100µl of this suspension was transferred into each of 4 FACS tubes labeled 'unstained' 'Annexin only' 'PI only' and 'Annexin V-FITC /PI'. 5µl (5µg) of ready made Annexin V-FITC dye was added to the appropriate tubes an incubated for 15 minutes under foil at room temperature. To account for background staining, Annexin V-FITC was added at 2.5 and 1.25 µl (2.5 and 1.25µg respectively). The effect of non-

specific binding was alleviated by pre blocking the cells with recombinant Annexin V (5μg) (BD Bioscience) for an incubation period of 15 minutes at RT before a subsequent stain in Annexin V-FITC. The cells were then gently vortexed half way through this incubation to disperse the dye through out the sample. After incubation, 300μl of binding buffer was added to each tube. Flow cytometry was carried out using FACScan cytometer equipped with a 488 nm laser and analysed using CellQuest software (Becton Dickinson).

2.6.5 DAPI staining

WTE14, KIT Null or BCL2 over-expressing cells were centrifuged at 1200rpm for 5 minutes then resuspended at 5000 cells/μl in 4% PBS buffered paraformaldehyde solution containing 10 μg/ml 4'6-diamidino-2-phenylindole (DAPI, Sigma). 10 μl of this suspension was placed onto a glass slide and covered and secured with a coverslip. The morphology of the cells' nuclei was observed using fluorescence microscopy under fluorescent light on a Zeiss Axioskop2 microscope using a 63x oil emulsion objective. The camera used was a ProgRes C14 from Jenoptik and images modified and saved using Open*lab*TM software at an excitation wavelength 350 nm. Nuclei were considered to have the normal phenotype if displaying diffuse and homogenous fluorescence. Apoptotic nuclei were identified by condensed chromatin gathering at the periphery of the nuclear membrane or by a fragmented morphology of nuclear bodies. More than 150 cells were counted for each cell line and the percentage of apoptotic nuclei was determined. This experiment was carried out in triplicate.

2.6.6 FLICA staining

FLICA staining was carried out in accordance with Immunochemistry Technologies' PolyCaspase detection kit and manufacturer's instructions. Briefly, $1x10^6$ KIT Null cells, WTE14 cells, BCL2 over-expressing clones or PD169316 /ACK2/ZVAD/SP600125/UV/ isotype control treated cells were resuspended in 1ml of PBS. 300μl of this suspension

was placed into fresh 15 ml falcon tubes and 10µl of 10X FLICA reagent was added to each tube. Stain was dispersed throughout the cells by flicking the tube several times. Cells were incubated with the FLICA stain for 1 hour at 37°C at 5% CO2. The cells were gently flicked several times throughout this incubation. Following this, 1 ml of 1X wash buffer was added to the tube and spun at 1000rpm for 5 minutes. The supernatant was discarded. This was carried out a further two times. After the final wash, the cells were resuspended in 400µl of wash solution and were ready for flow cytometry analysis. Unstained cells were prepared concurrently for cytometer gating controls. Fluorescein was measured on the FL1 channel with the FACscan cytometer (laser 488nm).

2.6.7 UV induction

A 75cm^2 flask of confluent cells was exposed to maximum intensity UV light for 10 minutes. Following from this, the cells were placed back into an incubator at 37°C at 5% CO^2 for 20 hours. These cells acted as a positive control for the FLICA assay.

2.7 APOPTOSIS REGULATION

2.7.1 ZVAD

2.7.1.1 Annexin V-FITC/PI/FLICA staining

WT E14 and KIT Null cells were seeded at 0.5 x 10^6 in 25cm^2 tissue culture grade flasks with ZVAD +/- (Biomol Research) in the presence or absence of LIF. Flasks were incubated at 37°C at 5% CO^2 for 3 days before staining with Annexin V-FITC (WT E14 only) or FLIKA (WT E14 and KIT Null) as scribed above.

2.7.1.2 Clonal assays

500 WTE14 or KIT Null ES cells per well were plated in pre-gelatinised 6-well plates with ES cell medium containing LIF. The following day the wells were gently rinsed with PBS and the medium replaced LIF +/- and ZVAD +/- (40µM). The assay was carried out for further 5 days with a fluid change including ZVAD replenishment on day 3. On completion of the 5 day incubation, ES cell colonies were fixed and stained for alkaline phosphatase activity as 2.2.4.). The assay was carried out in triplicate (with two replicates per test) and analysed by either Student's t-test or one way analysis of variance (ANOVA).

2.7.2 P38 inhibitor PD169316

2.7.2.1 P169316 dose dependency assay

500 WTE14 ES cells per well were plated in pre-gelatinised 6-well plate with ES cell medium containing LIF. The following day, wells were gently rinsed with PBS and the medium replaced LIF+/- ; PD169316 +/- (Calbiochem) or DMSO control +/-. PD169316/DMSO control was added at a final concentration of 0, 5 10 and 15µM (PD169316) / 0, 0.1%, 0.2% and 0.3% (DMS0). After 5 days the assay fixed and stained for alkaline phosphatase activity, and colonies were scored as stem, mixed or differentiated as described (2.4.4).). The assay was carried out in triplicate (with two replicates per test) and analysed by either Student's t-test or one way analysis of variance (ANOVA).

2.7.2.2 PD169316 and Annexin/FLICA staining

WT E14 and KIT Null cells were seeded at 0.5 x 10^6 in 25cm^2 tissue culture grade flasks with PD169316 +/- (10µM) in the presence or absence of LIF. Flasks were incubated at 37°C at 5% CO^2 for 3 days before staining with Annexin V-FITC (WT E14 only) or FLIKA (WT E14 and KIT Null) as scribed above (2.6.4 and 2.6.6.)

2.7.2.3 PD169316 and clonal assays

500 WTE14 or KIT Null ES cells were plated per well in pre-gelatinised 6-well plates with ES cell medium containing LIF. The following day the wells were gently rinsed with PBS and the medium replaced LIF +/- and PD169316 +/- (10μM). The assay was carried out for further 5 days with a fluid change including PD169316 replenishment on day 3. On completion of the 5 day incubation, ES cell colonies were fixed and stained for alkaline phosphatase activity as described above (2.4.4).

2.7.2.4 SP600125 dose dependence assay

500 WTE14 ES cells were plated per well in pre-gelatinised 6-well plates with ES cell medium containing LIF. The following day the wells were gently rinsed with PBS and the medium replaced LIF+/- ; SP600125+/- (Calbiochem) or DMSO control +/-. SP600125/DMSO control was added at a final concentration of 0, 2.5 5, 10, 15 and 20μM After 5 days the assay was fixed and stained for alkaline phosphatase activity, and colonies were scored as stem, mixed or differentiated as described above (2.4.4).). The assay was carried out in triplicate (with two replicates per test) and analysed by either Student's t-test or one way analysis of variance (ANOVA).

2.7.2.5 SP600125 Western blot analysis

Western blot analysis was carried out a described above (2.5) but specifically, protein lysates were isolated from WT E14 cells grown in the presence or absence of LIF and the presence or absence of the inhibitor SP600125 (5μM) for three days. Proteins were tested with anti-GAPDH (Abcam), anti-JNK and anti phosphorylated -c-Jun (Santa Cruz Biotechnology).

2.7.2.6 SP600125 serum starve/SCF induction assay

$0.5x10^6$ cells were grown overnight at 37°C at 5% CO^2 on pre-gelatinised 6 well plates with the inclusion of LIF. The following day, the ES cell media was removed and the cell monolayers were washed twice with PBS. The ES cell media was then replenished with fresh media with serum+/- ; ACK2 +/- ; Isotype control +/-. All cells, except controls, were starved of serum for 12 hours and incubated at 37°C at 5% CO^2. SCF at 50ng/ml was added to the appropriate wells for 15 minutes at 37°C at 5% CO^2 to stimulate KIT signalling. After this incubation, cell lysates were immediately removed on ice as described above and proteins were prepared for Western blot analysis.

2.7.2.7 SP600125 Annexin/FLICA stain

WT E14 and KIT null cells were seeded at 0.5 x 10^6 in $25cm^2$ tissue culture grade flasks with SP600125 +/- (5µM) in the presence or absence of LIF. Flasks were incubated at 37°C at 5% CO^2 for 3 days before staining with Annexin V-FITC (WT E14 only) or FLIKA (WT E14 and KIT Null) as scribed above (2.6.4 and 2.6.6.)

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2.7.2.8 SP600125 clonal assays

500 WTE14 or KIT Null ES cells were plated per well in pre-gelatinised 6-well plates with ES cell medium containing LIF. The following day the wells were gently rinsed with PBS and the medium replaced LIF +/- and SP600125 +/- (5µM). The assay was carried out for further 5 days. On completion of the 5 day incubation, ES cell colonies were fixed and stained for alkaline phosphatase activity as described above (2.4.4)

2.7.2.8 SP600125 Prolonged exposure

WT E14 cells were passaged untreated or passaged with the inclusion of SP6001225 once (2 days) twice (4 days) or 20 times (40 days) before placing into a clonal assay.

Following this assays were fixed stained and analysed as described above (2.4.4). To observe the effect of JNK inhibition reversal, cells previously exposed to antibody were placed into assay without antibody or given one clear passage (2 days) without antibody before being placed into assay. The assay was carried out in triplicate (with two replicates per test) and analysed by one way analysis of variance (ANOVA).

2.8 BCL2 OVER-EXPRESSION

2.8.1 Generation of the EGFP-FLAG-BCL2 PCR insert

The EGFP-FLAG-BCL2 PCR insert was generated using the P513-kEGFP-Flag-BCL2 vector kindly donated by Helen Boeuf (Duval, Malaise et al. 2004) as a template. A PCR product was generated with *Not1* and *Xho1* restriction ends using the primer pair;

Bcl2 F (5'- GCGGCCGCATGGTGAGCAAGGGCGAGGAGCTGT T 3' forward)
Bcl2xho (5'- CTCGAGTCACTTGTGGCCCAGGTATGCACC 3' reverse)

PCR was carried out with PCR reagents from Cambio Epicentre and included a proofreading polymerase enzyme with buffer J. Specifically, PCR was carried out with the following protocol; (95°C for 5 minutes/ (94ºC, 55 ºC, 72 ºC) for 30 minutes and 72ºC for 15 minutes.

2.8.2 Topo cloning

Fresh PCR product was used to carry out the TOPO cloning (poly A tail to combine with linearised vector with T overhangs) in the PCR 8 GW TOPO vector (Invitrogen) in accordance with Invitrogen's cloning instruction manual. Topo clones were transformed into chemically competent Dh5α cells (Invitrogen top 10 K4750-10) and grown over night at 37°C on spectinomycin plates. Resulting clones were picked and Minipreped using QIAprep Spin Miniprep Kit as described above (2.1.2). Plasmid DNA (100ng) was

sent for sequencing as described above (2.1.8) with the inclusion of 10% DMSO due to the sequence being G-C rich. Sequencing was carried out using the following primers;

GW1 (5´- GTTGCAACAAATTGATGAGCAATGC-3´ forward)
GW2 (5' - GTTGCAACAAATTGATGAGCAATTA-3´ reverse)

The resulting correctly identified clone was called Topo clone 2–BCL2 which was maxi prepped as described above (2.1.2) then stored as a glycerol stock (2.1.3) (Appendix 1).

2.8.3 The Gateway Reaction

The plasmid RFCA Pcagsip- Ires Puro was designed and developed by Melany Jackson (Appendix 2) JHBL and was used as the destination vector during the gateway reaction (Figure 2.2). This vector includes ccdB F plasmid encoded gene that inhibits the growth of e-coli (Bernard and Couturier 1992). The entry clone was Topo clone 2-BCL2. A mixture of 150 ng of Clone 2-bcl2-puro-topo, 150 ng RFCA pCagsip, 5X LR clonase reaction buffer, 1µl of Gateway LR clonase II enzyme mix (invitrogen) and TE to a final volume of 20 microlitres was briefly vortexed and then incubated at 25°C for 2 hours. After incubation Proteinase K was added to the sample to terminate the enzyme reaction. The resulting product was transformed into DH5α cells and selected on LB plates containing ampicillin (37°C overnight). Colonies were picked and streaked on ampicillin, spectinmycin and chlramphenicol plates. Clones that were resistant to ampicillin only were miniprepped and sequenced. Bcl2 gateway clones were sequenced using the primers.

Oligo 164 (5' TGC TGG TTG TTG TGC TGT 3' forward)
Bcl2 Xho(5' CTC GAG TCA CTT GTG GCC CAG GTA TGC ACC 3' reverse)

Confirmed clones, called BCL2 Gateway (Appendix 3), were maxi-prepped and made into glycerol stocks as described above (2.1.2 and 2.1.3) .

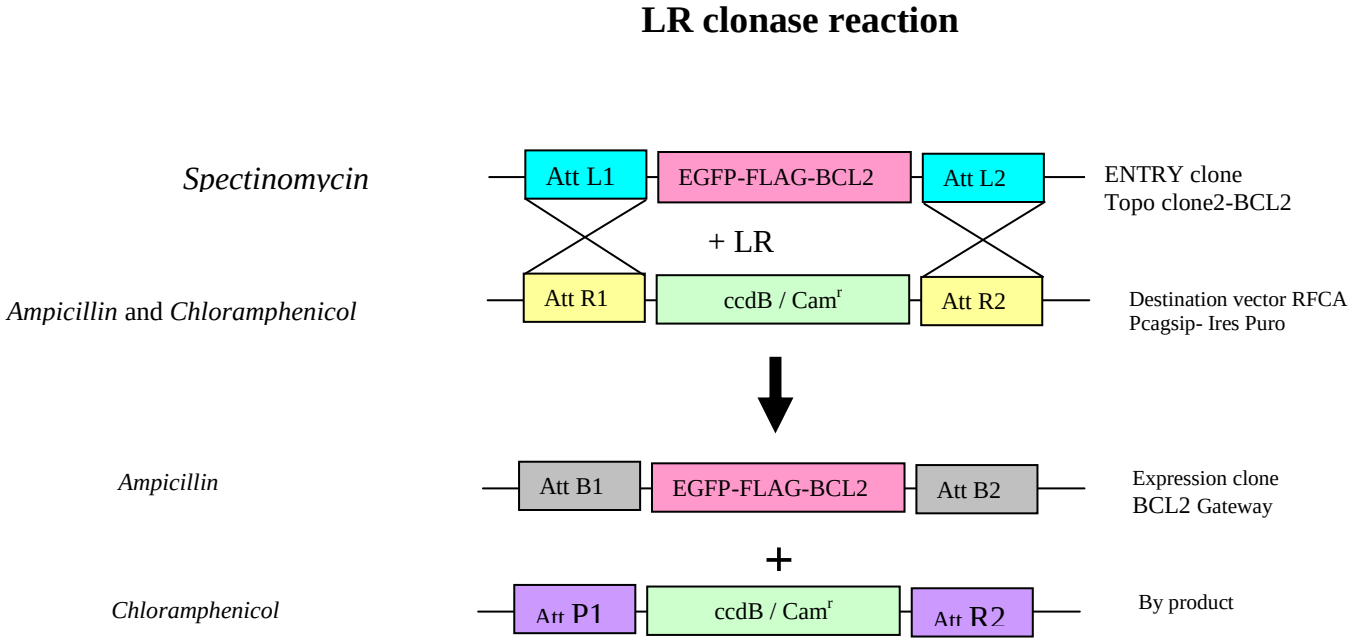

Figure 2.2 LR clonase reaction. The plasmid RFCA PCagsip is the destination vector during the gateway reaction and the entry clone is topo clone2-BCL2. Crossover at *att*L1 and *att*L2 sites for recombination transfers the gene of interest into any Gateway destination vector. Specifically designed primer binding sites within the *att* 1 and *att* 2 sites are used for sequencing with the GW1 and GW2 primers. Only Ampicillin resistant clones were selected and sequenced (diagram adapted from(Villefranc, Amigo et al. 2007)

2.8.4 BCL2 Gateway Electroporation

Electroporation was carried out as described above (2.2) but specifically, BCL2 Gateway was linearised with *Pvu1* and electroporated into NNC1cells and WTE14 cells. Linearised PCagsip was also electroporated into WT and KIT null cells as an empty vector control. KIT Null cells and WT E14 cells were grown in the presence of puromycin for selection (0.7 µg/ml) and were picked on their ability to form colonies after 9 days under puromycin (2.4.10) selection and their ability to fluoresce under UV (GFP) (*2.8.4.1).*

2.8.4.1 BCL2 EGFP Screen

Unstained BCL2 over-expressing clones and unstained empty vector control clones were screened initially by Flow Cytometry for EGFP expression using the FACScan flow cytometer on the FL1 channel (laser 488nm). EGFP positive clones were tested alongside WT E14/NNC1 control cells as well as empty vector control by Western blot as described above (2.5).

2.8.5 BCL2 over-expression and Annexin/FLICA staining

WT E14, KIT null and BCL2 over-expressing clones were seeded at 0.5 x 10^6 in 25cm^2 tissue culture grade flasks in the presence or absence of LIF and incubated at 37°C at 5% CO^2 for 3 days before staining with Annexin V-FITC or FLICA as scribed above (2.6.4 and 2.6.6.)

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2.8.6 BCL2 over expressing clonal assays

500 WT E14, KIT Null or BCL2 over-expressing ES cells per well were plated in pre-gelatinised 6-well plate with ES cell medium containing LIF. The following day the wells were gently rinsed with PBS and the medium was replaced with LIF +/- . The assay was carried out for further 5 days and completion of the 5 day incubation; ES cell colonies were fixed and stained for alkaline phosphatase activity as described above (2.4.4).). The assay was carried out in triplicate (with two replicates per test) and analysed by either student's t-test or one way analysis of variance (ANOVA).

2.9 MICRO ARRAY ANALYSIS

RNA was isolated from WTE14 and two independent KIT null cells lines (*KitW-lacZ/W-FKB and KitW-lacZ/W-lacZ*) lines grown in the presence of LIF and 36 hours after LIF withdrawal. This RNA was hybridised onto an Affymetrix chip and analysed by micro array (Bashamboo, unpublished data) in accordance with Schulz, et al 2009 and the Affymetrix Genechip Expression Analysis Technical Manual. Each cell line from each

condition was tested in triplicate and pair-wise comparisons were performed using GeneChip® Operating Software (GCOS) 1.4. Arrays were normalized by the log scale robust multi-array analysis (RMA) (Schulz, Kolde et al. 2009). The mean of each gene signal was calculated for each condition and differentially expressed genes were identified using the Student's t-test. The resulting data was managed using the KIT/SCF database generated by The University's of Edinburgh Bioinformatics. A gene was only included into the analysis if the differential fold change value was ≥ 1.5 and p value ≤ 0.05 when comparing the WTE14 cell line with the KIT Null cells lines. To ensure reproducibility, a gene had to be significantly up or down regulated in both KIT Null cells lines before their inclusion to the analysis and resulting gene lists. From the microarray analysis, a total of 45101 genes were returned of which 1,101 were differentially expressed when comparing WTE14 cells with both KIT Null cell lines. Complete gene listings can be found in Appendix 4-9. A low fold change indicates low gene expression in KIT Null cells when compared with WTE14 expression and the converse holds for genes with a high fold change. Genes were sorted into categories dependent on low and high fold changes in the presence or absence of LIF. Genes that were always high or low, regardless of LIF inclusion, were also identified (Appendix 10 and 11). Genes were also sorted on account of speculative compensatory mechanisms (Appendix 12), membrane perturbation (Appendix 13), apoptosis (Appendix 14), and JNK signalling (Appendix 15).

2.10 STATISTICAL ANALYSIS

Data from self renewal assays were initially performed using a Student's t-test and were reanalysed using one way ANOVA to confirm significance and increase sensitivity where p-values were borderline. The specific statistical analysis performed for each test condition was described in each figure legend. Self renewal assays were carried out in triplicate to obtain three independent replicates (n=3) where each replicate was carried out in duplicate to assess variance. Confidence intervals were calculated as the standard error of the mean (SEM) and were depicted as error bars on each graph. Statistical analysis was performed using the statistical analysis programme, Minitab 15.

Chapter 3

Assessing the role of c-*kit* in ES cells

Chapter 3

Assessing the role of c-KIT in ES cells

3.1 AIM

Determine the importance of the SCF/KIT pathway during ES cells self renewal and differentiation.

3.2 INTRODUCTION

KIT and its ligand SCF play an important role in the self renewal and differentiation of various stem and multipotent progenitor cell types. Bashamboo et al (2006) have previously reported a role for the SCF/KIT pathway in the early stages of ES cell differentiation. Using gene targeting techniques they generated a KIT null cell line that was able to self renew in the presence of leukaemia inhibitory factor (LIF) but was unable to survive when directed to differentiate *via* 2D monolayer culture. Following this, they hypothesised that the SCF/KIT signalling pathway was required for the survival of differentiating ES cells but was not essential for self renewal maintenance.

Conversely, Lu et al (2007) claimed that the KIT signalling pathway was involved in the maintenance of undifferentiated ES cells and could serve as a marker of ES cells with optimal differentiation. Their cell sorting experiments indicated that a KIT ^{hi} fraction displayed enhanced expression of pluripotency markers, produced increased stem cell numbers in clonal assays and gave enhanced differentiation in 3D culture when compared with their wild type, unsorted counterparts.

To determine if KIT signalling is important in maintaining the undifferentiated state of murine ESC, Bashamboo et al (2006) grew wild type (WT) E14 ES cells for one passage (2 days) in the presence of the KIT blocking antibody ACK2. Although these ACK2 treated cells exhibited a comparable death upon differentiation phenotype to KIT Null ES cells, both ACK2 treated cells and KIT Null cells displayed a non-significant reduction of

stem cell colonies when compared to their WT counterparts in the presence of LIF. This contrasted with the results reported by Lu et al (2006) where a 1.7 fold reduction in the number of stem cell colonies was observed when KIT signalling was blocked with the ACK2 antibody.

To further these investigations, the experiments described in this chapter were designed to assess the role of KIT signalling in ES cells and address the discrepancy between the results of Lu and Bashamboo.

3.3 EXPERIMENTAL STRATEGY

To assess the level of KIT expression during self renewal and differentiation, WT E14 cells were grown in the presence of LIF and then directed to differentiate *via* monolayer culture (2D) or Embryoid Body (3D) formation. Expression was semi-quantified using RT-PCR.

To confirm the KIT Null death upon differentiation phenotype reported by Bashamboo et al, we derived novel KIT null cell lines using gene targeting techniques and assessed their ability to maintain genetic stability, self renew and differentiate *via* monolayer culture. Self renewal and differentiation profiles of KIT Null cells were then compared with both WT E14 and Heterozygous cell lines.

To address the discrepancy shown between the studies carried out by Bashamboo and Lu, we neutralised the KIT receptor in WT E14 cells with ACK2 to analyse the effect of prolonged antibody exposure during the self renewal and differentiation process. To determine how different levels of KIT expression affects undifferentiated and differentiating cells, WT E14 cells were sorted into KIT ^{hi}, KIT ^{lo} and KIT ^{neg} populations using FACS analysis and their ability to form colonies in clonal assay was assessed.

3.4 RESULTS

3.4.1 KIT is expressed in both 2D and 3D differentiation systems

To determine the expression of c-*kit* in ES cells during self renewal and differentiation, RNA was isolated from WT E14 ES cells grown in the presence of LIF and from ES cells directed to differentiate *via* 3D EB formation or 2D monolayer culture. The products of semi-quantitative RT-PCR were normalised using *β-tubulin* (Figure 3.1).

c-*kit* is expressed by self renewing ES cells and upon differentiation, distinct patterns of c-*kit* expression are observed when comparing 2D and 3D cultures. Under monolayer conditions, c-*kit* expression remains consistent over a 5 day culture period regardless of LIF inclusion whereas c-*kit* expression during EB differentiation is consistent until day 2 (D2 -LIF) where expression is lost and is not regained until day 4 (D4 -LIF). The initial reduction of c-*kit* expression during EB formation may reflect a loss of pluripotency occurring with the onset of cellular differentiation and resulting functional changes as described by Glover *et al* (2006) who observed the development of specific cell types 48-72 hours after LIF withdrawal. The re-expression of c-*kit* in the EB system may represent the occurrence of differentiated cell lineages that are dependent on c-*kit* expression such as haematopoietic precursors or germ cells (Edling et al. 2007; Kehler 2007; McClanahan et al. 1993; Mckinny-Freeman et al. 2009). Each differentiation system provides specific, environmental cues to the ES cell population which may explain the discrepancy in timing and expression of c-*kit* between the two systems. The formation of EBs *in vitro* mimics embryonic development *in vivo* and provides a 3D framework with cellular interactions to support the differentiation of multiple cell lineages. Monolayer differentiation lacks this framework and the resulting cell lineages are likely to be limited.

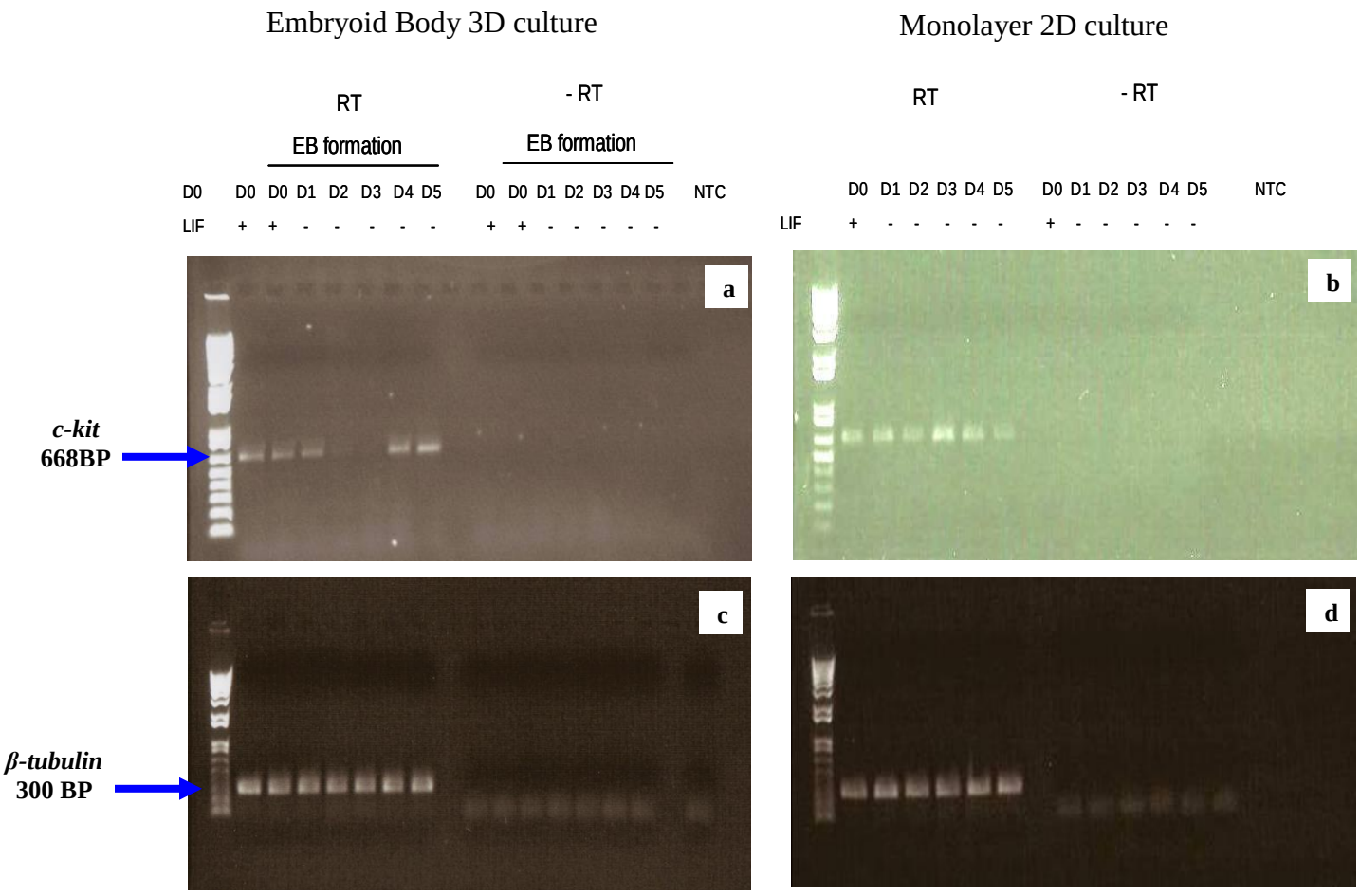


Figure 3.1 Semi-quantitative RT-PCR analysis of *c-kit* expression from RNA isolated during EB formation (3D culture a,c) and monolayer differentiation (2D culture b,d) of ES cells visualized by ethidium bromide stained gels. RNA was isolated from cells at daily time points (D0- D5) in the presence or absence of LIF as indicated. *β-tubulin* was used to normalise the concentration of DNA for semi Quantitative PCR for Embryoid body formation and monolayer differentiation (c,d)**.** The expected band for *c-kit* is 668bp and 300bp for *β-tubulin*. This is a representative experiment of two independent experiments. **RT**=Reverse Transcriptase; **-RT** = -Reverse Transcriptase control and **NTC**=Non Template Control.

3.4.2 Gene targeting and the generation of KIT Null cell lines

To confirm the KIT Null phenotype described by Bashamboo et al (2006) we knocked *Kit* out of WT E14 cells using the same gene targeting strategy employed by Bernex et al (1996) and Bashamboo et al (2006) (Figure 3.2a). Here, we employed a replacement vector (pGN- Δkit) which consisted of a 1.2 kb arm of 5' homology, a nls*lacZ*-neo cassette and a 6.1 region of 3' homology. Electroporation of this vector into WT E14 ES cells resulted in homologous recombination and a targeted deletion of codons 7 to 22 of *Kit* exon 1 and the first 200 bp of intron 1. This deleted region corresponded to the membrane signal peptide of c-*kit* and was replaced by the coding region of a nls*lacZ*-neo cassette which was in frame with the first coding bases of c-*kit* including the ATG (Figure 3.2 a). If targeted correctly, the nls*lacZ*neo cassette is under the control of the c-*kit* promoter and *lacz* expression and neomycin resistance can select for correctly targeted events. Of the 192 clones resistant to G418 only 48 stained positively (blue) by X-gal (Figure 3b and c). These clones were tested further by a multiplex PCR screening strategy to detect integration of the nls*lacZ*neo cassette as well as the unaltered WT allele (figure 3.2 d). To eliminate the possibility of random vector integration into an open, expressed chromosome region and therefore random gene expression, the precise genomic structure of resistant clones was analysed by Southern Blot (figure 3.2 e). Southern Blot confirmed that only 1 of the 48 screened clones successfully targeted the *Kit* locus to produce a heterozygous (*Kit* $^{w\text{-}lacZ/+}$*)* cell line. Karyotypic analysis indicated that this *Kit*$^{w\text{-}lacZ/+}$ clone was genetically stable and was employed to generate new KIT Null cell clones by initiating a gene conversion event *via* exposure to high G418 concentrations. Highly resistant clones were screened by multiplex PCR and genomic location was analysed by Southern Blot (Figure 3.2 e).

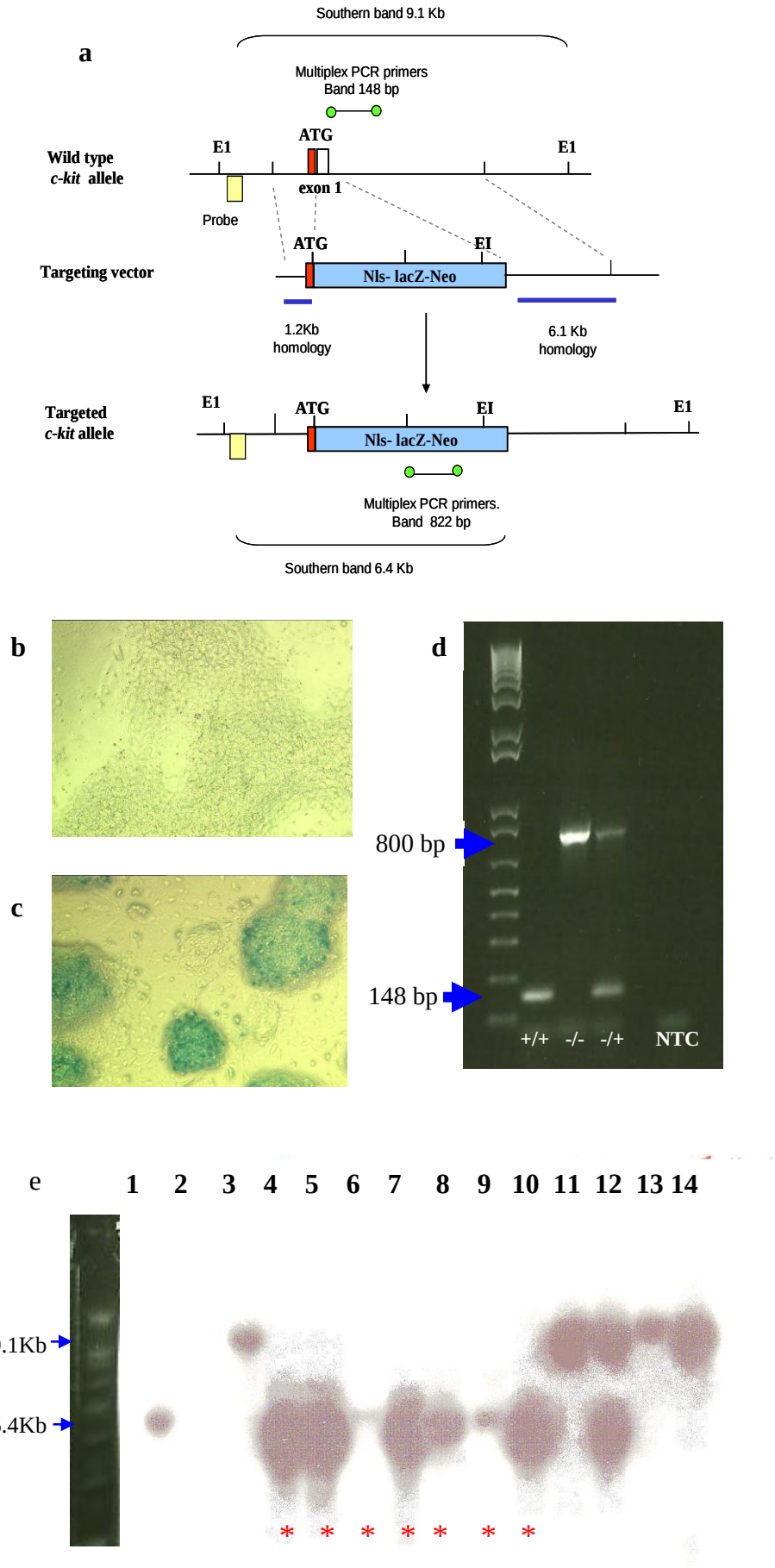


Figure 3.2 A targeting strategy to generate a heterozygous Kit $^{w\text{-}lacZ/+}$ cell line and novel Kit $^{w\text{-}lacZ/w\text{-}lacZ}$ cell lines from WT E14 ES cells (Kit $^{+/+}$). **(a)** Wild Type allele (WT) of c-kit which includes a region of DNA external to the coding region, exon 1, the ATG site and part of intron 1. The targeting vector (pGN- Δkit) containing a nls-lacZ neo cassette and the resulting targeted allele are also shown. The WT allele and targeting vector share a 5' 1.2 arm of homology and 3'.1 arm of homology as depicted by the broken lines. Multiplex PCR primers and resulting band lengths, EcoR 1 (E1) restriction points, Southern blot external probe and resulting Southern band lengths are displayed. X-Gal staining of **(b)** E14 control cells and cells targeted with **(c)** pGN- Δkit**. (d)** Multiplex PCR analysis of DNA isolated from *Kit*$^{+/+}$ (148 bp), and *Kit* $^{W\text{-}lacZ/W\text{-}lacZ}$ (-/- ,800 bp) *and Kit* $^{W\text{-}lacZ/+}$ *(-/+,* 800 and 148 bp). NTC, no template control.
(e) Southern blot analysis of *Eco*R1-digested DNA isolated from previously derived *Kit*$^{W\text{-}lacZ/W\text{-}lacZ}$ cells (lane 1, Bashamboo et al. 2006), E14 ES cells (lane 3), newly derived KIT null clones (lanes 4,5,6,7,8,9 and 10 band size 6.4Kb as shown by asterik) and newly derived *Kit* $^{W\text{-}lacZ/+}$ clone (lane12). WT bands are shown in lanes 11, 13 and 14 (9.1Kb). Band lengths were determined on the southern blot using a 1KB molecular weight ladder.

3.4.3 KIT protein analysis of KIT Null cells

To ensure that our targeting strategy was effective in ablating the *Kit* gene and its associated protein, we stained the newly derived KIT Null clones with an APC conjugated anti-KIT antibody (APC-ACK2) and analysed APC fluorescence using Flow Cytometry. WT E14 cells acted as a positive control whereas the KIT Null clone derived by Bashamboo et al (2006), named Clone 1, acted as a negative control. An APC conjugated isotype control was employed to eliminate the effect of non-specific immunoglobulin binding or background staining (Figure 3.3).

WT E14 cells express the KIT protein as demonstrated by a positive shift in APC-ACK2 staining when compared to unstained and isotope control populations. Conversely, KIT Null clones (Clone 1, NNC1, NNC2 and NNC3) do not express the KIT protein on the cell surface. Histograms of APC-ACK2 stained KIT Null populations are un-discernable from their unstained and isotype-APC stained counterparts. As the anti-KIT antibody ACK2 is specifically directed against the extracellular ligand binding region of KIT and Flow Cytometry has demonstrated that this region is absent in the KIT Null clones, we can conclude that conventional KIT signaling is eliminated through our gene targeting strategy.

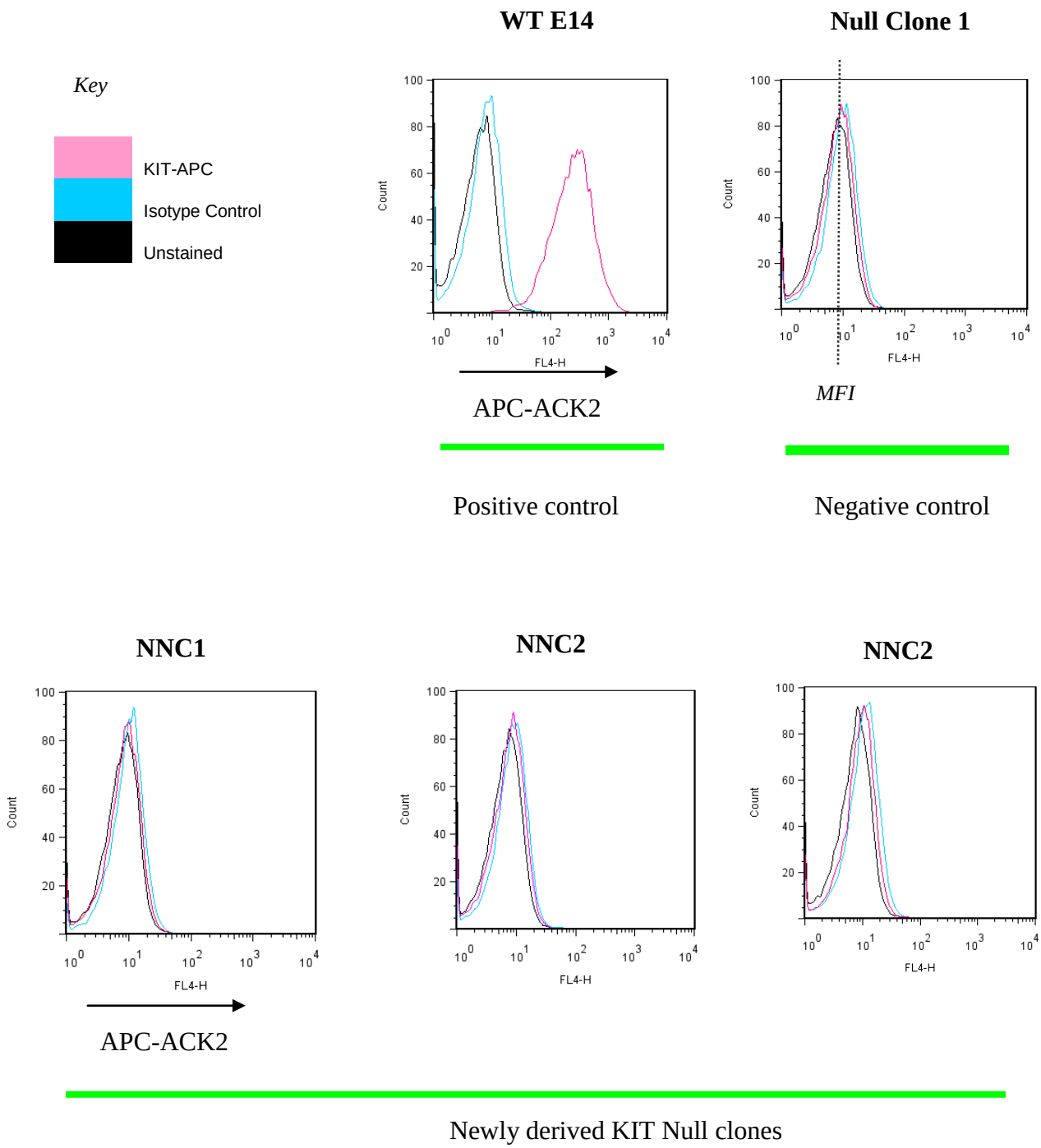


Figure 3.3 Flow cytometry histograms of APC-KIT stained New Null Clones NNC1, NNC2 and NNC3. WT E14 cells were stained as a positive control whilst Clone 1 Nulls (Bashamboo et al, 2006) were used as a negative control and an APC-isotype control antibody eliminates the effect of nonspecific staining. Unstained, APC-KIT stained and APC- isotype control stained populations are represented as individual histograms as defined by the accompanying coloured key. The histogram area represents the total cell number in each category and the mean fluorescence intensity (MFI) defines where the majority of the cell population lies.

3.4.4 Karyotypic analysis of parental and targeted cell lines

Mortensen et al (1992) reported that heterozygous ES cells expressing a single neo resistance gene can spontaneously duplicate the neo-targeted locus with a loss of heterozygosity of the WT allele to generate homozygous mutant cells. As G418 resistance correlates with *neo* expression (Paludan et al. 1989), spontaneous homozygous mutants can be selected if grown in culture with high G418 concentrations (Mortensen 1992). Lefebvre et al (2001) demonstrated that spontaneous homozygosity of the *neo* gene can occur through gene conversion events, mitotic recombination or chromosome loss and duplication. As these events can lead to genetic abnormalities such as aneuploidy which become evident after prolonged passage due to accumulation of chromosome abnormalities (Cooper et al. 2007; Corbet et al. 1999; Lefebvre et al. 2001), we subjected our newly derived KIT null cells to crude karyotypic analysis to determine their genetic status after gene targeting, high G418 selection and extended passage.

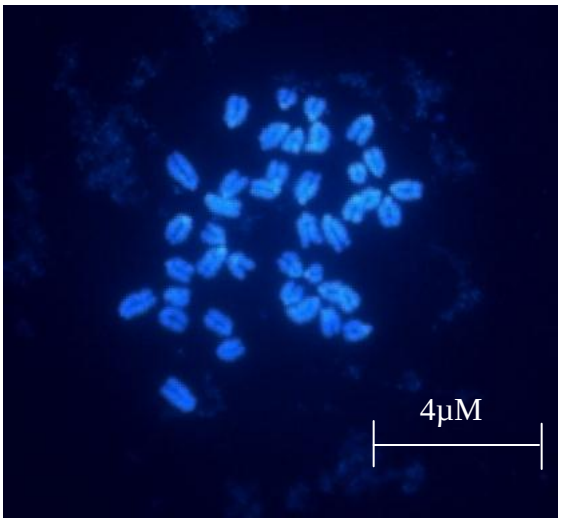
WT E14, Het and KIT Null cell lines were grown in prolonged culture and their chromosome numbers were counted after passage 5 (day 10), passage 10 (day 20) and passage 15 (day 30). At each passage interval, thirty chromosome spreads were counted per cell line and the proportion of euploid (n=40 figure 3.4 a) and aneuploid (n=41 figure 3.4 b) cells was recorded as a percentage (figure 3.4 c). A clone was considered euploid if greater than 75% of the metaphases displayed 40 chromosomes (Longo et al. 1997). After analysis of chromosome spreads, it became evident that the parental E14 cells were able to maintain a normal, euploid karyotype even after prolonged passage and these cells maintained genetic stability up to passage 36 (data not shown). The *Kit* $^{w\text{-}lacZ/+}$ heterozygous cell clone was able to maintain a normal karyotype when analysed at passage 15. However, it was evident that serial passage gave rise to the accumulation of an additional chromosome within the cell population. The effect of serial passage extending beyond 15 passages has not yet been investigated for this clone. The most striking effect of serial passage and genetic instability was observed for the three independently derived KIT Null clones. Although KIT Null cells are initially genetically stable, all three KIT Null cell clones lost euploidy, gaining an additional chromosome

(trisomy), on serial passage. This phenomenon was also displayed by the KIT Null clone derived by derived Bashamboo et al (2006) (data not shown).

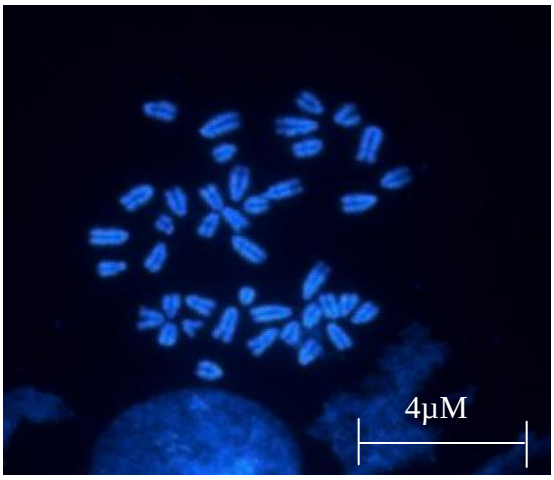
At passage 5, WT E14 cells have a significantly greater proportion of karyotypically normal cells when compared with the heterozygous and all newly derived KIT null clones ($p<0.05$). However, there is no significant difference when comparing chromosome spreads from the heterozygous clone with the KIT Null clones. At this stage, all clones are regarded as euploid. Upon serial passage (P15), again WT cells are significantly more euploid than the heterozygous and KIT null clones ($p<0.05$). However, at this passage interval the heterozygous clone maintains euploidy whereas all three KIT Null clones do not (with a significant reduction of KIT Null euploidy at $p<0,001$). Although this may associate a role for KIT or partial KIT expression with the maintenance of cellular karyotypic stability, it is difficult to separate this effect from accumulated genetic abnormalities arising as a response to adverse growth conditions over prolonged passage.

Several reports have demonstrated that prolonged murine ES cell *in vitro* culture results in an accumulation of chromosome abnormalities (Liu et al. 1997; Longo et al. 1997) and of theses abnormalities, trisomy of chromosome 8 and 11 are the most common. This additional chromosome is thought to give a growth advantage to the aneuploid cells (Liu et al. 1997; Sugawara et al. 2006) causing the rapid outgrowth and replacement of euploid cells in the culture (Liu et al., 1997; Sugawara et al., 2006). This theory corresponds with the report from Nichols et al (1990) who demonstrated that euploidy in an ES cell culture can deteriorate from 100% to 16% in only 5 passages (Nichols et al. 1990). This may account for the rapid reduction of euploid cells on serial passage of the KIT null cell clones which was exacerbated by targeting and selection in high G418 conditions (Corbet et al. 1999). All subsequent experimentation was carried out on KIT Null ES cells at low passage with a normal karyotype ensuring that all reported phenotypes were a result of *Kit* ablation rather than a manifestation of genetic engineering.

a



b



c

Wild type cell line	Heterozygous cell line	Null cell line	Passage 5		Passage 10		Passage 15	
			40 (%)	41 (%)	40 (%)	41 (%)	40 (%)	41 (%)
E14 **Kit** $^{+/+}$			100	0	99	1	99	1
	Kit $^{w\text{-}lacZ/+}$		92	8	92	7	88	11
		NNC1 **Kit** $^{w\text{-}lacZ/\ w\text{-}lacZ}$	86	14	71	29	61	39
		NNC2 **Kit** $^{w\text{-}lacZ/\ w\text{-}lacZ}$	89	11	75	25	60	40
		NNC3 **Kit** $^{w\text{-}lacZ/\ w\text{-}lacZ}$	90	10	71	29	59	41

Figure 3.4. Karyotype analysis of c-kit targeted ES cell clones derived from E14 cells. DAPI stained chromosome spreads with **(a)** a euploid karyotype where n=40 and **(b)** an aneuploid chromosome spread showing 41 chromosomes. **(c)** WT E14 cell s (*Kit* $^{+/+}$), Heterozygous (*Kit* $^{w\text{-}lacZ/+}$) and newly derived KIT Null Clones (NNC) (*Kit* $^{w\text{-}lacZ/\ w\text{-}lacZ}$)1,2 and 3 were grown for 5, 10 and 15 serial passages. At each passage interval, 30 chromosome spreads were counted per cell line. The proportion of ES cells cell displaying a euploid karyotype of 40 chromosomes or an aneuploidy Karyotype of 41 chromosomes (trisomy) at each passage was recorded as a percentage.

3.4.5 Self renewal analysis of parental and targeted cell lines

OCT4 and NANOG are key intrinsic factors that are essential for the self-renewal and maintenance of pluripotent ES cells (Chambers et al. 2004). We assessed the expression of *Oct4* and *Nanog* in newly derived heterozygous and KIT Null cells using QRT-PCR techniques (These experiments were performed by Gillian Cowan PhD thesis 2009 Figure 3.5). In three independent replicates, there was no significant difference in *Oct4* or *Nanog* expression when comparing targeted cells with control WT E14 cells ($P<0.05$). Even with reduced or ablated c-*kit* expression, ES cells have the potential to express the genes associated with self renewal. However, we did observe a distinct, yet non significant, reduction in *Nanog* expression when comparing KIT null clones with their WT E14 counterparts.

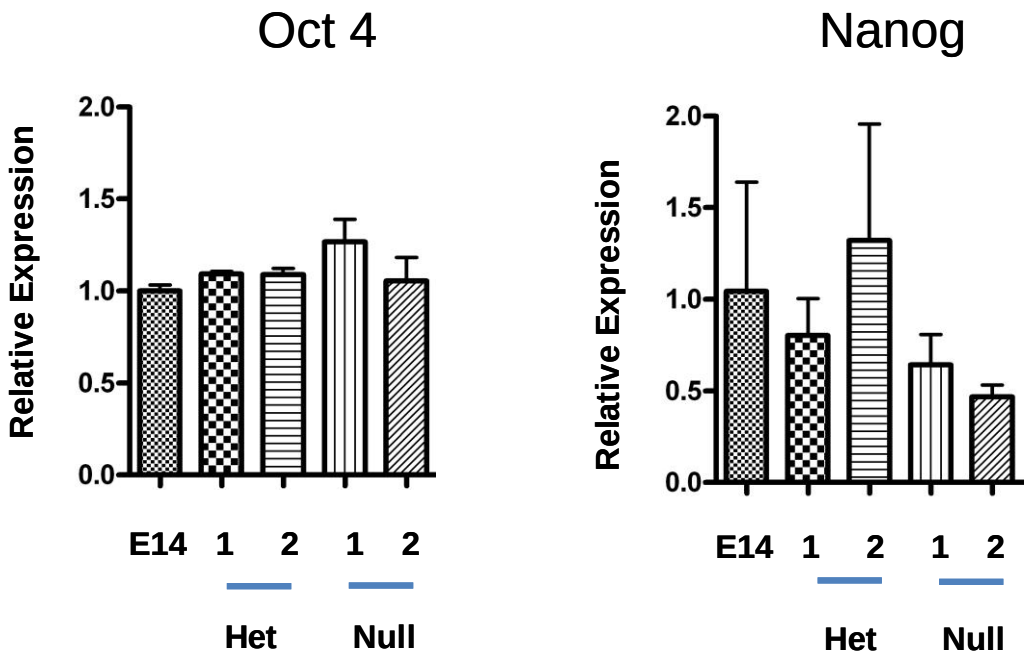


Figure 3.5 QRT-PCR analysis of *Oct4* and *Nanog* expression in WT E14, Heterozygous and KIT Null clones. All cells were grown in the presence of LIF and WT E14 control cells were compared with heterozygous and KIT null clones developed by Bashamboo et al 2006 (Het1 and Null 1) and with newly derived Heterozygous and KIT null clones (Het 2 and Null 2). In each case, the average relative gene expression from three independent replicates was represented as a histogram and standard deviations are represented by the error bars (n=3). The newly derived Kit null clone used in this assay was NNC1 (Cowan PhD thesis, 2009). (n=3. Error bars represent SEM. Analysed using a student's t-test where p>0.05)

3.4.6*KIT Null cells die upon differentiation*

Although the genes *Oct4* and *Nanog* were expressed in KIT null cells and levels of expression were comparable to WT E14 control cells, gene expression levels do not always relate to biological function. To analyse self renewal at the biological level, we set up 2D monolayer clonal assays to test the ability of newly derived KIT Null cells to self renew in the presence of LIF and their ability to form differentiated colonies upon LIF withdrawal (Figure 3.6).

In the presence of LIF, Heterozygous (Het) and KIT Null clones (Clone 1, NNC1, NNC2 and NNC3) cells were able to self renew and although there was a trend for the total number of stem cell colonies to reduce when comparing WT E14 cells to the targeted clones, this difference was not significant (Het $p=0.1$; Clone 1 $p=0.07$; NNC1 $p=0.07$;NNC2 $p= 0.06$ and NNC3 $p= 0.06$). When the cells were directed to differentiate on the removal of LIF only ES cells with full (WT E14) of partial KIT (Het) activity were able to form differentiated colonies. All targeted clones, whether Het or KIT Null, showed a significant reduction in their ability to form differentiated colonies when compared to the control WT E14 population (Het $p<0.001$) and Clone 1, NNC1, NNC2 and NNC3 ($p<0.001$). The KIT Null clones were unable to form differentiated colonies and appeared to die upon differentiation (Figure 3.6a). It is interesting to note that while total stem cell colony numbers do not decrease significantly, the size of the individual colonies were visibly and significantly ($p=0.03$ Appendix 16) reduced when comparing WT E14 cells with the KIT Null clones (Figure 3.6b and c). This would imply that the ability of single cells to form self renewing colonies was not impeded by the loss of the KIT receptor but the rate at which the cells self renewed was reduced. The effect of reduced proliferation rates in the absence of functional KIT signalling was further demonstrated when equal cell numbers ($1x10^6$) of each cell line were seeded in to flasks and the resulting cell numbers were compared after two days in culture (Figure 3.6d). Every KIT Null clone had significantly lower total cell numbers when compared with their WTE14 counterparts (Clone 1 $p=0.02$; NNC1 $p= 0.04$; NNC2 $p=0.02$ and NNC3 $p=0.03$) whereas heterozygous cells were non significantly reduced in comparison to the WT E14 control

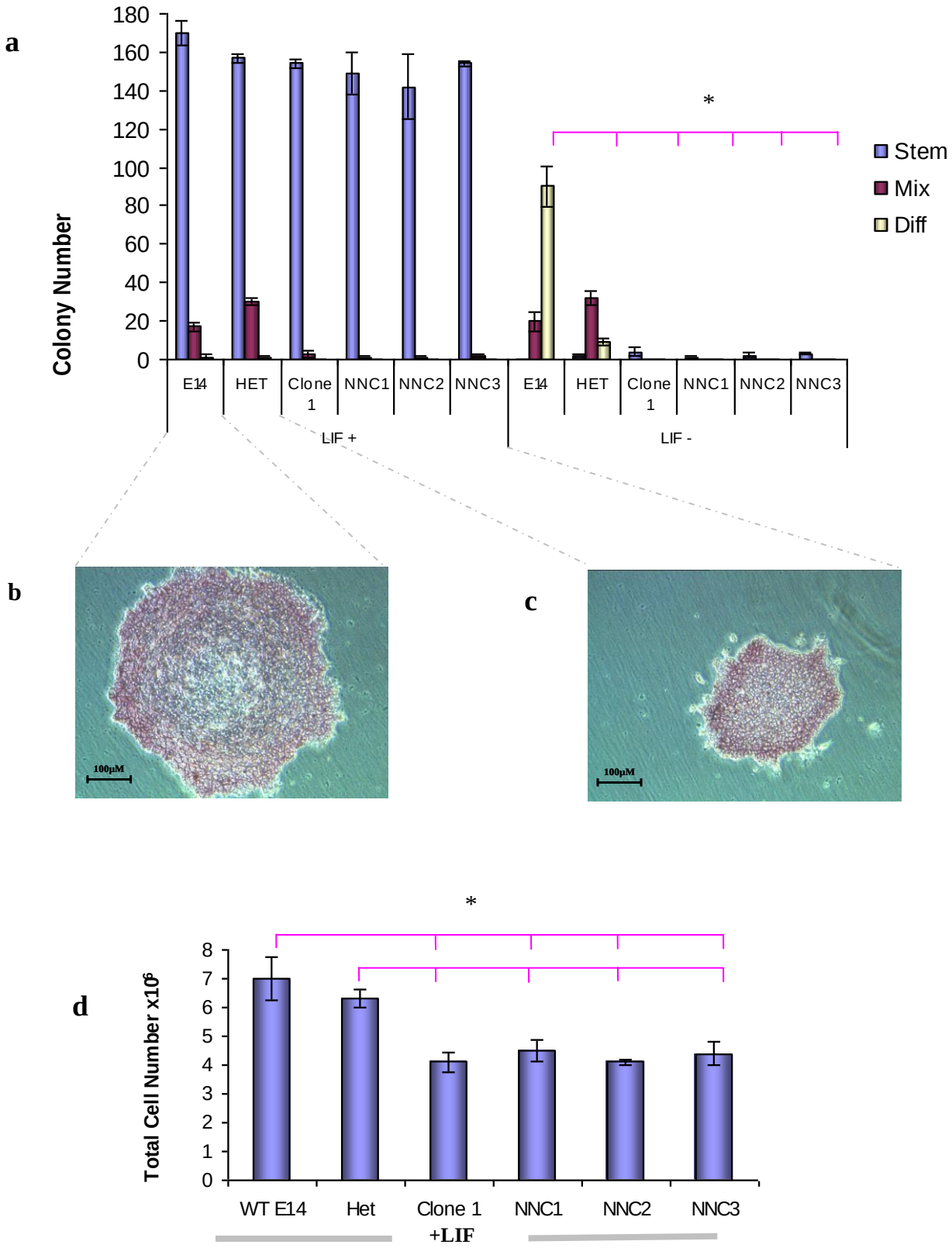


Figure 3.6 (a) Clonal assays showing the ability of control (WT E14) and targeted cells (Het, Clone 1, NNC1, NNC2 and NNC3) to self renew in the presence of LIF and their ability to differentiate in the absence of LIF(WT E14 vs Het $p<0.001$) and WT E14 vsClone 1, NNC1, NNC2 and NNC3 ($p<0.001$). Colonies were scored as Stem cell (blue bars) mixed (red bars) or differentiated (yellow bars). In each case, total colony numbers were recorded and presented as a series of histograms. A significant difference in colony number when comparing WT E14 control cells with targeted clones is represented by the pink bar and asterisk (*). Stem cell colonies of WT E14 cells **(b)** and KIT null cells **(c)** grown in the presence of LIF. Scale bar represents 100µM. **(d)** Histograms of total cell number from WT E14, Het and KIT Null clones after being seeded at a concentration of $1x10^6$ and grown for a two day period (n=3) WT vs Clone 1 $p=0.02$; NNC1 $p= 0.04$; NNC2 $p=0.02$ and NNC3 $p=0.03$. Error bars represent Standard error of the mean (SEM). Analysed using a Student's t-test.

3.4.7 KIT Null cells have limited differentiation in 3D culture.

As EB formation provides differentiating ES cells with a 3 dimensional framework and complex signaling networks that are not present in monolayer cultures, we tested the hypothesis that signaling molecules occurring throughout EB formation may compensate for a lack of KIT and prevent the death phenotype in differentiating KIT Null cells. Briefly, undifferentiated WT E14 and KIT Null ES cells were directed to differentiate under EB formation conditions and the resulting bods were placed into bacteriological grade Petri-dishes from days 0 (D0) to day (D5). At this point, EBs were either allowed to grow on the Petri-dishes until day 10 (D10) or individual EBs were placed into pre-gelatinised 24 well plates and were analysed for beating foci *via* cardiomyocyte assay (Figure 3.7 a). On D0 –D3 EB differentiation (Figure 3.7 b), KIT Null EBs were visibly smaller than their WT E14 counterparts and by D3 did not form dense central cores as shown by WT E14 EBs. By D5, WT E14 EBs has diffuse boundaries and adhered to other EBs in the culture dish whereas KIT null EBs, although forming dense cores, maintained their tight, spherical structures and did not attach to other EBs in the culture vessel. By D10, WT E14 EBs began to develop into complex, bubble like structures that began to beat whereas the KIT null EBs formed modest, motionless structures.

Under cardiomyocyte stimulating conditions, WT E14 EBs flattened onto the culture dish, differentiated extensively and approximately 90% of the EBs produced beating foci. Even though KIT Null EBs formed limited differentiation on the periphery of the flattened bod, none of the EBs formed beating foci in three independent experiments (Figure 3.7c). This differs from the monolayer differentiation system which did not sustain any KIT Null differentiation. Therefore, passing the KIT Null cells through an EB stage may provide the cells with additional signals that can aid differentiation but not compensate for a lack of KIT.

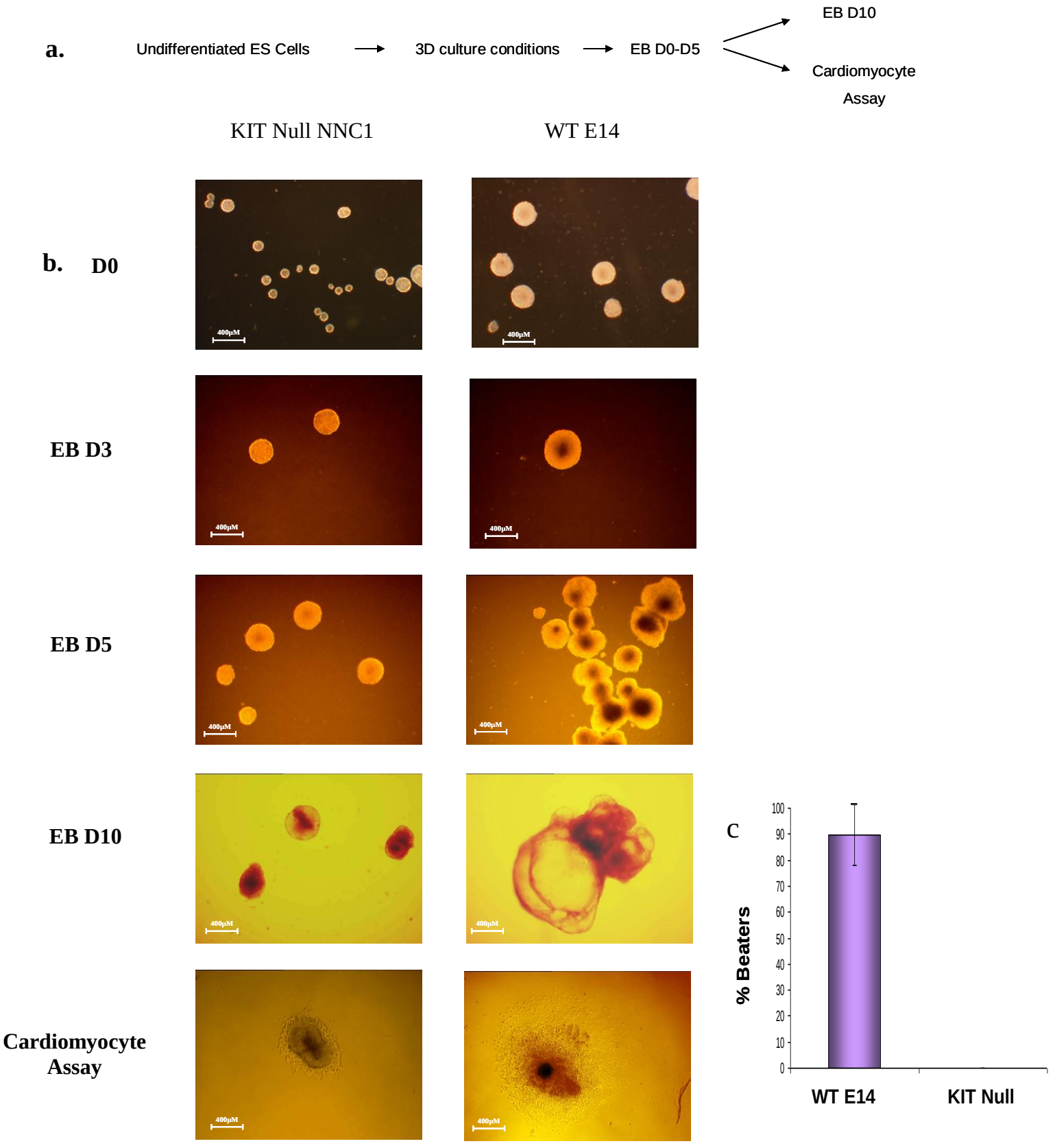


Figure 3.7 Differentiation of WT E14 cells and KIT Null cells *via* EB formation. **(a)** A flow diagram outlining the differentiation strategy of ES cell to cardiomyocyte assay. **(b)** Images taken at various time intervals during EB formation day 0 (D0); Day 3 (D3); day 5 (D5) and day 10 (D10). **(c)** Percentage of beating foci from three independent cardiomyocyte assays comparing WT E14 and KIT Null cells.Scale bar = 400µM. (n=3. Error bars represent SEM. Analysed using a Student's t-test where WT E14 vs Kit Null = p<0.001)

3.4.8 *Recapitulation of the KIT Null phenotype using ACK2*

WT E14 cells were cultured in the presence of a of a KIT neutralising antibody called ACK2. ACK2 binds specifically to the extracellular ligand binding region of the KIT receptor and blocks SCF from binding to the receptor and therefore, blocks all associated signalling downstream of KIT (Yoshinaga et al. 1991). WT E14 cells were grown in the presence of varying concentrations of ACK2 to determine an optimal antibody concentration for subsequent experimentation that was efficacious and did not cause any adverse reaction to the cells. Any non-specific immunoglobulin binding effect was eliminated by parallel testing with an isotype control. The initial dose-dependency assay involved a 2D monolayer self renewal and differentiation assay spanning antibody concentrations of 0-15µg where antibody was added directly to the cells on assay (Figure 3.8 a). Under self renewing conditions (+ LIF), there was a trend for the stem cell colonies to reduce in number as antibody concentration increased. However, this reduction in colony number was not significant when comparing each antibody concentration to the cell control ($p>0.05$). As the cells were directed to differentiate on the removal of LIF, increasing antibody concentration resulted in extensive cell death. This inhibition of differentiation become significantly different from the control at 10µg-15µg ($p=0.01$). There was no effect on self renewal or differentiation when the isotype control was added to the cells on assay (Figure 3.8 b). As ACK2 at 10µg caused no effect to the self renewal capacity of the WT ES cells (+LIF) and significantly reduced their ability to survive on differentiation (LIF-), similarly to the KIT Null phenotype, ACK2 was used at this concentration in subsequent assays.

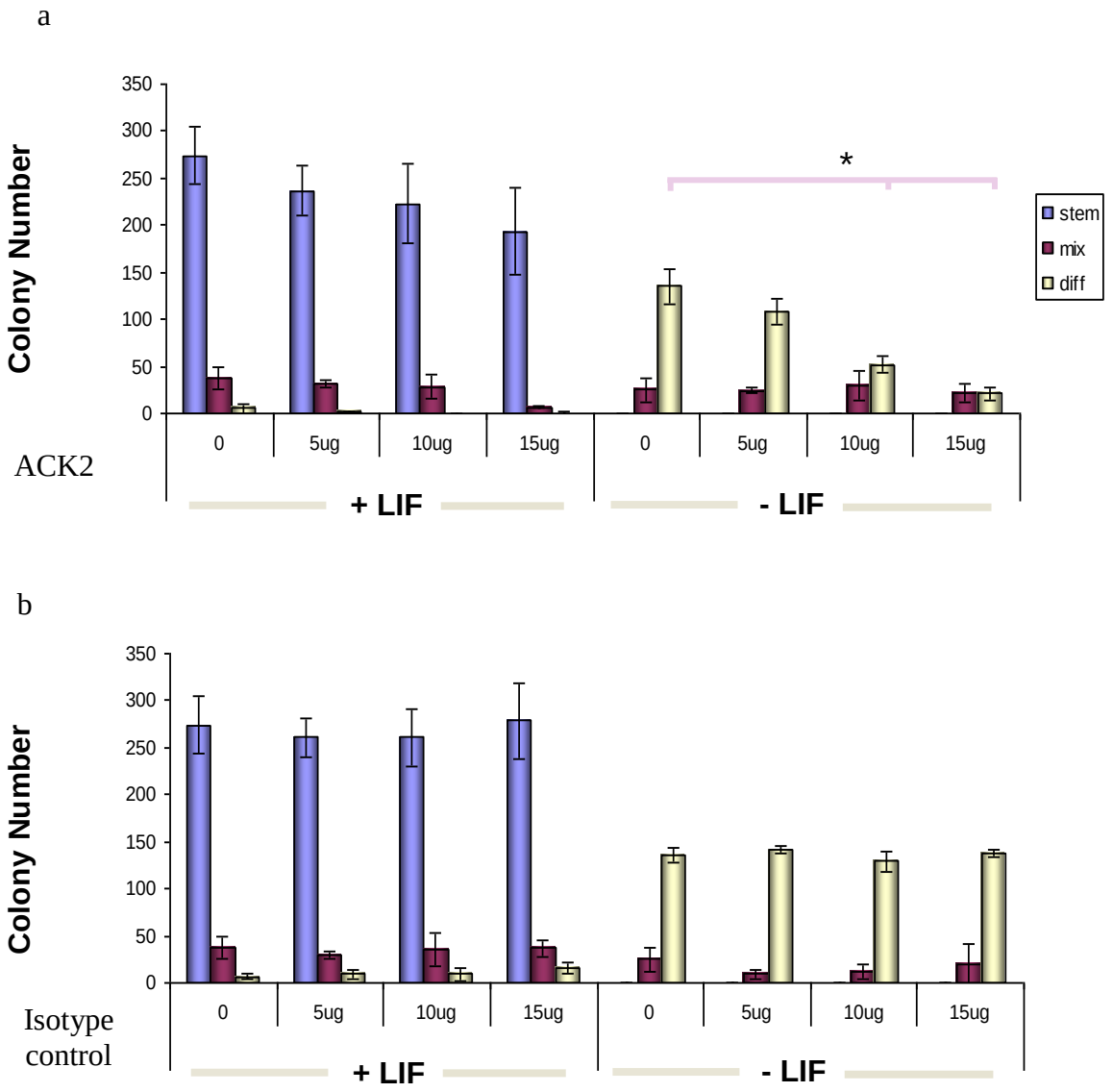


Figure 3.8 Monolayer self renewal (+LIF) and differentiation (-LIF) assay of WT E14 cells grown in the presence of ACK2 or an Isotype control. (a) ACK2 was added to the cells at a concentration of 5-15µg. A significant colony reduction (p=0.001) was only observed in differentiating WT E14 cells when ≥10µg of antibody was added on assay. This significance is shown by the pink bar and asterisk. (b) WT E14 cells were grown in the presence of an Isotype at a concentration of 5-15µg. 0 refers to a cell control where no antibody or isotype control was added to the cells on assay (n=3. Error bars represent SEM. Analysed using a Student's t-test)

3.4.9 *Prolonged exposure to ACK2 significantly reduces ES cell self renewal.*

Lu et al (2007) reported a 1.7 fold reduction in stem cell colony number when ES cells were grown in the presence of ACK2 and LIF whereas, Bashamboo et al found a non-significant reduction in stem cell colony number when cells were grown for one passage (2 days) in ACK2. To investigate this discrepancy further and to observe the effect of prolonged ACK2 exposure, WT E14 cells were grown in the presence of ACK2 for one passage (2 days), 2 passages (4 days) and greater than 10 passages (>20 days) and stem cell, mixed and differentiated colonies were counted and compared to cell and isotype controls (Figure 3.9)

After one passage (P1 +LIF) of the cells in ACK2, self renewal was unaffected but when LIF was removed (P1 LIF-) a significant reduction in differentiated colonies (P=0.03) was observed which was comparable to that previously described by Bashamboo et al 2006. This block on differentiation can be prevented if ACK2-treated cells were placed into the monlayer assay in the absence of antibody (ACK2-). After two passages in ACK2 (P2+LIF), self renewal is significantly reduced in comparison to controls (ACK2; P= 0.02) and self renewal is not restored on removal of ACK2 in the assay (ACK2 –; P= 0.006). Treated cells required one clear passage in the absence of ACK2 (P1 ACK2-) in order to restore stem cell colony numbers to control levels. On LIF withdrawal (P2 LIF-), a similar pattern was observed where differentiation was significantly blocked by ACK2 (ACK2; P=0.008) and was not restored (ACK2-; P=0.02) until treated cells were passaged for at least one ACK2-free passage. We also observed this effect for cells that were passaged in ACK2 for >10 passages (P>10) where ACK2 significantly reduced the ability of the ES cells to self renew (ACK2; P=0.01 and ACK2 - ; P=0.01) and self renewal was not restored until one ACK2-free passage. On removal of LIF, again ACK2 significantly inhibits the survival of differentiating WT E14 cells (ACK2; P=0.001 and ACK2-; p= 0.004) and survival was only restored with one ACK2-free passage. We believe that technical artefacts such as non-specific toxicity can be excluded as an explanation for this phenomenon because the cells were able to self renew albeit at a slower rate than their WT counterparts and that the isotype control with the same

conformation as ACK2 displayed no phenotype. It is also of interest to note that the morphology of the stem cell colonies grown in the presence of both LIF and ACK2 ($p=0.03$) were smaller than controls which also mimicked the KIT null phenotype (Figure 3.4.1).

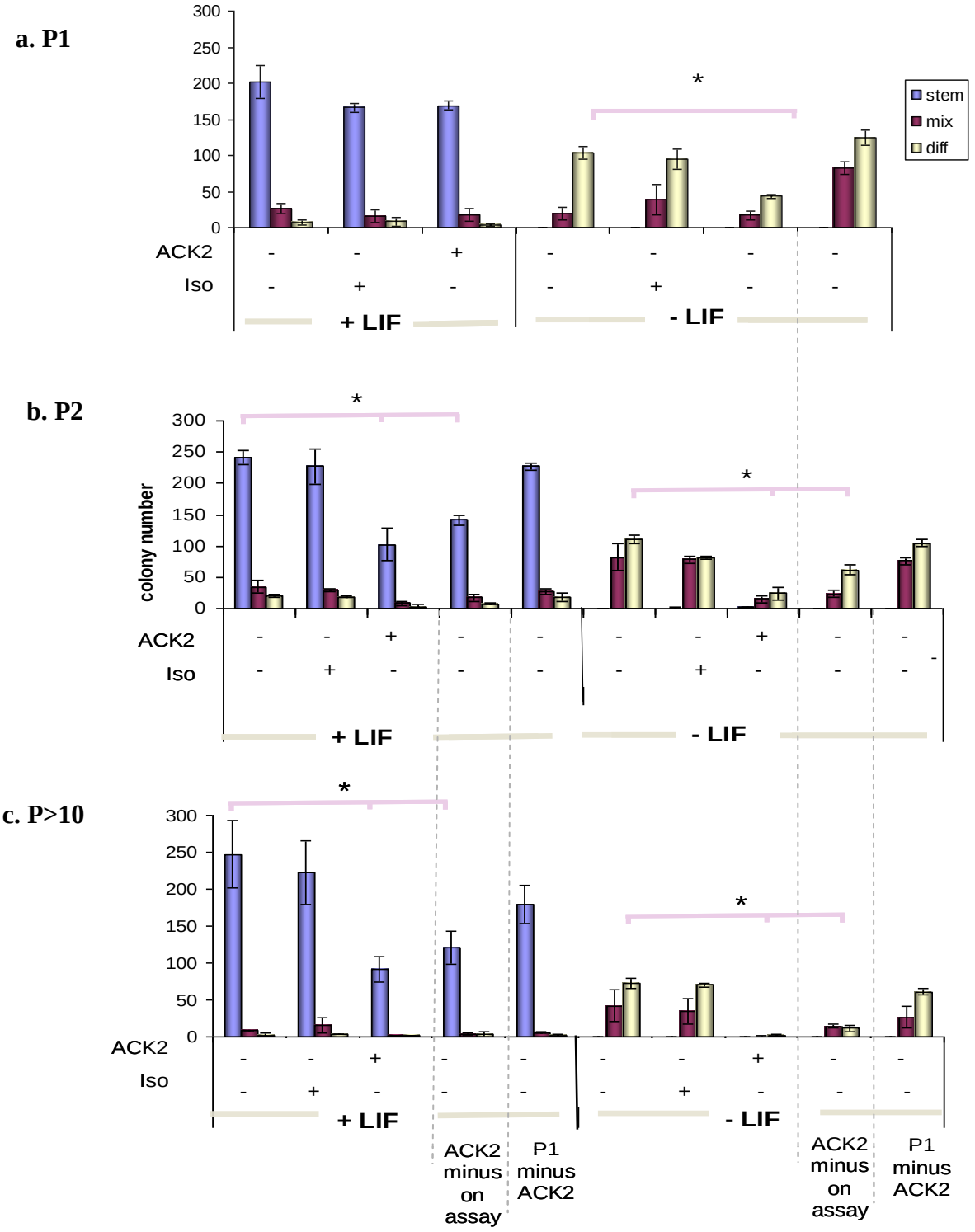


Figure 3.9 Clonal monolayer assay of WT E14 cells exposed to ACK2 (10µg). Cells were grown under self renewal (+LIF) and differentiation (-LIF) conditions. Cells were grown in ACK2 for 1 passage (a. P1), 2 passages (b. P2) and greater than 10 passages (c. P>10) before being placed into the clonal assay. Significant reductions ($p<0.05$) in colony number are denoted by the pink bars and asterisk. Refer to text for exact p-values (n=3. Error bars represent SEM. Analysed using a Student's t-test)

3.4.10 ES cells can be sorted into distinct populations dependent on KIT expression

Even under self renewal growth conditions, ES cell populations are a dynamic mixture of cell types which spontaneously self renew and differentiate into specific cell lineages dependent on expressed cell surface markers (Toyooka et al. 2008). Lu et al (2007) demonstrated that fractioning undifferentiated ES cells into KIT ^{hi}, ^{lo} and ^{neg} populations resulted in a KIT ^{hi} population with an increased capacity to self renew and differentiate. To investigate this finding and determine the role of the KIT receptor in our system, we sorted WT E14 cells into KIT ^{hi}, KIT ^{lo} and KIT ^{neg} fractions dependent on KIT expression using FACS analysis and an APC-KIT conjugated antibody (Figure 3.10 a). To ensure no overlap of the KIT ^{lo} and KIT ^{neg} population, the intermediate cell population, which minimally expressed KIT, was excluded from the analysis. To observe the effect of serial passage on KIT expression, each population was passaged once (2 days) twice (4 days) or three times (6 days) after the initial sort and their level of KIT expression was re-determined by Flow Cytometry (Figure 3.10b).

Even after serial passage, cells sorted by KIT expression remained in their specific compartments where KIT expression was significantly greater in the KIT ^{hi} population when compared with WT E14 cells ($p<0.001$), the KIT lo population ($p<0.001$), the kit neg population ($p<0.001$) and the KIT null clone NNC1 ($P<0.001$). Similarly to the results shown by Lu, the population of KIT neg cells began to re-express KIT post sort. This may be due to a cell population with extremely low KIT expression that were inadvertently included into the sorted KIT neg population or it could indicate that KIT expression fluctuates in an ES cell population grown under self renewing conditions and may represent different stages of early differentiation.

To address the function of KIT expression level during self renewal and differentiation, each sorted fraction was analysed by clonal assay (Figure 3.10c). In the presence of LIF, self renewal of the KIT ^{hi} fraction was significantly greater than the KIT ^{lo} ($p=0.01$), KIT ^{neg} ($p=0.02$) and NNC1 ($p=0.02$) cells. Although there was an increased level of self renewal when comparing KIT ^{hi} cells with WT E14 cells, this difference was non

significant. WT cells self renew significantly greater than KIT ^{Neg} (P=0.004) cells but similarly and non significantly different from KIT ^{lo} or KIT null cells.

The KIT Neg cells also showed a skewed clonal profile where significantly more cells were differentiating ($p<0.03$), even under self renewal conditions, when compared with the WT E14 cell control. This high level of differentiated and mixed colonies may imply that KIT Neg populations are representative of early differentiating cells rather than KIT Null equivalents. Unlike the KIT Neg fraction, KIT Null cells have a self renewal profile similar to and non significantly different from the WT E14 cells and are unable to survive upon differentiation.

In the absence of LIF, both KIT ^{hi} and WT E14 cell populations have significantly greater differentiated colonies than the KIT ^{lo}, KIT ^{neg} and KIT Null cells ($p<0.001$ in all comparisons). Both KIT ^{lo} and KIT ^{neg} cells have a significantly greater ability to differentiate than the KIT Null cells which may suggest that a reduction in KIT expression results in a reduction in cell survival upon differentiation similarly to the KIT Null phenotype. This would agree with the report from Lu et al (2007) who showed that an increase in KIT expression resulted in enhanced self renewal of the cells which, in turn, improved the ability of the cells to differentiate. However, it should also be noted that cell populations with weak KIT expression ($^{lo}/^{neg}$) are potentially in the early stages of differentiation as shown by their skewed clonal profile and their ability to differentiate in the presence of LIF. The inability of KIT $^{Neg}/^{lo}$ fractions to differentiate on the removal of LIF may be due to inadequate growth conditions provided by the clonal assay which cannot sustain their requirements for survival upon differentiation. Therefore, KIT ^{neg} cells are not equivalent to KIT Null cells as KIT Null cells are unable to sustain differentiation regardless of LIF inclusion.

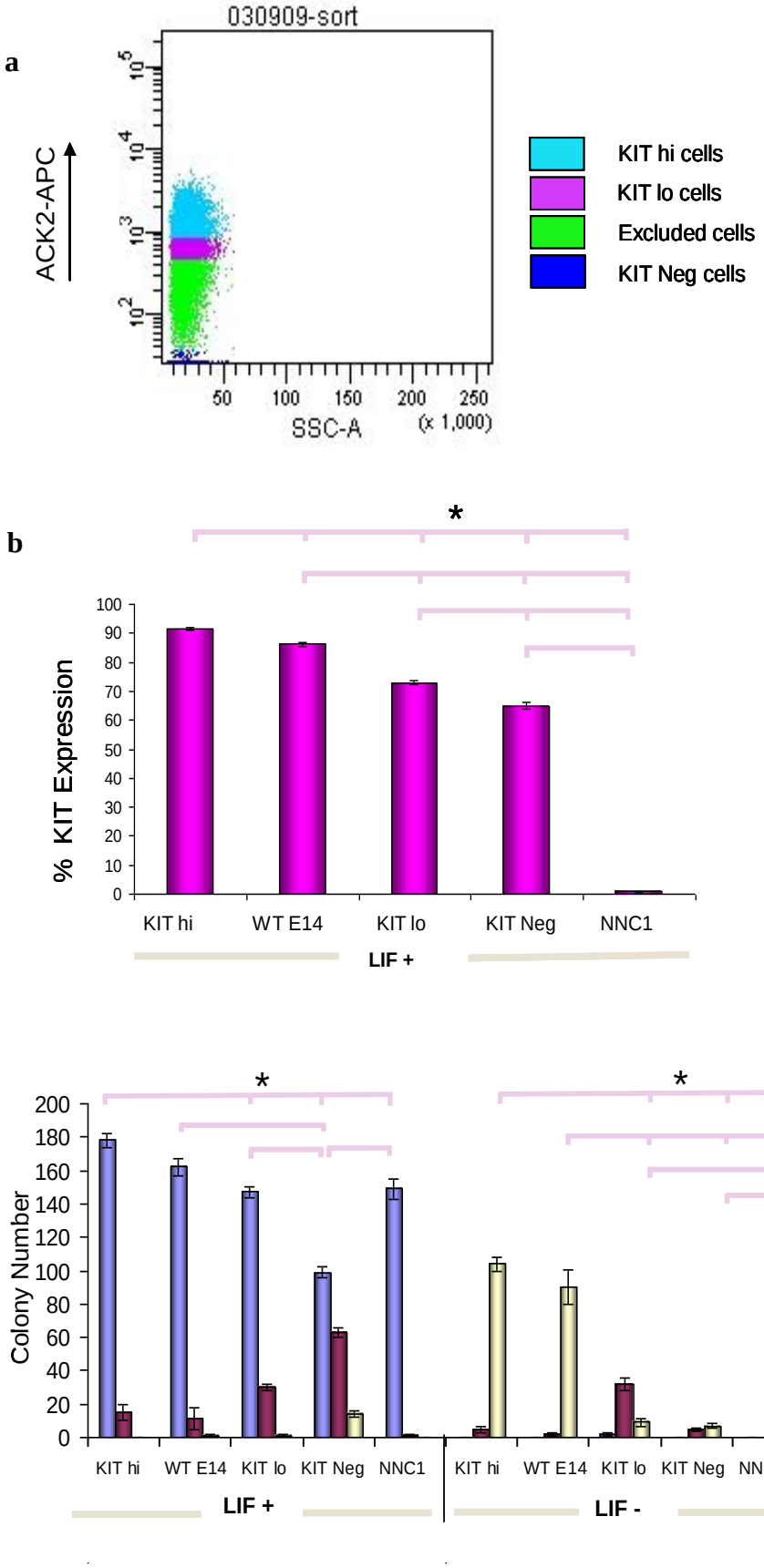


Figure 3.10 Sorting WT E14 ES cells according to KIT expression level. (a) FACS analysis of WT E14 cells grown under self renewing conditions and sorted with an ACK2-APC conjugated antibody into distinct populations of KIT ^{hi,} KIT ^{lo} and KIT ^{Neg} cells. Cells with minimal KIT expression were excluded from the analysis to avoid overlap of the KIT ^{lo} and KIT ^{Neg} categories. (b) Sorted cells grown under self renewing conditions (LIF +) were passaged for 6 days after the initial sort and then re-analysed for KIT expression with an ACK2-APC antibody via FACS analysis. WT E14 and KIT Null cells were tested concurrently as controls. KIT expression was recorded as a percentage. (c) Sorted cells, WT E14 and KIT Null cells were placed into a clonal assay and their ability to form stem, mixed or differentiated colonies was recorded. Cells were grown under both self renewal (+LIF) and differentiation (-LIF) conditions. Significant reductions ($p<0.05$) in colony number are denoted by the pink bars and asterisk. Refer to text for exact p-values. (n=3. Error bars represent SEM. Analysed using a Student's t-test)

3.5 DISCUSSION

3.5.1 *KIT and ES cell maintenance and proliferation*

Abrogation of KIT signalling *via* gene targeting techniques allowed the investigation of a KIT Null mutation and its affect on ES cell self renewal and differentiation. We report that KIT ablation did not affect the ability of KIT knock-out clones to express gene markers associated with self renewal or have an altered self renewal profile when compared with the parental WT E14 cell line. This is in agreement with the study conducted by Bashamboo et al (2006). Initially this may appear to contrast with the report by Lu et al (2007) who associated KIT signalling with enhanced ES cell self renewal and functionality, our extended analysis using a KIT Null neutralising antibody, FACS analysis and clonal assays allowed us to address this discrepancy.

Bashamboo et al (2006) reported that ACK2 treated WT E14 cells displayed a similar phenotype to KIT Null cells where stem cell colony numbers were non-significantly reduced when compared with non treated controls. However, Lu et al (2007) describe only a 1.7 fold reduction in stem cell colony number in the presence of the antibody. Similarly to Bashamboo et al (2006), we employed the WT E14 cell line in our study and found that on initial passage (2 days) with ACK2 only a slight and non significant reduction in stem cell colony number was observed. A significant reduction became evident after prolonged exposure (>4 days) of the cells to ACK2. It is conceivable that WT E14 cells require sustained exposure to ACK2 before a self-renewal phenotype is evident and that this phenotype was not observed by Bashamboo et al due to reduced cell-antibody exposure. Lu et al (2007) did not use the WT E14 cell line but the R1 ES cell line in their study, which may account for the difference in sensitivity to the antibody and the non-essential requirement for prolonged ACK2 exposure. The data presented in this study and the study by Lu et al (2007) showed that inhibition of KIT signalling by ACK2 in WT E14 cells reduced but did not completely prevent the process of self renewal. This implies an auxiliary role for KIT signalling during ES cell maintenance.

However, there is now a discrepancy in the self renewal profile displayed by WT E14 cells treated with ACK2 and the self renewal profile of KIT Null cells. The significant reduction in stem cell colony numbers when KIT is artificially blocked in WTE14 cells that is not displayed by the KIT knock-out clones may be explained by compensatory mechanisms that have arisen during the selection of KIT Null clones. Compensatory mechanisms for reduced or ablated KIT signalling by receptor/ligand pairs that converge with KIT associated downstream signalling pathways have been previously observed in other proliferating cell lineages such as the EPO receptor in erythroid cell development (Wu et al. 1995); the Cytokine receptor γ chain (γ_c) in thymocytes (Rodewald et al. 1997) and the *c-fms* receptor during blastocyst implantation (Arceci et al, 1992) as well as *c-fms* in mast cell mitosis and survival. (Dubreuil et al. 1991)

The distinct reduction in stem cell colony size upon clonal assay, the reduced cell number after passage with an initial $1x10^6$ cell seed and the reduction of EB diameter when comparing the KIT Null clones with WT E14 controls, may associate KIT signalling with a pronounced role in ES cell proliferation and rate of self renewal rather than the process of self renewal itself. This theory is supported as a similar effect on stem cell colony size and cell number was observed with WT E14 cells treated with ACK2.

3.5.2 *The effect of KIT upon differentiation*

Upon LIF withdrawal and the initiation of differentiation, it became evident that there was an absolute requirement for KIT signalling in order to sustain cell survival. This was demonstrated by complete cell death in differentiating KIT Null cells, inhibited differentiation in ACK2 treated WT E14 cells and limited differentiation during KIT Null EB development. This finding correlates with data described by Bashamboo et al (2006) who reported a cell death phenotype in differentiating KIT Null ES cells and WT E14 cells treated with ACK2. Although Lu et al (2007) did not recapitulate such a pronounced cell death phenotype (-LIF) using the ACK2 antibody (only a 1.6 fold reduction) they used it at a concentration of 10 ng/mL rather than 10μg/ml as employed by the present study and by Bashamboo et al (2006). It may be that ACK2 reduces in efficacy with reduced concentration. However, they achieved a 5.7 fold reduction in

EB formation when using the tyrosine kinase inhibitor Imatinib. Imatinib does not exclusively block KIT (Heinrich et al. 2000) but blocks related RTKs such as Abelson protein tyrosine kinase (abl) and platelet derived growth factor (PDGF) (Carroll et al 1997). Lu et al (2007) argued that this inhibitor was acceptable to use in their system as the R1 ES cell line employed in their experimentation did not exhibit abl or PDGF expression in the undifferentiated state as demonstrated by their microarray data.

Although cell lineages such as germ cells (De Miguel et al. 2002; Dolci et al. 1999; Yoshida et al. 1997) mast cells (Galli et al., 1995; Lemura et al. 2002) haematopoietic stem cells (Engstrom et al. 2003; Keller et al., 1995; Young et al. 2006) , interstitial cells of cajal (Maeda et al. 1992) and melanocytes (Ito et al. 1999) show an absolute requirement for KIT signalling for their migration, proliferation , survival or function, other *in vivo* signalling pathways have demonstrated KIT compensatory mechanisms. Wasow et al 2002 showed that over-expression of *Epo* in c-Kit$^{W/W}$ mice rescued this previously embryonic lethal phenotype by partial rescue of the red blood cell lineage. In this study, we demonstrated the partial differentiation of KIT Null cells as they were passed through EB culture which suggests that signalling pathways supplementary to rather than compensatory for KIT signalling may exist *in vitro*. Certainly *in vivo*, even the most severe *W* mutations affecting the kinase activity of c-*kit* and therefore reflect loss of KIT function, allow the progression of cell and tissue differentiation. However, affected animals often die perinatally due to severe macrocytic anaemia (Russell, 1979; Reith et al. 1990). Therefore, compensatory mechanisms for KIT signalling must exist *in vivo* which may be recapitulated *in vitro*.

3.5.3 *Cell sorting with KIT expression level*

Even under optimal self renewal growth conditions, a population of ES cells comprises of a dynamic range of cell types at different stages of the self renewal or differentiation process. (Toyooka et al. 2008). Sorting WT E14 cells based on KIT expression level resulted in distinct categories of KIT ^{hi}, ^{lo} or ^{neg} populations which remained in their compartments for up to 6 days (3 passages). Similarly to Lu et al (2007) we found that a

KIT ^{hi} population had a positive effect on the self renewal and differentiation ability of the ES cells by enhancing stem cell colony and differentiated colony formation upon clonal assay. Again, the increase in stem cell colony formation may reflect a KIT mediated effect on cell survival or increased proliferation rate rather than a direct effect upon self renewal.

Although Lu et al (2007) claim that undifferentiated ES cells with low KIT expression have reduced ability to self renew and differentiate, their clonal analysis only considered the formation of stem cell colonies and did not mention the clonal profile of the KIT Neg cells which was skewed towards differentiation as shown by our analysis. The comprehensive study of KIT expression throughout embryonic development by other groups (Edling et al. 2007; Keller et al. 2007; McClanachan et al. 1993 Mckinny-Freeman et al. 2009) and the crude analysis of KIT expression during EB formation by RT-PCR in our study demonstrated that KIT is expressed in waves and expression switches on and off dependent on differentiation model and stage of development. It is more likely that the KIT ^{neg} ES cells described by Lu et al (2007) are not a true KIT ^{Neg} population of undifferentiated ES cells but represent a population of ES cells that are in the initial stages of differentiation. This may account for their skewed differentiation profile under self renewing conditions in clonal and EB formation assays; their reduced expression of pluripotency markers and their reduced level of KIT expression. Our KIT Null clones and the Kit Null clone derived by Bashamboo et al (2006) expressed the gene markers associated with self renewal and were able to self renew within limits of control WT E14 cells. Therefore, a population of KIT ^{neg} cells is not equivalent to an undifferentiated KIT Null ES population which may account for the discrepancy in self renewal in potential on comparison of the two studies.

3.5.4 *Karyotypic analysis of KIT Null clones*

Upon serial passage, targeted cells both heterozygous and Null began to lose euploidy. The most striking and rapid loss of euploidy was observed in the in three independently derived KIT Null clones where each clone gained an extra chromosome. It is difficult to

discriminate if this effect was due to a loss of the KIT receptor and associated signalling events, accumulated genetic abnormalities in response to adverse growth conditions during prolonged passage or a growth advantage given to a particular cell population containing the additional chromosome. In the present study we have established that KIT Null cells although able to self renew, do so at a reduced rate when compared with the parental WT E14 cell line. During gene targeting events, cells were exposed to a high G418 concentration which had the potential to induce and select for genetic abnormalities such as trisomy. Upon serial passage, it is possible that a genetic abnormality, either arising spontaneously or induced via G418 exposure could give rise to a population of rapidly proliferating cells that would outgrow a slowly proliferating, euploid KIT null population. Therefore, it is essential that KIT null cells and stocks of KIT Null cells are maintained at minimal passage during subsequent experimentation and all experiments performed in this thesis were done on early passage, karyotypically normal cells. To determine if KIT signalling has a direct influence on karyotype instability, WT E14 cells which maintain a normal karyotype up to and potentially beyond 36 passages, could be passaged in the KIT blocking antibody ACK2 and karyotypic of analysis of untreated control, isotype antibody treated and ACK2 treated cells could be compared.

3.5.5 *Conclusion*

In summary, this study indicates the auxiliary role of KIT during the self renewal and an absolute role for KIT on cell survival during the onset of ES cell differentiation. KIT is involved in enhancing ES cell proliferation under self renewing and differentiation conditions as shown by colony size in clonal assay, increased cell number on passage and EB size and function upon cardiomyocyte assay analysis. The following chapter will explore the means by which the KIT Null cells perish upon differentiation and observe the effect of enhancing some of the previously reported survival pathways associated with KIT signalling in order to prevent the KIT Null death upon differentiation phenotype.

Chapter 4

Assessing apoptosis in KIT Null cells

Chapter 4

Assessing apoptosis in KIT Null cells

4.1 AIM

To investigate the KIT Null cell death mechanism and test potential inhibitors of the process.

4.2 INTRODUCTION

Apoptosis is a complex genetic programme by which the body eliminates damaged, redundant or infected cells in order to maintain an effective immune system (Murray et al. 2001) and allow the body to function at optimum (Kerr et al. 1972; Adams 2003). The activation of caspases, mitogen activated protein kinases (MAPK) and endonucleases contribute to the apoptotic process and recent publications suggest that the signals required for apoptosis are also required to trigger cell differentiation. Therefore, differentiation and cell survival depends on a balance between anti- apoptotic and pro-apoptotic factors (Duval et al. 2000; 2004; 2006). The protein family BCL2 consists of 20 apoptotic regulators that both contribute to and oppose apoptosis. (Lindsten et al. 2000; Cuconati et.al 2002). The founding member of this family is a pro-survival protein called BCL2 whose over-expression has been associated with malignancies via cell death inhibition which contrasts to other oncogenes that typically cause malignancies *via* promotion of cell proliferation (Vaux et al. 1988; Mead et al. 2008).

One of the classical methods of apoptosis detection is membrane asymmetry identification via Annexin V staining. This dye binds preferentially to phosphatidyl serine (PS) a phospholipid that resides on the inner leaflet of the cell plasma membrane. During the initial stages of apoptosis, PS is exposed to the outer layer of the membrane without loss of membrane integrity. As a result, Annexin V can be conjugated to a fluorescent marker and used in conjunction with an exclusion dye such as Propidium Iodide (PI) to distinguish early apoptotic cells from necrotic cells. It was using this apoptosis detection method that Bashamboo et al discovered that KIT Null cells (Clone 1) were constitutively

Annexin V positive. To investigate whether this Annexin V positivity was due to KIT null cells being predisposed to apoptosis or an anomaly of nonspecific staining we carried out a series of investigations using Clone 1 as well as the newly derived KIT Null clones (NNC1,NNC2 and NNC3) to analyse their apoptotic state under both self renewing and differentiating conditions.

MAPK such as c-Jun N-terminal Kinase (JNK) and p38 have been associated with both pro and anti-apoptotic programmes. Duval et al (2004) demonstrated that a third of differentiating ES cells apoptose due to P38 signaling and subsequent activation of the caspase cascade. (Takekawa et al. 2000; Duval et al 2004; Duval et al. 2006). As inhibition of the P38 pathway using the anti-apoptotic drug PD169316 significantly prevents cell death without affecting differentiation *via* an up regulation of BCL2 (Duval et al. 2004) we tested the hypothesis that inhibition of the P38 pathway could prevent the death associated with the KIT Null phenotype.

Bashamboo et al previously demonstrated that KIT signaling was required to support cells through the apoptotic crisis on the induction of differentiation *via* the pro-survival protein BCL2 (Bashamboo et al 2006) and in 2004, Duval et al showed that over-expression of the pro-survival protein BCl2 prevented apoptosis in differentiating ES cells. To test the hypothesis that BCL2 was implicated in KIT mediated survival, we over-expressed this protein in WT E14 and KIT null cells to observe its effect on cell survival during differentiation.

4.3 EXPERIMENTAL STRATEGY

Using Flow Cytometry analysis, Clone 1 cells were tested alongside the newly derived KIT Null clones NNC1, NNC2 and NNC3 to confirm the previously described constitutively Annexin V positive phenotype of the KIT null cells.

To asses if KIT Null Annexin V positivity was related to a predisposition to apoptose, self renewing and differentiating cells were analysed for apoptotic features such as caspase activation, nuclear fragmentation and chromatin condensation.

To determine whether KIT Null Annexin V positivity was due to a lack of KIT signaling or membrane asymmetry, KIT signaling was blocked in WT E14 cells using ACK2 in order to recapitulate the KIT Null phenotype.

To ascertain if KIT Null cell death followed the classical pathway of apoptosis, cell death was blocked in differentiating KIT Null and WT E14 cells using the broad range caspase inhibitor ZVAD.

To determine the effect of P38 inhibition during WT E14 and KIT Null differentiation, the P38 pathway was blocked in these cells using the chemical inhibitor PD169316.

To establish the role of BCL2 during WT E14 and KIT Null cell differentiation, BCL2 was over-expressed in both WT and KIT Null cells and its effect cell survival was determined via Flow Cytometry and clonal assay.

4.4 RESULTS

4.4.1 *Confirmation of the KIT Null Annexin V Positive phenotype*

Several newly derived KIT null clones (NNC1, NNC2 and NNC3) were employed to investigate Annexin V positivity and determine whether this phenotype was due to *Kit* ablation or a feature unique to the Bashamboo cell line, Clone 1 (Bashamboo et al. 2006). Clone 1 cells were employed as an Annexin V positive control whereas WT E14 cells acted as a negative control. All cells were grown under self renewal conditions with the inclusion of LIF.

Analysis of Flow Cytometry scatter plots of Annexin V-FITC and PI stained cells showed that every KIT Null clone was annexin positive with the majority of cells distributed to the lower right hand 'Annexin V positive' quadrant (Q3) similarly to the Clone 1 positive control (Figure 4.1a). Conversely, WT E14 cells have the majority of their distribution in the lower left 'Annexin V negative' quadrant (Q4). Quadrants 1 and 2 represent cells that are stained with Propidium Iodide (PI) a dye that is excluded from viable cells but stains cells in the late stags of apoptosis (Q2) and during necrosis (Q1)(Moore et al. 1998). As PI positive cells represent a non-viable population, these cells will be excluded from the analysis. On direct comparison WT E14 cells show significantly lower staining ($p<0.05$) than every Null clone indicating that Annexin V positivity is a specific and reproducible effect of c-*kit* ablation (Figure 4.1 b). Under usual circumstances this extent of Annexin V positivity would indicate that each KIT Null clone had entered an early phase of apoptosis. However, their ability to self renew and survive in the presence of LIF cells contradicts this theory.

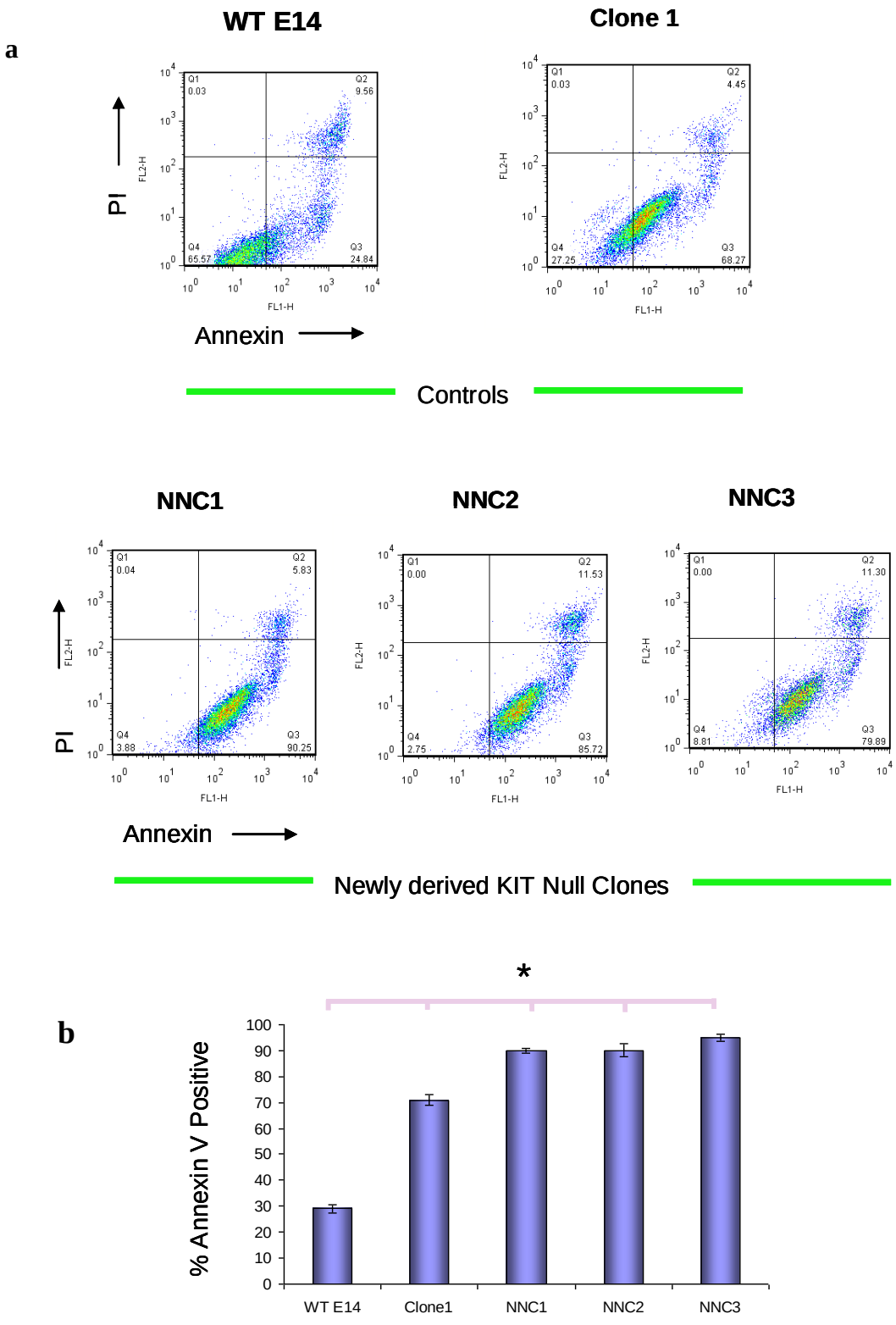


Figure 4.1 Annexin V-FITC Staining of KIT Null cells. **(a)** Scatter plots of newly derived KIT Null cells (NNC1, NNC2 and NNC3) stained with Annexin V-FITC (5µg) (x-axis) and Propidium Iodide (PI) (y-Axis) in the presence of LIF and analysed by Flow Cytometry. WT E14 cells and previously derived KIT null cells (Clone 1) acted as negative and positive controls respectively. All clones were analysed using the same gate. **(b)** Histograms depicting Annexin V positivity of WT E14 cells vs KIT Null clones in the presence of LIF. Significant differences ($p<0.05$) are shown as an asterisk and pink bars. (n=3. Error bars represent SEM. Analysed using a Student's t-test)

To ensure that the KIT Null Annexin V positive phenotype was due to specific staining of Annexin V rather than an artefact of non-specific staining caused by residual FITC, WT E14 and NNC1 cells were analysed with varying concentrations of Annexin V-FITC. Previously, (Figure 4.1a) WT E14 cells and NNC1 cells were stained with 5µg of Annexin V-FITC as recommended by the manufacturer. However, reducing the concentration of the stain by 50% (2.5 µg) and 75% (1.25 µg) still maintained the KIT Null Annexin V positive phenotype (figure 4.2). To elucidate if this staining was specific to Annexin V binding, WT E14 and KIT Null cells were pre-incubated and blocked with recombinant, un-conjugated Annexin V before counterstaining with Annexin V-FITC. In the pre-adsorbtion step, recombinant annexin binds to all available PS sites, Annexin V-FITC is unable to bind on counter stain and fluorescence is blocked. As fluorescence was blocked in both WT E14 and NNC1 cells, we can conclude that Annexin V binds specifically to these cells and the effect of non-specific fluorescence can be eliminated.

4.4.2 *Assessing the apoptotic status of KIT Null cells*.

As PS exposure, membrane asymmetry and Annexin V positivity are phenomena associated with early apoptosis, it may be hypothesised that KIT Null clones are either apoptotic or predisposed to apoptose and are awaiting the appropriate death signal. However, KIT Null cells have the ability to self renew in culture, clonal assays and remain viable in the presence of LIF. Furthermore, under self-renewing conditions they do not express other biochemical or morphological features associated with apoptosis such as nuclear fragmentation or chromatin condensation and activation of the caspase cascade, (Figure 4.3 ad 4.4). When comparing DAPI stained WT E14 cells with NNC1 (Figure 4.3a) cells under self- renewing conditions (day 0; LIF +), cell nuclei of both cells types are large, intact and refractile with minimal signs of apoptosis (Figure 4.3b). The onset of apoptosis is only triggered when KIT Null cells are directed to differentiate in the absence of LIF. Three days after LIF withdrawal NNC1 cells begin to show extensive signs of chromatin condensation and nuclear fragmentation (Figure 4.3c) and the percentage of apoptotic cells is significantly greater than their WT counterparts ($p<0.01$)

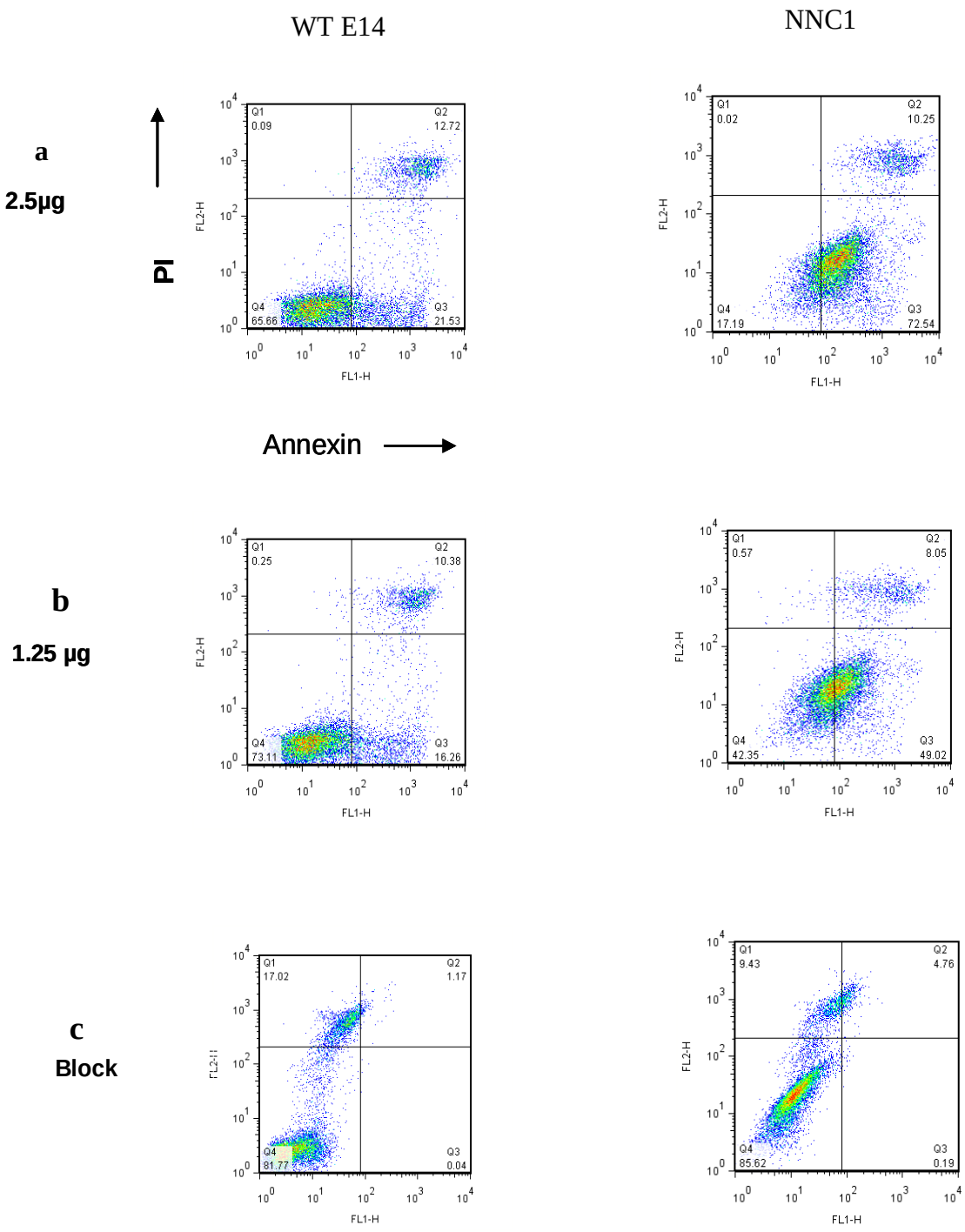


Figure 4.2 Reducing and blocking Annexin-V staining. Scatter plots of WT E14 cells and KIT Null cells (NNC1) stained with Annexin V-FITC (x-axis) and PI (y-axis) in differing concentrations of Annexin stain (**a**.2.5µg or **b**.1.25µg). Annexin V staining was blocked using recombinant Annexin V at 2.5µg **(c)** before staining with Annexin V-FITC at 2.5µg. PI staining was kept constant throughout at 0.5µg.

As KIT Null cells are constitutively annexin positive, another method of apoptosis detection was employed to quantify cell death. Caspases (cysteine, aspartic acid specific proteases) are a family of proteases that are present but inactive in viable cells. In response to apoptotic signals, initiator caspases (caspase -2,-8,-9,-10) are cleaved then form heterotetramers which in turn cleave, activate and amplify effector caspases (caspase -3,-6 and 7). Effector caspases cleave proteins within the cell that regulate cell survival ultimately resulting in cell death. (Arends et al. 1991, Salvesen et al. 1997. Sleeet al. 1999).

To detect activated caspases within the cell, we employed the cell permeable Fluorochrome Inhibitor of Caspases reagent (FLICA). FLICA is cell permeable, non-cytotoxic inhibitor which comprises of three main units; carboxyfluorescein (fam), Valine-Alanine- Asparagine (VAD) and fluoromethylketone (fmk). VAD is a protein sequence that is recognised by and binds to many activated caspases (caspase-1, -3, -4, -5, -6, -7, -8 and -9) and fmk is a protein moiety that covalently binds to a cysteine residue located within the activated caspase to inhibit its enzymatic function (Figure 4.4 a-e). As the FLICA reagent covalently binds to the activated caspase, it is retained within the cell and any unbound reagent is washed away during assay. The remaining signal is a direct measure of activated caspase within the cell (Bedner et al. 2000; Grabarek et al. 2002).

WT E14 and NNC1 cells grown in the presence of LIF (LIF +; day 0) or the absence of LIF (LIF-; day 3) were analysed by Flow Cytometry with the FLICA reagent (Figure 4.4f). In the absence of LIF there was a significant increase in activated caspases in both WT E14 and NNC1 when compared with their self-renewing counterparts ($p<0.01$ in both cases) indicating that a proportion of cells enter apoptosis on differentiation. A similar result was reported by Duval et al (2004) who showed that 30% of cells enter apoptosis on differentiation. When comparing differentiating WT E14 cell with differentiating NNC1 cells (day 0; LIF-), WT E14 cells have significantly lower activated caspase levels than NNC1 cells ($P<0.01$). This implies that the KIT null cells have enhanced apoptosis induction, even at early stages of differentiation. To act as a positive control for apoptosis and to ensure FLICA reagent functionality, WT E14 cells were

exposed to UV in the presence of LIF to induce apoptosis before staining. UV induced apoptosis was significantly greater than all other conditions regardless of cell type or differentiation status ($p<0.05$).

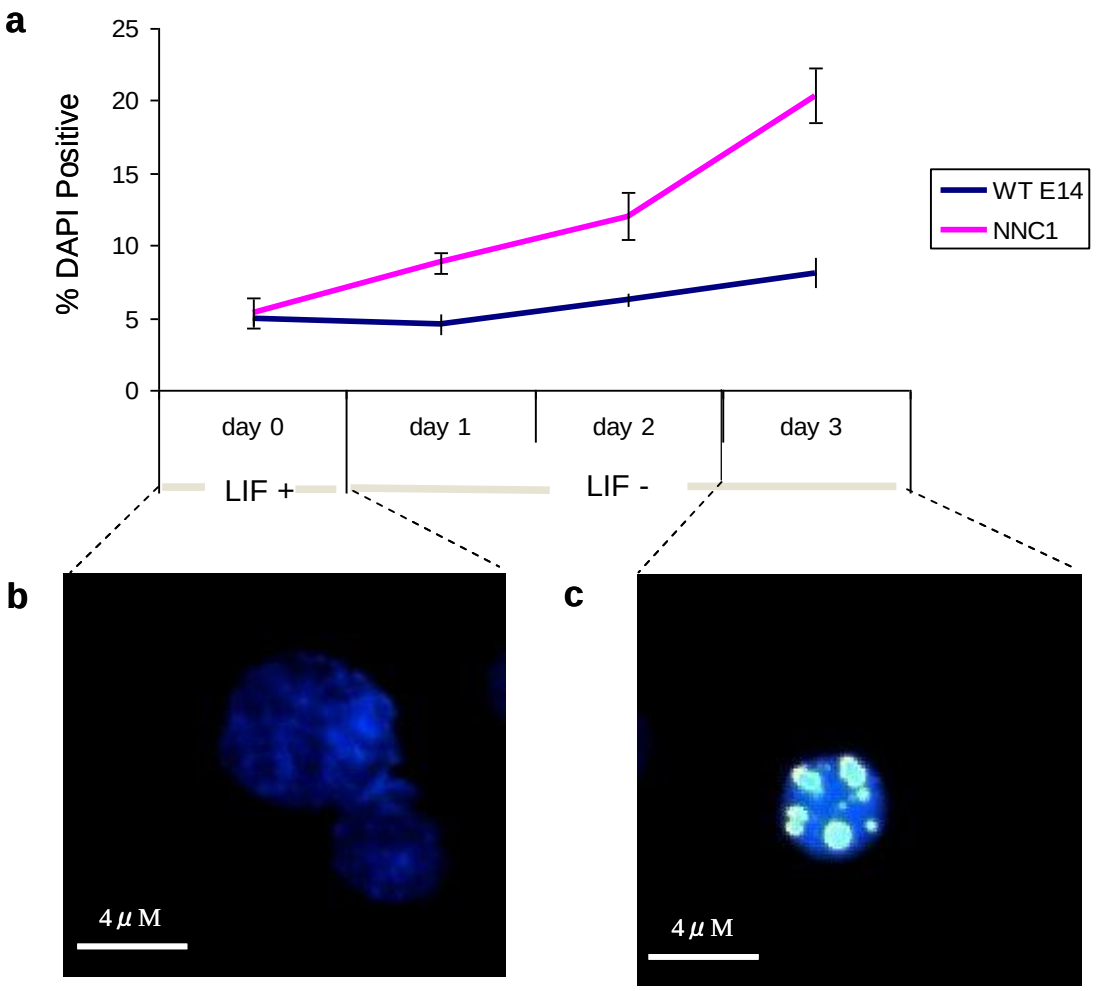
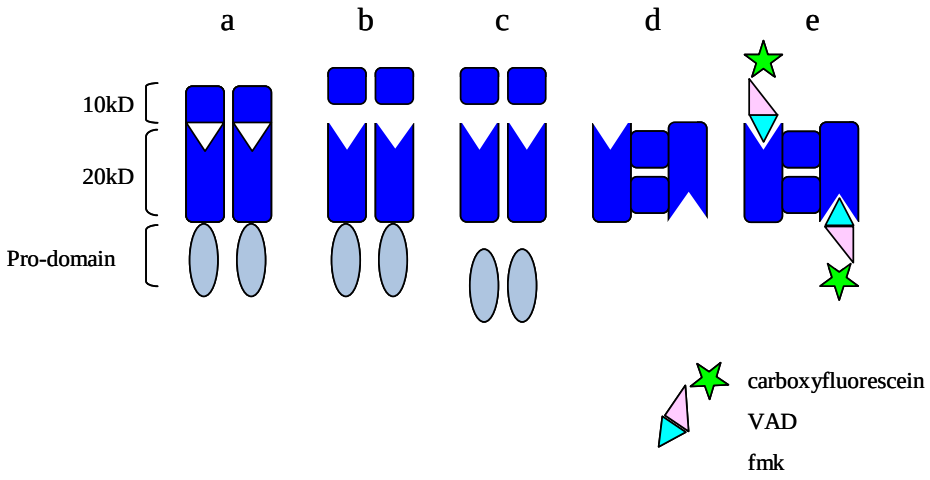


Figure 4.3 Detecting apoptotic features. (a) DAPI staining of WT E14 and KIT Null cells in the LIF+(day 0) and under differentiating conditions LIF- day1-day3. **(b)**Viable cells have large nuclei with uniform staining whereas **(c)** apoptotic cells have small brightly stained nuclei determined by nuclear fragmentation and chromatin condensation. Three days after LIF withdrawal NNC1 cells begin to show extensive signs of chromatin condensation and nuclear fragmentation and the percentage of apoptotic cells is significantly greater than their WT counterparts ($p<0.01$) . (n=3. Error bars represent SEM. Analysed using a Student's t-test)



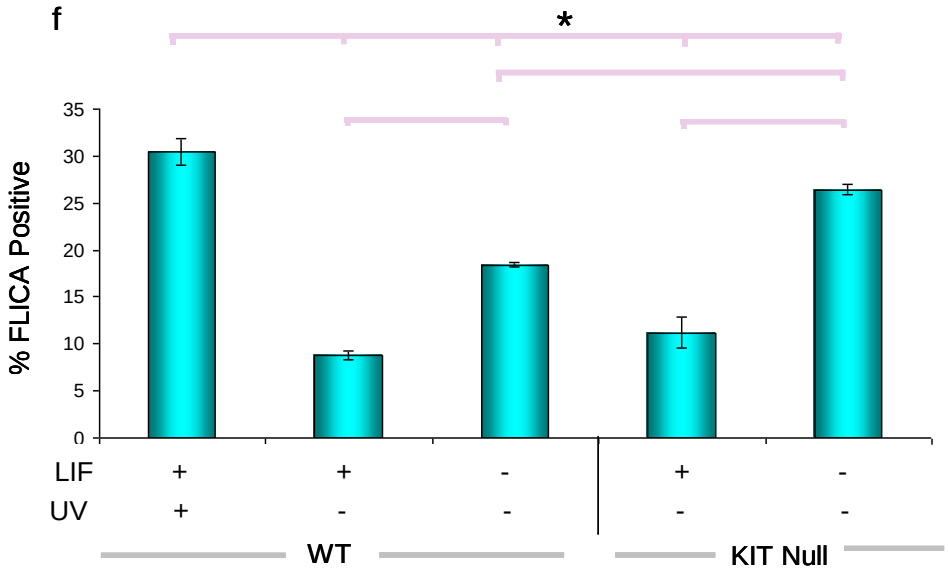


Figure 4.4 FLICA detection of activated caspase (a) inactivated caspases exist in viable cells in 3 subunits; an N-terminal prodomain, a 20 kD catalytic subunit and a 10 kD subunit. **(b)** Upon apoptosis induction, caspases are cleaved at an asparagine residue to release the 10kD subunit and then at a second asparagine residue **(c)** to release the pro-domain. **(d)** The 10kD and 20Kd subunits re-assemble to form heterotetramer and the activated caspase enzyme. The active centers containing the reactive cysteine residue to which the fmk protein moiety binds is depicted by the excised triangular ends. **(e)** VAD contains a sequence that is recognised by activated caspases and covalent binding of fmk to active caspase centres inhibits caspase enzymatic activity. Carboxyfluorescein acts a a green fluorescent tag for Flow Cytometry detection and analysis. **(f)** FLICA staining of WT E14 cells KIT null cells and UV treated cells grown in the presence (LIF+, day 0) or absence (LIF–, day3) of LIF. Significant differences between each condition are depicted by the asterisk and by pink bars ($p<0.05$) refer to the text for exact p-values . (n=3. Error bars represent SEM. Analysed using a Student's t-test)

4.4.3 *The effect of ACK 2 on the KIT Null Annexin V Positive phenotype.*

Under self-renewing conditions, NNC1 cells display minimal signs of apoptosis where levels of activated caspases and nuclear fragmentation were comparable to control WT E14 cells. (DAPI/FLICA staining Figure 4.3 and 4.4). This conflicts with the Annexin V positive state of the KIT Null cells which implies the onset of cell death. However, it may also suggest that KIT Null Annexin V positivity is a phenomenon that occurs independently from apoptosis as reported to occur in viable B-cells (Dillon et al, 2000) and activated platelets (Thiagarajan et al. 1990). To investigate this further, ACK2 (10µg) treated WT E14 cells were compared with isotype control treated cells (10µg) and untreated cells to observe the effect of a block in KIT signaling on Annexin V staining (Figure 4.5). Under self renewal conditions, ACK2 has no effect on Annexin V positivity. However, ACK2 significantly enhanced apoptosis in differentiating cells when compared with untreated and Isotype control cells where $p= 0.02$ and $p= 0.04$ respectively. As WT E14 cells treated with ACK2 did not display the constitutively Annexin V positive phenotype of the KIT Null cells, Annexin V positivity may be due to membrane perturbation caused by a physical loss of the KIT receptor to the membrane rather than a lack of KIT signaling. As expected, a block in KIT signaling enhanced apoptosis which correlates with the clonal assay analysis described in Chapter 3.

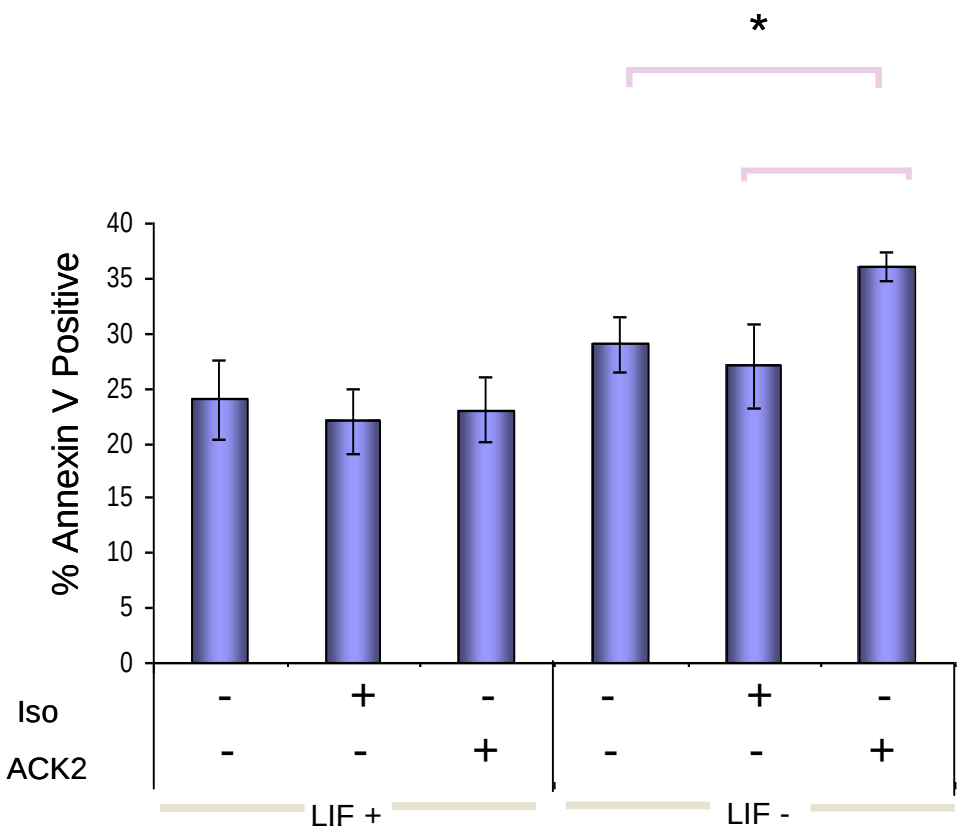


Figure 4.5 ACK2 inhibition of KIT signalling and Annexin V –FITC staining. Histograms of percentage annexin staining of WT E14 cells grown in the presence (LIF +) and absence (LIF-) of LIF with the inclusion of the KIT blocking antibody ACK2 or an Isotype control (iso) (both antibodies at 10µg). Significant differences between each condition are depicted by the asterisk and by pink bars ($p<0.05$). ACK2 significantly enhanced apoptosis in differentiating cells when compared with untreated and Isotype control cells where $p= 0.02$ and $p= 0.04$ respectively (n=3. Error bars represent SEM. Analysed using a Student's t-test)

4.4.4 Caspase inhibition and ES cell survival

As KIT Null cells appear to follow the classical pathway of apoptosis upon differentiation, we wanted to test the hypothesis that blocking apoptosis with the broad range caspase inhibitor, ZVAD would enhance KIT Null survival upon differentiation. To ensure that the ZVAD reagent was efficacious, differentiating WT E14 cells were tested in the presence and absence of ZVAD (40μM) and analysed by Flow Cytometry for Annexin V positivity (Figure 4.6a). As expected, differentiating WT E14 cells have greater annexin positivity than WT E14 cells grown under self renewing conditions ($p<0.01$). On ZVAD inclusion, Annexin V positivity is significantly reduced on comparison to both self-renewing ($P<0.01$) and differentiating cells ($P<0.01$) indicating inhibitor efficacy. To determine if ZVAD prevented the KIT Null death upon differentiation phenotype, ZVAD was added to differentiating KIT Null cells and analysed by Flow cytometry with FLICA staining (Figure 4.6b). WT E14 cells were tested concurrently as a staining control. When ZVAD was added to differentiating cell cultures, caspase activation was significantly reduced in both differentiating WT and KIT null cells ($p<0.01$ in both instances) when compared with controls. Therefore, ZVAD was able to prevent apoptosis in KIT Null cells.

However, apoptosis inhibition upon differentiation onset may not equate to successful, late stage differentiation. To observe if blocking apoptosis with ZVAD allowed KIT null cells to form differentiated colonies, clonal assays were established to observe the effect of ZVAD on cell survival (Figure 4.6 c and d). Under self-renewing conditions, ZVAD had no effect on WT E14 cells but as the cells were directed to differentiate, ZVAD significantly increased the proportion of surviving differentiated colonies ($p<0.01$) on comparison to controls. Although ZVAD significantly increased the proportion of KIT Null stem cell colonies in the presence of LIF ($p=0.01$), KIT Null cells were unable to survive when directed to differentiate.

Even though caspase activation was not completely inhibited with ZVAD, WT E14 cells treated with ZVAD displayed a significant increase in differentiated colonies when

compared with controls ($p<0.01$) indicating inhibitor efficacy. This effect was not was not observed in KIT Null cells grown under the same conditions. This data implies that KIT provides a pro-differentiation signal to cells which is not compensated with a block in apoptosis and may suggest that both pro -differentiation signals and anti-apoptotic signals are required for a cell to successfully differentiate.

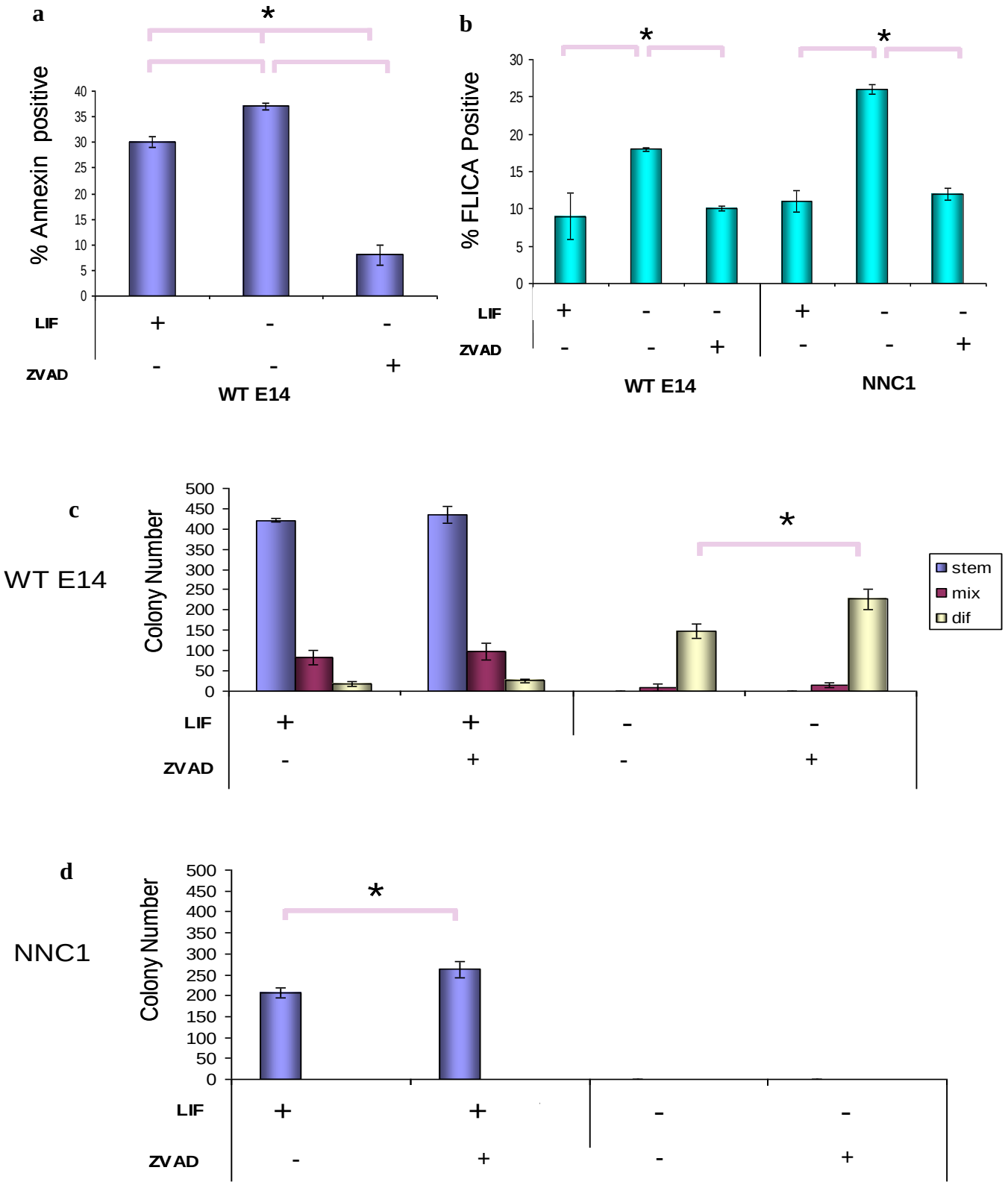


Figure 4.6 Caspase inhibition with ZVAD (40μM) and its effect on apoptosis. **(a)** WT E14 cells grown in the presence or absence of LIF and the presence or absence of the broad range caspase inhibitor ZVAD. Cells were tested by FLOW cytometry with Annexin V FITC staining and the result was depicted as a histogram of percentage Annexin V positivity. **(b)** WT E14 and KIT Null cells (NNC1) were tested for caspase activation then caspase activation inhibition using ZVAD in the presence and absence of LIF. Cells were tested by Flow Cytometry with FLICA and percentage caspase activation (fluorescence) was displayed as histograms. Clonal assays of WT E14 cells **(c)** and KIT null cells **(d)** grown under self renewing conditions (LIF+) and differentiating (LIF-) conditions with the inclusion or absence of ZVAD. Stem cell Mixed and differentiated colonies are depicted as histograms. Significant differences between each condition are depicted by the asterisk and by pink bars ($p>0.05$). Refer to text for exact p-values. (n=3. Error bars represent SEM. Analysed using a Student's t-test)

4.4.5 *Inhibition of P38 in ES cells*

Duval et al (2004) showed that 30% of differentiating ES cells died by apoptosis via activation of the mitogen activated protein kinase (MAPK) p38 pathway. Inhibition of this pathway using the inhibitor PD169316 resulted in an up regulation of BCL2 expression and survival of the differentiating cells. As KIT null cells apoptose on differentiation with a decrease in BCL2 expression (Bashamboo et al. 2006) we tested whether KIT-mediated survival upon differentiation was through inhibition of the P38 pathway. To test inhibitor efficacy and witness any dose dependent effect or toxicity, varying inhibitor concentrations were added to WT E14 cells on clonal assay. DMSO was added as a diluent control and tested concurrently (Figure 4.7a). Under self renewal conditions (LIF+), the P38 inhibitor had no effect on the ability of the cells to self renew. As the cells were directed to differentiate (LIF-), a significant increase in the number of differentiated colonies was seen only when the inhibitor was used at 10μM ($p=0.02$). Increasing volumes of the diluent control did not affect the self renewal or differentiation potential of the cells in this clonal assay.

To test the efficacy of the inhibitor further, untreated WT E14 cells and WT E14 cells treated with the inhibitor were directed to differentiate and their Annexin V positivity was analysed by Flow Cytometry (Figure 4.7b). As expected, differentiating WT E14 had significantly greater Annexin V positivity ($p<0.01$) than WT E14 cells grown under self-renewing conditions. When the PD169316 was added to differentiating cells, not only was Annexin V staining significantly reduced ($p<0.01$) in comparison to untreated differentiating cells, it was also significantly lower than the cell control under self renewing conditions ($P<0.01$). A similar effect was also displayed when activated caspases were investigated (Figure 4.7c). The inhibitor significantly reduced activated caspases on differentiation significantly lower than the cell controls in both WT E14 ($P<0.01$) and NNC1 KIT null cell clone ($P<0.05$). Although Duval et al experienced a 30% survival using this inhibitor our survival was 20% reduction on clonal assay, 65% reduction of Annexin V staining and a 60% reduction in caspase activation. This provided evidence for PD169316 functionality.

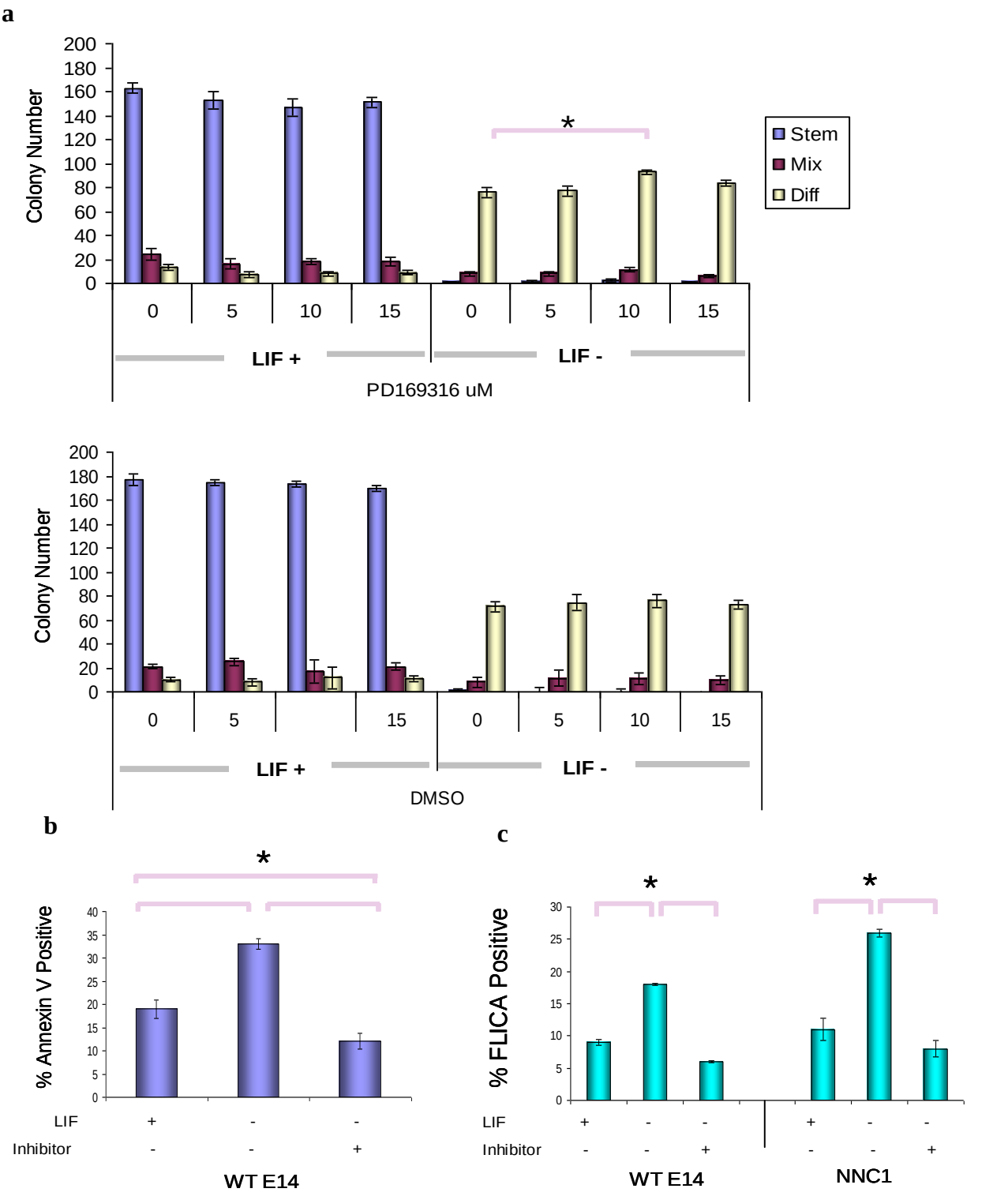


Figure 4.7 P38 inhibitor (PD169316) dose dependency assay. **(a)** Clonal assays of WT cells grown in the presence (5μM, 10μM or 15μM) or absence (0) of the P38 inhibitor PD169316 under self renewing (+LIF) of differentiating conditions (LIF-) Stem mixed and differentiating colonies were represented as histograms. As a diluent control, DMSO was added to the cells at the same volume as the inhibitor. **(b)** Annexin V-FITC staining of self renewing (LIF+) WT E14 cells or differentiating WT E14 cells (after 3 days, LIF-) with the presence or absence of PD169316 as shown by the key. **(c)** FLICA staining of WT E14 cells and NNC1 cells grown in the presence or absence of LIF or PD169316 as shown by the x-axis key. Histograms from **(b)** and **(c)** were generated from Flow Cytometry data and significant differences between each condition are depicted by the asterisk and by pink bars ($p>0.05$). Refer to text for exact p-values. (n=3. Error bars represent SEM. Analysed using a Student's t-test)

To determine if P38 inhibition was able to support KIT null cells through the apoptotic crisis and allow them to differentiate, clonal assays were performed on KIT Null and WT E14 cells under self-renewal and differentiation conditions (Figure 4.8). In the presence of LIF, PD169316 had no effect on the population of self-renewing WT E14 cells but in the absence of LIF, PD169316 treated cells had significantly higher survival rates than both cell and diluent controls (in both comparissons $p<0.001$) (Figure 4.8a).

Although KIT Null cells are unaffected by inhibitor inclusion under self-renewal conditions, there is a slight but non-significant prevention of apoptosis when PD169316 is added to the differentiating KIT Null cells on assay (Figure 4.8b and c). As this rescue was non-significant, we can conclude that KIT mediated survival does not act through inhibition of the P38 pathway. While P38 was able to prolong the survival of differentiating KIT Null cells, its inclusion was unable to prevent cell death providing further support for the requirement of both anti-apoptotic and pro-differentiation signals for the progression of cellular specialisation.

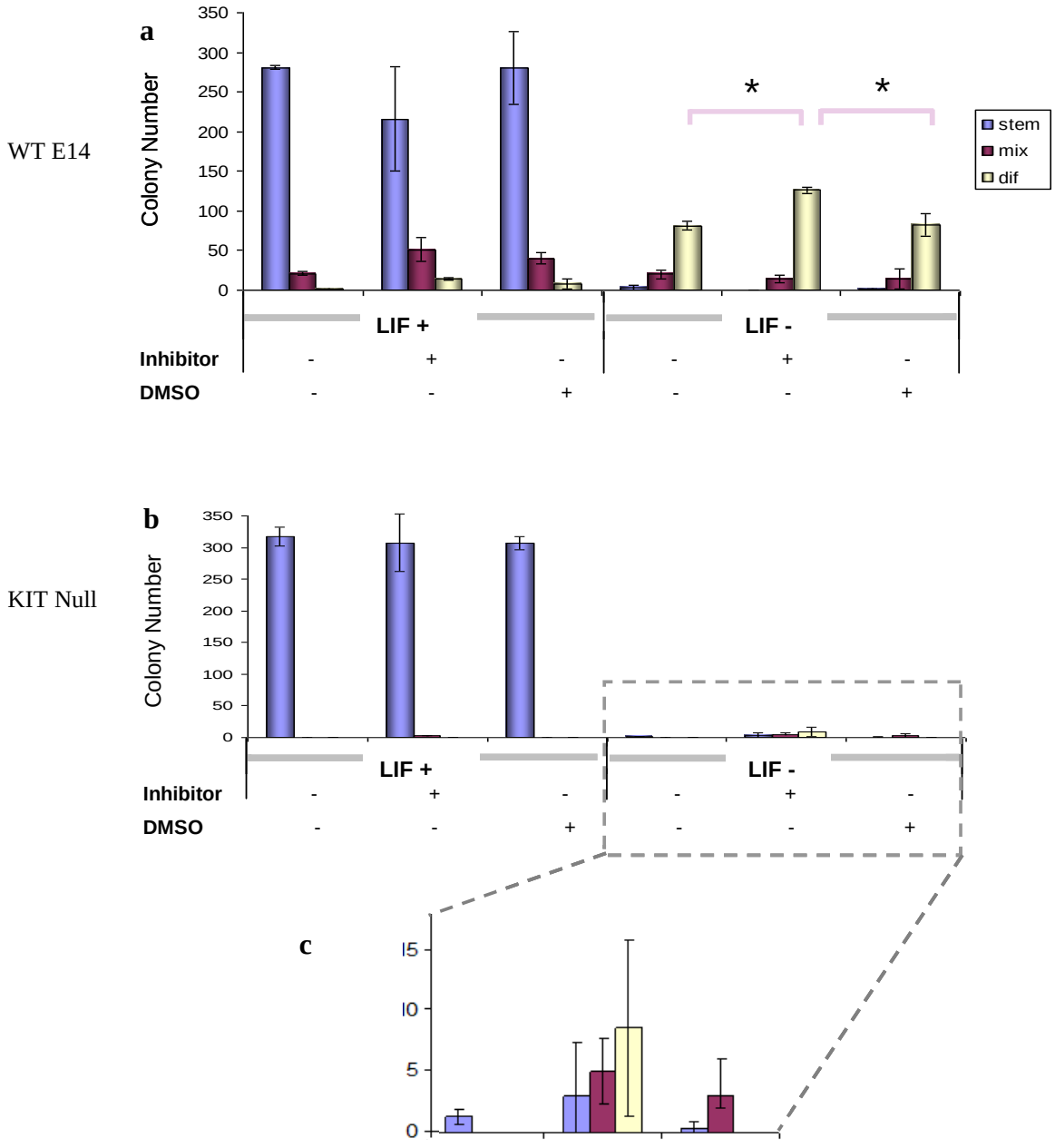


Figure 4.8 P38 inhibition (PD169316) and effect on apoptosis. Clonal assays of **(a)** WT E14 cells and **(b)** NNC1 cells grown in the presence or absence of PD169316 (10 µM) under self renewing (LIF+) or differentiating (LIF-) conditions. DMSO was added as a diluent control. **(c)** The magnified panel describes a slight but non-significant ($p<0.05$) prevention of apoptosis when the p38 is added to differentiating KIT null cells (NNC1). Significant differences between each condition are depicted by the asterisk and by pink bars ($p<0.05$). In the absence of LIF, PD169316 treated WTE14 cells had significantly higher survival rates than both cell and diluent controls (in both comparissons $p<0.001$) (n=3. Error bars represent SEM. Analysed using one way analysis of variance (ANOVA)

4.4.6 *The Generation of BCL2 over-expressing ES cells*

To confirm the involvement of BCL2 in KIT mediated survival (Bashamboo et al. 2006), WT E14 and KIT Null ES cell clones over-expressing the fusion protein EGFP-Flag-BCL2 were established (Figure 4.9 a). WT E14 and KIT Null ES clones containing only the vector backbone and no fusion protein insert were concurrently generated and employed as empty vector controls. Electroporated clones were selected in puromycin (0.7 µg/ml) and unstained clones were screened by Flow Cytometry for EGFP expression (Figure 4.9 b). EGFP positive clones were further analysed by Western blot using ant-Flag, anti-EGFP and anti-BCL2 antibodies (Figure 4.9 c) to detect the fusion protein of (55KDa). 50 puromycin resistant clones were selected for each construct (fusion protein or empty vector) and cell line of which 10 EGFP positive clones were tested further by Western blot. Figure 4.9 c is a representative sample of highly expressing WT BCL2 (F4) and Null Bcl2 clones (7B), negative empty vector controls (WT D3 and Null E1) and unmanipulated WT E14 and NNC1 cell controls.

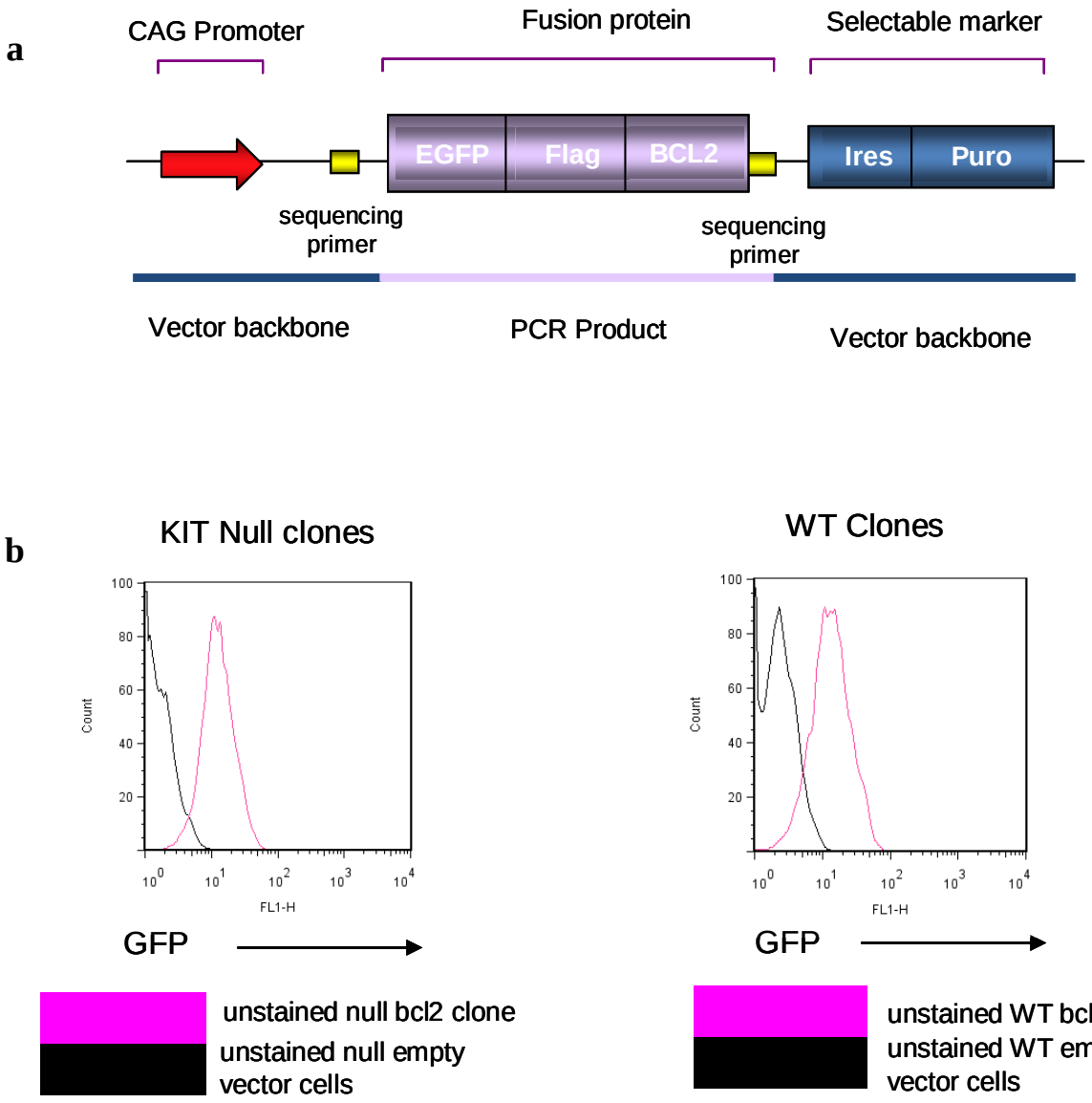


Figure 4.9 BCl2 over-expression. **(a)** A schematic showing the PCAGsip vector containing the 1484 bp cDNA EGFP-FLAG-BCL2 fragment generated by PCR. Location of the CAG promoter, selectable marker (puromycin), vector backbone and sequencing primers are also shown. **(b)** Over-expressing clones were selected by a positive shift in the FL1 channel (GFP) with Flow Cytometry analysis. WT E14 and NNC1 (black histogram) cells do not express the fusion protein whereas WT BCL2 and null BCL2 (pink histogram) ***constitutively*** express the fusion protein and fluoresce. **(c)** A Western Blot showing protein lysates (LIF+) from WT E14 and NNC1 cells electroporated with an empty vector containing only the vector back-bone and the BCL2 fusion protein vector . Protein lysates were tested for EGFP, BCL2 and Flag. 50µg of protein were added to each well and GAPDH was used as a loading control. CAG = CAG Promoter; IRES = Internal Ribosomal Entry Site; Puro = Puromycin; EGFP = Enhanced Green Fluorescent Protein.

4.4.7 *BCL2 over-expression in ES cells*

The pro-survival protein BCL2 was over-expressed in self renewing and differentiating WT E14 and KIT Null cells to observe its effect on ES cell survival. Cells were tested for nuclear fragmentation and condensation by DAPI stain, caspase activation by FLICA and membrane PS exposure by Annexin V FITC. Under self-renewing conditions, over-expression of BCL2 significantly reduced nuclear fragmentation and condensation in both WT E14 and KIT Null cells ($p=0.02$ and $p=0.01$ respectively; figure 4.10 a). On the removal of LIF and the onset of differentiation (day 3, LIF-) BCL2 over-expression significantly lowered the percentage of DAPI positive cells in KIT Null cells ($P<0.01$) when compared with their parental equivalent (NNC1) and have a cell death profile similar to and non significantly different from WT E14 cells and WT E14 cells over-expressing BCL2 (figure 4.10 a). BCL2 over expression in WT E14 did not significantly reduce the percentage of DAPI positive cells when compared with the parental line regardless of growth condition.

In the presence of LIF, the percentage of caspase activation was minimal in both KIT Null and WT E14 cells which may account for the slight and non-significant reduction of caspase activation with BCL2 over-expression (Figure 4.10 b). However, upon differentiation, BCL2 over-expression significantly reduced caspase activation in both WT (approximately 52% reduction, $P<0.01$) and KIT null cells (approximately 56% reduction, $p<0.01$) indicating that BCL2 plays a role in the survival of differentiating ES cells by inhibiting cell death in cells entering the apoptotic crisis. Duval et al report that this apoptotic crisis is initiated two days after LIF withdrawal and apoptotic signals produced at this time may be required to trigger the differentiation process. Concurrently, some of these cells may commit to irreversible apoptosis if apoptotic signals exceeded a survival threshold (Duval et al. 2000).

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As the assays described above monitor cell survival until day 3 of LIF withdrawal reflecting only the initial stages of differentiation, clonal assays were established to provide a quantitative analysis of cell survival resulting from BCL2 over-expression in

late stage differentiation (Figure 4.10 c). In the presence of LIF, over-expression of BCL2 has no effect on the ability of WT E14 or KIT Null cells to self-renew. Conversely, in the absence of LIF, BCL2 over-expression in WT E14 cells resulted in a significant increase in the number of differentiated colonies (approximately 28%) when compared with cell and empty vector controls ($p<0.01$). Duval et al (2004) reported a similar increase in survival of differentiating cells (approximately 30%) in ES cells over-expressing BCL2 compared to control cells. However, there was no cell survival when BCL2 was over-expressed in differentiating KIT Null cells (Figure 4.10 d). Therefore, BCL2 over-expression prolongs the survival of KIT Null cells during the initial stages of differentiation but does not prevent the KIT null phenotype of death upon differentiation in later differentiation events. We can conclude that there is an absolute requirement for KIT during the later stages of ES cells differentiation and KIT mediated survival does not act *via* the action of BCL2.

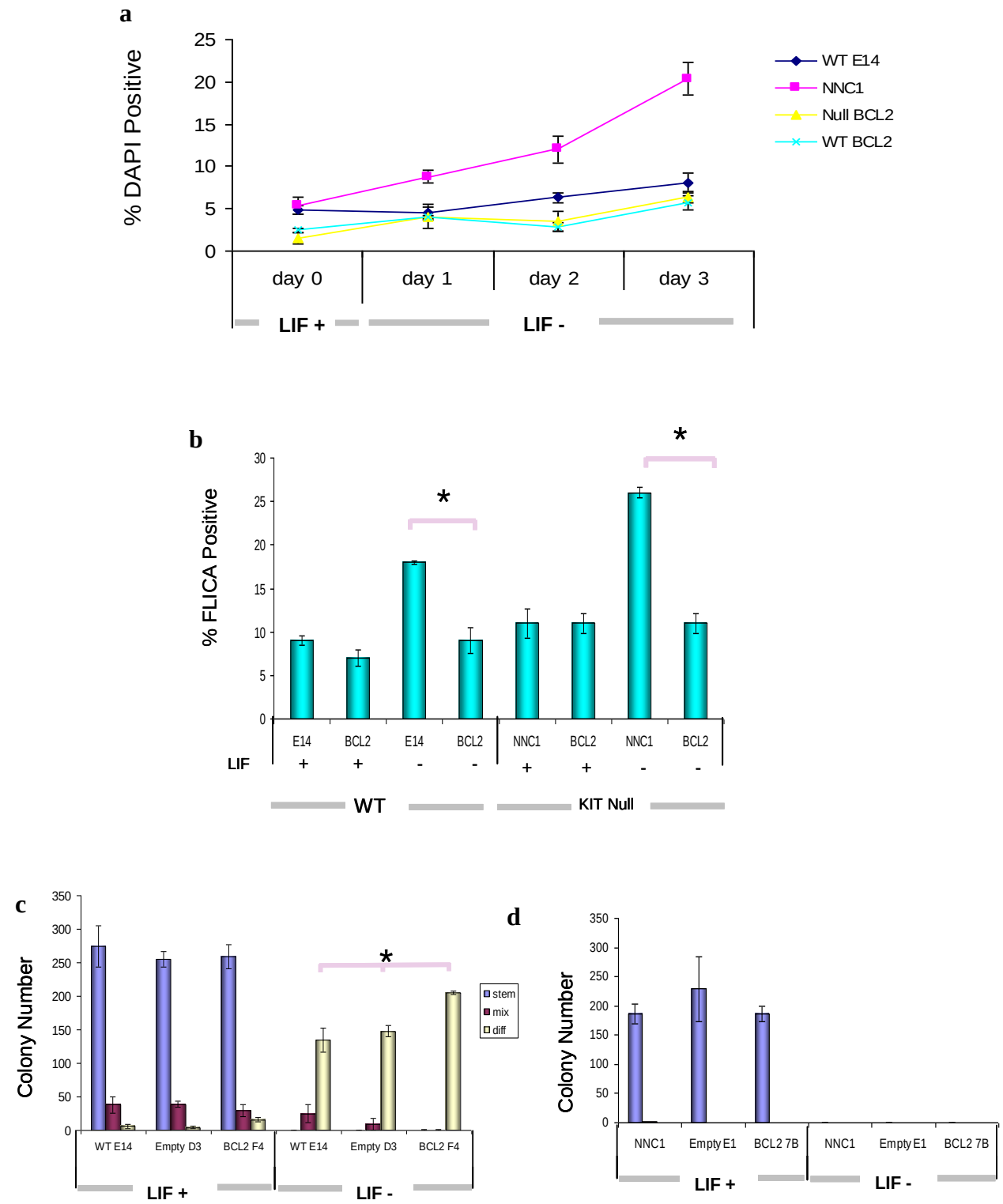

Figure 4.10 BCL2 over-expression and effect on cell survival. (a) Apoptosis detection by DAPI staining of WT E14 cells, NNC1 cells, WT BCL2 and Null BCL2 cells grown in the presence of LIF and absence of LIF for 3 days. Apoptotic cells were expressed as a percentage. (n=3. Error bars represent SEM. Analysed using a student's t-test(b) FLICA staining of control and BCL2 over-expressing WT and KIT Null cells in the presence of LIF (LIF +) of after 3 days after LIF withdrawal (LIF-). (c) WT and (d) KIT Null clonal assays of control and BCL2 over-expressing clones under self renewing and differentiating conditions. Significant differences between each condition are depicted by the asterisk and by pink bars ($p<0.05$). Refer to text for exact p-values. (n=3. Error bars represent SEM. Analysed using one way analysis of variance (ANOVA)

4.4.9 The effect of BCL2 over-expression on the KIT Null Annexin V positive phenotype.

As BCL2 has been associated with PS exposure inhibition (Uthaisang et al. 2003) we wanted to observe the effect of BCL2 over-expression on constituently annexin positive KIT Null cells (Figure 4.11). In the presence of LIF, BCL2 over-expression did not prevent the KIT Null Annexin V positive phenotype as KIT Null cells over expressing BCL2 displayed significantly greater Annexin V staining when compared with control WT E14 cells assayed under the same growth conditions ($p<0.01$). Upon differentiation, KIT Null cells (NNC1) were approximately 90% Annexin V positive, which significantly reduced to 60% positive with BCL2 over-expression ($p<0.01$). However, this reduction in Annexin V staining was not sufficient to be within limits of the WT E14 control cells grown under the same conditions ($P<0.01$).

In comparison, BCL2 over-expression significantly reduced the proportion of apoptosing WT E14 cells grown under both self renewing ($p<0.01$) and differentiation (approximately 31% reduction<0.01) conditions. This result is similar to that reported by Duval et al (2004) indicating efficacy of the BCL2 vector.

4.4.10 Apoptotic factors from micro array

To investigate the role of apoptotic factors further, a simple search was carried out on the differentially expressed genes using the SCF/KIT database to identify pro and apoptotic factors (Appendix 14). The identified apoptotic genes only contributed to 3.6% of the total differentially expressed genes and contained both pro and anti apoptotic factors of which only one BCL2 family member was down regulated. This was *Bclaf1* a transcriptional repressor that interacts with several members of the BCL-2 family. Over-expression of this protein induces apoptosis, and its down regulation at the protein level is associated with binding to pro-survival proteins such as BCL2 or BCL-XL (Kasof 1999). *Bclaf1* gene expression was down regulated during KIT Null differentiation and may be a potential gene candidate for further investigation.

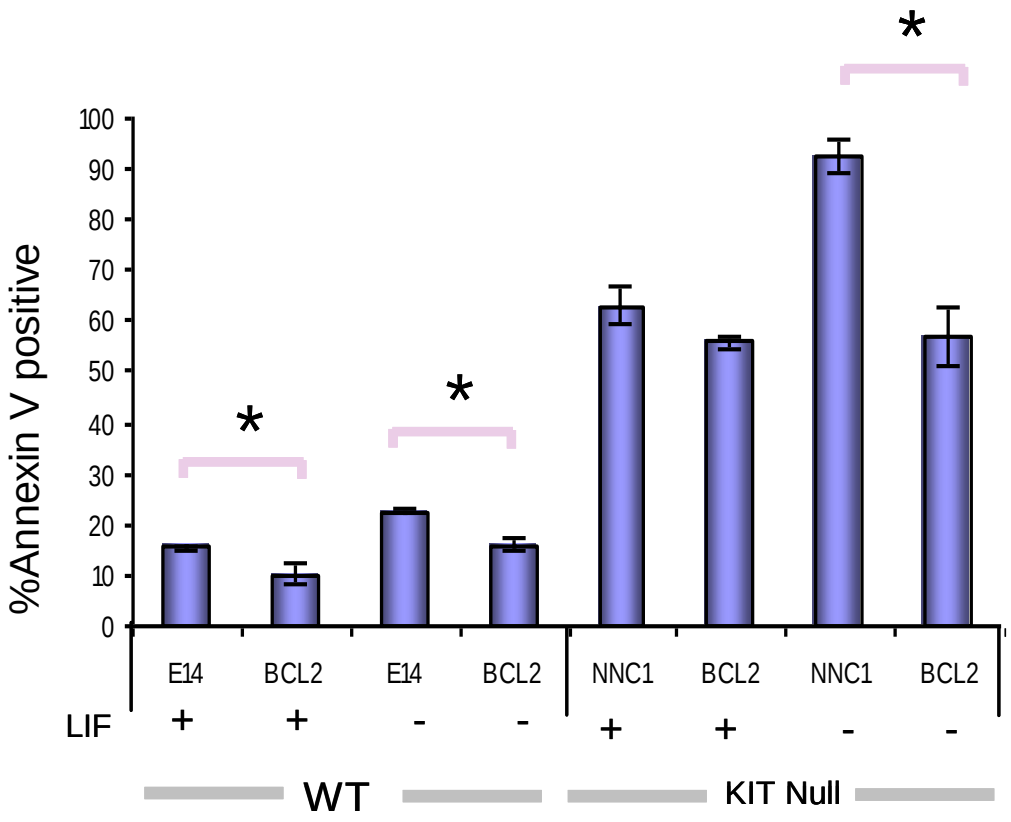

Figure 4.11 BCL2 over-expression and effect on KIT Null Annexin V positive phenotype. Annexin V staining of control and BCL2 over-expressing WT and KIT Null cells in the presence of LIF (LIF +) of after 3 days after LIF withdrawal (LIF-) Significant differences between each condition are depicted by the asterisk and by pink bars ($p<0.05$). Refer to text for exact p-values. (n=3. Error bars represent SEM. Analysed using a Student's t-test).

4.5 DISCUSSION

4.5.1 *Explaining the Annexin V positive phenotype*

The Annexin V positive phenotype was observed in all independently derived KIT Null clones where the effect of non-specific binding was eliminated with decreasing concentrations of Annexin V-FITC and specificity was confirmed with a pre-adsorption step with recombinant Annexin V. Determining whether this effect was caused by ablation of KIT signalling or due to a physical loss of the receptor causing membrane perturbation was only partially resolved with pre-treatment of WT E14 cells (+LIF) with ACK2. Here, a block in KIT signalling did not recapitulate the KIT Null Annexin V positive phenotype and alone, may not be involved in the maintenance of membrane asymmetry.

In mammalian cells, membrane composition is not uniform along the two leaflets of the plasma membrane where the distribution of phospholipids differs between the inner and outer layers. Choline containing lipids such as phosphatidylcholine (PC) and sphingomyelin (SM), are located primarily on the outer leaflet whereas amine-containing glycerophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS) are distributed primarily on the inner leaflet The inner leaflet also contains minor phospholipids such as phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate (PIP), and phosphatidylinositol-4,5-bisphosphate (PIP_2) (Hasegawa et al. 2006; Daleke et al. 2003). Phospholipid asymmetry is maintained by ATP dependent transporters called flippase and floppase as well as the ATP independent transporter, Scramblase. Flippase is highly selective for PS and functions to maintain this phospholipid to the inner cell surface. Floppase keeps phospholipids to the outer leaflet and Scramblase randomly distributes newly synthesised lipids throughout the membrane

Phosphatidylserine (PS) transfer to the outer leaflet of the plasma membrane is associated with early apoptosis onset and exposure acts as a phagocytotic trigger to circulating

macrophages in order to eliminate compromised material (Botto et al, 2004; Fadok et al. 1998). Although KIT Null cells express PS on their outer membrane leaflet, the theory of apoptosis predisposition can be eliminated as they have the ability to self renew, express gene markers associated with self renewal and do not display the morphological features associated with apoptosis under self renewing conditions.

Exposure of PS to the outer leaflet in viable cells is not exclusive to KIT Null cells and has been observed in activated platelets during cell-cell interactions enabling the process of coagulation; activated murine B-cells; T cells stimulated via the ATP receptor $P2X_7$; $CD4^+CD45RB^{lo}$ activated, memory T cells and during murine, embryonic myotube formation. Viable, PS exposing cells are thought to evade the phagogytotic process as macrophage chemotactic factors are not excreted concurrently alongside PS exposure (Van Den Eijnde et al. 2001). PS is also exposed in viable B-cells lacking the protein tyrosine phosphatase (PTP) called CD45. CD45 is located on all hematopoietic cells except erythrocytes and platelets and functions to regulate T and B cell signal transduction *via* SRC kinase (Kung et al. 2000). PS exposure on CD45 deficient B-cells was explained by deregulation of survival pathways due to a physical loss of CD45 as well as membrane distortion caused by decreased lipid packing (Elliott et al, 2006).

One membrane component that relies on dense lipid packing for correct function is the lipid raft. This domain is rich in densely packed cholesterol, sphingolipids and protein receptors and is also associated with PS surface exposure on both viable and apoptotic cells (Ishii et al. 2005; Dillon et al, 2000; Pike. 2003). Lipid rafts are enriched with signalling proteins and act as a platform to unite and promote these receptor-protein interactions (Calder et al. 2007). KIT and KIT associated signalling has been linked with lipid raft interaction and Jahn et al (2004) described how KIT recruitment to lipid rafts was required for efficient activation of the PI3-K/Akt pathway in order to mediate survival and proliferation. As previously stated, B-cells deficient in the transmembrane membrane protein CD45 are PS positive yet viable due to an altered membrane structure and reduced lipid packing. KIT Null cells are also PS positive yet viable. Could this phenomenon have arisen in the KIT Null cells due to a physical loss of the

transmembrane KIT protein resulting in lipid raft perturbation and PS exposure? Perhaps KIT signalling plays a more fundamental role and is involved in the expression of the Flippase, Floppase Scramblase genes required to maintain membrane asymmetry.

Upon investigation of the microarray data for genes responsible for membrane asymmetry maintenance (Scramblase, flippase and floppase), only floppase (*Abca3*) was up regulated in the absence of LIF. Although floppase is responsible for the externalisation of PI to the outer leaflet of the plasma membrane it was not highly expressed under growth conditions for pluripotency. If this gene was responsible for the Annexin V positive phenotype the expectation would be its significantly high expression under all growth conditions. However, it should be noted that in the presence of LIF, floppase has a fold change of 1.49 and p value of 0.06 which may merit further investigation of this gene (Q-RT-PCR) and resulting protein expression (Western blot) in explanation of the Annexin V positive phenotype.

BCL2 over-expression and prolonged survival did not prevent the KIT Null Annexin V positive phenotype (+LIF). Again, the Annexin V positive phenotype of the KIT Null cells may not be indicative of cells primed for apoptosis but a phenotype occurring independently from cell death potentially *via* membrane perturbation. As yet, the reason for KIT Null PS exposure is unknown but determination of function may be resolved by conducting a phagocytosis assay. KIT Null cells may only survive in culture *in vitro* as there is no means for their removal and destruction. If the function of PS exposure on KIT Null cells was to flag this defective cell population for macrophage clearance, the engulfment of stained KIT Null cells cultured alongside macrophages could be observed and quantitatively measured via Flow Cytometry.

4.5.2 *KIT Signalling and Cell Survival.*

The SCF-KIT signalling pathway has been implicated with survival in many stem and progenitor types and specific survival pathways appear to be cell type and developmental stage dependent and include PI3K activation in early haematopoietic progenitors (Wandzioch et al. 2004), PKB activation and inactivation of FoxO3a (Engstrom et al.

2003) and by activation of SRC Kinase in T and B cells independently from PI3K (Agosti et al. 2004). PGC survival is *via* activation of SRC and AKT independently from PI3K (Miguel et al. 2002; Mclaren. 2003) whereas mast cell survival is through PIK/SRC kinase activation of RAC1 and JNK (Timokhina et al. 1998). JNK activation *via* KIT has also been implicated in cell cycle arrest and survival in melanoma cells (Alexaki et al. 2008). The specific role of KIT mediated JNK activation and its role in ES cell differentiation will be discussed in Chapter 5.

Previous investigations have indicated that the specific action of KIT mediated survival is through up-regulation of the pro-survival protein BCL2 (Bashamboo et al. 2006; Carson et al. 1994; Zeuner et al. 2003). In this present study, BCL2 over-expression delayed the effects apoptosis in differentiating cells but did not prevent cell death caused by KIT receptor ablation. This delayed apoptosis yet failure to enhance cell survival was also demonstrated with P38 and Caspase activity inhibition. Functionality of the BCL2 over-expressing vector, ZVAD and PD169316 inhibitors was confirmed via rescue of approximately 30% of differentiating WT E14 in accordance with the result described by Duval et al (2004; 2006), FACS analysis with Annexin V-FITC and FLICA reagents.

The detection of early apoptotic markers could only be extended until day three after which time, all parental KIT Null cells perished and could not be compared with BCL2 P38 or Caspase inhibited cells. To give a quantitative measure of the later stages of apoptosis, clonal assays were conducted over a 5 day period where day 5 was the earliest time point at which distinct colonies could be observed in WT E14 control wells. However, the wells containing the KIT Null clones did not form colonies and all cells had perished by day 5. This was also the case for KIT Null clones over-expressing BCL2 or treated with inhibitors. Both treated and control KIT Null cells had the ability to adhere to the wells and began to flatten but were unable to sustain the later stages of differentiation upon LIF withdrawal. Although it may be surmised that a block in apoptosis should allow the cells to survive to some capacity in culture, the lack of an appropriate differentiation signal may result in cell death *via* caspase independent cell death (Kroemer el al. 2005) or secondary necrosis (Krysko et al. 2008)

Duval et al (2000; 2004) claimed that the signals required for differentiation were the same as those required for cell death. Therefore, cell survival upon differentiation depended on maintaining these death signals within a defined threshold. However, the data from this present study indicates that two signals may be required for differentiation progression; one for apoptosis inhibition and the other for differentiation. To test this hypothesis, KIT Null cells inhibited with ZVAD, PD169316 or BCL2 over-expressing clones could be taken through a 3D aggregation stage via embryoid body formation to observe if a survival signal (or a combination of survival signals using multiple inhibitors) with a differentiation signal could enhance and sustain survival upon differentiation in the absence of KIT signalling.

4.5.3 *Conclusion*

By knocking out the *Kit* gene in ES cells, we recapitulated the KIT Null death upon differentiation and constituently Annexin V positive KIT Null phenotype reported by Bashamboo et al 2006. Further to this, apoptosis was confirmed as the mechanism of cell death by detection of chromatin condensation, nuclear fragmentation and caspase activation. Although we were able to delay the effects of programmed cell death by inhibition of the P38 pathway, caspase inactivation and the over-expression of BCL2, this was insufficient to sustain the survival of KIT Null cells during the later stages of the differentiation process.

Chapter 5

Is there a link between KIT signalling, JNK activation and ES cell survival?

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Is there a link between KIT signalling, JNK activation and ES cell survival?

5.1 AIM

Investigate the role of JNK in ES cells and its relationship with KIT signalling

5.2 INTRODUCTION

JNK1, JNK2 and JNK3 along with their subsequent downstream effectors have been shown to promote proliferation and survival in several cell types including ES cells (Xu et al. 2010) erythroid cells (Jacobs et al. 2004), cells of the liver during regeneration (Schwabe et al. 2003) and Mouse Embryonic Fibroblasts (Tournier et al. 2000). Specifically, c-Jun, the major downstream effector of the JNK pathway has been associated with proliferation (Angel et al. 1991) and cells lacking c-jun or non-phophorylatable c-jun mutants, displayed defects in both proliferation and embryonic development (Behrens et al. 1999; Foltz et al. 1997; Hong et al. 2003; Timokhina et al. 1998).

Previous reports have shown that JNK is activated by SCF /KIT signaling to promote the proliferation and survival of haematopoietic cells (Foltz et al. 1997) and bone marrow derived mast cells (BMMC) (Timokhina et al. 1998). By expressing a mutated KIT receptor which contained point mutations at the major phosphorylation sites in KIT (tyrosines 567, 569, 702, 719, 728, 745, and 934) in 32D cells (cells that do not normally express KIT), and then adding back specific binding sites, Hong et al (2003) showed that KIT dependent JNK activation and subsequent cell survival was via SRC binding, rather than PI3K binding to the specific tyrosine residues 567 and 569 on the mutant KIT receptor. (Hong et al. 2003).

To determine whether there is a link between KIT signalling, JNK activation and ES cell survival upon differentiation we inhibited JNK in WT E14 cells using the chemical inhibitor SP600125. This JNK specific, cell permeable inhibitor is ATP competitive with a 300 fold greater affinity for JNK 1, 2 and 3 over other MAPKs such as P38 and ERK (Bennett et al. 2001). The effects and associated phenotype of JNK inhibition in differentiating ES cells were correlated with the KIT Null phenotype to determine if the KIT/JNK pathway is a potential avenue for further study.

5.3 EXPERIMENTAL STRATEGY

5.3.1 Determine the effect of JNK inhibition on ES cell self renewal and differentiation, and survival using the chemical inhibitor SP600125.

5.3.2 Analyse the downstream effect of KIT mediated JNK activation via detection of phosphorylated c-jun, a target of the JNK pathway.

5.4 RESULTS

5.4.1 JNK inhibitor SP600125 dose-dependency assay

As JNK has been associated with both anti- and pro apoptotic functions dependent on cell type and stage of differentiation (Nishina et al. 2003; Wada et al. 2004), we tested the effect of JNK inhibition on ES cells using the JNK inhibitor, SP600125. Here, WT E14 cells were grown in the presence of SP600125 from 0μ-20μM under self-renewal (+LIF) and differentiation (-LIF) conditions to ascertain the optimal inhibitor concentration for use in subsequent assays. The concentration at which the inhibitor caused minimal adverse effects to the cells under self-renewal conditions was selected. The initial dose dependency analysis involved a 2D monolayer clonal assay where the inhibitor was added directly to the cells on assay (Figure 5.1a). Under self-renewing conditions (+ LIF), there was a trend for stem cell colonies to reduce in number as inhibitor

concentration increased but this reduction became significant when the inhibitor concentration increased beyond 15μM ($p<0.01$). At this stage it was difficult to distinguish whether the significant reduction of stem cell colonies was due to inhibitor toxicity or a phenotype specific to JNK inhibition. Using the same inhibitor concentrations under differentiation conditions (-LIF), there was a trend for the number of differentiated colonies to decrease with inhibitor inclusion and this decrease became significant at 5μM ($p<0.01$). The unaffected self renewal potential of JNK-inhibited WT E14 cells; in conjunction with the death phenotype upon differentiation was of particular interest as it reflected the reported KIT Null phenotype under the same growth conditions. To investigate this speculative relationship further, the SP600125 inhibitor was then used at the non-toxic concentration of 5μM in subsequent assays. There was no effect on self-renewal or differentiation when the DMSO (diluent control) was added to the cells on assay (Figure 5.1b)

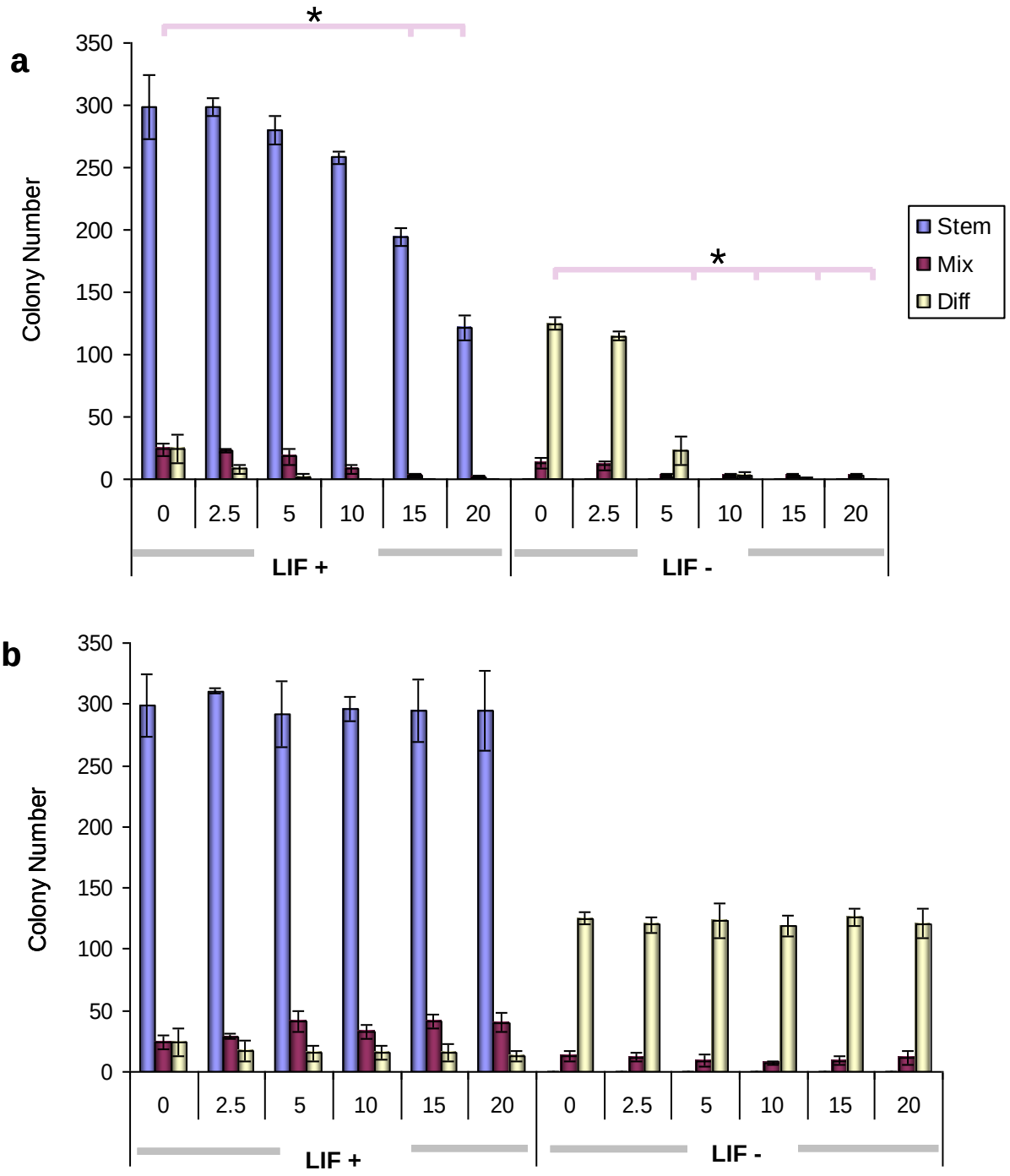

Figure 5.1 Clonal and dose-dependency assay to assess the effect of the JNK inhibitor SP600125 on WT E14 cells. WT E14 cells were grown in the presence of **(a)** SP600125 from 0-20μM or **(b)** a diluent control (DMSO) 0-20μL (0-0.2%) in the presence (LIF+) or absence (LIF-) of LIF. SP600125 or DMSO was added directly to the cells on assay. Significant differences (P<0.01) are represented by the pink bar and asterisk.DMSO will be used at a concentration of 0.1% insubsequent assays.). (n=3. Error bars represent SEM. Analysed using one way analysis of variance (ANOVA)

5.4.2.1 Blocking c-jun phosphorylation with SP600125

To determine inhibitor efficacy, protein lysates from untreated and SP600125 (5μM) treated WT E14 cells were tested by Western Blot for JNK and c-jun, a transcription factor phosphorylated by JNK. JNK phosphorylates c-jun on serine-63 and serine-73 to cause an increase in transcriptional activity of IFN-γ, IL-2, TNF-α and VEGF (Finkenzeller et al. 1995; Foletta et al. 1998; Jainet al. 1992; Pulverer et al. 1991) (Figure 5.2a). Although it is customary to test several down stream targets of a particular pathway when determining the effect of an inhibitor e.g. JNK/p-JNK/c-jun/p-c-jun, this was a preliminary investigation and was based on the work by Bennett et al (2001) who originally optimised the effect of SP600125 in Jurkat T-cells using p-c-jun. In the presence of LIF and the absence of SP600125, both JNK and phosphorylated c-jun were detected. When the cells were directed to differentiate on LIF withdrawal, there was a slight reduction in detected phosphorylated c-jun whereas JNK expression remained constant. On addition of the SP600125 inhibitor, the intensity of the phosphorylated c-jun band is visibly diminished compared with LIF-/inhibitor- control indicating efficacy of the inhibitor at the protein level during differentiation onset. However, total jun levels would have to be compared with p-c-jun levels before conclusions can be drawn on phosphorylation differences.

5.4.2.2 KIT signaling and down stream protein expression of c-jun

To assess whether c-jun was phosphorylated upon activation of the SCF/KIT pathway, an experiment was devised which compared p-c-jun expression in untreated WT E14 cells, isotype control antibody treated cells and WT E14 cells stimulated with SCF or blocked with ACK2. Untreated cells were grown in the presence of LIF and serum and GAPDH was used a loading control (Figure 5.2). A comprehensive description of this experiment is described in the materials and methods section.

In the absence of LIF, serum and SCF (Figure 5.2b), levels of phosphorylated c-jun are reduced when KIT is blocked with ACK2. As ACK2 specifically blocks KIT signaling

(Nishikawa et al. 1991) and causes cells to apoptose upon differentiation with a down regulation of p-c-Jun and because addition of the JNK inhibitor at 5μM to differentiating ES cells recapitulates the KIT null phenotype, these data may be consistent with the hypothesis that KIT signaling mediates the survival of differentiating ES cells by activating the JNK pathway.

Phosphorylated c-jun was present in untreated, undifferentiated WT E14 cells which, as stated previously, may associate c-jun activation with ES cell self renewal. c-jun phosphorylation was also evident in serum- and LIF-starved cells stimulated with SCF, regardless of ACK2 inclusion. Here, ACK2 does not appear to have blocking effect on WT E14 cells when they are stimulated with additional SCF suggesting that either endogenous SCF produced by the ES cells (Palmqvist et al, 2005) or exogenous SCF, at a concentration of 10μg /ml, overrides the blocking effect of ACK2. Previously, we have shown that ES cells require at least two passages in ACK2 in order to cause a significant apoptotic effect in differentiating WT E14 ES cells. It may be that these cells require a longer incubation time with ACK2 or an additional passage with the antibody prior to assay in order to contend with a SCF challenge and to observe an ACK2 dependent inhibition effect at the protein level.

5.4.2.3 SP600125 treated WTE14 cells die by apoptosis upon differentiation

To determine the manner in which the WT E14 cells were dying in response to SP600125 treatment, WT E14 cells were tested by Flow Cytometry for Annexin V (Figure 5.2c) and FLICA (Figure 5.2d expression). The percentage of Annexin V positive cells was significantly greater in differentiating SP600125 treated WT E14 cells than both self-renewing cells ($p=0.01$) and untreated differentiating WT E14 cells ($p=0.01$). When WT E14 cells were tested for activated caspases, again differentiating SP600125 treated cells were significantly more FLICA positive than both undifferentiated cells ($p<0.01$) and untreated differentiating cells ($p<0.01$). As addition of SP600125 to differentiating WT E14 cells enhanced Annexin V positivity as well as caspase activity this associates

SP600125 induced death with apoptosis. Again, this phenotype is consistent with KIT Null apoptosis upon differentiation phenotype and the hypothesis of a pro-survival role for JNK activation in differentiating ES cells.

5.4.2.4 *The effect of JNK inhibition on KIT Null cells.*

It was hypothesised that a block in JNK activation would increase the level of apoptosis in differentiating KIT Null cells if JNK activation was working independently from KIT signalling. To test this hypothesis NNC1 cells were primarily tested by clonal assay to observe the effect of JNK inhibition during self renewal and colony formation (Figure 5.3a). In the presence of LIF there was no significant difference in the ability of the cells to form stem cell colonies and in the absence of LIF no stem, mixed or differentiated colonies were detected. As expected, JNK inhibition did not appear to affect the self renewal capacity or differentiation capacity of the KIT Null cells.

To test whether JNK inhibition increased the level of apoptosis in differentiating KIT Null cells, parallel testing of KIT null cells in their self renewing state were tested alongside differentiating (day3, LIF-) KIT Null cells and KIT Null cells allowed to differentiate in the presence of SP600125 (Day3, LIF-, SP600125+). As KIT Null cells are constitutively Annexin V positive, apoptosis was identified by activated caspase detection with the FLICA reagent (Figure 5.3b). Although the percentage of FLICA positive cells significantly increased in differentiating KIT Null cells in comparison to cells grown under self renewing conditions ($P<0.01$), there was no significant increase of FLICA positive cells on addition of SP600125 on comparison to untreated, differentiating cells. Therefore, the hypothesis that there is no association between KIT signaling and JNK activation can be rejected as no additional caspase activation was observed when the KIT null cells were directed to differentiate in the presence of the inhibitor. Again, this correlates with the data presented from protein analysis and clonal assay from JNK inhibited WT E14 cells.

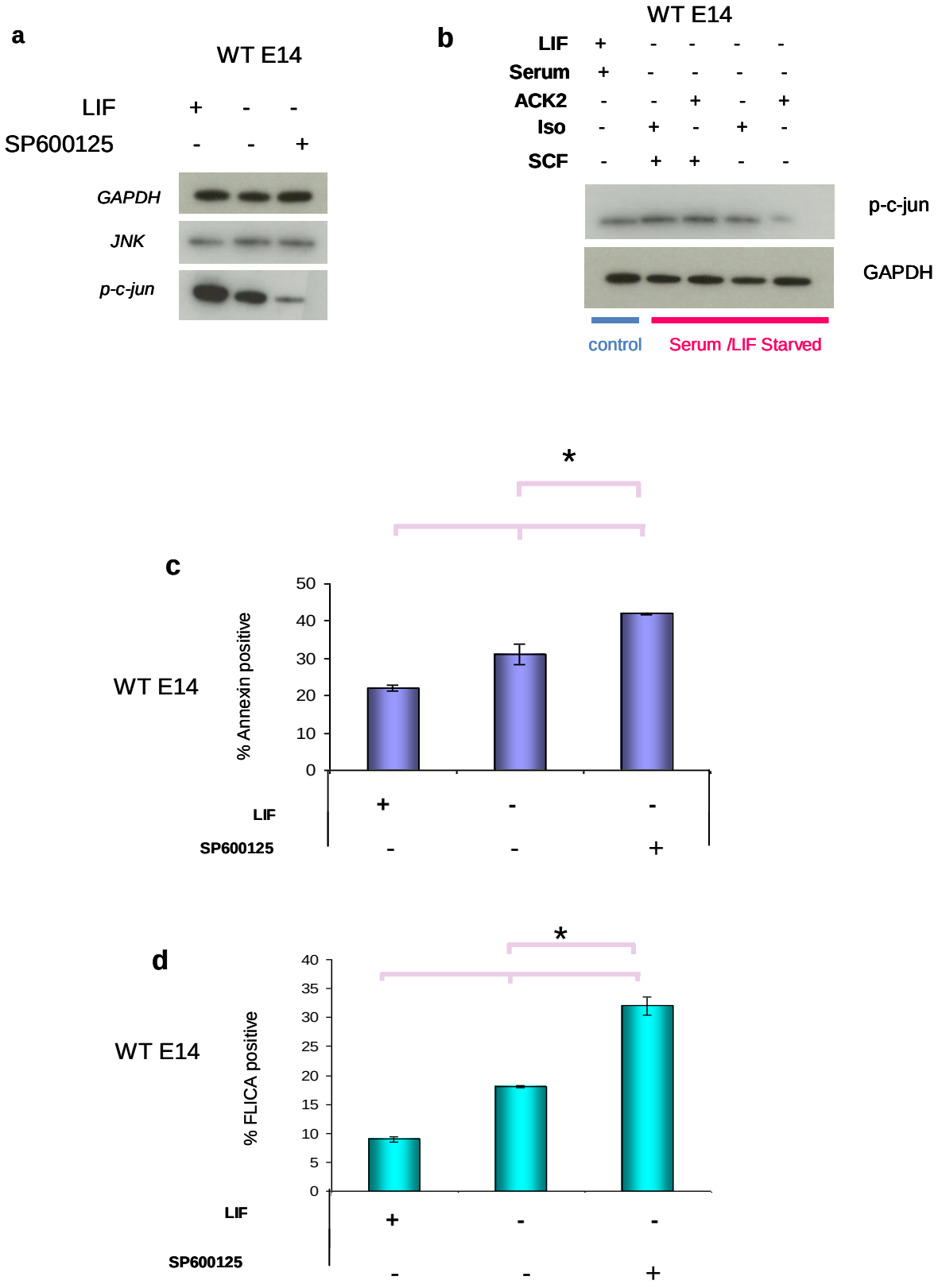

Figure 5.2 Testing the functionality of the of the SP600125 inhibitor in WT cells. (a) Protein lysates isolated from WT E14 cells grown in the presence (+) or after 3 days absence (-) of LIF and the presence or absence of the inhibitor SP600125 (5μM). Proteins were tested with anti-GAPDH (40), anti-JNK (46kD) and anti phosphorylated -c-Jun (p-c-jun, 45kD). **(b)** WT E14 cells grown in the presence or 3 days absence of LIF, Serum, SCF, ACK2 or an isotype control. Resulting protein lysates were tested for p-c-jun and GAPDH. **(c)** Annexin V-FITC staining of WT E14 cells +/- LIF and +/- SP600125. **(d)** FLICA staining of WT E14 cells +/- LIF and +/- SP600126. Significant differences are represented by the pink bars and asterisks where $p<0.05$. Refer to text for exact p-values. (n=3. Error bars represent SEM. Analysed using one way analysis of variance (ANOVA).

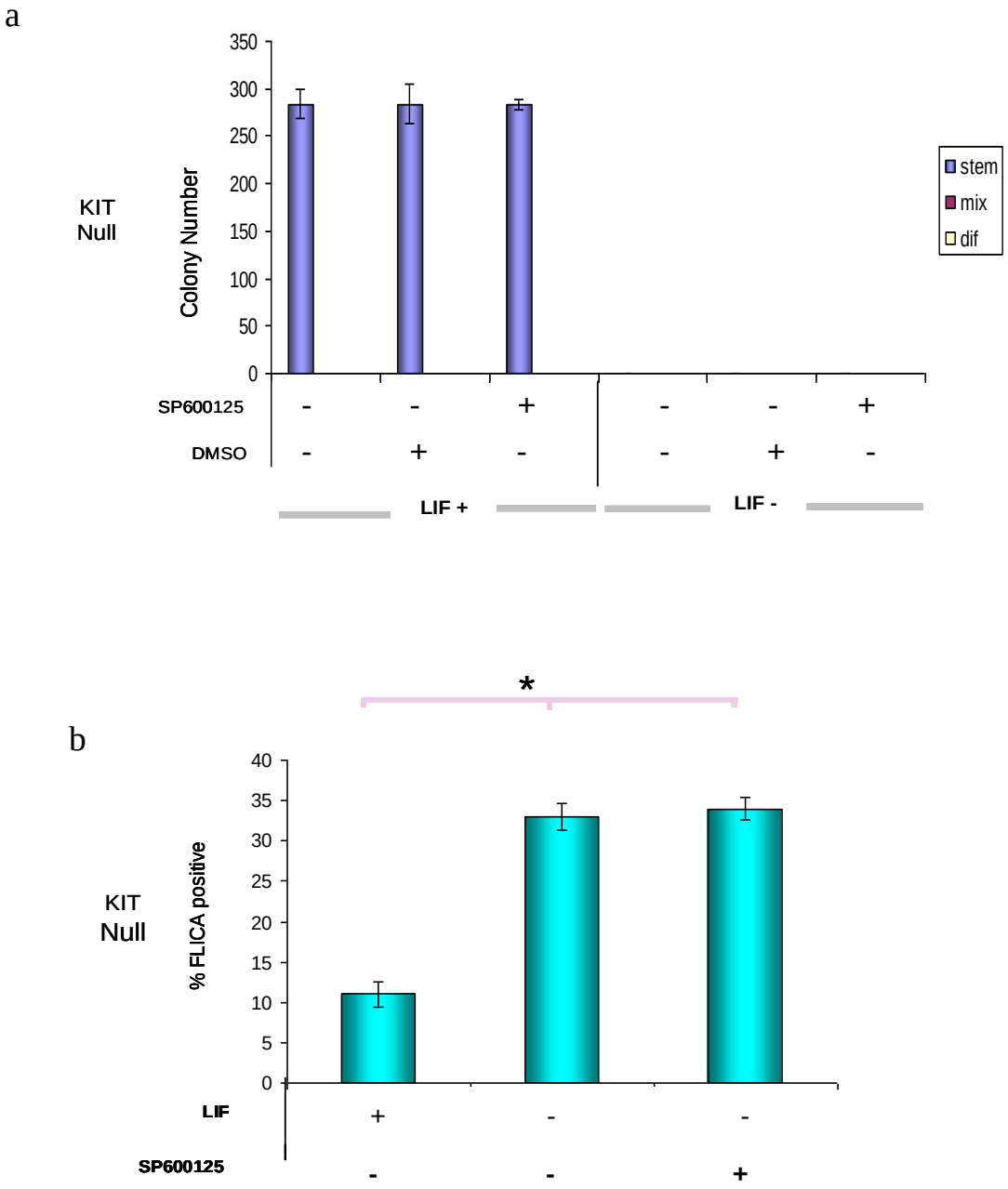

Figure 5.3 Testing the role of SP600125 inhibitor on KIT Null cells. (a) (c) KIT Null cells (NNC1) were tested by clonal assay in the presence and absence of LIF and the presence and absence of SP600125. DMSO was used as a diluent control. **(b)** FLICA of KIT Null (NNC1) cells +/- LIF and +/- SP600126. Cells were allowed to differentiate for 3 days in the absence of LIF. Significant differences ($p<0.01$) are represented by the pink bars and asterisks.). Refer to text (n=3. Error bars represent SEM. Analysed using one way analysis of variance (ANOVA)

5.4.2.5 Prolonged effect of JNK inhibition

As prolonged exposure of WTE14 cells to ACK2 revealed a role for KIT signalling during the self renewal process, we wanted to investigate the effect of prolonged JNK inhibition on ES cells. To accomplish this, WT E14 cells were grown in the presence and absence of LIF and the JNK inhibitor, then analysed by clonal assay over serial passage (Figure 5.4). Initially, SP600125 was added to the cells directly on assay (Figure 5.4 P0) and under self renewing conditions there was no significant difference in the ability of SP600125 treated cells to form stem cell colonies when compared with diluent control and untreated control cells. In the absence of LIF and the onset of differentiation, SP600125 treated cells form significantly fewer differentiated colonies ($P<0.01$) when compared with controls.

When the cells were passaged once (2 days) in the inhibitor (Figure 5.4 P1) there was no effect on stem cell colony formation. On the removal of LIF, SP600125 treatment resulted in a significant reduction ($p<0.01$) the number of surviving differentiated colonies. As SP600125 treated cells were able to self renew in the presence of LIF, the potential effect of inhibitor toxicity was eliminated. To test if SP600125 induced death was reversible, SP600125 was removed from inhibitor-treated cells on assay and the ability of the cells to survive upon differentiation was recorded. After removal of the inhibitor on assay, cell death was prevented and cells were able to survive upon differentiation similarly to and non-significantly different from control cells.

When WT E14 cells were passaged twice (4 days) in the presence of the inhibitor, again there was no effect on self-renewal in the presence of LIF but the number of differentiated colonies was significantly lower ($p<0.01$) than controls when LIF was removed. However, unlike the observation made from P1, exclusion of SP600125 from inhibitor treated cells on assay did not significantly prevent cell death ($p<0.01$). Here, SP600125 treated cells required one clear passage (2 days without inhibitor) prior to clonal assay inclusion before the number of differentiated colonies was comparable to control cells (Figure 5.4 P2).

When the cells were passaged twenty times in the inhibitor (40 days), results comparable to P2 were obtained (Figure 5.4 P20). Self renewal was unaffected, the number of differentiated cells was significantly reduced ($p<0.01$) and SP600125 treated cells required one passage in the absence of the inhibitor before differentiation potential was restored to the cells. These data implies that prolonged exposure to SP600125 in differentiating ES cells causes a cell death phenotype similar to that of differentiating KIT Null cells, which is fully reversible and does not affect self-renewal. Therefore, a block in JNK signaling does not play a role in the self-renewal of ES cells but may contribute their survival upon differentiation.

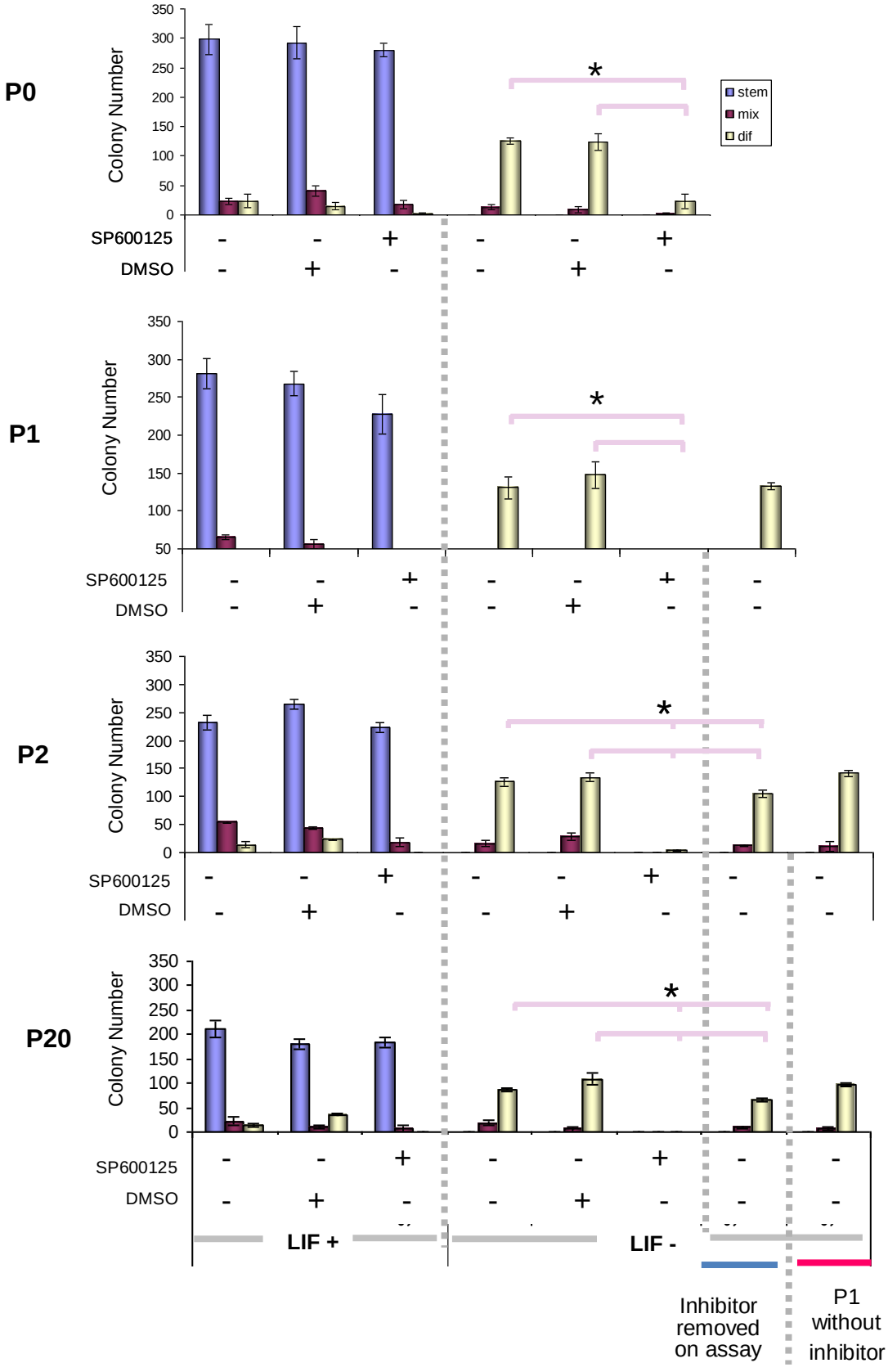

Figure 5.4 Serial passage of SP600125 (5μM) in WT E14 cells. WT E14 cells were grown in +/- LIF and +/- DMSO (diluent control) and placed into clonal assays. SP600125 was added on assay (P0), after passage 1 (P1), passage 2 (P2) or passage 20 (P20). To prevent cell death caused by SP600125 inclusion, the inhibitor was removed from cells on assay (Inhibitor removed on assay; blue bar) or the cells were given one clear passage without the inhibitor (P1 without inhibitor; red bar).Significant differences are represented by the pink bar and asterisk where $p<0.01$ in all cases. (n=3. Error bars represent SEM. Analysed using one way analysis of variance (ANOVA).

5.5 DISCUSSION

5.5.1 JNK and self renewal

Although phosphorylated c-jun was evident under self renewal conditions and also reduced on LIF withdrawal, a block in JNK did not negatively affect the ability of WT E14 cells or KIT null cells to proliferate when grown under self renewing conditions. However, further experimentation comparing total c-jun with p-c-jun must be carried out in order to confirm this result. Even so, this preliminary result agrees with a recent study by Xu (2010) who demonstrated that ES cells that were deficient in either JNK1 ($JNK1^{-/-}$) or JNK2 ($JNK2^{-/-}$) as well as double mutant ES cells ($JNK1^{-/-}$ $JNK2^{-/-}$) did not display a phenotype that negatively affected ES cells self renewal. Interestingly, their study reported that a deficiency in JNK2 alone caused an increase in ES cell proliferation as $JNK2^{-/-}$ mutants proliferated more rapidly than wild type control, $JNK1^{-/-}$ and the JNK1/2 double knock out cells. In regard to rapid proliferation, we did not observe an increase of stem cell colony number with SP600125 treatment.

Increasing inhibitor concentrations during the dose dependency assay produced fewer stem cell colonies than controls. This reduction may be attributed to SP600125 toxicity at increased inhibitor concentrations (Bennett et al. 2001) rather than a direct effect of JNK inhibition on ES cell self renewal. Toxicity rather than proliferation inhibition is also supported by the fact that prolonged inhibitor exposure had no significant effect on the development of stem cell colonies or the ability of the cells to self renew during serial passage.

5.5.2 JNK, differentiation and apoptosis

Using the JNK inhibitor SP600125, we have shown an absolute requirement for JNK activity during the onset of differentiation in WT E14 ES cells. A similar result was reported by Xu (2010) who employed JNK knock out ES cells to demonstrate the requirement of JNK during EB formation and differentiation where genes involved in

mesoderm formation (*Brachyury*,), endoderm development (*Sox17* and *Hnf1*,) and ectoderm differentiation (*Wnt1* and *Pax2)* were down-regulated. A fundamental role for JNK activation has also been reported for successful T-cell (Dong et al. 1998) and erythroid differentiation (Hietakangas et al. 2001).

The detection of elevated PS exposure (Annexin V positive) and activated caspase (FLICA positive) levels confirmed that apoptosis was the means by which WT E14 cells perished upon differentiation onset when JNK activity was inhibited. Taking advantage of the reversible action of SP600125 we could also determine that a block in JNK during self renewal followed by a period of inhibitor withdrawal did not permanently affect the potential of the cells to differentiate in subsequent assays indicating that the block in JNK becomes important upon differentiation onset rather than a manifestation of inhibitor toxicity. However, WT E14 cells with extensive exposure to the SP600125 required at least one passage free of the inhibitor before differentiation potential was restored to control levels. This reversibility and also the delay in reversibility may be explained by the chemical composition of the inhibitor. SP600125 binds to the ATP binding pocket of JNK without permanently altering the conformation of the JNK protein and is therefore fully reversible (Bennett et al. 2001). SP600125 comprises of 3 aromatic, one pyrazole and three aromatic rings and as such, was assigned a low disassociation constant (ki = 190nm) which implies a strong bond between the inhibitor and the substrate. Therefore, it is likely that inhibitor and substrate may take several days to dissociate and become fully metabolized from the cells (Bennett et al 2001; Kar, 2005).

5.5.3 Drawing parallels between the KIT Null model and JNK inhibition

The KIT Null ES cell model and the WT E14/ACK2 inhibition model (Chapter 3) demonstrate an absolute requirement for KIT mediated survival during differentiation onset. Using WT E14 cells inhibited with SP600125 we have also demonstrated an absolute requirement for JNK during apoptosis onset. As KIT has been linked to JNK activation and cell survival in other cell systems (Foltz et al. 1997; Hong et al. 2003; Timokhina et al. 1998) we wanted to investigate if KIT mediated ES cell survival acted

through activation of the JNK pathway. Although the results presented only give a correlative association of KIT with JNK activation for example, a reduction of phosphorylated c-jun in the presence of the KIT inhibitor ACK2 when compared to controls; the similarities in phenotype of KIT null cells and the SP600125 treated WT14 cells upon differentiation; as well as the non additive effect of SP600125 on apoptosis upon KIT Null differentiation; this study has provided preliminary evidence for a potential KIT mediated survival pathway that could be investigated further. Unfortunately time did not permit me to carry out a full analysis of expression of signalling molecules of the JNK pathway, the levels of both total and phosphorylated JNK and c-jun proteins (e.g. JNK/p-JNK/c-jun/p-c-jun), need to be investigated in WTE14, SP600125 treated WTE14 cells and KIT null cells under both conditions for self renewal and differentiation. This would clarify the role of KIT signalling with JNK activation and survival.

When sorting the micro array data for MAPK associated genes, it became evident that regulators of the JNK pathway were most predominant (Appendix 15). Only one gene, P2rx7, associated with ERK activation, was down regulated in the absence of LIF. Many genes involved with JNK activation were down regulated in KIT Null cells, even in the presence of substrates that usually cause induction of this pathway such as *Adm* and *Gadd45b*. Both *Adm* and *Gadd45b* were highly expressed regardless of LIF inclusion. In the absence of LIF, the gene *Als2cr2* was highly expressed and functions to inhibit apoptosis via the JNK signalling pathway.

Ask1 and *Tak1* are upstream positive activators of the JNK pathway that interact with the scaffold protein *Sh3rf1*. These MAPKKK have been linked to KIT signalling pathways *via* PI3K, RAC and SRC phosphorylation. In the absence of LIF and the onset of differentiation these upstream regulators significantly reduce in expression level ($p<0.05$) in parallel with an up-regulation of factors that are usually inhibited (*Nupr1*) or negatively regulated (*Nup62* and *Efna1*) by the JNK pathway. Genes that are always negatively regulated regardless of self renewal or differentiation status include (*Ndst1*

and *Fgd4*). No striking differences were observed when cells were grown under conditions for pluripotency (LIF+).

As there is a potential association between KIT signalling, JNK activation upon differentiation and cell survival (Chapter 5) and previous research has shown that JNK expression was not required for ES cell self renewal but became an important factor upon LIF withdrawal (Xu 2010), the microarray analysis presented may provide additional support for the theory that KIT mediated survival is *via* the JNK pathway.

Confirmation of expression levels of the key regulating genes involved in this pathway with Q-RT-PCR in conjunction with Western blot analysis and perhaps gene expression knock down using siRNA techniques in WTE14 cells to investigate JNK up/down stream effectors would also provide evidence for the role of JNK during KIT mediated survival.

Stimulants such as mustard oil or gonadotropin elevate the transcription and phosphorylation activity of c-jun in neurons (Jin woo 2008 and evi 1998) by driving upstream effectors of JNK. Over-expression of upstream effectors such as MLK3, a direct activator of MKK4, has also proven successful in JNK/c-jun activation of murine fibroblastic NIH 3T3 cells and human embryonic kidney (HEK) 293 cells (Hartcamp et al. 1999). Activation of JNK phosphorylation events in the KIT Null cell line using these stimulators alongside Western blot analysis to verify JNK activation at the molecular level and effect of the stimulation at the biological level using clonal assays would be a simple experiment to observe a restoration of differentiation in the KIT null cells.

5.5.4 Conclusion

Both pro-and anti-apoptotic roles have been assigned to JNK activation in various cell lineages and this study associates JNK signaling with ES cell survival upon differentiation. The correlation of the KIT Null phenotype with the SP600125 inhibited WT E14 phenotype upon differentiation as well as the preliminary result from microarray provides an intriguing KIT/JNK survival pathway for further study.

Chapter 6

Summary and Future Prospectives

Chapter 6

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6.1 SUMMARY

Regenerative medicine encompasses the establishment of functional tissues or cells *in vitro* in order to alleviate the problems of damaged or diseased tissues and organs *in vivo*. This field involves a biomedical approach to clinical therapy such as organ and tissue engineering as well as progenitor and stem cell technology (Mason et al. 2008). ES cells have provided an exciting avenue for regenerative medicine due to their ability to self renew, proliferate and differentiate into cells of the three germ layers including germ cells (Evans et al. 1981; Martin, 1981).

The self renewal ability and genetic status of the founding ES cell population is of particular importance in the development of novel cellular therapies to ensure a continuous supply of cells that can be differentiated reliably and safely into specified lineages using defined protocols with defined growth factor and cytokine cocktails. To avoid the potential risk of zoonotic pathogen transmission from animal derived products and the addition of undetermined growth constituents to the culture media, ES cells must be grown feeder layer free without the inclusion of animal serum. As the molecular mechanisms controlling ES cell differentiation is still poorly understood it is difficult to supplement serum and feeder free systems with the correct balance of growth factors for optimal self renewal, pluripotency, proliferation, differentiation and lineage specific survival.

The SCF-KIT pathway has been correlated to pluripotency in ES cells (Palmqvist et al. 2005; Lu et al 2007) and implicated in the survival of many stem and progenitor lineages. Specifically, the research by Bashamboo et al (2006) associated the KIT receptor with a key role in murine ES cell survival upon differentiation and was the founding paper on

which this study was based. This project aimed to further investigate the findings by Bashamboo et al by defining the role of the SCF/KIT signalling pathway during ES cell self renewal and differentiation with specific emphasis on KIT mediated survival upon differentiation.

6.1.1 Self renewal and differentiation

Using gene targeting techniques, we generated a murine ES cell model that could directly compare the effect of a KIT Null mutation with their wild type counterparts at both the cellular and molecular level under conditions for both self renewal and differentiation. Following from this, we were able to compare our findings with the work by Bashamboo and other related publications. Although there was some debate over the role of KIT during ES cell maintenance (Lu et al. 2007; Bashamboo et al. 2006) we support the theory that KIT signalling is required for the proliferation of self renewing ES cells rather than the process of self renewal itself. We can substantiate this claim as the gene markers for pluripotency (*Oct4* and *Nanog*) had similar expression levels in both KIT Null and wild type cells and when each cell type was placed into clonal assay, they produced comparable stem cell colony numbers yet, colony size, growth rates and cell number after passage were significantly lower in KIT null cells than wild type cells. Additionally, this phenotype of small colony size was recapitulated in ACK2 treated wild type cells when placed into a clonal assay. However, prolonged exposure of ACK2 to wild type cells caused a reduction in stem cell colonies on clonal assay indicating a non-essential, auxiliary role for KIT during ES cell self renewal.

Enriching a population of ES cells based on KIT expression not only resulted in a positive effect on self renewal (this study; Lu et al. 2007) but enhanced differentiation in clonal assay (this study and Lu et al. 2007) and EB formation (Lu et al. 2007). Conversely, a block in KIT signalling caused by gene targeting or neutralising antibody demonstrated a key requirement for KIT upon monolayer differentiation with the severest phenotype being KIT ablation where all differentiating cells perished by apoptosis.

When cultured as embryoid bodies, KIT Null cells produced smaller bods then limited differentiation upon cardiomyocyte differentiation. This limited differentiation may be due to receptor cross talk where a similar receptor with converging pathways could signal in place of KIT. One potential candidate was the Platelet Derived Growth Factor b (PDGFb) receptor. This receptor is of the same receptor family as KIT and was up regulated at all times upon microarray analysis. A compensatory receptor such as PDGFb may also account for the comparable level of self renewal potential that was observed in both the KIT null cells and the wild type control when compared with the reduced stem cell colony numbers in the ACK2 treated cells.

KIT signalling appears to have a profound effect on the ability of undifferentiated ES cells to proliferate in culture and perhaps a minor role in the process of self renewal itself. However it is likely that another receptor may compensate for KIT under conditions for self renewal and differentiation. Even so, it may be beneficial to add SCF as a supplement to undifferentiated cells in the absence of serum to promote proliferation. At this stage, the consequences of compensatory mechanism activation are not defined. Would activation of the PDGFb in place of KIT result in an alteration of the differentiated cell types produced?

6.1.2 Apoptosis

Bashamboo et al were the first to associate KIT signalling with BCL2 mediated survival of differentiating ES cells. They also defined apoptosis as the mechanism of cell death caused by KIT ablation. We confirmed this cell death mechanism by detection of caspase activation (FLICA), chromatin condensation and DNA fragmentation (DAPI) in differentiating KIT Null cells as well as recapitulating apoptosis detection in differentiating ACK2 treated wild type cells. However, blocking apoptosis in KIT Null cells by over-expressing the pro-survival protein BCL2 and inhibiting caspase activation (ZVAD) and P38 activation prolonged survival but did not support the cells through the apoptotic crisis initiated by the differentiation process. Although microarray data identified several genes associated with both positive and negative regulation of

apoptosis, genes with the highest differential expression in KIT Null cells encoded stimulators of signalling pathways leading to differentiation (*Adrenomedullin and Gadd45b*) rather than anti-apoptotic proteins or members of the Bcl2 family. This indication from microarray analysis, along with the inability to progress differentiation by inhibition of apoptosis, raises the hypothesis that KIT signalling provides a pro-differentiation signal rather than an anti-apoptotic signal upon LIF withdrawal and may imply that the relationship between KIT and BCL2 may be a consequence of two independent processes occurring at the same time. Although the processes stimulated by KIT signalling and BCL2 activation may work in conjunction to drive optimal survival in differentiating cells, one may not depend exclusively on the other.

KIT Null cells grown under conditions for pluripotency are viable and have the ability to self renew, yet display an Annexin V positive phenotype that could not be explained by non-specific staining. This phenotype was consistent across four independently derived KIT Null clones and as caspase activation, chromatin condensation or DNA fragmentation was not observed in these self renewing cells, it appeared to be a phenomenon occurring independently from apoptosis. As self renewing, ACK2-treated wild type cells did not recapitulate this phenotype it may be hypothesised that the Annexin V positive phenotype of the Null cells is due to a physical loss of the receptor causing membrane perturbation rather than a block in KIT signalling. One potential explanation may be disrupted lipid packing at lipid raft sites resulting in surface PS exposure. Microarray data has indicated that the gene responsible for externalisation of phospholipids to the outer membrane *Abc3r* (floppase) has elevated expression (both LIF+/LIF-) in two independent KIT null cell lines when compared with controls and may be a potential candidate to explain the KIT Null Annexin V positive phenotype. However, it is unclear if this phenotype that we have observed in our experimental model has any relevance *in vivo*. Does this phenotype arise *in vivo* and is it a mechanism to rid the body of potentially harmful, non-functional cells caused by genetic aberrations?

6.1.3 Cell sorting

Sorting WT E14 cells based on KIT expression resulted in a population of KIT negative cells that became KIT positive over serial passage under self renewing conditions. Even though KIT was not restored to control limits, this identified the fluctuation of *Kit* expression during self renewal. A similar observation of KIT restoration was observed by Lu et al upon serial passage of KIT neg grown under conditions for self renewal.

Upon differentiation, we demonstrated that KIT ablation was not equivalent to a population of KIT negative cells as KIT negative cells displayed a phenotype skewed towards differentiation, even in the presence of LIF, whereas the KIT Null cells did not survive upon differentiation. Similar phenotypes of fluctuating expression during self renewal and skewed differentiation have been observed when sorting wild type populations for *Nanog* and *Hex.*

To monitor the expression of *Nanog* in undifferentiated and differentiating ES cells, Chambers et al generated an ES cell line with GFP targeted to *Nanog*. They found that under conditions for self renewal GFP^-, and therefore *Nanog* negative, cells became GFP^+ and remained positive even after serial passage. When GFP^- cells were placed into clonal assay and initiated to differentiate, they produced fewer differentiated colonies with a lower rate of proliferation. Therefore, down regulation of *Nanog* predisposed ES cells to differentiate but does not mark their commitment to differentiate upon re-expression of *Nanog* (Chambers, 2007)

In another study, Canham et al (2010) used an enhanced yellow fluorescent protein (Venus) to monitor the expression of the early endodermal marker *Hex* in undifferentiated ES cells. Sorting ES cells by Venus (V) expression and the expression of the ES cells marker SSEA-1 (S), allowed the detection of an early primitive endoderm precursor (V^+S^+) as well as a population of (V-S+) cells. Under self renewing conditions, these populations exist in equilibrium where each cell type can switch to their counterparts. However, when these fractions were directed to differentiate, the V^+S^+

population produced primitive endoderm lineages whereas the $V^{-}S^{+}$ cells developed into epiblast (Canham, 2010). This presents the possibility that wild type ES cells could be sorted depending on KIT expression to give rise to specific cell lineages.

6.1.4 Signalling

Signals relayed by JNK through c-Jun regulate a range of cellular process including cell proliferation, tumourigenesis, apoptosis and embryonic development (Dunn et al. 2002; Timokhina *et al*. 1998; Wiltshire *et al*. 2002). JNK has also been associated with KIT related cancers such as mastocytosis and small cell lung carcinomas (SCLC) (Shinoda et al. 2005). In particular, JNK 1 and 2 cause a cellular proliferative effect when stimulated by the constituently active D816V c-kit mutant during mastocytosis progression (Timokhina et al. 1998; Wang et al. 2006) whereas JNK provides an anti-apoptotic effect that enhances multi drug resistance during the chemotherapy treatment of small cell lung carcinomas (SCLC) (Shinoda et al. 2005). As both of these cancers are associated with defects in KIT signalling, the JNK pathway may be an important target in the treatment of KIT mediated disease.

To observe the effect of JNK activation on the self renewal, proliferation and differentiation capacity of ES cells and find evidence of an association with KIT signalling and JNK activation, JNK was blocked in ES cells using the JNK inhibitor SP600125 and the resulting phenotypes were correlated with our KIT Null/WTE14 model. From this analysis, there were several parallels drawn between the KIT null phenotype and the phenotype of JNK inhibited ES cells. Similarly to KIT ablation, SP600125 inhibition revealed a non-essential role for JNK during ES cell self renewal but an essential role during differentiation onset. Upon differentiation, the mechanism of cell death was confirmed as apoptosis via detection of PS exposure and activated caspases. Further to this, ACK2 blocked ES cells displayed a down regulation of c-jun expression at the protein level similarly to SP600125 treated cells in the absence of LIF. Therefore, the hypothesis that 'KIT signaling mediates the survival of differentiating ES cells by activating the JNK pathway' was formulated. To add support to this claim, JNK

inhibition did not increase the proportion of apoptotic cells during KIT Null differentiation. An increase in cell death would have been observed with inhibitor inclusion if JNK were acting independently from KIT. At the molecular level, analysis of micro array data also highlighted that major upstream regulators of the JNK pathway, such as *Ask1* and *Tak1*, were down regulated in differentiating KIT Null cells reinforcing the theory that this signalling pathway could be involved in KIT mediated survival.

Finding a pathway downstream of the KIT receptor that is specifically responsible for the aberrant growth of a particular cell linage is particularly important in development of new pharmacological therapies. In particular, mastocytosis arises from the *Kit* mutation Asp 816 Val, an activating mutation that is resistant to Imatinib mesylate STI571. Blocking the JNK/c-Jun pathway would provide selective approach disease suppression and avoid the side effects associated with non-specific tyrosine-kinase receptor inhibition.

6.1.5 Genetic stability and methylation

Although ES cells have the ability to remain pluripotent and can self renew for prolonged periods in culture, extensive passages of both murine and human ES cell lines have the potential to accumulate karyotypic abnormalities and changes in methylation patterns. (Liu et al. 1997; Longo et al. 1997; Draper et al. 2004; Inzunza et al. 2004) The rate of genetic abnormality accumulation and can be enhanced with clonal selection or alteration of culture method. Weaning human ES cells from feeder layers resulted in trisomy of chromosome 17 or 12 (Draper et al. 2004; Inzunza et al. 2004) and changes in growth media altered the methylation patterns of the maternally expressed H19 gene. Here, a loss of methylation on the paternal allele was observed in embryos cultured in Whittens's medium but not KSOM medium supplemented with amino acids indicating that culture conditions can selectively affect imprinted gene expression (Doherty et al. 2000). These problems will have to be addressed during the development of human ES cell based transplantation and cellular therapies to ensure product safety.

In our model, karyotypic analysis of KIT Null cells demonstrated an increase in chromosome number after serial passage. Although the data presented by this study was unable to elucidate the means by which this process occurred. Further investigation will clarify whether karyotipic instability arose from adverse growth conditions during selection or via a direct effect of KIT ablation. If KIT is involved in the maintenance of genetic stability, this would have implications on the addition of SCF to ES cell culture media during their routine culture.

DNA methylation involves the modification of gene expression through interaction with DNA methyltransferase (DNMT). In mammals there are three active DNMTs (1, 3a and 3b) which covalently binds a methyl group to DNA at CpG islands in regulatory regions or promoter regions of DNA. As a result binding sites for transcription factors are blocked and consequently repress gene expression. DNMT1 is a maintenance methyltransferase, which preferentially recognizes hemimethylated DNA and methylates newly synthesized DNA whereas DNMT3a and 3b are de novo methyltransferases which create new methylation patterns on non-methylated DNA (Bestor, 2000). DNA methylation is an epigenetic regulator of parent-specific genomic imprinting (Reik, 2001). Imprinting relies on both maternal and paternal methylation patterning and plays a significant role in regulating embryonic development, placental function and behavioral processes (McGrath,2005; Lefebvre 1997). *In vivo*, parental methylation patterns are erased during embryogenesis and re-established during gametogenesis. The fertilized embryo is also initially methylated on both imprinted and non imprinted genes from male and female origin and then de-methylates prior to implantation with the exception of imprinted genes which remain methylated. Re-methylation of the blastocyst ICM occurs by de novo DNMT 3a/3b (McLaren et al. 1999). *In vitro*, ES cells are derived from ICM cells harvested during the initial stages of re-methylation and ES cells quickly become methylated upon cell line establishment. Therefore, these cells may not reflect a cell population that exists *in vivo* (Latham et al. 2007). Although ES cell with Dnmt1−/− and Dnmt [3a−/−,3b−/−] mutations are hypomethylated, they are able to self renew successfully but are unable to terminally differentiate into specific lineages on the removal of LIF (Jackson et al. 2004)

Microarray analysis presented another theory of cell survival by means of KIT regulated methylation patterns. The gene that displayed the greatest fold change (-22.89 Appendix 4) and was down regulated in both the presence and absence of LIF was the mesoderm specific transcript gene *Mest* (Peg1) which encodes an endoplasmic reticulum expressed protein. *Mest* is an imprinted gene expressed only from the paternal allele and due to its unmethylated state is expressed during all stages of male germ cell development (swales 2005). The female allele becomes highly methylated upon oocyte maturation by de novo DNMT 3a/3b (Lucifero et al. 2004) and remains silent. In the embryo, the *Mest* gene is exclusively expressed in embryonic and extra-embryonic mesoderm in early post implantation embryos (Sado et al. 1993), Upon differentiation and lineage specification *Mest* expression is reduced. However, complete loss of *Mest* during the early stages of development causes embryonic lethality and *Mest* deficiency or inappropriate expression causes growth retardation of embryonic structures and behavioral defects in the adult (Kanwar et al. 2002; Lefebvre et al. 1997).

The data presented from this array data shows that Mest expression is reduced at all times in the KIT null cells with a concurrent 2 fold increase of *DNMT3a*. As de novo methylation is responsible for *Mest* methylation and therefore silencing *in vivo*, it is possible that KIT Null cells are hypermethylated. Further to this the other paternally expressed genes *Peg3 ,Snrpn , Znf127, Ndn,* and *Impact* genes as well as maternally expressed *Igf2r* and *p57KIP2* genes were investigated but returned no differential expression. This may link KIT signalling specifically to *Mest* expression via *DNMT3a.* Further experimentation confirming the expression levels of *Mest* and *DNMT3a* in KIT Null cells as well as establishing the methylation status of the KIT Null cells would be advantageous in testing this theory.

6.2 FUTURE PROSPECTIVES

6.2.1 The generation of an inducible vector

The generation of a KIT Null ES cell line has been invaluable in demonstrating the importance of KIT signalling during ES cell differentiation *in vitro*. However, this is 'an all or nothing' model and can not be employed to investigate the effect of KIT activation or exclusion at specific time points during lineage specification. To account for this, Bashamboo et al generated a pharmacologically inducible KIT Null cell line (FKB/KIT) which comprised of two FK506-binding domains (FKBP12) fused to KIT- intracellular kinase signalling domains (Jin et al. 1998). Stimulation of the FK506 binding domains by the drug AP20187 caused dimerisation and auto-phosphorylation of the intracellular kinase domains of KIT and activation of signal transduction that was comparable to the wild type receptor. This system allowed the reversible and specific activation of KIT at desired periods during cellular differentiation. Bashamboo et al (2006) generated the inducible cell line by knocking the FKB/KIT gene into a heterozygous $Kit^{W\text{-}lacZ/+}$ clone resulting in the $Kit^{W\text{-}lacZ/W\text{-}FKB}$ cell line. Although $Kit^{W\text{-}lacZ/W\text{-}FKB}$ partially rescued the KIT Null phenotype with the administration of AP20187, this cell line did not grow well after extensive culture possibly due to the accumulation of chromosomal abnormalities as previously discussed. Unfortunately, this cell line was lost due to poor cell growth before karyotypic analysis could commence to test this hypothesis.

As the vector used to generate the inducible line had a low targeting efficiency of only 1% it would be of use to re-design the targeting vector using a *Kit* containing bacterial artificial chromosome (BAC) in conjunction with recombineering techniques (Copelamd et al. 2001) to lengthen the targeting arms and increase vector efficiency. Designing the vector with a neomycin cassette flanked by two loxP sites in frame with the inducible gene would allow targeting directly into wild type cells without the requirement of the heterozygous line. After clonal selection and targeting confirmation using Southern blot, the neomycin cassette could be removed using cre-recombinase. Following this, the

newly derived KIT $^{+/FKB}$ cell line could be retargeted with the same vector to produce a fully inducible cell line avoiding the requirement for high G418 exposure or extensive passage in the absence of functional KIT signalling.

Chapter 3 gave a crude indication that KIT was expressed at varying levels throughout differentiation *in vitro*. Interestingly, this *in vitro* model mimics the waves of *Kit* expression observed during developmental processes such as melanogenesis, gametogenesis and haematopoiesis *in vivo*. An inducible cell line would allow the specific analysis of the effects of *Kit* expression during lineage specification and in conjunction with Q-RT-PCR give a quantitative measure of lineage specific genes that are up or down regulated upon *Kit* induction or elimination. Protein analysis carried out on cell lysates isolated during the different differentiation strategies would confirm the results from Q-RT-PCR as well as identify distinct KIT mediated signalling pathways that are active during lineage specification. Again WT and KIT Null cells could serve as positive and negative controls for these processes.

6.2.3 Receptor compensation

Low level differentiation was observed in KIT Null cells when directed to differentiate via embryoid body formation. This was in contrast to the monolayer system in which all cells died by apoptosis upon differentiation. Early microarray analysis has indicated that the *Pdgfb* was elevated in both the presence and absence of LIF in two independent KIT Null cell lines. As this receptor is of the same receptor family as KIT it would be of interest to establish if this receptor could aid differentiation in the absence of KIT signalling by engaging in convergent signalling pathways. No differential expression was shown for in vivo compensatory receptors such as the *Epo, IL-3* , *Tpo* or *flt-3* associated with erythroid rescue or hepatic stem cell activation in W/W^{v} mutant cells (Antonchuk et al. 2004; Goldwasser et al. 1990; Omori et al. 1997) or colony-stimulating factor-1 (CSF-1)/c-*fms* in murine peri-implantation blastocysts (Arceci et al. 1992) or *c-fms* during mast cell survival (Dubreuil et al. 1991).

Q-RT-PCR analysis of KIT Null cells would allow the confirmation of elevated *Pdgfb* expression and subsequent Western blot of cell lysates or Flow Cytometry analysis of intact cells would confirm elevated expression at the protein level in comparison to wild type cell controls. To determine if a switch in receptor signalling from KIT to PDGFb causes an alteration in lineage specification, Q-RT-PCR could be employed to identify genes that are on / off during at several stages of EB formation or cardiomyocyte assay and compare the wild type model with that displayed by the KIT Null clone.

6.2.4 Cell populations

The observation that undifferentiated ES cells could be segregated into categories based on KIT expression and that these sorted cells remained in their specific categories over serial passage was used to formulate the hypothesis that 'specific undifferentiated cellular compartments may represent specific progenitor lineages'.

During her PhD study Gillian Cowan carried out provisional Q-RT-PCR analysis of WTE14 cells sorted on the basis of KIT ^{hi} and KIT ^{neg} expression (Gillian Cowan, PhD thesis 2009). This study indicated that a KIT ^{Hi} population positively correlated with early germ cell gene markers such as *Blimp1*, *Fragilis* and *Stella*. It would be of importance to pursue this hypothesis to investigate the possibility that an undifferentiated population of ES cells could be sorted on the basis of KIT expression in order to enrich for an early germ cell progenitor.

6.2.5 Membrane asymmetry

The constituently Annexin V positive phenotype of the KIT null cells may be explained by the up-regulation of the gene floppase (*Abc3r*) as indicated by early micro array analysis. Confirmation of *Abc3r* up-regulation by Q-RT-PCR and protein analysis would provide support for the mechanism. However, it is not certain whether this PS exposure

phenotype has function. As PS exposure is associated with apoptosis onset and phagocytotic engulfment, it would be of interest to observe the uptake of KIT Null cells upon co-culture with macrophages and compare this engulfment, if any, with Annexin V low wild type ES cells. Engulfment could be observed and quantified via dual staining of ES cells and macrophages and sorted with FACS analysis.

6.2.6 JNK

One pathway that has linked KIT signalling with the positive regulation of tumourigenesis is the JNK pathway (Vivanco et al. 2007; Sancho et al. 2009). Although the results presented by this study only demonstrate a correlative association of KIT with JNK activation, a comprehensive screen of JNK downstream signalling molecules e.g. JNK/p-JNK/c-jun/p-c-jun, in WTE14, SP600125 treated WTE14 cells and KIT null cells under both conditions for self renewal and differentiation would clarify this association and relate KIT signalling with JNK activation and ES cell survival.

Already micro array analysis indicates that genes encoding proteins that mediate JNK activation are down regulated upon differentiation onset in two independent KIT Null clones. Confirmation of this result with Q-RT-PCR in conjunction with Western blot analysis and functional assays would provide evidence for the role of JNK during KIT mediated survival of differentiating ES cells.

6.2.7 Gene regulation

To determine the role of KIT in the maintenance of chromosome stability, wild type ES cells may be grown in the presence of the KIT neutralising antibody ACK2 for prolonged passage then analysed karyotypically. Concurrent passage of untreated wild type ES

cells could serve as a control. In the event of generating an inducible cell line, a similar experimental procedure could commence but with the addition or absence of the stimulating drug, AP20187.

Epigenetic changes brought about by changes in DNA methylation patterns is an intriguing line of investigation for KIT mediated signalling pathways. Many cancers show aberrant methylation patterns, and methylation is central to many other important processes, such as embryonic development and cell cycle regulation.
The data presented from this array data implies that *Mest* expression is reduced at all times in the KIT null cells with a concurrent 2 fold increase of *DNMT3a*. As de novo methylation is responsible for *Mest* methylation and therefore silencing *in vivo*, it is possible that KIT Null cells are hypermethylated. Again, confirmation of the down regulation of *Mest* and up-regulation of *DNMT3a* using Q-RT-PCR as well as protein analysis could confirm the microarray findings as well as provide support for the hypothesis that KIT signalling is involved with methylation pattern establishment.

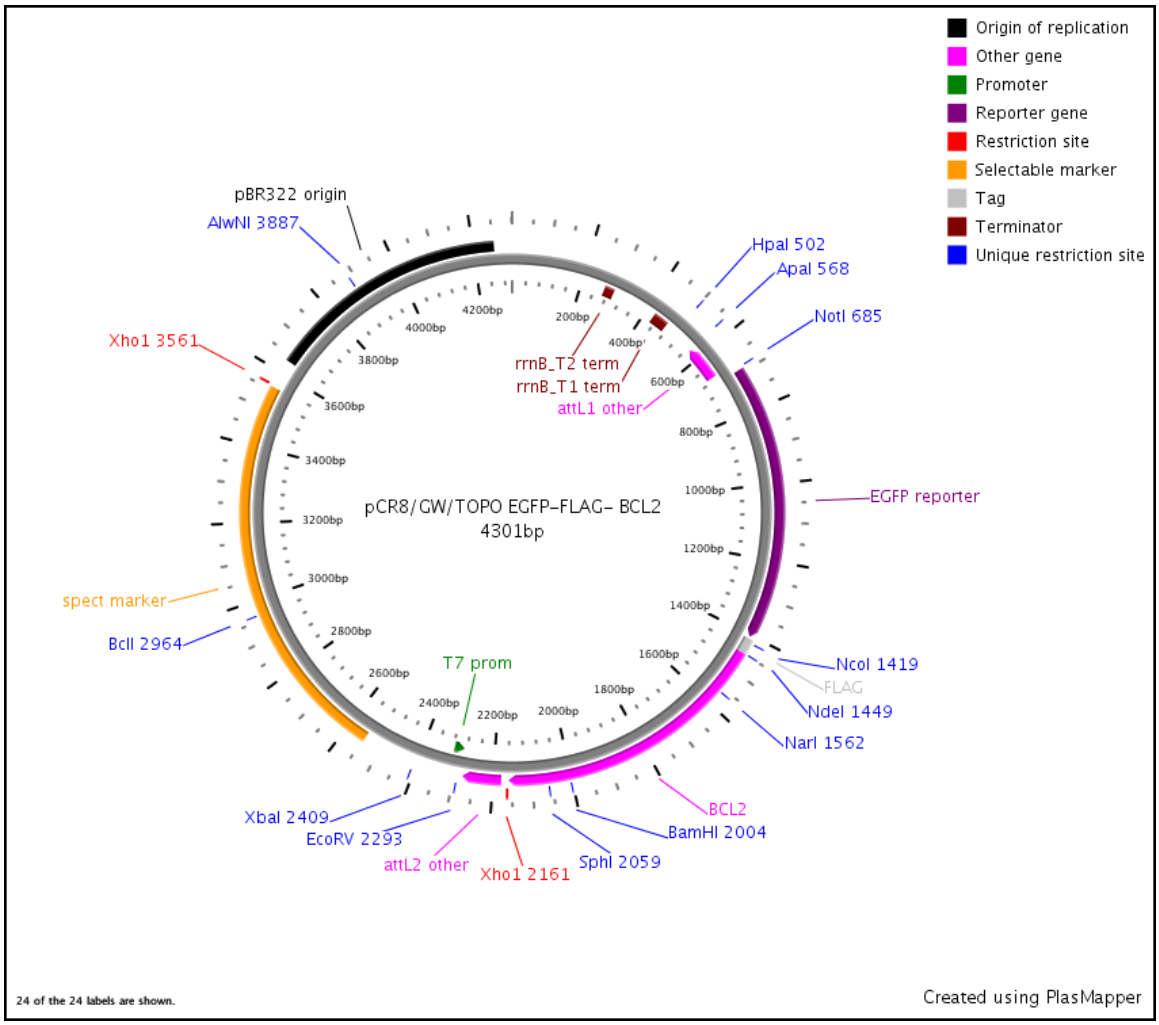
6.2.8 Final thought

This study has presented a plethora of intriguing phenotypes which have inspired the formulation of novel hypotheses for further exploration. Investigation of the KIT receptor using our knock out model has allowed the association of KIT signalling with proliferation as well as survival upon differentiation via a pro differentiation signal rather than an anti-apoptotic signal. We have also identified the JNK pathway as a potential target of KIT mediated survival in addition to a hypothetical link with DNA methylation, gene expression regulation, membrane asymmetry regulation and chromosome stability. *Kit* is a gene responsible for many pleotrophic effects *in vivo* as reflected by its varied phenotypes *in vitro* and thus, should be considered during ES cell maintenance, differentiation, lineage specification and during the derivation of cellular or pharmacological therapies.

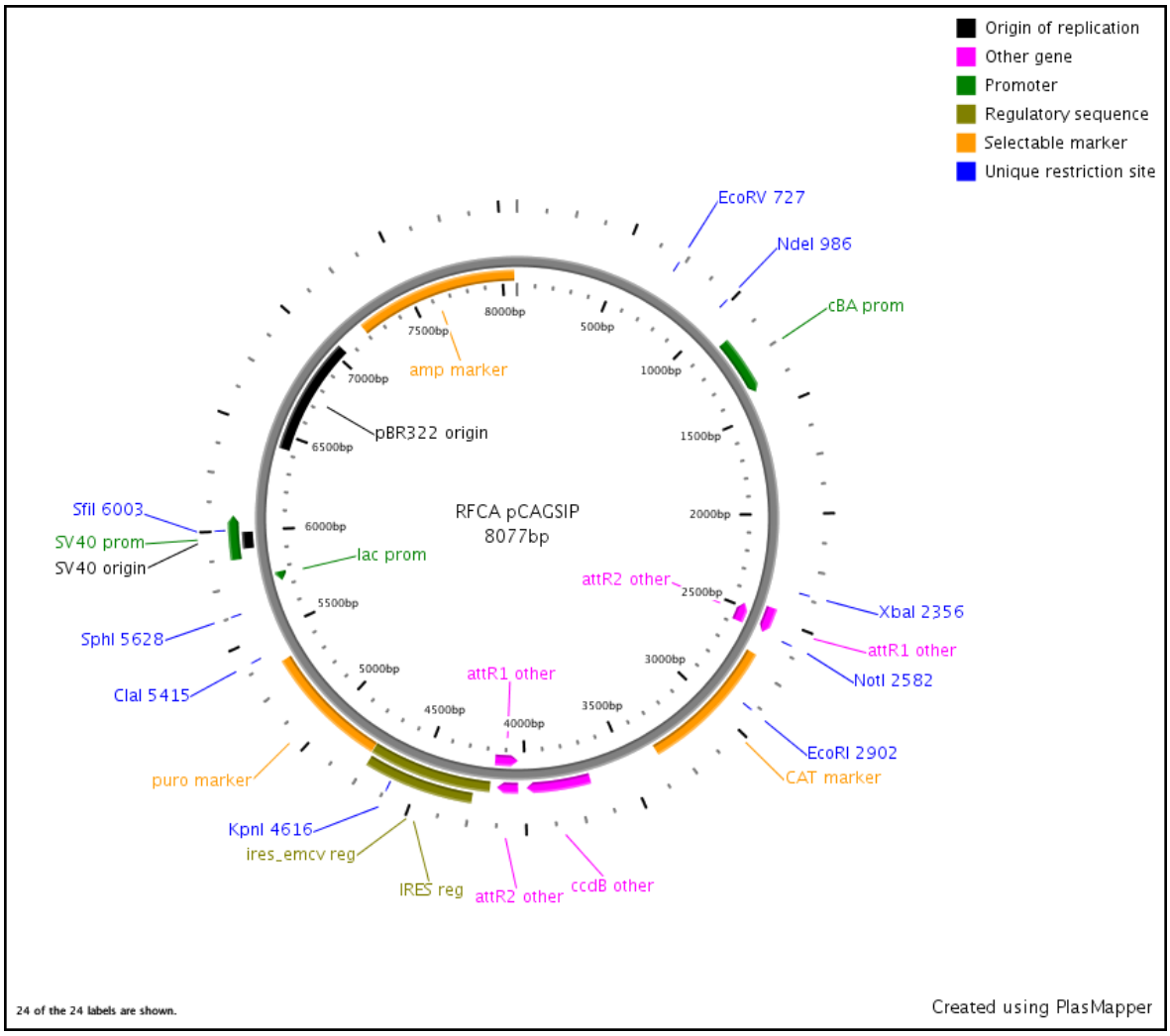
APPENDICES

Appendix 1 - pCR8/GW/TOPO bcl2

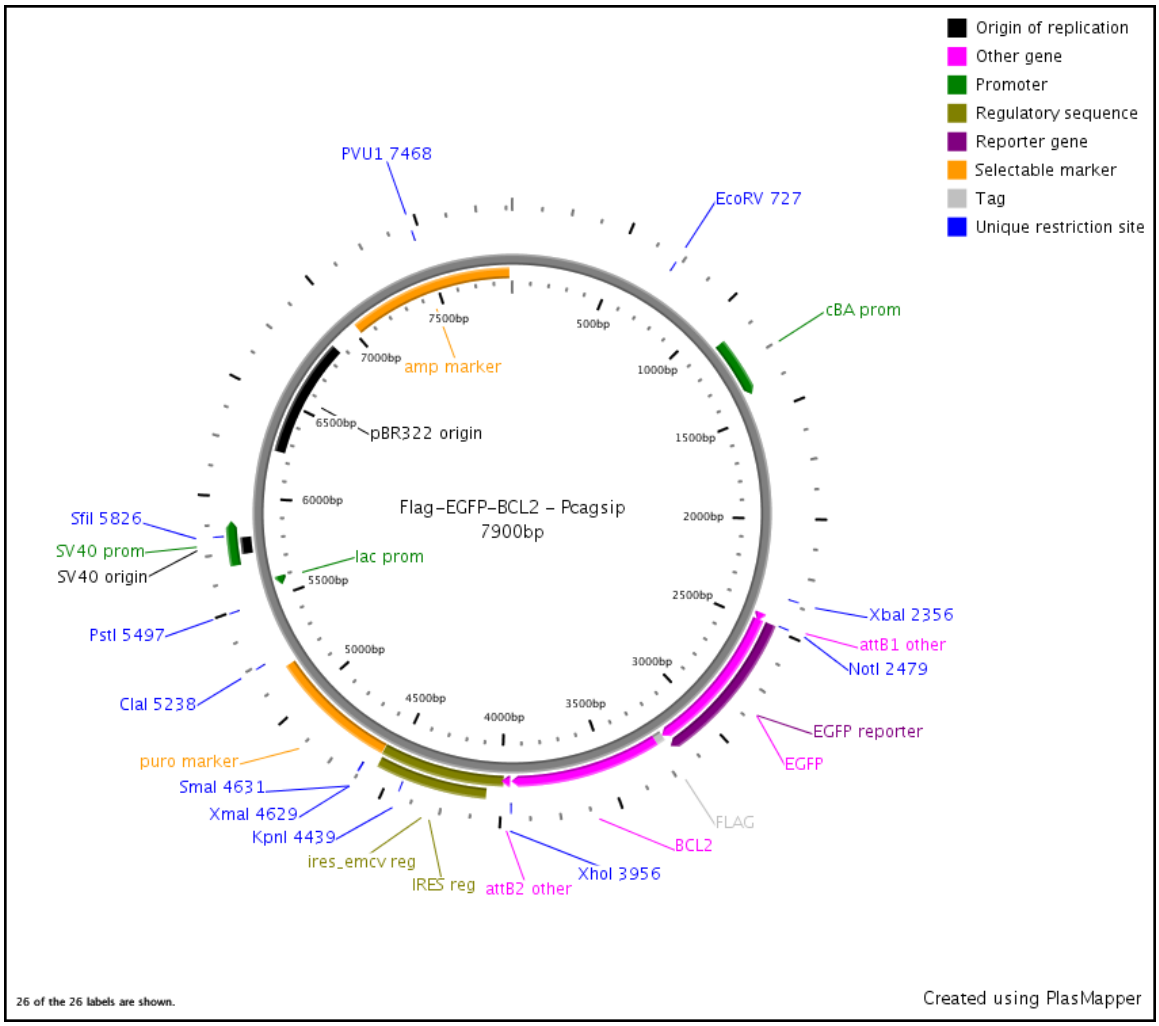
Topo Clone 2 BCL2



Appendix 2 – RFCA PcagSip



Appendix 3 – BCL2 gateway



Appendix 4

Complete Gene Listings – Micro Array Data

Genes that are always low

Symbol	Entrez ID	Fold change	p-value
Mest	17294	-22.89	8.46691E-06
Kit	16590	-11.27	0.000131969
Tox3	244579	-5.38	0.000510852
Hmga2	15364	-4.48	0.00244546
Lefty1	13590	-4.39	0.0466286
2610528J11Rik	66451	-3.83	0.00460529
Car2	12349	-3.55	0.0105087
Ube1y1	22202	-3.46	0.00391346
Uty	22290	-3.15	0.00172268
Ddx3y	26900	-3.08	0.000606614
Bmi1	12151	-3.05	0.00020175
Ddx3y	26900	-3.04	0.000568738
Lefty1	13590	-2.87	0.000559163
Aff3	16764	-2.82	0.00298331
Mat2a	232087	-2.74	0.0405987
Plac8	231507	-2.63	0.00274024
Eif2s3y	26908	-2.61	0.00456108
Alox5ap	11690	-2.54	0.000816223
Uty	22290	-2.53	0.00608592
Pof1b	69693	-2.52	0.0128626
Fgd4	224014	-2.5	0.0351006
Pcolce2	76477	-2.49	0.00034343
EG667823	667823	-2.42	0.0251314
Rpl30	19946	-2.4	0.018457
Gpr177	68151	-2.39	0.0120792
4930526L06Rik	75138	-2.33	0.00268558
2610528J11Rik	66451	-2.33	0.00414445
Plac8	231507	-2.32	0.00169168
4930526L06Rik	75138	-2.27	0.0358052
Odz4	23966	-2.24	0.0353817
Eif2s3y	26908	-2.21	0.00182436
Rgnef	110596	-2.17	0.00329201
Car2	12349	-2.15	0.00188042
2700023E23Rik	70036	-2.15	0.015266
Stx3	20908	-2.14	0.00115826
Kcnk1	16525	-2.14	0.0161313
Rgnef	110596	-2.11	0.00868319
EG667823	667823	-2.1	0.00214902
Gpr177	68151	-2.1	0.000497039
Galnt7	108150	-2.01	0.0481532
Tox3	244579	-2	0.00278207
Efs	13644	-2	0.00632446
Hmga2	15364	-1.94	0.00108337

Hmgb1	15289	-1.94	0.0142479
2700023E23Rik	70036	-1.93	0.00739123
Stx3	20908	-1.93	0.0266332
Zfy2	22768	-1.92	0.00139377
Fgd4	224014	-1.92	0.00795374
Tmem144	70652	-1.9	0.0295257
Sfrs12	218543	-1.9	0.015665
Mtdh	67154	-1.89	0.0150715
Zfy2	22768	-1.88	0.0133547
Chmp4c	66371	-1.87	0.00199394
Bmi1	12151	-1.86	0.0231236
Marveld1	277010	-1.86	0.0159641
Galnt7	108150	-1.84	0.0214465
Chmp4c	66371	-1.82	0.0188394
Pcolce2	76477	-1.8	0.0182913
Rpl30	19946	-1.79	0.00196609
Pttg1	30939	-1.79	0.00385103
5730601F06Rik	77519	-1.77	0.0260964
Odz4	23966	-1.76	0.0131493
Mat2a	232087	-1.76	0.0121224
Sfrs12	218543	-1.74	0.0363009
Aff3	16764	-1.73	0.0304486
Xpo1	103573	-1.73	0.00391355
Marveld1	277010	-1.73	0.0150785
Alox5ap	11690	-1.72	0.0068364
Pof1b	69693	-1.72	0.0230529
Uspl1	231915	-1.71	0.0025972
Kcnk1	16525	-1.67	0.00922084
Pttg1	30939	-1.65	0.0340946
Hmgb1	15289	-1.63	0.00100996
Ndst1	15531	-1.62	0.00872428
Itgb5	16419	-1.58	0.00670989
Mtdh	67154	-1.58	0.00133284
5730601F06Rik	77519	-1.57	0.0138925
Xpo1	103573	-1.56	0.00355708
Itgb5	16419	-1.55	0.0190201
Efs	13644	-1.53	0.0242852
Tmem144	70652	-1.52	0.024223

Appendix 5

Complete Gene Listings – Micro Array Data

Genes that are low LIF+

Symbol	Entrez ID	fold change	p-value
Serpinb6c	97848	-3.13	2.56E-05
Lefty2	320202	-2.93	0.002403
Dppa3	73708	-2.81	0.008322
Aoah	27052	-2.7	0.00052
Enah	13800	-2.5	7.38E-05
Cphx	105594	-2.5	0.01397
Vwf	22371	-2.49	0.018866
672498	672498	-2.47	0.00266
EG668339	668339	-2.38	0.001481
Apobec2	11811	-2.27	0.002513
Slc23a1	20522	-2.27	0.033908
Calml4	75600	-2.27	0.004967
Notum	77583	-2.24	0.006423
Lpar4	78134	-2.23	0.010817
Trim2	80890	-2.23	0.000516
Gpa33	59290	-2.18	0.001787
Magohb	66441	-2.14	0.000896
Rbm35a	207920	-2.14	0.003232
Patl2	67578	-2.12	0.013564
Tacstd1	17075	-2.08	0.00095
2210409E12Rik	72381	-2.01	0.008096
Si	20431	-2	0.016195
Dpys	64705	-2	0.011006
C430002E04Rik	78706	-1.99	0.003142
Obox6	252830	-1.99	0.000785
AW061290	381110	-1.98	0.013781
Pga5	58803	-1.96	0.004373
Hesx1	15209	-1.95	0.000381
Pou4f2	18997	-1.94	0.003401
Aldh3a1	11670	-1.93	0.009362
Nat2	17961	-1.93	0.011506
1190005I06Rik	68918	-1.92	0.001761
D1Pas1	110957	-1.91	0.003641
Tmem64	100201	-1.9	0.000188
Msc	17681	-1.88	0.007444
Slc40a1	53945	-1.88	0.0051
3021401C12Rik	70678	-1.87	0.003689
Pdzd4	245469	-1.85	0.000148
Zscan5b	170734	-1.84	0.007297
Slfn9	237886	-1.84	0.001019
Brd4	57261	-1.82	0.001107
OTTMUSG00000002042	432591	-1.82	0.039722

Mypn	68802	-1.81	0.013675
Calcoco2	76815	-1.81	0.004712
BC049349	234413	-1.81	0.008461
Ldhc	16833	-1.8	0.000289
Lad1	16763	-1.79	0.023699
Epha4	13838	-1.77	0.004836
Spp1	20750	-1.76	0.00255
Pramel7	347712	-1.76	0.037836
Terf1	21749	-1.75	0.004492
Ier2	15936	-1.73	0.001907
Dpp4	13482	-1.72	0.001814
Tmem40	94346	-1.72	5.07E-05
1500011B03Rik	66236	-1.71	0.011704
Duxbl	278672	-1.71	0.022354
Efhd1	98363	-1.69	0.010199
Ppap2b	67916	-1.67	0.005386
Kcmf1	74287	-1.67	0.002227
Scarb2	12492	-1.66	0.00583
Tsx	22127	-1.65	0.023659
B3galtl	381694	-1.64	0.000956
Ddr2	18214	-1.63	0.038155
Xlr3a	22445	-1.63	0.038874
EG626058	626058	-1.62	0.006667
Tfpi	21788	-1.6	0.01845
Dmrtc1c	71083	-1.59	0.016547
Aurkc	20871	-1.58	0.001552
Chic1	12212	-1.56	0.008671
Rex2	19715	-1.56	0.010866
Lpar1	14745	-1.55	8.96E-06
Rpl39l	68172	-1.55	0.000145
Acss1	68738	-1.55	0.024295
Rhox13	73614	-1.55	0.015154
Birc3	11796	-1.51	0.007559
Klhl13	67455	-1.51	0.001359

Appendix 6

Complete Gene Listings – Micro Array Data

Genes that are low LIF-

Symbol	Entrez ID	fold change	p-value
1300003B13Rik	74149	-4.63	0.004526
Hs6st2	50786	-4.37	0.008932
1110012J17Rik	68617	-3.9	0.021317
Has2	15117	-3.67	0.002887
Pftk1	18647	-3.49	0.009212
Irs1	16367	-3.37	0.006214
Dscaml1	114873	-3.31	0.002029
Grhl2	252973	-3.26	0.021473
B230206F22Rik	78878	-3.22	0.006588
Slc16a2	20502	-3.21	0.020233
Mcoln3	171166	-3.21	0.00145
Pphln1	223828	-3.08	0.000916
2700008G24Rik	72545	-3.01	0.001721
Cdh9	12565	-2.99	0.010782
Prkcbp1	228880	-2.96	2.96E-05
P2ry5	67168	-2.91	0.02685
2610008E11Rik	72128	-2.83	0.006625
Ptpn13	19249	-2.79	0.019421
Hivep2	15273	-2.77	2.54E-05
Marveld3	73608	-2.77	0.002929
Lrp2	14725	-2.75	0.020445
Peli2	93834	-2.75	0.009335
Dnase2a	13423	-2.74	0.04551
Arl4c	320982	-2.74	0.001502
Cacna1b	12287	-2.69	0.00137
Id1	15901	-2.65	0.006057
Car4	12351	-2.64	0.001113
Dab2	13132	-2.64	0.014852
Setd1b	208043	-2.63	0.033169
Fat1	14107	-2.62	0.025426
Gfra1	14585	-2.61	0.003505
Zfp467	68910	-2.57	0.029875
Irs4	16370	-2.56	0.006105
Pou3f1	18991	-2.56	0.042136
Wnt8a	20890	-2.55	0.002471
Thbs1	21825	-2.55	0.001591
2610019E17Rik	75614	-2.55	0.046492
Prss8	76560	-2.53	0.002669
Cpeb1	12877	-2.51	0.003853
Rbm39	170791	-2.51	0.016205
Wt1	22431	-2.48	0.008853
Myl9	98932	-2.48	0.017298
Cdh2	12558	-2.46	0.003841

Wbscr27	79565	-2.44	0.001457
Klhl23	277396	-2.44	0.024422
Cobll1	319876	-2.44	0.010882
Slc6a15	103098	-2.43	0.034368
Centd1	212285	-2.43	0.019124
2410014A08Rik	109154	-2.42	0.043674
Hdgfrp3	29877	-2.41	0.001173
Rbm35b	77411	-2.4	0.002412
Ripk1	19766	-2.38	0.027257
Ms4a4d	66607	-2.37	0.004601
Plch1	269437	-2.34	0.018138
Cachd1	320508	-2.34	0.024622
Fbxo32	67731	-2.33	0.007874
Pdf	68023	-2.33	0.033294
Chd7	320790	-2.33	0.001028
Zfp758	224598	-2.32	0.042235
Irs2	384783	-2.3	0.004224
Cited2	17684	-2.29	0.03814
Plxdc2	67448	-2.29	0.0059
6430527G18Rik	238330	-2.29	0.010347
Marveld2	218518	-2.28	0.005277
Sema3c	20348	-2.27	0.000154
Smarca1	93761	-2.27	0.00228
Lrrn4	320974	-2.27	0.002532
Plcxd1	403178	-2.27	0.012106
Rbm3	19652	-2.25	0.000213
Riok2	67045	-2.24	0.034541
Lrfn4	225875	-2.24	0.001177
2210010L05Rik	98682	-2.23	0.002364
1810032O08Rik	66293	-2.22	0.003471
Katnal2	71206	-2.22	0.011697
Slc12a2	20496	-2.21	0.041235
AA409316	105732	-2.21	0.025703
Amotl2	56332	-2.2	0.005538
Kctd12b	207474	-2.19	0.003363
Gramd1c	207798	-2.18	0.007781
Inadl	12695	-2.17	0.005962
Kctd15	233107	-2.17	0.031149
Kif1a	16560	-2.16	0.011096
Kdelc2	68304	-2.16	0.011168
Atf7ip2	75329	-2.16	0.035758
A730035I17Rik	77630	-2.16	0.02576
Ccno	218630	-2.16	0.006081
C530008M17Rik	320827	-2.16	0.046789
Zfhx3	11906	-2.15	0.016242
Zbtb2	381990	-2.15	0.034374
Fign	60344	-2.13	0.002315
Erap1	80898	-2.13	0.029607
2310045A20Rik	231238	-2.13	0.015188
Gabra4	14397	-2.12	0.000154

Cyr61	16007	-2.12	0.042656
Rnpc3	67225	-2.12	0.033816
Zfp423	94187	-2.12	0.016947
Socs2	216233	-2.12	0.01527
Foxa1	15375	-2.11	0.016496
Stk11	20869	-2.11	0.042437
Malat1	72289	-2.11	0.015565
Josd3	75316	-2.1	0.040201
Met	17295	-2.09	0.013785
Itsn2	20403	-2.09	0.026876
AI314180	230249	-2.08	0.049732
Nr3c1	14815	-2.07	0.003118
Psmb8	16913	-2.06	0.031928
Rpo1-4	20019	-2.06	0.009543
Zfp52	22710	-2.06	0.001667
Trim44	80985	-2.06	0.007409
P2rx7	18439	-2.05	0.005816
Atrx	22589	-2.04	0.045986
Rpl3	27367	-2.03	0.002841
Rps9	76846	-2.03	0.009922
Zfp608	269023	-2.03	0.036834
Mettl2	52686	-2.02	0.000597
Jmy	57748	-2.02	0.006914
Eif4g3	230861	-2.02	0.000255
Gli3	14634	-2.01	0.029701
Sox3	20675	-2.01	0.030476
Slc4a4	54403	-2.01	0.005603
Crebzf	233490	-2.01	0.023509
Zfp36l2	12193	-2	0.046417
Map3k5	26408	-2	0.003993
Dapk1	69635	-2	0.002536
Pdlim3	53318	-1.99	0.004742
Lemd3	380664	-1.99	0.037843
Vamp8	22320	-1.98	0.002052
Irx5	54352	-1.98	0.007005
Ubxn2b	68053	-1.97	0.000574
AI504432	229694	-1.97	0.040809
Cdc14a	229776	-1.97	0.021854
Rif1	51869	-1.96	0.008918
Mrps25	64658	-1.96	0.048512
1110059G02Rik	68786	-1.96	0.022116
Tulp4	68842	-1.96	0.027811
4930426D05Rik	74644	-1.96	0.010737
Zfp386	56220	-1.95	0.021763
Afap1l2	226250	-1.95	0.00613
Ebf1	13591	-1.94	0.038235
D5Wsu178e	28042	-1.94	0.033293
Hnrnpa2b1	53379	-1.94	0.001769
Bclaf1	72567	-1.94	0.046322
Rbm45	241490	-1.94	0.025121

Sall1	58198	-1.93	0.036832
2310010M24Rik	71897	-1.93	0.007746
9030204A07Rik	109199	-1.93	0.013572
Crabp2	12904	-1.92	0.028561
Crebbp	12914	-1.92	0.014931
Fndc4	64339	-1.92	0.002796
5730408K05Rik	67531	-1.92	0.010929
Larp4	207214	-1.92	0.045947
Zfp362	230761	-1.92	0.018905
Pon2	330260	-1.92	0.003686
Cpsf6	432508	-1.92	0.006211
Fst	14313	-1.91	0.01336
Acsl5	433256	-1.91	0.004396
Rragd	52187	-1.9	0.028614
Dpp7	83768	-1.9	0.022809
Thap6	381650	-1.9	0.019976
Rbmx	19655	-1.89	0.000468
Zcrb1	67197	-1.89	0.001541
Pwwp2b	101631	-1.89	0.001716
Sestd1	228071	-1.89	0.012336
Bach2	12014	-1.88	0.040329
Cox7c	12867	-1.88	0.011271
Mfhas1	52065	-1.88	0.000975
Mdn1	100019	-1.88	0.042976
App	11820	-1.87	0.000785
1200003I07Rik	66869	-1.87	0.045248
Ttc17	74569	-1.87	0.012215
Fzd6	14368	-1.86	0.047647
Slc35b4	58246	-1.86	0.019068
2610002M06Rik	67028	-1.86	0.002091
Neto2	74513	-1.86	0.008583
9430029L20Rik	109249	-1.86	0.03924
Zfp709	236193	-1.86	0.026503
Smcr8	237782	-1.86	0.016321
Rnf217	268291	-1.86	0.000913
Ank	11732	-1.85	0.035763
Ctnnd2	18163	-1.85	0.011483
Atf7ip	54343	-1.85	0.015468
Prkd1	18760	-1.84	0.009879
Axud1	215418	-1.84	0.013703
Ddx51	69663	-1.83	0.024551
2810403A07Rik	74200	-1.83	0.034454
Tcf3	21415	-1.82	0.004259
Naalad2	72560	-1.82	0.009571
Vgll3	73569	-1.82	0.028932
Rps15a	267019	-1.82	0.014427
Wac	225131	-1.81	0.007293
Lypd6	320343	-1.81	0.00621
Dtna	13527	-1.8	0.007242
Ktn1	16709	-1.8	0.02844

Nab1	17936	-1.8	0.001827
Dlg2	23859	-1.8	0.014706
Phlda3	27280	-1.8	0.001501
Atxn1l	52335	-1.8	0.0294
3300001P08Rik	67684	-1.8	0.002263
Rpl41	67945	-1.8	0.009395
Stox2	71069	-1.8	0.017063
Dos	216164	-1.8	0.039114
Trub2	227682	-1.8	0.00235
Tmem150	232086	-1.8	0.040777
D030011O10Rik	320560	-1.8	0.011531
Csf2ra	12982	-1.79	0.006994
Mef2a	17258	-1.79	0.026205
Notch1	18128	-1.79	0.007693
Matr3	17184	-1.78	0.001189
Plxnc1	54712	-1.78	0.014776
1810029B16Rik	66282	-1.78	0.010106
Rbm12b	72397	-1.78	0.025485
Csnk1d	104318	-1.78	0.013869
Ssh1	231637	-1.78	0.011649
C77370	245555	-1.78	0.01951
Ctgf	14219	-1.77	0.024081
Pabpc1	18458	-1.77	0.038973
2810474O19Rik	67246	-1.77	0.017511
Tnrc6c	217351	-1.77	0.016622
Cpne8	66871	-1.76	0.009872
Cdc2l5	69562	-1.76	0.02757
Depdc2	109294	-1.76	0.017031
D8Ertd82e	244418	-1.76	0.005533
AI747699	381236	-1.76	0.000519
Rai1	19377	-1.75	0.032389
Soat1	20652	-1.75	0.016879
Rpl35	66489	-1.75	0.029639
Zcchc3	67917	-1.75	0.018492
Lysmd2	70082	-1.75	0.01475
1700013H16Rik	75514	-1.75	0.000783
6820401H01Rik	75743	-1.75	0.007905
Sertad4	214791	-1.75	0.01064
Med13	327987	-1.75	0.01668
Rpl9	20005	-1.74	0.01825
Map3k7	26409	-1.74	0.037242
Reep3	28193	-1.74	0.020636
Zdhhc2	70546	-1.74	0.002655
Nrip1	268903	-1.74	0.028645
F830028O17Rik	320071	-1.74	0.007797
Ldoc1	434784	-1.74	0.00856
Dab1	13131	-1.73	0.016795
Dst	13518	-1.73	0.008659
Epc1	13831	-1.73	0.009848
Satb1	20230	-1.73	0.021319

Nr2c2	22026	-1.73	0.008863
Zfp644	52397	-1.73	0.017882
Wars2	70560	-1.73	0.046215
Cds2	110911	-1.73	0.001189
Dusp4	319520	-1.73	0.029857
2810432L12Rik	67063	-1.72	0.022967
Rps10	67097	-1.72	0.016953
Akap13	75547	-1.72	0.040414
Rbm16	106583	-1.72	0.019852
Ahdc1	230793	-1.72	0.039746
Suhw4	235469	-1.72	0.006194
Rbm4	19653	-1.71	0.021568
Snhg1	83673	-1.71	0.021721
Tmem5	216395	-1.71	0.00673
Dsg2	13511	-1.7	0.023819
Sh3kbp1	58194	-1.7	0.038918
Sfpq	71514	-1.7	0.00345
4930432O21Rik	74670	-1.7	0.037117
Gt(ROSA)26Sor	14910	-1.69	0.00548
Zfp566	72556	-1.69	0.019449
Spopl	76857	-1.69	0.02712
Elf1	13709	-1.68	0.008512
Epb4.1l3	13823	-1.68	0.020388
2810002O09Rik	72345	-1.68	0.000362
Wdr40b	245404	-1.68	0.020668
Spin2	278240	-1.68	0.010828
Sos2	20663	-1.67	0.010213
Wdr4	57773	-1.67	0.031317
Rpl11	67025	-1.67	0.014557
ENSMUSG00000073019	331484	-1.67	0.003968
Pgr	18667	-1.66	0.004369
Slc20a2	20516	-1.66	0.000442
Gtf3c6	67371	-1.66	0.012083
Ipo4	75751	-1.66	0.006757
Hif1a	15251	-1.65	0.005518
AU044157	100334	-1.65	0.043483
Lzts2	226154	-1.65	0.000404
Lpgat1	226856	-1.65	0.017078
Phf20l1	239510	-1.65	0.036475
Setx	269254	-1.65	0.024398
Auts2	319974	-1.65	0.014278
Ssbp2	66970	-1.64	0.004692
Cdc2l6	78334	-1.64	0.018137
Wdr6	83669	-1.64	0.022616
Zfp771	244216	-1.64	0.022527
L1cam	16728	-1.63	0.041958
Smndc1	76479	-1.63	0.001829
AW112037	98667	-1.63	0.011406
D330050I23Rik	399603	-1.63	0.031703
Pde3b	18576	-1.62	0.00094

Rnpepl1	108657	-1.62	0.016373
AI661453	224833	-1.62	0.035331
Peo1	226153	-1.62	0.017713
Id4	15904	-1.61	0.03739
Prlr	19116	-1.61	0.042153
Tbk1	56480	-1.61	0.021725
Wdr12	57750	-1.61	0.035871
Iws1	73473	-1.61	0.007295
Gns	75612	-1.61	0.041008
Cyp7b1	13123	-1.6	0.019112
Bcl11a	14025	-1.6	0.042175
Gpatch4	66614	-1.6	0.03109
2610044O15Rik	72139	-1.6	0.008891
Bicc1	83675	-1.6	0.03284
Dcun1d2	102323	-1.6	0.029049
Ttc13	234875	-1.6	0.040359
LOC552906	552906	-1.6	0.048088
Bub3	12237	-1.59	0.018072
Pbx3	18516	-1.59	0.026522
Thap2	66816	-1.59	0.015543
Dph2	67728	-1.59	0.038339
Wdr81	192652	-1.59	0.014507
Dse	212898	-1.59	0.029448
4930513N10Rik	319960	-1.59	0.018207
Naaladl1	381204	-1.59	0.006749
BC023969	407828	-1.59	0.000565
Bicd1	12121	-1.58	0.012318
1300014I06Rik	66895	-1.58	0.020053
Yaf2	67057	-1.58	0.043175
Zfp513	101023	-1.58	0.012258
Bptf	207165	-1.58	0.021384
B230354K17Rik	320063	-1.58	0.03487
Slc20a1	20515	-1.57	0.00052
Gcs1	57377	-1.57	0.003518
Sh3rf1	59009	-1.57	0.005608
2700038G22Rik	67194	-1.57	0.012308
1110032E23Rik	68659	-1.57	0.02246
Wdr7	104082	-1.57	0.026449
Strn	268980	-1.57	0.005311
Prpf4b	19134	-1.56	0.005859
Renbp	19703	-1.56	0.018493
Ubl4	27643	-1.56	9.72E-05
Tbl1xr1	81004	-1.56	0.049166
Bmpr1a	12166	-1.55	0.001585
Ccdc59	52713	-1.55	0.011403
St6gal2	240119	-1.55	0.008692
Dpysl3	22240	-1.54	0.041711
Akt3	23797	-1.54	0.004841
2810022L02Rik	67198	-1.54	0.009148
2810427A07Rik	72767	-1.54	0.037273

Prtg	235472	-1.54	0.031536
Nmi	64685	-1.53	0.027957
Zfp787	67109	-1.53	0.035728
Car13	71934	-1.53	0.032171
Zfp618	72701	-1.53	0.008747
Hnrnpr	74326	-1.53	0.033721
Pnpla7	241274	-1.53	0.025188
Insm1	53626	-1.52	0.029562
Alg13	67574	-1.52	0.019283
Fjx1	14221	-1.51	0.001668
Zfp830	66983	-1.51	0.043395
Grip1	74053	-1.51	0.034598

Appendix 7

Complete Gene Listings – Micro Array Data

Genes that are always high

Symbol	Entrez ID	fold change	p-value
EG653016	653016	10.71	0.000273
Adm	11535	6.97	0.006384
Gadd45b	17873	6.94	0.035357
Ier3	15937	6.57	0.000353
Txnip	56338	5.84	0.008172
A2m	232345	5.47	0.001829
Zc3h6	78751	4.64	0.013729
Ppp1r3c	53412	4.59	0.002643
4930583H14Rik	67749	4.42	0.002927
Dnaic2	432611	4.41	0.008241
Cadm4	260299	4.23	0.010215
Lrrc2	74249	4.04	0.00466
Ndrg1	17988	4.04	0.009897
Aoc3	11754	3.95	0.008563
Dynlt3	67117	3.91	0.016584
Gpi1	14751	3.79	0.000544
Adm	11535	3.78	0.002924
Hoxa1	15394	3.43	0.023056
Cdc42ep2	104252	3.41	0.003107
Rnf128	66889	3.34	0.018156
Pdgfrb	18596	3.23	0.019588
Porcn	53627	3.13	0.009091
H2-Bl	14963	3.11	0.01823
Hoxb13	15408	3.11	0.008702
Sult6b1	73671	3.01	0.012964
Dhrs7	66375	2.99	0.018137
Gabbr1	54393	2.97	0.045829
Frmd4b	232288	2.96	0.010593
Polr3gl	69870	2.96	0.002411
Zc3h6	78751	2.96	0.001466
Arrdc4	66412	2.93	0.005904
Txnip	56338	2.91	0.00049
Rnf128	66889	2.9	0.016913
Dnaic2	432611	2.8	0.005974
Ppp1r3c	53412	2.78	0.003005
Bhlhb2	20893	2.75	0.026594
Ier3	15937	2.71	0.000389
Bhlhb2	20893	2.7	0.000772
Pdgfrb	18596	2.66	0.003827
Etv4	18612	2.63	0.00332
Dixdc1	330938	2.57	0.008344

Tcf19	106795	2.54	0.002402
Aldh1l2	216188	2.52	0.01821
Dhrs7	66375	2.51	0.001464
Slc16a6	104681	2.5	0.00035
Otub2	68149	2.49	0.01027
Fkbp1b	14226	2.45	0.0028
Ero1l	50527	2.43	0.001103
Lrrc2	74249	2.42	0.028081
Stag3	50878	2.41	0.014322
Pygl	110095	2.4	0.002447
Lyrm5	67636	2.39	0.00494
Dynlt3	67117	2.38	0.001346
Krt19	16669	2.38	0.033157
Asns	27053	2.37	0.000292
Ccnd2	12444	2.35	0.018804
Cd68	12514	2.34	0.02154
Cd47	16423	2.32	0.011272
Cd99	673094	2.27	0.000192
Armc8	74125	2.24	0.026344
Asns	27053	2.24	0.000168
1810020D17Rik	66273	2.23	0.000743
Ndrg1	17988	2.23	0.000347
Ndufs4	17993	2.23	0.009174
Entpd5	12499	2.21	5.59E-05
Slc9a3r2	65962	2.19	0.006779
EG653016	653016	2.17	3.99E-05
Hoxb13	15408	2.17	0.022618
Hoxa1	15394	2.16	0.008629
Matn3	17182	2.14	0.000877
Nudt12	67993	2.13	0.008692
Tmem106a	217203	2.13	0.006064
Acpl2	235534	2.12	0.008781
Ccnd2	12444	2.12	0.044662
B230380D07Rik	235461	2.11	0.01962
Dna2	327762	2.11	0.039302
4931406C07Rik	70984	2.09	0.000526
Rab39	270160	2.09	0.013352
Dna2	327762	2.08	0.003533
Ndrg1	17988	2.08	0.002102
Aldh1l2	216188	2.07	0.004112
Dnmt3a	13435	2.07	0.004359
Sipa1l1	217692	2.07	0.002547
Gadd45b	17873	2.06	0.005904
Lrrc51	69358	2.06	0.018316
Cadm4	260299	2.04	0.001439
6030408C04Rik	217558	2.03	2.23E-05
9130227C08Rik	219094	2.03	0.017057
A2m	232345	2.03	0.003055
Krt19	16669	2.02	0.006582
3110057O12Rik	269423	2.01	0.011358

Acpl2	235534	2	0.025816
Cdc42ep2	104252	2	0.014979
Gabbr1	54393	2	0.003305
Snupn	66069	2	0.029402
Gpi1	14751	1.98	0.005695
Rab39	270160	1.98	0.003219
6030408C04Rik	217558	1.96	0.00156
Arrdc3	105171	1.96	0.033445
D130020L05Rik	319760	1.95	0.029142
Etv4	18612	1.94	0.001792
Ift122	81896	1.94	0.018169
Cd99	673094	1.93	0.048918
Cd47	16423	1.92	0.006694
Dnmt3a	13435	1.92	0.005178
Papola	18789	1.92	0.014338
Frmd4b	232288	1.91	0.015018
Pygl	110095	1.9	0.009339
Wipi1	52639	1.89	0.043501
Arrdc3	105171	1.88	0.015682
Lrrc51	69358	1.88	0.03579
Tspyl4	72480	1.88	0.001951
Dixdc1	330938	1.86	0.004225
Gbe1	74185	1.86	0.017887
Lyrm5	67636	1.86	0.011213
4930583H14Rik	67749	1.83	0.001126
Atp5s	68055	1.83	0.002423
Aoc3	11754	1.82	0.020256
Rbks	71336	1.82	0.012451
Crkrs	69131	1.81	0.010894
Fkbp1b	14226	1.81	0.000981
Spg3a	73991	1.81	0.00653
Spg3a	73991	1.81	0.02979
9130227C08Rik	219094	1.8	0.023247
Atp5s	68055	1.79	0.012762
Ero1l	50527	1.79	0.001683
Sipa1l1	217692	1.79	0.003231
B230380D07Rik	235461	1.78	0.000796
Matn3	17182	1.78	0.009102
Nudt12	67993	1.78	0.009555
Plekhh1	211945	1.78	0.011372
Crkrs	69131	1.77	0.017515
Stag3	50878	1.77	0.003532
Lrrc2	74249	1.76	0.013819
Atp1a3	232975	1.75	0.01029
Entpd5	12499	1.75	0.002937
H2-Bl	14963	1.75	0.007363
Pgm2	72157	1.75	0.007316
Snupn	66069	1.75	0.012054
Polr3gl	69870	1.74	0.003656
Tspyl4	72480	1.74	0.008403

D930009K15Rik	399585	1.72	0.020164
Tcf19	106795	1.71	0.002466
6720401G13Rik	103012	1.7	0.025637
Arrdc4	66412	1.69	0.01386
Plekhh1	211945	1.69	0.002939
Ppapdc1b	71910	1.69	0.025386
Ptgr2	77219	1.69	0.0152
Siae	22619	1.69	0.011741
Trf	22041	1.69	0.032928
4931406C07Rik	70984	1.68	0.00235
Bcap29	12033	1.68	0.018346
D930009K15Rik	399585	1.68	0.02577
Rdh11	17252	1.67	0.047991
Ift122	81896	1.66	0.006838
Ppapdc1b	71910	1.66	0.01128
Pter	19212	1.66	0.010471
Rdh11	17252	1.66	0.038102
Trf	22041	1.66	0.025571
Rbks	71336	1.65	0.006665
Siae	22619	1.64	0.020386
Papola	18789	1.63	0.038948
Slc9a3r2	65962	1.63	0.007508
Armc8	74125	1.61	0.033766
Bcap29	12033	1.61	0.015003
Gbe1	74185	1.61	0.000225
Pter	19212	1.61	0.008718
Wipi1	52639	1.61	0.040633
Pgm2	72157	1.6	0.003464
Sult6b1	73671	1.6	0.001899
Ptgr2	77219	1.59	0.000646
Tcte3	21647	1.59	0.039618
6720401G13Rik	103012	1.57	0.006303
Cd68	12514	1.56	0.025475
D130020L05Rik	319760	1.56	0.007154
Slc16a6	104681	1.56	0.046957
Atp1a3	232975	1.55	0.029894
Otub2	68149	1.53	0.00344
Tmem106a	217203	1.53	0.005295
1810020D17Rik	66273	1.51	0.03354
3110057O12Rik	269423	1.51	0.029398
Porcn	53627	1.51	0.015398

Appendix 8

Complete Gene Listings – Micro Array Data

Genes that are high LIF -

Symbol	Entrez ID	fold change	p-value
Igfbp4	16010	6.12	0.022218
Atp12a	192113	5.2	0.034683
Nupr1	56312	4.17	0.015656
LOC624112	624112	3.7	0.040737
Stra8	20899	3.36	0.014634
Ypel2	77864	3.34	0.039338
Selenbp1	20341	3.29	0.038505
Cidea	12683	3.12	0.011504
Icam1	15894	3.12	0.030684
Smarcd3	66993	3.09	0.034352
Cyp2s1	74134	3.05	0.031639
Actn3	11474	3.04	0.001613
Gdpd1	66569	3.04	0.046835
Anxa2	12306	3.01	0.003859
Plekha4	69217	3	0.004953
Irf8	15900	2.96	0.020435
Mapt	17762	2.94	0.012047
Stmn2	20257	2.93	0.014065
Pvr	52118	2.91	0.015788
Acss2	60525	2.86	0.019339
Gchfr	320415	2.82	0.018241
Acp5	11433	2.8	0.001765
Aldoc	11676	2.78	0.01476
P4ha1	18451	2.77	0.0427
Higd1a	56295	2.75	0.020308
Cox7a1	12865	2.73	0.031978
Tcfcp2l1	81879	2.72	0.015299
Hck	15162	2.71	0.034658
Rmcs5	114888	2.68	0.024608
Padi2	18600	2.66	0.038446
H2-Ab1	14961	2.65	0.002661
Ppp1r3b	244416	2.64	0.046883
Ankrd37	654824	2.63	0.004227
Slc1a1	20510	2.63	0.006072
Cobl	12808	2.59	0.003299
Esrrb	26380	2.59	0.007544
Fbxo2	230904	2.59	0.029233
3100002J23Rik	75429	2.58	0.029909
Nid1	18073	2.58	0.004186
Mfge8	17304	2.56	0.039232
Xpr1	19775	2.56	0.014739
Anln	68743	2.54	0.011499

Tcfap2c	21420	2.53	0.002008
Gprc5a	232431	2.5	0.001512
Clgn	12745	2.48	0.001241
AU015836	385493	2.47	0.021281
Gstm1	14862	2.47	0.001181
Pcsk1n	30052	2.47	0.008586
2600009P04Rik	66572	2.46	0.004789
Stx12	100226	2.46	0.01343
Aldh1b1	72535	2.44	0.003883
Socs3	12702	2.42	0.020476
Atp4a	11944	2.41	0.035251
Col5a3	53867	2.39	0.011917
Pde9a	18585	2.38	0.005756
Ncan	13004	2.35	0.037107
Pcolce	18542	2.35	0.042962
Tnnt1	21955	2.33	0.005799
Scarb1	20778	2.32	0.004194
Vegfa	22339	2.31	0.009503
A430033K04Rik	243308	2.28	0.002621
Dgat2	67800	2.27	0.047929
Iqcg	69707	2.27	0.005036
Ppm1j	71887	2.27	0.005225
Pycr1	209027	2.25	0.006009
Rasa4	54153	2.25	0.003958
Tex14	83560	2.25	0.011632
Gsn	227753	2.23	0.022017
Cth	107869	2.21	0.001413
Immp2l	93757	2.21	0.0172
Cib2	56506	2.2	0.020862
Ott	18422	2.2	0.01682
4933437F05Rik	71275	2.19	0.027026
BC032203	210982	2.19	0.03356
Ndufa4l2	407790	2.19	0.004847
Sat2	69215	2.19	0.049855
EG432988	432988	2.18	0.003272
Guca1a	14913	2.18	0.035346
4930504H06Rik	75040	2.17	0.049715
2200002K05Rik	69137	2.16	0.004355
Paqr5	74090	2.16	0.020257
Sc5d	235293	2.16	0.015719
Wfdc2	67701	2.16	0.021002
Pfkfb3	170768	2.15	0.027795
Stat3	20848	2.15	0.031919
Selenbp2	20342	2.14	0.0274
E2f1	13555	2.13	0.029204
Fstl1	14314	2.13	0.018477
Sep-06	56526	2.12	0.002222
Figf	14205	2.12	0.011667
Specc1	432572	2.12	0.025742
Stc1	20855	2.12	0.017517

Tmem38b	52076	2.12	0.015017
3632451O06Rik	67419	2.11	0.002186
Capns1	12336	2.11	0.003017
Snapc1	75627	2.11	0.006183
Tpmt	22017	2.11	0.030061
Ccbl2	229905	2.1	0.020929
Ldhb	16832	2.09	0.03285
Abca3	27410	2.08	0.025979
Ephx1	13849	2.08	0.003231
Insig1	231070	2.07	0.043779
Aifm2	71361	2.06	0.015417
Ccng2	12452	2.06	0.00383
Exdl2	97827	2.06	0.006268
Hey1	15213	2.06	0.001701
Sox15	20670	2.06	0.002339
Syce1	74075	2.06	0.012615
Ak7	78801	2.05	0.017344
Eif2s2	67204	2.05	0.011072
Bhlhb8	17341	2.04	0.004515
Rab11fip5	52055	2.04	0.017136
Acp6	66659	2.03	0.026024
Hsdl2	72479	2.03	0.038328
Lrrc34	71827	2.03	0.003325
Csnk1e	27373	2.02	0.003751
Hebp1	15199	2.02	0.00523
Clybl	69634	2.01	0.000462
Spa17	20686	2.01	0.02366
Triml1	244448	2.01	0.000372
Zap70	22637	2.01	0.022789
Cd74	16149	2	0.005854
Irgq	210146	2	0.010122
Lss	16987	2	0.006271
Stxbp1	20910	2	0.031058
Slc29a1	63959	1.99	0.005465
Trim40	195359	1.99	0.02251
Lgmn	19141	1.98	0.009602
Psph	100678	1.98	0.008011
Tbrg1	21376	1.98	0.004715
Tmem50b	77975	1.98	0.008255
1700025K23Rik	66337	1.97	0.001485
4930455F23Rik	74895	1.97	0.029788
BC028528	229600	1.97	0.017511
Hsf2bp	74377	1.97	0.024533
Rad9b	231724	1.97	0.006333
Hnrnpd	11991	1.96	0.004962
Smtnl2	276829	1.96	0.007735
Cntnap2	66797	1.95	0.002582
Mfap5	50530	1.95	0.021274
Pik3r5	320207	1.95	0.039169
Rcn3	52377	1.95	0.010249

B4galnt2	14422	1.94	0.00324
Mad2l2	71890	1.94	0.009165
Neil1	72774	1.94	0.032404
1810010H24Rik	69066	1.93	0.041192
Insl6	27356	1.93	0.027684
Nptx2	53324	1.93	0.012713
Zfp383	73729	1.92	0.003421
4930430E16Rik	73873	1.91	0.008748
Ccbl1	70266	1.91	0.010579
Rab27a	11891	1.91	0.007181
Slc31a1	20529	1.91	0.040794
Vash1	238328	1.91	0.023742
Pgrmc2	70804	1.9	0.003811
2610029I01Rik	77032	1.89	0.00254
Akap2	11641	1.89	0.016684
Dock6	319899	1.89	0.015528
Ucp2	22228	1.89	0.008649
1810058I24Rik	67705	1.88	0.030583
Agtr1a	11607	1.88	0.002982
Glo1	109801	1.88	0.018006
1110007C09Rik	68480	1.87	0.025206
Angptl6	70726	1.87	0.032281
Cnot6l	231464	1.87	0.021588
Nubpl	76826	1.87	0.00179
Rage	26448	1.87	0.005516
1110001A16Rik	68554	1.86	0.044764
Cdc42	12540	1.86	0.013565
Elovl2	54326	1.86	0.015013
Fip1l1	66899	1.86	0.000345
Pigyl	66268	1.86	0.004214
Psat1	107272	1.86	0.011776
D330028D13Rik	231946	1.85	0.008407
Timp3	21859	1.85	0.008882
Adamtsl4	229595	1.84	0.0147
Sccpdh	109232	1.84	0.011449
Slc7a1	11987	1.84	0.006586
Tmem146	106757	1.84	0.003951
1110054O05Rik	66209	1.83	0.030574
Bcat1	12035	1.83	0.002595
Nicn1	66257	1.83	0.042381
4632404H22Rik	78755	1.82	0.001022
Ahi1	52906	1.82	0.042244
Cry2	12953	1.82	0.01294
Hk1	15275	1.82	0.031258
2610318N02Rik	70458	1.81	0.00042
Calca	12310	1.81	0.0094
Hspb11	72938	1.81	0.020496
Pck2	74551	1.81	0.001846
Tmed10	68581	1.81	0.001157
Cdyl2	75796	1.8	0.004146

Chrna5	110835	1.8	0.003756
Elmod2	244548	1.8	0.027534
Fam126a	84652	1.8	0.002802
Sox12	20667	1.8	0.014061
Tm7sf2	73166	1.8	0.008103
Tnfaip8	106869	1.8	0.009941
Als2cr2	227154	1.79	0.008427
Krtap16-7	170656	1.79	0.008619
Nmnat2	226518	1.79	0.021941
Syne2	319565	1.79	0.007462
Prelid2	77619	1.78	0.008813
Prkcb	18751	1.78	0.011058
Slc38a4	69354	1.78	0.001146
EG434402	434402	1.77	0.015788
Nos1	18125	1.77	0.000234
Rims3	242662	1.77	0.025583
Twf2	23999	1.77	0.015661
1810063B07Rik	67509	1.76	0.022821
Mef2b	17259	1.76	0.010416
Pgm1	66681	1.76	0.006415
Ppp2r5c	26931	1.76	0.007371
Rad51l1	19363	1.76	0.006263
Rrm2b	382985	1.76	0.009535
Tcp11	21463	1.76	0.003093
Trfr2	50765	1.76	0.010893
Nrgn	64011	1.75	0.004952
Rnf19a	30945	1.75	0.001716
Slc1a5	20514	1.75	0.017482
1200009F10Rik	67454	1.74	0.045097
Aldh6a1	104776	1.74	0.00147
Brp44l	55951	1.74	0.007187
Gch1	14528	1.74	0.010924
Nup62	18226	1.74	0.015255
1700009P17Rik	75472	1.73	0.023309
Slc13a5	237831	1.73	0.019783
Idh3a	67834	1.72	0.002847
Ppp4r2	232314	1.72	0.020654
Scube2	56788	1.72	0.012785
Anxa4	11746	1.71	0.001216
Ctdspl2	329506	1.71	0.014671
E430028B21Rik	211948	1.71	0.032819
Ncor1	20185	1.71	0.032632
Zrsr2	22184	1.71	0.038364
Chrna9	231252	1.7	0.019961
Cmtm6	67213	1.7	0.011321
Gys1	14936	1.7	0.023935
Itpk1	217837	1.7	0.004879
Sub1	20024	1.7	0.007947
Ccs	12460	1.69	0.005756
Coq3	230027	1.69	0.009128

Gpr160	71862	1.69	0.001763
Lgals8	56048	1.69	0.021231
Litaf	56722	1.69	0.007004
Wwtr1	97064	1.69	0.018756
Zfp277	246196	1.69	0.002789
Zfyve21	68520	1.69	0.001358
Nek6	59126	1.67	0.007397
Prss36	77613	1.67	0.027111
1810049H13Rik	66431	1.66	0.006666
Sars	20226	1.66	0.001311
Slc3a2	17254	1.66	0.00056
Acyp1	66204	1.65	0.028218
Efhc1	71877	1.65	0.023729
Gfpt2	14584	1.65	0.003253
Mett5d1	76894	1.65	0.027774
Ostf1	20409	1.65	0.008331
Aqp11	66333	1.64	0.001954
Chmp2a	68953	1.64	0.024304
Cxcl12	20315	1.64	0.015802
Nars	70223	1.64	0.001036
Oas1c	114643	1.64	0.029671
Pde2a	207728	1.64	0.024873
Slc8a3	110893	1.64	0.00787
Spns2	216892	1.64	0.048073
Tsga10	211484	1.64	0.004505
2210010C17Rik	70080	1.63	0.014964
2410017P09Rik	69709	1.63	0.017082
Bnip3	12176	1.63	0.001583
Gstm3	14864	1.63	0.025601
Phox2a	11859	1.63	0.033617
Tmhs	328789	1.63	0.040443
Cryz	12972	1.62	0.003147
Mbp	17196	1.62	0.022546
Zfp185	22673	1.62	0.000785
Creld1	171508	1.61	0.004894
Ndrg2	29811	1.61	0.001409
Psmb9	16912	1.61	0.032814
Synj2bp	24071	1.61	0.025054
Gng2	14702	1.6	0.005
Pdzd8	107368	1.6	0.017999
1810030N24Rik	66291	1.59	0.002264
Adamts9	101401	1.59	0.015991
Asrgl1	66514	1.59	0.014575
Ccdc77	67200	1.59	0.014364
Rpl22	19934	1.59	0.004935
Slc25a12	78830	1.59	0.006434
Derl3	70377	1.58	0.011386
Slc6a3	13162	1.58	0.00979
Cars	27267	1.57	0.045623
Plod2	26432	1.57	0.024527

Tfrc	22042	1.57	0.013896
Ttll8	239591	1.57	0.027051
Gstz1	14874	1.56	0.012366
Phf21a	192285	1.56	0.001687
Prps1	19139	1.56	0.041858
Lnp	69605	1.55	0.032316
Asl	109900	1.54	0.003171
Bphl	68021	1.54	0.002992
Gstm4	14865	1.54	0.032619
Narf	67608	1.53	0.030505
Sf3b1	81898	1.53	0.004896
Mlh3	217716	1.52	0.00017
Rbpj	19664	1.52	0.000515
Efna1	13636	1.51	0.028847
Usp14	59025	1.51	0.00062

Appendix 9

Complete Gene Listings – Micro Array Data

Genes that are high LIF +

Symbol	Entrez ID	fold change	p-value
Ovgp1	12659	4.07	0.004275
1700026D08Rik	75556	4	0.019638
Atr	245000	2.89	5.46E-05
BC022960	237246	2.75	0.000608
Zfpm2	22762	2.69	0.006111
Ccdc82	66396	2.67	0.002865
Scd1	20249	2.66	0.004388
H3f3b	15081	2.5	0.012424
Scyl1	78891	2.49	0.004529
Fgf11	14166	2.49	0.007133
Neil3	234258	2.47	0.003587
6720463M24Rik	77744	2.41	0.001516
AI842396	103844	2.33	0.004312
Ltbp3	16998	2.31	0.006902
2510009E07Rik	72190	2.31	0.000626
4921513D23Rik	223989	2.27	0.008547
Ddit3	13198	2.25	0.010153
Trpc2	22064	2.22	0.001896
Pla2g7	27226	2.16	0.001925
Atxn10	54138	2.13	0.011031
Rarb	218772	2.1	0.008266
Chrnb1	11443	2.09	0.000959
Itga8	241226	2.05	0.013982
Fkbp7	14231	2.05	0.007565
6030426L16Rik	628850	2.04	0.001479
Ccdc58	381045	2.03	0.002476
Prmt8	381813	2.02	0.010599
Meg3	17263	1.99	0.000305
Ppil6	73075	1.98	0.027742
Mid1	17318	1.97	0.01297
Nisch	64652	1.96	0.000429
Greb1	268527	1.96	0.005735
Ddit4	74747	1.96	0.002082
Ccdc76	229780	1.96	0.004392
Gdap10	14546	1.93	0.000671
Nrcam	319504	1.92	0.044349
D4Wsu53e	27981	1.92	0.001463
Chchd7	66433	1.91	0.00192
Hook1	77963	1.88	0.006116
4933439C20Rik	66776	1.88	0.01452
Cryzl1	66609	1.87	0.003032
5430406J06Rik	73848	1.87	0.004895

Mirg	373070	1.85	0.029337
Kank3	80880	1.85	0.002039
Dennd4a	102442	1.83	2.10E-05
Atp5c1	11949	1.83	0.010186
Sema5b	20357	1.82	0.005647
Cald1	109624	1.82	0.014786
Npepps	19155	1.81	0.015435
2310005E10Rik	67861	1.8	0.014729
1500012D20Rik	78327	1.79	0.000951
Tet2	214133	1.78	0.017184
Ylpm1	56531	1.77	0.031684
Phf20l1	239510	1.76	0.021539
5830418K08Rik	319675	1.75	0.006386
Clk1	12747	1.74	0.005234
Zfp53	24132	1.73	0.014735
Appl2	216190	1.73	0.005218
Lpin1	14245	1.72	0.002979
Iah1	67732	1.71	0.016962
Ube2b	22210	1.7	0.005517
Sbsn	282619	1.69	0.012074
Mapk4	225724	1.69	0.031152
Hrbl	231801	1.68	0.036437
Erdr1	170942	1.68	0.002117
Timm9	30056	1.67	0.019481
Senp8	71599	1.67	0.001166
Nipa1	233280	1.67	0.029954
Ccdc28b	66264	1.67	0.011386
Mbnl2	105559	1.66	0.0017
Hist2h2be	319190	1.66	0.010461
Rbm25	67039	1.65	0.031137
Map3k2	26405	1.65	0.025672
Ndufab1	70316	1.64	0.043186
Gstt1	14871	1.64	0.001387
Ctr9	22083	1.63	0.038507
B630005N14Rik	101148	1.62	0.002384
2010004M13Rik	76511	1.62	0.003477
Zfp36l1	12192	1.61	0.002395
Mfsd11	69900	1.6	0.001594
4930422N03Rik	76871	1.58	0.001073
Sf3b2	319322	1.57	0.013359
Eml5	319670	1.57	0.030955
Chrnb4	108015	1.56	0.005618
Uevld	54122	1.54	0.002433
Cbfa2t2	12396	1.54	0.030809
Bnip2	12175	1.54	0.004358
1110006E14Rik	76286	1.54	0.037052
Suv39h1	20937	1.53	0.000604
Rbbp4	19646	1.53	0.004865
Hells	15201	1.53	0.01175
2810417H13Rik	68026	1.53	0.00143

2700097O09Rik	72658	1.53	0.001651
Ncapd2	68298	1.52	0.013731
Cdadc1	71891	1.52	0.007863
Slc43a2	215113	1.51	0.006726
Six6os1	75801	1.51	0.00298
Rgs11	50782	1.51	0.027371
Gmpr2	105446	1.51	0.018461
Calu	12321	1.51	0.001387

Appendix 10

Micro Array Data

Genes expressing the greatest fold changes

From the microarray analysis, a total of 45101 genes were returned of which 1,101 were differentially expressed when comparing WTE14 cells with two independently derived KIT Null cell lines. Genes were sorted into categories dependent on low and high fold changes in the presence or absence of LIF. A "low" fold change indicates that the gene expression is lower in KIT Null cells compared with the level of expression in WT E14 cells and a "high" fold change indicates that the gene is expressed at a higher level in KIT null cells compared to controls. Genes that were expressed at a higher or lower level, regardless of LIF inclusion, were also identified.

It is important to realise the limitations of the microarray data that has been presented and the theoretical nature of the discussed hypotheses. This data only gives a snapshot of the cells genetic composition at a given time point and is therefore only an estimation of gene transcription and expression. As the RNA was extracted from the cells after 36 hours, this time interval may not have been long enough to observe any linage specific expression changes. The cells were also grown in monolayer cultures so may not provide the differentiating cells with the correct growth requirements for differentiation. To add support to the genes isolated from micro array analysis and the theories presented, further experimentation must be carried out to relate function to gene expression. This experimentation should initially include Q-RT-PCR analysis to confirm expression levels of the selected genes a well as differences in protein levels. However, caution must be taken with protein level in this instance as the time taken for RNA expression may not equate to the time required for post translation modification at the protein level. Further experimentation on WTE14 cells using 'knock down' experimentation with siRNA in conjunction with clonal assays could confirm the effect of the gene at the biological level. Even so, this data search has presented several candidate genes and a potential signalling pathway for further study.

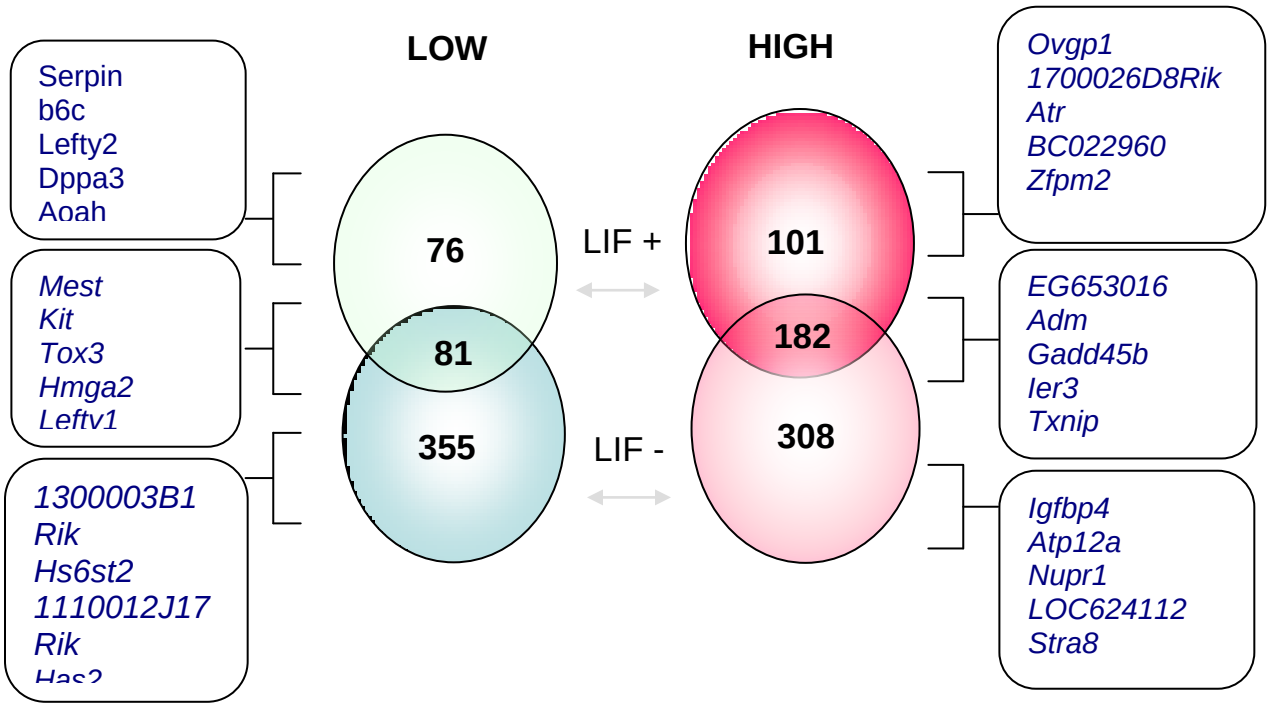
Appendix 10 Genes expressing the greatest expression fold changes (5 genes per category)

Condition	*Gene*	*Fold change*	*p-value*	*Function*
Low LIF+	Serpin	-3.13	2.56E-05	Gametogenesis
	Lefty2	-2.93	0.00240333	Controls left-right embryo/heart patterning
	Dppa3	-2.81	0.00832241	Expressed in primordial germ cells (Alias Stella)
	Aoah	-2.7	0.000519558	Prevents prolonged inflammatory reactions to Gram-negative bacteria and LPS
	Enah	-2.5	7.38E-05	Links signal transduction pathways and is required for normal neural development
Always low	Mest	-22.89	8.46691E-06	Expressed in all major fetal organs and tissues and is involved in gene imptinting/methylation
	Kit	-11.27	0.000131969	A receptor tyrosine kinase
	Tox3	-5.38	0.000510852	Involved in alteration of chromatin structure.
	Hmga2	-4.48	0.00244546	High mobility group (HMG)
	Lefty1	-4.39	0.0466286	Involved in left-right asymmetry determination of organ systems during development
Low LIF-	1300003B13Rik	-4.63	0.00452621	Zinc ion binding
	Hs6st2	-4.37	0.00893246	A cell surface component that influences cell growth, differentiation, adhesion, and migration
	1110012J17Rik	-3.9	0.021317	Expressed during embryonic brain development E6-E9.5
	Has2	-3.67	0.00288673	Involved with embryonic development leukocyte homing and tumor metastasis.
	Pftk1	-3.49	0.00921185	A serine/threonine-protein kinase involved in the control of the eukaryotic cell cycle,
High LIF +	Ovgp1	4.07	0.00427506	Encodes a secreted protein involved with ovulated oocytes, blastomeres and spermatozoan acrosomal regions
	1700026D8Rik	4	0.0196383	Unknown but expressed at E1 and in germ cell of the adult
	Atr	2.89	5.46E-05	PI3K family member that signals in response to DNA strand breaks/ replication errors to maintain cell viability/chromosomal stability
	BC022960	2.75	0.000608366	Expressed on the postnatal ovary and on the retina
	Zfpm2	2.69	0.00611125	Regulates haematopoiesis and cardio genesis
Always high	EG653016	10.71	0.000273241	Signals downstream of Oct 4 expressedin pluripotent cell lines
	Adm	6.97	0.00638398	A survival factor against hypoxic cell death by suppressing reactive oxygen species and is PI3K regulated.
	Gadd45b	6.94	0.0353568	Inhibits upstream regulators of JNK
	Ier3	6.57	0.000352653	This gene functions to protect cells from Fas- or tumor necrosis factor type alpha-induced apoptosis.
	Txnip	5.84	0.00817171	Functions as a transcriptional repressor and over-expression will induce G0/G1 cell cycle arrest.
High -LIF	Igfbp4	4.07	0.00427506	Encodes an IGF-binding protein that prolongs the half-life of the IGFs to stimulate cell culture growth.
	Atp12a	4	0.0196383	Catalyzes the hydrolysis of ATP
	Nupr1	2.89	5.46E-05	Responsible for potassium absorption in various tissues
	LOC624112	2.75	0.000608366	Hypothetical protein
	Stra8	2.69	0.00611125	Required for the transition into meiosis for both female and male germ cells.

Appendix 11

Micro Array Data

Genes expressing the greatest fold changes



Venn diagram showing numbers of differentially expressed genes in the following categories; High gene expression in Kit null cells compared to WT ET 14 cells (red) Low gene expression in Kit null cells compared to WT E14 cells(blue) in the presence (+LIF) and absence (- LIF) of LIF. Overlapping areas refer to genes that are high or low both in self-renewing (+LIF) and differentiating (-LIF) ES cells.

Appendix 12

Micro Array Data

Potential Compensatory Receptors

Condition	Receptor	Gene	Entrez	Fc	p-value	Function
Always high	PDGF	Pdgfrb	18596	3.23	0.0195876	Transmembrane receptor tyrosine kinase
High Lif -	Flt	Vegfa	22339	2.31	0.00950305	Binds and activates Flt 1
		Figf	14205	2.12	0.0116674	Binds and activates Flt 1
High Lif +	FGF	Fgf11	14166	2.49	0.00713286	Induces broad mitogenic and cell survival activities
		Fgfr1op2	77744	2.41	0.00151649	Binds to a transmembrane regions of FGF1R to promote ligand independent dimerisation
		Fgf17	103844	2.33	0.00431222	Mitogen that binds to FGFR1-4
		Fgf13	72190	2.31	0.00062621	Mitogen that binds to FGFR1-4
		Fgfr4	223989	2.27	0.00854738	Transmembrane receptor tyrosine kinase
		Fgf1	22064	2.22	0.00189606	Mitogen that binds to FGFR1-4 Transmembrane receptor
Low LIF -	HGFR	Met	17295	-2.09	0.0137854	Tyrosine kinase

Differentially expressed genes with a role in receptor signalling and potential compensatory mechanisms for c-kit deficiency (Fc= foldchange).

Appendix 13

Micro Array Data

Membrane Perturbation

Condition	Gene	Entrez	Fc	p-value
Always high	*Rab39*	270160	2.09	0.013352
	Cd99	673094	2.27	0.000192
	Cd68	12514	2.34	0.02154
	Ero1l	50527	2.43	0.001103
	Frmd4b	232288	2.96	0.010593
	H2-Bl	14963	3.11	0.01823
	Porcn	53627	3.13	0.009091
	Pdgfrb	18596	3.23	0.019588
	Rnf128	66889	3.34	0.018156
	Cdc42ep2	104252	3.41	0.003107
	Cadm4	260299	4.23	0.010215
	4930583H14Rik	67749	4.42	0.002927
	Ier3	15937	6.57	0.000353
	Ier3	15937	6.57	0.000353
High LIF+	*Timm9*	30056	1.67	0.019481
	Atp5c1	11949	1.83	0.010186
High LIF-	*Abca3*	27410	2.08	0.025979
	Figf	14205	2.12	0.011667
	2200002K05Rik	69137	2.16	0.004355
	Xpr1	19775	2.56	0.014739
	H2-Ab1	14961	2.65	0.002661
	Rmcs5	114888	2.68	0.024608
	Hck	15162	2.71	0.034658
	Higd1a	56295	2.75	0.020308
	Pvr	52118	2.91	0.015788
	Stmn2	20257	2.93	0.014065
	Anxa2	12306	3.01	0.003859
	Selenbp1	20341	3.29	0.038505
	Atp12a	192113	5.2	0.034683
Always low	*Mest*	17294	-22.89	8.47E-06
	Kit	16590	-11.27	0.000132
	2610528J11Rik	66451	-3.83	0.004605
	Odz4	23966	-2.24	0.035382
	Stx3	20908	-2.14	0.001158
	Marveld1	277010	-1.86	0.015964
Low LIF +	*Has2*	15117	-3.67	0.002887

	Vwf	22371	-2.49	0.018866
	Slc23a1	20522	-2.27	0.033908
	Epha4	13838	-1.77	0.004836
Low LIF-	*Dscaml1*	114873	-3.31	0.002029
	Slc16a2	20502	-3.21	0.020233
	Mcoln3	171166	-3.21	0.00145
	Cdh9	12565	-2.99	0.010782
	Peli2	93834	-2.75	0.009335
	Cacna1b	12287	-2.69	0.00137
	Cdh2	12558	-2.46	0.003841
	Plch1	269437	-2.34	0.018138
	Cachd1	320508	-2.34	0.024622
	Lrfn4	225875	-2.24	0.001177
	Slc12a2	20496	-2.21	0.041235
	Cox7c	12867	-1.88	0.011271
	Reep3	28193	-1.74	0.020636
	Lpgat1	226856	-1.65	0.017078
	Prlr	19116	-1.61	0.042153
	Aoc3	11754	3.95	0.008563
	Hs6st2	50786	-4.37	0.008932
	Marveld3	73608	-2.77	0.002929
	Lrp2	14725	-2.75	0.020445
	Gfra1	14585	-2.61	0.003505

Appendix 14

Micro Array Data

Apoptotic regulators

Condition	Gene	Entrez	Fc	p-value	Apoptosis
Always high	Adm	11535	6.97	0.00638398	anti
	Gadd45b	17873	6.94	0.0353568	anti
High LIF-	Nupr1	56312	4.17	0.0156559	anti
	Vegfa	22339	2.31	0.00950305	pro-
	E2f1	13555	2.13	0.0292042	pro-
	Cidea	12683	3.12	0.0115041	pro-
	Aifm2	71361	2.06	0.0154174	pro-
	Agtr1a	11607	1.88	0.00298249	pro-
	1110007C09Rik	68480	1.87	0.0252057	anti
	Adamtsl4	229595	1.84	0.0146997	pro-
	Tnfaip8	106869	1.8	0.00994052	anti-
	Als2cr2	227154	1.79	0.00842711	anti-
	Rrm2b	382985	1.76	0.00953504	anti
	1200009F10Rik	67454	1.74	0.0450971	pro-
	Litaf	56722	1.69	0.00700436	pro-
	Bcap29	12033	1.68	0.0183457	pro-
	Nek6	59126	1.67	0.00739737	anti-
	Efhc1	71877	1.65	0.0237288	pro-
	Bnip3	12176	1.63	0.00158255	pro-
	Bnip2	12175	1.54	0.00435788	anti-
High LIF +	Hells	15201	1.53	0.0117503	anti-
	Ddit3	13198	2.25	0.0101532	pro-
	Rarb	218772	2.1	0.00826633	pro-
	Ddit4	74747	1.96	0.00208198	pro-
Low LIF-	Bcl11a	14025	-1.6	0.0421745	anti
	Smndc1	76479	-1.63	0.00182944	anti
	Sh3kbp1	58194	-1.7	0.0389175	pro-
	Notch1	18128	-1.79	0.00769348	anti
	Map3k5	26408	-2	0.00399269	pro/anti
	Jmy	57748	-2.02	0.00691351	pro-
	P2rx7	18439	-2.05	0.00581636	pro-
	Ripk1	19766	-2.38	0.0272567	pro-
	Dnase2a	13423	-2.74	0.0455096	pro-
	Hif1a	15251	-1.65	0.00551823	pro-
	Map3k7	26409	-1.74	0.0372419	pro/anti
	Axud1	215418	-1.84	0.0137026	pro
	App	11820	-1.87	0.000784599	pro-
	Bclaf1	72567	-1.94	0.0463219	pro-
	Dapk1	69635	-2	0.00253603	pro-
	Birc3	11796	-1.51	0.00755898	anti-
Low LIF+	Spp1	20750	-1.76	0.0025503	pro/anti

Appendix 15

Micro Array Data

MAPK regulators

Condition	*Gene*	*Entrez*	*Fc*	*p-value*	*Function*	*reference*
Always high	Adm	11535	6.97	0.00638398	Activates JNK	Oufik 2009
	Gadd45b	17873	6.94	0.0353568	Activate MKK4 upstream of JNK and P38	Takekawa 1998
High LIF-	Nupr1	56312	4.17	0.0156559	Downregulated by JNK	Malicet 2003
	Als2cr2	227154	1.79	0.00842711	Antiapoptotic activation of JNK	Sanna 2002
	Nup62	18226	1.74	0.0152551	Negative regulator of MAPK pathways	Di Cristofano 2001
	Efna1	13636	1.51	0.028847	Inhibits the EGFR-RAS-MAPK pathway	Neve 2006
Low LIF -	Map3k7(TaK 1)	26409	-1.74	0.0372419	Activates MAPK8/JNK, MAP2K4/MKK4,	Takatsu 2000
	Map3k5 (ASK1)	26408	-2	0.00399269	Activates the MAPK pathway	Nakagawa 2008
	P2rx7	18439	-2.05	0.00581636	Activates the MAPK pathway (ERK1/2)	Gavala 2008
	Sh3rf1	59009	-1.57	0.00560794	A scaffold protei for the JNK /c-jun pathway	Lyons 2007
Always low	Ndst1	15531	-1.62	0.00872428	Enhances MAPK signalling	Pallerla 2007
	Fgd4	224014	-2.5	0.0351006	Activates JNK	Obaishi 1998

Differentially expressed genes with a role in MAPK signalling (Fc= foldchange

Appendix 16

Colony size of WTE14 and KIT Null cells

	Cell Size uM	
Replicate	*WT E14*	*KIT Null*
1	540	215
	590	153
	520	141
2	870	213
	870	266
	642	213
3	701	215
	730	240
	700	276

Comparison of colony size between WTE14 and KIT Null cell clones

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